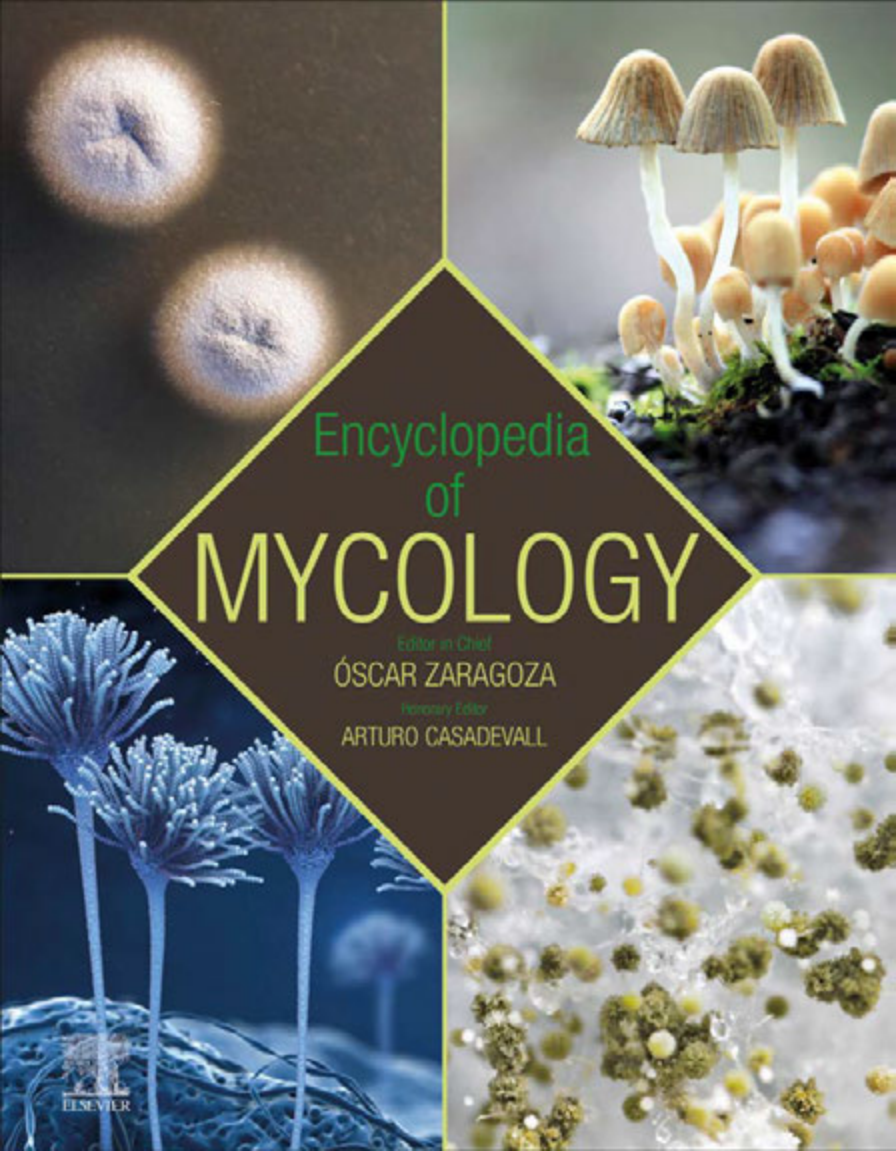

Encyclopedia of MYCOLOGY

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ENCYCLOPEDIA OF MYCOLOGY

Volume 1

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Dr Óscar Zaragoza received his degree in Biology in 1995, and in 1996 he started his scientific career, which has been focused on microscopic fungi. He started his PhD under the supervision of Dr Juana María Gancedo (Biomedical Research Institute, CSIC, Madrid, Spain) in 1996. He obtained his PhD in 2000, and in his thesis, he studied catabolite repression in the yeast *Saccharomyces cerevisiae*. During this period, and under the supervision of Dr Carlos Gancedo, Dr Zaragoza started working with human pathogenic fungi, using *Candida albicans* as model.

In 2001 he started his postdoctoral training in the laboratory of Dr Arturo Casadevall (Albert Einstein College of Medicine, New York), who is one of the most recognized scientists in the field of Clinical Mycology. During this period, he focused his research on human pathogenic fungi, and in particular, on *Cryptococcus neoformans*, which has a significant prevalence among HIV+ patients. In 2006, he returned to Spain, and began his work as Principal Investigator at the Mycology Reference Laboratory of the National Centre for Microbiology of the National Health Institute Carlos III (Madrid, Spain), directed by Dr Rodríguez-Tudela and Dr Cuenca-Estrella.

Dr Zaragoza's group main interest is the investigation of human fungal pathogens. These microorganisms affect mainly immunosuppressed patients, and they are associated with high mortality rates and a significant increase in the economic cost of patient treatment. In the last decade, he has directed two main research lines. One is focused on the mechanisms of fungal adaptation to the host, using the pathogen *C. neoformans* as model. In addition, the group is interested in the investigation of antifungal agents (action mechanisms and resistance). Dr Zaragoza is currently working at the Mycology Reference of the National Centre for Microbiology of the National Health Institute Carlos III, where he also supervises different tasks aimed to support the National Health System (identification and antifungal susceptibility of clinical isolates).

His research has authored more than 80 publications in some of the most important international journals of the field (*PLOS Pathogens*, *mBio*, *Infection and Immunity*, *mBio*, *Cellular Microbiology*, *Antimicrobial Agents and Chemotherapy*, among others), and several chapters in specialized books. Dr Zaragoza is also member of the editorial boards of *PLOS ONE*, *BMC Microbiology*, *Frontiers in Microbiology* (*Fungi and their development*) and *Mycopathologia*.

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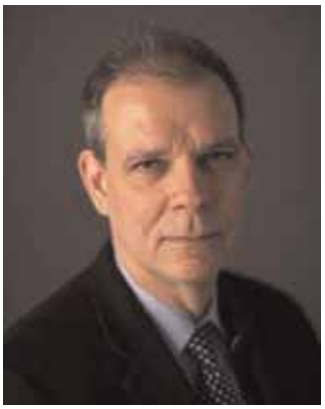

Arturo Casadevall, M.D., Ph.D., is a Bloomberg Distinguished Professor and Alfred and Jill Summer Chair of the Molecular Microbiology and Immunology at Johns Hopkins School of Public Health. He received his M.D. and Ph.D. degrees from New York University. He completed his internship/residency in internal medicine at Bellevue Hospital and specialized in Infectious Diseases at the Albert Einstein College of Medicine. The author of over 800 papers, books and chapters, his major research interests are in fungal pathogenesis and the mechanisms of antibody action. He is editor-in-chief of mBio, Deputy Editor of the Journal of Clinical Investigation and serves on several editorial boards. He has received numerous honors including election to the American Society for Clinical Investigation, American Academy of Physicians, American Academy of Microbiology, Fellow of the American Academy for the Advancement of Science, American Academy of Arts and Sciences and the National Academy of Medicine.

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Raffaella Maria Balestrini. Biologist, PhD in Fungal Biology and Biotechnology. With CNR since 1998, Raffaella is currently a Research Director at CNR-IPSP. She has expertise on studies aimed to highlight the cellular and molecular bases of plant-soil microorganism interactions, mainly focusing on cell wall changes and nutritional exchanges. She mainly contributed to elucidate different aspects related to the interface creation and, by applying a laser microdissection (LMD) approach, she contributed to obtain new knowledge on the cell-specificity in arbuscular mycorrhizal (AM) roots. Thanks to the participation at diverse international consortia, she has contributed to highlight the genome features of different mycorrhizal fungi. Current research interests mainly address crop responses to environmental stresses and the role of root-associated microorganisms (e.g., mycorrhizal fungi) in improving tolerance. Scientific activities have been carried out within national and international projects including EU projects. Member of the editorial board for different scientific journals and she reviewed several project proposals for different national and international Institutions. She is member of diverse Editorial Boards of international peer-reviewed scientific journals. She has published over 100 papers in peer-reviewed indexed journals and many book chapters.

Miia R. Mäkelä is Principal Investigator and Adjunct Professor at the Department of Microbiology, University of Helsinki, Finland. Her main research interest is to understand fungal plant biomass conversion and degradation at the molecular level and apply this knowledge in biotechnology.

She obtained her PhD in Microbiology from the Faculty of Agriculture and Forestry, University of Helsinki. Her postdoctoral projects addressed various aspects of fungal lignocellulose degradation, including genome mining and characterization of novel enzymes for plant biomass conversion and valorization. During her position at Westerdijk Fungal Biodiversity Institute, The Netherlands, she focused on regulation of plant biomass conversion and metabolic engineering in ascomycete fungi. She received the prestigious Academy of Finland Research Fellowship position in 2017, which allowed her to develop a research line on the basidiomycete regulatory systems controlling plant biomass conversion process. Functional genomics and post-genomic approaches as well as genome editing are integral to her research.

She has (co-)authored over 100 peer-reviewed publications and has been a guest-editor for two special issues of the journal Fungal Genetics and Biology. She is currently an editorial board member for Biotechnology Letters, and an associate editor for Frontiers in Fungal Biology and Frontiers in Microbiology.

Josh Nosanchuk, MD is Professor of Medicine (Infectious Diseases) and Microbiology & Immunology as well as Senior Associate Dean for Medical Education at Albert Einstein College of Medicine in New York City. In his Dean position, Dr Nosanchuk strives to integrate basic, clinical and health system science across the curriculum of the medical school. Dr Nosanchuk's laboratory focuses on pathogenic fungi and novel therapeutics. Major pathogenic fungi studied include *Histoplasma capsulatum*, *Candida parapsilosis*, *C. auris*, *C. albicans*, and *Cryptococcus neoformans*. In particular, the laboratory investigates 1) how antibody can modify disease outcomes, 2) the role of melanin production on pathogenesis, and 3) the effects of extracellular release of vesicles from fungi, which contain numerous products associated with virulence. Novel therapeutics developed in the Nosanchuk laboratory include melanin-binding antibodies that have been used in a clinical trial for melanoma and pre-clinical compounds such as nitric oxide-releasing nanoparticles and siRNA targeting fidgetin-like 2, a microtubule severing enzyme that regulates wound healing.

Carla Viegas is a full Professor at Lisbon School of Health Technology, Director of the Occupational Health Master's course and researcher at H&TRC- Health & Technology Research Center from ESTeSL-IPL, NOVA National School of Public Health, Public Health Research Centre, Universidade NOVA de Lisboa and Comprehensive Health Research Center (CHRC). She is graduated in Environmental Health from Lisbon School of Health Technology – Polytechnic Institute of Lisbon has a Masters degree in Safety and Ergonomics from Lisbon University and PhD in Occupational and Environmental Health from New University of Lisbon. Her major field of study is occupational and environmental mycology leading and participating in several national and international projects about both areas of expertise. Special interests are occupational exposure to fungi in highly contaminated settings and complementarity of culture based- methods and molecular tools to assess fungal contamination. Additionally, she has expertise in sampling campaigns performed in different occupational environments using wide sampling and analyses approach to assess multiple microbiologic agents. Carla has authored has several publications/communications in the referred areas of specialization.

Alfredo Vizzini is a biologist with over 30 years' experience in Systematic Mycology. He graduated with a PhD in Biology and Biotechnology of fungi from the Department of Plant Biology of Torino, Italy. He is now an Associate Professor of Systematic Botany at the University of Torino (Department of Life Sciences and Systems Biology) where he teaches biodiversity of fungi and terrestrial plants. His research interests focus on mushroom taxonomy, especially agaricoid and boletoid species, using morphological and molecular approaches.

He is the author of over 200 publications on peer-reviewed international journals. Alfredo is currently associate/section editor of the journals Ascomycete.org, Biodiversity Data Journal, Bulletin of Environmental and Life Sciences, Italian Botanist, MycoKeys, Mycosphere, Phytotaxa, Studies in Fungi, Taxonomy, and a curator of the webpages Basidiomycota (https://basidio.org/), and Faces of Fungi (https://www.facesoffungi.org/).

Ronald P. de Vries. Group Leader Fungal Physiology at Westerdijk Fungal Biodiversity Institute (The Netherlands) and Professor in Fungal Molecular Physiology at Utrecht University (The Netherlands).

His research addresses the conversion of biomass (plant and marine) by fungi, focusing on the extracellular enzymes, the intracellular metabolic pathways and the transcriptional regulators that control these processes, as well as the application of fungi for the biobased economy. It contains a strong (post-)genomic component combined with modern and established experimental methodologies, such as genome editing, enzymology, metabolic engineering and physiology.

He graduated at Wageningen University (The Netherlands) and spent some time there and at Institut Pasteur (Paris, France) as a postdoc, after which he moved to Utrecht University as a postdoc and senior scientist, before taking up his current position. He obtained the prestigious Dutch VIDI (2005) and VICI (2013) grants and became an honorary member of the Hungarian Microbiological Society in 2017. He is a visiting professor at University of Helsinki (Finland) and a member of the fungal advisory board of the Joint Genome Institute, USA.

PREFACE

Fungi are organisms that have a profound significance in multiple processes of great importance for our daily life. But, apart from their influence in human affairs, fungi are fascinating organisms to study in themselves. They comprise both macroscopic and microscopic organisms, and it is estimated that there are somewhere between one and five million species of fungi. Fungi are found in most of ecosystems and are in constant contact with multiple other organisms, being involved in many commensal, symbiotic and parasitic interactions.

From a biological point of view, fungi are heterotrophic eukaryotic organisms that have a cell wall that contains chitin and several other polysaccharides (glucan, galactomannan, etc). They can reproduce by both sexual and asexual cycles. Despite the great variation among fungal morphology (macroscopic and microscopic), genetic tools reveal close relationships from an evolutionary point of view, and are closer to animals than to plants. But still, the Fungal kingdom contains a great variability in structure, shape, colour, reproduction and biology.

Most readers are already familiar with macroscopic fungi (mushrooms). Their colours and shape could be considered as one of the beautiful creations of nature, and many people look forward to those seasons of the year when they can enjoy viewing them in fields and forests. But in addition, macroscopic fungi have been enigmatic organisms for centuries. Some of them are edible, others poisonous or hallucinogenic, and still others have medical properties. Nowadays, they have multiple applications, not only as a delicious food source, but also in biotechnology, and there is a great evidence of their role in geochemical balance in the environment.

A major part of mycology involves microscopic fungi, which are divided in two main classes: yeasts and filamentous fungi (or moulds). And although invisible to the eyes, their biology and effects are also fascinating. In fact, we could express the importance of microscopic fungi by remembering one of the most famous quotes from the "The little Prince", when the Fox revealed that the essential things in life are invisible to the eyes. When Antoine de Saint-Exupéry wrote this quote, he did not refer to microscopic fungi, but when we consider all the effects that they have on our world, we could certainly consider them as essential. How would the world be today without fungi? We will never know, but the fact that fungi are found in most ecosystems and establish interactions with all kind of living organisms indicate that they have played a role in evolution to select and shape our current world and way of living. Even in the case of humans, fungi have influenced the evolution of our immune systems, microbiota and other aspects such as body temperature. For thousands of years, we have taken advantage of fungal biology to produce cheese, wine, bread, beer and many other products. Paradoxically, we have not understood the basis of their action until relatively recently in our history, towards the end of XIXth century, and nowadays they are a powerful tool in today's biotechnology. Fungi triggered a revolution in the medicine when Alexander Flemming found that they can be the source of antibiotics. Since the discovery of penicillin from the fungus *Penicillium notatum*, the search for many other compounds with biological activities produced by any kind of microorganism has been continuous and successful. Today fungi produce numerous important drugs including antimicrobial drugs and statins. In addition, fungi are used as microscopic factories to produce many compounds of interest or acquire new metabolic functions that provide multiple biotechnological applications.

Research in microscopic fungi has been classically promoted by their important role in biotechnology. This interest began during the XXth century as the industry developed and some species became important model organisms (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* or *Neurospora crassa*, and *Aspergillus nidulans*), which allowed studies that led to the unravelling of key aspects in metabolism, genetics, cell cycle regulation, circadian cycles and many others are process. Even the first eukaryotic microorganism that was fully sequenced was the yeast *S. cerevisiae*, which highlight the importance of these microorganisms in research.

Despite their innumerable beneficial effects, the interaction of fungi with other living organism such as plants and animals can also result in deleterious outcomes and disease. We could explain the role of fungi as potential pathogens by revealing the secret that the letter "F" hides in the name Fungi, since they can be considered as either Friends or Foes. In the case of humans, microscopic fungi can cause many different diseases. Some of these pathogenic fungi live as inoffensive commensal in our body, but in some cases, depending on multiple factors, mainly related to our immune system, they can produce damage and disease. In some way, we could envision them as the main character from the classic written by Robert L. Stevenson, so these fungi could resemble to the kind and nice Dr. Jekyll that sometimes turns into the hideous and evil Mr. Hyde. In other cases, fungi that cause disease are acquired from the environment and they do not belong to our microbiota. This poses the challenge of how environmental changes (global warming, release of antimicrobial, use of antifungals, environmental interactions, etc) affect the virulence of the fungi that we constantly acquire from external sources. Most of fungal diseases are superficial and non-life threatening, but they can cause great discomfort. The incidence of these types of fungal diseases, such as infections of the skin (i.e., athletes foot), eye and mucosae such as the vagina (mainly vaginal candidiasis) or oral cavity is enormous, reaching around one hundred millions of people per year. In addition, some fungal species can be the causative agents of invasive disease, mainly among immunocompromised patients. As historical curiosities, the first cases of HIV+ was originally diagnosed with invasive fungal diseases. And even more anecdotic, fungi were in some moment linked to the mythical "Tutankhamun's Curse" and related to the deaths of some of the discoverers of the tomb of the young Pharaoh. Nowadays, it is estimated that around 1.5 million people die every year due to fungal pathogens, a number that is unacceptably high. Moreover, beyond humans, fungi can also cause devastating plagues in the environment in animals and plants. As examples of their negative effects, the economic impact of fungi as crops contaminants is estimated in billions of dollars every year. And among animals, many fungi are common causes of disease

in multiple species, both vertebrates and invertebrates, and there are some species of amphibians have been driven to extinction by fungal diseases. In the United States the so called 'white nose syndrome' fungal disease threaten to drive several bat species to extinction.

In conclusion, the biology of fungi and their multiple effects in our world justified the organization of this Encyclopaedia, which has been possible to develop thanks to the dedicated work of the editors that have organized the five sections in which this work is organized: **Dr. Carla Viegas** (Lisbon School of Health Technology/Polytechnic Institute of Lisbon, Portugal, editor of Environmental Mycology), **Dr. Alfredo Vizzini** (University of Torino, Italy, editor of Macroscopic fungi), **Dr. Raffaella Ballestrini** (Fungal Biology), **Dr. Miia R. Mäkelä** (University of Helsinki, Finland) and **Dr. Ronald P de Vries** (Westerdijk Fungal Biodiversity Institute/Utrecht University, both editors of the Fungal Biotechnology section) and **Dr. Josh Nosanchuk** (Einstein College of Medicine, Ney York, editor of Medical Mycology). This work has been also possible due to the engagement of a large number of authors who are leading experts in their respective fields. We would like to stress that the development of this project took place during COVID19 pandemic, which has all our lives. We are aware of how difficult it has been for many authors to continue with their regular work (academia, research, health assistance, etc) with the restrictions caused by the pandemic. We are sure that for all of them, participation in this Encyclopaedia required a great deal of extra effort, and for this, and we are very grateful for their dedication and engagement in this enterprise. We also acknowledge and thank the Elsevier staff for their support and their assistance with such a challenging project. We warmly thank all the people involved in the completion of this Encyclopaedia, and we are proud that the final result provides a nice and comprehensive work on one of the most exciting topics in Biology.

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Next Generation Sequencing: Transcriptomics

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The Rise of NGS Transcriptomics in Fungi

Genomics and its applications are some of the most advanced areas of current biological research. However, the genome sequencing represents just the first step toward a broad knowledge of an organism. The integration of functional, structural and cellular approaches that, all together, form the so-called *post-genomic* activities, is necessary for the deeply understanding of the role and the network of genes/proteins, and to the assignment of functions to the whole gene dataset discovered into organisms. Transcriptomics is one of the most developed fields in this current *post-genomic* era. By mirroring the differential gene expression during development and different phases of organisms life cycle, as well as during their responses to biotic and abiotic factors, the transcriptome constitutes the dynamic link between the static genome of an organism, a tissue or a cell, and its phenotype, at a specific time point (Meijueiro *et al.*, 2014; Stark *et al.*, 2019). In fact, morphogenetic changes of organisms might often depend on corresponding changes of activity of large numbers of genes. Several studies on single transcripts were carried out many years before any transcriptomics approaches were available. Traditional techniques included cDNA Amplified Fragment Length Polymorphisms (cDNA-AFLP), low-throughput Sanger sequencing of random transcripts from complementary DNA (cDNA) libraries [namely, expressed sequence tags (ESTs)], and PCR-based techniques, i.e. retro-transcriptase quantitative PCR (RT-qPCR) (Bhadauria *et al.*, 2007; Lowe *et al.*, 2017). Nevertheless, only after the development and the improvement of higher-throughput techniques it was possible to investigate how an organism transcriptome as a whole is finely regulated.

Fungal transcriptomics is a rising field which has gained more and more attention in the last two decades. The first fungal transcriptome, based on serial analysis of gene expression (SAGE), was released in 1997 for yeast (Velculescu *et al.*, 1997), and after that, thanks to the development and the availability of modern large-scale molecular tools, an exponential increasing number of fungal transcriptomes was published (Nowrousian, 2013; Meijueiro *et al.*, 2014). Currently, even if some other recent promising techniques has been proven to be useful in transcriptomics i.e., high throughput-SuperSAGE (HT-SuperSAGE) (Soanes *et al.*, 2012), the two most popular large-scale transcritptomic approaches applied in fungi remained microarrays (Nowrousian, 2007) and RNA sequencing (RNA-seq) (Wang *et al.*, 2010b). Microarrays refers to a collection of thousands of oligonucleotide spots, defined as probes, attached to a solid surface, which can hybridize a cDNA, defined as target, under high-stringency conditions (Schulze and Downward, 2001). The cDNA sequences, each of them representing a single transcript, are labeled and hybridize to the corresponding gene sequences on the array, and relative abundance of cDNA in the target is quantified by fluorescence detection and quantification (Schulze and Downward, 2001). This technology allowed for the first time the simultaneous analysis of thousands of transcripts. Pioneering fungal microarray studies were performed in yeast more than 20 years ago (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997). Microarrays have been produced for several species of filamentous fungi, mainly by using PCR amplicons from EST libraries (Breakspear and Momany, 2007). The application of microarray technology to decipher transcriptomic profiles of fungi subjected to different conditions has proven to be useful for extending the knowledge on how fungi face with their surrounding environment. For example, *Cryphonectria parasitica* cDNA microarrays were used to monitor the changes in transcriptional profile when infected by a hypovirus (Allen *et al.*, 2003). A significant reprogramming of the *C. parasitica* transcriptome was observed and differentially expressed genes (DEGs) related to stress responses, carbon metabolism, and transcriptional regulation were detected (Allen *et al.*, 2003). Another classical example is represented by microarrays for *Aspergillus nidulans,* fabricated by using PCR products from a EST libraries generated during conidial germination, and used to assess the transcriptomic response of the fungus to different nutrient sources (Sims *et al.*, 2004). In the symbiotic Ascomycete *Tuber melanosporum*, microarrays allowed to track the transcriptomic changes occurring in its life cycle, in particular during the shift from free living mycelium to the formation of ectomycorrhizas and fruit bodies (Martin *et al.*, 2010).

However, it should be noted that microarray-based technology can be affected by some important limitations. First, for the customization of arrays, a reference genome sequence or a comprehensive set of EST sequences must be available, and second, non-specific or repeated cDNA sequences with high similarity hybridization to targets might strongly affect the dynamic range of the recorded signal (Lowe *et al.*, 2017). Moreover, not characterized transcripts (e.g., non-coding transcripts) and RNA species missing in EST libraries or in genome annotations cannot be detected by using this technology which is based on the availability of well-known sequences (Lowe *et al.*, 2017).

Currently, the emergence and the democratization of high throughput next-generation sequencing (NGS) techniques as substitute to Sanger sequencing for large-scale genomic analysis provided an opportunity to further explore and understand the differential gene expression at an exceptional resolution (Marconi *et al.*, 2014). In contrast to traditional Sanger sequencing, NGS allowed high throughput parallel sequencing of over millions of DNA nucleotides from multiple samples at reduced cost and time (Metzker, 2010). International sequencing activities, such as the ongoing "1000-fungal genomes" project (see "Relevant Websites section") of the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) (Grigoriev *et al.*, 2014), are aimed at increasing the dimension of fungal genome sequence databases by covering all fungal taxonomic units through sequence characterization of the most representative isolates. Remarkably, for almost all genome projects currently finished or in progress, a related large scale gene expression dataset is provided (Kuske *et al.*, 2015). Dedicated databases containing manually annotated fungal transcript sequences (e.g., see "Relevant Websites section"; Stajich *et al.*, 2012) are also in ongoing development.

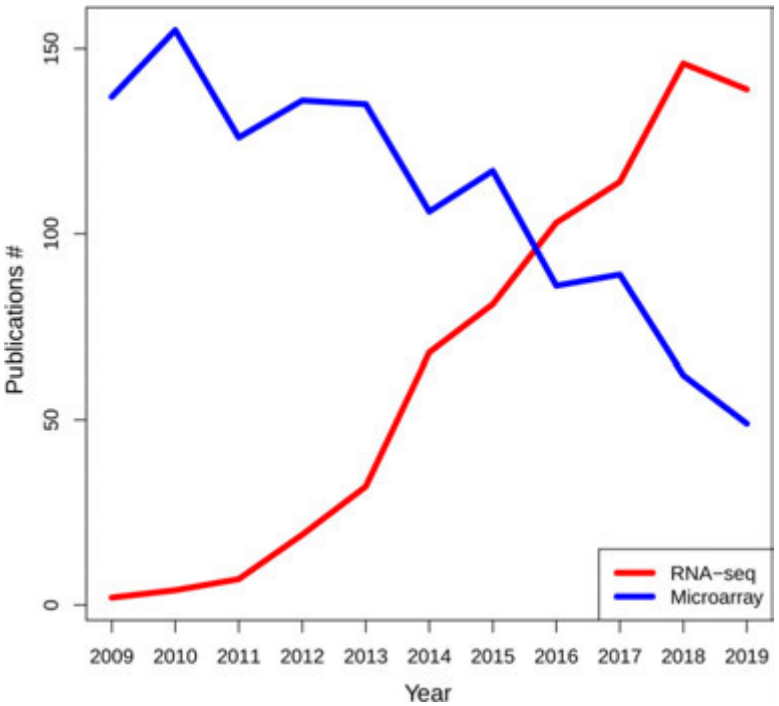

Fig. 1 Results of a search on Scopus database (www.scopus.com) with the terms "microarray AND fungi" and "RNA-seq AND fungi". Number of publications and years were reported.

In this line, RNA-Seq refers to the sequencing, through NGS, of transcripts converted into cDNA sequences, in which abundance is directly proportional to the number of counts from each transcript (Stark *et al.*, 2019). Compared to microarrays, RNA-seq does not require a priori knowledge of the genome sequence and this method can potentially be used for the detection of quantification of each RNA species present in samples, including non-coding or uncharacterized RNAs. RNA-seq overcomes microarray technology because of its intrinsic potential to discover transcript variants, i.e., transcripts showing SNPs and mutations (Stark *et al.*, 2019). Comparative transcriptomics analyses using RNA-seq provided high accurate determination of quantitative expression levels, the identification of tissue-specific transcript splicing variants and isoforms, and the detection of small and large non-coding RNAs involved in gene expression regulation (Stark *et al.*, 2019). For all these reasons, RNA-seq has become the gold standard for whole-transcriptome gene expression quantification (**Fig. 1**).

Transcriptomics Through RNA-Seq

The first step of a RNA-seq, as for microarray, includes the isolation of RNA from the samples, either from single fungal isolates and from environmental matrices. Commercial kits available for plant so far have started to be optimized also for fungal materials, and some protocols for extraction of high-quality RNA with sequencing purposes have been published (Patyshakuliyeva *et al.*, 2014; Cortés-Maldonado *et al.*, 2020). This step is crucial since fungal cells, in some cases even more than plant cells, may contain nasty chemicals such as melanin and phenolic compounds as well as high levels of polysaccharides which can interfere with the downstream analysis, especially those PCR-based. In addition, the yield of fungal RNA in some samples such as plant or food samples is often very low (Argumedo-Delira *et al.*, 2008). Optimal starting amount of RNA for RNA-seq may range from 100 ng to 1000 ng, but there are kits available for ultra-low RNA input such as 10 pg–10 ng (Tariq *et al.*, 2011). Once RNA is isolated, purified and its quality is checked, several NGS platforms are available. One of the first NGS technology serving transcriptomic research was pyrosequencing, a method involving sequencing-by-synthesis followed by detection of pyrophosphate release during the activity of DNA polymerase through chemiluminescent enzymes, such as luciferases (Ronaghi, 2001). Pyrosequencing was licensed by 454 Life Sciences and allowed to obtain sequences of approximately 500 bp in size, but, as becoming noncompetitive for costs, the platform was discontinued in 2013. Currently, the two most popular and established NGS platforms used for RNA-seq in fungi are manufactured by Illumina (MiSeq and HiSeq) and Ion Torrent (Lahens *et al.*, 2017). The two platforms mainly differ for the basic sequencing technologies: while for Illumina amplified individual library DNA molecules were spotted on a solid surface and a fluorescence-based system for reading the bases in a nucleotide sequence is used (sequencing by synthesis), for Ion Torrent emulsion PCR libraries were used and detection is achieved by electronic sensors reading bases through the H^+ ion generated during nucleotide incorporation (Lahens *et al.*, 2017). Despite these NGS methods, defined as of second generation (the first included Sanger and Maxwell sequencing methods), proven to be powerful, they still retain some pitfalls, including the generation of short reads, i.e., ranging from 100 to 500 bp, which often might hinder the correct reconstruction of

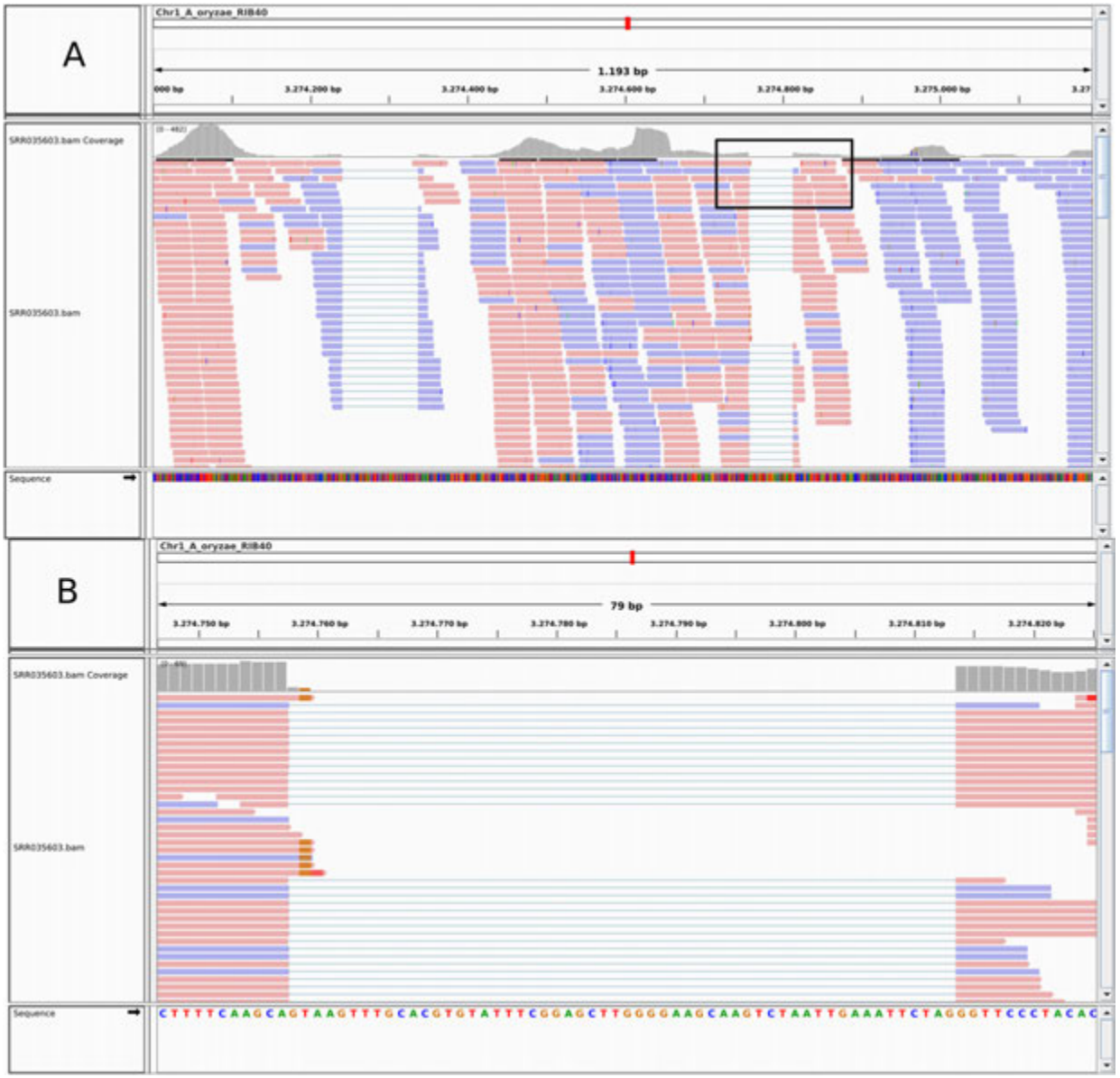

Fig. 2 Example of visualization of aligned reads from a RNA-seq experiment in IGV. In (A), the chromosome 1 of *Aspergillus oryzae* isolate RIB40 is used as reference for the alignment and the pileup of reads embedded in a BAM file (SRR035603.bam) is showed. Coverage is represented as gray histogram on the top of the alignment. Red and blue arrow-blocks represent first strand and complementary paired-end reads, respectively. Black squared section is detailed in (B). In (B), structure of an intron is showed. The intronic region was not covered by reads from the transcript, and reads over-spanning the exon boundaries. A cyan line, representing the intron, links the two exons. Data for this figure were obtained from Nowrousian, M., 2013. Fungal gene expression levels do not display a common mode of distribution. BMC Research Notes 6, 55, and freely available in SRA database.

full-length transcripts (Lowe *et al.*, 2017). Recently, the so called third-generation/long-read methods appeared on the market. Third generation sequencing works by reading the nucleotide sequences at the single molecule level, in contrast to existing methods which require breaking DNA strands into small fragments and the subsequent amplification (Van Dijk *et al.*, 2014). In this scenario, the company PacBio developed the sequencing platform of single molecule real time sequencing (SMRT), where signals of fluorescent light emission from each nucleotide incorporated by a DNA polymerase bound to the bottom of a chip, characterized by an optical waveguide (zero-mode waveguide), were detected and sequences were resolved (Roberts *et al.*, 2013).

Whereas each platform requires specific protocols of library preparation and nucleotide detection, the second generation sequencing platforms are characterized by the need to prepare amplified sequencing libraries before proceeding to the sequencing of the amplified cDNA clones (Lowe *et al.*, 2017). A standard RNA-seq protocol shared among all platforms include the cDNA library preparation, which consists of nucleic acid fragmentation, cDNA synthesis from RNA using random or oligo (dT) primers, adapter ligation, fragment size selection, and PCR amplification. By contrast, third generation sequencing platforms does not need

the prior amplification of cDNA (Van Dijk *et al.*, 2014). It is worth noting that transcriptomics primary focus on coding RNA translated into proteins (mRNA) which typically represent the 2%–3% of the total RNA in a cell. Other RNA species are indeed present into cells, including ribosomal RNA (rRNA) and tRNA (which are approximately 95% of a cell), and non-coding RNA such as snRNA, siRNA, snoRNA and microRNA (Meijueiro *et al.*, 2014). Several strategies of enrichment of a specific RNA population can be used to trigger the transcriptomic analysis depending on the focus of the experiment. For example, mRNA is usually isolated and converted into cDNA by using reverse transcriptase and oligodT primers, whereas noncoding RNA species can be included in libraries by using immunoprecipitated RNA-binding proteins or by selectively ligating 3′ and 5′ adapters before the reverse transcription to cDNA (Lowe *et al.*, 2017).

Irrespective of NGS platform used, transcripts deriving from RNA-seq are provided as short (less than 1.5K bp) sequences called *reads*, which can be aligned against a reference genome or *de novo* assembled into longer sequences when a reference genome is not available. However, Illumina and Ion Torrent platforms showed some differences in the generated data: for the first one all reads generated in a single run have the same lengths, while the read lengths of the second are variable (Lowe *et al.*, 2017). Paired end reads, i.e. reads from both ends of a fragment, can be also produced if the utilized NGS technology allowed it. Reads are typically provided as unsorted FASTQ files, a file format similar to FASTA but comprehensive of all the information about base-calling and sequence quality (Marconi *et al.*, 2014). Reads with low quality levels, i.e., low *phred* scores, or with sequence errors should be purged and sequences of vectors, adapters, tags, and tails that were added experimentally during the preparation of the libraries should be trimmed by specific bioinformatic tools (Kulski, 2016). Filtered reads are then assembled into longer sequences called *contigs* representing complete transcripts, their fragments or isoforms (Kulski, 2016). Presence of isoforms and alternative splicing in fungi has been indeed demonstrated so far (Grützmann *et al.*, 2014). Several bioinformatic tools are used to map the reads to genomic sequences, chromosomes and scaffolds. Compared to aligners of DNA sequences, RNA-seq mappers need to cope with intronic sequences which lead large gaps in the alignment. In fungi, the most established read mappers for this purpose include TopHat (Trapnell *et al.*, 2009), STAR (Dobin *et al.*, 2013), Trinity (Haas *et al*, 2013), and MapSplice (Wang *et al.*, 2010a). Commercial softwares such as CLC Genomics Workbench (CLC bio, Cambridge, MA, USA) and Newbler (Roche 454 Life Science, Branford, CT, USA) are also available. A common output of the read alignment process is a sortable SAM (Sequence Alignment/Map) file or its compressed binary version BAM. Barcodes, read quality, amplicon tags and other information are embedded in this type of files (Li *et al.*, 2009). These format files can be used as input for the popular software SAMtools (Li *et al.*, 2009), which not only allows at calculating read coverage by performing read pileup on reference genome, but also can be useful for statistics and filtering purposes (Li *et al.*, 2009). Visualization of reads aligned to reference sequences can be achieved by using Integrate Genomic Viewer (IGV; Thorvaldsdóttir *et al.*, 2013) which has been developed as an open source easy to understand tool for the dealing of NGS data, with a fashionable graphical user interface (GUI) (**Fig. 2**).

On the other hand, as stated before, without the availability of a reference genome, reads need to be assembled into longer contigs through a *de novo* assembly process. This strategy is generally based on the generation of De Bruijn graphs, where overlapping sequences are represented as paths which overlap, in order to reconstruct the original transcript sequences (Grabherr *et al.*, 2011). This approach is also recommended as complementary analysis of reference-guided mapping, in order to identify transcripts missed by alignment processes and to correct errors that may occur during the annotation (Hölzer and Marz, 2019). *De novo* assembly of RNA-seq data is more challenging than *de novo* genome assembly because of difference on gene expression, potentially masking low expressed loci, and presence of splicing events, making the reconstruction of exons in genes in the assembly paths complicated (Hölzer and Marz, 2019). Several powerful free tools such as Trinity (Grabherr *et al.*, 2011), Oases (Schulz *et al.*, 2012), and SoapDeNovo (Xie *et al.*, 2014) were developed for *de novo* transcriptome reconstruction. Completeness of *de novo* assembly can be verified through different bioinformatic tools included in specific pipelines, such as BUSCO (Simão *et al.*, 2015). This step is pivotal because of presence of large gaps and missing transcripts are know to likely occurring during *de novo* assembly processes.

Transcript sequences deriving from RNA-seq analysis may allow to strongly improve *in silico* annotations of putative genes and gene structures, as well as to train bioinformatic tools like gene predictors (e.g., AUGUSTUS; Stanke *et al.*, 2006). Thanks to alignment of reads on the available genomes, assembled transcripts are utilized to detect intron-exon boundaries in predicted/annotated genes (Lowe *et al.*, 2017), thus reducing annotation errors. A study in *Laccaria bicolor* highlighted that approximately the 69% of predicted gene models deviated from the real transcript sequences derived by RNA-seq data (Larsen *et al.*, 2010). Characterization of transcriptome can be carried out through gene ontology (GO) mapping of sequences, via specific tools such as Blast2Go (Conesa *et al.*, 2005). Basically, the GO mapping processes result in the assignment of defined GO terms, which provide information on involved biological process, molecular functions and predicted cellular components, of annotated transcripts, in order to characterize them (Conesa *et al.*, 2005).

In addition to the *in silico* characterization of transcripts, the second main goal of RNA-seq experiments is to compare the expression level of genes in two or more different conditions. Quantification of gene expression can be achieved since the total number of reads per transcript is directly proportional to the level of a transcript multiplied by transcript length (Haas *et al.*, 2013). However, it is worth noting that transcripts with same expression levels may have different chance to be sequenced depending on their size. In order to reduce this issue, expression levels are frequently normalized by counting the number of reads or fragments per kilobase per million reads (RPKM and FPKM, respectively) (Mortazavi *et al.*, 2008). Normalizing data into RPKM also provide an indication of relative expression levels between transcripts in a single library or between two libraries showing different sequencing depth (Lowe *et al.*, 2017). Quantitative differences in transcriptional levels can be also normalized and calculated on raw read count through the use of dedicated tools, including the two R packages DESeq2 (Love *et al.*, 2014) and

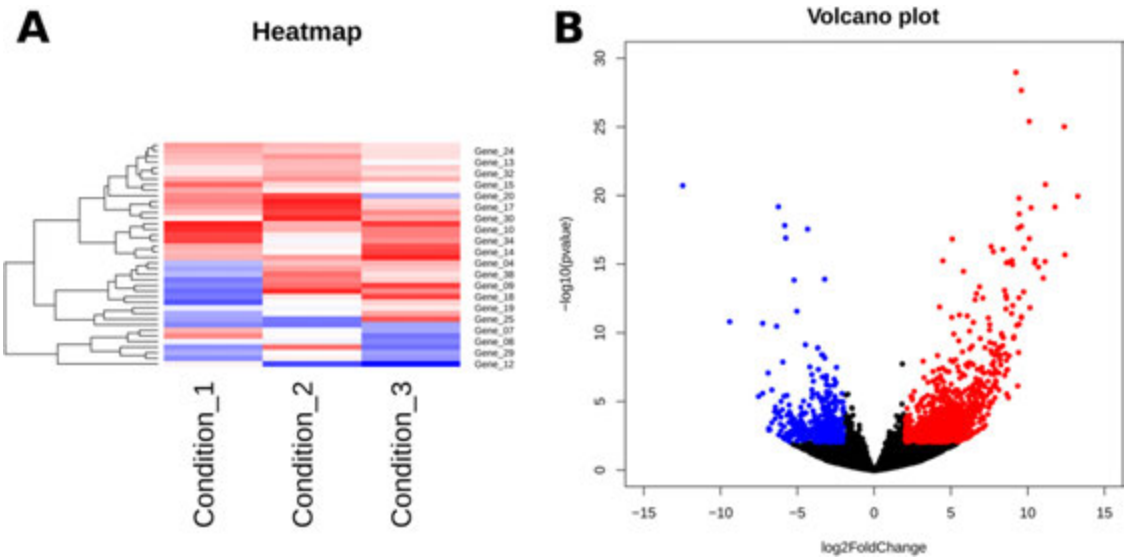

Fig. 3 Example of visualization output of RNA-seq data. In (A), example of a heatmap. Gene expression pattern of each sample/condition is represented in columns, while rows represent expression of each specific gene. Transcript abundance is indicated by different color: high-expression (red), average expression (white) and low or no expression (blue), e.g., expression of Gene_20 is high on Condition_2, and very low in Condition_3. Genes with similar expression profiles can be hierarchical clustered. In (B), a volcano plot showing DEGs. Significant up-regulated genes were represented by red dots (log2fold change > 2, p-value < 0.05), while down regulated genes were represented by blue dots (log2fold change < -2, p-value < 0.05). Black dots represent genes slightly regulated and/or not significantly expressed. Both p-value and fold changes were log2-transformed, as usually carried out for this type of data. Data for this figure were random generated.

edgeR (Robinson *et al.*, 2010), as well as the software Cuffdiff (Trapnell *et al.*, 2010), which test for differential expression using parametric approaches based on a negative binomial distribution model. The suite of tools Cufflinks embedded the algorithms for assembling transcripts, estimating their abundances, and assessing for differential expression (Trapnell *et al.*, 2012). The package *limma*, intended to be used for microarray data, has recently upgraded in order to perform differential gene expression analysis using RNA-seq data (Ritchie *et al.*, 2015). Recently, other softwares, e.g., Salmon, were developed to quantitatively and statistically assess DEGs by RNA-seq data (Patro *et al.*, 2017).

The outcomes of comparative analysis of differences in gene expression are often visualized as heatmaps, in which a color gradient for each gene represents its specific expression level in a determined condition/time/treatment (**Fig. 3**). Hierarchical clustering of genes showing similar patterns of expression may also be performed, allowing at fast tracking transcriptional changes at cluster level and at identifying gene co-expression patterns across different samples/conditions (Ben-Dor *et al.*, 1999; Lowe *et al.*, 2017). Volcano plots representing the whole set of DEGs are also commonly utilized to show a broad picture of the transcritpomic data (**Fig. 3**).

The raw sequence data from RNA-seq analysis are mostly submitted to dedicated sequence databases such as the NCBI Sequence Read Archive (SRA) database and the Gene Expression Omnibus and ArrayExpress, in order to obtain a database accession number useful for tracking data in publications (Shumway *et al.*, 2010). The number of accessions of RNA-seq studies in fungi in SRA database is increasing day by day (**Fig. 4**).

It should be mentioned that results from a large scale transcriptomic approach may be intrinsically prone to errors and attention should be focused during characterization of absolute gene expression levels. Several researchers agree that data from RNA-seq analysis have to be eventually validated using a target approach, such as the quantitative RT-PCR, by considering specific reference genes for normalization of expression, by following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, and by possibly using additional biological replicates (Bustin *et al.*, 2009; Fang and Cui, 2011).

NGS Transcriptomics to Identify Changes During Development and Growth Under Different Environmental Conditions

Experiments performed in fungi by using RNA-seq techniques allowed primarily to expand the knowledge on their basic biology, especially on shifts between the different phases of their life cycle. For instance, the cellular and molecular remodeling of mycelia to form complex reproductive structures such as fruit bodies have been studied in the light of changes in transcriptional landscapes. Several Basidiomycetes producing macroscopic fruit bodies were used as models to elucidating the mechanisms of the development of the reproductive structures. Through a comparative analysis of transcriptional patterns among six Agaricomycetes species, it has been demonstrated that, in fungi, fruit bodies development involves a major reprogramming of gene expression (Krizsán *et al.*, 2019). In *Schizophyllum commune*, for example, approximately 60% of total genes were expressed during specific developmental stage, including fruit body formation (Ohm *et al.*, 2010), while in *Auricularia polytricha*, about 9% of the total transcript catalogue were significantly differentially expressed during the shift from free living mycelium to fruit body

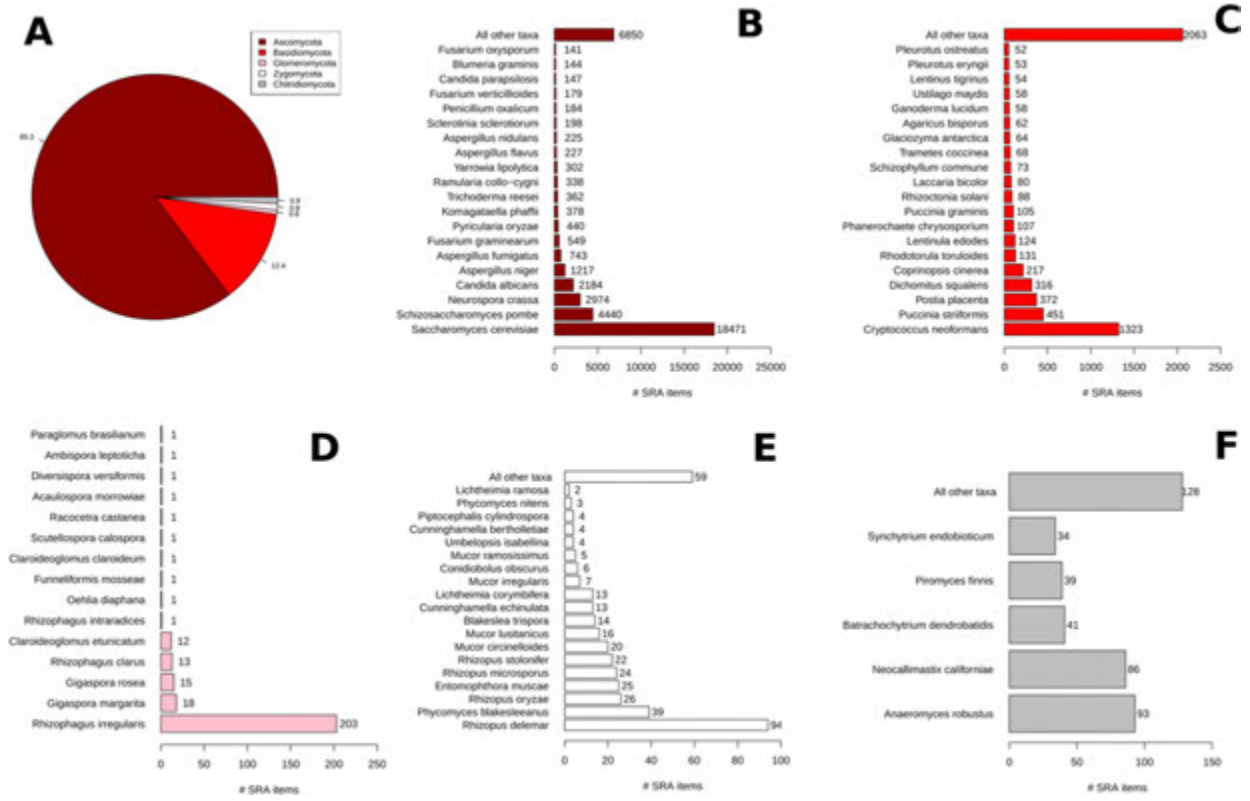

Fig. 4 Outcomes of a search on NCBI SRA database using the words "RNA-seq" and "organism: fungi" (search performed on March 2020). In (A), distribution of the main fungal phyla in the whole dataset of SRA accessions. Percentage (%) for each phylum is reported. In (B), (C), (D), (E) and (F) list of Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, and Chitridiomycota species, respectively, with high number of accessions in NCBI SRA database.

(Zhou *et al.*, 2014). Common families of DEGs detected among Basidiomycetes during fruit body production are represented by genes coding for Carbohydrate Active Enzymes (CAZymes) affecting main components of the fungal cell wall, i.e., chitin and glucans, glycoside hydrolases (GH), hydrophobins, expansin-like proteins, and cerato-platanins (Krizsán *et al.*, 2019). The displacement of genes encoding cell wall related proteins seem to be required during the transition from simple to complex multicellular structures like macroscopic fruit bodies, which implied a dramatic cell wall remodeling. RNA-seq data also provide evidence of the crucial role in sporulation of several conserved key multicellularity-related genes including transcription factors, which are developmentally regulated in fruiting bodies (Pelkmans *et al.*, 2017; Krizsán *et al.*, 2019), and species-specific defense-related genes, suggesting that also chemical defense might be a function involved in a fruiting body production (Krizsán *et al.*, 2019). In *Morchella importuna*, the gene expression profile detected by RNA-seq analysis showed that carbohydrate catabolism and energy metabolism related pathways were finely regulated during fruit body formation, as well as genes encoding for heat shock proteins, thus suggesting a role of environmental temperature in sporulation (Hao *et al.*, 2019). From RNA-seq data, it was also observed that antisense transcripts may play a role in the formation of reproductive structures of *Coprinopsis cinerea* (Muraguchi *et al.*, 2015). A comparative large-scale analysis among three related smut species, i.e., *U. maydis* (common smut of corn), *Ustilago hordei* (covered smut of barley), and *Sporisorium reilianum* (head smut of corn), suggested that antisense RNAs could have a genome-wide influence on gene expression in smut fungi (Donaldson *et al.*, 2017). Antisense expression of genes appeared to have a role in growth and development of *Schizophyllum commune* (Ohm *et al.*, 2010), in *Aspergillus nidulans* (Sibthorp *et al.*, 2013), in the rice blast pathogen *Magnaporthe grisea* (Gowda *et al.*, 2006), and in *Neurospora crassa* (Arthanari *et al.*, 2014).

Sexual development transcriptional profiles were studied by high-throughput transcriptomic approach also in several Ascomycetes. Using *Botrytits cinerea* as a model system, whole transcritpome of apothecia was obtained and results allowed at inferring a possible priming role of gene involved in plant infection during fruit body generation (Rodenburg *et al.*, 2018). Comparative analysis of transcriptomic levels of orthologous gene repertoire between closely related species allowed at elucidating other ecological and evolutionary aspects of fungal development. For example, by coupling morphological characterization with RNA-seq analyses, it was possible to provide useful information on the ecological differences between two plant pathogenic fungi, *Fusarium verticillioides* and *F. graminearum* (Sikhakolli *et al.*, 2012). The comparison of the regulation of orthologous genes in a time-point experiment highlighted the different role of fruiting bodies in the ecology and epidemiology of the two pathogenic species (Sikhakolli *et al.*, 2012). A comparative RNA-seq analysis of several different truffle species (*Tuber* spp.) demonstrated that volatile organic compounds of their edible fruit bodies rely on the differential expression of a plethora of existing genes not strictly specific to *Tuber* species (Murat *et al,*

2018). More recently, a transcriptomic profiling of the edible Ascomycete *Tuber magnatum* Pico (white truffle) has been obtained from fruit bodies collected under different environmental conditions, and a link between RNA-seq data and metabolomic profile has been performed (Vita *et al.*, 2020). Developmental transitions during the life cycle of *Tuber melanosporum*, i.e., free living mycelium, fruit body production and ectomycorrhizae, was also studied by RNA-seq (Tisserant *et al.*, 2011).

During adaptations to diverse environments, fungi seems to finely regulate different fractions of their transcriptomic pattern (Meijueiro *et al.*, 2014). For example, the genes and pathways associated to protein production in different culture media were identified in *A. nidulans* and *A. niger* by a RNA-seq analysis (Pullan *et al.*, 2014; Brown *et al.*, 2016). A RNA-seq study on the entomopathogenic fungus *Beauveria bassiana* suggested that the fungus adapted to different environmental niches by activating well-defined gene sets: for instance, when locust hind wings were used as substrate for growth, proteases were found to be induced, whereas the use of corn root exudates enhanced the expression of carbohydrate hydrolases (Xiao *et al.*, 2012). Colonization of wood by the brown rot agent *Postia placenta*, explored through a time course RNA-seq and coupled with enzymatic assays, allowed at detecting a unique fungal "pretreatment" strategy acting during the first phases of wood degradation, providing insights on a natural efficient conversion of woody plant materials into cellulosic compounds (Zhang *et al.*, 2016). In *Grosmannia clavigera*, RNA-seq data highlighted that the fungus can use products of detoxification of terpenoids as a carbon source, resulting in a transcriptional reprogramming of this species when subjected to environmental stresses (DiGuistini *et al.*, 2011).

NGS Transcriptomics to Decipher the Molecular Mechanism of Interactions With Other Organisms

Transcriptomic approaches based on NGS and aimed at dissecting the molecular mechanisms of interaction between fungi and other organisms has been successfully used in many different research fields. Given that the output of RNA-seq simultaneously comprises reads deriving from transcripts of both partners, and the comparison of these sequences against dedicated databases allow at distinguishing the origins of each transcript inside the RNA pool (Westermann *et al.*, 2012), this method was applied to study with high accuracy the pathways involved in the interaction dual experimental systems.

In the last years, large scale transcriptomics contributed to address important questions on human, animal and plant pathology, as well as on symbiosis between fungi and plants. A remarkable example is represented by a study on the opportunistic pathogenic yeast *Cryptococcus neoformans*, which causes human meningitis and it is responsible for thousands of deaths among immunodepressed people every years (Janbon *et al.*, 2014). By using RNA-seq, it was analyzed and compared the gene expression of two strains of *C. neoformans* collected from the cerebrospinal fluid of infected patients, and pathways that were crucial for the survival of *C. neoformans* in the central nervous system were identified. This allow at elucidating the genetic basis of the fungal disease (Chen *et al.*, 2014). Other important transcriptomic analysis performed on this basidiomycetous pathogenic yeast provided insights on *C. neoformans* response to stress, mating efficiency, and virulence (Janbon *et al.*, 2014; Yu *et al.*, 2020).

Plant disease were also studied from a fungal transcriptomic point of view. Transcriptomic profile of predicted secreted proteins strongly improved the ability to identify putative genes coding for small molecule selectively binding to plant proteins and regulating their biological activity, called effectors. It has been well documented that effectors play a key role during infection processes in compatible/incompatible interactions and in susceptibility/resistance to diseases, as well as in symbiosis (Alfano, 2009). Dual RNA-seq analysis on Norway spruce trees naturally infected by the forest pathogens *Heterobasidion* spp. revealed a specific repertoire of effector-like genes up-regulated during host colonization, in addition to genes encoding carbohydrate- and lignin-degrading enzymes (Kovalchuk *et al.*, 2019). The transcriptomic profile by NGS of the rice blast agent *Magnaporthe oryzae* during host infection allowed at identifying 240 transcripts encoding putative secreted proteins probably involved as effectors (Kawahara *et al.*, 2012). Up-regulation of genes encoding glycosyl hydrolases, cutinases and LysM domain-containing proteins were observed in the blast fungus, while expression of pathogenesis-related and phytoalexin biosynthetic genes was observed in rice (Kawahara *et al.*, 2012). Candidate effectors were discovered through dual RNA-seq in several important plant pathogens, e.g., *Cronartium ribicola* (Liu *et al.*, 2015), *Erysiphe pisi* (Gupta *et al.*, 2020), *Parastagonospora nodorum* (Jones *et al.*, 2019), *Puccinia striiformis* f. sp. *tritici* (Dobon *et al.*, 2016), and the number of new reported putative effector-like genes in different pathosystems is still rising.

Symbiosis between plants and fungi has been deeply studied through transcriptomic analysis. By using 454 pyrosequencing of transcripts isolated from the orchid mycorrhizal fungus *Tulasnella calospora* and its plant host *Serapias vomeracea*, an inventory of plant and fungal genes expressed in mycorrhizal protocorms was generated and the symbiotic process was characterized from both host and fungal sides (Balestrini *et al.*, 2014). The candidate effectors involved in the mutualistic symbiosis between the ectomycorrhizal fungus *Laccaria bicolor* and its hosts were also identified thanks to a large scale transcriptomic approach, and numerous small secreted proteins (SSPs) putatively involved in the establishment of the symbiosis were characterized (Martin *et al.*, 2008; Plett *et al.*, 2015). RNA-Seq data from fully formed ectomycorrhiza between *L. bicolor* and poplar roots were also used to predict the ectomycorrhizal metabolome (Larsen *et al.*, 2011). Large scale transcriptomics have been performed on other important ectomycorrhizal fungi, including *T. melanosporum* and *Paxillus involutus* (Kohler and Tisserant, 2014). Arbuscular mycorrhizal (AM) fungi has been also characterized through deep transcriptome sequencing. A RNA-Seq approach on the AM fungus *Rhizophagus irregularis* was performed, allowing at exploring the gene expression profile upon symbiosis with the plant *Medicago truncatula* (Tisserant *et al.*, 2013) . About 4,7% of its genes were induced in colonized roots, and most of them were characterized as involved in signal transduction, energy production and conversion, secondary metabolism, transport and metabolism (Tisserant *et al.*, 2013). As for ectomycorrhizal fungi (Martin *et al.*, 2008), several genes coding for SSPs were found to be highly expressed during AM symbiosis interaction (Tisserant *et al.*, 2013).

Perspectives of NGS Transcriptomics in Fungi

Thanks of the exponential availability of specific protocols and reagents, in the last years NGS methods have been coupled to other novel molecular approaches in order to improve the resolution of analyses. A recent tool which has been proven to be useful when coupled to RNA-seq is laser microdissection (LM) technology. Laser microdissection is a powerful technology that allows the rapid isolation of selected cell populations from a section of heterogeneous tissues in a way conducive to the extraction of several cellular compounds, including nucleic acids, proteins and metabolites (Balestrini *et al.*, 2009). LM in fact combines the use of the microscope and the application of a manual guided (PC assisted) laser to separate different cytological components from specimen sections on a microscope slide. This technology was used in studies on mycorrhizal fungal symbioses in order to isolate cortical cells from roots of several plant species colonized by arbuscular mycorrhizal fungi in combination with gene expression target approaches (Balestrini and Fiorilli, 2020 for a review) and large scale transcriptomics (Gaude *et al.*, 2012) or from truffle/hazelnut ectomycorrhizae (Hacquard *et al.*, 2013), and in studies on plant pathogenic fungi in order to dissect the tissue-specific responses of plants to the infection (Hacquard *et al.*, 2010; Chandran *et al.*, 2010). Recently, one interesting study performed on the Ascomycete *Sordaria macrospora* has revealed the high potential of this tool for elucidating the various transcriptomic patterns of different fungal tissues (Teichert *et al.*, 2012). By selectively isolating RNA from protoperithecia and free living mycelia of *S. macrospora* through a LM approach, and by analyzing it with RNA-seq, significant difference in gene expression between the two components were detected (Teichert *et al.*, 2012).

Despite LM is useful for tissue-specific analysis, and tailored RNA isolation protocols are available for extraction of nucleic acid from chemically-treated samples like paraffine-embedded specimens (Takahashi *et al.*, 2010), a high number of cells are needed as starting biological material. In some cases, e.g., to investigate on differential expression of a spore population with different or similar genetic background, the RNA sequencing of a single cell [Single-cell RNA-seq (scRNA-seq; Saliba *et al.*, 2014)] is required. Several methods to isolate single cell from tissues and cell populations, i.e., multiparametric flow cytometry and sorting based on a fluorescence gating strategy, micromanipulation with a glass pipette, optical tweezers laser-based, were successfully used in several studies (Saliba *et al.*, 2014). In the last years, thanks to SMRT provided by third generation sequencing technology, scRNA-seq has started to be an established method for uncovering the individual transcriptomic complexity within populations of yeasts (Gasch *et al.*, 2017; Nadal-Ribelles *et al.*, 2019; Saint *et al.*, 2019). Results were promising and it is likely that this approach will be applied in future for other microorganisms, including filamentous fungi.

An emerging and promising field taking advantage by the rapid development of NGS tools and aimed at expanding the knowledge on the identification of multipartite metabolic interactions occurring between two or more organisms is represented by metatranscriptomics. Metabarcoding studies on DNA from environmental samples demonstrated without doubt to be very useful to study fungal community in the field. However, metabarcoding surveys may be biased by PCR amplification and not all species present in the samples have the same chance to be detected (Tedersoo *et al.*, 2015). Moreover, both dead and living organisms are detected by DNA sequencing, potentially masking the real structure of the living populations in some particular samples, e.g., in stool or soil samples, where contaminant DNA cannot be distinguished from DNA of the living microbial community (Marcelino *et al.*, 2019). The sequencing by RNA-seq of transcripts, representing the whole activity of genes of all organisms living in the samples, may circumvent this issue, helping researchers in ecological studies of complex fungal community (Kuske *et al.*, 2015; Marcelino *et al.*, 2019). In addition, as for dual RNA-seq, metatranscriptomics can allow to decipher the interactions among fungi and other organisms. Metatranscriptomics of some mycorrhizal communities have been recently performed, allowing a deeper understanding of the functional roles of these fungi in nature (Gonzalez *et al.*, 2018; Liao *et al.*, 2014). However, as for transcriptomics of a single organism, the interpretation of RNA-seq results from metatranscriptomics is still challenging, because of differences in transcript turnover rates and of limitations of using homology-based gene assignments during function prediction (Kuske *et al.*, 2015). The increased availability of annotated transcriptomes of fungal species in validated databases will aid to fill the missing information needed by future metatranscriptomics studies (Kuske *et al.*, 2015).

Conclusions

In the last years, thanks to NGS strategies, fungal transcriptomics has improved the understanding of how genomes are expressed during growth, development and under environmental stresses, what genes were involved in pathogenic and symbiotic interactions, and what is the role of different RNA species, such as non-coding RNA and antisense transcripts, in fungal life cycles. The growing availability of large-scale transcriptomics data allowed comparative analyses aimed at addressing important questions on functional, ecological and evolutionary aspects of fungi. Both small scale laboratories and big consortia have access to these NGS data, and thanks to the reduction of costs of NGS technology, they will be able to perform new transcriptomics studies in order to improve the databases and to strength the network between transcriptomics and other *omics* approaches. The new challenge for high throughput NGS transcriptomics, in fact, will be the growing request of computational resources for analyzing and storing big data coming from RNA-seq. Future improvement of sequencing technologies and bioinformatic tools will be pivotal to keep this positive trend, along with the proportional enhancement of bioinformatic facilities and sequence databases.

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The Cell Wall of Medically Relevant Yeasts and Molds

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Introduction

Thus far, an estimated 1.5 million fungal species have been classified, and 300 out of there affect human beings (Fisher *et al.*, 2020). Fungal infections are acquiring relevance in the last years because most of them affect individuals already with serious underlying illnesses and are challenging to treat. The main risk factors for developing a fungal infection include anti-cancer, immunosuppressive and antibiotic therapies, solid organ and hematopoietic stem cell transplantations, HIV infection, diabetes mellitus, and the use of intravenous lines (Lanternier *et al.*, 2013). Moreover, the socio-economic and geo-ecological characteristics of a population can increase fungal disease risk factors, incidence, and prevalence (Bongomin *et al.*, 2017).

The fungal cell wall (CW) is a key player in the pathogenic process, being important in the fungal adhesion to the host, and contains pathogen-associated molecular patterns that can interact with the pattern recognition receptors of the host immune cells. The fungal CW is located above the plasma membrane, acts as a permeability barrier, contributes to cell shape and protection from osmotic and mechanic stresses, and is essential for cell integrity and viability (Díaz-Jiménez *et al.*, 2012). The fungal cell growth can be radial or polar, and this defines the final cell morphology of yeast and filamentous fungi, respectively. Molds grow by polarized and apical extension, forming filamentous and vegetative cells known as hyphae, whose continued growth forms the hyphal network name as mycelium. Thus, the hyphae's biological traits, including the CW structure and organization are closer to that found in molds.

The *Candida albicans* Cell Wall

Candida albicans is a dimorphic and opportunistic organism, and one of the leading etiological agents of nosocomial fungal infections (Brown *et al.*, 2012). The *C. albicans* CW is likely the most thoroughly studied fungal structure from medically relevant fungal species and will be used here as an example of CW composition, organization, and synthesis (**Fig. 1**). Its wall is a layered structure, with a homogeneous inner layer of about 100 nm and the outermost layer of about 180 nm and composed of proteins (Klis *et al.*, 2001). The main components of the CW are carbohydrates, contributing to 80%–90% of the CW dry weight (Díaz-Jiménez *et al.*, 2012; Mora-Montes *et al.*, 2009). These saccharides are chitin, β-1,3- and β-1,6-glucans, and mannose oligosaccharides (mannan) covalently associated with proteins (Mora-Montes *et al.*, 2009). Proteins and lipids contribute to about 6%–25% and 1%–7% of the CW dry weight (Chaffin *et al.*, 1998) (see **Fig. 1**).

Chitin and β-glucans are the main components of the wall inner layer, are covered by the outer layer components, except in the budding scars and the hypha primary septum (Perez-Garcia *et al.*, 2011), and are the wall skeleton that provides physical strength (Chaffin *et al.*, 1998). Chitin is a linear polymer and is arranged in an antiparallel fashion, associated with each other by hydrogen

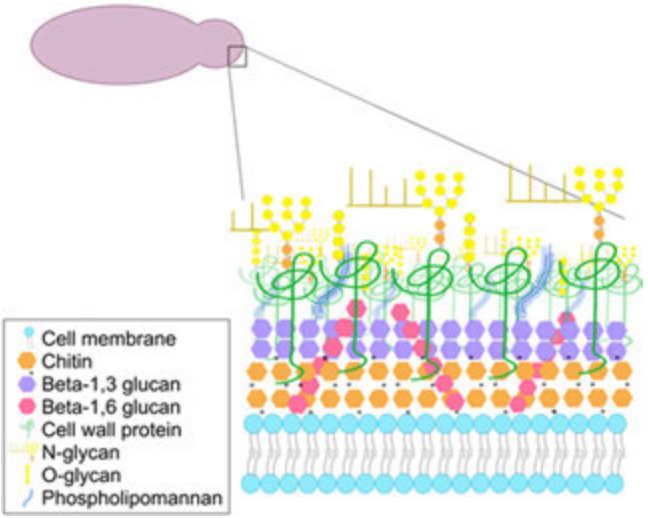

Fig. 1 Representation of *C. albicans* cell wall. The inner layer of the cell wall (close to the cell membrane) is composed of antiparallel chitin chains, followed by β-1,3- and β-1,6-glucans. The outermost cell wall layer is composed of a thick mannoproteins layer that contains *N*-linked and *O*-linked mannans, and phospholipomannans.

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bonds (Shepherd, 1987); while β-1,3-glucans are aligned and kept together by hydrogen bonds (Klis *et al.*, 2001). The β-1,6-glucans can be of variable lengths and distribution and act as molecular linkers between wall proteins and β-1,3-glucans and chitin (Klis *et al.*, 2001; Garcia-Rubio *et al.*, 2020). The outermost layer is composed of mannoproteins, which account for about 20%–30% of the total CW weight (Mora-Montes *et al.*, 2009), and may contain glycosylphosphatidylinositol (GPI) anchors that cross-link them to β-1,6-glucans (Garcia-Rubio *et al.*, 2020). They also contain *O*-linked and *N*-linked oligosaccharides attached to serine/threonine or asparagine residues, respectively (**Fig. 1**). The phospholipomannan is currently the main glycolipid characterized in this CW and contains β-1,2-oligomannosides linked to phytoceramide associated with phytosphingosine and hydroxy fatty acids (Trinel *et al.*, 2002).

Chitin Synthesis

Chitin is the second most abundant organic compound on earth, after cellulose, and is synthesized by a wide variety of organisms from different taxonomic groups, including pathogenic fungi (Merzendorfer, 2011). This is a homopolymer of N-acetylglucosamine (GlcNAc) that is linked by β-1,4- glycosidic bonds and folds on itself to form antiparallel chains of about twenty units. This arrangement allows compaction in strong microfibrils, more resistant than any other molecule in nature (Lenardon *et al.*, 2010).

A chitin proportion found in pathogenic fungi is deacetylated to chitosan by one or more chitin deacetylases (Klis *et al.*, 2006). In *C. albicans*, 5% of the chitin is deacetylated to chitosan, while in *Cryptococcus neoformans* more than 60% chitin is deacetylated (Baker *et al.*, 2007). Chitin synthesis is highly conserved in fungi and the process involves a defined number of enzymatic reactions that convert different sugars into a GlcNAc polymer. The sugar main source is glucose or its storage compounds -glycogen or trehalose (François and Parrou, 2001). The synthesis route is divided into three main reactions, the first leads to GlcNAc formation (**Fig. 2**), the second one follows a variant of the Leloir route that gives rise to uridine diphosphate (UDP)-GlcNAc (**Fig. 2**), and the last reaction involves chitin polymerization using UDP-GlcNAc as the activated sugar donor (**Fig. 2**). The first two reactions occur in the cytoplasm; while the third step takes place in specialized microdomains of the plasma membrane (Merzendorfer, 2011).

The presence of glutamine-fructose-6-phosphate amidotransferase, UDP-GlcNAc, and chitin synthase (CHS) are determinants for chitin synthesis. The CHS activity is specifically associated with chitin biosynthesis, and is divided into seven classes (I-VII); although the functional importance and presence of all these classes seem to be species-specific (Niño-Vega *et al.*, 2004) (**Table 1**). Class I and II enzymes produce only a small chitin fraction but are essential for the primary septum formation (Munro *et al.*, 2001). Class IV, V, and VII enzymes often produce considerable chitin amounts and share sequence homology; whereas class V and some class VII enzymes contain myosin-like domains (Niño-Vega *et al.*, 2004). Class III, V, VI, and VII enzymes have only been identified in filamentous fungi and are absent in *C. albicans* (Lenardon *et al.*, 2010) (**Table 1**). The multiplicity of CHS enzymes in various organisms suggests that they may have redundant functions in chitin synthesis.

The CHS are membrane proteins and the catalytic domain, which faces the cytoplasm, contains conserved saccharide- and nucleotide-binding sites (Merzendorfer, 2011). For this reason, nascent chitin chains must translocate through the plasma membrane before they assemble into microfibrils and are deposited on the cell surface. The chitin translocation process is thought to be a CHS intrinsic property that involves transmembrane segments of the C-terminal region (Lenardon *et al.*, 2010; Merzendorfer, 2011).

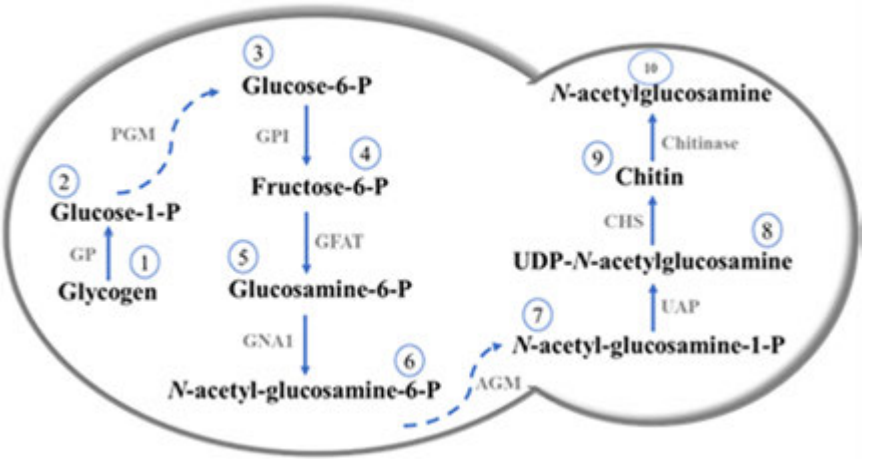

Fig. 2 Simplified chitin synthesis pathway in fungi, starting from glycogen. The acronyms in the figure refer to Glycogen phosphorylase (GP), phosphoglucomutase (PGM), glucose-6-phosphate isomerase (GPI), glutamine-fructose-6-phosphate amidotransferase (GFAT), glucosamine-6-phosphate-*N*-acetyltransferase (GNA1), phosphoacetylglucosamine mutase (AGM), UDP-*N*- GlcNAc pyrophosphorylase (UAP), chitin synthase (CHS) and chitinase.

Table 1 Classification of chitin synthases in fungi of medical interest

	CHS classes							
Organism	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*Function*
C. albicans	Chs2 Chs8	Chs1	–	Chs3	–	–	–	*Chs1*: primary septum synthesis *Chs2 and Chs8*: protection of the nascent cell wall during polarized growth *Chs3*: synthesizes the majority of chitin found in the cell wall as well as the chitin ring at division sites (Lenardon *et al.*, 2010)
A. fumigatus	ChsA	ChsB	ChsC ChsG	ChsF	CsmA	ChsD	CsmB	*ChsA and ChsC*: cell wall chitin compensation *ChsB*: function at polarized growth sites and forming septa during hyphal growth and conidia development *CsmA and CsmB*: these enzymes appear to localize themselves to sites of polarized cell wall expansion in an actin-dependent manner *ChsD, ChsG, ChsF*: unknown functions (Lenardon *et al.*, 2010)

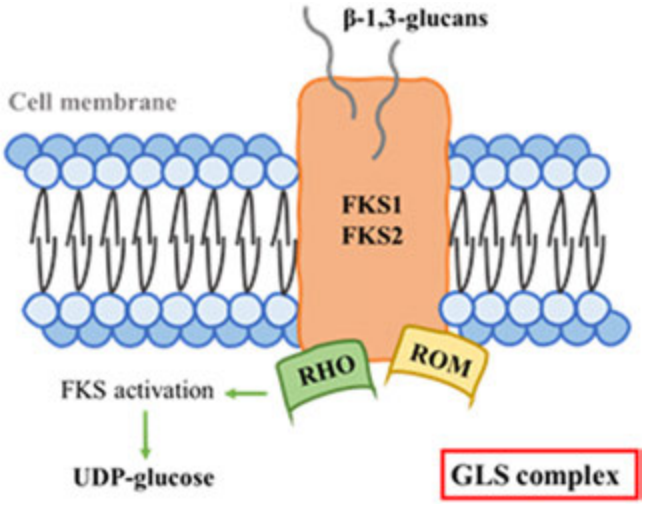

Fig. 3 Schematic representation of the GLS complex, which is involved in the β-1,3-glucan synthesis. The FKS subunit catalyzes the elongation of growing β-glucan chains by the addition of UDP-glucose monomers. RHO is a GTP-dephosphorylase protein that regulates Fks1 and Fks2 by GDP. ROM is a cell wall-associated GDP-GTP exchange protein responsible for the GTP regeneration.

β-Glucans Synthesis

Most of the fungal β-glucans are made up of β-1,3-linked glucose units with varying amounts of β-1,6-linked branches. The β-1,3-glucans are the most important and abundant polysaccharides in the fungal CW, reaching around 65%–90% of total glucan content (Bowman and Free, 2006).

The initial step in the β-1,3-glucan synthesis is the cytoplasmic generation of the precursor UDP-glucose (UDP-Glc), used as a sugar donor by glucan synthases. In fungi, the GLS membrane protein complex (UDP-Glc:1,3-β-D-glucan 3-β-D-glucosyltransferase), is in charge of catalyzing the reaction of β-1,3-glucan synthesis, using UDP-Glc as a substrate (Latgé, 2007). The elongation continues with the addition of UDP-Glc monomers at the non-reducing end of the growing glucan chain, and each new polymer is transported from the plasma membrane to the CW, through a channel of transmembrane domains. The GLS complex is composed of two subunits, a catalytic FKS unit and a regulatory RHO unit with guanosine triphosphatase activity (Beauvais *et al.*, 2001) (**Fig. 3**). The gene encoding for Fks1 of the FKS subunit is expressed in vegetative growth under optimal conditions; while *FKS2* is induced by environmental stress or during sporulation. Various evidence indicates that Fks1 and Fks2 are the β-1,3-glucan synthase catalytic subunits (Dijkgraaf *et al.*, 2002; Nogami and Ohya, 2009).

It is known that RHO is a regulatory G protein, which fulfills the function of activating FKS by GTP dephosphorylation. A Rho1 post-translational modification is necessary for β-1,3-glucan synthesis, the C-terminal end of RHO-type GTPase is modified with a geranylgeraline group by the type I geranylgeranyltransferase enzyme, this modification is required for binding and activation of Fks1 (Ohya *et al.*, 1993; Nogami and Ohya, 2009). The ROM protein is also involved in this process, since allows GDP-GTP exchange in the wall and modulates RHO activity (Teparić and Mrsa, 2013). The cytosolic domains of FKS are responsible for

Table 2 Genes involved in the synthesis of β-1,3- and β-1,6-glucans in medically relevant fungi

S. cerevisiae gene[a]	*C. albicans*	*A. fumigatus*	*Function*
KRE5	*KRE5*	AFUA_2602360	UDP-glucose: glycoprotein glucosyltransferase, 1,6-β-D-glucan biosynthesis
KRE6	*KRE6*	BA78_1578	Essential β-1,6-glucan synthase subunit
SKN1	*SKN1*	BA78_1578	Protein with a role in β-1,6-glucan synthesis; probable *N*-glycosylated type II membrane protein
KRE9	*KRE9*	CDV57_05718	Protein of β-1,6-glucan biosynthesis; required for hyphal growth.
KNH1	*KNH1*	CDV57_05718	Protein involved in cell wall β-1,6-glucan synthesis, similar to Kre9
KRE1	*KRE1*	Not found	Cell wall glycoprotein; β-glucan synthesis
RHO1	*RHO1*	RHO1	Small GTPase of Rho family; regulates β-1,3-glucan synthesis activity
FKS1	*MEQ_00226*	FKS1	Essential β-1,3-glucan synthase subunit
FKS2	*GSL2*	FKSP	Protein similar to β-1,3-glucan synthase
ROM2	*ROM2*	ROM2	Putative GDP/GTP exchange factor

[a]*Notes*: The synthesis of β-glucans has been extensively studied in the fungus *Saccharomyces cerevisiae* and the genes involved in this synthetic pathway are almost completely characterized. Therefore, using a blast analysis, taking as reference the *S. cerevisiae* genes, a search was carried out to determine the presence of these genes in the selected organisms. Gene nomenclature corresponds to accession codes of the GeneBank database (https://www.ncbi.nlm.nih.gov/genbank/).

catalysis, and therefore β-glucans synthesis takes place in the cytosol. Subsequently, the biomolecules go to the periplasmic space, where they are incorporated into the CW (Reverberi *et al.*, 2004; Bowman and Free, 2006). Other proteins, such as Pma1 have been found near the GLS complex, and this particular protein maintains an acidic environment in the CW closer to the membrane, which is important for glucan synthesis (Schimoler-O'Rourke *et al.*, 2003) (see **Table 2**).

The β-1,6-glucan synthesis is likely to occur on the cell surface, although the mechanism behind is not well dissected yet. The first step in its synthesis may involve an endoplasmic reticulum (ER) hypothetical protein acceptor, followed by the synthesis of polysaccharide chains and branching in later stages of the secretory pathway on the cell surface. However, the activity of the identified gene products remains unknown, so it is not clear how and to what extent they are involved in the synthesis of this polymer (Nogami and Ohya, 2009). Thus far, a cell surface β-1,6-glucan synthase has not been found, but several proteins of the Golgi apparatus and ER seem to be involved in this synthetic pathway in an unknown way (Shahinian and Bussey, 2000). The *KRE5*, *CWH41*/*GLS1*, *ROT2*/*GLS2*, and *CNE1* encode ER proteins that participate in the β-1,6-glucan synthesis (Shahinian *et al.*, 1998). In the case of Kre5, this has a similarity to UDP-glucose: glycoprotein glucosyltransferase and has been suggested that may play a role that indirectly contributes to glucan synthesis (Shahinian and Bussey, 2000) (see **Table 2**).

Glycoproteins Synthesis

The carbohydrate part of glycoproteins is known as glycans and are linked to the polypeptide backbone in a process known as protein glycosylation pathway. This posttranslational modification plays an important role in protein structure and function, affecting solubility, folding, stability, and intracellular trafficking (Helenius and Aebi, 2001). Some glycoproteins are covalently attached to the CW matrix and are known as "integral CW proteins", which often are produced as GPI-anchored proteins; while the "non-integral" proteins are weakly associated with the wall (Castillo *et al.*, 2008).

According to the glycan-binding site on the protein, there are two types of glycans, the *N*-linked and *O*-linked glycans. The *O*-linked glycans are short and simple oligosaccharides attached to the -OH group of serine or threonine side chain by an acyl bond, whereas the *N*-linked glycans are high-weight and highly branched oligosaccharides attached to asparagine residues within the sequon Asn-X-Ser/Thr (Mora-Montes *et al.*, 2009). The biosynthesis of both *N*-linked and *O*-linked glycans is a multi-step process that takes place in the ER and Golgi complex and involves glycosyltransferases, glycosidases, and other carbohydrate modifying enzymes (Mora-Montes *et al.*, 2009; Martinez-Duncker *et al.*, 2014).

The *N*-linked glycan synthesis begins with the assembly of a dolichol-linked oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, in the ER and its subsequent transfer in a co-translational mechanism by an oligosaccharyltransferase complex to the amide side chain of the asparagine of a nascent polypeptide (Martinez-Duncker *et al.*, 2014). Then, this precursor is processed to $Glc_1Man_9GlcNAc_2$, which is key for protein folding in the glycoprotein quality control mediated by the calnexin/calreticulin chaperones (Helenius and Aebi, 2001). Once the polypeptide is properly folded, the oligosaccharide is further processed to $Man_8GlcNAc_2$, which is directed to the Golgi complex for additional processing by mannosyltransferases, generating high mannose content *N*-linked glycans (Martinez-Duncker *et al.*, 2014). In *C. albicans*, the synthesis of an outer chain, and attachment to $Man_8GlcNAc_2$ takes place in the Golgi complex. This process begins with the α-1,6-mannosyltransferase Och1, which is responsible for adding one α-1,6-mannose unit to $Man_8GlcNAc_2$ (Martinez-Duncker *et al.*, 2014). Then, a chain containing 50 or more α-1,6-mannose residues is built on the initial mannose added by Och1, generating the outer chain backbone (Bates *et al.*, 2006). The enzymes involved in the backbone elongation are Van1, Anp1, Mnn9, Mnn10, and Mnn11 (Martinez-Duncker *et al.*, 2014). Then, lateral oligosaccharides containing α-1,2-mannose units branched the backbone and may be decorated with α-1,3-mannose or β-1,2-mannose units (Mora-Montes *et al.*, 2009). The enzymes required for the synthesis of this outer chain are listed in **Table 3**. In *C. neoformans*, the *N*-linked glycans contain xylomannan, having a long nucleus of α-1,6-mannoses with side chains consisting of α-1,2-mannoses ending in a xylose residue (Park *et al.*, 2013). In *Aspergillus fumigatus*, the outer chain has a different structure,

Table 3 Enzymes involved in the biosynthesis of fungal *N*-linked and *O*-linked glycans

Enzyme	*C. albicans*	*A. fumigatus*	*Function*
Och1	✓	✓	α-1,6-Mannosyltransferase; initiates *N*-glycan outer chain branch addition
Van1	✓	✓	Member of Mnn9 family of mannosyltransferases
Anp1	✓	✓	Putative Golgi-resident mannosyltransferase; member of Mnn9p family
Mnn9	✓	✓	Mannosyltransferase involved in the *N*-linked outer-chain mannan biosynthesis
Mnn10	✓	✓	α-1,6-Mannosyltransferase involved in biosynthesis and organization of cell wall polysaccharides
Mnn11	✓	✓	Role in protein *N*-linked glycosylation, protein glycosylation, and α-1,6-mannosyltransferase complex localization
Mnn2	✓	✓	α-1,2-Mannosyltransferase, required for normal cell wall mannan content
Mnn6	✓	✓	Role in protein glycosylation and Golgi apparatus localization
Mnn1	✓	✓	Putative α-1,3-mannosyltransferase; of the mannosyltransferase complex
Pmt1-Pmt6	✓	✓	Protein mannosyltransferase, member of the PMT family which includes Pmt1p, Pmt2p, Pmt4p, Pmt5p, and Pmt6p
Mnt1/Ktr1	✓	✓	α-1,2-Mannosyl transferase; predicted type II Golgi membrane protein
Mnt2	✓	✓	α-1,2-Mannosyl transferase, partially redundant with Mnt1
Mnt3	✓	✓	Mannosyltransferase

Notes: The presence or absence of the enzymes involved in the synthesis of *N*-linked and *O*-linked glycans in *Candida albicans* and *Aspergillus fumigatus* was determined. The presence of these enzymes was investigated in their respective genome databases (http://www.candidagenome.org/, http://www.aspgd.org/).

instead of a linear nucleus of α-1,6-mannose units there is a repeated tetrameric mannan composed of α-1,2-mannose-α-1,6-mannose-α-1,2-mannose-α-1,2-mannose with β-1,5-galactofuranose side chains (Henry *et al.*, 2016; Engel *et al.*, 2012) (see **Table 3**).

The *O*-linked glycosylation pathway also begins in the ER, where the Pmt1-Pmt6, a family of protein *O*-mannosyltransferases (PMT) takes a mannose unit from dolichol-phosphate-mannose and transfer it to an acceptor protein (Martinez-Duncker *et al.*, 2014; Mora-Montes *et al.*, 2009). Then, additional mannoses are added to the oligosaccharide by Golgi resident α-1,2-mannosyltransferases, generating a short chain of α-1,2-mannose residues that may contain up to seven mannose units (see **Table 3**) (Diaz-Jimenez *et al.*, 2012). *A. fumigatus* has *O*-linked mannogalactans, and there is evidence that the mannose chain has α-1,6- rather than α-1,2- bonds, although this fungus has Mnt1, the enzyme responsible for the production of α-1,2-mannan (Leitão *et al.*, 2003). In *C. albicans* and *C. neoformans*, Pmt2 is essential for cell viability (Willger *et al.*, 2009).

The *Candida albicans* Cell Wall Proteome

There are quantitative and qualitative differences in the *C. albicans* CW protein composition in both yeast and hyphae. The different proteins can be attached to the wall by a GPI anchor (Klis *et al.*, 2001; Satala *et al.*, 2020), these account for about 88% of all covalently linked proteins, and aspartyl proteinases, chitinases, glucanases, phospholipases, adhesins, and proteins for β-1,6-glucan biosynthesis are among the best GPI-containing wall proteins (Richard and Plaine, 2007). Moreover, it has been predicted that other 115 proteins with unknown functions are attached to the wall *via* a GPI anchor (Richard and Plaine, 2007).

Another type of wall proteins is released by extraction from intact cells with reducing agents, suggesting they are linked through disulfide bridges to other wall proteins (Klis *et al.*, 2001). Among these proteins, Eng1 (endo-β-1,3-glucanase), Gca1, Bgl2 (β-1,3-glucosyltransferase), Pdi, Pir1, MP65 (glucan metabolism), Sim1 (cell wall maintenance), Tos1, Pra1, and Iff2/Hyr3 have been identified (Caminero *et al.*, 2014). The last type of wall proteins are those linked directly to β-1,3-glucan through an alkali-sensitive linkage (without an interconnecting β-1,6-glucan moiety), the proteins have internal repeats and have been designated as Pir (Klis *et al.*, 2001).

The proteins mentioned above contain a signal sequence at the N-terminus that allows transportation to the cell surface, *via* the conventional secretory pathway, but other proteins lacking that signal and non-covalently associated with the wall are also found (Satala *et al.*, 2020). In *C. albicans*, the presence of moonlight proteins in the CW has been described, and include Cdc25 (CDC25 cell division cycle), Aco1 (aconitate hydratase), Adh1 (alcohol dehydrogenase), Fba1 (fructose bisphosphate), Gap (glyceraldehyde-3-phosphate dehydrogenase), Icl1 (isocitrate lyase), Pdc11 (pyruvate decarboxylase), Act1 (actin), various Rpl (ribosomal proteins), Hsp90 and Hsp70 (heat shock protein), Tef1 (translation elongation factor eEF1 α-A chain) Atp1 (ATP synthase alpha subunit) Cit1 (citrate synthase), among others (Satala *et al.*, 2020). Some of these proteins have been described to have adhesion properties but others an unknown function in the CW (Satala *et al.*, 2020; Castillo *et al.*, 2008).

The Cell Wall Composition in Other *Candida* Species

Although the structure and composition of the *C. albicans* CW are well known, this is not the case for other *Candida* species, such as *Candida guilliermondii*, *Candida tropicalis*, *Candida krusei*, *Candida auris*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida glabrata*, *Candida lusitaniae*, and *Candida orthopsilosis*, mainly because it was thought that the *C. albicans* wall model could be applied to all these species (Navarro-Arias *et al.*, 2019).

Our group has determined the basic cell wall composition of *C. tropicalis*, *C. krusei*, *C. guilliermondii*, and *C. auris* (Navarro-Arias *et al.*, 2019). The walls from the analyzed species had differences in the levels of chitin, glucan, mannan, and wall proteins, when compared to *C. albicans*: (1) *C. auris* has higher chitin levels; (2) *C. krusei* contains higher chitin levels and low mannan, glucans, and wall proteins contents; (3) *C. guilliermondii* possesses higher mannan levels; (4) and *C. tropicalis* and *C. albicans* have similar amounts of all the mentioned components. When the content of cell wall *O*-linked and *N*-linked mannans were also analyzed, it was observed that *C. guilliermondii* had increased levels of both mannans, while *C. krusei* showed lower levels when compared to *C. albicans* (Navarro-Arias *et al.*, 2019). Two parameters associated with the mannan length, named phosphomannan content and wall porosity, were also evaluated: *C. albicans* and *C. auris* had similar phosphomannan levels; *C. tropicalis* and *C. guilliermondii* had a higher phosphomannan content, and *C. krusei* had a lower phosphomannan abundance (Navarro-Arias *et al.*, 2019). The CW porosity was similar for *C. tropicalis*, *C. guilliermondii,* and *C. krusei*, but it was lower in *C. albicans* and *C. auris* (Navarro-Arias *et al.*, 2019). Additionally, the experimental evidence suggested that both β-1,3-glucan and chitin are underneath the external mannan layer in all the analyzed species (Navarro-Arias *et al.*, 2019).

Another study also reported the CW composition of *C. tropicalis*, *C. glabrata*, *C. parapsilosis,* and *C. guilliermondii* (Walker and Munro, 2020). They found that all of the species have similar glucan levels, except for *C. tropicalis* that had reduced glucan content, and *C. glabrata*, which had a higher content when compared to *C. albicans* (Walker and Munro, 2020). Mannan content was reduced in *C. glabrata*, *C. parapsilosis*, and *C. guilliermondii*; while chitin was in higher amounts in *C. tropicalis* but in lower levels in *C. glabrata* and *C. guilliermondii* (Walker and Munro, 2020).

The CW mannan structure from different *Candida* species has also been reported. In *C. krusei*, it was found that *N*-linked and *O*-linked mannans have a similar composition than that reported for *C. albicans* (Kuraoka *et al.*, 2019). *C. tropicalis* and *C. albicans* were found to share certain mannan moieties; while *C. glabrata* mannans contain small branches with low α-mannan content and one or two α-1,2-linked mannose residues (Nguyen *et al.*, 2018). In *C. parapsilosis*, the *N*-linked mannans are shorter and less complex than in *C. albicans*; and the *O*-linked mannans play a more important role during the interaction with the host, representing about half of the total wall mannan content (Perez-Garcia *et al.*, 2016).

The *Aspergillus* Cell Wall

Aspergillus spp. encompass several environmental filamentous fungi considered as opportunistic, being *A. fumigatus* the main etiological agent of invasive aspergillosis, a disease linked with high mortality rates in immunocompromised patients (Garcia-Rubio *et al.*, 2020). They possess two different morphotypes, conidia, and hyphae, and although the same polysaccharides are found in both, the CW organization is different (Beauvais *et al.*, 2014). In general, the wall can be divided into an alkali-soluble fraction, which represents the wall outer layer, and an alkali-insoluble fraction, which is the inner layer. In the alkali-soluble fraction, linear α-1,3-glucans (92%), galactosaminogalactan (GAG) (7%), and galactomannan (3%) are found, while the insoluble fraction, which is thought to provide rigidity, is composed of galactomannan (8%), chitin (22%), chitosan (7%), β-1,3-glucans (51%) that can extend up to 1500 residues long, and a linear β-1,3/1,4-glucan (6%), component with an unknown role that has not been described in any other fungal species (Garcia-Rubio *et al.*, 2020). The wall core is the β-1,3-glucan polymer, highly branched with β-1,6-linkages, to which chitin, chitosan, galactomannan, and β-1,3/1,4-glucan are covalently linked (Beauvais *et al.*, 2014).

The β-1,3-glucan synthesis is essential for *A. fumigatus* viability, while defects in the chitin or galactomannan synthesis generate only sick cells with virulence attenuation (Bernard and Latgé, 2001; Schmalhorst *et al.*, 2008).

In *A. fumigatus*, two types of wall-associated proteins can be found: (1) not glycosylated or glycosylated proteins without galactofuranose residues, which have been found also in the culture medium; (2) and *N*-linked and *O*-linked glycosylated GPI proteins containing galactofuranose, such as Gel2 and Ecm33, which are thought to be indispensable for the wall synthesis and vital for fungal growth (Jin, 2012). Gel2 belongs to a family of β-1,3-glucanosyltransferases, and its absence leads to slower growth, abnormal conidiogenesis, altered wall composition, and reduced virulence (Jin, 2012); while Ecm33 has an unknown function but participates in maintaining the correct wall architecture, and its disruption results in conidial separation defects, chitin accumulation, rapid conidia germination, and increased virulence (Jin, 2012; Romano *et al.*, 2006). No proteins covalently bound to the *A. fumigatus* wall polysaccharides have been found, however, a proteomic analysis of wall-associated proteins described that an acid phosphatase is the major GPI-anchored protein strongly associated with β-1,3-glucan, although the presence of a covalent link was not established (Bernard *et al.*, 2002).

The conidial CW is a two-layered structure that can be observed as a dense pigmented outer layer and as a translucent inner layer (Bernard and Latgé, 2001). It is known that this morphotype is covered by a superficial rodlet layer composed of the hydrophobic proteins RodA and RodB, known as hydrophobins (Beauvais *et al.*, 2014; Bernard and Latgé, 2001), organized in an amyloid configuration making the wall waterproof (Latge *et al.*, 2017). There is a dihydroxy naphthalene (DHN) melanin layer underneath the hydrophobins, important for the CW structuration and stiffness (Latge *et al.*, 2017). These structures are required for conidia survival and dispersion into the air, and both overlap with the α-1,3-glucans (Latge *et al.*, 2017).

The rodlet layer also confers immunological inertness to the conidia by functioning as a masking mechanism to avoid the recognition of β-1,3-glucans by dectin-1 (Beauvais *et al.*, 2014), and RodA was reported as the only essential protein for rodlet structure formation (Latge *et al.*, 2017). When RodA is missing from the surface, these morphotype is easily recognized by immune cells (Aimanianda *et al.*, 2009). However, conidia without hydrophobins can still bind to the host cells *in vitro* and *in vivo*, with no changes in the virulence (Girardin *et al.*, 1999).

Melanin presence in the wall helps conidia to counteract the effect of reactive oxygen species since albino conidia are more susceptible to these radicals and show a lower survival rate within phagocytic cells (Pihet *et al.*, 2009). It was demonstrated that melanin also plays an indirect role in the fungal pathogenesis, allowing the correct assembly of the wall layers of resting conidia (Pihet *et al.*, 2009). Analysis of albino mutants showed considerable changes in the conidial wall organization, with the loss of the outermost electron-dense layer, the absence of the rodlet layer, and a decrease in the cell electronegative charge (Pihet *et al.*, 2009).

The presence of sialic acid has also been reported in the conidial wall, being recognized by the *Sambucus nigra* agglutinin, a specific lectin that binds N-acetyl-neuraminic acid (Warwas *et al.*, 2007). High-performance liquid chromatography and mass spectrometry analyses confirmed the presence of this component in the conidia wall, and digestion with neuraminidase demonstrated that it helps the fungus to bind to the host extracellular matrix (Warwas *et al.*, 2007).

When conidia germinate due to water entrance and glycerol accumulation, the isodiametric growth gets replaced by polarized growth, causing the conidial wall rupture to form a germ tube with a wall that extends from the inner layer of the conidia. In consequence, several structures change in the cell, including the loss of the rodlet and melanin layers, which turn the hydrophobic surface of the conidium into hydrophilic; and the exposure of the β-glucans in the surface of the swollen conidia, which increases the surface adhesive properties (Latge *et al.*, 2017). The α-glucans exposed interact with each other, causing the swollen conidia aggregation (Latge *et al.*, 2017; Beauvais *et al.*, 2014).

In mycelia, around 90% of the CW is composed of polysaccharides, some of which are structurally and covalently bound to the β-1,3-glucan core; while others, such as α-1,3-glucan and GAG, fill up the pores between fibrillar polysaccharides (Latge *et al.*, 2017). After swelling, conidia germinate and develop into hyphae that can grow differently depending on the conditions, (1) as a network of agglutinated hydrophobic hyphae, under static and aerated conditions; (2) or as separate hyphae, under shaken liquid conditions. Electron microscopy analysis has shown that in the first case the wall looks like a single electron-translucent layer; while in the second case, it looks like a thin electron-dense layer with extracellular material covering the CW (Latge *et al.*, 2017).

In addition to the differences in the CW polysaccharides composition and organization in the two morphologies, these may also vary depending on the growth and nutritional conditions. For example, when the fungus is growing as a biofilm, the α-1,3-glucans are found on the surface of the mycelial wall, but when the growth is under shaken and submerged conditions, they are localized on the CW inner layer (Beauvais *et al.*, 2014). Also, *A. fumigatus* growth under limiting glucose concentrations results in a reduction of the β-1,3-glucan synthase activity, with a consequent reduction of CW β-1,3-glucan levels (Clavaud *et al.*, 2012). On the other hand, hypoxia is associated with an increment in β-1,3-glucans and chitin, and with the reduction of α-1,3-glucans (Shepardson *et al.*, 2013).

GAG is a heterogeneous linear polymer composed of α-1,4-linked galactose, N-acetyl-galactosamine, and galactopyranose, and is secreted by growing hyphae (Speth *et al.*, 2019). It is an important component during biofilm formation since it binds to the hyphal surface, creating a polysaccharide envelope that covers and protects the growing fungus, forming an extracellular matrix between hyphae (Speth *et al.*, 2019). When GAG deficient strains were evaluated in mouse models of invasive aspergillosis, they were incapable of forming biofilms and showed an attenuated virulence (Gravelat *et al.*, 2013; Speth *et al.*, 2019). Also, GAG is a major hypha adhesin, because mutants incapable of synthesizing this compound do not form biofilms, and exhibit a reduced adherence to pulmonary epithelial cells (Speth *et al.*, 2019; Gravelat *et al.*, 2013). Moreover, strains that overexpress this wall component exhibit an increased binding to the host cells and other surfaces (Neves *et al.*, 2017).

It is also known that GAG needs to be deacetylated by the enzyme Agd3, not only to bind to the hyphae but also to bind to other surfaces (Lee *et al.*, 2016). Agd3 mutants showed adhesion and virulence defects both *in vitro* and *in vivo* (Lee *et al.*, 2016). This wall component has been described in several *Aspergillus* species, including *A. fumigatus*, *Aspergillus nidulans*, *Aspergillus niger*, and *Aspergillus parasiticus* (Lee and Sheppard, 2016).

The Fungal Cell Wall and the Development of Antifungal Drugs

Since the CW has an essential role in maintaining cell integrity, this structure represents an attractive target for the development of antifungal drugs. Additionally, many wall components are not synthesized by the mammalian host, potentially reducing the collateral effects when developing a compound with antifungal properties. Besides the selection of the target, antifungals should ideally meet some criteria: (1) the activity of the antifungal must have a broad spectrum against yeast and filamentous fungi; (2) it should be fungicidal rather than fungistatic; (3) to be directed at a specific fungal target and to have no interference with host targets; (4) to have multiple delivery methods, particularly oral availability; and (5) to have minimal side effects or toxicities (Mazu *et al.*, 2016).

The chitin synthesis inhibitors act as analogs of the UDP-GlcNAc substrate for Chs. Among these inhibitors, we found polyoxins, which are closely related to nucleotide antibiotics that are produced by *Streptomyces cacaoi* var *asoensis* (Endo *et al.*, 1970). The polyoxin D action mechanism is the competitive inhibition of Chs for UDP-GlcNAc (Endo *et al.*, 1970). The polyoxins are dipeptidyl or tripeptidyl nucleosides that are transported into the cell *via* a peptide transport system (Naider *et al.*, 1983). Nikkomycins X and Z are also competitive inhibitors of fungal Chs that are structurally similar to polyoxins. These compounds showed *in vivo* and *in vitro* antifungal activity against *Blastomyces dermatitidis* and *Coccidioides immitis* (Hector *et al.*, 1990). However, some fungi like *Candida* spp., *C. neoformans*, *Aspergillus* spp. have been reported to be resistant to nikkomycins (Hasim and Coleman, 2019).

Other Chs inhibitors are HWY-289, arthrichitin (activity against *Candida* spp., *Trichophyton* spp.), and FR-9000403 (Mazu *et al.*, 2016). A recent study found two benzothiazole compounds, described as IMB-D10 and IMB-F4, which inhibited Chs *in vitro*, reduced chitin levels in yeast cells, and inhibited the *S. cerevisiae* and *C. albicans* growth (Li *et al.*, 2019). Nikkomycin Z has shown to have a synergistic interaction with fluconazole and itraconazole against *C. albicans, C. parapsilosis, C. neoformans,* and *C. immitis;* while combined with itraconazole, Nikkomycin Z showed synergism against *A. fumigatus* and *Aspergillus flavus* (Li and Rinaldi, 1999).

Echinocandins are semisynthetic lipopeptides produced from fungal precursors: caspofungin from *Glarea lozoyonensis* pneumocandin B_0, anidulafungin from *A. nidulans* echinocandin B_0, and micafungin from *Coleophoma empedra* hexapeptide FR901370 (Eschenauer *et al.*, 2007). Echinocandins are hexapeptide with *N*-linked acyl fatty acid chains that intercalate in the cell membrane phospholipids (Lima *et al.*, 2019). The echinocandin's action mechanism is the noncompetitive inhibition of β-1,3-glucan synthase, which causes the decrease in the β-1,3- and β-1,6-glucan network, leading to a disordered and osmotically unstable CW and fungal death (Lima *et al.*, 2019). Echinocandins inhibit the Fsk subunit of the glucan synthase complex and mutations in this subunit lead to the resistance showed in some *Candida* species (Perlin, 2011).

Ibrexafungerp (SCY-078 formerly MK-3118) is a semisynthetic orally bioavailable enfumafungin derivative, structurally distinct from echinocandins. This compound is a potent inhibitor of glucan synthase, with activity even in echinocandin-resistant *Candida* strains (Pfaller *et al.*, 2017). Moreover, Ibrexafungerp exhibited antifungal activity against *C. auris* strains resistant to echinocandin and fluconazole (Arendrup *et al.*, 2020). In *C. glabrata*, spontaneous mutations in the β-1,3-glucan synthase *FKS* subunit showed partially overlapping but an independent binding sites for Ibrexafungerp and echinocandins (Jiménez-Ortigosa *et al.*, 2017).

The combination of nikkomycin Z and echinocandin (anidulafungin or micafungin) showed synergistic effects against *C. albicans* isolates and laboratory-derived echinocandin-resistant *fks* mutants (Cheung and Hui, 2017); while a similar observation was reported with echinocandin FK463 in *A. fumigatus* (Chiou *et al.*, 2001). The combination of different compounds with distinct targets may be a good solution for the arising problem of fungal resistance against antifungal drugs.

One of the proteins involved in GPI synthesis is Gwt1 (GPI-anchored wall protein transfer 1), an inositol acyltransferase that catalyzes inositol acylation (Lima *et al.*, 2019). The compound 1-[4-butylbenzyl]isoquinoline (BIQ) inhibits the expression of GPI-mannoproteins in both *S. cerevisiae* and *C. albicans*, resulting in growth inhibition (Tsukahara *et al.*, 2003). A missense mutation in *GWT1* suppressed the drug-induced growth-inhibitory phenotype, suggesting that the encoded protein is the target of this compound (Tsukahara *et al.*, 2003). A derivative compound from BIQ, E1210, has a broad-spectrum antifungal activity that also inhibits the fungal GPI biosynthesis and showed potent antifungal activity against *C. albicans, C. tropicalis, A. flavus, A. fumigatus,* and *Fusarium solani* (Hata *et al.*, 2011).

Another class of compounds are members of the pradimicin family. Pramidicins appear to act by calcium-dependent complexing with the saccharide portion of cell surface mannoproteins, which leads to the perturbation of the cell membrane, leakage of intracellular contents and cell death (Gonzalez *et al.*, 1998). A derivative of paramedicine, BMS-181184, was proven to have *in vitro* antifungal activity against *Candida* spp., *C. neoformans, A. fumigatus,* dermatophytes, and *Sporothrix schenckii* but it had no activity against *A. niger, A. flavus, Malassezia furfur, Fusarium* spp, and *Pseudallescheria boydii* (Fung-Tomc *et al.*, 1995).

Thus, the fungal CW components and their biosynthetic pathways still are novel and important targets for the development of new antifungal drugs.

Immune Sensing and the Fungal Cell Wall

The immune system has developed very efficient strategies to control fungal pathogens, generally ensuring a result in our favor (Hernandez-Chavez *et al.*, 2017). Once the pathogen has entered the host, it is likely eliminated by phagocytic cells, such as macrophages and neutrophils of the innate immune system that belong to the first defense line (Hernandez-Chavez *et al.*, 2017; Erwig and Gow, 2016). Both kinds of phagocytic cells are efficient in fungal recognition, mainly through interaction with the CW, which contains molecules that cannot be synthesized by the host, and therefore are detected as pathogen-associated molecular patterns by various patterns recognition receptors (Díaz-Jiménez *et al.*, 2012; Becker *et al.*, 2015). It is known that polysaccharides are the most abundant CW components, and almost all of them can be recognized by specific receptors and stimulate immune responses (Erwig and Gow, 2016). However, to avoid host defense mechanisms, fungi have developed strategies to escape immune sensing (Hernandez-Chavez *et al.*, 2017).

The CW changes both composition and architecture when environmental conditions are adverse, and when the cell is under high-stress levels, contributing to the strategies required for adaptation and survival (Díaz-Jiménez *et al.*, 2012; Gow *et al.*, 2017). This is considered an advantage for some fungi since those changes can provide advantages to resist or disguise the action of immune effectors (Hernandez-Chavez *et al.*, 2017).

The recognition of CW components is essential to trigger a host protective immune response, and the outcome of this will depend on the ligands-receptors engaged and the downstream signaling pathways activated (Hernandez-Chavez *et al.*, 2017). The pattern recognition receptors are classified into four families: Toll-like receptors (TLR), NOD-like receptors (NLR), retinoic acid-inducible gene I (RIG-I), and C-type lectins (CLR), most of which are expressed in dendritic and other myeloid cells (Netea *et al.*, 2008). When interacting with *C. albicans*, TLR2 recognizes phospholipomannans, TLR4 recognizes *O*-linked mannan, TLR6 is involved in zymosan recognition, and TLR9 detects fungal DNA (Netea *et al.*, 2008). C-type lectin receptors are primarily membrane-bound receptors that recognize polysaccharide structures. Dectin-1 recognizes β-1,3-glucans, mannose receptor and DC-SIGN recognize *N*-linked mannans, the macrophage-inducible C-type lectin (Mincle) recognizes α-mannans, and galectin-3

Table 4 Pathogen-associated molecular patterns (PAMPs) present in some pathogenic fungi and recognition of fungal components by pattern recognition receptors (PRRs)

Pathogens	*PAMPs*	*PRRs that recognize PAMPs*
C. albicans	β-1,3-Glucans, β-1,6-glucans, *N*-linked mannans, *O*-linked mannans, α-mannans, α-mannose oligosaccharides, β-1,2- oligomannans, chitin.	TLR4, TLR2, TLR9, Dectin-1, Dectin-2, Dectin-3, DC-SIGN, MR, Galectin-3, Mincle
A. fumigatus	Galactomannan, α-1,3-glucans, β-1,3-glucans, β-1,6-glucans, β-1,4-glucans	DC-SIGN, Dectin-1, Dectin-2, TLR2, TLR4

detects mannans and β-1,2-oligomannans (Drummond *et al.*, 2011) (see **Table 4**). Several studies have shown that Dectin-1 mediates a wide variety of antifungal cellular responses, such as phagocytosis, cytokine, and chemokine production, which contribute to a strong inflammatory immune response. Some of these activities are stimulated when both dectin-1 and TLR2 interact with β-1,3-glucan and establish a co-stimulatory effect that positively boosts those immunological elicitors (Dennehy *et al.*, 2008; Netea *et al.*, 2008).

As mentioned, CW polysaccharides are organized in different layers, and to avoid its sensing by immune cells, fungi manage to "hide" and cover structural polysaccharides, in particular the β-1,3-glucans and chitin, with different molecules such as mannans, working as a protective shield (Heinsbroek *et al.*, 2005; Hernandez-Chavez *et al.*, 2017). This mechanism is not exclusive of *C. albicans* though. In *A. fumigatus* conidia, the hydrophobins and melanin mask the β-glucans, evading the host defense system (Steele *et al.*, 2005). The *C. neoformans* capsule masks the recognition of cell wall mannan and β-1,3-glucan; however, the cell does not go completely unnoticed by the innate immune system, because the capsule can be recognized by TLRs that trigger an inflammatory response (Marcos *et al.*, 2016). Dectin-2 and Dectin-3 receptors are also transmembrane proteins of the C-type lectin family that recognize α-mannans (Zhu *et al.*, 2013).

Chitin interacts with different receptors, in a size- and concentration-dependent manner (Da Silva *et al.*, 2009; Wagener *et al.*, 2014). At low concentrations, small particles of 1–10 μm can induce an anti-inflammatory response through the mannose receptor, in conjunction with TLR9 and NOD2, leading to secretion IL-10 (Wagener *et al.*, 2014). Particles of intermediate size (40–70 μm) induce a pro-inflammatory response, triggering the production of TNFα and IL-17 (Marcos *et al.*, 2016). Very large chitin particles (70–100 μm) and very small particles < 2 μm are inert and do not trigger an immune reaction (Marcos *et al.*, 2016). It is likely that during the infection process, and assuming that the chitin particles that remain are of intermediate size, an inflammatory process is induced and disrupts fungal cells, and from these, small particles arise to trigger an anti-inflammatory response, limiting the immunological reaction (Da Silva *et al.*, 2009).

Melanins, which are complex amorphous polymerized phenolic compounds, are also found in the CW of some dimorphic fungi. These compounds prevent complement activation, neutralize antimicrobial peptides, and protect cells from oxidative damage (Nosanchuk and Casadevall, 2006). Because of its importance in fungal virulence, the host immune system has evolved antifungal strategies that allow melanin detection (Smith and Casadevall, 2019). One of the strategies is the use of C-type lectins such as the melanin receptor MelLec, responsible for recognizing the naphthalene-diol unit of DHN-melanin. MelLec recognizes melanin from cell surface such as that found on *A. fumigatus* conidia and in other DHN-melanized fungi (Stappers *et al.*, 2018).

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The Fungal Chitinases

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Introduction

Chitin derives from the Greek word chiton (*χιτών*) meaning garment, and constitutes one of the most abundant biopolymers in nature. It consists of β-1–4 linked N-acetyl-D-glucosamine (GlcNAc) residues, forming a linear long chain and it is a structural component in many organisms. Chitin is synthesized by chitin synthases, which are plasma membrane-associated enzymes transferred by small vesicles called chitosomes (Bartnicki-Garcia, 1987). These enzymes are responsible for catalyzing the transfer of GlcNAc from uridine (UDP)-N-acetylglucosamine to the growing chitin chain. The chitin chain then inserts into the cell wall adjacent to plasma membrane, aided by hydrogen bonds (Bowman and Free, 2006).

The fungal cell wall is a dynamic compartment, contributing to hyphal ability to cope with different environmental stresses. Its composition is mostly β-1.3 and β-1.6 glucans, chitin and glycoproteins and differs from those in oomycetes and plants, where chitin is absent or exists in very small amounts (Melida *et al.*, 2013; Hinkel and Ospina-Giraldo, 2017). Chitin plays an important role in fungal cell wall rigidity and plasticity, although the percentage of chitin in filamentous fungi does not exceed 10%–20% of the total cell wall dry biomass (Specht *et al.*, 1996; de Nobel *et al.*, 2000), while in yeast-like fungi the proportion is even less (1%–2%) (Klis *et al.*, 2002). Chitin plays also a key role in fungal-plant interactions since it is recognized by plant receptors and triggers basal plant defense mechanisms resulting in pattern-triggered immunity (PTI) (Boller and Felix, 2009). Except fungal cell walls, chitin can be found in insects and nematodes (Hill *et al.*, 1991; Merzendorfer and Zimoch, 2003), in crustacean and mollusks shells (Peters, 1972; Kurita, 2006), in protozoa and algae (Mulisch, 1993; Kapaun and Reisser, 1995). However, it is absent from vertebrates and plants, meaning that it can be an appropriate target for anti-fungal drugs (Chaudhary *et al.*, 2013).

Chitinases (EC 3.2.1.52) are hydrolytic enzymes, responsible for chitin degradation. They cleave the β-1.4-bond releasing oligomeric, dimeric (chitobiose), and polymeric GlcNAc products. Yeast-like fungi contain fewer chitinase genes than the filamentous ones, and the number highly varies between species; from only one in the yeast *Schizosaccharomyces pombe* to 36 in *Trichoderma virens*. Chitinases are categorized into two glycoside hydrolase (GH) families; 18 and 19. Chitinases from filamentous fungi belong exclusively to GH18, while GH19 chitinases have been identified in the microsporidia phylum (Henrissat, 1991; Rönnebäumer *et al.*, 2006). Chitinases in GH18 and GH19 families display limited sequence similarity, differences in their three-dimensional structures and in their catalytic mechanisms (Perrakis *et al.*, 1994; Brameld *et al.*, 1998). Fungal species also contain hydrolytic enzymes that degrade chitobiose to monomers. These enzymes are called *N*-acetylhexosaminidases (NAGases) and belong to the GH20 family (Cantarel *et al.*, 2009).

Chitinases can be classified as endochitinases or exochitinases depending on their cleavage patterns (**Fig. 1**). Exochitinases can cleave the chitin chain from the ends producing chitobiose, while endochitinases cleave the chitin chain at random positions (Horn *et al.*, 2006; **Fig. 1**). Endo- and exochitinases also show differences in their catalytic clefts; exochitinases have tunnel-shaped clefts while the substrate cleft in endochitinases are open and shallow (van Aalten *et al.*, 2001; Hurtado-Guerrero and van Aalten, 2007). Chitinase binding sites are long and contain at least five sugar units. All GH18 proteins contain a $(\alpha/\beta)_8$ barrel (TIM barrel) fold and the substrate-binding amino acids are located in loops extending from the $(\alpha/\beta)_8$ barrel (Lienemann *et al.*, 2009). Finally, GH18 have been reported to be involved in transglycosylation process as well (Boer *et al.*, 2004).

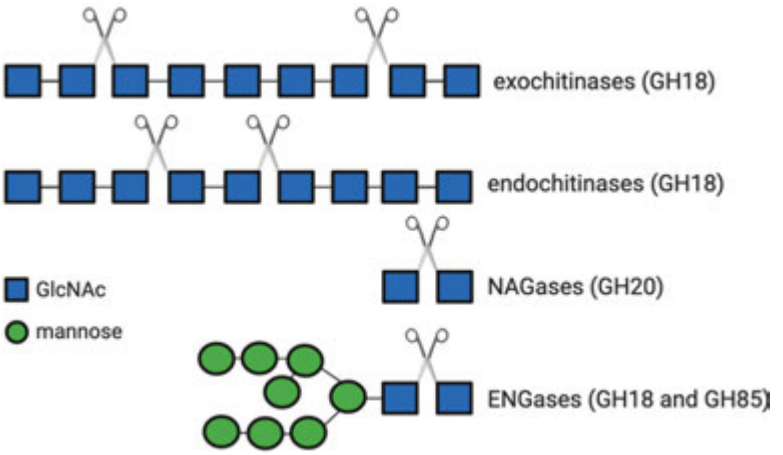

Fig. 1 The enzymatic activity of GH18s and GH20s in fungi. Endochitinases cleave the chitin chain randomly, exochitinases cleave from the open ends producing chitobiose (dimers) further cleaved by GH20 enzymes. ENGases are responsible for the deglycosylation of *N*-glycoproteins, cleaving the *N*, *N*-diacetylchiotobiose moiety from high mannose N-linked glycans. Figure was created with BioRender.

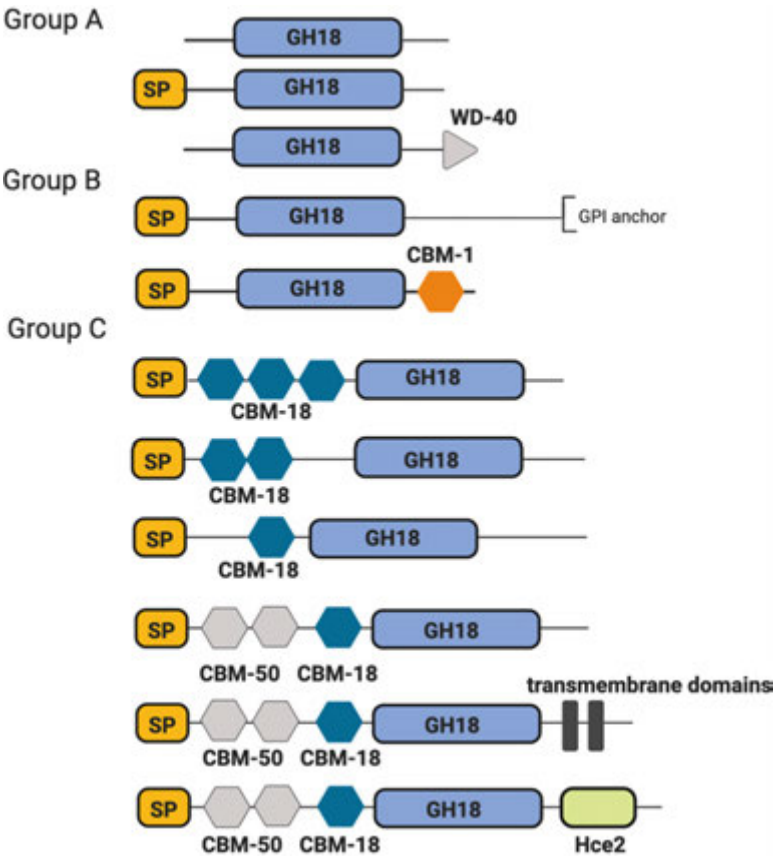

Fig. 2 The domain structure of fungal chitinases. Abbreviations: SP: signal peptide, GH18: glycoside hydrolases family 18, CBM-1: carbohydrate binding module family 1; cellulose-binding, CBM-18: carbohydrate binding module family 18; chitin-binding, CBM-50: carbohydrate binding module family 50; LysM, GPI: glycosylphosphatidylinositol structure. Figure was created with BioRender.

Phylogenetic analyses of fungal GH18 enzymes show that they cluster in three distinct groups; A, B and C (Seidl *et al.*, 2005; Karlsson and Stenlid, 2008; Karlsson *et al.*, 2016). These groups are further subdivided in several subgroups: Group A contains four subgroups (A-II, A-III, A-IV, and A-V), group B contains six subgroups (B-I, B-II, B-III, B-IV, B-V, and B-VI) while group C is divided into two subgroups (C-I and C-II). In certain fungal species, a chitinase of bacterial origin has been identified, probably as a result from horizontal gene transfer (Ubhayasekera and Karlsson, 2012).

Chitinases in all three groups typically contain a GH18 module with the catalytic DxxDxDxE motif, necessary for chitinolytic activity (van Aalten *et al.*, 2001; Gruber *et al.*, 2011a; Tzelepis *et al.*, 2012). In addition to their catalytic module, many GH18 enzymes contain additional domains such as the carbohydrate binding module family 1 (CBM1) or CBM50 (peptidoglycan binding LysM domain) (Seidl *et al.*, 2005; Tzelepis *et al.*, 2012; **Fig. 2**). These modules are not involved in the catalytic activity but enhance the enzymes ability for binding to crystalline chitin (Suzuki *et al.*, 1999).

Group A Chitinases

Most chitinases in this group are predicted to have exochitinase activity since they typically have a narrow substrate catalytic cleft. Many members contain a signal peptide at their N-termini, indicating that they are targeted to the endoplasmic reticulum (ER) and are possibly secreted (**Fig. 2**). In addition to the GH18 catalytic module, a WD40 domain has been found in chitinases from the entomopathogenic fungus *Beauveria bassiana* (Agrawal *et al.*, 2015) (**Fig. 2**). This domain is involved in a variety of cellular processes, such as protein-protein or protein-DNA interactions (Xu and Min, 2011). Fungal genomes contain variable numbers of genes encoding group A chitinases. For instance, the basidiomycete *Ustilago maydis*, which causes smut disease in maize, contains only two genes (Kamper *et al.*, 2006), while the soil-borne pathogen *Fusarium oxysporum* contains 12 genes. The well-studied mycoparasitic *Trichoderma* species contain an average number of seven group A chitinase genes (Kubicek *et al.*, 2011), while the model species *Neurospora crassa* has five (Tzelepis *et al.*, 2012).

One of the best studied group A chitinases is Ech42 from *Trichoderma*. Its enzymatic function has been characterized and is atypical for group A as it is shown to possess endo-activity (Carsolio *et al.*, 1994; Hayes *et al.*, 1994). It contains at least seven subsites and cleave the chitin chain preferably between the second and the third sugar from the reducing end and it can degrade chitohexose

$(GlcNAc)_6$ into three $(GlcNAc)_2$ units (Hartl *et al.*, 2012). When it comes to regulation and function, the *ech42* gene was highly induced under different conditions such as fungal-fungal interactions, starvation, autolysis and during cell wall degradation (Gruber and Seidl-Seiboth, 2012). Deletion of this gene did not affect *Trichoderma* mycoparasitic ability against *Rhizoctonia solani* or *Sclerotium rolfsi* (Carsolio *et al.*, 1999), while reduced anti-fungal activity against *Botrytis cinerea* was observed (Woo *et al.*, 1999). In *T. reesei*, three additional group A chitinase genes were expressed during similar conditions as *ech42* (Seidl *et al.*, 2005).

In the mycoparasitic species *Clonostachys rosea*, the homolog to the *ech42* gene was also induced during interactions with *B. cinerea* and on media where chitin was the sole carbon source (Mamarabadi *et al.*, 2008a; Tzelepis *et al.*, 2015). Deletion of this gene led to lower *in vitro* antagonistic activity but did not reduce *C. rosea* biocontrol ability (Mamarabadi *et al.*, 2008b). In addition to *ech42*, *C. rosea* contains seven other genes in group A (Karlsson *et al.*, 2015; Tzelepis *et al.*, 2015). None of these genes were induced during interactions between *C. rosea* and *B. cinerea*, *R. solani* or *F. graminearum*, while some of them were up-regulated when *C. rosea* was exposed to carbon starvation or during growth on chitin-medium, as compared to carbon rich medium (Tzelepis *et al.*, 2015). In the entomopathogenic species *Metarhizium anisopliae*, nine group A chitinases have been identified (Junges *et al.*, 2014). The *chimaA1* gene was up-regulated on media containing chitin or GlcNAc, while high transcript levels of *chimaA7* were measured during growth on high glucose medium (Junges *et al.*, 2014). Furthermore, three and two genes were induced at the conidial stage or during appressoria formation, respectively (Junges *et al.*, 2014).

In *N. crassa*, deletion of group A chitinases did not have any impact in fungal phenotypes, such as growth rate, conidiation or colony morphology, while variable transcription patterns were observed (Tzelepis *et al.*, 2012). For instance, two genes (*gh18–3* and *gh18–4*) were induced during interaction between *N. crassa* and *F. sporotrichoides*, (Tzelepis *et al.*, 2012). In the opportunistic human pathogen *Aspergillus fumigatus*, deletion of the group A *chiB1* chitinase gene did not cause any changes in colony morphology but resulted in lower chitinolytic activity during the autolytic phase in batch cultures (Jaques *et al.*, 2003). However, a functional analysis of the group A ChiB chitinase in *A. nidulans* revealed a crucial role in autolysis (Yamazaki *et al.*, 2007; Pocsi *et al.*, 2009).

In the ectomycorrhizal species (ECM) *Laccaria bicolor* there are three putatively secreted group A chitinases, which are induced at different time points of ECM development, indicating a continuous degradation of chitin during the establishment of the mutualistic relationship (Veneault-Fourrey *et al.*, 2014).

In yeast-like fungi, the number of genes encoding chitinases is lower compared to filamentous species. In the smut fungus *U. maydis*, the group A chitinase Cts1 is involved in cytokinesis (Langner *et al.*, 2015). Deletion of the *cts1* gene led to changes in colony morphology and sedimentation rate. Cts1 was also shown to be essential during separation of mother and daughter cells and during filamentous growth (Langner *et al.*, 2015), possibly by degrading chitin in the cell wall. The model yeast species *Saccharomyces cerevisiae* contains only one chitinase gene in group A (*ScCTS2*) that is involved in asci formation (Giaever *et al.*, 2002). Deletion of the group A *AgCTS2* gene from the plant pathogenic fungus *Ashbya gossypii*, homologous to *ScCTS2*, impacted the phenotype of spores (Dünkler *et al.*, 2008). The wild-type spores have a hyaline appearance, while spores from the *AgCTS2* mutants were speckled with dot-like, vesicular bodies. (Dünkler *et al.*, 2008). However, deletion of the *AgCTS2* gene did not affect spore germination or polarity (Dünkler *et al.*, 2008). Interestingly, complementation of *AgCTS2* with the homologs *ScCTS2* and *CaCTS4* from *S. cerevisiae* and *Candida albicans* respectively, restored the phenotype of spores indicating a conserved function in sporulation over species boundaries (Dünkler *et al.*, 2008). In *C. albicans*, *CaCTS4* is induced during the yeast growth phase but down-regulated during the hyphal growth phase (McCreath *et al.*, 1996). However, deletion of this gene did not cause any obvious phenotype (Dünkler *et al.*, 2005). Finally, the opportunistic human pathogen *Cryptococcus neoformans* contains three homologs to the *ScCTS2* gene, which are involved in sexual reproduction of this species (Baker *et al.*, 2009).

Certain predicted group A GH18 proteins contain mutations in the catalytic DxxDxDxE motif, indicating loss of enzymatic activity. For instance, *T. reesei* contains four genes in subgroup A-II and A-IV with these type of amino acid substitutions (Karlsson and Stenlid, 2009). The A-IV subgroup CHI18–3 protein is predicted to be localized in the mitochondria (Seidl *et al.*, 2005) and contains two S-globulin domains, often associated with GH18 modules (Shewry and Halford, 2002). Genome analyses revealed that there are orthologs in other fungal species as well (Seidl *et al.*, 2005; Tzelepis *et al.*, 2012, 2015), indicating that the presence of a putative mitochondrial non-chitinolytic GH18 protein is widespread among the Sordariomycetes. It is possible that these enzymatically inactive GH18 proteins retain the ability for chitin binding, i.e., evolve into lectins, as was recently shown for the group A protein MpChi from the basidiomycete cacao pathogen *Moniliophthora perniciosa*. The enzymatically inactive MpChi binds to chitin and thereby suppresses chitin-triggered plant immunity and facilitates infection (Fiorin *et al.*, 2018). A paralog to MpChi in the closely related cacao pathogen *M. roreri* carries a single substitution in its catalytic motif that results in reduced chitinolytic activity and was shown to suppress plant immunity in a similar fashion as MpChi (Fiorin *et al.*, 2018). Genome mining revealed that many plant pathogens contain predicted GH18 proteins with degenerate catalytic motifs, suggesting that recognition of chitin by plant pattern recognition receptors drives evolution of chitin-scavenging lectins in plant pathogenic fungi (Fiorin *et al.*, 2018).

Group B Chitinases

GH18 enzymes in this group are divided in six subgroups (B-I through B-VI) (Seidl *et al.*, 2005; Karlsson *et al.*, 2016). They are predicted to have a more shallow and wider catalytic cleft as compared to chitinases in group A, indicating endochitinase activity (**Fig. 2**). However, certain enzymes from the B-V subgroup have a mannosyl glycoprotein endo-N-acetyl-β-D-glucosaminidase (ENGase) type activity (Stals *et al.*, 2010; **Fig. 2**). The number of group B chitinase genes varies between one in *S. cerevisiae* to 11 in mycoparasitic *Trichoderma* species (Kuranda and Robbins, 1991; Seidl *et al.*, 2005). They are also predicted to vary in size, from small

(30–40 kDa) to large (80–90 kDa) proteins. They typically contain a signal peptide at the N-terminus, indicating that they are targeted to the ER. Cellulose-binding domains (CBM1), which also bind chitin (Boraston *et al.*, 2004), are often found in group B chitinases. *Trichoderma* species contain high numbers of chitinases with CBM1 domains (Seidl *et al.*, 2005). Experimental addition of CBM1 domains to group B chitinases in *Trichoderma* resulted in an increase of its antifungal activity (Limon *et al.*, 2001, 2004), suggesting a role in fungal-fungal interactions. Certain large group B chitinases contain a glycosylphosphatidylinositol structure (GPI anchor) in the C-terminus indicating that they are localized in the cell wall (Yamazaki *et al.*, 2008; Tzelepis *et al.*, 2012). The group B chitinase Chit33 from *Trichoderma* has been enzymatically characterized. It is a typical endochitinase having a shallow and open substrate-binding site with at least six subsites, producing $(GlcNAc)_2$ and $(GlcNAc)_4$ (Hartl *et al.*, 2012).

In *T. reesei*, the *chi18–13* gene was expressed in presence of *R. solani* cell wall components and during confrontation with *B. cinerea* (Seidl *et al.*, 2005). Similarly, its homolog in *T. atroviride* was highly induced before and during interactions with *R. solani*, indicating that group B chitinases are involved in mycoparasitic interactions (Seidl *et al.*, 2005). The group B chitinase Chi18–15/Chit36/Ech37 is an interesting example of a horizontal gene transfer event, from a bacterial donor (likely related to *Streptomyces*) to a hypocrealean acceptor, as it is found in both *Trichoderma* and *Clonostachys* (Karlsson and Stenlid, 2009; Ubhayasekera and Karlsson, 2012). Gene expression analysis showed that *chi18–15* was induced during growth on chitin and fungal cell walls, perhaps suggesting a function in nutrient acquisition (Viterbo *et al.*, 2002). Besides the *ech37* gene, the *C. rosea* genome contains two additional group B chitinases, *chiB1* and *chiB2* (Tzelepis *et al.*, 2015). The *chiB1* gene was highly induced during interactions with *R. solani*, while both genes were up-regulated on media where chitin was the sole carbon source (Tzelepis *et al.*, 2015). The saprotrophic species *N. crassa* also contains two genes in group B and one of them, *chit-1*, was induced on chitin media as well (Tzelepis *et al.*, 2012). Analysis of a *chit-1* deletion strain showed that this mutant grew slower as compared to the wild type strain, indicating a role in hyphal growth (Tzelepis *et al.*, 2012).

The fungus *M. anisopliae* contains seven genes in this group (Junges *et al.*, 2014). Among those, *chiMaB7* showed significantly higher transcript levels during infection-related conditions (blastospores, tick cuticles and appressoria), while the *chimaB4* gene was up-regulated during growth on media contained chitin or GlcNAc as sole carbon sources (Junges *et al.*, 2014). Furthermore, the *M. anisopliae* B group endochitinase CHI2 seems to be involved in virulence, since overexpression of this chitinase led to an increased efficacy of the fungus to kill the insect *Dysdercus peruvianus* while gene deletion resulted in a reduced fungal infection efficiency (Boldo *et al.*, 2009). Similarly, the CHI30 chitinase is also shown to be involved in virulence and heat shock adaptation in *M. anisopliae* (Staats *et al.*, 2013).

In the ectomycorrhizal species *Tuber melanosporum* two B group chitinases, *TmelCHT2.1* and *TmelCHT2.2*, are highly induced in the symbiotic stage. This indicates the importance of cell wall modification during the switch from the free-living to symbiotic stage (Balestrini *et al.*, 2012). Moreover, in *L. bicolor* there is a chitinase gene in this group that was up-regulated at different time points of ECM development, similar to the group A genes mentioned previously (Veneault-Fourrey *et al.*, 2014).

Regarding yeast-like species, the group B *S. cerevisiae CTS1* chitinase gene was expressed in the mother and daughter cells during the early stages of separation (Langner and Gohre, 2016). Deletion of the *CTS1* gene led to a defect in cell separation and formation of multicellular aggregates (Kuranda and Robbins, 1991). The *C. albicans* genome contains three group B chitinase homologs to *CTS1*, where deletion of *CaCHIT1* and *CaCHIT2* resulted in increased hyphal growth on solid media (Dünkler *et al.*, 2005). CaCHIT2 is a typical large group B chitinase with a GPI anchor at the C-terminus (Dünkler *et al.*, 2005). Deletion of the *CaCHIT3* resulted in formation of chains of non-separated cells similar to the *S. cerevisiae CTS1* deletion phenotype, indicating a role of these genes in cytokinesis (Dünkler *et al.*, 2005). The group B endochitinase KlCTS1 from the yeast *Kluyveromyces lactis* appears to provide the same function in cytokinesis, since deletion of this gene led to inefficient cell separation (Colussi *et al.*, 2005).

As mentioned above, certain members of the B-V subgroup are shown to exhibit ENGase (EC 3.2.1.96) activity (Stals *et al.*, 2010) (**Fig. 2**). They contain a GH18 module with a conserved catalytic motif similar to active chitinases (DxxDxDxE) and likely evolved from an ancestral chitinolytic enzyme through neofunctionalization (Karlsson and Stenlid, 2008, 2009). Some of these enzymes are involved in deglycosylation of misfolded *N*-glycoproteins in the ER-Associated Degradation (ERAD) process, cleaving the glycoside bond in the *N*,*N*-diacetylchiotobiose moiety as shown in **Fig. 1** (Suzuki *et al.*, 2002). Filamentous ascomycetes typically contain a cytosolic ENGase, while a second, putatively secreted protein has been identified in certain species (Tzelepis *et al.*, 2017). In general, GH18 ENGases are absent from yeast-like species, such as *Saccharomyces*, *Candida*, and *Cryptococcus* (Tzelepis *et al.*, 2017). In Basidiomycetes, the situation is mixed with ENGases being absent from *Heterobasidium*, *Ustilago*, and *Puccinia* genera, while *Schizophyllum* and *Stereum* species have one putative ENGase in their genomes (Tzelepis *et al.*, 2017). Deletion of cytosolic ENGases led to severe phenotypic changes in filamentous ascomycetes (Dubey *et al.*, 2012; Tzelepis *et al.*, 2012). In *N. crassa*, deletion of the cytosolic *gh18–10* ENGase resulted in slower mycelial growth, increased conidiation and tolerance to abiotic cell wall stress agents, and reduced amount of secreted proteins (Tzelepis *et al.*, 2012). Similar results were observed in *T. atroviride* Δ*Eng18B* strains, which is homolog to the *gh18–10* gene (Dubey *et al.*, 2012). Interestingly, this mutant lost its antagonistic ability against *B. cinerea* as well, possibly as an effect from the reduced secretion phenotype (Dubey *et al.*, 2012). Further analysis on Eng18B enzymatic function revealed that it is an active de-glycosylating enzyme, involved in the ERAD process (Tzelepis *et al.*, 2014a).

Group C Chitinases

Chitinases in this group are predicted to exhibit exochitinase activity although no member from this group has yet been biochemically characterized. They are present only in filamentous ascomycetes and are subdivided to two subgroups (C-I and C-II)

(Karlsson and Stenlid, 2008; Gruber *et al.*, 2011a). They show sequence similarities to the α/β subunits of the killer toxin zymocin produced from the diary yeast *K. lactis* and are therefore often referred to as "killer toxin-like" chitinases (Magliani *et al.*, 1997). In *K. lactis*, the function of the α subunit chitinase is to degrade the antagonist cell wall in order to facilitate penetration of the γ subunit, which is the active toxin (Butler *et al.*, 1991). A similar function has been hypothesized for the group C chitinases (Tzelepis and Karlsson, 2019). Genome analysis in *Trichoderma* species revealed that aggressive mycoparasitic species, such as *T. virens* and *T. atroviride*, contain higher number of C group chitinase genes, as compared to the weak mycoparasite *T. reesei* (Ihrmark *et al.*, 2010).

Most C group chitinases are predicted to have an intact catalytic domain, while few of them contain mutations that would render them devoid of chitinolytic activity (Karlsson and Stenlid, 2009; Gruber *et al.*, 2011b). As it was shown in **Fig. 2**, group C chitinases contain a signal peptide at the N-terminal and multiple domains (Tzelepis and Karlsson, 2019). In the C-I subgroup, chitinases usually contain one to three carbohydrate-binding family 18 (CBM18) together with the catalytic GH18 module, both located at the N-terminus (**Fig. 2**). Members in the C-II subgroup typically contain one CBM18 and two LysM CBM50 domains (Gruber *et al.*, 2011a,b; Tzelepis *et al.*, 2012, 2014b). The CBM18 domains bind to chitin and it is suggested to increase adherence of chitinases to the substrate (Boraston *et al.*, 2004). The CBM50 modules have peptidoglycan and chitin binding affinity (Buist *et al.*, 2008) and their role in fungal-plant interactions is well established, since they bind to chitin and stealth fungal hyphae from plant recognition (de Jonge *et al.*, 2010). This mechanism seems to be universal in the fungal kingdom, since it has been described in both ascomycete and basidiomycete phytopathogenic species (Mentlak *et al.*, 2012; Takahara *et al.*, 2016; Dölfors *et al.*, 2019). Furthermore, some members in the C-II subgroup contain transmembrane domains at the C-terminus, indicating a plasma membrane localization (Tzelepis *et al.*, 2012; 2014b; **Fig. 2**). Finally, it has been reported that a small protein domain (Hce2) is fused to the C-termini of certain C-II chitinases (Stergiopoulos *et al.*, 2012). This protein domain is homologous to the necrosis-inducing Ecp2 effector, which has previously been found in phytopathogenic species such as *Mycosphaerella graminicola* and *M. fijiensis* (Stergiopoulos *et al.*, 2010). A chitinase from *T. reesei* is predicted to contain an epidermal growth factor-1-like module at the C-terminus, potentially involved in protein-protein interactions (Wouters *et al.*, 2005). Although the exact roles of Hce2 and EFG-1 remain to be elucidated, it is speculated that they serve a role similar to the zymocin γ-subunit (Tzelepis and Karlsson, 2019).

When it comes to regulation of C-group chitinases, it was initially speculated that the primary function of these enzymes was in fungal-fungal interactions. However, gene expression data later suggested their involvement in other aspects of fungal biology as well. In the *N. crassa* genome, there are three members in the C group. The C-I member *chi18–9* was induced during carbon starvation conditions and not during interactions with fungal species, in contrast to the other C-II members, *chi18–6* and *chi18–8*, which were induced during interspecific interactions and during self-interactions (Tzelepis *et al.*, 2012). However, expression patterns differed during interactions with different fungal species, ascomycetes (*B. cinerea*) verses basidiomycetes (*R. solani*), suggesting that differences in cell wall composition may control regulation (Tzelepis *et al.*, 2012). The *A. nidulans* genome contains four genes in C-II subgroup and gene expression analysis showed that all of them were highly induced during interactions either with ascomycetes (*B. cinerea*) or basidiomycetes (*R. solani*), while there were not induced during interactions with *Phytophthora* species, which lacks chitin in the cell wall, or when dead cell wall material was the only carbon source (Tzelepis *et al.*, 2014b). Expression studies of C-group chitinases have been described in other fungal species as well, such as in the thermophile *Myceliophthora thermophile* where up-regulation was observed during growth on straw (Kolbusz *et al.*, 2014), and in the mycoparasite *Tolypocladium ophioglossoides* during parasitism of truffle tissue (Quandt *et al.*, 2016).

Analysis of expression patterns in *Trichoderma* species support the concept that C-group chitinases are involved in different aspects of fungal life and not only in interspecific interactions (Seidl *et al.*, 2005; Gruber *et al.*, 2011a,b). For instance, in *T. atroviride*, all eight C-group chitinase genes in *T. atroviride* were up-regulated during interactions with *B. cinerea* but not with *R. solani*, and no induction was observed on dead *R. solani* cell wall material (Gruber *et al.*, 2011a). Furthermore, four genes were induced on media where chitin was the sole carbon source, indicating their involvement in nutrient acquisition, while two of them were induced in the central part of the fungal colony, with older hyphae, compared to younger hyphae in periphery, suggesting an additional role in hyphal branching and autolysis (Gruber *et al.*, 2011b). In contrast, gene expression analyses in *T. virens* showed that only four of totally 14 C-group chitinase genes were induced during interactions with either *B. cinerea* or *R. solani*, while 11 of them were induced on fungal cell wall material (Gruber *et al.*, 2011b).

The structure of chitin also seems to influence the induction of these genes, since different genes were up-regulated by different forms of chitin (crude or colloidal) (Gruber *et al.*, 2011b). Finally, *Trichoderma* genome analyses revealed that *tal* genes, predicted to contain only CBM50 modules without any catalytic domain, are often clustered and regulated together with C-group chitinase genes (Gruber *et al.*, 2011a). Functional analysis showed that they are involved in hyphal growth (Seidl-Seiboth *et al.*, 2014). Furthermore, Tal6 protects hyphae from chitinases, increases *Trichoderma* virulence and modulates the activation of plant immunity (Romero-Contreras *et al.*, 2019). In contrast, the mycoparasite *C. rosea* contains a reduced number of genes encoding C-group chitinases as compared to *Trichoderma* mycoparasitic species. There is one member in the C-I and one in the C-II subgroup (Tzelepis *et al.*, 2015). Transcriptional analysis in this species showed that none of these genes were induced in interaction with *Fusarium* species, while the C-I member was only induced on chitin media (Tzelepis *et al.*, 2015). *Metarhizium anisopliae* contains four genes in this group that appeared to be constitutively expressed (Junges *et al.*, 2014).

Functional analyses of C-group chitinases have been conducted in model filamentous species, such as *N. crassa* and *A. nidulans*. In *N. crassa*, deletion of either C-II subgroup genes did not affect its growth or tolerance to abiotic stress conditions (Tzelepis *et al.*, 2012). In *A. nidulans*, deletion of either of the four C-II subgroup genes resulted in increased biomass production in liquid cultures. Deletion of *chiC2–2*, predicted to contain the Hce2 domain, resulted in mutants with reduced *in vitro* antagonistic ability against *B. cinerea* (Tzelepis *et al.*, 2014b). Moreover, three deletion strains showed to be slightly more tolerant in abiotic stress conditions (Tzelepis *et al.*, 2014b).

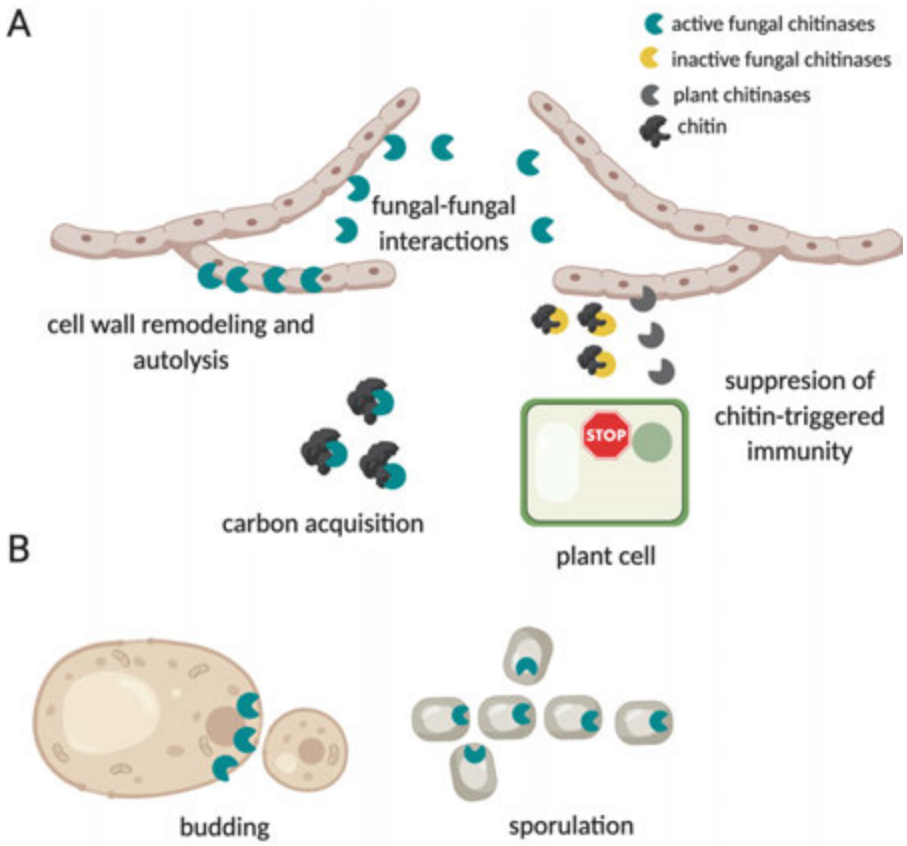

Fig. 3 Proposed functions of fungal chitinases. (A) chitinases in filamentous species have a function in fungal-fungal interactions, degradation of exogenous chitin for nutrient acquisition, autolysis and hyphal branching. Inactive fungal chitinases are involved in perturbation of chitin-triggered immunity during fungal-plant interactions. (B) chitinases in yeast-like fungal species are involved in cytokinesis, such as separation of mother and daughter cells during budding and separation and formation of spores. Figure was created with BioRender.

In *Trichoderma* mycoparasitic species, functional studies are limited. It is possible that gene redundancy in these species prevents observable phenotypes. The only study comes from deletion of a C group gene in *T. atroviride*, where deletion led to reduced conidiation (Seidl-Seiboth *et al.*, 2014). However, this gene carries mutations in the catalytic motif indicating that its chitinolytic activity has been abolished (Gruber *et al.*, 2011a). In contrast, deletion of the *C. rosea* C-II chitinase *chiC2* gene resulted in mutants with reduced *in vitro* antagonistic activity against *B. cinerea* and *R. solani*, but not against *Fusarium graminearum* (Tzelepis *et al.*, 2015). However, deletion of *chiC2* did not affect the biocontrol ability of *C. rosea* against *B. cinerea* (Tzelepis *et al.*, 2015).

Concluding Remarks

Chitinolytic enzymes are present in all fungal species and they are involved in degradation of chitin, an important structural component of fungal cell walls. Fungal chitinases from filamentous fungi belong to GH18, while a single GH19 chitinase has been described in the microsporidial species *Encephalitozoon cuniculi* (Rönnebäumer *et al.*, 2006), a very basal fungal group. Based on their catalytic activities, they can be categorized as exochitinases, which cleave the chitin chain from the ends, or as endochitinases, which cleave the chitin at random positions. Exochitinases release chitobiose, which is further degraded by GH20 NAGases. Phylogenetically, fungal GH18 genes are divided into three groups A, B, and C, further sub-divided in many subgroups. Since gene redundancy is one of the main problems in chitinase functional studies, their precise roles in fungal biology still remain unclear. From the available studies, data show that chitinases play roles in different aspects of fungal physiology, such as degradation of exogenous chitin for nutrient release, in mycoparasitic interactions, during carbon starvation and autolysis, in cell wall remodeling and hyphal growth (**Fig. 3(A)**). In yeast-like species, the number of chitinase genes is lower, typically between one and four, and hence more functional data are available. In these species, chitinases are involved in cytokinesis, such as mother and daughter cell separation during budding (**Fig. 3(B)**), and in sporulation, such as spore formation and separation (**Fig. 3(B)**). The killer toxin-like chitinases in group C is an interesting case. They are present only in filamentous ascomycetes and contain multiple domains such as CBM50 and CBM18, and all of them are predicted to be secreted (**Fig. 2**). In some of them, the Hce2 domain, homologous to the Ecp2 effector, is present. Many aspects of these chitinases remain to be elucidated. Since transcriptional data show that

induction of these genes is often triggered in fungal-fungal interactions, important questions concerns host specificity and cell wall composition or elicitors produced by the living cells that may trigger their expression. Furthermore, the role of the CBM50 and Hce2 modules should be clarified. It is speculated that they may be released as individual peptides after secretion through proteolytic activity, and may function in self-protection and as a toxin, respectively.

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GTPases in Hyphal Growth

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Hyphal Growth in Filamentous Fungi

Filamentous fungi are an evolutionarily flourishing group of microorganisms of significant ecological importance (Evans and Hedger, 2001). They have a considerable impact on our economy and ecosystem because important enzymes derive from fungi with applications in food, textile, recycling and other industries (Cairns *et al.*, 2018). In addition, many fungi are human and plant pathogens that pose a threat to public health and agriculture (Fisher *et al.*, 2012). Among the filamentous fungi there are also the mycorrhizal fungi, which bring countless benefits to the host plant (Smith and David, 2008), and edible fungi, such as truffle, one of the most valuable foods (Hall *et al.*, 2003). Last but not least, fungi are extremely important for understanding many functions of eukaryotic cells in vastly researches and their secondary metabolism is of significant pharmaceutical relevance (Keller *et al.*, 2005).

A better understanding of cytoplasmic organization and behavior can lead to higher and more diverse levels of insight to biology of cells and their evolutionary histories.

The dominant cell type of filamentous fungi are hyphae, which are filamentous of elongated cells that expand at the apex of the tip cell (Steele and Trinci, 1975). The processes involved in the growth of hyphae have affected many scientists for many years. Thanks to the progress in efficient technological DNA sequencing and gene deletion methods, today there is a drastic increase in the number of data. Despite these new resources, filamentous growth is a critical aspect of fungal biology that is not yet comprehensively understood (Meyer *et al.*, 2016). Hyphae exhibit a highly polarized form of cell growth that requires the regulation of numerous processes including cell wall synthesis, polarized vesicle transport, exocytosis, endocytosis, cytoskeletal function, turgor pressure, organelle positioning and bulk cytoplasmic flow. These actions result in apically growing tube-shaped hyphae.

Cell Wall Synthesis

Hyphal growth is accompanied by the secretion of exoenzymes that participate in lysis of the substrate or are involved in the synthesis of the fungal cell wall (Archer and Wood, 1995). The fungal cell wall plays a significant role in development and integrity of the fundamental architecture required for survival and proliferation of the filamentous fungus. The structure of the cell wall is composed typically of polysaccharides and glycoproteins, that can be considered as an extracellular gel-like matrix (Ruiz-Herrera and Ortiz-Castellanos, 2019). The cell wall of most filamentous fungi containsβ (1,3)- β (1,4)- β (1,6)- β (1,7)- glucans, α (1,3)-glucans, chitin, galactomannoproteins, and other less well-characterized glucans. It has been seen that in the alkaline-insoluble fraction are present fibrils of β (1,3)- and β (1,7)-glucans and chitin. Glycoproteins together with alkaline-soluble polysaccharides form an amorphous matrix (Klis *et al.*, 2006). These matrix glycoproteins are presynthesized and packaged into vesicles at the Golgi and delivered along cytoskeleton to the specific points of wall growth. After exocytosis glycosylphosphatidylinositol (GPI) anchors glycoproteins to the plasma membrane (Steinberg *et al.*, 2017).

The polysaccharides are synthesized by enzymes chitin synthases (CHS) and glucan synthases (GS), which are transported on the cell membrane in an inactive form within vesicles, where the enzymes are inserted to synthesize in situ the chitin and β (1,3)-glucans (Sanchez-Leon *et al.*, 2011). Chitin is thought to be the major polysaccharide found in the *Neurospora crassa* septae (Potapova, 2014). Filamentous fungi generally contain several genes encoding CHSs and they are organized into seven different groups or classes, as opposed to three classes of CHSs in yeast or dimorphic species, which correlates with the lowest chitin content in their cell wall (Riquelme and Bartnicki-Garcìa, 2008). The 1–3-glucan synthase complex (GSC) synthesizes β (1,3)-glucans: it contains a catalytic subunit (Fks) and a regulatory subunit (Rho1). Only one essential *Fks* gene has been identified in filamentous fungi. While in *Saccharomyces cerevisiae* there are two *Fsk* genes and they haven't distinct functions. Catalysis products operated by Fsk transmembrane protein poured out of the plasma membrane. Rho1 is a GTPase which is synthesized in the endoplasmic reticulum and inserted into plasma membrane by geranylgeranylation (Inoue *et al.*, 1999).

Today the most accepted view proposes that cell wall-loosening enzymes, such as chitinases and glucanases, participate in the breakage of polysaccharide chains, such as chitin and β (1,3)-glucans, allowing the addition of newly arrived material and generating free ends, substrate for cross-linking enzymes, that rigidify the cell wall (Riquelme *et al.*, 2018).

The Spitzenkörper and Vesicles Pathway

The apex of filamentous fungi is a highly dynamic region where cell growth and morphogenesis occur through the coordinated events of exocytosis, cytoskeletal dynamics and cell wall synthesis. Spitzenkörper (Spk) is where secretory vesicles self-assemble. Using phase-contrast light microscopy, the Spk is observed as a phase-dark body, in most taxa, is partially or completely surrounded a phase-bright central core (Roberson *et al.*, 2010). The Spk is important for hyphal growth and its position in the growing hypha determines the directionality of growth (Riquelme *et al.*, 2000). At the ultrastructural levels the Spk contains vesicles of different sizes,

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actin, microtubules and above all ribosomes, suggesting that translation of mRNA and protein synthesis happen in the hyphal apex (Howard and Aist, 1979). It is thought that the Spk functions as a vesicles supply center. It receives Golgi derived vesicles that releases exocytic vesicles in a controlled manner: in fact, the Golgi cisternae are distributed along the hyphae (Riquelme *et al.*, 2018). It determines an exocytosis gradient that fixes the shape of the fungal apex (Bartnicki-Garcìa *et al.*, 1989). In *N. crassa* the enzymes responsible for the synthesis of the cell wall, CHS and GCS, were located in the Spk. Specifically, CHS was identified at the core of Spk, where microvesicles concentrate and GCS at the Spk outer layer (Riquelme *et al.*, 2007). A biochemical stratification of Spk was detected in *Aspergillus nidulans*, but not the precise location of CHS synthetases (Takeshita *et al.*, 2015).

The biogenesis and release of vesicles take place through different passages, as in other eukaryotes: scission from the donor membrane-vesicle formation, vesicle transport, vesicle docking and vesicle fusion. There are involved coat complexes, which promote vesicle formation and recognize cargo-sorting signals; tethers which interact with coat proteins and mediate docking; and SNAREs (soluble NSF [N-ethylmaleimide-sensitive factor] attachment protein [SNAP]receptors) which facilitate the fusion between the donor and target membrane (Bonifacino and Glick, 2004). Rab GTPases are molecular switches to regulate these steps of vesicular transport along cytoskeletal elements. In *N. crassa* homologous genes have been identified that encode for Rab GTPases that occupy two distinct parts of Spk (Gould and Lippincott-Schwartz, 2009): YPT-1Rab1 was found in the core, while SEC-4Rab8 and YPT-31Rab11 were located in the outer layer. This suggests that distinct Rabs regulate the traffic of the different vesicle populations to the Spitzenkörper (Sánchez-León *et al.*, 2015). FRAP (fluorescence recovery after photobleaching) experiments allowed the identification of the time of the vesicular turnover at the Spk, which is rates from 20 to 40s (Sánchez-León *et al.*, 2015). Scientists speculate that the vesicles of Rab that reach the Spk derive from the apparatus of Golgi (Pantazopoulou *et al.*, 2014).

Once the vesicles have reached the membrane, they are fused through exocysts. In *A. nidulans*, *Candida. albicans* and other filamentous fungi, fluorescently imprinted exocysts components localized at growth sites, but if there is a tethering mechanism of exocytosis it is little understood (Riquelme *et al.*, 2014). After exocytosis, the last stage of secretory pathway, vesicles fuse with their target membrane by SNAREs interactions. The synaptobrevin vesicular SNARE protein SynA was observed at the Spk of *A. nidulans* and at the apical plasma membrane (Taheri-Talesh *et al.*, 2008).

It seems that exocytosis and endocytosis work in tandem for hyphal morphogenesis (Delgado-Alvarez *et al.*, 2010; Upadhyay and Shaw, 2008). Endocytosis is the result of the extra plasma membrane released by exocytosis (Riquelme *et al.*, 2018).

Structure and Action Mechanism of Small GTPases

Structure

The small GTPases proteins have been studied by crystallographic analysis, and it has emerged that they consist of five fairly conserved domains responsible for binding with GTP, from G1 to G5. G1 motif (I) is a purine nucleotide binding signal; G2 motif (E) is in one of two segments that redirects with GDP or GTP binding function and provides major component of the effector binding surface; G3 motif (II) is involved in Mg^{2+} binding; G4 (III) motif brings the hydrogen bond into contact with the guanine ring; the G5 (IV) motif creates indirect associations with the guanine nucleotide (Wennerberg *et al.*, 2005; Colicelli, 2004; Goitre *et al.*, 2014).

Action Mechanism

Small GTPases perform their physiological activity switching the molecular structure in two forms which support mutual transformation, GTP-binding activated state and GDP-binding non-activated state, which can also be called as "ON" state and "OFF" state, respectively (Jhonson and Chen, 2012).

The multiple biological functions of the small GTPases proteins are mediated through a highly regulated GTP/GDP binding cycle. Three different classes of proteins are required for the regulation: (1) guanine nucleotide exchange factors (GEFs), which stimulate the GTP–GDP exchange reaction; (2) GTPase-activating proteins (GAPs), which stimulate the GTP-hydrolyzing reaction; and (3) guanine nucleotide dissociation inhibitors (GDIs), which antagonize the actions of GEFs and GAPs and regulate the subcellular localization and the cycling of GTPases between membrane and cytosol (Hoffman *et al.*, 2000; Rivero *et al.*, 2002). The active GTP-bound GTPases interact with a myriad of effectors that relay upstream signals, inducing a number of downstream events, including rearrangements of the actin cytoskeleton network and protein kinase-dependent induction of transcription (Hall, 1998; Jaffe and Hall, 2005).

GEFs and GAPs coexist in most cells, increasing the diversity of signals that regulate small GTPases activity (Goitre *et al.*, 2014). Guanine nucleotide dissociation inhibitors (GDIs) are contrary to exchange factors (Menotta *et al.*, 2008). GDI specifically binds GDP-bound GTPase and inhibits GDP release (Rak *et al.*, 2003; Malagnac *et al.*, 2013).

Classification

Ras proteins were the first to be discovered, as their mutation led to various forms of cancer. Subsequently, the researches brought to light several GTPase enzymes with a 3D structure similar to Ras. To date, over 150 members of this family are known, therefore it is counted among the superfamilies.

Ras-like proteins have been named small G proteins. They are divided into five subfamilies, according to their amino acid sequence, their structure and function (Kahn *et al.*, 1992).

In the various organisms these proteins belonging to each subfamily have been extensively studied, and it has been found that they can have overlapping functions. Ras are involved in cell proliferation, Rho in cell morphology, Ran plays a role in nuclear transport and Rab and Arf in vesicular traffic (Goitre *et al.*, 2014).

Ras

Ras superfamily is the largest and most diverse superfamily. In mammals it mainly plays a role in regulating the mechanisms of immunity and inflammation (Colicelli, 2004; Goitre *et al.*, 2014). The GTPases of this group are a general component of the eukaryotic signaling pathway and contribute to the processes of development, proliferation, differentiation and survival of the eukaryotic cell.

It has also been shown that the different isoforms (the highly conserved H-, K- and N-Ras) perform specific biological functions (Haigis *et al.*, 2008; Karnoub and Weinberg, 2008).

Rho

Small GTPases of the Rho family function as molecular switches in separate or correlative signaling pathways that tightly regulate diverse cellular functions (Karnoub *et al.*, 2004). These signaling pathways link the cytoplasmic receptors to activate cytoskeleton reorganization and the subsequent biological effect. They are ubiquitously expressed in eukaryotes and are grouped in three major subfamilies: Rho, Rac, and Cdc42.

The Rho GTPases were thought to be primarily involved in the regulation of cytoskeleton organization (Hall, 1998) required for vesicle trafficking, motility, adhesion and morphogenesis (Kaibuchi *et al.*, 1999; Rivero and Somesh, 2002). Moreover, they play the same significant role in modulating cell polarity, genetic transcription, cell cycle progression, extracellular matrix (ECM) remodeling apoptosis, tumorigenesis (Johnson, 1999; Jaffe and Hall, 2005) and various enzymatic activities (using NADPH oxidase activity to generate the reactive oxygen, ROS) (Li *et al.*, 2014).

Rab

Rab subfamily is the largest subfamily in small GTPase Protein family (Pereira-Leal and Seabra, 2001). In *Arabidopsis* 93 small GTPases family members were detected, and among them 57 belong to Rab subfamily. Rab GTPases modulate membrane traffic processes (vesicle formation, vesicle movement along actin and tubulin networks, membrane fusion). Through these processes the surface proteins are transported from the Golgi apparatus to the plasma membrane and are then recycled.

Over 60 types of Rab GTPases have been found, with the function of stimulating and regulating the docking and fusion of the trafficking vesicles to the membranes; at least one is found for each cytoplasmic organelle and the different membranes have different Rab proteins.

Arf

The Arf protein (ADP ribosylation factor) is the main regulator of the biosynthesis of trafficking vesicle in eukaryotic cells (Donaldson and Jackson, 2011; Ma *et al.*, 2020). It has strong homology with Rab GTPase, but unlike this, which acts in a single step in the transport process to the membrane, Arf acts in several steps. For example, Arf1 protein functions in retrograde transport from Golgi to the ER through recruitment of COPI coated vesicle proteins and in the Trans-Golgi-Network (TNG, ie the formation of vesicles) (Beck *et al.*, 2009); Arf1 also regulates recruitment of clathrin through AP-1, AP-3 and AP-4 complexes (Wennerberg *et al.*, 2005).

Ran

Small GTPase Ran is one of the most expressed in eukaryotes (Moore, 1998). It is involved in nucleocytoplasmic transport, participating both to the import and the export from the nucleus of proteins and RNAs, modulate the formation of cell spindle apparatus, cell cycle progression, structure and function of nucleoplasm, cell redox reaction, RNA synthesis and processing, etc. (Sazer and Dasso, 2000). Nuclear import receptors such as importin beta bind their substrates only in the absence of GTP-bound RAN, forming the tripolymer, and release them upon direct interaction with GTP-bound RAN, while export receptors behave in the opposite way. Thereby, Ran controls cargo loading and release by transport receptors in the proper compartment and ensures the directionality of the transport. Similarly Ran regulates the export of the core (Kim *et al.*, 2001).

Function of Small GTPase in Hyphal Growth

GTPases are molecular switches or timers in many cellular processes (Gilman, 1987). They are involved in signal transduction in response to activation of cell surface receptors, including transmembrane receptors; protein biosynthesis at the ribosome; regulation of cell differentiation, proliferation, division and movement; translocation of proteins through membranes; transport of vesicles within the cell, and vesicle-mediated secretion and uptake, through GTPase control of vesicle coat assembly (Threadgill *et al.*, 1997; Parri and Chiarugi, 2010).

In general, we can summarize from numerous scientific researches, that the different GTPses have the following prevalent roles: Ras sub- family modulation of the gene expression; Rho subfamily regulation of cytoskeleton reorganization, cell wall synthesis,

cell cytokinesis, and MAP kinase cascade pathway; Rab sub-family and Sar/Arf subfamily modulate the trafficking and formation of coated vesicles; Ran subfamily acts in transport into and out of the cell nucleus during interphase and also in mitosis.

Numerous studies have concerned their involvement in hyphal growth in filamentous fungi, in the acquisition of the fundamental polarity for growth directionality and in the underlying cytoskeletal morphological changes.

Ras

Ras GTPases are fundamental for detecting and responding to environmental signals in all eukaryotes (Goitre *et al.*, 2014; Arkowitz and Bassilana, 2015). Their action is expressed through the activation of numerous effectors belonging to kinases pathways (such as the conserved Ras/cAMP/protein kinase A, PKA) (D'Souza and Heitman, 2001; Fimia and Sassone-Corsi, 2001; Gerits *et al.*, 2008). Many studies, using mutant deletion, have shown that Ras plays a key role in hyphal growth and morphogenesis.

S. cerevisiae has two Ras (Ras1 and Ras2), one Rap (Rsr1/Bud1) and one Rheb (Rhb1) ortholog, whereas *Schizosaccharomyces pombe* has only one Ras, one Rheb, but no apparent Rap ortholog.

In particular Ras 2 in *S. cerevisiae* is involved in hyphal growth consequent to starvation (Mösch *et al.*, 1996). Its connection to nutrient availability in fission yeast has been shown also by Chen *et al.* (2019) in 2019. Since the conserved NDR/LATS kinase Orb6 responds to nutritional cues, Orb6 increases the protein levels of a Ras1 GTPase activator, the guanine nucleotide exchange factor Efc25 and regulates Ras1 GTPase activity. These evidences remark the involvement of Ras in promoting cell adaptation, balancing the opposing demands of promoting cell growth and extending chronological lifespan.

In Daniels (2012) it was shown that the formation of the peculiar finger structure, induced in *C. albicans* by the presence of CO_2, is coordinated by Ras 1, and also in *Cryptococcus neoformans* Ras 1 is involved in hypoxia-induced growth (Chang *et al.*, 2014).

In filamentous fungi the role of Ras is crucial for hyphal growth, as reported in literature (Arkowitz and Bassilana, 2015; Kanauchi *et al.*, 1997; Feng *et al.*, 1999; Leberer *et al.*, 2001; Zhu *et al.*, 2009; Huang *et al.*, 2009; Som and Kolaparthi, 1994; Fortwendel *et al.*, 2004; Truesdell *et al.*, 1999; Alspaugh *et al.*, 2000; Waugh *et al.*, 2002; Lee and Kronstad, 2002; Boyce *et al.*, 2005; Bluhm *et al.*, 2007; Zhang *et al.*, 2012a,b; Knabe *et al.*, 2013; Minz Dub *et al.*, 2013).

In filamentous fungi the activation mechanism of Ras, dependent on the GTP/GDP bound cycle, has been studied by means of mutants and the corresponding GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins) have been shed light on.

Numerous studies on the Cdc25 homolog in *Ustilago maydis*, *Yarrowia lipolytica*, *C. albicans*, *A. nidulans* and *Colletotrichum orbiculare* have highlighted its importance in the onset of pathogenesis (in *U. maydis*, Müller *et al.*, 2003), in filamentous growth (*C. albicans*, Shapiro *et al.*, 2009), in the maintenance of polarity (in *A. nidulans* Harispe *et al.*, 2008), in hyphal morphology (*C. orbiculare*, Schubert *et al.*, 2006; Harata and KuboRas, 2014). Therefore Ras, like all GTPases, depends on the GTP/GDP cycling.

GTPase Ras is localized at the level of plasma membrane through the farnesylation of a cysteine in the conserved CAAX tail, in combination with a palmitoylation (Eisenberg *et al.*, 2013; Wright and Philips, 2006). In yeasts and *Candida*, the palmitoylation occurs on a single cysteine residue that allows the membrane anchoring. In filamentous fungi, two conserved palmitoylation cysteines have been identified (Fortwendel *et al.*, 2012; Nichols *et al.*, 2009). Ras localization is spread evenly across the membrane in *C. albicans* and in *Aspergillus fumigatus* (Fortwendel *et al.*, 2012; Piispanen *et al.*, 2011).

The palmitoylation is crucial for several cellular events, e.g. mating in *S. pombe* (Onken *et al.*, 2006), hyphal growth at extreme temperature in *C. neoformans* (Nichols *et al.*, 2009), while in *A. fumigatus* is necessary for hyphal growth, cell wall development, and virulence (Fortwendel *et al.*, 2012). Ras, depending on its location, has specific effectors in the different membrane compartments.

A Ras GTPase associated protein, MadC a GTPase activating protein (GAP), affects the circadian clock output in *N. crassa*, as reported in Polaino *et al.*, 2017, thus it is a target for photoreponses.

Moreover in pathogen filamentous fungi such as *Magnaporthe oryzae* Ras is involved in appressorium formation and in fungal infection. In fact, as shown in Hendy *et al.* 2019, the action of farnesyltransferase β-subunit gene, RAM1, that regulate post-translational farnesylation process can affect the proper localization of many proteins in signal transduction, including Ras, and consequently can modulate hyphal growth and sporulation.

In Martin-Vicente *et al.* 2019, another Ras regulator, a Ras-subfamily-specific guanine nucleotide exchange factors (RasGEFs) in the human pathogen *A. fumigatus*, has shown to affect the properly timed polarity establishment during early growth and branch emergence as well as for cell wall stability, in that it is essential for the integration of multiple signaling networks performed by Ras.

Rho subfamily

Rho

Cdc42 and Rho1 were shown to be required for viability and for cell polarization in *S. cerevisiae* and *S. pombe* (Bi and Park, 2012; Perez and Rincón, 2010; Arkowitz and Bassilana, 2015; Brauns *et al.*, 2020). Moreover Rho1 is involved in cell wall synthesis, in that it modulates glucan synthesis and the specific MAP-kinase pathway.

Among filamentous fungi, Rho 1 is essential for viability and hyphal growth in *C. albicans* and *U. maydis* (Arkowitz and Bassilana, 2019; Wakade *et al.*, 2020; Lu *et al.*, 2014; Corvest *et al.*, 2013; Pham *et al.*, 2012; Smith *et al.*, 2002; Dünkler and Wendland, 2007), while not in *Y. lipolytica* and *Fusarium oxysporum* where it is nonessential (León *et al.*, 2003; Martínez-Rocha *et al.*, 2008). Cell wall

integrity (Arkowitz and Bassilana, 2019; Corvest *et al.*, 2013; Edlind *et al.*, 2005) and virulence (Roemer *et al.*, 2003) of *C. albicans* depends on Rho1; it is the regulatory component of a complex (β-1,3-glucan synthase complex, GSC) responsible of β-1,3-Glucans synthesis (Arkowitz and Bassilana, 2019).

In *U. maydis* the expression alteration of Rho1 determinates growth defects. In Paul *et al.*, 2014 an interaction between Rho1 and an ammonium transporter crucial for hyphal growth regulation has been reported. Studies on *C. neoformans* revealed the presence of three Rho1 homologs, Rho1, Rho10 and Rho11 (Lam *et al.*, 2013), with different roles, such as viability, temperature reactivity and cell wall integrity. Dominant active forms of Rho1 are somewhat defective in capsule formation, suggesting this GTPase may also be important for virulence. Mutations in Rho1 GEF, rom2, exhibited alterations in temperature sensitive growth, actin organization, cell morphology and infectious capacity (Fuchs *et al.*, 2007; Tang *et al.*, 2005).

Conditional rho1 mutants provoke swell at the hyphal tip, apical lyses at the non-permissive temperature, hypersensitivity to cell wall damage and limitation in cell wall integrity MAP kinase signaling in *N. crassa*, (Richthammer *et al.*, 2012). The mutation of GAP and GEF Rho regulators causes similar morphological effects, in addition, a defect in sub-apical branching and sensitivity is observed in GAP mutants if exposed to an actin depolymerization substance (Richthammer *et al.*, 2012; Vogt and Seiler, 2008).

In *A. nidulans* the hydrolysis blockage and the alteration of the GAP domain induces a serious defect in germ tube emergence. In *A. fumigatus*, Rho1 is crucial (Dichtl *et al.*, 2012) and its GEF regulator (Rom2) is fundamental in cell wall integrity (Samantaray *et al.*, 2013). In *Ashbya gossypii*, there are two Rho1 homologs, Rho1a (RhoH) and Rho1b (Rho1): the presence of only one required for viability, but they are both critical for cell wall integrity and remodeling in dimorphic and filamentous fungi (Köhli *et al.*, 2008; Walther and Wendland, 2005; Wendland and Philippsen, 2001). Similar effects are shown in the Tus1 GEF mutant (Lengeler *et al.*, 2013).

In *C. albicans*, *U. maydis*, *A. fumigatus* and *N. crassa*, Rho1 and/or its GEF Rom2 are localized to the hyphal apex (Caballero-Lima *et al.*, 2013; Dichtl *et al.*, 2010; Pham *et al.*, 2012; Richthammer *et al.*, 2012; Samantaray *et al.*, 2013). In *N. crassa* the presence of Rho1 GAP Lrg1 to the hyphal tip is dependent on the growth rate (Vogt and Seiler, 2008).

In *C. albicans* active Rho1 was broadly associated with the hyphal tip, consistent with the Rom2 distribution, and a $\sim$10-fold increase in the levels of active Rho1 was observed at the septum upon cell division (Corvest *et al.*, 2013). In *A. gossypii*, Rho1b was localized predominantly to the hyphal tip, whereas Rho1a was localized to the septum at 25°C (Köhli *et al.*, 2008), while after heat shock conditions both Rho GTPases were localized to the entire hyphal cortex, demonstrating their different roles.

Cdc42

Cdc42 is the main regulator of cell polarity and has been studied in numerous filamentous fungi.

Cdc42 and Rac have similar roles, as shown in *A. nidulans* (ModA and RacA), *N. crassa* (CDC-42 and RAC-1), and *U. maydis* (Cdc42 and Rac1) (Virag *et al.*, 2007; Araujo-Palomares *et al.*, 2011; Lichius *et al.*, 2014; Harris and Momany, 2004; Riquelme *et al.*, 2018) in particular as concern cell polarity machinery. Both anchor the membrane via a typical C-terminal CAAX motif. In *N. crassa*, the spatial distribution of these Rho GTPases changes during development. Cdc42 and Rac regulate the chemotropism exhibited during germ tube development and together with Cdc24, Cdc42, are localized at the apical dome in mature hyphae.

In *Penicillium marneffei* and *C. albicans* Cdc42 is necessary for viability, (Bassilana *et al.*, 2003; Boyce *et al.*, 2003, Boyce *et al.*, 2001; Mahlert *et al.*, 2006; Michel *et al.*, 2002; Ushinsky *et al.*, 2002), as well as cell polarization. The GEF Don1 is critical for cell separation and localizes to endosomal vesicles (Hlubek *et al.*, 2008; Schink and Bölker, 2009; Weinzierl *et al.*, 2002).

In *U. maydis*, Cdc42 is required for virulence and cell separation during budding, yet this protein does not appear to be essential for cell polarity (Mahlert *et al.*, 2006).

Cdc42 has a role in polarized growth in *C. albicans* and strains with reduced levels of Cdc42 arrested growth (Bassilana *et al.*, 2003; Arkowitz and Bassilana, 2015; Ushinsky *et al.*, 2002). Furthermore, Cdc42 and its activator Cdc24 are required for the yeast to hyphal transition, tropic responses and virulence (Bassilana *et al.*, 2003; Brand *et al.*, 2008; VandenBerg *et al.*, 2004), and the level of active Cdc42 is critical for the initiation and maintenance of hyphal growth (Bassilana *et al.*, 2005), as shown in Corvest *et al.* 2013. Also the localization of its GAP is important for maintaining active Cdc42 at apical level (Zheng *et al.*, 2007a,b).

In *A. nidulans* functional analysis of the homologs of the yeast GEF Cdc24 and the yeast GAP Rga1 shown that Cdc24 is important for the establishment of hyphal polarity and localizes to hyphal tips, and that Rga1 is necessary for the suppression of branching in developing conidiophores.

Results showed in Menotta *et al.*, 2007 suggest a fundamental role of Cdc42 in cell polarity development in *Tuber borchii* Vittad. Immunolocalization experiments revealed an accumulation of Cdc42 in the apical tips of the growing hyphae, and very interestingly, the expression of the constitutively active TbCdc42 Q63L transformed in yeast cells switched on a series of evident morphological modifications, since elongated shape, and giant cells and cell aggregations were present.

Moreover, in a few fungi, this highly conserved small GTPase also plays roles in cell separation and/or cytokinesis, which may reflect its importance in septin-dependent processes. In fact, in yeast the ring diameter is set through the dynamic interplay of septin recruitment and Cdc42 polarization, establishing it as a model for size homeostasis of self-assembling organelles (Kukhtevich *et al.*, 2020).

Also in *C. neoformans* Cdc42 is necessary for septin localization (Ballou *et al.*, 2010).

In *A. gossypii*, Cdc42 is essential for spore germination (Wendland and Philippsen, 2001).

In contrast, Cdc42 is not required for viability in other filamentous fungi, e.g. *N. crassa*, *A. nidulans*, *A. niger*, *Magnaporthe grisea* and *Claviceps purpurea*. In conditional cdc42 mutants *N. crassa* hyphae grew in an uncoordinated, zig-zag fashion (Seiler and Plamann, 2003), were distorted (loss of polarity) and periodic reinitiation of growth at the swollen tips were visible

(Araujo-Palomares *et al.*, 2011). Moreover cdc24 mutants exhibited increased tip branching and bem1 mutants resulted in chains of spherical cells (Seiler and Plamann, 2003), even if subsequent studies concluded that bem1 is not critical for hyphal polarity establishment in *N. crassa* (Schürg *et al.*, 2012).

Cdc42 was found to be important also for hyphal branching in *A. nidulans*, *Curvularia trifolii* and *Schizophyllum commune* (Chen *et al.*, 2006; Virag *et al.*, 2007; Weber *et al.*, 2005; Si *et al.*, 2016). In *A. niger* a reduced and delayed germ tube formation was observed in the cdc42Δ mutant, while no significant effects were observed in hyphal morphology (Kwon *et al.*, 2011). It has been reported a role in virulence mechanisms in *M. grisea*, where cdc42 is probably involved in appressorium formation (Zheng *et al.*, 2009; Dagdas *et al.*, 2012). In the plant pathogen *C. purpurea*, Cdc42 was found to be important for pathogenicity, branching and conidation (Scheffer *et al.*, 2005).

In some filamentous fungi Cdc42 is important for mycorrhiza development, as shown in Menotta *et al.*, 2007, where RT-real-time PCR analyses revealed an increased expression of Tb*cdc42* during the phase preparative to the instauration of symbiosis, in particular after stimulation with root exudate extracts.

From research carried out on filamentous fungi, it is shown that in most cases cdc42 has a fundamental role in hyphal growth and hyphal branching.

As concern Cdc42 localization, in *C. albicans* this GTPase is located at apical tip level (Bassilana and Arkowitz, 2006; Crampin *et al.*, 2005; Hazan and Liu, 2002) in F-actin dependent way (Hazan and Liu, 2002), as well as its coordinators Cdc24 and Bem1 (Bassilana *et al.*, 2005; Pulver *et al.*, 2013). The GAP Bem3 localized to a diffuse patch at the hyphal tip, which sometimes appeared as a ring, whereas the GAP Rga2 was largely cytoplasmic (Court and Sudbery, 2007; Zheng *et al.*, 2007a,b). Also in *A. gossypii*, *N. crassa*, *C. purpurea* and *S. commune* Cdc42 is visualized a cortical cap or crescent at the hyphal tip (Araujo-Palomares *et al.*, 2011, Herrmann *et al.*, 2014; Köhli *et al.*, 2008; Weber *et al.*, 2005). Cdc24 and Bem1 are located at the hyphal tip in *A. gossypii* and in *Epichloë festucae*; while in *N. crassa* Cdc24 and Bem1 localized similarly to the hyphal tip (Köhli *et al.*, 2008; Takemoto *et al.*, 2011) and they appear more broadly distributed (Araujo-Palomares *et al.*, 2011; Schürg *et al.*, 2012). While Cdc42 was observed at the hyphal septa, active Cdc42 was not detected at this location (Corvest *et al.*, 2013; Hazan and Liu, 2002).

Rac

The Rac small GTPase is highly homologous to Cdc42, has similar functions but also specific roles. In *P. marneffei* and *C. albicans*, Rac1 is not essential for viability but is important for filamentous growth (Bassilana and Arkowitz, 2006; Boyce *et al.*, 2003; Hurtado *et al.*, 2000). In *P. marneffei*, a rac1 deletion strain produced aerial hyphae, extensive apical branching and altered actin cytoskeleton (Boyce *et al.*, 2003). In *C. albicans*, it seems that the lacking of active rac1 does not cause an alteration in the actin cytoskeleton, but can compromise the invasive growth (Bassilana and Arkowitz, 2006). Mahlert *et al.* (2006) reported that Rac1 is not essential in *U. maydis*, but has a role in cell morphology and hyphal growth.

Two Rac paralogs (Rac1 and Rac2) were found in *C. neoformans* (Ballou *et al.*, 2013). Defective Rac1 impaired completely the hyphal growth (Vallim *et al.*, 2005), but Rac1 nor Rac2 were necessary for virulence in an inhalation model of cryptococcosis (Ballou *et al.*, 2013; Vallim *et al.*, 2005). Rac has a less important role in hyphal branching while it is fundamental in polarized growth during response to specific inducers.

Rac mutants generated hyperbranching at apical tip in *N. crassa*. Rac and Cdc42 together were found to be necessary for polarity establishment and maintenance (Araujo-Palomares *et al.*, 2011).

Rac alone is not essential for viability in the three *Aspergillus* species, *A. fumigatus*, *A. nidulans* and *A. niger*, (Kwon *et al.*, 2011; Virag *et al.*, 2007; Si *et al.*, 2016), but double rac, cdc42 mutants were inviable in *A. nidulans* and *A. niger* (Kwon *et al.*, 2011; Virag *et al.*, 2007). In *A. fumigatus* and *Aspergillus niger* rac deletion mutants exhibited multiple axes of polarity, with increased apical branching (Kwon *et al.*, 2011).

In *M. grisea*, rac1 defect was compatible with life, while conidial and appressorium development was repressed (Chen *et al.*, 2008). In *C. purpurea*, hyphae of both rac and cla4 mutants were shorter and wider than wild-type cells and exhibited hyper-branching (Rolke and Tudzynski, 2008).

The p21-activated kinase Cla4 is an important effector of Rac in fungi (Cotteret and Chernoff, 2002; Boyce and Andrianopoulos, 2011; Bustelo *et al.*, 2007). BcRac, the Rac homolog of the gray mold fungus *Botrytis cinerea*, was found to have Cla4 as effector; BcCla4 protein was found to mediate all the Rac driven processes, including hyphal growth and morphogenesis, conidia production and pathogenicity (Minz-Dub and Sharon, 2017).

In *Trichoderma reesei* Rac1 defect induces hyperbranching, apolar growth and impacts on cellulase activity (Fitz *et al.*, 2019).

Therefore, in filamentous fungi, Rac is central for filament branching.

As regards the localization in *P. marneffei* Rac1 it is found at the level of the hyphal apices and in the division sites, while in *C. albicans* it is widespread in the cell cytoplasm. Instead Rac1 was observed in the nucleus in *C. albicans* (Bassilana and Arkowitz, 2006; Boyce *et al.*, 2003; Vauchelles *et al.*, 2010). The two forms of rac present in *C. neoformans*, on the other hand, are localized at the membrane level, both cellular and organellar (Ballou *et al.*, 2013). The two forms of Rac in *N. crassa* are located in a differentiated way under the apical region, reflecting the different functions attributed to them (Araujo-Palomares *et al.*, 2011).

Rab

Rab GTPases are the largest group of the small GTPases family, plays a pivotal role in the secretion of proteins and serve as regulators of the intracellular membrane trafficking system (Mizuno-Yamasaki *et al.*, 2012; Li and Marlin, 2015; Pfeffer, 2017).

Fungal hyphae extend by apical growth and this process requires continuous polarized trafficking of secretory vesicles to a special structure called Spitzenkörper (SPK).

Rab family orchestrates the vesicle secretion in all eukaryotic cells, in coordination with protein coats, molecular motors, tethering factors and SNAREs (Grosshans *et al.*, 2006). Rab proteins are as signaling molecules, safeguarding the arrival of vesicles to the specific domains (Jin *et al.*, 2011; Hervé and Bourmeyster, 2018).

In addition, in *N.crassa* YPT-1 has been shown to be involved in the CHS traffic activity through tests of immunoprecipitation followed by mass spectrometry that supposed YPT-1 is one of the CHS-1, CHS-4 and CHS-5 interacting proteins (Fajardo-Somera *et al.*, 2015) and through characterization by density gradient centrifugation (Verdín *et al.*, 2015). Benli *et al.* (1996) indicated Ypt31 and Ypt32 (Rab11 orthologs) in *S. cerevisiae* as helpers of vesicular carriers.

RabE, orthologue of Rab11, in *A. nidulans* has been seen to play a role in the transformation of the Golgi's cisternae into post-Golgi (Pantazopoulou *et al.*, 2014). Sánchez-León *et al.* (2015) have found the different localization of the homologs of *S. cerevisiae* YPT-1 (Rab1) and YPT-31 (Rab11) in *N. crassa*: laser scanning confocal microscopy showed that YPT-1 occupied the Spk microvesicular core and YPT-31 was at the macrovesicular layer of the Spk. This research confirmed that distinct Rabs participate in micro and macro vesicles pathway.

Other studies suggested that YPT-31 is not significant for hyphal growth, because through FRAP analysis, the YPT-31 levels in some fungal systems are similar in *N. crassa* where the growth was lower (Jones and Sudbery, 2010; Pantazopoulou *et al.*, 2014).

With quantitative superresolution localization microscopy of live *A. nidulans* cells it has discovered that chitin synthase ChsB, located in Spk, is transported with two different speeds for hyphal growth (Zhou *et al.*, 2018). These findings provide the characteristic intermittent cell growth and shed light on how downstream regulators coordinate the vesicles release. In *S. cerevisiae*, Sec4p is the Rab8 homolog that is involved in the last anterograde vesicles transport (Walworth *et al.*, 1992).

Sec4p, as YPT-31, GS-1 and FKS-1, in *N. crassa* is allocated at the marginal layer of the Spk (Sánchez-León *et al.*, 2015). Sec4p associates with Sec15p and this interaction can control the exocyst assembling (Guo *et al.*, 1999). But the location seen by Sánchez-León *et al* in 2015, suggests that Sec4p can also takes part in the transport of GSC (Sánchez-León and Riquelme, 2015).

Nevertheless, further investigation is necessary to explain the role of Sec4p for GSC and especially how the different Rabs come into play in the pre-exocitary phase.

Arf

In all eukaryotes, membrane/protein trafficking to the plasma membrane is mediated by vesicular transport between different cellular compartments (Donaldson and Jackson, 2011). The small GTPases of the Arf (ADP-ribosylation factor) family guide membrane/protein trafficking. In *S. cerevisiae* has been describing the processes controlled by the ADP ribosylation factors of the Arf/Sar family, in particular vesicle formation and trafficking, cytoskeletal rearrangements, cell polarity and budding (Roth, 1999; Lambert *et al.*, 2007; Suda *et al.*, 2018). *S. cerevisiae* Arf family includes seven members: Arf1, Arf2, Arf3, Arl1, Arl3, Cin4, and Sar1. Ar1 and Arf2 together with Ar11 have regulating role in the secretory pathway. Specifically Arf1/2 are involved in the formation of COPI vesicles and clathrin coated vesicles at cis and trans Golgi cisternae, respectively (Roth, 1999; Suda *et al.*, 2018).

In contrast to *S. cerevisiae*, there are still unknown aspects of Arf/Arl proteins in filamentous fungi. Labbaoui *et al.* (2017) investigated Arf/Arl proteins in *C. albicans* and they identified Arf2 and Arl1 as key regulators of membrane traffic, critical for this fungal hyphal growth and virulence. However ArfB in *A. nidulans* is the *S. cerevisiae* Arf3 homolog, and it is involved in polarized growth and endocytosis (Lee *et al.*, 2008), also as its homolog in *M. oryzae*, Arf6, during asexual development (Labbaoui *et al.*, 2017). Also within the Nematode-trapping (NT) fungus *Arthrobotrys oligospora* was found an ortholog of Arf-GAP of *S. cerevisiae*, Aoglo3, to be involved within the regulation of multiple cellular processes such as mycelial growth, conidiation, environmental adaption, endocytosis and pathogenicity. Moreover, the deletion of Aoglo3 gene, in particular, shown growth defects, a rise in hyphal septation and the Aoglo3 mutant sporulation capacity decreased (Ma *et al.*, 2020).

Fiedler *et al.* (2018) characterized ArfA in *A. niger* and demonstrated that it impacts fungal growth rates, hyphal tip morphology and protein secretion. Subcellular localization experiments of fluorescently labeled proteins associated with cytoskeletal elements provided evidence that the position of the endocytic actin ring is impacted by both lowered and elevated levels of ArfA expression, and ArfA secretion at septa level was hypothesized. Fluorescent microscopy shown that the position of the actin ring at the hyphal tip is affected by the ArfA expression in *A. niger*.

Moreover it was reported in yeast, that Arf3 regulates the Bud1 GTPase activating protein Bud2p and the GTP/GDP exchange factor Bud5; since Bud1 affects Cdc24, and subsequently Cdc42, Arf3 is upstream an important hierarchical GTPase cascade that regulate actin dynamics necessary for polar growth (Hsu and Lee, 2013).

The actin ring is the site of endocytosis, and the location of this cytoskeletal apparatus at the hyphal tip is vital for polar growth (Taheri-Talesh *et al.*, 2008). In *A. nidulans*, the actin ring is maintained precisely 1–2 μm behind the hyphal apex, even in rapidly growing hyphae (Taheri-Talesh *et al.*, 2008). It is seen that the *A. niger* glucoamylase might not only be secreted at the hyphal tip, but also at hyphal septa as previously suspected (Gordon *et al.*, 2000). This is consistent with studies using *A. oryzae*, where the major extracellular protein alpha-amylase was observed to localize in the space between the plasma membrane and cell wall at septa [i.e., the septal periplasm (Hayakawa *et al.*, 2011)]. Septal exocytosis is required for secondary cell wall thickening, intercalary hyphal growth, and branch initiation in filamentous fungi (Hayakawa *et al.*, 2011; Read, 2011). Therefore, ArfA is also required for normal exocytic and/or endocytic processes at the hyphal septum.

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Membrane Transporters, an Overview of the Arbuscular Mycorrhizal Fungal Transportome

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Introduction

Membrane transport in fungi contributes to key aspects of their growth and development, and to their adaptation to multiple ever-changing environments. These transport systems, consisting of one or more proteins embedded in the cell membranes, enable the cell to ensure the uptake of essential nutrients and the efflux of toxic compounds, and play key roles in ion homeostasis and cell signaling. Transport proteins can be simple channels or pores created in the membrane, that facilitate diffusion of compounds down their concentration gradient, or active transporters that require metabolic energy to drive the transport of solutes against their concentration gradient (Busch and Saier, 2004).

One of the main changing extracellular conditions is nutrient availability. Adaptations to these conditions require nutrient-sensing mechanisms in order to supply specific nutrients and to adapt cellular metabolism, growth and development accordingly. One fundamental mechanism for this adaptation is regulated transport across cell membranes through the coordinated uptake or efflux of nutrients across the plasma membrane and intracellular membranes. Recently, the identification of transceptors, membrane proteins that act both as transporters and receptors, establishes a link between transport and signaling (Steyfkens *et al.*, 2018).

Membrane transport has been a main topic of research during the last century and remains an active field of study. Among fungi, most research has been performed in *Saccharomyces cerevisiae*. Even before the emergence of high-throughput genome sequencing techniques, genes encoding transport proteins of this model yeast were cloned and characterized. Progression into the genomics era led to rapid advances in the catalog of available fungal genomes for mining and *in silico* predictions of all membrane transporters of a fungal species (De Hertogh *et al.*, 2002; Grigoriev *et al.*, 2014). It is now known that approximately 10% of a yeast genome corresponds to membrane transporters (De Hertogh *et al.*, 2006) and it is firmly established that many of these transport proteins belong to protein families conserved in organisms ranging from bacteria to humans (André, 1995). The important transport processes operating in several fungal species have been described (Benito *et al.*, 2011; Hora'k, 2013; Dutta and Fliegel, 2018) and excellent comprehensive reviews have summarized the yeast transport processes (Conrad *et al.*, 2014; Ramos *et al.*, 2016). For a detailed description of the membrane transport proteins readers are referred to the Transporter Classification Database (TCDB) webpage (See Relevant Websites Section). This chapter summarizes the general types of transport processes that enable translocation of substrates across fungal membranes and provides an overview of the transport systems characterized so far in arbuscular mycorrhizal (AM) fungi, the most ancient and widespread fungal plant symbionts (Smith and Read, 2008). AM fungi are obligate biotrophs that enhance host plant nutrient acquisition in exchange for carbon compounds. This requires an efficient nutrient transport system in which the transporters are key actors.

Types of Transport Processes

Transport of solutes across biological membranes may occur via simple diffusion, facilitated diffusion or active transport (**Fig. 1**).

Simple Diffusion

Simple diffusion is a passive mechanism that does not involve the input of energy by the fungus to accomplish the movement of gases or uncharged small molecules, such as ethanol, across the membrane lipids. Simple diffusion can be affected the plasma membrane composition and proton motive force, but specific transporters are not usually involved.

Facilitated Diffusion

In facilitated diffusion solutes are transported across the membrane along a concentration with the assistance from membrane proteins, such as channels or transporters (also named facilitators, carriers, or permeases). No metabolic energy is expended since this transport process is governed by the concentrations of the molecule of interest on either side of the membrane. If the concentration on one side of the membrane barrier is higher than on the other side, the movement of molecules through the connecting channel or facilitator will naturally occur, in order to balance the concentrations on both sides of the membrane (Eddy and Barnett, 2007).

Channels

Membrane channels are complexes of membrane proteins or peptides that mediate passive transport of solutes by forming an aqueous diffusion pore. They can operate by different mechanisms, being the most common the gated channel, which requires a

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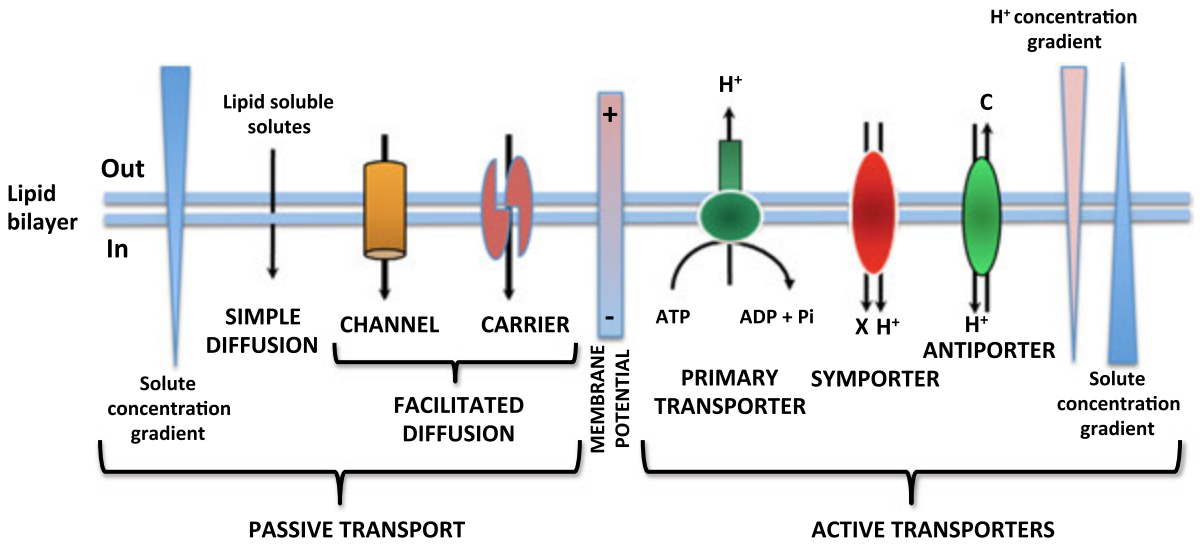

Fig. 1 Types of membrane transport processes. Passive transport processes do not require energy input. Lipid soluble solutes enter the cells via simple diffusion. In facilitated diffusion solutes are transported along a concentration gradient through channels or carriers. Solutes moving against a concentration gradient are transported at the expense of metabolic energy. Primary transporters couple ATP hydrolysis to the transport of a solute against its concentration. The plasma membrane H^+-ATPase generates the proton motive force required for the activity of the secondary transporters. Symporters transport two solutes in the same direction and antiporters in the opposite direction. X (anion or a solute moved against its concentration), C (cation).

trigger, such as a change in membrane potential in voltage-gated channels, to unlock or lock the pore opening. Ion channels play a key role in cation homeostasis, being the best characterized the *S. cerevisiae* efflux voltage-gated K^+ channel Tok1, which is activated by membrane depolarization (Ketchum *et al.*, 1995).

Aquaporins, belonging to the family of major intrinsic proteins, also form pores in the membrane facilitating mainly the transport of water into and out of the cell. Aquaporins form tetramers in the cell membrane, with each of the four monomers acting as a water channel. The driving force for water movement is the gradient of chemical potential of water (osmotic and/or hydrostatic pressure) between both sides of the membrane and predicted to occur in either direction (Finkelstein, 1984). In the fungal kingdom, there are five groups of fungal aquaporins, with two groups of classical aquaporins and three groups of aquaglyceroporins. Although water is the main substrate transported by aquaporin, they can transport other substrates, such as glycerol, H_2O_2, $NH_4{}^+$, boron, urea, and CO_2 (Sabir *et al.*, 2016).

Another important family of channels is the ammonium channel transporter (AMT) family. AMT proteins are homotrimers, in which each subunit contains a narrow pore through which substrate transport occurs. As expected for a channel, NH_3 uniport appears to occur by energy-independent, non-concentrative, bidirectional diffusion (Loque *et al.*, 2007), but $NH_4{}^+$ may be the true substrate requiring in this case metabolic energy (Fong *et al.*, 2007). AMT proteins appear to function as channel/carrier hybrids. *S. cerevisiae* has three AMT homologs named Mep1, Mep2, and Mep 3, that also transport methilammonium and present different affinities for ammonium. Mep2 has been shown to function both as a transporter and as a sensor, generating a signal that regulates filamentous growth in response to ammonium starvation (Lorenz and Heitman, 1998).

Copper (Cu^+) transporters of the Ctr family also function by a channel mechanism that mediate Cu^+ uptake by a passive, membrane potential-dependent mechanism (Dumay *et al.*, 2006). They function as trimmers forming a channel in the membrane. Three homologs have been described in *S. cerevisiae*, two located in the plasma membrane (Ctr1 and Ctr3) and one in the vacuolar membrane (Ctr2) (Puig and Thiele, 2002). However, multiple homologs have been identified in *Schizosaccharomyces pombe* (Beaudoin *et al.*, 2013).

Transporters or carriers

In contrast to a channel, a transporter is assumed to transfer the solute across the membrane by undergoing reversible conformational changes that expose its solute-binding site alternately on each side of the membrane. For example, in *S. cerevisiae* hexoses are taken up by facilitated diffusion through hexose transporters. The high rates of phosphorylation of hexoses together with its subsequent metabolism provide the driving force for continued translocation into the cell. The *S. cerevisiae* hexose transporter family is comprised of 17 members, with different substrate affinities for glucose (Leandro *et al.*, 2009). Given that facilitated diffusion systems operate optimally around their substrate affinity value or Km, it is believed that the wide range of substrate affinities of the *S. cerevisiae* hexose transporters (ranging from 1 to 100 mM) will enable uptake of glucose and other hexoses across the wide range of hexose concentrations present in the yeast environment. However, in other organisms uptake of hexoses requires metabolic energy, as it is presented below for the AM fungal hexose RiMST2 (Helber *et al.*, 2011).

Active Transport

Active transport is the process of moving solutes across a membrane against a concentration gradient and requires metabolic energy. There are two types of active transporters, primary transporters that use adenosine triphosphate (ATP), as a source of energy and secondary transporters that use an electrochemical gradient.

Primary active transporters

Primary transporters are integral membrane proteins that couple ATP hydrolysis to the transport of a solute against its concentration. These transporters play a key role in the generation of gradients of protons and cations across the membranes and in the detoxification of toxic compounds. There are four types of primary transporters: P-type ATPases, V-ATPases, F-ATPases, and ABC transporters.

P-type ATPases

P-type ATPases are transmembrane proteins that couple ATP hydrolysis to the efflux of a cation out of the cytosol. They function as pumps for various cations (H^+, Ca^{2+}, $Cu^{+/2+}$, Zn^{2+}) across the plasma or intracellular membranes. The plasma membrane H^+-ATPase is by far the most extensively studied ATP-driven transport system in yeast (Serrano *et al.*, 1986) and in many other fungi (Ghislain and Goffeau, 1991; Kühlbrandt *et al.*, 2002). Its primary function is to provide an energy source for the transport of nutrients into the cell. The yeast PMA1 H^+-ATPase is an electrogenic enzyme since it extrudes positive charges forming a membrane potential (negative on the inside) that serves as a source of energy for the activity of secondary active transporters (Barnett, 2008).

A sub-family of P-type ATPases comprises the Ca^{2+}-transporting ATPases, which in *S. cerevisiae* play a key role in calcium storage in the vacuoles and in the secretory compartments of the endoplasmic reticulum and Golgi through the activity of the Pmc1 and Pmr1 Ca^{2+}-ATPases, respectively (Cunningham and Fink, 1994). A second sub-family of P-type ATPases is the P_{1B}-type ATPases also known as Heavy Metal ATPases that pump metals across membranes against their electrochemical gradient. The best-characterized member of this sub-family is the product of the *CCC2* gene that is required for exporting Cu^{2+} from the cytosol to the secretory pathway (Yuan *et al.*, 1995).

V-ATPases

V-ATPases consist of peripheral and integral membrane subunits. They are proton pumps that acidify organelles, such as vacuole, lysosomes, endosomes, and Golgi (Kane, 2006; Forgac, 2007). Together with the plasma membrane H^+-ATPase plays a key role in fungal pH and in the generation of the proton gradients that serve as a source of energy for secondary transporters.

F-type ATPases

F-type ATPases are located in the mitochondria and function mainly as an ATP synthase utilizing ADP, inorganic phosphate and an electrochemical gradient of protons.

ABC (ATP binding cassette) transporters

Members of the ATP-binding cassette (ABC) superfamily catalyze the ATP-dependent transport of chemically diverse compounds across the plasma membrane or intracellular membranes. They play key roles in the efflux of xenobiotic compounds, physiological substrates, and toxic intracellular metabolites. The yeast genome contains 30 ABC proteins that are classified in different sub-families (Paumi *et al.*, 2009). The best-characterized yeast ABC transporter is the vacuolar yeast cadmium factor that transports glutathione-complexes to the vacuole and plays a role in detoxifying metals (Li *et al.*, 1996).

Secondary transporters

Antiporters

An antiporter is a membrane protein that transports two molecules at the same time in the opposite direction. Usually the transport of one ion or molecule is against its electrochemical gradient and the movement is powered by the free energy stored in the proton gradient generated by the plasma membrane H^+-ATPase or the vacuolar ATPase. A primary example of this type of transporters is the family of Na^+/H^+ antiporters or Na^+/H^+ exchangers (NHEs), proteins essential to keep the Na concentrations low in the cytosol (Dutta and Fliegel, 2018). For example, the genome of *S. cerevisiae* encodes three Na^+/H^+ antiporters: the plasma membrane Nha1 that has similar affinity for Na^+ and K^+ (Bañuelos *et al.*, 1998), Nhx1 that is the main proton-coupled antiporter mediating potassium or sodium transport across the vacuolar membrane (Cagnac *et al.*, 2007) and ScKha1p that is localized in the Golgi (Maresova and Sychrova, 2005).

Two other examples of antiporters are the family of calcium proton exchangers that play a key role in calcium homeostasis (Cunningham and Fink, 1996) and the cation diffusion facilitator (CDF) family that function by H^+ antiport for metal efflux (Montanini *et al.*, 2007). Members of the CDF family transport heavy metals including cobalt, cadmium, iron, zinc and possibly nickel, copper and mercuric ions and are involved in metal tolerance/resistance by efflux (Paulsen and Saier, 1997).

Symporters

Symporters are proteins that simultaneously transport two molecules across a membrane in the same direction. The most widely held model for this process has the molecules binding to the transport protein that is exposed on the external surface of the

membrane. In an energy-dependent process, these molecules are driven through a central region of the protein to emerge on the opposite side of the membrane. The protein molecule remains stationary. Examples of symporters are the family of sulfate transporters that mediate transport of SO_4^{-2} by using the electrochemical gradient of protons (Cherest *et al.*, 1997) and the Pho family of phosphate transporters that also use the proton gradient as a driven force for translocation of phosphate ions in a symport manner (Bun-ya *et al.*, 1991). The potassium transporters of the Trk family normally function also as H^+/K^+ or Na^+/K^+ symporters and are thought to be driven by the membrane potential created by the plasma membrane H^+-ATPase. Fungi usually have two Trk systems (Corratgé-Faillie *et al.*, 2010). In *S. cerevisiae*, Trk1 and Trk2 strongly differ in their affinity for potassium, being Trk1 and high-affinity K transporter and Trk2 a low-affinity one (Ko and Gaber, 1991).

Amino acid transport is also a primary example of a symport transport process since amino acids are generally present in the natural environment at concentrations far lower than those found in the cytoplasm. Amino acids are, therefore, taken up against its concentration gradient by members of the amino acid transporter (AAT) family (Gournas *et al.*, 2018). Members of the proton-dependent oligopeptide transporter (POT/PRT) family also function by a proton symport process (Hauser *et al.*, 2001).

The mechanism of transport of several secondary transporters has not been determined yet. This is the case of the members of the Zinc (Zn^{2+})-Iron (Fe^{2+}) permease (ZIP) family (Gaither and Eide, 2001). The energy source for metal translocation is not ATP and several driving forces, including bicarbonate, pH dependence or phosphorylation, have been proposed.

Why Arbuscular Mycorrhizal Fungi?

Arbuscular Mycorrhizal (AM) fungi, belonging to the subphylum Glomeromycotina within the Mucoromycota (Spatafora *et al.*, 2016), are complex but extremely successful soil-borne microorganisms. They are obligate biotrophs that establish a compatible interaction with plants, called arbuscular mycorrhiza, by either avoiding or suppressing plant defense reactions while redirecting host metabolic flow to their benefit without being detrimental to their host (Gianinazzi-Pearson, 1996). AM fungi have accompanied land plants through evolution and survived across periods of important environmental change to become ecologically and agriculturally important symbionts that improve overall fitness of most land plants (Smith and Read, 2008). Substantial evidence supports their use as natural biofertilizers and bioprotectors in agriculture (Gianinazzi *et al.*, 2010; Berruti *et al.*, 2016).

The main benefit for the plant is an improved mineral nutrition, especially of phosphorus, nitrogen, zinc and copper. The fungus develops in the soil an extensive network of extraradical hyphae that overgrows the soil surrounding the plant roots and functions as an additional absorptive surface area for the plant increasing its capacity to forage nutrients beyond their depletion zone (**Fig. 2(A)** and **(B)**). In return, the plant provides to the fungus the carbon compounds, either in the form of carbohydrates or lipids, required to complete its life cycle. This nutrient exchange between partners occurs in the arbuscules, specialized and highly branched structures formed by the fungus in the root cortical cells (**Fig. 2(C)**). AM fungal nutrient uptake from the soil and coordinated exchanges between the plant and the fungus at the symbiotic interface formed in the cortical cells colonized by arbuscules is controlled by the coordinated regulation of specialized plant and fungal membrane transport systems. Despite nutrient exchange between partners and nutrients themselves seem to be at the heart of the evolutionary success of this symbiosis, the mechanisms underlying these processes are not yet fully understood. So far, molecular players controlling nutrient transport processes in the symbiosis have been mainly identified and characterized on the plant side (Wipf *et al.*, 2019). However, much less is known on the fungal side.

AM fungi are obligate biotrophs incalcitrant to pure culture in the absence of a host plant, possess coenocytic hyphae and multinucleated spores and so far no sexual reproduction has been described yet (Gianinazzi-Pearson *et al.*, 2012). These characteristics introduce inherent limitations in the application of standard techniques, such as genetic transformation and mutant generation/characterization, for their study, which greatly hinders advances in the knowledge about gene function in these crucial group of fungi. Nevertheless, significant progress has been made recently in our understanding of their biology through the release and subsequent mining of genome sequences. *Rhizophagus irregularis* (formerly named *Glomus intraradices*)

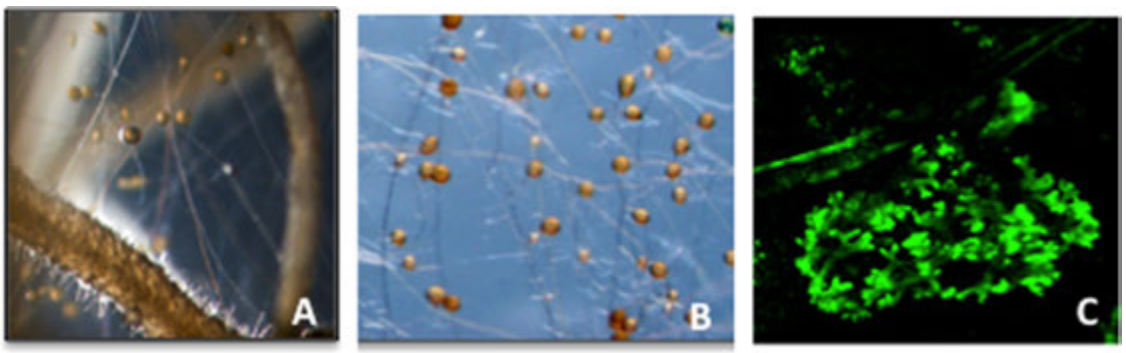

Fig. 2 (A) *In vitro* culture of *R. irregularis* in association with carrot roots. (B) Extraradical mycelium of *R. irregularis* showing hyphae and spores. (C) Arbuscule of *R. irregularis* detected by wheat germ agglutinin-FITC labeling on a root section.

DAOM197198 was the species chosen for the first genome-sequencing project on AM fungi (Tisserant *et al.*, 2012). Nowadays, the genomes or transcriptomes of several AM fungi, such as *Rhizophagus clarum* (Kobayashi *et al.*, 2018), *Diversispora epigaea* (formerly *Glomus versiforme*; Sun *et al.*, 2019), *Gigaspora margarita* (Salvioli *et al.*, 2016), and *Gigaspora rosea* (Tang *et al.*, 2016) have been sequenced and annotated. These gene repertoires have enabled the identification of genes potentially encoding for transport proteins in different AM fungal species.

AM Fungal Transportome

Current knowledge on the transportome in AM fungi, that is, the complete repertoire of fungal genes encoding membrane transporters is still low. Much of the information generated on the Glomeromycotina transportome focuses on the model species *R. irregularis*.

Primary Active Transporters

P-type ATPases

As mentioned above, P-ATPases are enzymes that couple ATP hydrolysis to the transport of a cation out of the cytosol, being the plasma membrane H^+-ATPase an essential protein for the functioning of the secondary transporters. In the Glomeromycotina, two H^+-ATPases genes have been characterized in the AM fungus *Funneliformis mosseae* (formerly, *Glomus mosseae*) (Ferrol *et al.*, 2000; Requena *et al.*, 2003). Both genes are differentially expressed in the different developmental stages of the fungus and are more highly expressed in the intraradical than in the extraradical mycelium. Expression of *GmHA5* is up-regulated by phosphate, the main nutrient transferred by the fungus to the plant, suggesting that this isoenzyme provides the proton motive force required for phosphate uptake by a symport transport process. Interestingly, *GmHA5* was found to be highly expressed in the arbuscules (Balestrini *et al.*, 2007). As found for *F. mosseae*, *R. irregularis* also expresses two plasma membrane H^+-ATPase genes (Tisserant *et al.*, 2012). A total of 35 genes putatively encoding P-type ATPases were identified in the *R. irregularis* transcriptome, ten potentially encoding Ca-ATPases likely involved in Ca homeostasis and four encoding heavy metal ATPases likely involved in copper/metal homeostasis (Tamayo *et al.*, 2014).

ABC transporters

The first ABC transporter described in the Glomeromycotina was GintABC1, an ortholog of the yeast cadmium factor (ycf1) that transports metal-glutathione complexes into the vacuoles and that was suggested to play a role Cu and Cd tolerance in *R. irregularis* (González-Guerrero *et al.*, 2010). Thirty-one more genes putatively encoding ABC transporters were identified in the *R. irregularis* transcriptome. They include ABC multidrug transporters, multidrug resistance-associated proteins, MRP-like ABC transporters and oligomycin resistance ATP-dependent permeases.

Transporters of Macronutrient Ions

Phosphate transporters

Improved phosphorus uptake is the main benefit of the AM symbiosis for the host plant. The first step of symbiotic phosphorus uptake is phosphate uptake from the soil by the extraradical mycelium, which is believed to occur through by a phosphate/proton symporter of the *S. cerevisiae* Pho84 family. Phosphate transporters have been described in *D. epigaea* (Harrison and van Buuren, 1995), *F. mosseae* (Benedetto *et al.*, 2005), *R. irregularis* (Maldonado-Mendoza *et al.*, 2001) and *G. margarita* (Xie *et al.*, 2016). These transporters are expressed in the extraradical mycelium, suggesting a role in phosphate uptake from the soil. However, this hypothesis has not been proven due to the lack of stable transformation systems for AM fungi. By using yeast as a heterologous expression system, it was shown that the *D. epigaea* and G. *margarita* phosphate transporters *GvPT* and *GigmPT* encode high-affinity transporters that are dependent on the metabolic energy generated by the H^+-ATPase. These transporters are also expressed in the intraradical mycelium suggesting a second role in phosphate reabsorption from the apoplast of the symbiotic interface created in the arbuscule-colonized cortical cells (Benedetto *et al.*, 2005). Inactivation of *GigmPT* by host-induced gene silencing impaired arbuscule development, supporting the view that phosphate itself acts as a signal in the establishment of the symbiosis and that GigmPT is a transceptor that is involved both in Pi transport and Pi sensing (Xie *et al.*, 2016).

The *R. irregularis* genome also has homologs of the *S. cerevisiae* Na^+/phosphate symporter Pho89p and of the low-affinity vacuolar phosphate transporter Pho91 (Tisserant *et al.*, 2012). However, these transporters have not been characterized yet.

Ammonium transporters

Two high-affinity ammonium transporters, GintAMT1 (López-Pedrosa *et al.*, 2006) and GintAMT2 (Pérez-Tienda *et al.*, 2011), and one low-affinity GintAMT3 (Calabrese *et al.*, 2016) have been characterized in *R. irregularis*. GintAMT1 is highly expressed in the extraradical mycelium and is believed to mediate soil NH_4^+ uptake when NH_4^+ is present in low concentrations, for example, in acidic conditions. However, GintAMT2 might be involved in the recovery of NH_4^+ leakage during fungal metabolism, as it is highly expressed in the arbuscules. Finally, the low-affinity ammonium transporter GinAMT3, which is also expressed in the

arbuscules, could be also involved in NH_4^+ retrieval form the symbiotic interface but when the NH_4^+ concentrations are high. Both the high-affinity and low-affinity NH_4^+ transport processes require metabolic energy provided by the plasma membrane H^+-ATPase, as both transport activities where inhibited when the extraradical mycelium was supplied with the ionophore carbonylcyanide m-chlorophenylhydrazone and the ATP-synthesis inhibitor 2,4-dinitrophenol (Pérez-Tienda *et al.*, 2012). Orthologs of the *R. irregularis* AMT genes have been identified in the genomes of *G. rosea*, *G. margarita*, and *D. epigea*, but they have not been characterized yet.

Nitrate transporters

Although AM fungi show a clear preference for NH_4^+ they are also able to take up nitrate (Villegas *et al.*, 1996). Only a high-affinity nitrate transporter (GiNT) has so far been partially characterized in *R. irregularis* (Tian *et al.*, 2010). A role for GiNT in transporting nitrate into the extraradical mycelium was suggested, as its expression is regulated by nitrate availability.

Other transporters of inorganic ions

Despite several studies have shown the ability of AM fungi to improve potassium or sulfur nutrition of their host plants (Smith and Read, 2008), the fungal transporters involved in the symbiotic transport process of these nutrients have not been described yet. Genes encoding putative potassium transporters of the Trk (acronym of transport of K) and HAK (high-affinity K uptake) families and sulfate transporters have been identified in the genomes of the sequenced AM fungi. However, further research is needed to elucidate their role in the mycorrhizal transport pathway of these macronutrients.

Metal Transporters

Metal ions are essential micronutrients for fungal metabolism, as they are essential components of a wide variety of metalloproteins, transcription factors, and other proteins. Fungi possess a repertoire of metal transport proteins mediating active uptake of metals when present at low concentrations (Radisky and Kaplan, 1999) and intracellular transport systems delivering metals to the different organelles (Luk *et al.*, 2003). However, high metal ion concentrations are toxic. Under these conditions, transport systems pumping metals out of the cytosol and compartmentalizing excess metal in the vacuoles contribute to metal detoxification (Ruotolo *et al.*, 2008). The importance of metal transporters in AM fungi relies not only on their role on AM fungal homeostasis but also on the importance of these fungi on host plant homeostasis (Ferrol *et al.*, 2016). In fact, AM fungi increase the uptake of low mobility metal micronutrients when plants grow in soils deficient in these elements and, on the other hand, to alleviate metal toxicity in contaminated soils. Several genes putatively encoding proteins implicated have been identified in the genomes of the sequenced AM fungi and mined in the genome of *R. irregularis* (Tamayo *et al.*, 2014).

Zinc transporters

Two families of Zn transporters have been described in *R. irregularis*: the ZIP and the CDF families. The *R. irregularis* ZIP family is composed of five members that, although have not been characterized yet, are likely involved in transport of Zn and/or other metal ion substrates from the extracellular space or organellar lumen into the cytoplasm. In contrast, CDF proteins transport Zn and/or other metal ions from the cytoplasm into the lumen of intracellular organelles or to the outside of the cell (Eide, 2006). Out of the six genes potentially encoding CDF transporters in *R. irregularis* only *GintZnT*1 has been functionally characterized (González-Guerrero *et al.*, 2005). GintZnT1 was shown to be involved in Zn detoxification, as its expression in yeast decreased Zn cytosolic levels and its expression pattern in response to Zn correlated with the accumulation pattern of Zn in the fungal vacuoles.

Copper transporters

The *R. irregularis* genome has two genes encoding Cu transporters of the Ctr family and a CTR-like protein (Gómez-Gallego *et al.*, 2019). Members of this family mediate Cu^+ uptake by a passive, membrane potential-dependent mechanism. Functional analyses in yeast and gene expression patterns in response to copper revealed that *RiCTR1* encodes a plasma membrane Cu transporter that is involved in Cu uptake by the extraradical mycelium and *RiCTR2* a vacuolar transporter involved in Cu mobilization of vacuolar stores. The third gene RiCTR3 produces, as a consequence of an alternatively spliced event, two transcripts, RiCTR3A and RiCTR3B. RiCTR3B was suggested to be a receptor involved in Cu tolerance, as it confers copper tolerance to the metal sensitive Δ*yap-1* mutant yeast and its expression is highly induced by Cu toxicity in the extraradical mycelium (Gómez-Gallego *et al.*, 2019).

Iron transporters

Three members of the reductive pathway of Fe assimilation, the ferric reductase (RiFRE1) and the high-affinity Fe permeases (RiFTR1–2), have been characterized in the model fungus *R. irregularis* (Tamayo *et al.*, 2018). In this high-affinity uptake process, the metal is reduced from Fe^{3+} to Fe^{2+} by membrane-bound ferrireductases, and then it is rapidly internalized by the concerted action of a ferroxidase and a permease that form a plasma membrane protein complex (Kwok *et al.*, 2006). Functional analyses in yeast mutants and gene expression patterns in response to Fe indicate a role for the plasma membrane RiFTR1 in Fe acquisition by both the extraradical and intraradical mycelium and for FiFTR2 in Fe homeostasis under Fe-deficient conditions.

Arsenite efflux pump

Although specific transporters for non-essential metals, such as arsenate, unlikely exist in AM fungi, uptake of these non-essential and toxic metals is mediated by transporters of essential metals or even through other type of transporters. For example, in *R. irregularis* uptake of the metalloid arsenate occurs via the high-affinity phosphate transporter GiPT (González-Chávez *et al.*, 2011). However, arsenite detoxification is partially mediated via the specific efflux pump of arsenite GiArsA (González-Chávez *et al.*, 2014).

Transporters of Organic Compounds

Organic nitrogen transporters

Besides inorganic nitrogen, AM fungi can take up from the soil organic nitrogen in the form of amino acids, such as glycine, glutamic acid and arginine, and in the form of small peptides (Hawkins *et al.*, 2000). So far, an amino acid permease (GmosAPP1) and a dipeptide transporter (RiPT2) have been characterized in *F. mosseae* and *R. irregularis*, respectively. GmosAPP1 is expressed in the extraradical mycelium and transports non-polar and hydrophobic amino acids, such as proline, serine, glycine, and glutamine, by a proton-coupled, pH and energy-dependent process (Cappellazzo *et al.*, 2008). The *R. irregularis* dipeptide transporter RiPTR2 is expressed both in the extraradical and intraradical mycelium and has been suggested to play a role in the uptake of small peptides from the soil and the reuptake of peptides from the interfacial apoplast (Belmondo *et al.*, 2014). As yet uncharacterized oligopeptide transporter was also found to be overexpressed in the intraradical mycelium of *G. rosea* (Tang *et al.*, 2016).

Sugar transporters

As obiligate biotrophs, AM fungi need the supply of carbon compounds from the host plant for their growth and metabolism. Based on stable isotope labeling experiments, it has long been considered that AM fungi receive carbohydrates and specifically glucose from the plant (Pfeffer *et al.*, 1999; Trepanier *et al.*, 2005). However, it has been recently shown that despite lipids comprise up to 95% of spore dry weight (Bécard *et al.*, 1991) and up to 47% of hyphal volume in some regions of the extraradical mycelium (Bago *et al.*, 2002), AM fungi are auxotrophs for fatty acids and they also receive lipids from the plant host (Bravo *et al.*, 2017; Jiang *et al.*, 2017); however, the fungal transporter mediating lipid uptake remains uncharacterized.

Sugar uptake from the apoplast of the symbiotic interface created in the arbuscule-colonized cortical cell is mediated *in R. irregularis* by the monosaccharide transporter RiMST2 (Helber *et al.*, 2011). RiMST2 is a high-affinity monosaccharide transporter that operates by a H^+ cotransport process with a broad substrate spectrum. Besides glucose, it is able to transport xylose, mannose, and fructose with decreasing affinity in that order. RiMST2 is primarily expressed in intraradical fungal structures, and its silencing results in impaired formation, malformed arbuscules. Additional monosaccharide transporters (RiMST3, RiMST4, RiMST5 and RiMST6) and a putative sucrose transporter (RiSUC1) have been identified in *R. irregularis* (Helber *et al.*, 2011). However, their contribution to sugar uptake has not been clarified yet.

Water Channels

Aquaporins have been functionally characterized in *R. irregularis* and *R. clarus*. In *R. irregularis*, the two identified aquaporins RiAQP1 and RiAQP2 were found to be expressed both in the extraradical mycelium and in the arbuscules (Li *et al.*, 2013). Three genes potentially encoding aquaporins have been identified in *R. clarus*. *RAQP3 aquaporin 3* was most highly expressed in intraradical mycelia and encodes an aquaglyceroporin responsible for water transport across the plasma membrane. Knockdown of *RcAQP3* by virus-induced gene silencing revealed a role for this water channel in phosphate translocation from the outer to the inner hyphae (Kikuchi *et al.*, 2016).

Concluding Remarks

During the last decades research on membrane transport has made continuous progress and still remains an active field of study. A tremendous number of integral membrane proteins have been characterized in different fungi, especially in the model yeast *S. cerevisiae*, and the mechanisms by which their function is regulated have been elucidated.

Despite nutrient transport has been one of the more extensively studied aspects of the AM symbiosis; current knowledge on AM fungal transporters is still in its infancy. Significant progress has been made recently through the release and subsequent mining of genome sequences. The repertoire of annotated genes potentially encoding membrane transport proteins represents valuable sequence data for further functional validation. *S. cerevisiae* has been shown to be a useful tool to determine the function of the AM fungal transporters identified so far. Although AM fungi can not be genetically modified, development of host-induced and virus-induced gene silencing techniques of AM fungal genes has allowed functional analysis of a few genes expressed in the intraradical mycelium. It is expected that developments and advances in technologies, such as – omics, live cell imaging, stable isotope tracking and genetic manipulation, will provide a holistic view of the transport systems operating in AM fungi. This knowledge will be crucial to understand their biology and adaptation to environmental stresses.

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Fungal Secondary Metabolism

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Introduction

Primary metabolism is referred to the biochemical reactions that lead to metabolites which are required for the growth and maintenance of cellular functions. These natural compounds are the precursors for the formation of essential biomolecules such as nucleic acids, proteins, carbohydrates and lipids. Vital primary metabolism is related with the phase of rapid microbial growth, the so-called logarithmic growth phase. A maximum accumulation of primary metabolites is present at the end of that phase, while in the following stationary phase the metabolites derived from primary metabolism may be further transformed to other products including secondary metabolites (SMs).

SMs are low molecular-weight compounds derived from primary metabolites via specialized pathways. They are produced mainly by microorganisms and plants, typically only by a limited range of species (Vinale *et al.*, 2009).

The fungi produce a huge number and variety of SMs, many of which have interesting biological activities. They serve important functions such as: (1) competitive weapons against bacteria, fungi, plants, insects and animals; (2) metal transporting agents; (3) agents of symbiosis between microbes and plants, nematodes, insects, etc.; (4) sexual hormones (Demain and Fang, 2000).

SMs are not essential for vegetative growth in pure culture but are commonly obtained during the stationary phase, which is often related to differentiation and sporulation. Batch rather than continuous culture usually favors secondary metabolite production (Demain and Fang, 2000).

These natural compounds show an enormous variety of structures and biosynthetic origins, and there have been many examples of SMs with chemical features previously unreported. Moreover, the production is limited to one or a few organisms, which may not be closely related, and it differs significantly among diverse strains of the same species (Herbert, 1994).

Fungi have been exploited to yield various SMs useful for application and subsequent production of valuable supplies such as antibiotics, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, and hormones. Fungal SMs used in medicine include the cyclosporines (immunosuppressants) from *Tolypocladium inflatum*, the cholesterol-reducing compound lovastatin, produced by *Aspergillus terreus* and the antibiotic penicillin biosynthesized by *Penicillium chrysogenum*. *Gibberella fujikuroi* releases gibberellins, that are plant hormones also known as virulence factor of this fungus on plants. Although interest in these beneficial metabolites is significant to humankind (in medicine, industry and agriculture), fungi also produce deleterious SMs with toxic effects (e.g., mycotoxins) (Demain and Fang, 2000). These include the cytotoxic and antiproliferative aspyridones from *Aspergillus nidulans*, the aflatoxins produced by *Aspergillus flavus*, and gliotoxin produced by *Aspergillus fumigatus*, for which toxicity has been ascribed to a disulfide bridge present in this metabolite (Brakhage, 2013).

Many examples of fungal metabolites with toxic effects (mycotoxicosis) on animal organisms have been reported in the veterinary literature. Starting from 1970 several cases have been recorded and these findings made a major impact on the scientific community, resulting in the present concern about the effects of mycotoxins on human health (Campbell and Stoloff, 1974; Marroquín-Cardona *et al.*, 2014). Aflatoxins, ochratoxins, fumonisins, deoxynivalenol, and zearalenone are the major mycotoxins in terms of public health concern. These fungal metabolites can cause adverse effects in humans and due to the cosmopolitan nature of mycotoxigenic fungi contribute to the worldwide incidence of mycotoxins in food and feed (Lee and Ryu, 2017).

Biochemistry and Biological Impact of Fungal Secondary Metabolites

Fungal SMs are formed as families of closely related compounds resulting from biosynthetic enzymes less specific to their substrate than those of primary metabolism (Hanson, 2003). The yield of different family members can be modified by changing the composition of the growth medium, the environmental conditions and by mimicking biotic interactions (i.e., co-cultures) (Vinale *et al.*, 2017; Pan *et al.*, 2019). Different members of a family frequently demonstrate diverse biological activities (Hanson, 2003).

The majority of SMs belong to one of the following families: (1) Polyketides and fatty acids; (2) Terpenoids and steroids; (3) Phenylpropanoids; (4) Alkaloids; (5) Specialized amino acids and peptides (Hanson, 2003).

Though not crucial for their survival, fungi bio-synthetize numerous SMs of the mentioned families with different chemical structures and properties. Especially filamentous Asco- and Basidiomycota are rich sources of polyketides, non-ribosomal peptides, terpenes and indole alkaloids that are biosynthesized via specialized pathways (Herbert, 1994).

Fungal secondary metabolism often is connected to specific stages of morphological differentiation such as asexual sporulation. The produced SMs are incorporated into structural elements of the cell or released into the environment as volatiles or dissoluble substances. They hence play important roles in regulating interactions between organisms. Examples of fungal compounds involved in biotic interactions are phytotoxins, produced by fungal pathogens as virulence factors for plants, mycotoxins,

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produced during the colonization of crops by specific fungi and capable of causing disease and death in humans and other animals, pigments, with antioxidant activity, and antibiotics, that are considered natural products capable of killing or inhibiting microbes. It is important to underline that biological activities are not essentially limited to one specific group or single metabolites and a single fungal metabolite can produce different effects on specific targets (Karlovsky, 2008).

Antibiotics are produced by fungi for gaining an advantage in competition, permitting the microbe to occupy space and to acquire access to nutrients. Most of the useful fungal SMs have been isolated and characterized via a screening approach following "bioassay-guided fractionation", meaning that the bioactivity of the compound(s) are constantly monitored during the isolation procedure by employing *in vitro* or *in vivo* test systems for detecting the biological activity of an extract or a pure substance (bioassay). All generated fractions are tested for biological activity, and those showing the desired bioactivity are further processed until the bioactive compound is obtained in a pure form (Ghisalberti, 2003; Colegate and Molyneux, 2007).

Main Classes of Fungal Secondary Metabolites

Polyketides

Polyketides are the amplest class of fungal SMs and are biosynthesized by type I polyketide synthases (PKSs). Short-chain carboxylic acids, typically acetyl-coenzyme A (acetyl-CoA) and malonyl-CoA, are condensed to form carbon chains of variable lengths. The main difference between polyketides and fatty acids is the full reduction of the β-carbon in fatty acids, which is an optional event in polyketide synthesis. The variety of fungal polyketide chemical structures is a consequence of many iteration reactions, the number of reduction reactions, which extender unit is used and, in the case of aromatic polyketides, cyclization of the nascent polyketide chain. Other structures are related to the introduction of diverse post-polyketide-synthesis steps.

Non-ribosomal peptides

Non-ribosomal peptides may contain non-proteinogenic amino acids and are synthetized by enzymes named non-ribosomal peptide synthetases (NRPSs). The diversity among non-ribosomal peptides depends on the number of amino acids (peptide length), if the peptide is cyclized, and variations in the functions of the NRPS domains.

Terpenes and terpenoids

Terpenes are linear or cyclic compounds composed of isoprene units which can be saturated or unsaturated, and modified in various ways. Terpenoids are derivatives of terpenes that contain additional functional groups and oxidized methyl groups moved or removed at various positions. Terpenoids are divided into monoterpenes, sesquiterpenes, diterpenes, sesterpenes, and triterpenes depending on their carbon units. Monoterpenes are produced from geranyl pyrophosphate, sesquiterpenes are generated from farnesyl pyrophosphate, and diterpenes and carotenoids are biosynthesized from geranylgeranyl pyrophosphate. The defining enzyme in terpene biosynthesis is terpene cyclase, which generates different terpenes from different diphosphates.

Indole alkaloids

Indole alkaloids are typically resulting from tryptophan and dimethylallyl pyrophosphate. Occasionally other amino acids than tryptophan are used as precursors (Keller *et al.*, 2005).

The overall pathway of carbon from sugars up to the formation of the main classes of fungal SMs is given in **Fig. 1**. In particular, it is possible to follow the biosynthetic relationship of these natural products (Hanson, 2003).

Biological Functions and Ecological Roles of Fungal Secondary Metabolites

SMs can provide self-protection, they may act as mediators for communication with other organisms and are virulence factors for plant and animal pathogens. Moreover, they serve as defense molecules against other microbes (antibiotics) thus contributing to the organization of microbial consortia.

The role of fungal SMs in microbial interactions is mainly referred to the production of antibiotics, recognized as weapons to kill or inhibit competitors. For example, penicillin was discovered for the antibiosis of *Penicillium* spp. contaminating *Staphylococcus* spp. Recently their function as signaling molecules in microbial consortia has been demonstrated and several microorganisms have established diverse mechanisms to cope with antimicrobial molecules produced by antagonists. Moreover, some antibiotics also promote the growth of other microbes, act as chemoattractants, or function in a specific communication process (quorum sensing, with the secretion of virulence factors and/or biofilm formation). Another example is that production of 2,4-diacetylphloroglucinol, a specific antibiotic released by *Pseudomonas fluorescens*, is reduced by fusaric acid formed by *Fusarium oxysporum* through repression of the responsible biosynthetic genes. In contrast, fusaric acid and other metabolites promote the colonization of *F. oxysporum* hyphae by *P. fluorescens* (Fox and Howlett, 2008).

Some examples of fungal SMs are given in **Fig. 2**.

Close physical interactions among fungi and bacteria results in a specific regulation of fungal secondary metabolism that may increase or decrease the production of specific molecules. In some cases, these interactions may also induce the ex-novo production of a single metabolite (i.e., the production of orsellinic acid-derived polyphenols by *Aspergillus nidulans*) (Kusari *et al.*, 2012).

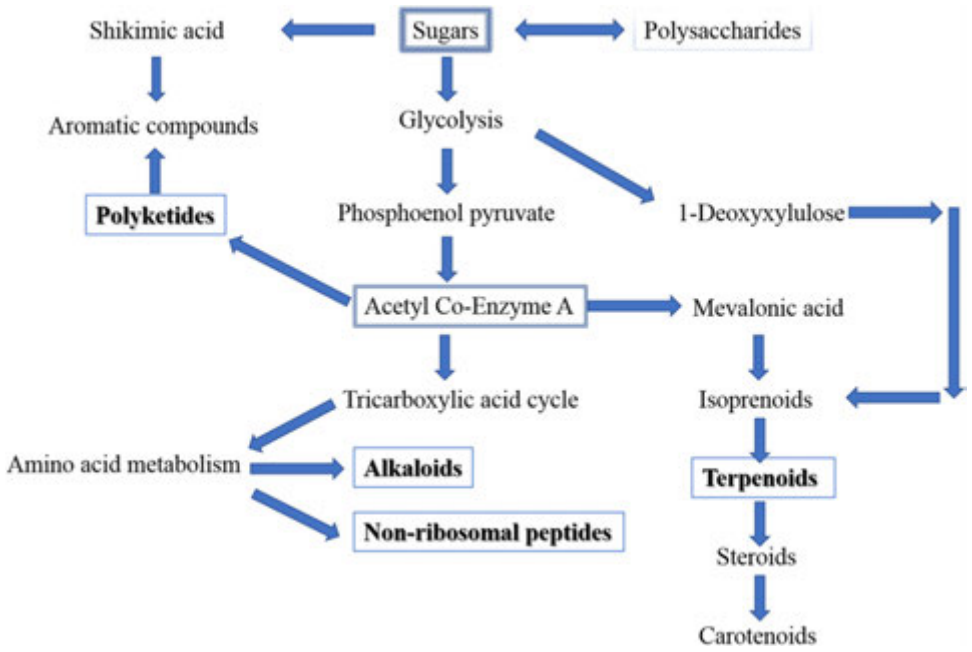

Fig. 1 Biosynthetic relationship of fungal secondary metabolites. The main classes of fungal secondary metabolites are given in bold. Acetyl Co-Enzyme A is the molecule from which the main metabolites are derived.

SMs help fungi to be competitive in an ecological niche. In this context it is important to underline that the effects of natural products are depending on the ecological concentrations and a single metabolite can function as a signal rather than as a toxin. Gradient-dependent effects of phenazine from *Pseudomonas aeruginosa* on *Aspergillus* spp. have been demonstrated. A high concentration of this compound is antibiotic while a low to moderate concentration induces sporulation in the fungus. Many examples of induction of fungal SMs when fungi are confronted by other microbes are reported in literature that represent a sort of microbial 'language' (Keller, 2019).

Dose-effect responses of fungal metabolites on plant growth and development have also been recognized. This is the case for some *Trichoderma*- derived SMs (i.e., 6-pentyl-α-pyrone, harzianolide, harzianic acid) that may act as auxin-like compounds. Typically, these molecules have an optimum activity among 10^{-5} and 10^{-6} M while are inhibitory at higher concentrations (Vinale *et al.*, 2008; Vinale and Sivasithamparam, 2020).

SMs are further known as virulence factors in plant-fungal interactions and their role in the interaction with animals is also recognized, although not completely elucidated. Moreover, fungal metabolites are involved in the interactions of symbiotic and mycorrhizal or endophytic fungi with plants.

The communication with insects also elicits biosynthesis of SMs in fungi. This interaction is well described in entomopathogenic fungi, especially in *Metarhizium* spp., *Beauveria bassiana* and *Pochonia* spp. Examples of such compounds are destruxins, a cyclic depsipeptide from *Metarhizium anisopliae* with different modes of actions (i.e., inhibiting V-ATPase, changing ion transport in gut and epithelial tissues, even making behavioral changes), tenellin, beauvericin, and bassianolide from *B. bassiana*.

Fungal Genes Involved in Secondary Metabolite Biosynthesis

Most of the core genes coding for enzymes that act in a discrete pathway for the biosynthesis of a specific secondary metabolite are located in physical vicinity in the fungal genome. This grouping of functionally linked genes into biosynthetic gene clusters (BGC) is a distinctive feature of fungal secondary metabolism.

The central part of a secondary BGC typically codes for a backbone-generating enzyme such as polyketide synthase (PKS), non-ribosomal peptide synthetase (NRPS), hybrid NRPS-PKS, dimethylallyl tryptophan synthase, or terpene cyclase. PKS and NRPS core enzymes consist of multiple domains that progressively assemble the metabolite backbone. Their genes show high diversity and discontinuous distribution across fungal genomes, respectively, and evolve rapidly. In addition, accessory enzymes involved in chemical modification of the generated product to generate the final compound, transporters for product export, and transcription factors for cluster regulation may be encoded by cluster-resident genes (**Fig. 3**). Although secondary BGC often are self-contained units for the biosynthesis of a specific substance, this is not necessarily always the case. There is no standard composition of these gene clusters but diversity regarding structure, content and size even among different strains of the same fungal species. Some clusters for example encode multiple backbone genes while others lack certain genes such as those coding for transcription factors that regulate biosynthetic gene expression. The penicillin biosynthesis cluster of *Penicillium chrysogenum* exemplifies the latter case.

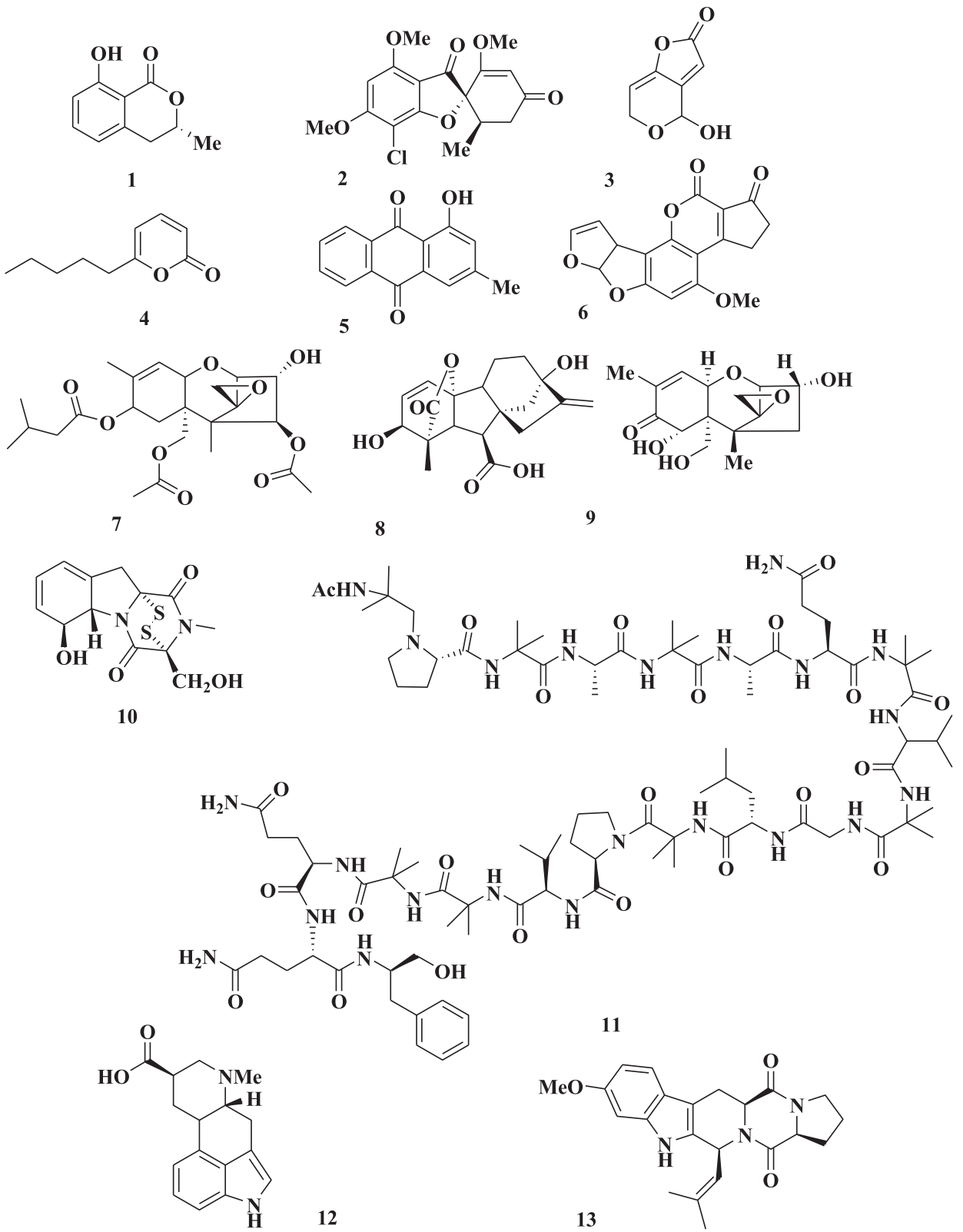

Fig. 2 Examples of fungal secondary metabolites belonging to the main groups of natural products. Polyketides: (**1**) Mellein from *Aspergillus melleus*; (**2**) Griseofulvin from *Penicillium griseofulvum*; (**3**) Patulin from *Penicillium* spp.; (**4**) 6-pentyl-α-pyrone from *Trichoderma* spp.; (**5**) 1–hydroxy-3–methyl-anthraquinone from *Trichoderma harzianum*; (**6**) Aflatoxin B1 from *Aspergillus flavus*. Terpenes and terpenoids: (**7**) T2 Toxin from *Fusarium* spp.; (**8**) Gibberellin GA3 from *Gibberella fujikuroi*; (**9**) Deoxynivalenol from *Fusarium* spp. Non-ribosomal peptides: (**10**) Gliotoxin from *Trichoderma* and *Gliocladium* spp.; (**11**) Alameticin, from *Trichoderma viride*. Indole alkaloids: (**12**) Ergotamine from *Claviceps purpurea*; (**13**) Fumitremorgin C from *Aspergillus fumigatus*.

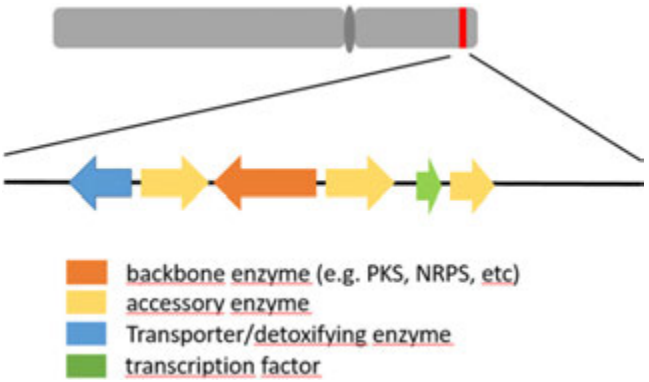

Fig. 3 Scheme of a prototypical secondary metabolism gene cluster and its subtelomeric localization on the chromosome.

A pathway-specific regulator is absent from the gene cluster; instead, penicillin production is subject to regulation by a network of global regulatory proteins that are responding to diverse nutritional and environmental cues and simultaneously govern various cellular processes including metabolism and development. The biosynthesis of a given secondary metabolite may also involve more than one gene cluster. This is the case for cephalosporin production by *Acremonium chrysogenum*, to which two clusters are contributing (Martin *et al.*, 2014; Zeilinger *et al.*, 2015).

A further hallmark of many fungal secondary BGC is their enrichment near to the ends of chromosomes, at subtelomeric regions. As the areas adjacent to the telomeres contain densely packed nucleosomes which renders them transcriptionally silent, these secondary BGC are subject to chromatin-level control. Nucleosome repositioning as well as DNA and histone modifications are involved in their transcriptional activation, which occurs in response to pleiotropic environmental triggers (Collemare and Seidl, 2019).

Besides impacting their expression, the subtelomeric localization of secondary BGC is believed to affect evolution and horizontal gene transfer as these regions usually are highly prone to recombination. While gene duplication had a prominent role in the evolution and subsequent diversification of secondary metabolic genes, several BGC seem to have originated from the horizontal transfer of genes from bacteria to fungi. The clustering of secondary metabolism genes then further facilitates their intra-kingdom horizontal transfer between different fungal species and contributes to metabolic diversity (Osbourn, 2010).

The metabolic potential of a given fungus can be explored directly from its genome sequence by screening for backbone genes and gene clusters using bioinformatics approaches. Among the different groups of fungi, filamentous ascomycetes are especially rich in secondary metabolism BGC. In these organisms, which typically harbor dozens of these clusters, a significant portion of their genome is devoted to secondary metabolite biosynthesis and its regulation. However, many gene clusters for secondary metabolism pathways are unexpressed and hence no product is present under standard laboratory culture conditions, as these do not mimic the required physiological triggers the producing fungus encounters in its natural habitat. The activation of these unexpressed BGC is a promising avenue to further exploit fungi as a source of bioactive natural products, including novel drug leads. Hitherto successful approaches tackled cluster composition and regulation by using genetic engineering, chromatin structure modification by using epigenetic modifiers, as well as simulation of the natural habitat by using co-cultivation and synthetic microbial communities (Keller, 2019).

Regulation of Fungal Secondary Metabolism

Fungal secondary metabolism is regulated by a complicated network of signals, some of them activated as a response to particular nutritional or environmental conditions, and that usually affect in a wider form to the fungal metabolism, not specifically to the secondary metabolism (**Fig. 4**) (Brakhage, 2013; Keller, 2019; Macheleidt *et al.*, 2016; Yin and Keller, 2011).

The New Generation Sequencing and Bioinformatic techniques/tools currently available emphasize the potential of fungi to produce an enormous variety of secondary metabolies. However, it has been also shown that for a particular growth and/or environmental condition only a minimal fraction of the BGC identified on a fungal genome are expressed and their products detected, remaining the rest in a cryptic or quasi-cryptic state.

Pathway-Specific Regulators

Pathway-specific regulator genes are usually located inside the BGC, or at least in one of the clusters of genes involved in its biosynthesis. The most common transcription factors (TF) regulating fungal SMs production are those of the Zn_2Cys_6 (=C6-Zn) binuclear type, but Cys_2His_2 Zinc finger TFs are also common, even when they are less known (**Fig. 4**). Other, less frequent TFs in fungi are basic region leucine zipper (bZIP) and winged helix proteins. bZIP are involved in stress response and regulation of SM biosynthesis (Shaaban *et al.*, 2010), and winged helix TFs, have been related to the production of cephalosporin C in *A. chrysogenum* (Schmitt and Kück, 2000).

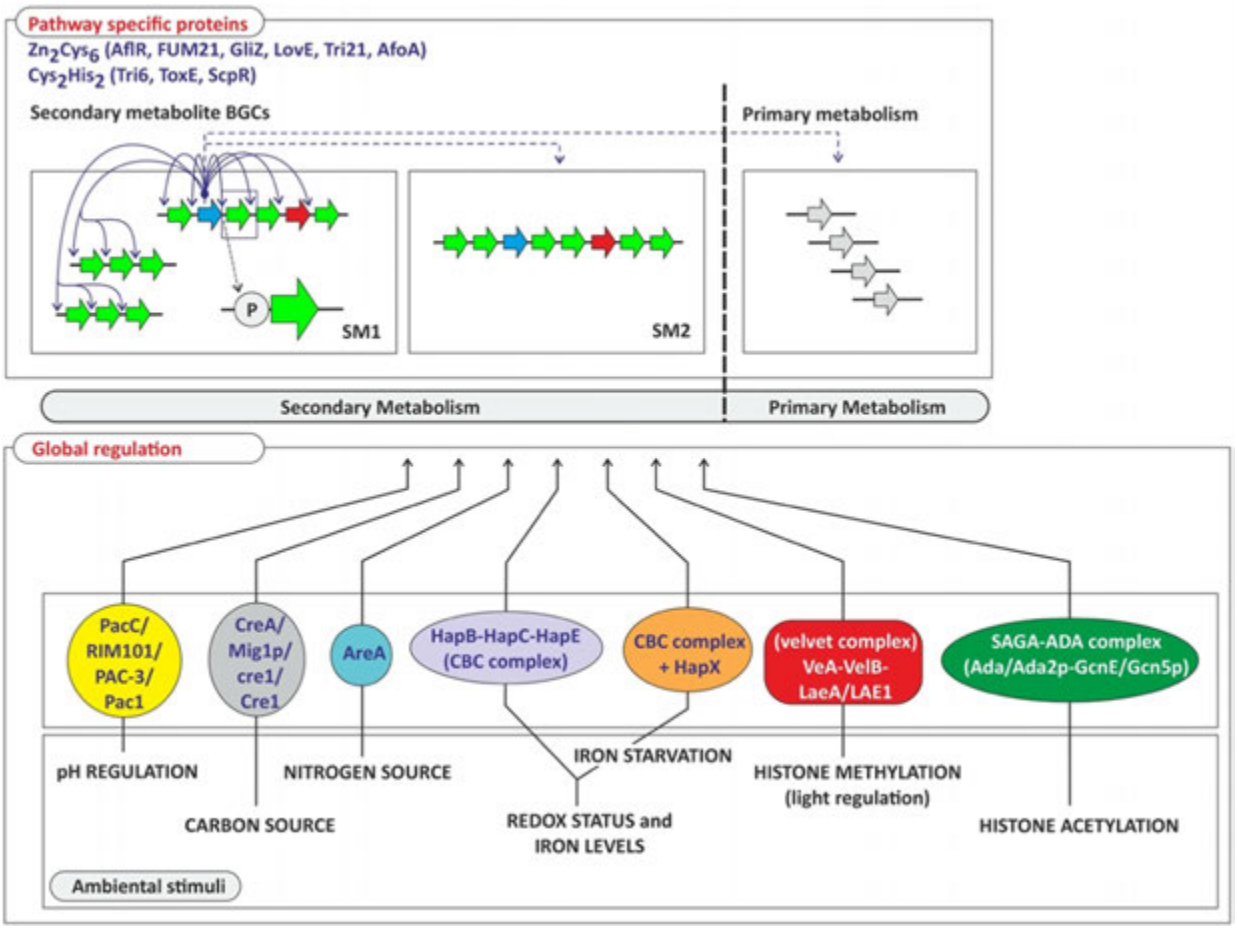

Fig. 4 Main Pathway specific (upper panel) and Global (lower panel) regulation protein-complexes involved in regulation of secondary metabolism. Thick blue arrow in the upper panel corresponds to a gene encoding a pathway specific regulator inside the biosynthetic gene cluster (BGC). Red arrow represents a gene encoding one of the key enzymes usually found in the secondary metabolite BGCs, i.e., PKS, NRPS, hybrid PKS-NRPS, terpene synthase. In the upper panel, dashed lines indicate the possibility that one particular pathway specific regulator would have a more global effect, regulating other secondary metabolite (SM) BGCs and also eventually some genes involved in primary metabolism. P = Promoter region.

C6-Zn TFs recognize palindromic motifs in BGC promoters (Fernandes *et al.*, 1998). Nowadays, there are known many of these regulators, being some of the most studied examples: AflR from *Aspergillus* species, which is involved in the biosynthesis of sterigmatocystin/aflatoxin (AF/ST) (Chang *et al.*, 1995); GliZ for regulation of gliotoxin biosynthesis in *A. fumigatus* (Bok *et al.*, 2006), LovE for lovastatin biosynthesis regulation in *A. terreus*, FUM21 for fumonisin biosynthesis regulation in *Fusarium verticilloides* (Brown *et al.*, 2007), and AfoA for regulation of asperfuranone in *A. nidulans* (Grau *et al.*, 2018) (**Fig. 4**). These TFs are usually required for expression of most, if not all, the other genes involved in the SM biosynthesis. For example, in the gliotoxin biosynthesis, all the genes present in the BGC, except *gliT*, are regulated by GliZ. GliT, is a gliotoxin oxidase, which confers resistance to gliotoxin (Schrettl *et al.*, 2010).

A recently reported exception to these C6-Zn TFs is Tri21. Its encoding gene is present in one of the *TRI* BGCs in species belonging to the *Fusarium incarnatum-equiseti* species complex (FIESC). Tri21 regulates only the late genes involved in the biosynthesis of the trichothecene deacetoxyscirpenol (Brown *et al.*, 2020).

Cys_2Hys_2 Zn finger proteins constitute the other main type of fungal pathway-specific regulators. Some examples are: Tri6 (Proctor *et al.*, 1995), involved in regulation of trichothecene mycotoxins biosynthesis; ToxE for production of HC-toxin, a cyclic tetrapeptide, in *Cochliobolus carbonum* (Pedley and Walton, 2001), and ScpR, involved in synthesis of asperfuranone in *A. nidulans* (Bergmann *et al.*, 2010) (**Fig. 4**). Tri6 is an unusual zinc finger protein that regulates all genes involved in trichothecene biosynthesis and transport. However, its effect is not restricted to genes belonging to trichothecene BGCs. In *F. graminearum* Tri6 behaves, at least in some physiological conditions, more as a global regulator than as a pathway specific TF. This conclusion was supported by the description that more than 20% of the genes in the genome of *Trichoderma arundinaceum* were negatively regulated in a *tri6*-deletion background (Lindo *et al.*, 2018). Furthermore, Tri6 also acts as an autoinducer, and requires for wild-type expression of *TRI* genes a second regulator, Tri10, whose encoding gene is also located in one of the trichothecene BGC.

Regulation of Secondary Metabolism by Nutritional and/or Environmental Conditions

pH regulation

The global regulator PacC/RIM101/PAC-3/Pac1 is a Cys_2His_2 zinc finger transcription factor that is processed to its mature form only at alkaline pH (Peñalva *et al.*, 2008). PacC positively regulates the expression of all three structural genes involved in penicillin biosynthesis at alkaline pH. PacC was reported to regulate also other diverse SM biosynthetic pathways in other fungi, e.g., it negatively regulates the expression of genes involved in sterigmatocystin biosynthesis in *A. nidulans* (Keller *et al.*, 1997).

Regulation by carbon and nitrogen sources

Presence of easily assimilable carbon sources usually results in a repression of a wide variety of genes in fungal BGCs. In these conditions fungal growth prevails versus differentiation and production of SM. This mechanism, known as carbon catabolite repression, is mediated by CreA. This TF possesses a single Cys_2Cys_2 GATA Zn finger module, and was first described in *A. nidulans* (Dowzer and Kelly, 1991), but homologs have been identified in a wide diversity of fungi, including *A. flavus*, *A. fumigatus*, *A. niger*, *A. oryzae*, *A. chrysogenum* (*cre1*), *Candida albicans* (*Mig1*), *Cryptococcus neoformans*, *Saccharomyces cerevisiae* (*Mig1p*), and *Trichoderma reesei* (*Cre1*), as representative examples (**Fig. 4**).

Some of the SMs whose biosynthesis is known to be repressed by CreA include cephalosporin and penicillins in *A. chrysogenum* and *Penicillium chrysogenum* or *A. nidulans*, respectively (Martín *et al.*, 1999; Espeso and Peñalva, 1992). The reduction in production of these antibiotics, mediated by CreA was observed by a down-regulation of all genes involved in penicillin biosynthesis, i.e., *pcbAB*, *pcbC*, *penDE*, in *P. chrysogenum*, or *pcbC* and *cefEF* genes for cephalosporin biosynthesis in *A. chrysogenum* in the presence of glucose (Gutiérrez *et al.*, 1999; Jekosch and Kück, 2000).

Regulation of SM production by the nitrogen source is mediated by the global nitrogen regulator AreA (**Fig. 4**), which is also a Cys_2His_2 Zn finger TF that mediates nitrogen repression in response to $NH4^+$ or glutamine levels (Wilson and Arst, 1998). AreA is responsible for nitrogen-mediated repression of the gibberellin BGC in *F. fujikuroi* (Tudzynski *et al.*, 1999), and in the opposite it is required for the production of fumonisin B1 in *F. verticillioides* (Kim and Woloshuk, 2008). In a recent study, a genomic approach led to identify at least 4 NRPS, 3 PKS and 4 terpene synthases, involved in the synthesis of six different SM (ferrichrome, beauvericin, fusarins, fumonisins, gibberellins, acorenol), whose biosynthesis might be regulated by AreA in *F. fujikuroi* (Janevska and Tudzynski, 2018), thus emphasizing the general role of AreA in fungal SM BGC regulation.

Redox status and iron levels

The ability to maintain redox balance and iron homeostasis is crucial to all organisms. The CCAAT-binding complex (CBC) consists of three subunits, named HapB, HapC, and HapE in *A. nidulans* (**Fig. 4**), which are responsible for DNA binding, and is described as involved in sensing of reactive oxygen species (ROS) through oxidative modifications of cysteine residues of the HapC component, and hence represent a major mechanism of redox regulation (Hostschansky *et al.*, 2007). Furthermore, oxidation of these cysteines blocks CBC assembly. This complex would have a general regulatory role independent of the iron status, positively regulating utilization of nitrogen and carbon sources, and production of SMs such as penicillin in *A. nidulans* (Brakhage *et al.*, 1999), and aflatoxins in *A. parasiticus* (Reverberi *et al.*, 2008).

Iron is an essential cofactor for multiple cellular processes, e.g., respiration, biosynthesis of amino-acids, synthesis of sterols. However, excess of iron can potentially catalyze formation of ROS. Thus, iron levels must be tightly regulated. For sensing iron levels, a fourth peptide, HapX (**Fig. 4**), interacts with CBC complex in iron starvation conditions. Binding of HapX to CBC results in transcriptional repression of iron-dependent pathways and activation of the siderophore system to increase iron acquisition, such as siderophore biosynthesis (Hostschansky *et al.*, 2007). A repression of ergosterol biosynthetic genes by CBC complex has been also described in *A. fumigatus* in iron starvation conditions, which correlates with the high demand for heme-bound iron as a cofactor for ergosterol biosynthesis. Note that a huge number of fungal SMs require intermediates of the ergosterol biosynthetic pathway as precursors for their biosynthesis, e.g., sesquiterpenes, triterpenes, etc.

Regulation Mediated by Chromatin Modifications

Histone methylation

LaeA/LAE1 (loss of *aflR* expression A) is a nuclear methyltransferase-domain containing protein that interacts with VeA and VelB to form the velvet heterotrimeric complex. This complex synchronizes secondary metabolism and fungal development in relation to the light levels. LaeA is a global regulator of fungal secondary metabolism that was firstly described in the genus *Aspergillus*. It is required for the biosynthesis of several SM in different species of this genus, e.g., sterigmatocystin, penicillin, gliotoxin, terrequinose A, lovastatin, aflatoxin, mycelial pigments,… (Bok and Keller, 2004), but also in other fungal species, e.g., *Alternaria alternata* (Estiarte *et al.*, 2016), *Monascus ruber* (Liu *et al.*, 2016), *A. fumigatus* (Perrin *et al.*, 2007), *A. niger* (Wang *et al.*, 2018), and *F. verticillioides* (Butchko *et al.*, 2012). Light blocks the formation of the velvet complex, then repressing velvet-dependent functions, such as those related with secondary metabolism (Brakhage, 2013).

The sequence similarity of LaeA to histones and arginine methyltransferases, together with the analysis of mutants in genes encoding for the later enzymes, reveal that expression of SM genes should be related with the removal of heterochromatin marks in the genomic regions where these BGCs are located (Yin and Keller, 2011) (**Fig. 5**). Thus, the effect of LaeA, and hence of the velvet complex, is associated with chromatin modifications that would counteract the establishment of heterochromatin marks, i.e.,

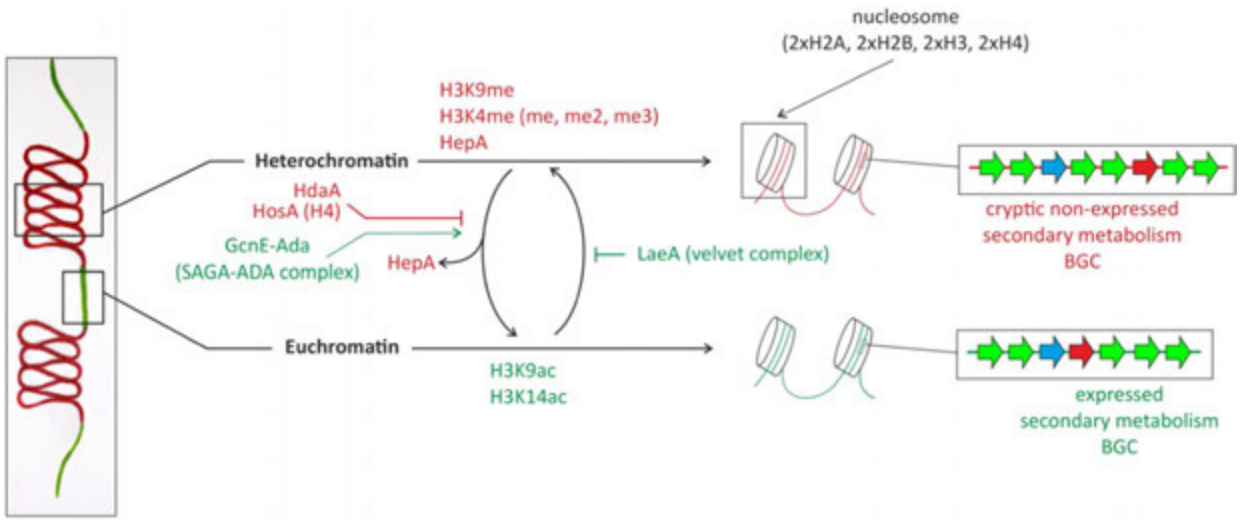

Fig. 5 Regulation of secondary metabolite BGC expression mediated by chromatin modifications. Complexes/proteins involved in that regulation are shown. Those marks and enzymes related to heterochromatin (non-expressed BGCs) or euchromatin (expressed BGCs) are written in red and green, respectively. H3K9me= methylation of lysine 9 (K9) in histone 3 (H3); H3K4me, me2, me3= mono, di-, and tri-methylation of lysine 4 in histone 3; H3K9ac and H3K14ac= acetylation of lysine 9 and lysine 14, respectively, in histone 3. Green arrow indicates induction of euchromatin conformation by SAGA-ADA complex. Truncated red line points to the suppression of euchromatin conformation by histone deacetylase enzymes (e.g., HdaA, HosA), and truncated green line points to the block of histone methylation carried out by LaeA. Heterochromatin and euchromatin are represented in red and green colors, respectively. Note that HosA is involved in deacetylation of H4 and H3.

histone methylation (**Fig. 5**). These data suggest that the expression of SM BGCs is under an epigenetic control by LaeA removing the heterochromatin marks, and by HepA binding and the consequent histone methylation to establish a heterochromatin structure. *hepA* encodes a putative heterochromatin protein, and its deletion leads to upregulation of SM biosynthetic genes, e.g., genes for aflatoxin, penicillin, terraquinone A biosynthesis in *Aspergillus* species (Reyes-Dominguez *et al.*, 2010).

Histone acetylation

The SAGA/ADA (Spt-Ada-Gcn5-acetyltransferase) complex was initially described as involved in regulation of fungal response to stress, e.g., oxidative stress and resistance to antimycotic compounds (Sellam *et al.*, 2009). This role is exerted through histone acetylation, which would have an opposite role to histone methylation in the regulation of the chromatin structural conformation (**Fig. 5**). GcnE/Gcn5p, a histone acetyl transferase should be the complex-subunit responsible for those acetylations, but this function should be carried out in interaction with Ada/Ada2p (Alteration/deficiency in activation), another component of the SAGA/ADA complex (Sellam *et al.*, 2009).

In *A. nidulans*, GcnE was shown to induce acetylation of histone 3 lysine 14 in orsellinic BGC, but interestingly only in interaction with *Streptomyces rapamycinicus* (Netzker *et al.*, 2015). Thus, this interaction induces the expression of the cryptic orsellinic acid BGC by promoting a change in the histone acetylation profile. Furthermore, acetylation of histone 3 in lysine 9 had a wider effect on the *A. nidulans* secondary metabolism, upregulating genes belonging both to the orsellinic acid and to the sterigmatocistin BGCs (Nützmann *et al.*, 2011).

Histone deacetylases would be involved in the removal of acetyl groups from the amino-terminal tail of core histones (Tribus *et al.*, 2005). Thus, their function will enhance the heterochromatin conformation, and eventually will negatively affect the expression of SM BGCs. Several enzymes with this function have been characterized, e.g., HdaA (histone 3 deacetylase) and HosA (histone 3, histone 4 deacetylase). Furthermore, deletion of *hosA* gene in *A. nidulans* resulted in an induction of expression of BGC involved in orsellinic acid and derivatives, and also in cichorine and aspercryptin biosynthesis (Pidroni *et al.*, 2018).

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Mycotoxins and Mycotoxigenic Fungi: Risk and Management. A Challenge for Future Global Food Safety and Security

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Introduction

Molds are microscopic filamentous fungi, which cause food and feed spoilage. Moldy foods and feeds may pose a serious risk to the health of consumers due to fungal toxic metabolites produced in the course of spoilage, referred to as mycotoxins. Mycotoxins are by definition "fungal metabolites which when ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death in man or domestic animals, including birds" (Pitt, 1996). By convention, the mushroom toxins and compounds that are toxic only to lower animals, such as invertebrates, are not regarded as mycotoxins. Toxigenic molds may act as plant parasites that colonize living plants in the field and/or may grow saprophytically on the products after harvest and throughout the entire production chain if environmental conditions, particularly temperature and moisture, are conducive to molding. Mycotoxins can be synthesized during either one or both of these invasive processes, as products of secondary metabolism of molds. Mycotoxins in food and feed may be fatal or cause severe illness at very small concentrations, often measured in parts per million (ppm) or parts per billion (ppb). Besides acute toxicoses due to high-dose mycotoxin exposure, the prolonged exposure to low and sub-lethal doses of mycotoxin results in chronic toxicosis, that is cumulative damages to specific tissues, organs or systems, sometimes associated to development of cancer (International Agency for Research on Cancer IARC, 2012a,b). Chemically, mycotoxins are structurally diverse compounds of low molecular weight that are synthesized from few precursors generated within the primary metabolism (Zeilinger *et al.*, 2015). The Food and Agriculture Organization (FAO) has estimated that 25% of the global food and feed output is contaminated by mycotoxins each year, with estimated losses of around 1 billion metric tons of foods and food products. Locally, these figures may increase, even considerably, in particular years when climatic conditions favorable to crop disease outbreak occur.

Contamination of foods and feeds with mycotoxins has been recognized as a matter of global concern since the early 1960s when a severe outbreak in poultry, the Turkey "X" disease, which killed 100,000 turkeys in the UK was discovered to be caused by consumption of moldy feedstuff contaminated with aflatoxin. However, the association between consumption of moldy grains and some human and animal diseases has been recognized since historical times (Pitt and Miller, 2017). Just to cite a few examples, the ergotism, a human disease resulting from the infection of rye by the fungus *Claviceps purpurea* is mentioned in the Old Testament and is recognized as an important cause of human mortality in Europe during the middle age. Also the trichothecenes, a group of mycotoxins produced by *Fusarium* species that infect wheat and other cereal grains, are thought to be linked to middle age plague epidemics. A human toxicosis (a pathology caused by exposure to a toxin) from consumption of *Fusarium*-infected grains occurred in southern Japan at the end of 1800; symptoms consisted in nausea, vomiting, diarrhea, abdominal pain, fever, and throat irritation. Similar toxicoses in humans, associated to the ingestion of moldy grains infected by *Fusarium* species, were also reported at the beginning of 1900 in China, Korea and Russia. The heart-attacking paralysis known as "acute cardiac beriberi", was reported in Japan and in a number of other Asian areas from the 17th century on. Beriberi was correlated epidemiologically to the consumption of moldy rice contaminated with the *Penicillium* mycotoxin citreoviridin. More recently, serious human toxicoses occurred in Kenya in the years 2004, 2005 and 2006, with hundreds of affected people and almost 200 fatalities recorded as a consequence of aflatoxin poisoning from contaminated maize (Schmale and Munkvold, 2014a).

This chapter offers an overview on the main mycotoxins and the relevant producing molds, including their economic and health impact, epidemiology, biosynthesis and genetics, regulation and management. The methods for the analysis of mycotoxins are out the scope of this chapter; comprehensive reviews and up to date information on that topic can be found in Maragos and Busman (2010) and Tittlemier *et al.* (2019).

Social and Economic Impact of Mycotoxins

Aside from the obvious social implications for public health (see the toxic effects of individual mycotoxins, later on in this chapter), the occurrence of mycotoxigenic fungi and mycotoxins in food and feed chains has also major economic impacts to human society. These include (1) reduction of crop output due to plant diseases and from the removal of infected and/or damaged product (sorting), (2) reduced quality and commercial value of produce, (3) reduced animal productivity due to health problems associated with consumption of mycotoxin-contaminated feed, and (4) costs for humans and animals health care (Schmale and Munkvold, 2014b). The extra production expenses for the control of mycotoxigenic fungi outbreaks, such as the use of selected resistant cultivars and fungicides spraying or for the adoption of specific measures of prevention (e.g., biological control) have also to be taken into account. At post-harvest, the multiple control strategies that have been developed to mitigate the mycotoxin risks in the course of storage, transportation or processing and the cost for the organization and functioning of a surveillance network are all additional burdens for farmers, handlers, processors and, ultimately, for consumers.

Estimations of the costs of mycotoxins are not available for all the world regions. In the USA, the total cost has been estimated 0.5–1.5 billion USD per year; Council for Agricultural Science and Technology CAST (2003) reports a yearly cost of 0.9 billion for

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crop losses of corn, wheat, and peanuts plus an additional 0.5 billion for regulatory enforcement, testing, and quality control measures. In developing countries, it is likely that losses exceed those in the USA. For instance, in three Asian countries (Indonesia, Philippines, and Thailand) annual losses were estimated at 0.9 billion USD for aflatoxins only (Schmale and Munkvold, 2014b).

Even if prevention measures are enforced, sometimes these are not effective enough to avoid the occurrence of mycotoxins at levels close or above the maximum allowable levels for food, resulting in low quality and reduced price of the produce or, in the worst scenarios, in rejection of lots or diversion towards alternative and low revenue uses, such as feed production or conversion to bioethanol. Many staple agricultural commodities such as wheat, rice, barley, corn, sorghum, soybeans, groundnuts and oilseeds are at high risk of mycotoxin contamination. For this reason, regulations on allowable levels of mycotoxins in staple food have been set and are strictly enforced by most countries. However, standards vary greatly among countries, particularly between developed and developing countries, and this difference may cause major trade issues in the globalized market. For some developing countries, where agricultural commodities account for a large part of the total national export, the economic impact of rejection of mycotoxin-contaminated products, as for both lost trade and additional costs for inspection, disposal and compensation in the case of claims, is considerable (Gbashi *et al.*, 2019; Taniwaki *et al.*, 2019). Also, the stricter mycotoxin standards for imported products applied in the developed countries may prompt the developing countries to export their best quality foods and keep the more heavily contaminated foods for domestic consumption, resulting in higher exposure of people to mycotoxins in those countries. This situation raises ethical and social issues and highlights the need for a more effective international cooperation on the fronts of standards harmonization and scientific research (Logrieco *et al.*, 2018). The climatic conditions in developing countries of the tropical zone favor proliferation of mycotoxigenic fungi and the accumulation of mycotoxins. Mycotoxin mitigation measures are costly and the control strategies should be implemented at multiple points of the production chain, through an organized and coordinated infrastructural system of control, surveillance and communication, which in some developing countries may be difficult to set (International Agency for Research on Cancer IARC, 2012b). To successfully implement such a system it is important that the expected benefits, in terms of trade and health, exceed their costs. In this sense, the role of scientific research for the development of effective low-cost mitigation strategies is of utmost importance.

While the human health losses from adverse effects of mycotoxins are primarily relevant to the social impact of mycotoxins, they have also economic impacts that can be evaluated by the tools of health economics. This cost can be calculated in two ways. The first is cost of illness, which is mainly relevant to developed countries because a large portion of the cost is health-care related. The second is disability-adjusted life years, which is applicable to both developed and developing countries (International Agency for Research on Cancer IARC, 2012b). Vardon *et al.* (2003) estimated the total losses due to the three main mycotoxins, aflatoxin, fumonisin and deoxynivalenol, in the USA to be as high as 1 billion USD per year; this cost was largely due to market losses, because human health effects are almost negligible in countries where a strict regulation and an effective surveillance are enforced. On the contrary, in the developing countries the loss should be calculated as a combination of market impacts, mainly due to rejection of contaminated lots, and adverse health effects on local consumers (Gbashi *et al.*, 2019).

Regulated Mycotoxins and Associated Fungi

Virtually all the plant pathogenic fungi are able to produce, in appropriate conditions, secondary metabolites that are toxic to invertebrate or vertebrate animals. To date, approximately 400 toxic secondary metabolites of fungal plant pathogens have been discovered. Many of these have been isolated from laboratory cultures and only a relatively limited number of them have been found to occur under natural conditions, where they play the roles of chemical weapons against competing microorganisms and grazing animals, of virulence factors, or of signaling molecules within ecological interactions. Among the naturally occurring fungal toxins only few are considered mycotoxins of significance for human and animal health because of their high toxicity, levels produced (especially in certain epidemiological conditions), or chances of exposure and intake. The most important mycotoxigenic fungal species belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*, which can contaminate staple crops and widely consumed foods (**Table 1**), such as cereals (maize, wheat, barley, oats and rice), nuts, oilseeds, fruits, dried fruits, vegetables, cocoa and coffee beans, wine, beer, herbs, and spices and therefore pose a risk to a large number of people and animals (See "Relevant Website section"). To protect consumers from health risks, many countries have adopted regulations to limit exposure to the major mycotoxins. A study from 2003 by the United Nations' Food and Agriculture Organization (FAO) on worldwide regulations for mycotoxins reported that at that time approximately 100 countries had established guidance and regulations on mycotoxin in food and feed (FAO, 2004). To date, the figures are possibly higher, but still there are countries, including a number of African countries, which do not have specific regulation for food safety (Matumba *et al.*, 2015). Also, many countries have regulated the tolerable levels for at least aflatoxin B_1 (AfB_1) or the sum of aflatoxins B_1, B_2, G_1 and G_2 in human food, but relatively few countries have established strict limits for other mycotoxins such as fumonisin (FUM), ochratoxin A (OTA), and deoxynivalenol (DON). For, instance, in the USA, maximum tolerated levels have been set by the Food and Drug Administration (FDA) for aflatoxins in food and feed and for patulin, but only guidance levels are given for DON and FUM in food and feed (FDA, 2000).

The various regulations set the limits for the content of mycotoxins in food and feed and, naturally, they have profound implications for health safety as well as for economic interests of producers and traders. However, the different perceptions of tolerable health risks of mycotoxins at global level, mainly associated to the level of development and susceptibility of crops to contamination in different countries, has led to lack of consensus about standards for regulated mycotoxins in food and feed (**Tables 2** and **3**). For example, of the 48 countries which in 2003 had established limits for total aflatoxins in food, standards ranged

Table 1 Major mycotoxins and mycotoxigenic fungi

Mycotoxin	*Main producing mold (s)*	*Commodities*	*Animals affected*	*Toxic effect (s)*
Aflatoxins (B_1, B_2, G_1, G_2, M_1, M_2)	*Aspergillus flavus*, *A. parasiticus*	Corn, peanuts, cottonseed, pistachios, figs, tree nuts, wheat, rice, spices, copra, dairy products	Humans, cattle, sheep, swine, aviary species, dogs, cats	Liver tumors in humans (IARC, Group 1), hepatotoxicity, bile duct hyperplasia, hemorrhage of the intestinal tract and kidneys, teratogenicity, immune suppressive
Ergot alkaloids	*Claviceps purpurea, C. africana, C. sorghi*	Rye, sorghum, cereals, pasture grasses	Cattle, sheep, horses, humans (rare)	Hallucinations, gangrene, lameness, agalactia, reduced weight gain, abortion
Fumonisin B_1	*Fusarium verticillioides*, *F. proliferatum*	Corn, corn silage	Humans, horses, swine	Esophageal cancer in humans (IARC Group 2B), carcinogenic, neural tube defects, liver damage, heart failure, encephalomalacia (horses), pulmonary edema (swine),
Ochratoxin A	*Aspergillus carbonarius, A. niger, A. ochraceus*	Grapes, raisins, wine, coffee, tree nuts, figs	Humans, swine, poultry	Kidney dysfunction (nephropatia), tumors in urinary tract (IARC, Group 2B)
	Penicillium verrucusum, P. nordicum	cereal grains, spices,cheese, meat		Nephropathy, feed refusal
Patulin	*Penicillium expansum*, *P. crustosum*, *P. urticae*, *P. griseofulvum*; *Aspergillus clavatus*, *A. terreus*	Apples, plums, grapes, peaches, pears, apricots, fruit juices	Humans	Carcinogenic in laboratory animals, damage in the brain, liver and kidneys of experimental animals, neurotoxic, immunotoxic, genotoxic and teratogenic effects of chronic exposure
Trichothecenes	Type B (deoxynivalenol, nivalenol, fusarenon-X): *Fusarium graminearum*, *F. cerealis/crookwellense*, *F. culmorum*	Wheat, barley, oats, corn	Swine, dairy cattle, poultry, horses, humans	feed refusal in swine, reduced weight gain and vomiting; gastrointestinal problems, diarrhea
	Type A (T-2 toxin, HT-2 toxin, neosolaniol, 4,15-diacetoxyscirpenol): *F. sporotrichioides*, *F. langsethii*, *F. sambucinum*, *F. equiseti*		Humans	nausea, fever, headaches, vomiting, leucopenia (aleukia), hemorrhaging and degeneration of bone marrow, skin inflammation
Zearalenone, Zearalenols	*F. graminearum*, *F. culmorum*, *F. equiseti*, *F. cerealis/ crookwellense*	Corn, wheat, hay	Swine, cattle	Vulvovaginitis, vaginal prolapse, rectal prolapse, anestrus and fertility problems, abortion, malformation of testicles and ovaries

Table 2 Standards for mycotoxins in food adopted in some countries and recommended by the Codex Alimentarius

Country/Regulation[a]	*Food*	*Max. Levels (μg/Kg = ppb, unless otherwise specified)*
AFLATOXINS (sum of $B_1 + B_2 + G_1 + G_2$)		
Brazil	Cereals and cereal-based food except maize, beans, cocoa and cocoa products	5
	Maize and maize products	20
	Spices	20
	Chestnuts, hazelnuts, almonds, pistachios, dried fruits	10
	Brazil nuts	15 in nuts for further processing
		10 in nuts for direct consumption
China	Wheat, barley, other grains, beans, nuts and seeds	5
	Rice and rice oil	10
	Corn and peanuts	20
	Formulated food for infants	0.5
Codex	Peanut, almond, hazelnut, pistachios, dried figs	15 in nuts for further processing
		10 in nuts for direct consumption and dried figs
European Union (EU)	Groundnuts, oilseeds	8 (AfB_1) and 15 (total) in products for further processing
		2 (AfB_1) and 4 (total) in products for direct consumption
	Hazelnuts, brazil nuts	8 (AfB_1) and 15 (total) in products for further processing
		5 (AfB_1) and 10 (total) in products for direct consumption
	Almond, pistachios, apricot kernels	12 (AfB_1) and 15 (total) in products for further processing
		8 (AfB_1) and 10 (total) in products for direct consumption
	Dried fruits other than figs, other tree nuts	5 (AfB_1) and 10 (total) in products for further processing
		2 (AfB_1) and 4 (total) in products for direct consumption
	Cereals and cereal-based products	2 (AfB_1) and 4 (total)
	Processed cereal-based foods and baby foods for infants and young children	0.1 (AfB_1)
United States Of America (USA)	All foods except milk	20
AFLATOXIN M_1		
Brazil	Liquid milk - cheese - powdered milk	0.5–2.5 - 5
China	Liquid milk and dairy products, powdered milk for infants	0.5
Codex	Liquid milk	0.5 (a concentration factor applies to partially or wholly dehydrated milks)
EU	Milk - milk for infants	0.05–0.025
USA	Milk, milk products	0.5
ERGOT ALKALOIDS		
EU	Unprocessed cereals with the exception of corn and rice	0.5 g of ergot sclerotia/Kg
FUMONISINS B_1, B_2, B_3		
Brazil	Maize flour and maize meal - raw maize	2000–4000
USA	Maize and maize products, sorghum, asparagus	2000–4000
Codex	Raw maize	4000 ($B_1 + B_2$)
	Maize flour, maize meal	2000 ($B_1 + B_2$)
EC	Unprocessed maize	4000
	Maize intended for direct human consumption	1000
	Maize-based breakfast cereals and maize-based snacks	800
	Processed maize-based foods and baby foods for infants and young children	200

(*Continued*)

Table 2 Continued

Country/Regulation[a]	*Food*	*Max. Levels (μg/Kg = ppb, unless otherwise specified)*
OCHRATOXIN A		
China	Roasted and ground coffee, roasted coffee beans, beans, cereals and ground cereal products	5
	Grape wine	2
Codex	Raw wheat, barley and rye	5
EC	Cereals and cereal-derived products for adults	3 (unprocessed) – 5 (processed)
	Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5
	Wine, aromatized wine, grape juice	2
	Processed cereal-based foods and baby foods for infants and young children	0.50
USA		Not set
PATULIN		
China	Fruits and fruit products (except haw rolls), fruit and vegetable juices/beverages, cider and haw wine	50
Codex	Apple juice	50
USA	Apples, apple juice and concentrate	50
EC	Fruit juices, concentrated fruit juices, spirit drinks, cider and other fermented drinks derived from apples	50
	Solid apple products	25
	Apple juice and apple puree for infants and young children, baby food	10
TRICHOTHECENES		
DEOXYNIVALENOL (DON)		
China	Corn and corn products, barley, wheat and wheat products	1000
Codex	Cereal grains for further processing	2000
	Flour, semolina and flakes derived from wheat, maize or barley	1000
	Cereal based food for infants and young children	200
EC	Unprocessed cereals and maize	1750 (durum wheat, oats and maize) – 1250 (other cereals)
	Cereals intended for direct human consumption, pasta	750
	Processed cereal-based foods and baby foods for infants and young children	200
USA	Cereals, cereal products	1000
ZEARALENONE (ZEA)		
China	wheat and wheat flour, corn and corn products (flour, flakes and grits)	50
EC	Unprocessed cereals and maize	100 (cereals other than maize) – 350 (maize)
	Cereals, cereal flour, bran and germ intended for direct human consumption	75
USA		Not set

[a]Brazil, República Federativa Do Brasil. Ministério Da Saúde. Resolução, Rdc Nº 7, De 18 De Fevereiro De 2011; China, GB2761-2017. (2017). National food safety standard: Maximum levels of mycotoxins in foods. National Standards of the Peoples's Republic of China, 1–8; Codex, Codex Alimentarius Commission. General Standard for Contaminants and Toxins in Food and Feed, Codex Stan CXS 193–1995 (CxS_193-2015). FAO and WHO. Available online: www.fao.org/input/download/standards/17/CXS_193e_2015.pdf (accessed on 13 August 2020); EU, European Commission (EC). 2006. No 1881/2006 of 19/12/2006. Setting maximum levels for certain contaminants in foodstuffs. USA, U.S. Food and Drug Administration. Guidance for natural toxins and mycotoxins. Retrievable online: https://www.fda.gov/food/chemicals-metals-pesticides-food/natural-toxins-and-mycotoxins.

Table 3 Standards for mycotoxins in feed adopted in some countries

Country/Regulation	*Feed*	*Max. Levels (mg/Kg = ppm)*
AFLATOXIN B_1		
China[a]	Corn-derived feed ingredients; Feed products	0.01–0.05
Codex[b]		Not set
European Union (EU)[c]	Feed materials, except compound feed for dairy and young animals	0.02 (relative to a feed with a moisture content of 12%)
	Complementary and complete feed	0.01 (relative to a feed with a moisture content of 12%)
	Compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	0.005 (relative to a feed with a moisture content of 12%)
United States of America (USA)[d]	Corn and peanut products intended for breeding or finishing: Cottonseed meal intended for beef cattle, swine, or poultry	0.2–0.3
	Corn, corn products, peanut products, cottonseed meal, and other animal feeds and feed ingredients for immature and dairy animals	0.02
ERGOT SCLEROTIA		
EU	Feed materials and compound feed containing unground cereals	1000 (relative to a feed with a moisture content of 12%)
FUMONISINS $B_1 + B_2$		
China	Corn and corn products, corn silage and stover; concentrate supplements for ruminants; concentrated and compound feeds for poultry	20–60
	Concentrate supplements for horse and rabbit, concentrated feeds for swine, compound feeds for swine, rabbit, and horse	5
EU	Maize and maize-derived feed materials	60 (relative to a feed with a moisture content of 12%)
	Complementary and complete feedingstuffs	5–50 (relative to a feed with a moisture content of 12%)
USA	Corn and corn by-products intended for equids, swine, breeding ruminants, breeding poultry and breeding mink (includes lactating dairy cattle and hens laying eggs for human consumption)	5–30
	Poultry; All other species or classes of livestock and pet animals	10–100
OCHRATOXIN A (OTA)		
China	Grains and grain products; compound feeds	0.1
EU	Feed materials (cereals and cereal products)	0.25 (relative to a feed with a moisture content of 12%)
	Complementary and complete feedingstuffs	0.05 – 0.1 (relative to a feed with a moisture content of 12%)
TRICHOTHECENES		
DEOXYNIVALENOL (DON)		
China	Plant feed ingredients	5
	Compound feeds; concentrate supplements	1–3
EU	Feed materials	8–12 (relative to a feed with a moisture content of 12%)
	Complementary and complete feedingstuffs	0.9 – 5 (relative to a feed with a moisture content of 12%)
USA	Grains and grain by-products	10 (ruminating beef and chickens), 5 (swine and other animals)

(*Continued*)

Table 3 Continued

Country/Regulation	*Feed*	*Max. Levels (mg/Kg = ppm)*
T-2 TOXIN (T-2)		
China	Plant feed ingredients; Formula feeds for swine and poultry	0.5
SUM T-2 + HT-2 TOXIN		
EU[e]	Cereal products for feed and compound feed	0.25–2 (relative to a feed with a moisture content of 12%)
ZEARALENONE (ZEA)		
China	Feed ingredients	0.5–1.5
	Formula feeds for young sow, piglet, swine; Concentrate supplements for calf, lamb, and animals in lactation	0.1–0.5
EU	Feed materials	2–3 (relative to a feed with a moisture content of 12%)
	Complementary and complete feedingstuffs	0.1– 0.5 (relative to a feed with a moisture content of 12%)

[a]GB13078-2017. (2017). Hygienical standard for feeds. National Standards of the Peoples's Republic of China, 1–7.
[b]Codex Alimentarius Commission. Code of Practice for the Reduction of Aflatoxin B1 in Raw Materials and Supplemental Feedingstuffs for Milk Producing Animals (CAC/RCP 45–1997).
[c]Directive 2002/32/EC of EU Parliament and of the Council on undesirable substances in animal feed. O_. J. 2002, L140, 1–17.
[d]Guidance levels. FDA (2000). Conference on mycotoxins in animal feeds, grains and food related to human and animal health. Rockville: Maryland.
[e]Indicative levels. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding (Text with EEA relevance) (2006/576/EC).

from 0 to 50 μg/Kg (ppb) (Dohlman, 2003). Most of the limits are set for human foods. Typically, higher regulatory levels are used for animal feed.

One more complication may arise from some countries that under the "precautionary principle" may want to set standards on certain mycotoxins for which scientific evidence of a health risk is not yet well-established. In this respect, harmonization of regulations and guidelines for production and surveillance is a crucial issue, with the aim to find an acceptable balance between health benefits and economic and trade costs. For this reason, a joint scientific expert committee (Joint FAO/WHO Expert Committee on Food Additives, JECFA) of the FAO and the World Health Organization (WHO), has been instituted as the international body responsible for evaluating the health risk from mycotoxins. International standards and codes of practice to limit exposure to mycotoxins from certain foods have been established by the Codex Alimentarius Commission based on JECFA assessments (Codex Alimentarius, 2015). However Codex standards are advisory, not mandatory, and the national standards may divert significantly from the Codex recommendations.

Aflatoxins

Aflatoxins (AfTs) are the most important mycotoxins worldwide, for toxicity, occurrence and economic impact. Different forms of AfTs exist and the main types are aflatoxin B_1 (AfB_1) which is the major toxin produced, B_2 (AfB_2), G_1 (AfG_1), and G_2 (AfG_2); farm animals metabolize these forms and transform them in the less harmful aflatoxins M_1 (AfM_1) and M_2 (AfM_2), which are found in milk, meat and other dairy products. AfTs are produced primarily by the fungi *Aspergillus flavus* (AfB_1 and AfB_2) and *A. parasiticus* (AfB_1, AfB_2, AfG_1 and AfG_2). Other *Aspergillus* species, viz. *A. nomius*, *A. sergii*, *A. bombycis*, *A. minisclerotigenes*, *A. parvisclerotigenus*, *A. pseudocaelatus*, *A. pseudotamari*, and *A. ochraceoroseus* also are aflatoxigenic (Varga *et al.*, 2011), but their significance for mycotoxin occurrence is negligible. Generally regarded as saprophytes, *Aspergillus* spp. grow on a large number of substrates. Under favorable conditions, some species exhibit limited parasitic abilities and can colonize crops in the field. *Aspergillus flavus* and *A. parasiticus* are xerophyte species that can grow well and proliferate in environments with low water activity ($aw \leq 0.85$) and under drought condition.

AfT contamination occurs in major crops such as maize, peanuts, cottonseed, pistachios, figs, tree nuts, cereal grains and spices, and derived foods and feeds (Council for Agricultural Science and Technology CAST, 2003). In maize, *A. flavus* develops on the surface of the ears, with the infected parts that become covered with masses of gray-green spores. AfTs may be produced by the fungus in the field until the kernel reaches about 15% of moisture. Cottonseed, may become contaminated with AfTs if the seed-bearing capsules are damaged in conjunction with high humidity and warm temperatures before or after harvest. Tree nuts such as pistachios and almonds may also be infected following injuries, such as the splitting of hulls. In peanut *A. flavus* and *A. parasiticus* cause a disease known as yellow mold, and high levels of AfT contamination may result following relatively high temperatures and moderate humidity. Infection of crops by aflatoxigenic species and production of AfTs in the field are affected by various factors including environmental conditions (hot and humid weather), drought plant stress in conjunction with high temperatures, mechanical injuries caused by plant grazing insects, and plant genotype (Diener *et al.*, 1987; Council for Agricultural Science and Technology CAST, 2003). Williams *et al.* (2004) defined the sub tropical and warm temperate regions located at latitudes between 40°N and 40°S as the "hot zones" for AfT risk, and estimated that 4.5 billion people living in those areas are chronically exposed to AfTs. It is expected that in the future temperate regions will become more subjected to AfT outbreaks, because of global Earth warming. AfTs often occur together with cyclopiazonic acid, another *A. flavus* mycotoxin, in groundnuts and with cyclopiazonic acid and the *Fusarium* toxins fumonisins in maize.

Usually, field contamination increase during postharvest activities, e.g., crop drying, or in storage if conditions, particularly moisture, are favorable to molding. However, in some cases, molding and AfTs contamination can occur even in the absence of previous field infection, if the commodity is stored improperly. AfTs are temperature-stable compounds and can resist to heat treatments used for food and feed processing, such as ultrahigh-temperature (UHT) treatment, pasteurization, roasting, and baking, and also to cold storage.

The AfTs are potent liver toxins and most animal species exposed to AfTs show signs of liver disease, ranging from acute to chronic. AfB_1 is a potent hepatocarcinogen and is classified as a Group 1 compound (carcinogenenic to humans) by the International Agency for Research on Cancer (International Agency for Research on Cancer IARC, 2012a). AfB_1 is also an immune suppressor and cause malnutrition and growth impairment in both humans and animals (Rushing and Selim, 2019). The kidney can also be affected in pigs and goats.

AfTs have been linked to cases of acute poisoning and chronic aflatoxicosis in humans and animals (Pitt *et al.*, 2012). In humans, AfTs have caused outbreaks of acute hepatitis. The association of ingestion of AfT-contaminated grains with inflammation of the liver is well supported by epidemiological evidences, most notably by the observations of outbreaks of acute hepatitis associated with consumption of maize heavily contaminated with aflatoxin in Kenya in 1981 and 2004, which resulted in several hundred deaths. Previously, sporadic cases of aflatoxicosis were reported in Western India (1974) and Malaysia (1988). The intake of total AfTs estimated to result in a risk of fatality was >1 mg/day, i.e., >20 μg/kg body weight (bw)/ day in adults, while aflatoxicosis without fatality may occur with 5–10-fold lower doses (Wild and Gong, 2010). Among the livestock, the most sensitive animals for acute toxicity are the avian species, primarily ducklings, followed by turkeys, chicks and quail. In other farm animals, decreasing level of sensitivity is shown by rabbits, young pigs, calves, mature cattle and sheep (Pitt *et al.*, 2012). Dogs are also quite sensitive to acute toxicity. The susceptibility of a species to toxicity of AfTs depends on its ability to metabolize the toxin efficiently and quickly to less toxic or nontoxic metabolites. Undernourished or stressed animals are more sensitive to AfTs.

Ergot Alkaloids

The ergot alkaloids (EAs) are mycotoxins produced by several species in the genus *Claviceps*, pathogens of cereals and grasses. There are four main groups of EAs, viz., the clavines, the lysergic acids, the lysergic acid amides, and the ergopeptides. The most important compound is ergotamine that belongs to the group of ergopeptides and is precursor of the psychoactive drug lysergic acid diethylamide (LSD). EAs accumulate in specialized resting structures (sclerotia) formed by the fungus in plant heads and spikes which are infected during cool, wet weather. Infection of flowers of susceptible hosts by *Claviceps* spp. results in replacement of the ovary by a sclerotium made of a hard and dense mass of fungal hyphae, trivially called, as well as the disease itself, "ergot" (Alderman *et al.*, 1999). The name *ergot* means 'spur' in French, since grains colonized with *Claviceps* spp. often resemble the spurs on the legs of a rooster. The sclerotia are usually white, black, or tan and are 1–4 times as large as the seeds they replace. The most important species is *C. purpurea*, which forms dark purple to black sclerotia. *Claviceps purpurea* is distributed worldwide in temperate climates and has a host range of more than 200 grass species. It is most prevalent on rye, but also occurs on barley, oats, and wheat, as well as wild and cultivated grasses, but not on sorghum or maize. *Claviceps africana*, also of importance in terms of mycotoxins, is a cause of a serious disease resulting in malesterile sorghum seed.

Ergots may be harvested with the grain and processed into flour or used for feed, resulting in high levels of contamination with EAs and in human and animal mycotoxicosis known as ergotism. EAs have biological properties of neurotransmitters and are vasoconstrictors; thus ergotism may have two different forms, that is gangrenous, affecting blood supply to extremities, and convulsive, affecting the central nervous system. Clinical symptoms are hallucinations, the feeling of itchy and burning skin, gangrene, loss of hands and feet, and even death. The biological activity of the sclerotia of *Claviceps* spp. was known in China more than 5000 years ago and ergotism is one of the oldest known human diseases caused by mycotoxins. The historical disease from the Middle Ages called St. Anthony's Fire, characterized by symptoms such as intense inflammation of the skin, sensation of insects crawling under their skin, gangrene and loss of hands and feet is thought to be the outcome of ergot poisoning. Also, EAs might have been the cause of hallucinations associated with witchcraft collective hysteria in the surrounding of Salem, Massachusetts, in the 1690s. It has been speculated that ergotism may have killed hundreds of thousands of people in Europe during the last millennium (Smith and Moss, 1985). Modern methods of grain sorting have significantly reduced the occurrence of ergotism as a human disease, but EAs are still an important issue in respect to contamination of feeds and mycotoxicosis in livestock. In the last century, cases of ergotism were reported in pigs, cattle sheep and horses consuming sorghum-based feeds or feeds based on small grains, such as rye and barley, or foraging on infected grasses contaminated (Council for Agricultural Science and Technology CAST, 2003; Cross, 2003). The symptoms include lameness, gangrene of the extremities (feet, ears, and tail), reduced milk production (agalactia), reduced weight gain, abortion, hypersensitivity, ataxia, convulsions, and, in sheep, intestinal inflammation. According to the European Food Safety Authority EFSA (2012) opinion, there is no evidence that EAs are transferred from animal feed to animal products, such as milk and meat and therefore these are unlikely to be an important source of human exposure to EAs. The EU has not set standard for EAs, but adopted a limit of 0.5 g/Kg of ergot sclerotia for oats, wheat, durum wheat and other unprocessed cereal grains except maize and rice, intended for human consumption (**Table 2**). However, the content of ergot sclerotia not always correlates well with the content in EAs, since it is possible high levels of EAs occur in grain with very little visible sclerotia, due to their fragmentation during harvesting and processing. Rye ergot in feed materials and compound feed containing unground cereals is also restricted to a maximum content of 1000 mg/kg relative to moisture content of 12%. Few countries besides the EU have standards for EAs or ergot sclerotia. Australia and New Zealand set a limit of 0.5 g/kg of ergot sclerotia in cereal grains. In Canada, guidelines for ergot sclerotia in cereals establish a quality grading, ranging from 0.01% for the highest quality, to 0.1% for the lowest quality. Also, maximum levels of EAs have been established for pig feed (6 mg/kg), feed for cattle, sheep and horses (3 mg/kg) and feed for chicks (9 mg/kg).

Fumonisins

The fumonisins (FUMs) are a group of mycotoxins produced mainly by *Fusarium verticillioides* and *F. proliferatum* and by a few other *Fusarium* species of minor importance (Pitt *et al.*, 2012). The species name *F. moniliforme*, often associated to production of FUMs in the earliest reports, is no longer in use. Fumonisin B_2 and B_4 are produced also by the species *Aspergillus niger* (Frisvad *et al.*, 2007; Mogensen *et al.*, 2010). Recent information indicates that FUM production by *A. niger* is rather common. Since *A. niger* is common in some fresh fruits, particularly berries, and on onions, FUMs produced by *A. niger* can be expected to be more widespread than currently assumed (Pitt *et al.*, 2012). To date, 28 types of FUM have been identified, which are classified into four groups: FUMs A, B, C, and P. Fumonisin B_1 (FB_1) is the most common and economically important form, followed by fumonisins B_2 and B_3 (Escrivá *et al.*, 2015). *Fusarium verticillioides* and *F. proliferatum* are ubiquitous species and the most common fungi associated with maize, where they cause the "Fusarium ear rot". *Fusarium verticilioides* has an endophytic nature; it can infect maize plants systemically without causing visible disease symptoms, and is found in symptomless systemically-infected seeds. FUMs can be found in a few other crops, typically at low levels, but their economic importance is mainly in maize. Epidemiological studies have clearly demonstrated the importance of drought stress, insect damage, hybrid genotype and, most importantly, temperature for FUM occurrence. Contamination of maize and maize-based products destined for human and animal consumption with FUMs are most likely to occur in the tropical, subtropical and warm areas of the world and in temperate growing regions, including those in North America and Europe. FUMs are often co-produced together with other toxins, such as fusaric acid, fusarins and moniliformin (Desjardins and Proctor, 2001). The International Agency for Research on Cancer (IARC) identified FB_1 as possibly

carcinogenic to humans (group 2B) and it has been proven to be carcinogenic to laboratory animals. Some studies indicate that FB_1 causes an increased prevalence of esophageal cancer in humans and toxic effects against nervous and cardiovascular systems, liver, lung and kidney in animals (Stockmann-Juvala and Savolainen, 2008). FUMs were characterized in the late 1980s, as a result of studies into the causes of esophageal cancer in Africa, coupled with outbreaks of equine leukoencephalomalacia and porcine pulmonary edema in the U.S., both associated with the consumption of corn-based feeds. Leukoencephalomalacia is a fatal diseases of horses with symptoms that include drowsiness, blindness, staggering, and liquefaction of brain tissue. In swine, symptoms of FUM poisoning are reduced feed intake and weight gain, liver damage; poisoned pigs can develop a fatal disease known as pulmonary edema, i.e., fluid accumulation in the tissue and air spaces of the lungs.

Ochratoxin A

Ochratoxins are mycotoxins produced by several species of *Aspergillus* and *Penicillium* (Wang *et al.*, 2016). There are three types of ochratoxins, namely A, B, and C. Particularly, ochratoxin A (OTA) is the most common and important one for public and animal health; ochratoxins B and C are less toxic and less common. OTA was originally described as a metabolite of *Aspergillus ochraceus* and subsequently isolated from a number of different *Aspergillus* species. However, the first report of natural occurrence of OTA was from a *Penicillium viridicatum* strain, later re-identified as *P. verrucosum*. The most important producers of OTA are the "black aspergilli" (*Aspergillus* Section *Nigri*) *A. carbonarius* and *A. niger*, which are commonly found in grapes, dried vine fruits, wine, and coffee. *Aspergillus ochraceus* and related species, which produce this toxin in coffee and sometimes in stored grains are less common producers. The only *Penicillium* species that produce OTA are *P. verrucosum*, and the closely related *P. nordicum*. *Penicillium verrucosum* commonly occurs in cereals in cool temperate climates, whereas *P. nordicum* has been isolated, uncommonly, from processed meats.

Contamination of commodities with OTA generally occurs because of poor storage conditions or non-optimal drying practices. OTA is a chemically stable compound; hence, ordinary food processing do not reduce its presence in foods and beverages significantly (Malir *et al.*, 2016). OTA has proven to induce kidney and liver tumors in laboratory animals and is classified by IARC as a Group 2B compound (possible human carcinogen) (International Agency for Research on Cancer IARC, 1993). OTA is mainly a nephrotoxin and is thought to be the cause of a chronic kidney disease in humans known as Balkan endemic nephropathy that occurred in the early 1970s. Recent studies have provided a link between ochratoxin exposure and human testicular cancer in Europe and tumors in the human urinary tract. Cases of ochratoxicoses of farm animals have been reported in pigs and poultry, with main clinical signs being renal dysfunction (nephropathy), edema and feed refusal. In pigs, excessive thirst (polydipsia) and passage of large volumes of urine (polyuria) are characteristic signs of this disease. Ochratoxins assumed with feed are transferred to blood, milk and muscle tissue of animals (Bui-Klimke and Wu, 2015) and thus may be found in animal-derived-products (meat, milk and eggs). According to the European Food Safety Agency (EFSA, 2006), the estimated source of OTA exposure in adults in Europe is: 44% cereals, 10% wine, 9% coffee, 7% beer, 5% cacao, 4% dried fruits, 3% meat, 3% spices, and 15% others foods.

Patulin

Patulin (PAT) is a toxin produced primarily by *Penicillium expansum*, and secondarily by numerous other *Penicillium* and *Aspergillus* species that affect fruits. Compared to other mycotoxins PAT is regarded as less risky, because the production of significant levels of PAT is accompanied by visible rotting of the fruit, so nearly all the toxin can be removed by rejection of rotting fruits through selection and sorting. Nevertheless, concentrations of patulin in foods are subject to regulatory control in some countries. PAT-producing fungi have been isolated from various moldy fruits and vegetables. PAT has been reported primarily in apple and apple-based foods, and occasionally in other fruits, such as pears, plums, peaches, figs, oranges and grapes. Toxicological studies on PAT have shown that effects of ingestion include convulsions, agitation, ulceration, edema, intestinal inflammation, vomiting and DNA damage in the brain, liver and kidneys of experimental animals. Chronic exposure have also neurotoxic, immunotoxic, genotoxic and teratogenic effects. However, based on the lack of evidences in humans, IARC has classified PAT in Group 3 as not carcinogenic to humans (International Agency for Research on Cancer IARC, 1986). PAT is destroyed by the fermentation process and therefore it is not found in fermented apple beverages, such as cider, and its occurrence concerns primarily juices, which are produced with less strictly selected fruits. The European Community sets the limits to the concentrations of PAT in food products at 50 μg/kg in all fruit juices, fruit concentrates and fermented drinks derived from apples or containing apple juice, at 25 μg/kg in solid apple products used for direct consumption, and at 10 μg/kg in apple juice and solid apple products for infants and young children (European Commission (EC), 2006).

Trichothecenes

Trichothecenes are a large class of fungal sesquiterpenoid secondary metabolites produced by several species of *Fusarium*, *Myrothecium*, *Spicellum*, *Stachybotrys*, *Cephalosporium*, *Trichoderma*, and *Trichothecium* (Cole *et al.*, 2003). More than 200 trichothecenes are known to date, but only few of them have economic and safety importance because may contaminate staple products and cause mytoxicoses in humans and farm animals, all of them produced by *Fusarium* spp. Trichothecenes are classified into four

types (A–D), based on substitutions on the 12,13-epoxytrichothec-9-ene core structure (McCormick *et al.*, 2011). *Fusarium* species produce either type A or type B trichothecenes in cereals (Foroud and Eudes, 2009) The type B trichothecenes, characterized by a C-8 keto group, are produced mainly by *F. graminearum* and *F. culmorum*, and include the important trichothecenes deoxynivalenol (DON, a.k.a. vomitoxin because of its deleterious effects on the digestive system of monogastric animals), nivalenol (NIV), 4-acetylnivalenol (a.k.a. fusarenon-X, FUS-X) and acetylated derivatives thereof. Of these, by far the most common contaminant of food and feed is DON, but it has been reported that in Japan NIV is as prevalent as DON in wheat and barley (Nagashima, 2015). Type A trichothecenes include compounds that have at C-8 a hydroxyl group, an ester functional group, or no oxygen substitution. Type A trichothecenes are produced mainly by the species *F. sporotrichioides* and *F. langsethiae* and include the mycotoxins neosolaniol (NEOS), T-2 toxin (T-2), HT-2 toxin (HT-2) and 4,15-diacetoxyscirpenol (DAS). Trichothecene contamination is economically important in wheat, barley, oats, maize and triticale. In temperate climate regions there is a prevalence of *F. graminearum* and, less frequently *F. culmorum*, *F.crookwellense* and *F. avenaceum*. *Fusarium graminearum* (sexual stage *Gibberella zeae*) causes a disease of wheat and other small grains known as Fusarium head blight (FHB) and on maize a disease known as Gibberella ear rot (GER). In wheat, infected spikes exhibit premature bleaching as the pathogen progresses within the head, while on maize the ears infected with *F. graminearum* appear covered with a pinkish fungal mycelium. The infected grains and corn become contaminated with DON and other type B trichothecenes, and often also with the estrogenic mycotoxin zearalenone, produced by the same *Fusarium* species. Under cooler conditions, the type A trichothecene-producing species *F. sporotrichioides*, *F. langsethii*, and *F. equiseti* predominate. However, the regional and annual relative abundance of the above *Fusarium* species and the associated mycotoxins may vary, mostly affected by temperature.

The mechanism of toxicity of trichothecenes to eukaryotic cells is based on the inhibition of protein synthesis by interfering with initiation, elongation, and termination stages of the process; the mycotoxin affects peptidyl transferase enzyme binding the 60S ribosomal subunit, thus causing the inhibition of protein translation. In general, the trichothecenes are more cytotoxic than other *Fusarium* metabolites and the type A trichothecenes have a higher cytotoxic effect than type B trichothecenes. However, trichothecenes are not carcinogenic to humans, according to IARC evaluation (International Agency for Research on Cancer IARC, 1993). Most trichothecene toxicology studies have focused primarily on T-2 and DON (Pestka, 2010; Li *et al.*, 2011). In animals, trichothecenes aberrantly activate proinflammatory gene expression, disrupt gastrointestinal function and interfere with growth hormone action. Acute exposures to high trichothecene dosages in experimental animals induce anorexia, diarrhea, and vomiting; at extremely high doses, even more severe effects have been observed, including gastrointestinal hemorrhage, leukocytosis, circulatory shock, reduced cardiac output, and ultimately death. Chronic exposure of animals to moderate doses of trichothecenes induces food refusal and weight loss, impairment of the immune system function, and developmental diseases.

Trichothecenes are among the most important mycotoxin as for economic impact and food and feed safety. The IPCS/WHO reported that trichothecenes are cause of fatal and chronic intoxications on human and livestock (International Program on Chemical Safety IPCS & World Health Organization WHO, 1990). Human disease outbreaks associated with the consumption of DON-contaminated cereal grains and flour were reported in China and India. The symptoms included nausea, fever, headaches, and vomiting. In the 1940s in Russia, T-2 caused a fatal disease of humans known as alimentary toxic aleukia (ATA). Symptoms of ATA in humans included nausea, vomiting, diarrhea, leucopenia (aleukia), hemorrhaging and degeneration of bone marrow, skin inflammation, and sometimes death. In the 1970s-1990s,T-2 was used in Southeast Asia as a chemical warfare agent released by plane-spray and hence called "yellow rain". As for livestock, trichothecenes, particularly DON, have been implicated in field outbreaks of pigs, cattle and chickens. The most frequently observed effect of DON in farm animals is feed refusal resulting in reduced performance. In the field, concentrations as low as 1 mg/kg have been associated with feed refusal in pigs, which is the most sensitive animal. More typically concentrations of >2–5 mg/kg are required for decreased feed intake and reduced weight gain and concentrations of >20 mg/kg diet for vomiting and feed refusal in ruminants (Pitt *et al.*, 2012). Dogs and cats are also sensitive to the emetic effects of DON.

Zearalenone

Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by several *Fusarium* species, but primarily by *F. graminearum* (teleomorph *G. zeae)* and *F. culmorum*, in maize and small grains (wheat, oats, barley, rye, sorghum, millet, and rice). Contamination of grains and silage usually comes from the infection of stems, stalks and heads before harvest, but may also occur at post harvest if the crop is not dried properly. Low levels of the ZEA reduced derivatives α-zearalenol (α-ZEOL), which exhibit increased estrogenic activity, and β-zearalenol (β-ZEOL) of about the same potency of ZEA, have been identified in corn, corn by-products, corn silage and soya meal (Schollenberger *et al.*, 2006). The same ZEA-producing species also produce the trichotecene toxin DON and in maize have been found in association with the endophytic fumonisin-producing species *F. proliferatum*. Therefore, grain lots contaminated with ZEA are frequently also contaminated with DON and, in corn, sometimes also with FUMs (Ali *et al.*, 1998; Doko *et al.*, 1996). ZEA mimics the reproductive hormone estrogen and has high affinity for estrogen receptors, causing reproduction and fertility disorders, known as estrogenism, in sensitive animals. A synthetic analog of ZEA, called zeranol was marketed as an anabolic agent for growth promotion of sheep and cattle. Zeranol is now banned in Europe.

Experimentally, ZEA treatment increased incidences of liver cell and pituitary tumors in mice but not in rats. To date, the human health effects remain largely undefined. Based on the currently available data, IARC has categorized ZEA as Group 3, not carcinogenic to humans (International Agency for Research on Cancer IARC, 1993). Swine are the most commonly affected farm

animals. Pre-pubertal female pigs are the most susceptible animal to the estrogenic effects of ZEA. The sensitivity of pigs may be due to a higher affinity of their estrogen receptors for α-ZEOL that is formed in the liver and in the intestines by metabolization of ZEA. Cattle and sheep are much less sensitive than pigs, and poultry are regarded as resistant. The clinical effects of zearalenone may include an enlarged uterus, swelling of the vulva and vagina (known as vulvovaginitis), enlarged mammary glands, anestrus (periods of infertility), and abortion. ZEA may be passed to nursing piglets through the mother's milk. Field outbreaks of estrogenic syndrome in pigs have been reported in North America, Europe, Africa, Asia, and Australia (Zinedine *et al.*, 2007).

Co-Occurrence of Mycotoxins

While regulations enforced by various countries have set the limits for the content of individual mycotoxins or total congeneric mycotoxins (e.g., aflatoxins) in food and feed, contamination with only one single mycotoxin is the exception rather than the rule. More frequently, two or more mycotoxins co-occur simultaneously, especially in feed. As a matter of facts, many mycotoxigenic fungi are able to produce different mycotoxins at a time; in addition, commodities can be contaminated by different mycotoxigenic fungi, and feeds are usually made by mixtures of various commodities, each of which can be contaminated with different mycotoxins. A three-year worldwide survey indicated that 48% of 7049 analyzed feedstuffs samples were contaminated by two or more mycotoxins (Rodrigues and Naehrer, 2012). With regard to the major regulated mycotoxins (AfTs, DON and other trichothecenes, ZEA, FUMs, and OTA), a survey of the literature concerning the co-occurrence in cereals and cereal-derived products, resulted in 127 different mycotoxin combinations reported (Smith *et al.*, 2016). The combinations AfTs + FUM, DON + ZEA, AfTs + OTA, and FUMs + ZEA were the most frequently reported. Although the significance is limited by the low numbers of literature data, the following combinations have also been reported: AfTs + OTA on herbs and spices, AfTs + OTA in dried fruits, and combinations of different trichothecenes in oilseeds (nuts, tree nuts, soy, olives) (Smith *et al.*, 2016). In cereals and meat products, OTA more or less frequently co-occurs with minor *Aspergillus* and *Penicillium* mycotoxins such as citrinin, and penicillic acid, which also possess nephrotoxic and carcinogenic activity, with AfTs or with the *Fusarium* mycotoxin FB_1 (Šegvić Klarić *et al.*, 2013).

Since most studies have focused on the occurrence and toxicology of a single mycotoxin, the health risk from this multi-exposure is not well-characterized. The exposure to multiple mycotoxins may lead to additive, synergistic or antagonistic toxic effects that cannot be always predicted based on the individual toxicities (Grenier and Oswald, 2011). Most studies have been based on in vitro experiments in cell models of human and animal origin, mostly using binary mixtures of mycotoxins. The results of these experiments are not always consistent, and different effects were reported for one particular combination, depending on the used cell model, the test concentration, the time of exposure and the ratio of mycotoxins in the combination. For instance, Alassane-Kpembi *et al.* (2013, 2015) reported that DON in combination with its acetylated derivatives (3-AcetylDON and/or 15-AcetylDON) resulted in synergistic cytotoxicity at low inhibitory concentration levels (Alassane-Kpembi *et al.*, 2013) and in additive effects at higher doses (Alassane-Kpembi *et al.*, 2015). As for the most common trichothecene combination DON + NIV, different effects (synergistic, additive or antagonistic) have been reported by different authors, depending on the cell line and test concentration (Alassane-Kpembi *et al.*, 2013, 2015; Marzocco *et al.*, 2009; Wan *et al.*, 2013). Likewise, variations of the effects depending on the model and tested concentrations were reported also for the mixtures of fusariotoxins DON + FUS-X and NIV + FUS- X + FUS-X (Alassane-Kpembi *et al.*, 2013, 2015), DON + T2 and DON + beauvericin (BEA) (Ruiz *et al.*, 2011; Ficheux *et al.*, 2012). With regard to another very common combination, DON + ZEA, Kouadio *et al.* (2007) and Ficheux *et al.* (2012) found additive cytotoxicity on Caco-2 and CFU-GM cell lines respectively, whereas Wan *et al.* (2013) and Bensassi *et al.* (2014) reported antagonism on IPEC-J2 and human HCT116 cells, respectively. Concerning the mixtures involving ZEA, FB_1 and emerging mycotoxins such as BEA and enniatins (ENNs), the majority of reports suggest antagonistic or additive cytotoxic effects (Smith *et al.*, 2016). Most of the studies addressing the effects of OTA + PAT, OTA + FB_1, and OTA + AfTs combinations have shown additive or synergistic interactions. However, less than additive and antagonistic interactions were also observed particularly for the OTA + AfTs combination (Šegvić Klarić *et al.*, 2013). The picture is even more complex when ternary, quaternary or multiple combinations are considered.

At the whole, from the knowledge currently available, it's not possible to draw definitive and solid conclusions about the toxic effects of co-occurring mycotoxins and hence regulations throughout the world currently do not take the effects of mixtures of mycotoxins into account. Nevertheless, it is nowadays apparent that the regulated mycotoxins often co-occur and interact toxicologically with each other and also with other less studied compounds, such as masked and emerging mycotoxins (Ficheux *et al.*, 2012). In general, most of the mycotoxin mixtures lead to additive or synergistic effects, highlighting both a significant threat to human and animal health and a critical regulatory issue that needs to be addressed urgently.

Modified (Masked) Mycotoxins

Myotoxigenic fungi produce mycotoxins in living plants during the infection process. In turn, plants can metabolize compounds produced by plant pathogens, including mycotoxins, as a mechanism of self-defense. Plant metabolites of mycotoxins are called masked mycotoxins (Berthiller *et al.*, 2013). Detoxification of metabolites produced by plant pathogens involves the chemical transformation of toxic compounds by plant enzymes (Phase I), followed by the covalent binding of phase I activated metabolites

(Phase II) that results in products with reduced or no toxicity (Coleman *et al.*, 1997). Phase I reactions usually involve hydrolysis or oxidation and are typical for lipophilic toxins, while most of the hydrophilic compounds are not affected by this phase. Hydrolysis in phase I is catalyzed by esterases and amidases; oxidations are in the majority of cases catalyzed by the cytochrome P-450 system (Coleman *et al.*, 1997). The reaction product of phase I are not necessarily less toxic than the parent compounds and in some cases the plant metabolite may be as toxic or even more toxic than the parent compound. In phase II, the plant enzymes catalise the covalent binding of hydrophilic phase I-generated metabolites with different residues, including glucose, malonic acid and glutathione (GSH, γ-glutamyl-cysteinyl-glycine) (Coleman *et al.*, 1997). Glucosyl residues conjugate with hydroxy, thiol, amino and carboxy groups. Malonyl residues conjugate with hydroxyl and amino groups. GSH residues have an affinity with electrophilic sites in the molecule. These phase II reactions are catalyzed by glucosyl-, malonyl- and glutathione-*S*-transferases (GSTs), respectively. Phase II products are usually either non-toxic or less toxic than the parent compounds. Finally, the phase II toxic metabolites are either excreted from the plant cytosol via membrane-bound transporters into the apoplast or compartmentalized into the vacuolar space. The phase III of plant detoxification involve sequestration of compounds conjugated to glucose or GSH into the vacuole or the irreversible binding to the cell wall. In this way, detoxification products are permanently stored in the plant tissue rather than excreted. The majority of toxins conjugated with GSH are compartmentalized in the vacuole, where the conjugates may undergo further transformations.

Plant metabolites have been described for some regulated mycotoxins, including the *Fusarium* mycotoxins DON, ZEA FUMs, NIV, FUS-X, T-2, HT-2 and the *Aspergillus* and *Penicillium* toxins OTA and PAT (Berthiller *et al.*, 2013). The modified mycotoxins are not detected by the common analytical methods utilized for assessment of crop and food contamination, because of the mycotoxin structure modification. Therefore, the extractable conjugated or nonextractable bound mycotoxins remain in the plant tissues and, although potentially harmful, they are neither routinely searched, nor regulated by law. Toxicological data on modified mycotoxins are still scarce, but several studies suggest that a potential threat to food safety may come from these substances (Gratz, 2017; Zhang *et al.*, 2020). Conjugation of mycotoxins with epoxides, lactones or aldehyde groups are mostly irreversible, but conjugation to glucose can be reversed by the glycosidase enzymes present in plants and/or in the digestive system of animals and are, therefore, of higher concern for food and feed safety. To date, only metabolites of some fusariotoxins have been found to occur in natural conditions. Particularly, glycosylated forms of DON, T-2, HT-2, NIV, ZEA, ZEOLs have been proven to occur in naturally infected cereals such as wheat, barley, oats and maize (Schneweis *et al.*, 2002; Berthiller *et al.*, 2009; Lattanzio *et al.*, 2012; Nathanail *et al.*, 2015), mostly co-existing with free forms. Currently, deoxynivalenol-3-β-D-glucoside (DON-3G) is the most extensively studied masked mycotoxin. DON-3G has been reported to occur in cereal grains (wheat, maize, barley and oats) and cereal flours, as well as in cereal-derived processed products such as bread, snacks, biscuits, pasta (Vendl *et al.*, 2010; Desmarchelier and Seefelder, 2011), malt and beer (Kostelanska *et al.*, 2009), and in feeds (Kovalsky *et al.*, 2016). The European Food Safety Authority (EFSA) considers the toxicity of DON-3G to be similar to that of DON and, due to the large rate of transformation of DON-3G in DON during digestion, has suggested that DON-3G is added to the total DON exposure for risk assessment. A tolerable daily intake (TDI) of 1 μg/kg body weight/day for the sum of DON, 3-acetyl-DON, 15-acetyl-DON and DON-3G has been recommended for chronic exposure of humans (European Food Safety Authority EFSA, 2017).

A few studies have demonstrated the presence of zearalenone-14-β-D-glucoside (ZEA-14G, a.k.a. zearalenone-4-β-D-glucoside), a metabolite of the estrogenic mycotoxin ZEA, in crop plants (Schneweis *et al.*, 2002). ZEN-14G and the ZEOLs derivatives α-zearalenol-14-β-D-glucoside (α-ZEOL-14G) or β-zearalenol-14-β-D-glucoside (β-ZEOL-14G) have been found in grains and cereal-based foods (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015). Another ZEA metabolite, namely zearalenone-14-sulfate (a.k.a. zearalenone-4-sulfate) has been identified in a survey of different cereal-based products (wheat flour, whole-meal wheat bread, maize meal, biscuits, wheat flakes, bran flakes, muesli, crackers, cereal snack bars and polenta), although only in 13 out of 84 analyzed samples and at low concentrations, ranging from <1–6.1 μg/kg (Vendl *et al.*, 2010).

Emerging Mycotoxins

Besides the regulated mycotoxins, there is a number of other fungal toxic compounds produced by spoilage agents which are neither routinely determined, nor legislatively regulated because of their lesser occurrence or uncertain toxicity in vivo. Some of these metabolites are produced by the main mycotoxigenic species and are often co-occurring with regulated mycotoxins. The term "emerging mycotoxins" is used for such compounds. Emerging mycotoxins may contribute to the overall health risk of the contaminated commodities, either because of their direct toxic effects or because of biological interaction with other mycotoxins, in additive or synergistic manner. Currently there are no regulations enforced that set standards for the presence of these mycotoxins in foods, since the available information about their occurrence, levels of contamination and toxicity is not sufficient to proof health risks for humans and animals. Lately, more research efforts have been done to assess the health risks they actually pose. The increasing diffusion of analytical methods based on liquid chromatography – mass spectrometry (LC-MS) that allow the sensitive and fast simultaneous determination of multiple fungal metabolites in various matrices is expected to provide more information about the occurrence and co-occurrence of emerging mycotoxins in foods and feeds (Tittlemier *et al.*, 2019).

Emerging *Fusarium* Mycotoxins

The most significant and frequently occurring emerging mycotoxins are produced by *Fusarium* spp. The main mycotoxins in this group are enniatins (ENNs), beauvericin (BEA), fusaproliferin (FP) and moniliformin (MON).

Enniatins and beauvericin

A recent study showed that BEA was the emergent mycotoxin with the highest prevalence in feed and feed ingredients, followed by ENNs (Streit *et al.*, 2013). ENNs and BEA are structurally and mechanistically similar molecules that are biosynthesized by the non-ribosomal multifunctional enzymes enniatin and beauvericin synthetases, hybrid enzymes of a peptide synthetase and an integrated N-methyltransferase. They are cyclic hexadepsipeptides consisting of three D-α-hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) alternating with three amino acid units. In BEA, the three amino acid residues are aromatic N-methyl-phenylalanines, whereas in the enniatins of type A and B the amino acid residues are aliphatic N-methyl-valine or–isoleucine or mixtures thereof. BEA was first isolated from the culture of the entomophagous fungus *Beauverina bassiana* and is produced by various *Fusarium* species, including *F. proliferatum* and *F. temperatum* (Logrieco *et al.*, 1998; Jestoi, 2008). ENNs, originally discovered from cultures of *F. orthoceras* var. *enniatum* (later renamed *F. oxysporum*) are produced by several *Fusarium* species, including *F. avenaceum*, *F. sambucinum*, *F. poae*, *F. sporotrichioides* and *F. tricinctum*, which infect cereals and other commodities.

The primary toxic action of BEA and ENNs is thought to be related to their ionophoric properties; they can form stable and lipophilic complexes with cations and transport them into lipophilic matrices such as cell membrane, resulting in disturbances of the cell osmotic balance. In addition, both ENNs and BEA form cation-selective channels in cell membranes, which impair the membrane functions (Kouri *et al.*, 2003; Kamyar *et al.*, 2004). ENNs and BEA exhibit a number of different toxic effects in vitro, but toxicity in vivo is generally low; this is thought to be due to their rapid metabolization rather than low bioavailability.

ENNs have antimicrobial, phytotoxic and insecticidal properties and low micromolar concentrations were shown to be cytotoxic to different animal cell lines (mouse macrophages, porcine kidney cells, *Spodoptera frugiperda* SF-9 cell line), and to reduce the motility of boar spermatozoa. Cell death was shown to be mediated by the induction of apoptosis via the mitochondrial pathway (Tonshin *et al.*, 2010) or by the induction of necrosis linked to lysosomal damage (Ivanova *et al.*, 2012). Several studies investigated the in vivo toxicity of ENNs in rodents. Most of these studies showed low toxicity. A single dose of 50 mg ENNs/kg body weight did not induce signs of toxicity in rats (Bosch *et al.*, 1989). Twenty-nine forms of ENNs are known (Sy-Cordero *et al.*, 2012), but ENNs A, A1, B, and B1 are the most frequently detected in food. In a survey of the occurrence of ENNs in Europe in the years 2000–2013, ENNs were detected in 37%, 68%, and 76% of food (n = 4251), feed (n = 3640), and unprocessed grain (n = 2647) samples (European Food Safety Authority EFSA, 2014). Maximum reported concentrations in grains were 950, 2000, 18,300, and 5720 μg/kg for ENN A, ENN A1, ENN B, and ENN B1, respectively. Based on these data, EFSA concluded that acute exposure to ENNs is not a concern to human health.

Antimicrobial activity against Gram (+) bacteria, cytotoxicity to human (e.g., HeLa, HEP G2, IARC BL-41, U937), animal (e.g., murine tumor, mouse macrophages, porcine kidney) and insect (e.g., SF-9) cell lines and pro-apoptotic effects (Wätjen *et al.*, 2014) in in vitro tests have been reported for BEA (Mallebrera *et al.*, 2018). BEA was not mutagenic in the Ames test (Fotso and Smith, 2003). In vivo, the reported LD_{50} values of BEA in mice were $\geq$100 and $\geq$10 mg/kg for oral and intraperitoneal (i.p.) administration, respectively (Omura *et al.*, 1991). In a survey of the occurrence of BEA in Europe in the years 2000–2013, BEA was detected in 20%, 21%, and 54% of food (n = 732), feed (n = 861), and unprocessed grain (n = 554) samples. Maximum concentrations for BEA determined in grains and in cereal-based food were 6400 and 844 μg/kg, respectively (European Food Safety Authority EFSA, 2014). In their scientific opinion, the EFSA concluded that acute exposure to BEA is not a concern to human health. BEA was also found to occur with high frequency (98% of 83 analyzed samples) in feed and feed raw materials samples, with maximum concentrations of 2330 μg/kg (Streit *et al.*, 2013), and in Chinese medicinal herbs (20% of 60 samples) with a maximum concentration of 125 μg/kg (Hu and Rychlik, 2014).

To date, no conclusion has been drawn with respect to health risk associated to chronic exposure to ENNs or BEA, due to insufficient data on their in vivo toxicity.

Fusaproliferin

FP is produced by a limited number of *Fusarium* species, most notably by the members of the *Gibberella fujikuroi* species complex, such as *F. proliferatum* and *F. subglutinans.* High levels of FP occasionally occur in grains and grain-based food and feed. However, toxicity and mode of action of FP have not been thoroughly investigated, so far. FP is moderately cytotoxic to lepidopteran SF-9 cells and to human B lymphocyte IARC/LCL 171 cells (Logrieco *et al.*, 1996). In vivo, FP has been found to be toxic in the brine shrimp (*Artemia salina*) larvae bioassay, an invertebrate model often used for studies of zootoxicity of mycotoxins and of environmental toxicology, with a level of toxicity (50% lethal dose, LD50 = 53.4 μM or 23.7 μg/mL) comparable to that of some other mycotoxins such as AfB_1 and DON (Logrieco *et al.*, 1996). FP exerted teratogenic and pathogenic effects on chicken embryos when applied at concentrations of 1 or 5 mM (Ritieni *et al.*, 1997a). FP has been found in grains and grain-based foodstuffs (Santini *et al.*, 2012), at concentrations that in maize may be as high as 500 mg/kg (Ritieni *et al.*, 1997b). FP was detected in 17% of samples (n = 83) of feed and feed raw materials with median and maximum concentrations of 2.6 and 14.8 mg/kg, respectively (Streit *et al.*, 2013). FP has been found to co-occur with BEA, FB_1 and FB_2 in maize from Europe, USA and South Africa (Ritieni *et al.*, 1997b; Munkvold *et al.*, 1998; Shephard *et al.*, 1999).

Moniliformin

MON was first isolated in 1973 from cultures of a *Fusarium moniliforme* isolate which was later identified as *F. proliferatum*. The list of MON-producing species includes *F. acuminatum*, *F. avenaceum*, *F. fujikuroi*, *F. oxysporum*, *F. proliferatum*, *F. sambucinum*, *F. subglutinans* and *F. tricinctum* (Jestoi, 2008). MON is an organic acid that in nature exists as sodium or potassium salt and is therefore strongly water-soluble. The primary mode of action of MON seems to be the interference with the tricarboxylic acid cycle by inhibition of thiamin pyrophosphatase dependent enzymes and of several other enzymes sharing thiamin as common cofactor (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, pyruvate decarboxylase, and acetohydroxy acid synthase) (Pirrung *et al.*, 1996). The authors suggested that MON functions as an active site-directed, irreversible affinity label for enzymes utilizing thiamin. MON was phytotoxic to wheat, tobacco and maize; it mostly showed low levels of cytotoxicity in vitro, except in lymphocytes, skeletomyocytes and cardiomyocytes (Jestoi, 2008), possibly due to the limited penetration of the hydrophilic molecule through the cell membrane. MON was not mutagenic in the Ames test indicating that it is not a genotoxic carcinogen (Knassmúller *et al.*, 1997). However, a study that investigated the genotoxic effects of MON on human peripheral blood lymphocytes, reported chromosomal aberrations, sister-chromatid exchanges, and micronucleus frequencies significantly increasing in a dose-dependent manner after treatment of lymphocytes with MON concentrations between 2.5 and 25 μM (Celik *et al.*, 2009). MON shows more severe effects in vivo, with birds and mink being the most sensitive animals to both oral and i.p. administrations (Jestoi, 2008). The main symptoms of acute moniliformin toxication are muscular weakness, respiratory stress, myocardial degeneration, accompanied by histopathological changes in organs such as the kidneys, the lungs and the pancreas and ultimately coma and death (Kriek *et al.*, 1977). Some in vivo studies with broiler chickens and turkey poultries fed with MON-contaminated culture material suggest that MON may have immunosuppressive effects (Li *et al.*, 2000a,b). MON has been found to occur in maize and maize-based products in South Africa, EU, UK, China, and Switzerland (Jestoi, 2008) in concentrations as high as 530 mg/kg of *Fusarium*-damaged kernels (Lew *et al.*, 1996). Occurrence of MON in cereal grains (wheat, rye, oats, barley) and cereal based products is also frequent in various regions of the world, albeit at lower levels than in maize (Jestoi, 2008).

Emerging *Aspergillus* and *Penicillium* Mycotoxins

The genus *Aspergillus* comprises more than 300 species and is characterized by a high level of chemodiversity. Besides AfTs, OTA, FUM and PAT, a number of other toxic substances have been isolated from *Aspergillus* spp. (Wilson, 1966; Varga *et al.*, 2015). Of major interest as possible food and feed contaminants are sterigmatocystin (STE) and cyclopiazonic acid (CPA).

STE is a precursor of aflatoxin biosynthesis produced by many *Aspergillus* species belonging to the sections *Versicolores*, *Usti*, *Aenei*, *Ochraceorosei*, *Cremei* and *Nidulantes* of the genus. While aflatoxin producing species in the section *Flavi* do not accumulate sterigmatocystin, which is efficiently converted to aflatoxins, species belonging to the sections *Ochraceorosei* and *Nidulantes* produce AfTs and STE simultaneously (Samson *et al.*, 2014). The main producers of STE are *A. flavus*, *A. parasiticus*, *A. nidulans*, and, most importantly, *A. versicolor* and its related species (EFSA, 2013). The toxic effect of STE is mediated by its furofuran ring structure, which forms DNA adducts after metabolic activation to an epoxide (Wang and Groopman, 1999). STE exerts genotoxic and cytotoxic effects on different cell lines, e.g., immortalized ovarian hamster cells (CHO-K1 cells; Zouaoui *et al.*, 2016), liver hepatocellular cells (HepG2; Gao *et al.*, 2015), and human lung adenocarcinoma cells (A549; Bünger *et al.*, 2004). STE is classified by IARC as a group 2b carcinogen (possibly carcinogenic to humans; IARC, 1987). In vivo studies have been carried out in livestock, fish, and other animals. Whereas the in vivo effects of STE are similar to those of AfB_1, its acute toxicity is much lower. Studies in ruminants found harmful effects of a STE-contaminated diet in cattle (Vesonder and Horn, 1985), but not in sheep (Böhm and Sayed, 1994). Data on the natural occurrence of STE are sparse. STE was found to occur in green coffee beans, spices, nuts, cereal grains, beer, and the outer layer of hard cheese colonized by *A. versicolor* (Versilovskis and De Saeger, 2010). The JECFA is currently working on a safety assessment of STE, following the recommendation of the Codex Committee on Contaminants in Foods (JEFCA, 2015).

CPA is an indol–tetramic acid biosynthesized by a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) enzyme from tryptophan, mevalonate, and two molecules of acetate. It was originally isolated from *Penicillium cyclopium*. While the main producers of CPA are *Penicillium* spp. (e.g., *P. camemberti*, *P. chrysogenum*, *P. griseofulvum*, *P. urticae*,; Frisvad *et al.*, 2004), it can be produced also by *Aspergillus* species in the section *Flavi*, including *A. flavus* and *A. oryzae*, and by *A. versicolor* (section *Versicolores)* and *A. lentulus* and *A. fumisynnematus* (section *Fumigati*, Varga *et al.*, 2011). CPA is a potent, specific, and reversible inhibitor of Ca^{2+}- activated ATPase in cell sarcoplasmic and endoplasmic reticulum (Plenge-Tellechea *et al.*, 1997). This mode of action has been demonstrated in various tissues and cells, including smooth, skeletal, and cardiac muscle, cultured renal epithelial cells, rat thymic lymphocytes, murine peritoneal macrophages, guinea pig ureter, and many others (Burdock and Flamm, 2000). In muscle tissues, the effect of CPA results in negative inotropic and chronotropic alterations that become apparent in the symptoms of CPA toxicity (Norred *et al.*, 1985). Chelation of such cations as calcium, magnesium, and iron may be also an important mechanism of toxicity of CPA. CPA is not considered to be a potent acute toxin (for instance, its oral LD_{50} in rodents is in the range of 30–70 mg/kg). Clinical signs of acute toxicity in mammals were similar in all studies and include ptosis, hypokinesia, ataxia, hypothermia, action tremor, convulsions, and cessation of food and water intake. The chronic toxicity of CPA has been investigated in many animal species, including chicken, rabbit, dog, pig, and rat (Burdock and Flamm, 2000). Effects of ingestion of CPA-contaminated food include severe gastrointestinal distress and neurological disorders and the digestive tract, liver, kidney and heart, show degenerative changes and necrosis. Since CPA is produced by several molds that commonly occur on agricultural commodities or that are used in certain food fermentations, such as some *Penicillium* species which are used in the production of

fermented sausages in Europe or *P. camemberti* used to produce Camembert cheese and *A. oryzae* used to produce fermented soy sauces, its occurrence warrants attention. CPA may occur naturally in corn and peanuts and in a type of millet (kodo) that reportedly caused human intoxication in India. Few mycotoxicoses attributable to CPA have been reported so far, primarily because of the benign nature of the intoxication. It was suggested that CPA was involved along with AfTs in the Turkey X disease in the United Kingdom in 1960.

Emerging *Alternaria* Mycotoxins

Alternaria is an ubiquitous fungal genus belonging to the division *Ascomycota*, which comprise about 300 described species. Several *Alternaria* spp. (e.g., *A. alternata*, *A. tenuissima*, *A. solani*, and *A. infectoria*, the so-called black molds) are considered as both saprophytes and plant pathogens that can cause diseases of a number of agricultural crops, viz., cereals (wheat, barley, and sorghum), tomatoes, sunflower seeds, citrus fruits, apples, grapes, and olives, and considerable economic losses (Logrieco *et al.*, 2009). Due to the ability of *Alternaria* spp. to grow at low temperatures, fungal infection of crops may also occur at post-harvest, even if storage or transport are under refrigeration. *Alternaria* spp. may produce in moldy foodstuff metabolites that are mutagenic and genotoxic in vitro and that potentially represent a serious health risk for both humans and animals. The most important of the *Alternaria* toxins are alternariol (AOH), alternariol monomethyl ether (AME) and tenuazonic acid. (TeA). It is worth to mention that Schwarz *et al.* (2012) reported that AOH, AME, and TeA made only a minor contribution to the genotoxicity of *A. alternata* extracts. Based on this finding, other metabolites (e.g., altertoxins) or not yet identified compounds may also be important for the assessment of the health risk associated to *Alternaria* food spoilage.

In vitro, AOH and AME were shown to be cytotoxic to human HeLa and colon carcinoma cell lines (Pero *et al.*, 1973; Bensassi *et al.*, 2012). Cytotoxicity appears to be mediated by the activation of the mitochondrial pathway of apoptosis (Bensassi *et al.*, 2012). AOH and AME induced DNA strand breaks and chromosomal aberration in different cell lines (Pfeiffer *et al.*, 2007). Also, AOH induced oxidative stress that resulted in oxidative DNA damage (Fernandez-Blanco *et al.*, 2015) and was shown to be mutagenic in mammalian cell lines (Brugger *et al.*, 2006). A slightly estrogenic potential of AOH was detected in a human endometrial adenocarcinoma cell line and synergistic estrogenic effects of AOH in combination with ZEN or its metabolite α-ZEOL were reported (Vejdovszky *et al.*, 2017). AOH and AME specifically inhibited progesterone secretion in cultured porcine granulosa cells, which suggests that these mycotoxins may affect the reproductive performance of pigs and, possibly, of other mammalian species (Tiemann *et al.*, 2009). Studies on in vivo toxicity of AOH or AME generally have resulted in no evidence of toxicity after oral administration, in chicken, rats and mice (Ostry, 2008). An in vivo study of AOH toxicokinetics revealed the toxin undergo low systemic absorption and rapid metabolization, and therefore the target organ toxicity is likely restricted to the gastrointestinal tract (Schuchardt *et al.*, 2014). In 2011 EFSA opinion concluded that the estimated chronic dietary exposure of humans to AOH and AME exceeds the threshold of toxicological concern for potentially genotoxic substances (EFSA, 2011). However, administration of high doses of the toxins did not elicit toxic or genotoxic effects in in vivo models. Nevertheless, the significant genotoxic potential shown in in vitro tests warrants more investigation of in vivo toxicity of AOH and AME.

TeA is produced by *Alternaria* species that grow on vegetables, fruits, and cereals (e.g., *A. alternata*, *A. tenuissima*, *A. citri*, *A. mali*, *A. oryzae*, *A. solani*) and by the plant pathogenic fungi *Phoma sorghina* and *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*) (Ostry, 2008). The toxic effect of TeA seems to be due to its interference with protein biosynthesis (Shigeura and Gordon, 1963). The compound was not mutagenic in the Ames test (Schrader *et al.*, 2006). In vivo, TeA was toxic to mice, rats, dogs and monkeys, and observed symptoms included diarrhea, muscle tremor, vomiting, and hemorrhages and, in some cases, death (Woody and Chu, 1992). Feeding broilers with feed contaminated with TeA at levels of 10 mg/kg for 3 weeks or daily oral administration of ≥ 1.25 mg/kg of body weight to broilers and layers caused lesions in different organs and decreased weight gain and feed efficiency (Giambrone *et al.*, 1978). Sorghum grain colonized by *Phoma sorghina* that contained TeA was associated with the human hematological disorder known as "Onyalai" (Bottalico and Logrieco, 1998). High levels of TeA (up to 1200 μg/kg) were found in Sorghum-based infant food (Asam and Rychlik, 2013). In 2011, based on a survey of literature, EFSA concluded that the dietary exposure estimate for TeA is lower than the relevant threshold of toxicological concern value and therefore TeA is unlikely to be a human health concern (EFSA, 2011). However, some risk seems to exist for chickens and for infants consuming Sorghum-based food.

Control and Management of Mycotoxins

Mycotoxigenic fungi can be roughly classified into "field fungi" (black aspergilli, fusaria, ergot) and "storage fungi" (aspergilli, penicillia), based on their ecological requirements for growth. Field fungi infect crops and produce mycotoxins primarily before harvest. Storage fungi, which have the capability to grow at low water activities and temperatures, colonize the commodities mainly after harvest, during storage, transportation, or processing. Some fungi, such as *A. flavus*, belong to both classes and may colonize the agricultural products both before and after harvest. Measures of control and management of mycotoxigenic fungi and mycotoxins can be put into effect along the whole food or feed chains; while the pre harvest management strategies aim mainly at prevention of infection and contamination of crops, the post-harvest strategies include prevention, remediation and surveillance practices. Surveillance include regulation and monitoring of mycotoxins in food- and feedstuffs in order to avoid that already contaminated commodities are consumed as such and are, instead, properly discarded, detoxified or diverted to alternative uses.

Finally, dietary interventions can be applied to reduce the livestock intake of mycotoxins with diet by reducing mycotoxin bioavailability or by degradation of mycotoxins to not toxic or less toxic compounds in animal's gastrointestinal tract.

Pre-Harvest Interventions

The pre-harvest approach consists of strategies designed to reduce crop infection by mycotoxigenic plant pathogens. These interventions include breeding of resistant plant cultivars, application of good agricultural practice, and crop protection by chemical pesticides or biological control. In recent years, there has been increasing interest in the development of information and communications technologies (ICT) tools for the prevention of mycotoxin outbreaks based on weather forecasting data monitoring systems associated to prediction models. These tools would support decision making about appropriate interventions that need to be enforced when weather conditions are favorable to fungal/mycotoxins outbreaks. Both empiric and mechanistic models have been used to predict mycotoxin contamination in crops, with a prevalence of the former. The empiric approach describes the relation between the driving variables (i.e., rain during flowering) and the event of interest, that is mycotoxin contamination in grain at harvest, using statistical analysis. On the contrary, predictions of mechanistic models are based on mathematical functions that describe the relationship between driving variables and the different steps of fungal infection cycle (i.e., sporulation, infection or toxin production), which functions are developed mainly by trials in controlled conditions. Forecasting systems have been experimented for *Fusarium* toxins (DON, T-2 and HT-2) in wheat and for FUMs and AfTs in maize (Battilani, 2016). Minor efforts have been devoted to AfT contamination in nuts. AfloMan is a forecasting system for the formation of aflatoxin in groundnuts, in use in Australia.

Breeding crop plants for resistance to mycotoxigenic fungi is a straightforward strategy for reduction of contamination before harvest. The problem of ergot contamination of cereals and millets has been successfully minimized in the past by cultivating varieties of rye, wheat and pearl millet that are resistant to the disease. However, the genetics of resistance to other mycotoxigenic fungi is complex, and high levels of resistance are not yet available in high-yielding commercial cultivars. Maize hybrids and wheat and barley varieties with at least partial resistance to mycotoxin-producing fungi are currently available, but the commercial breeding process involves significant technologies and cost, which raise questions of access and affordability for low income countries. Resistance to *A. flavus*, especially in nut crops and oily seeds, has been studied. However, the development of commercial cultivars that are less subjected to *A. flavus* infections or aflatoxin accumulation encounters hurdles, because of lack of resistant genotypes in some crop species (e.g., cotton), long time required for breeding programs and questionable stability of the resistance conferred by the currently available genes (Rajasekaran *et al.*, 2006). Improved resistance to drought, insect damages, or other abiotic and biotic stresses that predispose plants to pre-harvest formation of aflatoxin have been experimented in groundnuts and maize. Infection of maize kernels by *F. verticillioides* and *A. flavus* is enhanced by insect injury; the resistance to insects of transgenic *Bt* hybrids that express one of the insecticidal proteins from the bacterium *Bacillus thuringiensis* has resulted in reduced fumonisins and, less consistently, aflatoxins levels in maize kernels (Munkvold, 2003), but transgenic approaches are not universally accepted. Leaving *Bt* hybrids apart, differential resistance to aflatoxin contamination among corn hybrids has been found (Windham and Williams, 1998). This, along with significant advances in the identification of natural resistance mechanisms and traits (Brown *et al.*, 2001) have prompted breeding programs aiming at enhancing maize resistance to aflatoxin. However, resistance to aflatoxin contamination involves multiple chromosome regions and several genes. Therefore, selection for resistance, while maintaining essential agronomic characteristics, has been slow.

Wheat cultivars with reasonable resistance to Fusarium head blight (FHB) and accumulation of DON and, secondarily, of ZEA are in use in the USA, Canada, and Europe. Trichothecenes have been reported to act as virulence factors that contribute to the virulence of *Fusaria* to wheat and maize (Desjardins *et al.*, 1996). Hence, increased plant resistance to the toxins would result in increased plant resistance to the pathogen. However, a direct relationship between resistance to FHB and resistance to toxin contamination of the infected grain remains a subject of controversy. Although generally the higher the resistance of a genotype to FHB the lower the toxin accumulation, varieties with low FHB severity and high DON content, and vice versa, have also been described (Mesterházy *et al.*, 1999). No FHB resistant varieties are yet commercially available for durum wheat, but different sensitivities to trichothecenes type B accumulation was demonstrated among lines of *Triticum turgidum* subsp. *durum* which showed only slight differences in the levels of fungal infection (Favre *et al.*, 2004). Those wheat lines may contain endogenous compounds able to either inhibit trichothecene biosynthesis or to degrade or modify the toxins. In wheat, the resistance to FHB can be categorized into five types: type I resistance that operates against initial infection; type II resistance that operates against the spread of the pathogen within the host; type III that is the ability to resist to kernel infection; type IV that is tolerance to infection; and type V resistance to DON accumulation (Boutigny *et al.*, 2008). Type V resistance to *Fusarium* can be subdivided into two classes. Class 1 includes mechanisms by which the plants degrade or detoxify trichothecenes. This includes glycosylation, a natural process already reported in wheat (Berthiller *et al.*, 2005), and two other detoxification processes, acetylation and de-epoxidation. Detoxification by glycosylation in wheat has also been described for zearalenone (Schneweis *et al.*, 2002). Acetylation is a detoxification process used by *Fusarium* species to protect themselves from their own toxins. Kimura *et al.* (1998) isolated from *F. graminearum* the Tri101 gene, which encodes a trichothecene 3-O-acetyltransferase able to convert trichothecenes to less toxic derivatives. De-epoxidation activity has been found in bacteria of ruminal or intestinal flora and an epoxidase from a strain of *Eubacterium* sp. was able to enzymatically reduce different type A trichothecenes and deoxynivalenol to non-toxic de-epoxide metabolites (Fuchs *et al.*, 2002). These findings open the possibility of transgenic plants containing heterologous genes for

detoxification of trichothecenes, as shown in tobacco (Muhitch *et al.*, 2000), wheat and barley (Okubara *et al.*, 2000). However, the genes involved in the detoxification pathways mostly remain non-identified. Class 2 comprises mechanisms that lead to reduced mycotoxin accumulation by inhibition of their biosynthesis through the action of plant endogenous compounds. These include many plant compounds with antioxidant properties, like phenolic compounds, peptides or carotenoids, and with pro-oxidant properties, like hydrogen peroxide or linoleic acid-derived hydroperoxides, which have been described as "modulators" of mycotoxin biosynthesis. Trichothecenes are synthesized from trichodiene by a series of oxygenation reactions which require molecular oxygen. Therefore, changes in the oxidative status of the plant tissues can affect the secondary metabolism of the fungus and modulate the levels of trichothecene biosynthesis (Ponts *et al.*, 2006). A breeding strategy that combines both resistance to pathogen infection and toxin accumulation would probably lead to new wheat genotypes able to limit efficiently the occurrence of *Fusarium* mycotoxins.

Good Agricultural Practice (GAP) is the primary strategy to reduce mycotoxins in cereals and cereal based products. GAP consists of good farm management. In general, all the practices that reduce the biotic and the abiotic stresses of crops may be regarded as a part of a GAP strategy for prevention of mycotoxins. GAP includes planting at the appropriate time (to avoid high temperature and drought stress during the period of seed development and maturation) and with the right spacing, avoiding excess of watering and fertilization, control of weeds, cleaning from debris of preceding crop, crop rotation to reduce tiring of soil and soil pest population increase, and harvesting of crops at or before full maturity, since over-mature crops are more subjected to damages and hence fungal spoilage. Besides a general effect on plant vigor and health, these measures may also have a direct effect on survival, spread and infection of mycotoxigenic fungi. A code for GAP practices for the management of mycotoxins contamination in cereals has been issued by FAO/WHO (Codex Alimentarius, 2003). Wheat and maize have been found to be particularly susceptible to *Fusarium* species and they should not be used in rotation with each other. Crops such as potato, other vegetables, clover and alfalfa that are not hosts to *Fusarium* species should be used in rotation to reduce the inoculum in the field. FHB and maize ear rot are primarily initiated at the crop flowering season by the sexual spores (ascospores) of *F. graminearum* (*Gibberella zeae*) that are formed inside the fruit bodies (perithecia) of the fungus in the colonized stubble and then forcibly ejected up to plant heads. Therefore, the seed bed for each new crop should be prepared by plowing under or by destroying or removing old seed heads, stalks, and other debris that may have served, or may potentially serve as substrates for the growth and spread of mycotoxin-producing fungi. Excess precipitation during anthesis (flowering) makes conditions favorable for dissemination and infection by *Fusarium* spp.; thus irrigation during anthesis and during the ripening of the crops, specifically wheat, barley, and rye, should be avoided. Cereals should be harvested at full maturity, so that damage to the grain is minimized and moisture levels are lower than those conducive to mold growth during storage (generally 15%). Delayed harvest of grain already infected by *Fusarium* species may cause a significant increase in the mycotoxin content of the crop.

The use of pesticides for the control of mycotoxigenic fungi and the reduction of mycotoxins is controversial. Some in vitro studies have showed that fungicides might stimulate mycotoxin production when administered at sub-lethal concentrations (D'Mello *et al.*, 2001; Matthies *et al.*, 1999). In the field, the triazole fungicides metconazole and tebuconazole and the strobilurin fungicide azoxystrobin are effective against *Fusarium* species and FHB, but their use has resulted in inconsistent effects on DON contamination. In some field trails, the application of azoxystrobin resulted in an increase of DON content of grain (Simpson *et al.*, 2001). A biological approach for control of FHB and DON, consisting of the treatment of infected maize stalks with the microbial antagonists *Trichoderma atrobrunneum* and *Clonostachys rosea* to reduce FHB in subsequently grown wheat has been recently experimented, with promising results (Gimeno *et al.*, 2020). Biocontrol of AfTs relies on competitive exclusion of toxin-producing *A. flavus* by a non-toxigenic strain of *A. flavus*. The non-toxigenic strains of *A. flavus* are strains incapable of producing AfTs because are defective for a polyketide synthase gene necessary for AfT biosynthesis (Ehrlich and Cotty, 2004). The non-toxigenic *A. flavus* strain is spread on the soil surface on a carrier substrate that permits growth of the fungus and the consequent production of high numbers of spores and, with time, it displaces the native aflatoxigenic genotypes by a mechanism of competition (Cotty, 2006). This method has proven to be successful in several instances, leading up to 80% reduction of aflatoxin contamination; it is currently utilized in cotton- and maize-growing areas of the USA, and also in Kenya and has led to the development of the commercial biopesticides AF36 (non-aflatoxigenic strain NRRL 18543) and Afla-Guard® (non-aflatoxigenic strain NRRL 21882).

Post-Harvest Interventions

After harvest, it is of utmost importance to observe good manufacturing practice (GMP), that is a range of practices that prevent fungal growth and hence reduce mycotoxin formation. GMP should be implemented during handling, storage, processing, and distribution of commodities for human food and animal feed (Codex Alimentarius, 2003). Drying and maintaining proper storage conditions are key elements for mycotoxin management at post-harvest. Immediately after harvest, moisture levels of the crop should be determined and, if necessary, it should be dried in such a manner that damage to the grain is minimized and moisture levels are lower than those required to support mold growth during storage (generally less than 15% for cereals). On the farm, a first cleaning can be done by removal of defects, including immature nuts or grains, infected and/or insect damaged kernels and weed seeds, debris, stones, soil, husks. Grain or nuts infected by mycotoxin-producing fungi often have visual symptoms or other physical characteristics that allow them to be separated from the healthy product. This can be done by hand sorting, air-screen cleaners, optical sorters, and density separation (Grenier *et al.*, 2014). Hand sorting of moldy, shriveled or insect-infested

nuts or kernels has proven to be an effective method of removing mycotoxin-contaminated food components without removing a large proportion of the product (Turner *et al.*, 2005; Van der Westhuizen *et al.*, 2011). The de-hulling of grains has been shown to reduce mycotoxin contamination significantly, since most of mycotoxins are concentrated in the grain pericarp (Siwela *et al.*, 2005). Washing methods are used to remove water-soluble mycotoxins from the outer surface of grains. Soaking maize in a 0.1 M sodium carbonate solution for 24–72 h can remove significant amount of DON, ZEA and FUM from kernels (Grenier *et al.*, 2014). A drawback of this method is that the grain must be dried before it can be stored.

After harvest, crops should be dried to safe moisture levels and cooled as quickly as possible. Freshly harvested commodities should not be left piling or heaping wet for more than a few hours prior to drying. Sun drying of some commodities in high humidity may result in fungal infection; forced air circulation to aerate the commodities and prevent high moisture and raise of temperature in the pile is advisable. The storage facility should be dry, well-vented and protected from rain, drainage or ground water. Where possible, grains and seeds should be aerated by circulation of air through the storage area to maintain proper and uniform temperature levels throughout the storage area. During the storage period, moisture content and temperature in the stored product should be checked at regular intervals or monitored by electronic systems; these climatic parameters have to be held below the critical levels determined for the storage of cereal grains, beans, and nut crops. Control of molds and pests with appropriate registered chemicals or with alternative methods should be applied if needed, in order to reduce fungal spoilage and pest damage. The use of suitable, approved preservatives (e.g., organic acids such as propionic acid) exclusively in grains intended for animal feed may be effective in killing various fungi and thus prevent the production of mycotoxins. Care must be taken because these compounds can negatively affect the taste and odor of grains. Appropriate measures for the protection of the commodity from inappropriate humidity and temperature levels, as well as from parasites and pests should be observed also during transportation. The mycotoxin levels along these passages should be monitored, using appropriate methods of sampling and analysis (Maragos and Busman, 2010; Tittlemier *et al.*, 2019). The above practices are usually implemented in developed countries, but can be very challenging in developing countries where adequate drying and storage facilities are not common.

Highly contaminated lots, containing mycotoxins at levels above the regulatory levels of tolerance, must be diverted from use as human food or feed for sensitive species. In the EU, the blending of contaminated batches with batches of good quality to lower the average mycotoxin content of the materials is prohibited (Verstraete, 2008). Remediation of contaminated commodities can still be achieved by thermal, biological, or chemical means and detoxified products can be used for feeding less-sensitive livestock, such as mature beef cattle. Most mycotoxins are heat stable in the temperature range used for food or feed processing, therefore it can be difficult to reduce mycotoxin levels significantly by thermal inactivation, while maintaining product quality. Significant reductions in mycotoxin concentrations usually occur at temperatures above 160°C (Bullerman and Bianchini, 2007). Processes such as flaking, roasting toasting and canning of grains or flour can be used to reduce mycotoxin contamination. In maize, extrusion reduced AfTs by 50%–80%, FUMs by 34%–97%, DON by over 95% and ZEA by 60%–83%, depending on the extrusion parameters (Bullerman and Bianchini, 2007). Irradiations with ionizing radiation, i.e., solar radiation, γ-radiation, and microwaves were successful in reducing mycotoxin contamination of AfTs, OTA, FUMs, DON and ZEA in experimental trials, but the technology has not been implemented in food or feed processing, due to the high cost of irradiation units and because of a negative perception of consumers with regard to its safety (Calado *et al.*, 2014). Chemical detoxification methods could greatly reduce mycotoxin contamination, but their widespread use is limited because they not always comply with the FAO requirements. In many instances, mycotoxin degradation/transformation products have not been identified, raising questions about possible "hidden" mycotoxins which might survive the chemical process as transformed compounds with residual toxicity or that may revert to their native toxic forms after ingestion. Also, these processes may have an impact on nutritional value or palatability of feed. Internationally, the use of ammonia gas or ammonium sulfate to reduce AfTs levels in maize is widely accepted. The use of ammoniation for degradation of FB_1, DON and ZEA is more controversial. In fact, the process does not seem to reduce the toxicity of FB_1-contaminated products, in spite of the decrease in mycotoxin level, while in the cases of DON- or ZEA-detoxification, conclusive toxicity studies with decontaminated materials have not yet been conducted. Other chemical treatments have been used for mycotoxin detoxification with a certain degree of success. Ozonation is based on treatment of the products with ozone for a few seconds. Ozone is a powerful oxidizing agent that decomposes to form oxygen and is, thus, a nonpersistent and safe chemical. Ozonation has been successfully utilized for detoxification of AfTs, both in solution and in bulk maize and has also been reported to degrade trichothecenes, ZEA and OTA (Grenier *et al.*, 2014). Another strong oxidizing agent, hydrogen peroxide (H_2O_2), in combination with $NaHCO_3$ or $Ca(OH)_2$ can efficiently degrade AfTs, FUM and ZEA (Lopez-Garcia *et al.*, 1999). Other chemical treatments experimented for mycotoxin degradation include nixtamalization, (a traditional Mexican process for making tortillas and that consists of cooking corn in water and $Ca(OH)_2$ followed by steeping) for reduction of AfTs, FUM, ZEA and DON in maize, 0.5–2% sodium bisulfite against AfTs and DON, reducing sugars for control of FUM in maize and aqueous citric acid to degrade AfTs in feed (Grenier *et al.*, 2014).

Microorganisms have been identified that are capable of metabolizing certain mycotoxins, including AfTs, ZEA and DON, via production of extracellular lytic enzymes able to break or modify the mycotoxin molecules to non-toxic or less toxic compounds (Lyagin and Efremenko, 2019). The enzymes responsible of mycotoxin degradation may be extracted from microbial cultures and used in purified form (Loi *et al.*, 2018) for detoxification of commodities intended for feed production. The pros of this approach are the high efficiency and specificity of action of enzymes, the absence of toxicity to animals consuming the processed materials and the use, likewise all catalysts, in non-stoichiometric ratios with mycotoxins. One major con is the high cost of the enzyme purification process, which in the future might be overcome by the use of easy-to-obtain and inexpensive crude enzyme extracts (Branà *et al.*, 2020).

Dietary Interventions

Additives, known as detoxifying agents, may be added to the diet of animals (mainly of swine, poultry and cattle); these additives reduce the absorption of mycotoxins from the gastrointestinal tract and their distribution to blood, or transform the mycotoxins into less toxic metabolites. In the former case, the additives consist of materials that sequester mycotoxins by binding them on their surface (adsorption), in the latter case additives are microorganisms or microbial enzymes able to degrade or transform the mycotoxin's molecule (biotransformation). In the EU, feed additives for control of mycotoxins, defined as "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action", have been regulated as a new functional group in the category of feed additives (EC, 2009).

Mycotoxin binders, a.k.a. sequestering agents or adsorbing agents, may be mineral, organic or synthetic materials that have the property to bind mycotoxins by stable interactions such as hydrophobic binding, hydrogen bonds, electrostatic attraction or repulsion and coordination bonds (Di Gregorio *et al.*, 2014), which do not dissociate in the gastrointestinal tract of the animal, thus limiting their bioavailability after ingestion. The complexed mycotoxin is then eliminated with the feces. The most important property of binders is the capability to remain stably complexed at the varying pH values which are encountered along the digestive tract. This property is influenced by physical properties of the binder (total charge and charge distribution, the size of the pores, and the accessible surface area) and by toxins' physicochemical properties (polarity, solubility, and shape). Among the mineral binders, the most studied are the aluminosilicates (clays), which include phyllosilicates and tectosilicates. Phyllosilicates (bentonites, montmorillonites, smectites, kaolinites, and illites) can adsorb substances on their surface or within their interlaminar space. The tectosilicates include zeolites that function like both ion-exchange material and molecular sieve since provide a large and specific binding surface but can also differentiate molecules by size, shape and charge. Hydrated sodium calcium aluminosilicate and bentonite/montmorillonites clays are the best adsorbents for AfTs, but are generally less effective in binding of ZEA, OTA, FUM and trichothecenes, with variation amongst different types of clays. Clays could adsorb micronutrients and vitamins and have negative effects on their bioavailability. Therefore, an important element for the assessment of clays suitability as binders is the adsorbent's affinity for vitamins, minerals and other nutrients. Aluminosilicate mycotoxin-binders are currently commercialized worldwide.

Natural organic binders have been studied in the prospect to integrate clays as feed additives for mitigation of major mycotoxins other than AfTs. The combination of inorganic and organic adsorbents could be useful in the most frequent cases of multi-contaminated feeds. Such sorbents include activated charcoal, micronized fibers obtained from different plant materials, yeast and fungal cell walls and components thereof, and bacterial cells. Activated charcoal has been found very effective in adsorbing different mycotoxins (including DON) in in vitro tests, but the results of in vivo trials were controversial. Generally, the adsorption properties of activated charcoal depend on the feedstock materials used for its production, surface area and pore size distribution. Activated charcoal binding is unspecific, hence essential nutrients are also sequestered, particularly if their concentrations in feed are much higher compared to those of the target mycotoxin. Micronized fibers are obtained from different plant materials such as cereals or legumes (wheat, barley, alfafa, oat, pea hulls, dehydrated grape pomace) and consist mainly of cellulose, hemicellulose and lignin. Micronized wheat fibers exhibited beneficial effects against OTA adsorption. In vitro, organic sorbents have been demonstrated to be excellent in the simultaneous removal of several mycotoxins (AfB_1, ZEA, OTA and FUMs) from a liquid medium. However, cellulose materials seem to have less binding ability for AfB_1, compared to other inorganic adsorbents. Chlorophyll and its derivative chlorophyllin, which are natural constituents of green vegetables, can sequester aflatoxin in the gastrointestinal tract and impede its absorption. In addition, these compounds may have enzyme-inducing properties that contribute to mechanisms of detoxification. Interesting findings have been obtained from in vitro studies regarding humic acids, originating from natural decaying of organic plant materials, which have shown the capacity to adsorb mycotoxins, especially AfB_1, OTA and ZEA (Sabater-Vilar *et al.*, 2007). Biosorbents, based on yeast, fungi or bacteria cell walls are an environmentally friendly and rapidly biodegradable alternative to other sorbents. The adsorbing capability of microbial cell walls depends on the structure, especially pore shape and size, and the nature of the polysaccharides they contain. Yeast cell wall mainly consists of polysaccharides, i.e., ß-D-glucan and mannans, which exhibits a great variety of accessible mycotoxin adsorption loci by different binding mechanisms (hydrogen bonds, ionic or hydrophobic interactions). *Saccharomyces cerevisiae* cell wall has proven to be able to adsorb a wide spectrum of mycotoxins, such as ZEN, OTA, FB and trichothecenes (Yiannikouris *et al.*, 2006). Recently, Haidukowski *et al.* (2019) have shown that non-viable mycelium of the edible mushroom *Pleurotus eryngii* was able to efficiently adsorb up to 85% of AfB_1 in solution, in a range of pH values from 5 to 7, mainly by a mechanism of physical adsorption. Some strains of the lactic acid bacterium *Lactobacillus rhamnosus* have the ability to bind onto their cell wall some mycotoxins in animals' small intestine. Lactobacilli adsorb more efficiently non-polar toxins, such as ZEA and AfB_1, due to hydrophobicity of their cell wall surface (Kabak *et al.*, 2006). The cell wall components involved in the process are peptidoglycans, polysaccharides and teichoic acid. *Lactobacillus rhamnosus* is regarded as a safe and functional microorganism, currently used for the production of yogurt and healthy food.

Among the synthetic adsorbents, modified aluminosilicates have been produced in order to extend the binding efficacy of natural clays, mostly restricted to AfTs, to other mycotoxins, including ZEA, OTA and FUMs. The modifications consist of alterations of surface properties by exchange of structural charge-balance cations with high molecular weight quaternary amines, which results in an increased hydrophobicity. In vitro testing has confirmed the improved binding capability of modified clays (Papaioannou *et al.*, 2005). Also polymeric anion exchange resins, such as cholestyramine, divinylbenzene-styrene and polyvinylpyrrolidone (a highly polar

amphoteric polymer) have been demonstrated to bind mycotoxins, in vitro and in vivo. However, so far the practical application of synthetic binders has been limited because of the high cost.

Another strategy for control of mycotoxins in animals' diet is biotransformation, that is the degradation of mycotoxins into non-toxic metabolites by mycotoxin-degrading enzymes or by microorganisms producing such enzymes (Ji *et al.*, 2016). Several microbial species, including bacteria, yeast and fungi have been recognized for their ability to biotransform mycotoxins via (de)acetylation, oxygenation, ring/side chain cleavage, de-epoxidation, isomerization or glucosylation. However, this promising strategy has found so far few practical marketable applications, due to the limited knowledge of the nature of the biotransformation by-products and their toxicity.

Mycotoxins in a Global Warming Scenario

According to the Intergovernmental Panel on Climate Change IPCC (2014), the Earth's climate has been going through a period of global warming since the mid-20th century, mainly due to anthropogenic greenhouse gas (carbon dioxide, methane and nitrous oxide) emissions. In the climatic change scenario drawn by IPCC, the global surface temperature is likely to rise 0.3–1.7°C in a moderate foresight, or as much as 2.6–4.8°C in an extreme foresight. The global warming will result in rising of sea levels, regional changes in distribution and levels of rainfalls, with more frequent extreme weather events such as heat waves and heavy precipitations in a number of regions. Under the extreme scenario, annual precipitation will increase at high latitudes, many mid-latitude wet regions, and the equatorial Pacific, and decrease in many mid-latitude and subtropical dry regions with likely increase in the frequency of droughts.

The growth of mycotoxigenic fungi and mycotoxin biosynthesis are affected greatly by environmental parameters, especially temperature, relative humidity and water availability. Some mycotoxigenic species might shift their geographical distribution in response to global warming, leading to changes in the pattern of mycotoxin occurrence, both qualitatively and quantitatively. In addition, some studies suggest that the elevated atmospheric carbon dioxide levels associated to the global warming may enhance fungal colonization by *Aspergillus* and *Fusarium* species and thus contribute to increased mycotoxin production in crops (Bencze *et al.*, 2017; Medina *et al.*, 2014). Climate has also major effects on plant life cycle and plant susceptibility to stresses and fungal infections, as well as on insect life cycles and attacks. Global warming and changes in rainfall amount and distribution will probably modify the onset and duration of growing seasons and the geographical distribution of certain crops. In certain world areas, crops will be likely subjected to an increased number of biotic and abiotic stress combinations, particularly to the concurrent occurrence of drought and heat stress, and increases in pest reproduction rates and damages which facilitate infection by mycotoxigenic fungi. Since almost all of the mycotoxins present in food and feeds are produced in the field, in this scenario it may be expected that the occurrence, distribution and severity of mycotoxins in several areas of the world will change and, in some cases, increase (Magan *et al.*, 2011).

Recent quantitative estimations based on predictive models have shown that increased AfB_1 and DON contamination are expected in cereals in certain regions of Europe and Africa as a result of global warming. Longer and more severe droughts are foreseen to occur in West Africa and southern Europe in the next future, which may lead to an increase in AfTs occurrence in foods and feeds. Recent unusual outbreaks of AfTs have been reported in some regions of Europe as a result of drought stress of plants that made the crops more prone to infections by *A. flavus* and biosynthesis of AfTs (Miraglia *et al.*, 2009; Battilani *et al.*, 2016). According to some prediction models, the increase of Earth temperature would result in the geographical shift of *A. flavus*, which grows well under warm and dry weather, from southern Europe to more northern and eastern regions of Europe (Van der Fels-Klerx *et al.*, 2016). This implies that AfB_1 is likely to become a consistent food safety issue for crops, particularly maize, in Eastern Europe, Balkan Peninsula and the Mediterranean regions where AfB_1 outbreaks currently occur only occasionally. Also, increased contamination of wheat grains with DON is expected as a result of climate change. Some simulations have shown an increase of up to 3 times in DON presence in wheat in north-western Europe by 2040 (Van der Fels-Klerx *et al.*, 2012). Severe rains while plants are at the flowering stage are associated with increased infection of *Fusarium* spp. to wheat heads and corn ears and may cause higher levels of mycotoxins in kernels at harvest. In maize, the production of FUM has been associated with dry weather and late season rains, which are more likely to occur under the climate change scenario. Abundant rainfalls in the proximity of harvest may also induce farmers to harvest prematurely, when grains are not completely dry, hence favoring mold contamination and mycotoxin accumulation at post-harvest.

While the studies on the possible effect of climate change on mycotoxin occurrence have been so far focused on AfB_1 and DON, it is conceivable that changes in the distribution, frequency and levels of other regulated and not regulated mycotoxins would occur similarly, which prompt for more extended analyses to evaluate the mycotoxin risk in a world climate change scenario.

Biosynthesis and Genetic Regulation of Mycotoxins

Generally, the biosynthesis of mycotoxins is a multi-step pathway involving a series of enzymatic reactions to form the final molecular structure. For most of fungal mycotoxins, the genes encoding the proteins involved in the biosynthesis process are typically located next to one another in a biosynthetic gene cluster (BGC), facilitating a coordinated regulation of their expression (**Fig. 1**). According to the structural classification of mycotoxins as poliketides, ribosomal and nonribosomal peptides or

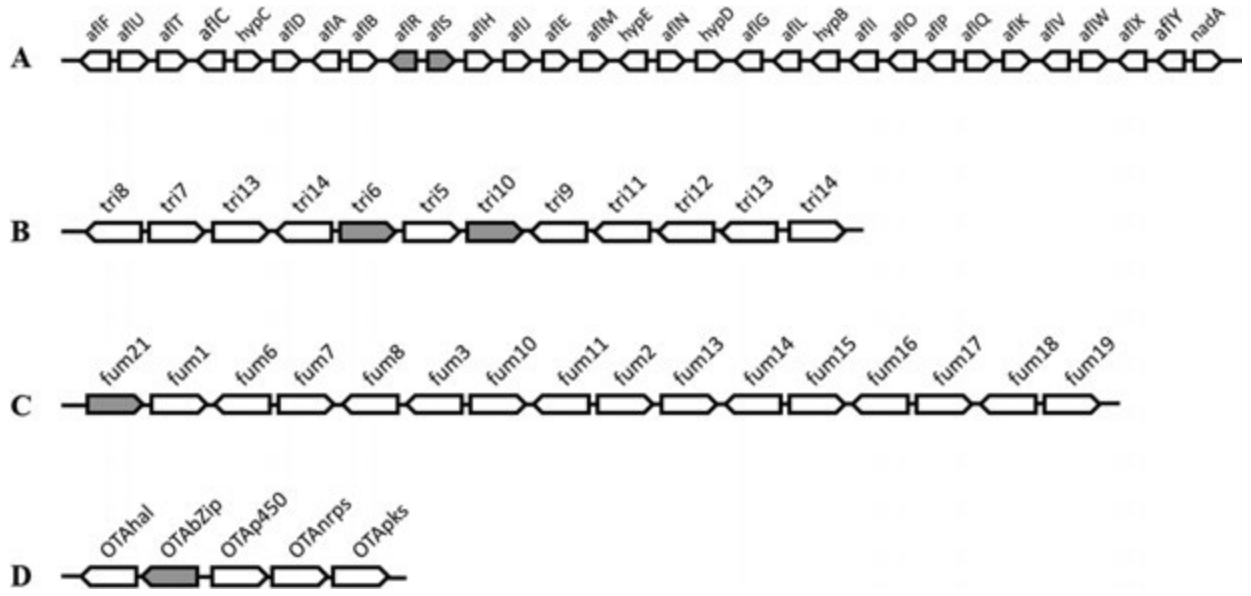

Fig. 1 Schematic rendition of mycotoxin biosynthetic clusters. Lengths of genes and intergenic regions are not drawn to scale; gray blocks represent regulatory genes. (A) aflatoxin cluster in *A. flavus*; (B) trichotecene cluster in *F. graminearum*; (C) fumonisin cluster in *F. oxysporum*, *F. proliferatum* and *F. verticillioides. A*; (D) ochratoxin A cluster in *A. carbonarius*.

terpenoids, a key gene encoding for a polyketide synthase (PKS), a non-ribosomal peptidase (NRPS) or a terpene synthase, is present in the cluster. Synthases or synthetases establish the molecular backbone of mycotoxin which is then modified by a variety of tailoring enzymes such as (de)hydratases, oxygenases, hydrolases, methylases, and others to form the final complex structure. The same biosynthetic pathway may give origin to the structural diversity of mycotoxins belonging to the same mycotoxins family, as a result of the absence or the non-functioning of a gene within a biosynthetic cluster, or the variation of enzymatic activity (Hoffmeister and Keller, 2007; Keller, 2019).

Other genes encoding metabolite transport proteins and transcription factors are generally present in the mycotoxin clusters. In particular, one or occasionally more transcription factors are required to regulate the expression of the other biosynthesis genes in the cluster (Osbourn, 2010; Brakhage, 2013). The most common type of cluster pathway regulators belongs to the class of C-6 zinc finger proteins that recognize palindrome motifs in cluster gene promoters. In addition to pathway-specific elements, the regulatory mechanism of mycotoxins is also composed of broad domain transcription factors and multiprotein complexes that transmit biotic and abiotic cues to biosynthesis mechanism; these regulatory processes are often interconnected and overlapping. Environmental parameters such as carbon and nitrogen sources, temperature, light, and pH, exert their influence on mycotoxin production through the mediation of global transcription factors such as CreA for carbon signaling, AreA for nitrogen signaling, and PacC for pH signaling (Brakhage, 2013). Several studies have reported both negative and positive regulation of biosynthetic genes by global regulators, turning off and on the energetically costly process of secondary metabolite production as an advantage to the producing fungus under certain environmental conditions. One of the most important multiprotein complexes of the regulatory mechanism is the Velvet complex. This heterotrimeric complex formed by VelB, VeA and LaeA, links sexual development with secondary metabolism in fungi in response to light, through a regulated spatial compartmentalization of the three proteins (Bayram *et al.*, 2008). Other regulation mechanisms could be responsible of the activation of biosynthetic genes that are based on signaling pathways or epigenetic control, the latter linked to chromatin remodeling proteins (Pfannenstiel and Keller, 2019).

The study of mycotoxin biosynthetic gene clusters is essential to gain a better comprehension of the regulatory process behind mycotoxin biosynthesis and the factors involved in mycotoxin production. Also, a thorough examination of the toxigenicity of fungal populations depending on the presence or the absence, in whole or in part, of the biosynthetic gene cluster has led to the development of successful biocontrol strategies based on the use of non-mycotoxigenic strains and is essential for the assessment of events of horizontal transmission of mycotoxin- producing capability among different populations. Finally, these studies have allowed the development of sensitive, specific, and high-throughput molecular diagnostic assays for mycotoxigenic fungi. The clusters of important mycotoxins, such as AfTs, trichothecenes, FUMs and ochratoxins, have been identified, even though the characterization of some of them is to be completed. The significant advance in fungal genome sequencing and bioinformatic analyses for metabolite mining have remarkably facilitated the characterization of gene clusters involved in the biosynthetic pathway, often starting from the identification of key synthase genes.

Aflatoxins

The aflatoxin biosynthesis gene cluster is probably the most widely studied fungal secondary metabolite gene cluster and extensive studies have been conducted on the regulatory mechanisms underlying AfTs production as well.

AfTs are produced by a polyketide pathway through one of the longest and most complex processes corresponding to a coordinated enzymatic cascade and leading to the production of stable intermediate molecules such as norsoloric acid, versicolorin A, sterigmatocystin and finally, AfB_1. The aflatoxin biosynthetic gene cluster includes 30 genes within a 75-kb genomic region, and to date, at least 27 enzymatic reactions have been shown to be involved in this process, whereas the roles of several other genes belonging to the cluster, remain unclear. In addition, a 7.5 kb cluster containing a group of four genes related to the utilization of sugar is located next to the aflatoxin gene cluster (Yu *et al.*, 2000; Bhatnagar *et al.*, 2006). The gene content and organization of the cluster is highly conserved among *Aspergillus* species in section *Flavi*, which includes *A. flavus* and *A. parasiticus*. Sequence variability and deletions in various genes/regions of the aflatoxin cluster have been used to assess variability in producing species and has provided markers that can be used to monitor variation in these *Aspergillus* species and to evaluate the risk they pose when present on food commodities. In addition, understanding the genetic variability in the aflatoxin cluster is a key to the selection of safe and effective aflatoxin-nonproducing strains for biological control efforts aimed at limiting contamination in crops (Donner *et al.*, 2010).

Regulation of aflatoxin biosynthesis is controlled by pathway-specific and globally-acting regulatory factors as well as epigenetic factors. The two cluster specific regulator genes *aflR* and *aflS* (previously named as *aflJ*) are adjacently located in the middle of the cluster, although they are divergently transcribed and share a 737 bp intergenic region. The *aflR* gene encodes a C-6 zinc finger transcription factor which binds to the promoter region of most of the aflatoxin structural genes, using a DNA-binding domain (Shimizu *et al.*, 2003). The AflR transcription factor acts as positive regulator by activating the transcription of genes and in turn the enzymatic process leading to the production of AfTs. Even though AflR is the main activator of the aflatoxin gene cluster, it interacts with AflS that plays the role of enhancer in the regulatory biosynthesis process (Chang, 2003). AflS does not present similarity to other proteins of known function and its exact role in aflatoxin biosynthesis is still under investigation. The *aflS* gene was shown to be regulated by AflR factor and it was also demonstrated that AflS interacts with AflR but not with the biosynthetic enzymes, revealing the co-active function of gene *aflS* in modulating the expression of aflatoxin early and mid-pathway genes in particular (Chang, 2003; Du *et al.*, 2007; Ehrlich, 2009).

Many biotic and abiotic environmental factors influence aflatoxin biosynthesis including nutritional sources; environmental effects such as water activity and temperature; physiological conditions such as pH and bioreactive agents (Yu and Keller, 2005). A complex regulation of genes guides the fungal response to environmental stimuli, affecting mycotoxin production. The activation or repression of several genes external to the aflatoxin cluster modulates aflatoxin gene expression and biosynthesis. The transcription factors CreA and AreA, which play crucial roles in the utilization of carbon and nitrogen sources, respectively, were found to be involved in the biosynthesis of AfTs (Georgianna and Payne, 2009; Caceres *et al.*, 2020). With regard to the influence of pH on the aflatoxin production, PacC seems to be the key factor involved in the related signaling pathway (Tilburn *et al.*, 1995). Globally-acting regulatory factors have been demonstrated to control the production of aflatoxins, like the Velvet complex which coordinates fungal development and secondary metabolism in a light-dependent manner (Chang *et al.*, 2012; Cary *et al.*, 2015), the global transcription factor genes *nsdC* and *nsdD* controlling both sclerotial, conidial and aflatoxin production (Cary *et al.*, 2012) as well as the recent identified global regulator Hbx1, encoding a homeobox transcription factor (Cary *et al.*, 2017). In addition, lipid metabolism especially that of fatty acid-derived oxylipins, has been shown to play a role in aflatoxin production (Tsitsigiannis and Keller, 2006). Some epigenetic mechanisms were also shown to regulate fungal development and aflatoxin production (Amare and Keller, 2014; Satterlee *et al.*, 2016).

Trichothecenes

Trichothecenes are a family of over 200 toxic sesquiterpenoids produced by species belonging to multiple fungal genera. In a recent work, Proctor *et al.* (2018) carried out a phylogenetic study on strains from nine trichothecene-producing fungal genera and reported how the diversity in trichothecene structures is largely due to gain, loss and change of function of genes during evolutionary processes. Therefore, the capability of producing trichothecene mycotoxins in certain *Fusarium* species and strains is likely the result of adaptive genetic changes, not yet clearly understood to date. All trichothecene-producing fungi examined to date have a trichothecene biosynthetic gene cluster that consists of 7–15 genes, but most of the fungi have additional trichothecene genes located in different genomic locations than cluster.

Trichothecenes are characterized by a core structure consisting of a three-ring molecule known as 12,13-epoxytrichothec- 9-ene (EPT) and then they are classified into four groups (Types A, B, C, and D) according to the substitution pattern of EPT (McCormick *et al.*, 2011). Biosynthesis of trichothecenes begins with the cyclization of farnesyl pyrophosphate, a primary metabolic intermediate, to form trichodiene through the reaction catalyzed by the terpene cyclase trichodiene synthase. Subsequently, a cytochrome P450 monooxygenase catalyzes oxygenation of trichodiene at three or four positions to yield isotrichodiol or isotrichotriol, which can cyclize non-enzymatically to form EPT or 3-hydroxy EPT (isotrichodermol), respectively. These latter molecules undergo one or more additional oxygenations, acylations and sometimes other modifications to form all trichothecene analogs. The trichodiene synthase gene (*tri5*) and the cytochrome P450 mono-oxygenase gene (*tri4*) encode the enzymes required for the essential steps that occur early in trichothecene biosynthesis (Hohn and Beremand, 1989; Kimura *et al.*, 2007).

The genetics and biochemistry of trichothecene biosynthesis have been studied most extensively in *Fusarium*. In *F. graminearum* and *F. sporotrichioides* the biosynthesis cluster includes, in addition to *tri5* and *tri4* genes, other 7 genes encoding enzymes responsible of modifications to the core EPT structure, i.e., a transporter protein (*tri12*) and the two regulatory genes *tri6* and *tri10* (Proctor *et al.*, 2018). Both species also have two smaller trichothecene loci, the first of these consists of the single gene *tri101*, while the second locus consists of the two genes *tri1* and *tri16*, which for their functional differences contribute to important structural differences of trichothecenes produced by the two species. Analysis of the *F. graminearum* genome sequence indicated that the three loci are located on different chromosomes. In other *Fusarium* species *tri1* and *tri101* are located in the main cluster (Alexander *et al.*, 2009; Proctor *et al.*, 2009). The regulatory genes *tri6* and *tri10* are predicted to encode a C-6 zinc finger transcription factor and a protein with a fungal transcription factor domain, respectively. Functional analyzes demonstrated *tri6* to be required for expression of other trichothecene genes, by binding to DNA sequence motifs in their promoter regions. The predicted TRI10 amino acid sequence does not include a DNA binding motif, anyway *tri10* gene also is required for expression of other biosynthesis genes, leading to the hypothesis that TRI10 interacts with TRI6 to induce trichothecene gene expression (Seong *et al.*, 2009). Both the *tri6* and *tri10* encoded proteins regulate the expression of most of the trichothecene biosynthesis genes and in addition of eight genes involved in the synthesis of farnesyl diphosphate, the metabolic precursor of trichothecenes and intermediate in the ergosterol and farnesol biosynthesis, thus acting as a global transcription regulators rather than pathway-specific regulators of trichothecene biosynthesis (Nasmith *et al.*, 2011).

Fumonisins

Fumonisins are structurally simple mycotoxins, but their biosynthesis requires a complex 42-kb long cluster containing 17 genes as described in *Fusarium* species. These mycotoxins consist of a linear carbon backbone with a nitrogen-containing group, one to four hydroxyl groups, and two tricarboxylate esters at various positions along the backbone. At least 28 different analogs of fumonisins were described and divided into four series designated A, B, C and P, due to the structural variation around the nitrogen atom and differences in the length of the carbon backbone (Ahangarkani *et al.*, 2014). The first described gene *fum1* is considered the key gene of this pathway, encoding a polyketide synthase which catalyzes the synthesis of the 18-carbon-long linear polyketide backbone structure. In the second step, the α-oxoamine synthase encoded by the *fum8* gene catalyzes the condensation of the polyketide with alanine to form the 20-carbon-long backbone of FB that is the most toxic member of the group, and FUMs A and P, while, during the biosynthesis of FUMs C, which is characterized by a 19-carbon-long backbone, the enzyme FUM8 catalyzes condensation of glycine to the polyketide. Several other structural genes contribute to the different structures of fumonisin analogs. As for the specific pathway regulator, the gene *fum21* is located adjacent to *fum1* and encodes a C-6 zinc finger DNA-binding transcription factor that positively regulates expression of *fum1* and *fum8*, confirming its involvement in the regulation of fumonisin synthesis. The cluster also includes the *fum19* gene encoding an ABC transporter that provides a sort of self-protection by exporting the toxin from the cell and reducing its cellular concentration (Proctor *et al.*, 2008). The order and orientations of genes within the three species *F. verticillioides*, *F. oxysporum*, and *F. proliferatum* are the same (Waalwijk *et al.*, 2004; Proctor *et al.*, 2013). However, the cluster is in a different genomic context in each species, suggesting that it has undergone translocation during the evolutionary history of fumonisin-producing *Fusaria*.

In species of *Aspergillus* section *Nigri* the composition of the fumonisin cluster is different than in *Fusarium* species and only presents 11 genes including the transcription factor *fum21*, which was found to regulate most of the cluster genes. The *A. niger* fumonisin cluster lacks the homolog of gene *fum2*, which is responsible for the final hydroxylation in the formation of FUM B_2 in *Fusarium*. Therefore, these *Aspergillus* species are only able to produce FUM B_2, B_4, and B_6, but not the most toxic one, FB_1 (Gil-Serna *et al.*, 2020). It was suggested that the fumonisin cluster in *Aspergillus* species was acquired via horizontal gene transfer from a common ancestor, by removing and/or reshuffling the genes (Khaldi and Wolfe, 2011; Aerts *et al.*, 2018).

Ochratoxins

The biosynthesis pathway of OTA has been largely clarified in recent years, essentially due to the great increasing of genome sequencing projects which led to identify the OTA clusters in several producing species belonging to *Aspergillus* and *Penicillium* genera. The key genes present in the biosynthesis cluster have been characterized and the study of their roles has resulted in a hypothesis about the biosynthesis mechanism and about the sequence of reactions along the pathway (Geisen *et al.*, 2006; Gallo *et al.*, 2012, 2017). OTA is a hybrid chlorinated molecule composed of a polyketide dihydroisocoumarin moiety which, according to this hypothesis, is firstly linked via amide bond to the amino acid phenylalanine to form ochratoxin B that then undergoes an halogenation reaction to add the chlorine atom to the molecule; the compound ochratoxin α may occur as a product of hydrolysis of OTA.

In the biosynthesis cluster, the OTA *pks* gene encodes the polyketide synthase required in the first step of the pathway, which is the formation of the isocoumarin pentaketide group from acetate and malonate. Adjacent to the *pks* gene, there is the OTA *nrps* gene encoding the peptide synthetase essential for the link between the dihydroisocoumarin and the phenylalanine synthesized via the shikimic acid pathway. In addition to the halogenase gene encoding the protein responsible of the introduction of the chlorine atom in the structure of OTA, two other genes are located in the cluster. They encode a P450 oxidase and a bZIP transcription factor that have been demonstrated to be involved in the pathway (Wang *et al.*, 2018). Other genes were annotated in OTA biosynthesis clusters of several producing species, such as a predicted transporter protein and one or two other fungal specific

transcription factors, whose role in the biosynthetic pathway needs to be investigated further. Therefore, length and composition of the OTA cluster remain not yet completely defined.

Also, the hypothesis of a possible involvement of two PKS proteins in the biosynthesis of OTA has been proposed, due to the identification of two putative OTA-related gene clusters, each of them containing a PKS gene, in *A. westerdijkiae* and *A. ochraceus*. Gene inactivation and expression studies suggested that one PKS is directly involved in biosynthesis of OTA while the other PKS might complement the expression of the former gene and be involved in the biosynthesis indirectly (Wang *et al.*, 2015; Han *et al.*, 2016).

At present, most of the regulatory aspects underlying the production of OTA remain unclear. It is likely that some of them are based on functioning models already identified for the production of other mycotoxins. The bZIP transcription factor positioned in the cluster probably acts as a pathway-specific regulator that controls production of OTA by regulating the other biosynthetic genes, but still its action mechanism remains undetermined. An additional transcription factor located adjacent to the biosynthetic genes is a zinc finger DNA-binding protein and was proved also to regulate the expression of some of the biosynthesis genes, although its inactivation merely reduces production of OTA (Wang *et al.*, 2018). Among the global regulators, the heterotrimeric Velvet complex has been the most studied in OTA producing fungi to clarify the link of the light-dependent fungal morphology and sexual development to production of OTA (Crespo-Sempere *et al.*, 2013). Moreover, the correlation between a NaCl rich environment and biosynthesis of OTA by *Penicillium* producing species was demonstrated by Schmidt-Heydt *et al.* (2012) to be mediated by the HOG MAP kinase signal cascade pathway under the hypothesis that the excretion of OTA carrying a chlorine in its molecule, contributes to the adaptation to salt rich environment by ensuring chloride homeostasis in the cell.

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Relevant Website

https://www.who.int/news-room/fact-sheets/detail/mycotoxins
Mycotoxins-World Health Organization.

RNA Interference in Fungi

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The Discovery of RNA Interference

RNA interference (RNAi) is a biological process, almost universally presents in eukaryotes, involved in the repression of gene expression (gene silencing) at transcription and/or post-transcription levels. In this process, specific RNA or DNA target molecules are recognized by short non-coding RNA molecules (20–30 nucleotides), known as small RNAs (sRNAs), and silenced by a multiple protein complex.

The first reported observations of RNAi date back to the early 1990s when two independent research groups, that were working on the over-expression of genes involved in pigment production in a plant (Napoli *et al.*, 1990) and a fungus (Romano and Macino, 1992), obtained unexpected results. In particular, they observed that a number of transformants, instead of being characterized by a more intense pigmentation, showed albino or variegated phenotypes. The peculiar feature was that those phenotypes were often transient and not always transmitted to the progeny; they called this phenomenon as "co-suppression" and "quelling", respectively. The molecular basis of this mechanism remained unclear until 1998 when Fire and colleagues understood that the nature of the silencing trigger was double-stranded RNA (dsRNA, Fire *et al.*, 1998). Their work brilliantly highlighted that, in nematodes, dsRNAs activate the sequence-specific silencing of endogenous genes, producing a much stronger effect compared to sense or antisense sequence-specific single-stranded RNAs (Fire *et al.*, 1998). During the following years, the molecular details of RNAi were elucidated giving rise to a number of biotechnological applications, from medicine to agriculture. For the impact of this discovery, in 2006 Fire and Mello were awarded the Nobel Prize in Physiology or Medicine.

The Core of RNAi Machinery: DCL, AGO and RdRp

In a simplified model valid for almost all eukaryotes, the general RNAi pathway can be described as a process involving three classes of proteins (**Fig. 1**): Dicer (or Dicer-like; DCL), Argonaute (AGO) and RNA-dependent RNA polymerase (RdRp; Ipsaro and Joshua-Tor, 2015).

DCL are enzymes that process dsRNAs or single-stranded hairpin RNAs into small RNA (sRNA) duplexes, typically 21–25 nucleotide(nt)-long (Bernstein *et al.*, 2001). The cleavage activity of DCL is provided by a pair of Ribonuclease III (RNase III) domains which act, together with a PAZ domain (primarily responsible for the recognition of the dsRNAs to be cleaved), as a molecular ruler to produce sRNA duplexes of the appropriate size, characterized by 2-nt overhangs at the 3′-ends and by a monophosphate group at the 5′-ends (MacRae and Doudna, 2007; Zhang *et al.*, 2004). The sRNA duplexes are then loaded onto AGO, the silencing effector proteins, which retain only one sRNA strand to become the minimal constituents of the (1) RNA-induced silencing complex (RISC; Cenik and Zamore, 2011), involved in post-transcriptional gene silencing (PTGS), or the (2) RNA-induced transcriptional silencing complex (RITS; Moazed, 2009), a nuclear form of RISC involved in transcriptional gene silencing (TGS). The sRNAs let the RISC or RITS to recognize, by sequence complementarity, their target nucleic acid molecules (RNA or DNA, respectively) that can therefore be silenced in different ways: (1) through mRNA cleavage or destabilization, or translation inhibition (Ipsaro and Joshua-Tor, 2015; Verdel *et al.*, 2004); or (2) through histone and/or RNA-directed DNA methylation (Moazed, 2009; **Fig. 1**).

AGO have a bilobal structure: one lobe is composed by a PAZ (the same found in DCL) and a N-terminal domains, the other by a MID and a PIWI domains (Kuhn and Joshua-Tor, 2013). The PAZ/N-terminal lobe recognizes the DCL-derived sRNA duplexes, thanks to their typical 2-nt 3′-overhangs, and binds only one strand of the sRNA duplex (the sRNA guide) at its 3′-end, discarding the other one. The second lobe, MID, is responsible for securing the sRNA guide to the protein by the aspecific binding of its 5′ monophosphate, while the PIWI domain, which possesses an RNAse-H fold, provides the RISC complex with the endonuclease catalytic effect directed against the target transcripts. Three paralogous classes of AGO are present in eukaryotes: the widespread AGO-like, the Piwi-like (only found in animals) and the *Caenorhabditis elegans*-specific group 3 AGO (Hutvagner and Simard, 2008; Wilson and Doudna, 2013). AGO are considered as the minimal and necessary determinant of RNAi; indeed, unlike DCL and RdRp, no AGO-independent RNAi pathways have been described so far (Ipsaro and Joshua-Tor, 2015).

The third class of proteins, the RdRP, is responsible for the "cascade effect": a fundamental property of RNAi through which a weak initial silencing signal can be drastically amplified in a positive feedback process (Baulcombe, 2015). In this way, few silencing RNA molecules can produce a massive silencing response, resulting sometimes in a huge reprogramming of the cell transcriptome. RdRp are commonly present in fungi and plants but absent, with the exception of nematodes, in the majority of animals. The RdRp-mediated silencing amplification can be accomplished in different ways: (1) RdRp can interact with AGO-cleaved mRNAs to synthesize their antisense fragments (in a sRNA primer-independent or -dependent manner), producing dsRNAs that are then recruited in the DCL circuit; or (2) they can directly produce sRNAs using an mRNA as a template. In plants RdRp are fundamental for defense against viral infections (Ghildiyal and Zamore, 2009). In addition to the amplification of

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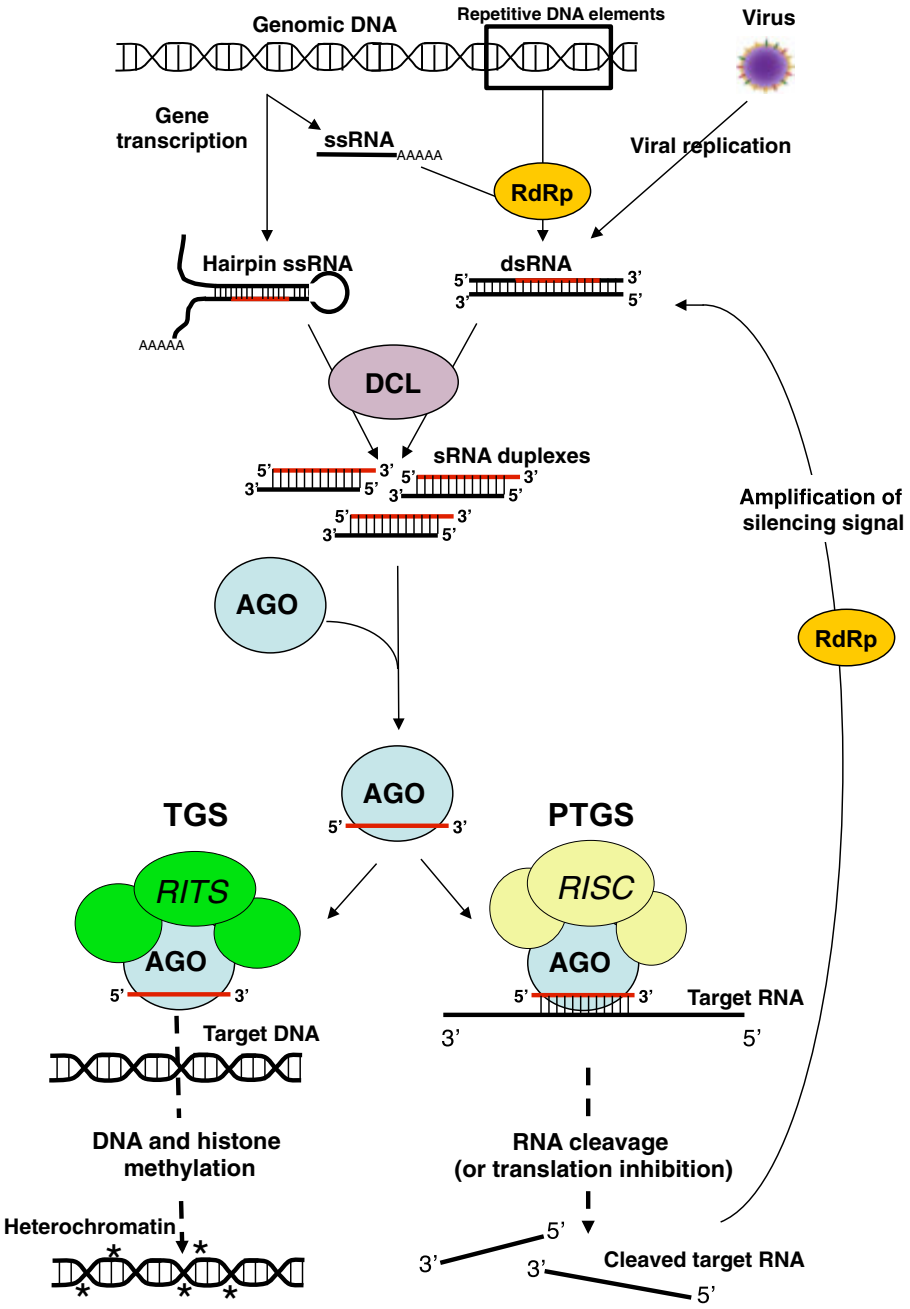

Fig. 1 *Simplified model of the RNAi pathway.* DCL (Dicer-like) enzymes process dsRNAs or single-stranded hairpin RNAs with different origins into small RNA (sRNA) duplexes. Only one sRNA strand (indicated in red) is then loaded onto AGO (Argonaute), the silencing effector proteins, to become the minimal constituents of the RNA-induced silencing complex (RISC) involved in post-transcriptional gene silencing (PTGS) which triggers the cleavage or the translation inhibition of target transcripts (right). AGO can be part of the RNA-induced transcriptional silencing complex (RITS) involved in transcriptional gene silencing (TGS) through DNA methylation and histone modifications of specific target DNA sequences (left). RdRp (RNA-dependent RNA polymerases) are responsible for the production of dsRNAs by the synthesis of antisense fragments from AGO-cleaved transcripts (amplification of silencing signal) or from non-degraded mRNA (*de novo* dsRNA production). Some classes of RdRp show a dual DNA/RNA-dependent RNA polymerase activity: they can recognize repetitive DNA elements (typical of transposable elements), synthetize ssRNA molecules and turn them into dsRNAs.

silencing signals, the RdRp may play a major role also in the activation of RNAi pathway by producing *de novo* dsRNAs from non-cleaved single-stranded (ss) RNA precursors but also, in fungi, in producing ssRNA precursors from DNA templates (Calo *et al.*, 2012; Lee *et al.*, 2010a).

In the simplified model proposed above, several accessory molecular components have been (intentionally) omitted. At molecular level, all these components cooperate to produce a complex and variegated system that can be differentiated, following a reductionist perspective, in a number of RNAi sub-pathways (Ghildiyal and Zamore, 2009).

Different Classes of sRNAs are Involved in RNAi

Small RNAs are the informational components of RNAi, directing its activity in a sequence-specific manner. Different classes of sRNAs have been proposed based on the proteins responsible for their biogenesis, on the RNAi pathway in which they are inserted, on their binding affinity with specific AGO proteins, on the species in which they have been discovered or on the biological functions they carry out (Ghildiyal and Zamore, 2009). However, because of the intrinsic variegated nature of RNAi, a classification system valid for the whole eukaryotic kingdom is missing. Historically, sRNAs have been divided in three major groups: (1) small-interfering RNAs (siRNAs), which include sRNAs deriving from dsRNA precursors, found in almost all RNAi-competents organisms; (2) microRNAs (miRNAs), which include those sRNAs precisely excised from ssRNA molecules with an intra-molecular self-complementary (hairpin) structure, typical of plants and animals; (3) PIWI (P-element-induced wimpy testes)-interacting siRNAs (piRNAs), which derive from ssRNA precursors to be loaded onto the Piwi-like AGO, specific of animals (Ghildiyal and Zamore, 2009).

miRNAs, siRNAs and piRNAs are regulated in vivo by specific modifications (Borges and Martienssen, 2015). Among them, two of the best characterized are the uridylation and the 2′-O-methylation of the 3′-ends of sRNAs, which have two opposite molecular meanings (Kim *et al.*, 2010). In plants, the 2′-O-methylation of sRNAs, which is carried out by the enzyme HEN1, is associated with sequence stability: indeed, the methylation prevents sRNAs from being uridylated by HESO1 and, consequently, from being degraded by SDN1 (Borges and Martienssen, 2015). For this reason, functional sRNAs are generally methylated during their biogenesis. HESO1 is associated with the RISC complex where it promotes (and this seems to be its primary function) the uridylation, and thus degradation, of the 3′-ends of the 5′ fragment of the AGO-cleaved mRNAs (Ren *et al.*, 2014). In this context, the 2′-O-methylation is necessary to avoid the unintentional uridylation by HESO1 of the AGO-loaded sRNA guide that would lead, after its degradation, to a loss of silencing information.

In plants a robust hierarchical classification system for sRNAs, based on their biogenesis, has been proposed (Axtell, 2013). Contrary to plants, in fungi sRNAs are mainly classified following the nomenclature of the specific RNAi pathway in which they have been first described. In the following sections, some specificities of the fungal RNAi and sRNA world will be discussed.

An Overview on RNAi and sRNAs in Fungi

RNAi is a widespread mechanism within the fungal kingdom, almost all fungal lineages possess the key components of RNAi machinery (reviewed in Nicolás and Garre, 2017; Torres-Martínez and Ruiz-Vázquez, 2017). Fungi often possess more than one gene encoding for the basic components of RNAi machinery, typically 1–2 DCL, 1–4 AGO and 1–4 RdRp (Chang *et al.*, 2012). A remarkable exception to this general rule is represented by the arbuscular mycorrhizal fungi (subphylum Glomeromycotina) which are characterized, with up to 40 genes depending on the species, by the expansion of AGO family (Lee *et al.*, 2018; Silvestri *et al.*, 2020, 2019).

The molecular details of fungal RNAi have extensively studied in few model species, in particular in the Ascomycetes *Neurospora crassa* (Dang *et al.*, 2014) and *Schizosaccharomyces pombe* (Martienssen and Moazed, 2015) and in the Mucoromycetes *Mucor circinelloides* (Torres-Martínez and Ruiz-Vázquez, 2016). In these three examples, different - or partially different - RNAi pathways involved in different biological functions have been discovered. The emerging picture seems to suggest that RNAi in fungi is, more than in plants, a highly variegated mechanism that even includes a number of species-specific sub-pathways. These fungal RNAi pathways can be divided, based on the biological function they perform, in two groups: (1) defense-related pathways and (2) endogenous regulatory RNAi pathways.

Defense-related RNAi Pathways

All living systems have to cope with a number of threats that can compromise their survival. Among them, specific genetic elements, such as viruses, transgenes/plasmids or (retro-)transposons, are generally dangerous for host organisms since their replication activity, if not actively thwarted, can be detrimental to cell homeostasis or genome integrity. In this context, RNAi plays a major role in cell defense by suppressing viral replication and the potential genome-damaging genetic elements. In fungi different defense-related RNAi pathways have been identified, some active during vegetative growth, others during sexual reproduction (reviewed in Chang *et al.*, 2012; Nicolás and Garre, 2017; Torres-Martínez and Ruiz-Vázquez, 2017).

Defense against mobile elements and transgenes

One of the very first characterized RNAi pathway was the “quelling” phenomenon in *N. crassa* (Romano and Macino, 1992). Quelling is a PTGS pathway active during vegetative growth that is involved in genome defense through the suppression of

transposon activity (Fulci and Macino, 2007). This pathway relies, considering only the three components of RNAi machinery presented above, on a peculiar DNA/RNA-dependent RNA polymerase (named QDE-1; Lee *et al.*, 2010a), on two functionally redundant DCL (DCL-1 and DCL-2; Catalanotto *et al.*, 2004) and an AGO (QDE-2; Cogoni and Macino, 1997). In quelling, QDE-1 is responsible for the initial triggering of RNAi pathway; indeed, it cooperates with other components, such as a RecQ DNA helicase (QDE-3) and a replication protein A (RPA), to recognize DNA tandem repeats - typical of transposable elements or multiple transgene copies - and to produce aberrant RNAs through its DNA-dependent RNA polymerase function. These molecules are then turned into dsRNAs by the same QDE-1, processed by DCL-1/2 into 25-nt long siRNAs and finally loaded onto QDE-2 for the PTGS of RNA target sequences corresponding to the original DNA tandem repeats.

In *N. crassa* another RNAi process, known as qiRNA (QDE-2-interacting small RNA) pathway, is involved in genome defense during the vegetative growth (Lee *et al.*, 2009). This system is mechanistically similar to quelling but it differs from the latter mainly because it gives rise to specific sRNAs (qiRNAs) only from the repetitive ribosomal DNA clusters. qiRNAs are produced as a specific response to dsDNA breaks and likely act as inhibitors of protein translation to protect the cell until the DNA damage is repaired.

Both these RNAi pathways are dependent on homologous recombination (HR) for their activation, with the difference that the qiRNA also needs a dsDNA break. In fact, contrary to what happens to other repetitive genomic regions, in the absence of DNA damage the ribosomal DNA clusters are generally protected from HR in order to prevent a potential detrimental loss of rDNA copy (Dang *et al.*, 2014).

Besides the case of N. crassa, quelling-like pathways have been identified and characterized in a number of other fungi, such as *M. circinelloides*, *Trichoderma atroviride*, *Fusarium graminearum*, *Aspergillus nidulans*, *Cryptococcus neoformans* and *Magnaporthe oryzae* (Torres-Martínez and Ruiz-Vázquez, 2017 and references therein). In all these examples the RNAi components cooperate to defend the cell against transposons, retrotransposons and transgenes, in processes molecularly similar to the *N. crassa* quelling. However, a remarkable exception to the all the systems listed above is the quelling-like mechanism of *M. circinelloides* in where, as a unique case so far described in the fungal kingdom, amplification of silencing signals has been documented (Calo *et al.*, 2012). In this organism, two non-redundant RdRp are active in the PTGS pathway: the first is required for RNAi initiation (RdRP-1; similarly to QDE-1) while the second (RdRP-2) for silencing amplification. RdRP-2 recognizes the targeted mRNA sequences and, using them as templates, synthesizes dsRNA molecules that give rise to new siRNAs (known as secondary siRNAs) through the DCL circuit.

In addition to the previous pathways, *N. crassa* is also characterized by a further PTGS RNAi process, active during sexual reproduction, that is known as meiotic silencing by unpaired DNA (MSUD; Shiu *et al.*, 2006, 2001). MSUD participates to the protection of genome integrity by recognizing and silencing, during meiosis, the unpaired sequences present only in one parental chromosome (likely mobile elements). In this pathway, a number of proteins initially cooperates to produce aRNAs from the unpaired sequences which are then subsequently converted into dsRNAs by the RdRp SAD-1 and into MSUD-associated siRNAs (masiRNAs; 25-nt long and enriched in uracil at their 5'end) by DCL-1. The AGO protein SMS-2 is finally involved in the silencing of target sequences. In the Basidiomycetes *C. neoformans*, a different RNAi pathway for defense against transposons during sexual reproduction, named sex-induced silencing (SIS), has been reported (Wang *et al.*, 2010).

For as regards the TGS pathway, the only example so far reported in fungi is the heterochromatin formation in *Schizosaccharomyces pombe* (Martienssen and Moazed, 2015; Verdel *et al.*, 2004). Heterochromatin is a condensed form of chromatin, fundamental for genome stability in eukaryotes. Heterochromatin ensures transcriptional suppression and is associated to those genomic regions characterized by highly repetitive elements, typically originated from the replicative activity of transposable elements. In the *S. pombe* heterochromatin formation pathway, nascent transcripts from non-coding genomic regions are turned into dsRNA by the unique RdRp and processed into siRNAs by the DCL. These siRNAs, after being loaded onto the single AGO, allow the recognition of the original DNA repeat regions. AGO-siRNA duplexes are included into RITS that is responsible, together with other recruited molecular components, of specific histone modifications (mainly methylation of histone H3 at the arginine position 9). This leads to the heterochromatin formation and thus to inhibition of transcriptional activity; in this way, the potential transposon detrimental action is suppressed.

Defense against viruses

One of the first functions assigned to RNAi was the defense against viral infections (Nicolás *et al.*, 2013). Fungi from all phyla are indeed infected by viruses (Herrero *et al.*, 2009). Fungal viruses (mycoviruses) are, in most cases, RNA entities encoding for at least one RdRp that is necessary for their replication (Ghabrial *et al.*, 2015). Mycoviral infections are generally asymptomatic (Roossinck, 2011) even if sometimes they can give rise to peculiar phenotypes, such as the case of *Cryphonectria parasitica*, the phytopathogenic agent of the chestnut blight. When infected by viruses of the genus *Hypovirus, C. parasitica* shows a reduced virulence (Milgroom and Cortesi, 2004). It is common for fungal endophytes of plants to host mycoviruses, which can sometimes play beneficial roles (Bao and Roossinck, 2013). An emblematic example is the tripartite mutualistic interaction between a plant, an endophytic fungus and its mycovirus, where the mycovirus was shown to be necessary to confer heat tolerance to the whole biological system (Márquez *et al.*, 2007).

The replication of almost all mycoviruses is characterized by a dsRNA intermediate; thus, during this phase, host DCL can process the sequences into viral sRNAs able to target the viral genome. A proof that fungal RNAi provides antiviral defense arises from the amount of new mycoviral genomes that have been discovered in the last years by exploiting small RNA-seq data (e.g., Nerva *et al.*, 2016). Nevertheless, the sRNA-seq approach is sometimes not sufficient to reconstruct *in silico* the whole genome sequence of mycoviruses, suggesting that some species can escape the host RNAi pathway (Nerva *et al.*, 2018). Anyway, direct

evidence of antiviral activity for fungal RNAi has been collected. This is the case of *C. parasitica* infected by *Cryphonectria hypovirus 1* (Segers *et al.*, 2007), *A. nidulans* infected by different viruses (Hammond *et al.*, 2008) and *Colletotrichum higginsianum* infected by *Colletotrichum higginsianum nonsegmented dsRNA virus 1* (Campo *et al.*, 2016). In analogy to viruses infecting non-fungal hosts, some mycoviruses have developed strategies to suppress the fungal anti-viral RNAi pathway (Hammond *et al.*, 2008; Segers *et al.*, 2006; Yaegashi *et al.*, 2013); this is a further indication that fungal RNAi serves as an antiviral defense system.

Endogenous Regulatory RNAi Pathways

In addition to defense against viruses, transgenes and mobile elements, which can be considered as exogenous genetic elements, RNAi plays a major role in eukaryotes also in the regulation of endogenous gene expression. However, in fungi the endogenous regulatory role of RNAi has been less widely studied compared to other organisms, such as plants or animals, mainly because fungal RNAi knock-out mutants often lack evident physiological or developmental phenotypes (Nicolás and Garre, 2017; Torres-Martínez and Ruiz-Vázquez, 2017). Most of the knowledge arises from studies conducted on *N. crassa* and *M. circinelloides*; in these two examples, different reverse genetics approaches, leading to the suppression of specific components of RNAi machinery, have allowed the identification of several classes of endogenous regulatory sRNAs and sRNA-generating processes.

A first group of fungal endogenous regulatory sRNAs is the class of miRNA-like identified in *N. crassa* (Lee *et al.*, 2010b). These miRNA-like share several features with plant miRNAs: they indeed originate through the precise excision of a sRNA fragment from an hairpin RNA precursor that is previously transcribed from a non-coding region. *N. crassa* miRNA-like are generally 25-nt long and enriched in uracil at their 5′-ends. Even if direct proof of their role in the regulation of gene expression is still lacking, the collected evidence supports this hypothesis; indeed, RNAi mutants show higher expression of the miRNA-like predicted target genes. Direct and indirect evidence of miRNA-like has also been collected in other Ascomycota and Basidiomycota species (Villalobos-Escobedo *et al.*, 2016) and, concerning basal fungi, in Glomeromycotina (Silvestri *et al.*, 2020, 2019).

A second group of endogenous sRNAs are the exon-derived siRNAs (*ex*-siRNAs) of *M. circinelloides* (reviewed in Torres-Martínez and Ruiz-Vázquez, 2016). The ex-siRNAs, that can be divided in 4 classes, originate from mature mRNAs in a DCL-dependent way. Class 1 and 2 ex-siRNAs are generally 23–24 nt-long sequences, are enriched in uracil at their 5′-ends and do not show a strand bias (they are both sense and anti-sense to the parental mRNA). These sRNAs are involved in the suppression of gene expression of the transcripts from which they originate. Class 3 and 4 ex-siRNAs are instead not characterized by specific length in nucleotide, are mostly sense to the originating exons and are not enriched in uracil at their 5′-ends; despite they are DCL-dependent, all these features suggest that they play a role in a non-canonical RNAi pathway.

M. circinelloides is also characterized by another group of gene regulator sRNAs, named rdRNAs, that are produced in a DCL-independent but RdRp-dependent way (reviewed in Torres-Martínez and Ruiz-Vázquez, 2016). These sRNAs share some features with class 3 ex-siRNAs: indeed they are almost all sense to mRNAs, show a random size distribution in terms of nucleotides and are not enriched in uracil at their 5′-ends but at the penultimate position of their 3′-ends. For these reasons, rdRNAs seem to derive from non-random degradation of mRNAs and likely share a partially similar biogenesis pathway with class 3 ex-siRNAs. Interestingly, a new class of RNAse III protein (R3B2) displaying unusual domain organization is involved in the biogenesis of rdRNAs. R3B2 have been only found in Mucorales and, for their similarity with RNAse III domains of bacteria belonging to Burkholderiales, they are probably a product of horizontal gene transfer event occurred between bacteria and a Mucorales ancestor (Trieu *et al.*, 2015).

Other classes of fungal sRNAs include the natural antisense transcript siRNAs (nat-siRNAs; Donaldson and Saville, 2012), that derive from the RNAi processing of dsRNAs formed by the overlapping of sense and antisense transcripts, and the *N. crassa* dicer-independent siRNAs (disiRNAs; Dang *et al.*, 2013) that, originating from gene-rich regions, they induce a specific type of transcription-dependent DNA methylation.

Loss of RNAi in Some Fungi

Even if the large majority of eukaryotes possess RNAi, there are some specific cases in which the evolution has led to the loss of key RNAi genes. In fungal kingdom, one of the best characterized example concerns the model yeast *Saccharomyces cerevisiae*, for which the loss of RNAi genes has been associated with the consistent infection with vertically transmitted dsRNA viruses (Drinnenberg *et al.*, 2011). Indeed, these viral entities, known as "killer viruses", encode for toxic proteins that, after being secreted into the yeast growth medium, are able to kill nearby virus-free cells but not the virus-infected ones, therefore providing a net selective advantage for the latter. However, the stable infection with killer viruses is incompatible with an active RNAi suppressing viral gene expression. Under these specific circumstances, it is plausible that the original *S. cerevisiae* ancestors who lost the RNAi competency were positively selected during evolution, as the killer phenotype more than offsets the disadvantage of lacking RNAi. Similar selective pressures have led to the loss of RNAi, as independent events, also in other phylogenetically distant species, such as the basidiomycete *Ustilago maydis* (Drinnenberg *et al.*, 2011).

Besides the killer phenotype, the lack of RNAi in other species is sometimes related to different benefits, as it has been observed for the animal pathogen *Cryptococcus deuterogattii* (Nicolás and Garre, 2017). This fungus has a strictly parasitic lifestyle and, for this reason, it needs to continuously evolve in order to escape the host defense. In this case, the lack of an active RNAi has been

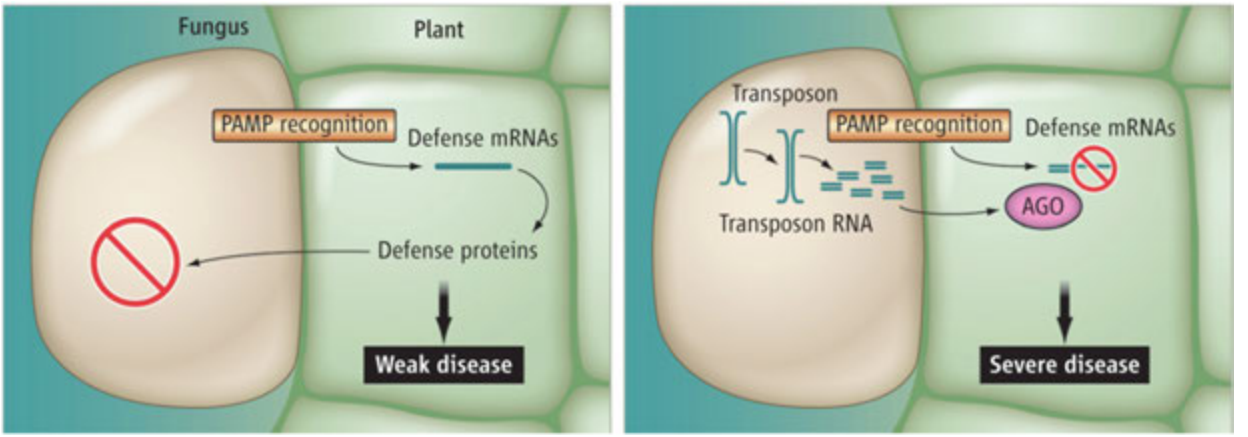

Fig. 2 *Fungal sRNAs can act as pathogen effectors.* Plants recognize the presence of fungal pathogens and activate the defense responses (left). Some fungal species can secrete sRNAs into the plant host cells, such as the case of transposon-derived *B. cinerea* sRNAs (Wang *et al.*, 2017; Weiberg *et al.*, 2013), silencing the host defense-related genes (right). These fungal sRNAs act as pathogen effectors by binding the plant AGO proteins. Adapted from Baulcombe, D., 2013. Small RNA – The secret of noble rot. Science 342, 45 with permission.

associated to the evolutionary advantage of having an increased genome mutation rate (due to the uncontrolled retrotransposons replication activity) and, consequently, a higher genetic variability that can be exploited by the pathogen as a counter-defense strategy (Feretzaki *et al.*, 2016).

The Role of sRNAs and RNAi in Inter-kingdom Interactions

In recent years it has been discovered that sRNAs play a role, beside endogenous gene regulation and genome protection, also in interspecies communication, in a process known as cross-kingdom RNAi (reviewed in Chaloner *et al.*, 2016; Huang *et al.*, 2019). Cross-kingdom RNAi was firstly observed in the pathogenic interaction between *Arabidopsis thaliana* and the necrotrophic fungus *Botrytis cinerea* (Weiberg *et al.*, 2013). In this work, the authors brilliantly demonstrated that, during infection, some 21–22 nt-long *B. cinerea* sRNAs, produced in a DCL-dependent way from long-terminal repeat retrotransposons, are transferred into the host cells (**Fig. 2**). Once in the plant cells, the fungal sRNAs are loaded onto plant AGO1 which silences, in a PTGS manner, specific defense-related genes, such as mitogen activated protein kinases (MAPKs), cell-wall-associated kinases, and genes involved in the accumulation of reactive oxygen species (ROS); in other words, the pathogen "hijacks" the host RNAi system at its own advantage.

Since this first discovery, cross-kingdom RNAi has been reported in several other pathogenic/parasitic interactions, in which sRNAs can behave both as "attack" and as "defense" molecules (i.e., can be transferred from the pathogen/parasite to the host or vice versa). For examples the fungal pathogens *Verticillium dahliae* (Wang *et al.*, 2016) and *Puccinia striiformis* f. sp. *tritici* (Wang *et al.*, 2017) and the parasitic plant *Cuscuta campestris* (Shahid *et al.*, 2018) exploit cross-kingdom RNAi to attack their host plants. On the other hand, plants can defend themselves by delivering sRNAs that target virulence genes in the pathogens, as it has been observed in plant interactions involving the fungi *V. dahliae* (Zhang *et al.*, 2016), *B. cinerea* (Cai *et al.*, 2018) or *Fusarium graminearum* (Zhang *et al.*, 2016; Jiao and Peng, 2018; Zhang *et al.*, 2016) or the oomycete *Phytophthora capsici* (Hou *et al.*, 2019).

The occurrence of cross-kingdom RNAi-like mechanisms is not limited to plants. The movement of sRNAs able to interfere with host gene expression has been also reported in the interactions between gastrointestinal nematodes (Buck *et al.*, 2014; Chow *et al.*, 2019) and mammals and between endobacteria (genus *Wolbachia*; Mayoral *et al.*, 2014) or the fungal pathogen *Beauveria bassiana* (Cui *et al.*, 2019) and insects. Furthermore, it has been shown that mobile sRNAs can also regulate gene expression in organism not provided with a functional RNAi machinery, such as the case of the movement of sRNAs from human cells to the malaria agent *Plasmodium falciparum* (Lamonte *et al.*, 2012) or gut bacteria (Liu *et al.*, 2016). Interestingly, gut microbiome is also influenced by dietary-derived plant sRNAs (Teng *et al.*, 2018). Recently, for the first time it has been demonstrated the involvement of cross-kingdom RNAi also in mutualistic interactions (Ren *et al.*, 2019), as bacteria of genus *Bradyrhizobium* secrete transfer RNA-derived sRNAs into cells of the host legume plant; these sRNAs, by binding plant AGO1, regulate the expression of nodulation-related host genes.

In 2018, Cai *et al.* (2018) clarified the mechanism through which plants send sRNAs into interacting fungi. In this work, it was shown that, at the *Arabidopsis-B. cinerea* contact interface, specific plant exosomes containing sRNAs are secreted into the apoplastic space and are then taken up by fungal cells. The plant knock-out mutants impaired in the production of exosomes were no longer able to defend themselves from the pathogen by means of sRNAs. Similarly, this exosome-mediated transfer mechanism has been reported also in a plant-oomycete interaction (Hou *et al.*, 2019) and in animal systems (Buck *et al.*, 2014; Chow *et al.*, 2019). In particular, the latter discovered that the nematode *Heligmosomoides baker* uses extracellular vesicles (EVs) to deliver, into the host cells, sRNAs together with AGO proteins; in this way, it is possible that few transferred sRNA molecules can have a very efficient

effect in the recipient organism since it is known that AGO-sRNA complexes can be stable for more than 3 weeks and, during this time, can maintain their silencing competency (Smibert *et al.*, 2013).

Cross-kingdom RNAi can also be artificially induced through the expression of RNAi constructs directed against interacting organisms. These techniques are known as host-induced (HIGS) or viral-induced (VIGS) gene silencing (reviewed in Baulcombe, 2015). Both approaches are powerful tools to protect plants from pathogens/parasites/insect pests but are also great technical opportunities for basic research, to obtain gene silencing in non-genetically transformable obligate plant biotrophs, such as arbuscular mycorrhizal fungi (Helber *et al.*, 2011; Kikuchi *et al.*, 2016; Tsuzuki *et al.*, 2016; Voß *et al.*, 2018; Xie *et al.*, 2016).

In addition to HIGS and VIGS, a further RNAi-based approach to control plant pathogens has been proposed: the spray-induced gene silencing (SIGS). In this case, it has been observed that the simple exogenous delivery onto crops of sRNAs/dsRNAs targeting fungal genes is sufficient to protect plants from pathogens (Koch *et al.*, 2016; McLoughlin *et al.*, 2018; Wang *et al.*, 2016). SIGS appears as a promising technique that could be likely used in agriculture in the next future (Huang *et al.*, 2019).

All these findings suggest that exchange of sRNAs between interacting organisms and cross-kingdom RNAi-like processes are likely extremely widespread natural communication strategies.

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The MAP Kinase Network As the Nervous System of Fungi

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All living organisms, from the simplest to the more complex, respond to external environmental changes through adaptive mechanisms. These mechanisms acquire special relevance in those microbes whose perpetuation depends on the establishment of an intimate relation with a host and that leads to a tight regulation of microbial genes that promote growth and survival (Biswas *et al.*, 2007; Cottier and Muhlschlegel, 2009; Alonso-Monge *et al.*, 2009b). Signal transduction cascades are essential molecular mechanisms that mediate this adaptation, and those involving mitogen activated protein kinases (MAPKs) are very relevant among eukaryotes. In fungi, they were first described in the *Saccharomyces cerevisiae* mating pathway, but gradually identified and characterized in many other fungi. In the case of pathogenic fungi, these signaling pathways play a key role in the adaptation of the microorganisms to the host environment and for the development of disease. This topic has been largely investigated in one of the main pathogenic yeasts, *Candida albicans* and, for this reason, it will be the main focus of the present chapter.

C. albicans is a pathogenic fungus of special significance to humans. Its first descriptions date from the first half of the 19th century, where several physicians described the presence of fungus in newborns oral thrush. *C. albicans* is frequently isolated in nosocomial blood-borne infections and is responsible for a wide range of diseases, collectively called candidiasis, which arise as a consequence of the inability of the host immune response to control fungal proliferation. This microorganism does not have a significant life cycle outside the host, and it is a frequent colonizer of humans, inhabiting the mucosa and especially the gastrointestinal and genitourinary tract of 30–70% of healthy individuals. *C. albicans* therefore behaves as an opportunistic pathogen, accounting for approximately 400,000 life-threatening infections per year (with a mortality rate exceeding 30%) and a far greater number of mucosal infections (Brown *et al.*, 2012). Clinical manifestations associated with candidiasis range from superficial (and relatively easy to handle through drug therapy) to mucosal and/or deep infections (oropharyngeal, vulvovaginal and invasive candidiasis) that may be of extreme severity among compromised individuals.

Despite the existence of some effective antifungals (polyenes, azoles and echinocandins), they all suffer problems related to cost, toxicity, pharmacokinetics or emergence of resistance (Kontoyiannis and Lewis, 2002; Akins, 2005) that severely limit its usefulness or may do it in a future. In addition, the eukaryotic nature of the fungus is another essential feature that limits the development of new antifungals. All these reasons support that fungal infections constitute a health problem yet to be solved; they also represent a challenge for basic and applied research where the identification of novel antifungal targets is extremely relevant. Understanding the mechanisms by which this fungus colonizes and causes disease is an important medical challenge and may provide ways to control its infections.

We intend here to highlight the main signaling pathways involving MAPKs in this organism. We will introduce, first, features related to the biology of this fungus regarding the recognition and immune response elicited by the host, and second, describe the main MAPK pathways characterized in this fungus and their connections with processes relevant in its biology, such as morphogenesis, cell wall construction and pathogenicity, as these processes represent an attractive target for the development of antifungal therapies. We refer the readers to other reviews which have outlined the role of these routes in other fungal models, not only *C. albicans* (Alonso-Monge *et al.*, 2009b; Román *et al.*, 2007; Qi and Elion, 2005; Chauhan *et al.*, 2006; Ikner and Shiozaki, 2005).

Introduction to *Candida albicans* Biology

Morphological Transitions

C. albicans was long considered to be a dimorphic fungus, that is, an organism that displays two different morphologies under different environmental conditions. The term dimorphic transition was coined to refer to the environmentally triggered conversion of yeast to hypha, which received substantial attention given its involvement in virulence (Mitchell, 1998; Whiteway and Oberholzer, 2004). However, it is a polymorphic fungus that can grow as yeast (unicellular), hyphae, pseudohyphae and chlamydospores (**Fig. 1**). Yeasts (also named blastopores) are ovoid cells, 3 to 6 μm in size, that reproduce in an asexual way by budding. Upon yeasts apical extension, germ tubes are formed which elongate in time originating cellular units separated by chitin septa, the hypha. In turn, hyphae can form yeasts by lateral budding (Odds, 1988). Pseudohyphal growth involves elongated cells that by a series of budding and separation remain attached to each other forming a chain (Gow, 1997). Under certain environmental conditions, as the absence of light, glucose limitation, low temperature and microaerophilia, thick-walled asexual structures, named chlamydospores, can be formed (Dujardin *et al.*, 1980; Montazeri and Hedrick, 1984). Although their biological function is unknown, they have been proposed to play a role in persistence (survival under unfavorable conditions) rather than dissemination.

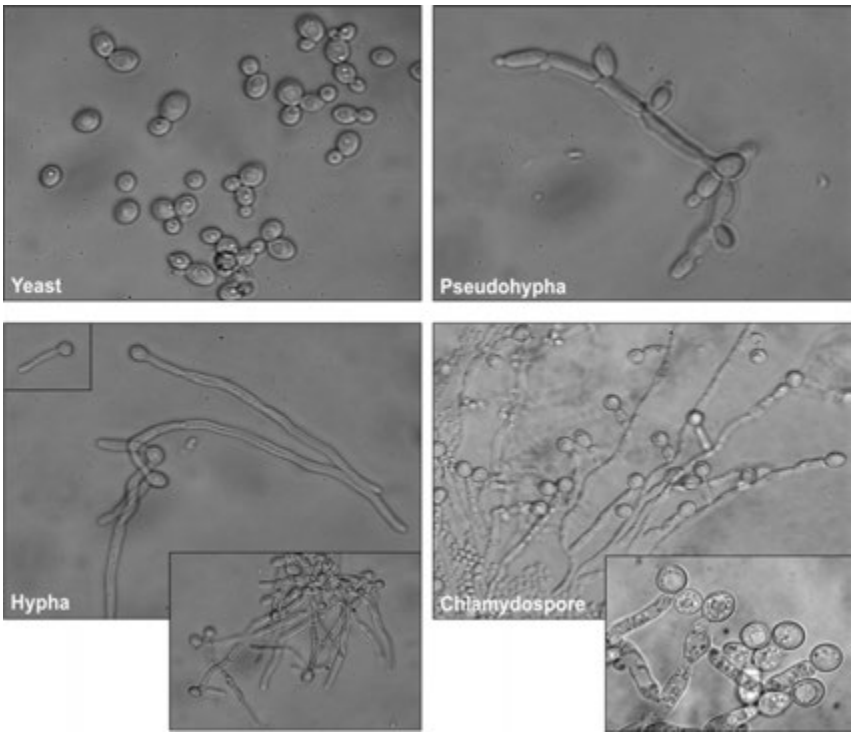

Fig. 1 Distinct morphological states of *Candida albicans*. The picture shows the microscopic appearance of several morphologies present in *C. albicans*. The blastopore or yeast is the unicellular morphology of *C. albicans*, characterized by its elliptical form and asexual reproduction through budding. By bud elongation a pseudohypha or a germ tube (which eventually develops into a hypha) can be formed. While hyphae present cellular units separated by septa (with no constrictions) and with parallel cell walls, pseudohyphae are formed by elongated cells with constrictions at the position of septa and show regular branching. Chlamydospores are round or oval refractive cells with a thicker cell wall and are larger than blastopores.

The dimorphic transition (ie, the transition between yeast and hypha morphologies) can be induced by a range of environmental cues. High temperature (37°C over 30°C) has been shown to facilitate this conversion (Lee *et al.*, 1975). The pH of the medium also influences morphogenesis and at low pH (<6) *C. albicans* cells predominantly grow in the yeast form, while at a high pH (>7), hyphal growth is induced (Soll, 1986). Nutritional factors such as *N*-acetylglucosamine (Simonetti *et al.*, 1974), proline (Dabrowa and Howard, 1981) or serum (Gow and Gooday, 1982) have been revealed to influence yeast-to-hypha conversion. Morphogenesis has also been shown to be regulated by quorum sensing, a mechanism of microbial communication that is also present in this fungus (Albuquerque and Casadevall, 2012). Farnesol (Hornby *et al.*, 2001), tyrosol (Chen *et al.*, 2004) and dodecanol (Hogan *et al.*, 2004) have been all described as quorum sensing molecules acting via different modes of action in this transition (Hall *et al.*, 2011) and may explain why high cell densities impair hyphal formation.

The morphological diversity of *C. albicans* has special relevance in the biology of this fungus due to its implication in virulence and pathogenicity (Lo *et al.*, 1997). It is believed that the distinct morphological presentations are required to enable the fungal adaptation to the new environmental conditions found within the human host, and the rapid colonization of its tissues as well as to facilitate the development of the infection to the entire host organism (Romani *et al.*, 2003; Saville *et al.*, 2003).

In addition to dimorphism, *C. albicans* is able to display great morphological variability. This observation on *C. albicans* colonies stored on agar at room temperature or in cold rooms for extended periods of time, led to the definition, in 1985 (Slutsky *et al.*, 1985), of the term "phenotypic switching," different from the yeast-to-hypha transition. This process is spontaneous and reversible and occurs only between two phases easily distinguishable by cellular morphology and staining with the dye phloxine B (Slutsky *et al.*, 1987). The white–opaque transition occurs at a relatively low frequency (approximately every 10^3–10^4 generations); however, it can be induced by certain environmental conditions (Morschhauser, 2010) such as the presence of CO_2 (Huang *et al.*, 2009), *N*-acetylglucosamine (Huang *et al.*, 2010), in anaerobic conditions (Ramirez-Zavala *et al.*, 2008) or by genotoxic and oxidative stress (Alby and Bennett, 2009). Although opaque cells had already been observed in 1987 (Slutsky *et al.*, 1987), only after the discovery of a mating-type locus (MTL) in *C. albicans* (Hull and Johnson, 1999) and the demonstration of its ability to mate (Hull *et al.*, 2000; Magee and Magee, 2000), the role of these cells in the fungal basic biology and pathogenesis, as well as the molecular mechanisms that regulate the white–opaque transition, became uncovered (reviewed in Soll, 2014).

Mating in *C. albicans* differs from other fungi. First, it requires that naturally frequent heterozygous cells at the MTL (a/α) become a/a or α/α. Second, MTL-homozygous cells must undergo an epigenetic switch from the predominant white to opaque phase, each cell type is heritable for many generations and switching occurs without a change in the DNA sequence of the genome

(Lockhart *et al.*, 2003). Opaque cells are the mating-competent form and are able to mate $\sim 10^6$ times more efficiently than white cells (Miller and Johnson, 2002). The tetraploid fusion product (a/a/α/α) then undergoes a random chromosome loss to generate recombinant progeny in a parasexual cycle (Bennett and Johnson, 2003; Forche *et al.*, 2008).

Rather paradoxical, the opaque phenotype is highly unstable at physiological temperature (Slutsky *et al.*, 1987; Rikkerink *et al.*, 1988), switching in masse to the white state when temperature is raised from below 25°C (optimal for opaque growth) to 37°C (Srikantha and Soll, 1993). This is in line with the fact that upon intravenously infection with these cells, mostly white cells were recovered from the kidneys of mice (Kvaal *et al.*, 1997). Nevertheless this is in agreement with the better colonization of skin (where temperature is lower) by the opaque phenotype. In addition, the presence of high levels of CO_2 (equivalent to those found in the host gastrointestinal tract and select tissues), *N*-acetylglucosamine (a monosaccharide produced primarily by gastrointestinal tract bacteria) and anaerobic conditions favor the white–opaque transition even at 37°C (Ramirez-Zavala *et al.*, 2008; Huang *et al.*, 2009, 2010).

Recently, other phenotypes have been also described in *C. albicans*. The passage though the mouse gastrointestinal tract triggers a novel phenotypic switch, resulting in the appearance of Gastrointestinally indUced Transition (GUT) cells (Pande *et al.*, 2013). These cells differ from the opaque phenotype by the absence of pimples at the cell surface and the inability to mate. They seem to express a transcriptome that is optimized for the adaptation to the digestive tract. In addition, a "white–gray–opaque" tristable phenotypic switching system has been also discovered (Tao *et al.*, 2014).

Fungal Recognition by Immune Cells and Activation of Host Defenses

As a commensal, *C. albicans* growth and location within the host is controlled by the normal microbiota, epithelial barriers and the innate immune system with which the fungus is continuously or transiently interacting (Hoffmann *et al.*, 1999; Mochon *et al.*, 2010). Although this arm of the immune system lacks the specificity and memory that characterizes the adaptive response, it can also distinguish self from nonself: fungal recognition by the innate immune system implies the identification of pathogen associated molecular patterns (PAMPs) by the corresponding receptors (PAMPs Recognition Receptors, PRR) present at the surface of the immune cells. From this dynamic interplay immune responses are generated that can be either beneficial, maintenance of the commensal state, or deleterious, favoring damage by the yeast (Jouault *et al.*, 2009).

The fungal cell wall is rich in PAMPs and while cell wall proteins have the key role of adhesion to surfaces, polysaccharides dominate immune recognition and contribute to the immunological signature of *C. albicans* (Netea *et al.*, 2008). Cell wall proteins are also relevant as antigens and have been used, in the last years, for the development of vaccines (reviewed in Vecchiarelli *et al.*, 2012) such as the *N*-terminal domains of Als1, Als3 (Spellberg *et al.*, 2006) and Hyr1 (Luo *et al.*, 2010). Studies have also paid attention to the role of the polysaccharide β-glucan, and it has been shown that passive vaccination with anti-β-glucan monoclonal antibody can be used to confer protection against vaginal infection by *C. albicans* (Torosantucci *et al.*, 2005; Pietrella *et al.*, 2010).

The polysaccharides of *C. albicans* cell wall can be recognized by two major classes of membrane-bound PRRs: the Toll-like receptors (TLRs) and the C-type lectin receptors (CLRs). TLR2 and TLR4 recognize phospholipomannans and O-linked mannans respectively, and they are the main TLRs involved in the signaling induced by *C. albicans* (Tada *et al.*, 2002; Jouault *et al.*, 2003; Netea *et al.*, 2006). Mannans from the fungal cell wall can also be recognized by CLRs. Specifically, α-mannans (Saijo *et al.*, 2010) and hypha-specific high mannose containing structures (Sato *et al.*, 2006) are recognized by dectin-2, while *N*-linked mannans are recognized by macrophage mannose receptors (MR) (Netea *et al.*, 2006) and by dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (Cambi *et al.*, 2008). CLRs play an important role in protective antifungal immunity (reviewed in Brown and Netea, 2012). Dectin-1 is also a CLR though it recognizes highly conserved β-glucans instead of mannans. Dectin-1 has been demonstrated to bind, in vitro, specifically to budding areas and bud scars of yeasts where β-(1,3)-glucan is more exposed, but not to hyphae (Gantner *et al.*, 2005). This result suggested that hyphae would be uniquely immune from dectin-1 mediated recognition; however, ex vivo results have shown that there is no morphotype-specific masking or unmasking of β-glucan (Wheeler *et al.*, 2008). An increased susceptibility of mice lacking dectin-1 to chemically induced colitis was described recently (Iliev *et al.*, 2012) which correlated with altered responses to commensal fungi.

Finally, other CLRs have been described. The soluble (not membrane-bounded) mannose-binding lectin (MBL), also recognizes mannans and mediates *Candida* opsonization and uptake (Brouwer *et al.*, 2008). The Mincle protein is a C-type lectin expressed predominantly on macrophages that was described to bind to *C. albicans*, although no specific ligand has been yet identified (Bugarcic *et al.*, 2008; Wells *et al.*, 2008). Galectin-3, a member of a β-galactoside-binding protein family, binds *Candida*-specific β-(1,2)-mannosides (Fradin *et al.*, 2000) allowing phagocytes to discriminate pathogenic from nonpathogenic yeasts and associates with TLR2 or dectin-1 upon macrophage–yeast interaction (Jouault *et al.*, 2006; Esteban *et al.*, 2011).

The recognition of *C. albicans* PAMPs by receptors of the immune system, leads to the activation of signaling pathways that ultimately stimulate cytokine production, phagocytosis and killing. The production of reactive oxygen species (ROS) is a major antifungal mechanism in phagocytes upon pathogen internalization (Missall *et al.*, 2004). Additionally, pathogens can be eliminated extracellularly through the action of secreted galectin-3 (Kohatsu *et al.*, 2006) and also by the release of antimicrobial peptides especially at the mucosa level, such as β-defensins, histatins (Hst 5) and cathelicidins (LL-37) (reviewed in Swidergall and Ernst, 2014). The innate response plays an essential role for the initiation and determination of the type of adaptive response that

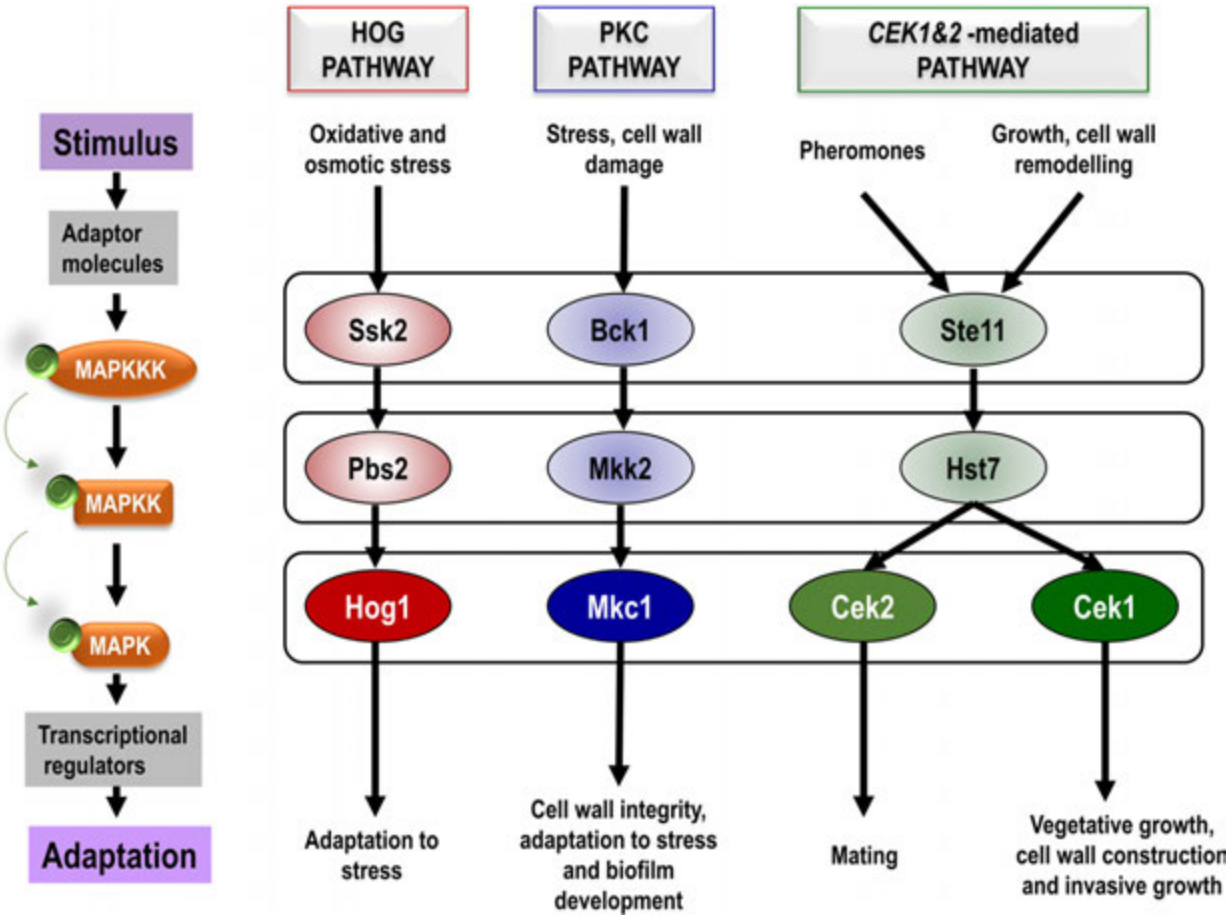

Fig. 2 MAP kinase mediated signaling in *Candida albicans*. A stimulus is perceived through specific sensors or receptors at the plasma membrane and transferred through an adaptor module to the MAPKs central core. By sequential phosphorylation the signal is transmitted to the MAP kinase that is finally translocated to the nucleus where it phosphorylates a transcription factor or a transcription factor complex which in turn allows the expression of target genes. The different stimuli known to trigger *C. albicans* MAPKs are shown as well as the main processes in which each pathway is involved. However, this figure should be considered as general scheme and only the main response is outlined for each pathway. As indicated in the text, some pathways participate through direct or indirect mechanisms in the processes here indicated as well as others.

will be generated upon the microorganism invasion. Recently, the Th17 adaptive immunity (characterized by the production of IL-17 and several other cytokines, including IL-22 and IL-23) has been identified and it appears to be primarily responsible for protection against fungal infections at the mucosa (reviewed in Hernandez-Santos and Gaffen, 2012).

Fungal Adaptation Through MAPKs-Mediated Signaling

MAPK pathways are highly conserved among eukaryotic organisms (Widmann *et al.*, 1999). They consist of a central module of three protein kinases that become sequentially activated by phosphorylation (Kultz, 1998): activated MAP kinase kinase kinases (MAPKKKs) phosphorylate MAP kinase kinases (MAPKKs), which in turn phosphorylate MAP kinases (MAPKs). These cascades mediate the transduction of signals perceived at the plasma membrane to the nucleus, where target genes are eventually regulated to allow the adaptive response (**Fig. 2**). The activating stimuli are first perceived by receptors (G-protein coupled receptors, histidine-aspartic phosphorylation sensors (two-component systems), integral membrane proteins or tyrosine kinase receptors) which transmit the signal to the MAPKs central core through upstream modules of intermediate proteins or adaptor molecules. The MAPKKKs, which are serine/threonine protein kinases become therefore, activated leading to the phosphorylation and activation of a MAPKK, which in turn stimulate MAPK activity. Each MAPKK can be activated by more than one MAPKKK, which is partially responsible for the interconnection or crosstalk among different routes (Cargnello and Roux, 2011). MAPKs (also called Extracellular-signal Regulated Kinases, ERKs) are dually phosphorylated on threonine and tyrosine residues within a conserved TXY motif (in which X represents glutamic acid, proline or glycine, depending on the MAPK) located in the activation loop of the catalytic subdomain VIII (Hanks *et al.*, 1988). Upon activation, the MAPK usually translocates to the nucleus where the activation

(or repression) of the corresponding targets such as transcription factors or other molecules, triggers specific cellular responses required for adaptation.

The activation of the pathway is transient and negative feedback mechanisms exist, such as those mediated by the action of specific phosphatases (Martin *et al.*, 2005). There are also positive feedback mechanisms that lead to an amplification of the signal. Other mechanisms used by the cell to regulate the pathways are through scaffold proteins whose main function is to bring other proteins together for them to interact. In this way, they improve signaling efficiency and ensure that signals are connected to the correct response while maintaining the specificity of the pathways. MAPK substrate selectivity is also achieved by the existence of specific interaction domains (docking sites) (reviewed in Saito, 2010).

Different MAPK pathways have been identified and characterized in the pathogenic yeast *C. albicans* (Román *et al.*, 2007; Alonso-Monge *et al.*, 2006) through the isolation of the MAPK genes *HOG1* (San José *et al.*, 1996), *MKC1* (Navarro-García *et al.*, 1995), *CEK1* (Csank *et al.*, 1998) and *CEK2* (Chen *et al.*, 2002), and the phenotype analysis of mutants defective in them. These studies initially resulted in defining a role for each of these routes/MAPKs in fungal physiology. Thus, the high osmolarity glycerol (HOG) pathway has been shown to be responsible for sensing stress, the Mkc1 route for cell wall integrity, Cek1 for invasion and Cek2 for mating. However, given the several connections and crosstalk mechanisms among these routes and others present in the cell, this is now realized as an oversimplification with most of the MAPK pathways playing a very pleiotropic role in fungi.

HOG Pathway: Sensing Environmental Stress

In *C. albicans*, the HOG pathway has been associated to many fundamental processes such as morphogenesis, cell wall biogenesis, virulence, although predominantly, in the response and adaptation to stress (Alonso-Monge *et al.*, 1999, 2003; Smith *et al.*, 2004; Enjalbert *et al.*, 2006). Recently, its role on *C. albicans* response to arsenate and arsenite has also been uncovered (Urrialde *et al.*, 2015). In fact, the MAP kinase of the pathway Hog1 (homolog to Hog1 in the yeast *S. cerevisiae*) becomes activated in response to a variety of stimuli such as high osmolarity or oxidative stress, which occurs in a Pbs2-dependent manner (the pathway canonical MAPKK) through Ssk2 (MAPKKK) mediated signaling (Arana *et al.*, 2005; Cheetham *et al.*, 2007). The activation of the HOG pathway leads to glycerol accumulation (San José *et al.*, 1996), as occurs in *S. cerevisiae* (Albertyn *et al.*, 1994), which counteracts cell dehydration caused by hyperosmotic environments. The activation of this route upon oxidative stress (Alonso-Monge *et al.*, 2003) is of special importance in *C. albicans* as it constitutes a type of stress probably more related to the environment this fungus encounters during its life cycle within the host. Translocation of Hog1 to the nucleus due to oxidative stress generates an adaptive transcriptional response which is partially overlapping, although distinguishable, from the one generated by the transcription factor Cap1 (Alonso-Monge *et al.*, 2003; Enjalbert *et al.*, 2006), homologue of *Sc*Yap1 in *C. albicans* and shown to play a role in multidrug and oxidative stress resistance (Alarco and Raymond, 1999; Zhang *et al.*, 2000). Farnesol and caspofungin have been also described to activate Hog1 (Bamford *et al.*, 2009; Kelly *et al.*, 2009), indicating that both treatments induce oxidative stress in this fungus which is sensed by the HOG pathway. The downstream molecular mechanisms that underlie Hog1-mediated oxidative stress resistance remain an area of active research. The transcriptional repressor Sko1 mediates in part this oxidative response (Alonso-Monge *et al.*, 2010).

In *S. cerevisiae*, the HOG pathway is activated upon osmotic stress via two different upstream branches: the *SLN1*-branch which is a two-component system relying on *SLN1*, *YPD1* and *SSK1* genes to mediate the activation of the functionally redundant Ssk2/Ssk22 MAPKKKs; and the *SHO1*-branch, the second input of this cascade, which converges at the Pbs2 MAPKK through the Ste20 PAK-like kinase (Posas *et al.*, 2000; de Nadal *et al.*, 2002; Hohmann, 2002). In contrast, although both branches do exist in *C. albicans*, only the orthologues of the mentioned genes for the *SLN1*-branch mediate the activation of Hog1 by oxidative stress (Chauhan *et al.*, 2003; Cheetham *et al.*, 2007). The *C. albicans SHO1*-branch is not involved in the activation of Hog1 but rather in the activation of Cek1, a homolog of the *S. cerevisiae KSS1*, upon osmotic stress (Román *et al.*, 2005). Deletion of upstream components of this pathway (mediated by Cek1), in combination with *SSK1*, does not impair activation of Hog1 or glycerol accumulation in response to osmotic stress, although cells remain sensitive to high osmolarity. Thus, although the *SHO1*-branch participates in the response to osmotic and oxidative stress, it does so through *HOG1*-independent mechanisms (Román *et al.*, 2005; Román *et al.*, 2009a). Integration of the oxidative stress response is, however, a complex process and the precise connections between the HOG pathway and other routes and oxidative defense mechanisms remain to be determined (Komalapriya *et al.*, 2015).

It has been also described the implication of the HOG pathway in different morphogenetic programs as well as in the biogenesis of the cell wall. Strains lacking genes from this signaling cascade such as *hog1*, *pbs2* or *ssk2* display enhanced true hyphal formation as evidenced under non-inducing conditions (Alonso-Monge *et al.*, 1999; Arana *et al.*, 2005; Cheetham *et al.*, 2007). In contrast, *ssk1* mutants show a reduced ability to filament even in the presence of serum, a phenotype that is not suppressed by *HOG1* overexpression (Alex *et al.*, 1998; Calera *et al.*, 2000). This is partially achieved via the activation of the Cek1-pathway (Eisman *et al.*, 2006), which promotes filamentation in this fungus. In addition, the Brg1 transcription factor and a positive regulator of filamentation, is expressed in *hog1*, *pbs2* and *ssk2* mutants independently of rapamycin, an activator of the TOR pathway, therefore suggesting that the TOR pathway is involved in hyphal elongation via the HOG pathway (Su *et al.*, 2013). Interestingly, *sko1* mutants, which are sensitive to oxidative stress and increase damage in *hog1* mutants, display increased filamentation (Alonso-Monge *et al.*, 2010). In addition to the yeast-to-hypha transition, Hog1 is also essential for chlamydospore formation, acting independently from Efg1 which was the first regulatory factor shown to be involved in this developmental

process (Sonneborn *et al.*, 1999). Both *efg1* and *hog1* are unable to form chlamydospores and overexpression of *EFG1* gene in a *hog1* mutant – and vice versa – did not restore the phenotype (Alonso-Monge *et al.*, 2003). Other genes that control chlamydospore development have been uncovered (Staib and Morschhauser, 2007). The Hog1 MAPK has been recently linked to another morphogenetic program in *C. albicans*, the white–opaque switching. In order to mate, homozygous cells for the MTL need to undergo an epigenetic switch from the standard white phenotype to the opaque and mating-competent state. The activation of the HOG pathway seems to be crucial for this process as the absence of Hog1, Pbs2 or Ssk2, as well as point mutations at the conserved phosphorylation sites of Hog1, increases the switching frequency and suppresses mating and pheromone-stimulated cell adhesion (Liang *et al.*, 2014; Chang *et al.*, 2016).

Regarding cell wall biogenesis, it was originally described that the deletion of *hog1* confers a resistant phenotype to certain compounds such as nikkomycin Z (which inhibits chitin synthesis) and Congo red or calcofluor white which alter the correct cell wall assembly (Alonso-Monge *et al.*, 1999; Román *et al.*, 2005). It has been proposed, based on transcriptional studies, that the HOG pathway regulates chitin synthesis coordinately with other pathways (Munro *et al.*, 2007). This could involve other MAPKs, such as Cek1, which has been also shown to be important in cell wall formation (Navarro-García *et al.*, 2005; Eisman *et al.*, 2006).

The HOG pathway also mediates metabolic adaptation in *C. albicans*. In fact *hog1* mutants are hypersensitive to inhibitors of the respiratory chain like azide and display an increased basal respiration, indicating that *hog1* mutants are more dependent on mitochondrial ATP synthesis (Alonso-Monge *et al.*, 2009a). Kaba and coworkers have further described a transient Hog1 phosphorylation under high iron concentrations, essential for the flocculent phenotype observed in a wild type strain, and a loss of viability of the *hog1* mutant when exposed to high concentrations of iron (Kaba *et al.*, 2013).

Since the HOG pathway plays so many crucial roles in the biology of *C. albicans*, it is not surprising the relevance of this pathway in fungal pathogenesis. In fact, though *hog1* mutants are hyperfilamentous (therefore, expecting to cause increased tissue damage), they show an attenuated virulence in a mouse model of systemic infection (Alonso-Monge *et al.*, 1999). A phenotype of *hog1* mutants that could explain this is their sensitivity to oxidative and nitrosative stress, even more pronounced in *pbs2* strains (Arana *et al.*, 2005). This could result in sensitivity to the phagocytic attack by macrophages and neutrophils which generate ROS or reactive nitrogen species (RNS) to allow fungal clearance (Missall *et al.*, 2004; Du *et al.*, 2005; Arana *et al.*, 2007). In addition, strains with a compromised HOG pathway are hypersensitive to some antimicrobial peptides such as the salivary cationic peptide histatin-5 (Hst 5) (Vylkova *et al.*, 2007) and β-defensins 2 and 3 (Argimon *et al.*, 2011). Hog1 (and Pbs2) have been also shown to play a critical role in the establishment of *C. albicans* in the mouse gut (colonization) which could be explained by their decreased adherence to the gut mucosa and/or sensitivity to bile salts (Prieto *et al.*, 2014).

Mkc1-Mediated Pathway: Maintaining Cell Integrity

The fungal cell wall suffers constant modifications in order to adapt to the different environmental conditions that *C. albicans* encounters. Defects in this adaptive response can lead to cell integrity loss which triggers a signaling pathway to revert the process (Blankenship *et al.*, 2010). The MAP kinase Mkc1 (homologue to Slt2/Mpk1 from *S. cerevisiae*) belongs to the so called cell integrity or PKC pathway. It has been demonstrated its role in cell wall biogenesis (Navarro-García *et al.*, 1995), in stress response (Navarro-García *et al.*, 2005), in morphogenesis (Navarro-García *et al.*, 1998), in biofilm formation (Kumamoto, 2005) and virulence (Díez-Orejas *et al.*, 1997). The cell integrity pathway includes a central module that incorporates the MAPKKK Bck1, the MAPKK Mkk2 and the MAPK Mkc1 (Alonso-Monge *et al.*, 2006) whose phosphorylation is dependent on the protein kinase C (Pkc1) which lies upstream of the MAPK core (Paravicini *et al.*, 1996; Navarro-García *et al.*, 2005). Functional studies using combined deletions of cell integrity and sterile vegetative growth (SVG) MAPKKs (*mkk2 hst7*) and MAPKs (*mkc1 cek1*) support a cooperative role for these pathways in regulating cell wall architecture under vegetative growth (Román *et al.*, 2015; Correia *et al.*, 2016). Mkc1 activation occurs in response to a wide range of external conditions such as cell wall damage, antifungal drugs and low temperature shocks (Navarro-García *et al.*, 2005). Mutants defective in *MKC1* present defects related to invasive growth under embedded conditions and in biofilm formation (Kumamoto, 2005) and are more sensitive to the action of cell wall degrading enzymes (such as zymolyase) and antifungals, developing superficial alterations in restrictive growth conditions such as high temperature (Navarro-García *et al.*, 1995). This pathway is especially relevant in the response of *C. albicans* to caspofungin. The echinocandins provide a safe and efficient therapy for systemic mycosis as they are noncompetitive inhibitors of β-(1,3) and β-(1,6)-glucan synthase which is involved in the synthesis of β-(1,3)-glucan, an essential component of fungal cell wall and absent from human cells. *C. albicans* can rapidly respond to the presence of echinocandins by elevating chitin content which has been described as a salvage mechanism of this fungus (Walker *et al.*, 2008). The chitin synthesis is coordinately regulated by the PKC, HOG and Ca^{2+} signaling pathways (Munro *et al.*, 2007) and, while both Mkc1 and Hog1 become activated in the presence of caspofungin, only calcineurin mutants and *MKC1* defective strains show a hypersensitivity to this compound.

This pathway has an important role in the cellular growth inside the host which is crucial for pathogenesis and Mkc1 is important for host interaction and fungal proliferation (Román *et al.*, 2007; Wachtler *et al.*, 2011). This kinase is also activated in response to oxidative stress, which is partially dependent on an intact HOG pathway (Navarro-García *et al.*, 2005). The fact that two MAPK pathways respond to oxidative stress reflects the relevance of this kind of stress in a pathogenic fungus (Herrero-de-Dios *et al.*, 2010). However, the absence of Mkc1 does not increase the sensitivity of *C. albicans* to killing by neutrophils or macrophages (Arana *et al.*, 2007), but does attenuate its virulence: *mkc1* mutants do not colonize target organs, and elicit an altered inflammatory response in infected animals (Díez-Orejas *et al.*, 1997).

Cek1-Mediated Pathway: The SVG Pathway

The Cek1 MAP kinase (homologue of *Sc*Kss1) was first identified by a functional screening with the aim of isolating *C. albicans* genes able to interfere in the *S. cerevisiae* pheromone-induced cell cycle arrest (Whiteway *et al.*, 1992). The pathway mediated by this kinase does indeed participate in the pheromone response, either through mating or biofilm formation, together with the MAP kinase Cek2 (homologue of *Sc*Fus3) (Chen *et al.*, 2002; Daniels *et al.*, 2006). The Cek1-mediated pathway is also involved in morphogenesis and hyphal formation, cell wall damage and glycosylation sensing (Monge *et al.*, 2006; Ernst and Pla, 2011); it also influences the response to oxidative and osmotic stress in *C. albicans* in cooperation with other pathways (Herrero-de-Dios *et al.*, 2010; Herrero de Dios *et al.*, 2013). Cek1 is activated through the MAPKKK Ste11 and MAKK Hst7 module (Csank *et al.*, 1998; Lee and Elion, 1999) and Cph1 works as a downstream effector (Liu *et al.*, 1994).

The pathway is also composed by other elements such as the Cst20 PAK (Leberer *et al.*, 1996), the upstream sensors Sho1, Msb2 and Opy2 (Román *et al.*, 2005; Herrero de Dios *et al.*, 2013; Román *et al.*, 2009b), and the VIH-type Cpp1 phosphatase that is believed to be involved in Cek1 dephosphorylation (Guhad *et al.*, 1998a). The elements of this cascade participate in the biogenesis of the cell wall through the so-called SVG pathway that promotes vegetative growth under basal (non-stressed) conditions. Strains defective in *CEK1*, *HST7*, *CST20*, *MSB2* or *SHO1* are susceptible to compounds such as Congo red, calcofluor white, echinocandins or the glucanase-enriched zymolyase (Román *et al.*, 2005; Eisman *et al.*, 2006; Arana *et al.*, 2009). The cell wall defects produced by the addition of either of these compounds or by specific mutations in cell wall genes, such in the ones related to *N* or *O*-glycosylation (Bates *et al.*, 2006; Cantero and Ernst, 2011; Ernst and Pla, 2011), lead to the activation of the pathway and ultimately to Cek1 phosphorylation. This process is mainly dependent on Sho1, except for tunicamycin-induced signaling (an inhibitor of bacterial and eukaryote *N*-acetylglucosamine transferases), which is specifically driven by Msb2 and cause an increase in the amount of Cek1 (Román *et al.*, 2009b; Román *et al.*, 2005). Cek1 can also be activated by iron and a role for Msb2 as an iron sensing protein has been hinted (Puri *et al.*, 2012). Cph1 (a target of Cek1) has also been described as an active chromatin remodeling transcription factor under iron-replete conditions (Puri *et al.*, 2014). Cek1, which is a short-lived protein, also responds to growth signals and is regulated by quorum sensing (Román *et al.*, 2009a). It becomes activated in conditions where an active growth is required, as the dilution in fresh medium of a stationary phase growing culture (resumption of growth). Induced cell wall remodeling upon Cek1 activation could explain, in part, the susceptibility of *C. albicans* cells to the antimicrobial peptide Hst 5 shown to bind to β-1,3-glucans exposed at the surface of the cell (Tati *et al.*, 2013). The induction of Cek1 phosphorylation, either by *N*-acetylglucosamine or serum, or its constitutive activation through deletion of Cpp1, increases the binding and uptake of Hst 5, rendering cells more sensitive to this antimicrobial peptide (Li *et al.*, 2015). On the other hand, a glyco fragment derived from the cleavage of the pathway sensor Msb2 is able to bind to Hst 5 and other antimicrobial peptides such as LL-37, human α- and β-defensins, mediating protection against their toxicity (Szafranski-Schneider *et al.*, 2012).

A morphogenetic role has been proposed for the Cek1-pathway: cells lacking some elements of this cascade such as *CST20*, *HST7* or *CPH1* are defective in hyphal formation on solid agar medium. In addition, *cek1*, although apparently normal in serum-induced liquid filamentation assays, is also required for this type of agar-invasive hypha formation where cells are first restrained in movement and nutrients may become limiting. In fact, nitrogen limitation has also been described as a triggering stimulus for the activation of this pathway (Köhler and Fink, 1996; Biswas and Morschhauser, 2005). The Sho1 adaptor and the Msb2 mucin were also described to play a role in agar invasion during starvation (Román *et al.*, 2009b) and it was demonstrated that Cek1 becomes phosphorylated during growth in contact to a semisolid surface, promoted in part by Dfi1, a plasma membrane protein (Zucchi *et al.*, 2010).

Contrary to Hog1, the Cek1-mediated pathway could exert a positive regulation on hyphal development, working in parallel with the protein kinase A (PKA) pathway. Activation of this latter pathway is dependent on the cyclic AMP (cAMP) and results in the phosphorylation of the transcriptional regulator Efg1 (Stoldt *et al.*, 1997; Bockmuhl and Ernst, 2001). In standard conditions, *efg1 cph1* mutants are totally incapable of forming filaments in vitro (Lo *et al.*, 1997), however, under hypoxia, Efg1 exerts a repressive role on hyphal development through the repression of the biosynthesis and activity of the Cek1 MAP kinase pathway (Desai *et al.*, 2015). Msb2 cleavage, which is necessary for Cek1 activation, has been related to biofilm formation (Puri *et al.*, 2012), while both Cek1 and Cph1 have been recently implicated in the process of pseudohyphal formation induced by sub-toxic concentrations of hydrogen peroxide (Srinivasa *et al.*, 2012).

Strains defective in elements of the Cek1-mediated pathway display a higher exposure of β-(1,3)-glucan at the cell surface with a consequent increase of the fungal cells binding to the immune receptor dectin-1, increase in phagocytosis and macrophage activation (Galan-Diez *et al.*, 2010). The *cek1* mutant is also associated to a reduced virulence in the mouse model for systemic candidiasis although it does not show hypersensitivity to macrophages or neutrophil-mediated killing (Guhad *et al.*, 1998b; Arana *et al.*, 2007).

Cek2: The Pheromone Response

The *CEK2* gene was cloned from an oligonucleotide probe-based screen used to find putative protein kinases from a *C. albicans* genomic library (Chen *et al.*, 2000). Additionally to the fact that shares 53% identity with *S. cerevisiae* Fus3, its expression is able to complement the mating defect of a *fus3 kss1* double mutant. Therefore, Cek2 is considered to be a functional homologue to *Sc*Fus3 and is implicated in the mating process of *C. albicans* (Chen *et al.*, 2002). Similar to *S. cerevisiae* where both MAPKs Kss1 (*Ca*Cek1 homologue) and Fus3 are necessary for an efficient mating, in *C. albicans* mating is also regulated by the orthologue protein kinase cascade in which Cek1 and Cek2 have an overlapping role. Although *cph1* and *hst7* mutants are totally incapable to mate, *cek1* has only a partial defect which becomes complete after the additional deletion of *CEK2* (Chen *et al.*, 2002; Magee *et al.*, 2002).

Opaque cells homozygous for the MTL locus can release pheromones that are sensed by opaque cells from the opposite mating type: opaque α-cells produce α-pheromone which binds to the Ste2 receptor on a-cells while opaque a-cells release a-pheromones which bind to the receptor Ste3 in α-cells (Bennett *et al.*, 2003; Tsong *et al.*, 2003). Upon pheromone sensing by the receptors, the signaling pathway is activated being responsible for the transduction of the pheromone signal to the nucleus to promote the appropriate transcriptional response. This cascade is composed by the trimeric G-protein complex (Cag1, Ste4, Ste18), the MAPK core Ste11, Hst7, Cek1 and Cek2 and the transcription factor Cph1 necessary to activate gene expression. As in *S. cerevisiae*, the mating pathway in *C. albicans* is composed by some of the genes that participate in the SVG pathway whose MAPK is Cek1. Furthermore, the same pathway is involved in the response of mating-incompetent white cells to pheromones released by opaque cells from the opposite mating type, culminating in enhanced biofilm formation (Daniels *et al.*, 2006). Because common elements are found in these pathways, specificity mechanisms such scaffold proteins must exist to prevent improper activation by environmental signals. The *C. albicans* gene *CST5* encodes the scaffold protein for the kinases in the pheromone response pathway of both opaque and white cells (Cote *et al.*, 2011; Yi *et al.*, 2011). Finally, orthologues of pheromone-inducible genes important for mating such as *FIG1* and *FUS1*, which encode membrane proteins activated by Cph1 or α-pheromone, have been also identified in the *C. albicans´* genome (Tzung *et al.*, 2001).

The first transcription factor involved in pheromone-induced biofilms was Tec1, one of the transcription targets (along with Efg1 and Bcr1) for conventional (a/α) biofilms. These biofilms in a/α cells are regulated by the Ras/cAMP pathway and not by the MAPK-mediated signaling (Sahni *et al.*, 2010; Yi *et al.*, 2011). The mating-related transcription factor Cph1 was found to play a minor role in this process. However, recent results obtained by Lin and coworkers (Lin *et al.*, 2013) point to a new model in which the same MAP kinase components and Cph1 are responsible for signal transduction in both white and opaque cells. According to these authors, Tec1 would have a general effect on biofilm formation, not specific to pheromone stimulation.

Cek2 is considered to be under the repressive influence of the *a1/α2* complex, not being expressed on heterozygous strains for the MTL locus. Indeed, *CEK2* RNA was only detected in *MTLa* and *MTLα* cells in a northern blot analysis (Chen *et al.*, 2002). Accordingly, Srikantha and coworkers (Srikantha *et al.*, 2006) demonstrated through chromatin immunoprecipitation-microarray analysis (ChIP-chip) that *CEK2* holds an *a1* binding site. Moreover, Cph1 overexpression regulates both Cek1 and Cek2 in *MTLa* and *MTLα* strains but not *MTLa/α* (Chen *et al.*, 2002). The deletion of *CEK2* in *MTLa/α* does not affect *C. albicans* survival to human neutrophils nor the binding to the immune receptor dectin-1, an opposite behavior to strains defective in *CEK1* or *HST7* (Arana *et al.*, 2007; Galan-Diez *et al.*, 2010). Furthermore, the a/α *cek2* mutant presents no defects in hyphal development on Spider and other inducing media (Chen *et al.*, 2002), again in contrast to other members of the MAP kinase cascade like Cst20, Hst7 (Köhler and Fink, 1996; Leberer *et al.*, 1996), Cek1 (Csank *et al.*, 1998) and Cph1 (Liu *et al.*, 1994). Therefore, not until recently, no significant phenotype for the *cek2* mutant had been described for the MTL heterozygous background. It has now been uncovered a cryptic role for this MAPK in cell wall biogenesis, as the absence of *CEK2* in *a/α* strains leads to a moderate sensitivity to cell wall disturbing agents and Cek2 can actually become phosphorylated by the same stimuli that activate the SVG pathway (Correia *et al.*, 2016).

Conclusions

We have highlighted here the main processes in which MAPK pathway are involved to allow adaptation to the environment. They include, among others, metabolic changes, responses to stress, alterations in the cell wall and invasion of solid surfaces. All of them are essential to establish a successful interaction with the host, either via a commensal program or a pathogenic relationship. We believe that a close analogy can be established with the nervous system of a mammalian organism where the main functions are sensory input, data integration and motor output. For the *C. albicans* MAPK pathways here outlined, these three features are present as MAPK routes sense external stimuli by membrane receptors, integrate these data through defined biochemical events and finally generate a response via transcriptional adaptation. To ensure specificity, a variety of mechanisms exist such as the presence of scaffold proteins, a defined compartmentalization (nucleus, cytoplasm, membrane) and enzymatic substrate specificity that ensure that the proper response in generated in a timely fashion.

Although we have focused here only in the fungal pathogen *C. albicans*, they also play an important role in other clinically relevant fungi such as *Cryptococcus* (Bahn *et al.*, 2005) or *Aspergillus* (May *et al.*, 2005). They are also crucial in phytopathogenic fungi (Perez-Nadales *et al.*, 2014; Turra *et al.*, 2014) and therefore important to establish relationships with other living communities in the environment. The development of chemicals that may target these routes appear therefore as a promising way to control fungal proliferation in different environments. We believe this area of research will be relevant during the following years connecting clinical and basic research.

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Communication With Plants

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Interaction of Fungi With the host

Mycorrhizal interactions with plants is described elsewhere, therefore this chapter will focus on the communication between plants and fungal pathogens. Fungal plant pathogens can be classified as *biotrophs*, *necrotrophs* or *hemibiotrophs* based on their lifestyle and interaction with the host (Bais *et al.*, 2006). Hemibiotrophic fungi represent the most interesting group of pathogens since they use sequential biotrophic and necrotrophic infection strategies to invade and colonize host plants. Transition from the asymptomatic biotrophic phase, characterized by intercellular thick primary hyphae, to the destructive necrotrophic phase, characterized by thin filamentous secondary hyphae, is referred to as the biotrophy-necrotrophy switch. The host can tailor the defense response according to the changing phases of the pathogen by several mechanism. It can be observed a change at transcriptional level, and also in the phytohormone signaling involving JA, ET or SA (Battilani *et al.*, 2018; Beccaccioli *et al.*, 2019). They represent an integral component of host-defense system against several fungal pathogens. The lifestyle of a pathogen often dictates the host's defense strategy, and the pathogen may even manipulate hormonal crosstalk for successful colonization. Plants adopt timely activation of suitable phytohormone signaling depending on the pathogen's lifestyle to restrict the infection process. Necrotrophism involved the action of lytic enzymes and phytotoxins secreted by the pathogen, and is opposed to that of biotrophic fungi that feed on the live host, generally without the action of degradative enzymes or phytotoxins, developing a form of parasitism which does not lead to tissue death. However, it is important to highlight that during the infection process pathogenic fungi activate a first biotrophic phase necessary for the success of the infection, although in the case of necrotrophs this phase is very short and is associated with the very early stages of the interaction. Process of infection of hemibiotrophic fungi is characterized by a first biotrophic phase of variable duration, and by a subsequent necrotrophic colonization. These two strategies show crucial differences in the cycle of the disease, in the morphology of the infection, in the mode of acquisition of nutrients, in the manifestation of symptoms, in the range of susceptible hosts and in the plant defense responses.

Necrotrophic Fungi

Necrotrophic fungi infect plant tissues by killing host cells and feeding by dead plant tissues. When the host plant fails to counteract the initial events of the infectious process, these fungal pathogens cause the formation of necrotic areas that culminate in the death of the affected organ or the entire plant. This occurs thanks to their ability to secrete a variety of hydrolytic enzymes and phytotoxic compounds before and during the colonization of the host. Due to the high secretion of cell wall degradative enzymes (CWDE), some necrotrophic fungi cause typical symptoms of extensive and soft rot. Necrotrophic fungi can be classified as optional parasites for the method of feeding, in fact they can live saprophytically and overwinter in the absence of the host even on dead plant substrates (Berkey *et al.*, 2012).

Necrotroph infection process includes the conidia germination, host penetration through the formation of appressorium and secretion of pathogenetic enzymes, such as CWDE, or the entering through wounds, stomata or lenticels. The penetration determines the formation of a primary lesion, which expanding will trigger the necrosis of the host tissues and fungal sporulation. After penetration, the plant tissues are further attacked by many pivotal molecules named Reactive Oxygen Species (ROS) and by low molecular weight metabolites produced by necrotrophic fungi during the infectious process. These metabolites can be specific and characteristic only of plant-fungus associations, or they can consist of phytotoxic metabolites with a broad spectrum of action. Necrotrophic fungi are also able to manipulate the plant's immune system by suppressing its resistance mechanisms and influencing hormone levels through the biosynthesis of its own hormones, disrupting the network of molecular signals necessary for the activation of defense. The multiple virulence factors and the wide diversity of necrotrophic fungal species, in turn, determine variations in host resistance strategies, often linked to the activation of many resistance genes, hormones, secondary metabolites and PR proteins (pathogenesis -related). Infection and nutrient acquisition strategies by necrotrophic fungi differs greatly from that of biotrophs. In fact, many defense mechanisms specific for the biotrophs, such as the HR, are instead generally considered susceptibility mechanisms, which promote infection and colonization by necrotrophic fungi. Necrotrophic plant resistance is controlled by numerous genes. The symptoms of necrotrophic infection consist of extensive necrosis, rot and death of the plant, for biotrophs instead they manifest themselves as a slow and gradual deterioration of the host, without compromising its survival. Necrotrophic fungi can be distinguished in two groups based on the specificity of interaction with the host: (1) host-specific necrotrophs or HSN (Host Specific Necrotrophs) with a very limited range of hosts and (2) Broad-range Host Necrotrophs (BHNs) with a wide range of hosts. HSN necrotrophic fungi develop the infection and produce host-specific toxins which function as strain-specific effectors, essential for their virulence and infection process. An example of this grouping is *Cochliobolus carbonum* (anamorph: *Helminthosporium carbonum*) which produces the HC toxin and is capable of developing the disease known as "leaf

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spot" only in susceptible maize genotypes. *Alternaria brassicicola* secretes a selective toxin or HST of a protein nature, known as toxin AB, and other phytotoxic metabolites of different nature such as Brassicicolin A, Brassicicenes A – F, Phomapyrone A/F/G. Host-specific resistance to these types of pathogens closely mirrors ETI and is based on the presence of proteins encoded by single genes capable of activating HST. The specific host toxin works as an effector because it suppresses the defense responses in sensitive hosts, but it is also essential for the host's response to attack. BHN necrotrophic fungi attack a wide range of hosts using lytic enzymes and toxins that are designed to knock out basic functions or common features to all plants. The fungal species that can be classified as BHN are very numerous and economically very important. Fungi that cause soft fruit rot, such as *Monilinia*, *Penicillium*, *Sclerotinia*, *Botrytis* and *Alternaria*, attack a large number of species. *Botrytis cinerea* is the most studied and known BHN because it is present all over the world, being a pathogen of numerous plants of agricultural interest. Different strains of *B. cinerea* can present various attack mechanisms that adapt to different hosts and tissues. *Sclerotinia sclerotiorum* is also able to attack a large number of hosts (more than 400) and is known to produce oxalic acid, an important virulence factor, together with several lytic enzymes such as glucanase, glycosidase, pectinase, cellulase, xylanase and protease. *B. cinerea* and *S. sclerotium*, together with the genera *Monilinia*, *Penicillium* and *Alternaria*, generally attack the aerial part of the host plant and spread through wind-blown spores or conidia.

Biotrophic Fungi

Biotrophs interacts with other organisms and are unable to survive as isolated entities. The organisms involved can share the same space, or they can develop processes in which one or both partners take advantage and improve their reproductive success. Trophic relationships are often used to classify interactions between plants and microbes, when plants live during nutrient exchange with the pathogen, we speak of "biotrophic interactions" and we refer to microbes as "biotrophs" (Bonfante and Genre, 2015). Interacting with live plants requires very complex mechanisms. Biotrophs have the ability to manage and take control of the plant's immune system, manipulate the host's metabolism and redirect nutrients to own advantage. To maintain this relation, many microbial species possess secreted effector proteins that exert various activities in the host plant. Biotrophs have developed very complex mechanisms to access the nutritional resources present in plant cells, and molecular mechanisms aimed at undermining plant immunity. Furthermore, biotrophic fungi generate specific morphological structures adapted to extract nutrients from plant cells: these structures are called haustoria. These are extensions of the terminal part of the hypha which penetrate through the cell walls of plant cells without affecting the plasma membranes. However, biotrophs are not limited to the formation of haustoria. There are many apoplastic biotrophs that do not produce haustoria such as *Cladosporium fulvum*, the green-blue mold agent of tomato, and *Ustilago maydis*, agent of corn coal. In this case, exchanges between the host and the pathogen take place in the apoplast. Sometimes, apoplastic signaling may also be relevant in interactions in which haustoria are integrated, and they are called "endophytic" microbes. Once this space is occupied, some microbes seem to have lost their original ability to grow in a saprophytic way, and they are called "obligate biotrophs". Examples of these are some arbuscular mycorrhizal fungi, common powdery mildew and some rusts. Biotrophic pathogen must invade the host tissue with minimal damage and evade recognition by plant cells. This goal is achieved through three interconnected strategies: (1) creation of interfacial layers rich in carbohydrates and containing proteins that separate the fungal cell walls from the plasma membrane of plants; (2) production of effectors able to modulate the host metabolism and suppress its defenses; (3) fungal cell wall shaping to evade the immunity triggered by PAMPs. The plant immune system has evolved to face the biotrophic pathogens. The first step of immunity is the perception of molecules derived from pathogens (molecular patterns) by specific membrane receptors (pattern recognition receptors - PRR). A second level of plant defense (effector-triggered immunity, ETI) is based on the direct or indirect recognition of the effectors secreted by pathogens (avirulence proteins) by cytoplasmic sensors, so-called resistance proteins, also called NOD-like receptors (nucleotide-binding oligomerization domain), which usually confer specific resistance. This defense response often involves a reorganization of the cytoskeleton and a conspicuous secretory activity. Plant resistance is also accompanied by the presence of defense phytohormones (jasmonic and salicylic acid). Salicylic acid-mediated responses are considered typical of reactions to biotrophic attack, while those mediated by jasmonic acid and ethylene are thought to be associated with the response to necrotrophic pathogens.

This distinction is now being questioned, some data revealing the crucial signaling role of jasmonic acid in the indisputably biotrophic interaction of grapevine with downy mildew (*Plasmopara viticola*). There are two main strategies that plants use to limit the invasion and growth of biotrophic fungal pathogens: resistance to penetration and resistance mediated by programmed cell death (PCD). Plant strengthens the cell wall and membrane to inhibit spore germination and prevent the formation of austori (resistance to penetration). The second mechanism of resistance (induction of PCD) is activated within the penetrated epidermal cells and interrupts the supply of nutrients to the fungi by blocking the further development of the fungus.

Hemibiotrophic Fungi

Hemibiotrophic fungi are classified in the middle between the necrotrophic and biotrophic fungi, being able to adopt both survival strategies: they first exploit the biotrophic phase as the beginning of the infection and, subsequently, carry out a necrotrophic attack to grow rapidly and finally reproduce. The reality is that there is no clear separation between these different lifestyles, even if their classification is a useful exercise for the scientist (Chowdhury *et al.*, 2017). For the agronomic importance, *Magnaporthe oryzae* together with the species belonging to the genus *Colletotrichum* represent today the most relevant models for the study of *hemibiotrophic* fungi. *Magnaporthe oryzae*, the causative agent of rice blast disease, is responsible for the most serious disease

affecting cultivated rice in the world. Instead, the genus *Colletotrichum*, composed of nearly 200 species organized in 14 phylogenetic groups, can infect a large number of plants of agronomic interest. Many phytopathogenic fungi develop a specialized structure, called the appressorium, to penetrate the plant epidermis. The appressorium can break the cuticle and the plant cell wall thanks to the high pressure and enzymatic action exerted on the host cell. During the biotrophic phase, the host's plasma membrane invaginates for the pressure exerted by the fungal hyphae, without penetration; at this stage, the host cell is still alive. The last phase of the infection, the necrotrophic one, involves the differentiation of thin hyphae which, growing quickly, kill and destroy the host tissues. The success of hemibiotrophic fungi, such as *Colletotrichum* sp. and *M. oryzae*, depends on the morphogenetic transition that allows them the formation of the appressorium and, therefore, the penetration of the host.

Host Sensing

How do Fungi Perceive the Host?

Fungi perceive their environment through light, chemical and physical signals. The perception of these signals activates signal transduction processes that induce changes in metabolism, cell organization and gene expression. These changes, in turn, lead to developmental and morphological processes such as sporulation and redirection of growth, induce the ability to degrade complex organic compounds and import nutrients and allow survival to stressful conditions: osmotic, oxidative, thermal stress and presence of antifungal substances. Pathogenic fungi, can synchronize all these responses as a result of the perception of the host in such a way as to be able to colonize it. All these responses can be modulated differently depending on the type of host or organ/tissue of the host attacked.

Plant Surface Detection

Pathogenic fungi that invade the above ground tissues (e.g., leaves, stems) recognize the surface of the plant, the cuticle, through its hydrophobicity and chemical composition. The cuticle is composed of cutin, a polymer of hydroxylated fatty acids and intracuticular and epicuticular waxes with complex compositions. In the pathogenic hemibiotrophic fungus *Magnaporthe oryzae* the membrane proteins Msb2 and Sho1 serve to recognize the rice cuticle (Christensen and Kolomiets, 2011). This molecular recognition leads to the formation of the appressorium. Msb2 is needed to detect hydrophobicity and cutin monomers while Sho1 is more important in the response to primary alcohols, which are the main components of waxes. Basidiomycete *Ustilago maydis* also depends on Msb2 and Sho1 for the development of appressoria on the surface of the maize leaf. Alongside the induction of appressoria, these surface signals induce the expression of effector genes associated with biotrophy (De Coninck *et al.*, 2015). Some authors have shown that in *M. oryzae* Msb2 has overlapping functions with another mucin, Cbp1, in the formation of appressoria and that extracellular and cytoplasmic domains of Msb2 have distinct roles in appressorium formation and invasive growth. *Botrytis cinerea* requires Msb2 for the formation of appressoria or so-called "infection cushions" on harder surfaces (e.g., stem), even though *msb2* mutants of this fungus are still virulent in various plant species (Gao *et al.*, 2007). The root invader *Fusarium oxysporum* does not encounter a cuticle but requires both Msb2 and Sho1 for invasive growth, root colonization and secretion of pectinolytic activity, apparently acting through the MAP kinase Fmk1 (Gebrie, 2016). Another radical pathogen, *Fusarium solani*, responds to cutin monomers by inducing the expression of a gene encoding for cutinase. The G-protein coupled receptor (G-protein) associated receptor, Pth11, is required for the development of appressoria in *M. oryzae* and is most likely involved in host surface recognition.

Cell Detection

By analyzing the gene expression of *Fusarium graminearum* (the causal agent of the Fusarium Head Blight in cereals) in the colonization of wheat kernels, a profound difference can be seen whether these are alive or dead. This suggests that the pathogen is able to specifically detect living plant cells. This has also been suggested by studies with the *F. oxysporum* effector gene SIX1, induced during root invasion but not in dead roots or by exudates or root extracts. Perhaps, living plant cells produce unstable compounds that are rapidly depleted after cell death and which can be detected by fungi. Another possibility is that living plant cells produce specific compounds only when they themselves detect the presence of microorganisms. An interesting example of the latter case is the accumulation of polyamine putrescine, a compound that in turn can induce the expression of genes such as *Tri5* that lead to the production of mycotoxin deoxynivalenol (DON) during the disease known as Fusarium Head Blight (FHB). If a polyamine is really the key factor for the activation of *Tri5*, it must be a very early response from the host because *Tri5* is already induced in the early stages of infection (in infection cushions).

Root Detection

In the soil, some fungi can sense the proximity of the roots through the compounds they release and respond by growing towards the source of those compounds, a process called chemotropism. A well-known example of a fungal response to compounds released by the roots is the branched hyphal response of arbuscular mycorrhizal fungi to strigolactones. Regarding pathogenic

fungi, a notable recent discovery is the response of the vascular pathogen *Fusarium oxysporum* to peroxidase released from the roots (described in the next paragraph).

Fungal Chemotropism

The term chemotropism is composed of the English word chemical and the Greek term trépomai "I turn around" and therefore designates a movement of organs in growth due to a chemical stimulus. In this paragraph, examples of fungal chemotropism directed towards the plant will be reported (Hemetsberger *et al.*, 2015). Chemotropism in this sense was reported for the first time in a work entitled "Comparative morphology and biology of fungi, mycetozoa and bacteria" of 1887, in which Anton de Bary, considered the founding father of modern plant pathology, described the directional growth of the germ tubes of the fungus *Uromyces appendiculatus* towards the stomata of the bean leaves. The stimulus can orient growth towards its origin (positive chemotropism) or in the opposite direction (negative chemotropism), or have no effect. The ability of substances to generate the stimulus also depends on their concentration. Growing hyphae are exposed to various chemotactic signals of different nature which can be divided into fungal signals (pheromones), nutrients and signals produced by the host (plant). The latter phenomenon can be observed in the rhizosphere, or in that portion of soil that surrounds the roots of plants. Numerous interactions with bacteria and fungi take place in it, in fact it constitutes one of the most complex terrestrial ecosystems. Considering the extremes, mycorrhizal fungi provide plants with mineral nutrients and increase their chances of survival in nutrient-limiting environments. On the contrary, pathogenic fungi lead to yield losses. Hence, the survival of plants within this complex ecosystem depends on their ability to distinguish beneficial fungi from parasites. Likewise, fungal survival is also based on identifying suitable host plants and the ability to overcome the host's defenses. The chemical environment established by the roots of plants influences the microbial composition of the rhizosphere. Plants are able to produce both attracting and repellent compounds, the former allow the establishment of beneficial relationships with microorganisms, the latter keep parasites at bay. A variety of compounds that contribute to plant-fungus communication are released from the roots of plants in their surroundings, i.e., in the so-called rhizosphere. These include low molecular weight substances such as ions, free oxygen, amino acids, organic acids, sugars, phenols and other secondary metabolites, as well as high molecular weight exudates such as mucilages (polysaccharides) and proteins. The rhizosphere therefore attracts both beneficial and harmful microbes thanks to an environment rich in sources of organic carbon; on the other hand, some volatile organic compounds emitted by the roots of plants act as underground defense substances that exert antimicrobial and anti-herbivorous activity. Root exudates can be produced both constitutively (the so-called phytoanticipins) and in response to stimuli such as an attack by a pathogen (the so-called phytoalexins). In addition to contributing to the chemical warfare between plants and their pathogens, root exudates are equally important as signaling molecules in plant communication with symbiotic microbes. The colonization of the root by mycorrhizal fungi occurs following the perception of the root exudates by the pre-symbiotic fungal mycelium. Strigolactones have been identified so far as the compounds responsible for attraction to the roots; these, are plant hormones derived from carotenoids present in the exudates of plants of different taxa and can be considered general signaling compounds that are essential for the establishment of mycorrhizal symbiosis. In mycorrhizal fungi, strigolactones act as hyphal branching factors thus stimulating root colonization. Some of the substances produced by the roots following stress (e.g., wounds) or microbial attacks can be released into the rhizosphere and act as a booster for (other) pathogens. For example, within the 17 different classes of defense-related proteins (PR), which are activated following both biotic and abiotic stress, PR9s contain members of class III peroxidases. These enzymes are secreted by the plant into the rhizosphere following injury or microbial attack.

Turrà *et al.* (2015) have shown that the phytopathogen *Fusarium oxysporum* perceives the activity of plant class III peroxidase and reorients the growth of its hypha towards the roots (**Fig. 1**) (Gebrie, 2016). The pathogen will start its infection from the roots, and from these it can penetrate and colonize the plant. Through in vitro assays it was shown that the exudate from tomato roots, containing catalytically active class III peroxidases, had a positive chemotropic effect on the growth of *F. oxysporum*. As is the case in several eukaryotic organisms, fungi can sense environmental stimuli through surface receptors, which activate and transmit these signals through signaling mediated by mitogen-activated protein kinases (MAPKs). For pathogenic fungi, the ability to perceive suitable hosts is crucial for parasitism. Thus, surface receptors and downstream signaling pathways specialized during the evolutionary process to detect nearby host plants. To identify the signaling pathway responsible for host perception (MAPK pathway) triggered by peroxidase, deletion mutants on the *F. oxysporum* MAP kinases were generated. The characterization of these mutants revealed that *F. oxysporum* exhibits several MAPK signaling pathways, specialized on the basis of the perceived stimulus which can be nutritional, linked to the mating type, or elicited by peroxidase. The study of the mutants highlighted a further discovery, namely that the same receptors responsible for the recognition of pheromones (which determine the mating type), were involved in the signaling elicited by peroxidase. *F. oxysporum*, like many other pathogenic fungi, enters the host tissue through the root wounds. When lesions occur in plant tissues, the production of reactive oxygen species (ROS) is triggered. The release of peroxidases, in the damaged sites, increases the toxicity towards the pathogens that are invading the tissue. Therefore, type III peroxidases, in addition to being linked to plant defense, are able to carry the pathogen towards the injured site of the host plant. It remains to be determined whether this concept can be generalized.

However, there remain some questions whose answers are still incomplete, for example it is not clear how *F. oxysporum* resist the antimicrobial activities of the plant, and if there is a mechanism that is able to tolerate this toxic environment for the pathogen. For root pathogens there is no evidence in this regard, but it is known that leaf pathogens to fight the defense responses of the

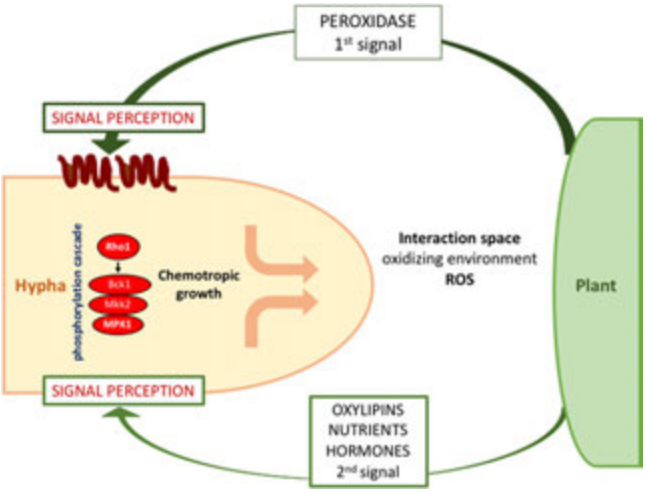

Fig. 1 Plant-pathogen interaction model. The fungal hyphae grow towards plant cells following the perception of peroxidase (first signal) which is perceived by specific receptors capable of activating the phosphorylative cascade in which the Mpk1 kinase is involved. Hyphae are stimulated to grow directionally in virtue of the oxidizing environment that is generated following the release of reactive oxygen species (ROS). Additional compounds released into the environment such as oxylipins, nutrients and hormones can act as a secondary stimulus of directional growth of the fungal hypha.

plant have evolved the ability to secrete molecules, enzymes and proteins known as effectors that alter the physiological processes and subvert the immune defenses of the plant. host plant, contributing to virulence. For example, the rice phytopathogen *Magnaporthe oryzae* produces a preserved glutathione peroxidase that neutralizes the hydrogen peroxide produced by plants. Fungal mutants, devoid of glutathione peroxidase, are more sensitive to reactive oxygen species, such as hydrogen peroxide, and are also altered in virulence. These enzymes are very conserved in filamentous fungi therefore it will be interesting to determine if *F. oxysporum* has a similar detoxification system. Another pathogen advancement mechanism that interferes with peroxidase has evolved in *Ustilago maydis*. An effector protein, called Pep1, secreted by *U. maydis* was found capable of inhibiting the activity of type III peroxidases and suppressing the oxidative burst during the first moments of the immune response. Like peroxidases, Pep1 also appears to be conserved in all carbon-causing biotrophic pathogens such as *U. maydis*.

In conclusion, the host-pathogen interface appears to be an area rich in interactions and signals conveyed by the environment, therefore understanding the chemotropic mechanisms underlying the recognition and progression of pathogenesis is essential for determining the fate of the coexistence of different organisms.

How Plants and Fungi Communicate

By-Passing Plant Defenses

Once recognized the host, fungi can enter the host only if able to bypass its defenses. In fact, beside the features of the pathogenic fungi discussed below, they can express or secrete molecules that trigger immune regulatory mechanisms; indeed, fungi can produce effectors that can modulate the plant immune system to their own advantage. To be pathogenic, fungi must access inside the plant, either directly through the leaf or root surface or through natural wounds or openings such as stomata. The first barrier for the infection is represented by preformed structural (i.e., cell wall of the plant) and biochemical defenses (i.e., phytoalexins). Furthermore, plant cells can quickly recognize a potentially dangerous microorganism and activate a wide range of responses aimed at killing it and limiting its spread in the host. Plants do not have circulating immune cells and do not have an adaptive immune system as in vertebrates; instead, each cell is able to recognize the presence of a pathogen, through autonomous biochemical mechanisms that resemble the innate immune system of animals. For this reason, we often talk about the innate immunity of plants to describe the complex of defense responses that are rapidly induced at the site of infection (Hogenhout *et al.*, 2009). Subsequently, longer-term responses may be observed not only in tissues directly in contact with the pathogen, but also in the rest of the plant, and acquired resistance to subsequent infections may occur. The immune response in plants consists of a general response triggered by pathogen-associated molecular pattern (PAMP) and a specific response triggered by effectors. The first type of response is known as PAMP triggered immunity (PTI) and the second as effector triggered immunity (ETI). Jones and Dangl (2006), developed a simplified model to explain the evolution of PTI and ETI in plants, known as the "zigzag model". This model is based on the results of previous studies carried out on plant interactions with biotrophic pathogens. According to the model, the first defense response is the PTI activated by PAMPs released by pathogens. Successful (hemi)biotrophic pathogens secrete effectors within the host cell, some of which can suppress PTI while others reprogram the physiology of the plant to promote host colonization. The host may possess resistance proteins (encoded by R genes) that can detect specific effectors or

monitor their activities, resulting in activation of ETI, a much more effective response and often (but not always) associated with the hypersensitive response (HR). These defenses are orchestrated by the tight relation among – at least – three defense-related hormones: jasmonic acid, salicylic acid and ethylene. In phase one of the HR, the activation of R genes triggers an ion flux, involving an efflux of hydroxide and potassium to the outside the cells, and an influx of calcium and hydrogen ions into the cells. In phase two, the cells involved in the HR generate an oxidative burst by producing reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide. These compounds affect cellular membrane function, in part by inducing lipid peroxidation. More generally, HR is a multifaceted response in which ROS, phytoalexins and PR proteins inter alia, are secreted into apoplast to challenge fungal invasion; fungi have to face these molecules to entertain a symbiotic (parasitic) relation with the host.

Lipid Signaling

When the pathogen and the host are in contact, an alteration of the composition of fatty acids occurs in both organisms. This alteration can be caused by the remodulation of the intake of fatty acids and the activity of enzymes involved in their synthesis and degradation. The expression of the lipid metabolism genes is induced during the host infection with consequent modification of the lipid profile. Enzymes that modify lipids regulate the production of lipid metabolites and consequently signal transduction and cell growth (Battilani *et al.*, 2018; Beccaccioli *et al.*, 2019).

Fatty Acids

Fatty acids are the building block of the lipid metabolism, they can be present as free fatty acids or can also be part of more complex lipid molecules such as acylglycerides, phospholipids and sphingolipids. The class of phospholipids includes phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The lipid molecules can be the substrate for different enzymes, such as the lipases secreted by different phytopathogenic agents that act as virulence factors; phospholipases generate molecules derived from phospholipids which are crucial signals in plant-pathogen interactions and act as modulators of many signal transduction pathways during plant-pathogen interaction. Changes in the content of phospholipids and the activity of phospholipases in the plant activate the defense and resistance responses to necrotrophic pathogens. Plant phospholipases are traditionally classified into phospholipases A, C or D, based on the location of the glycerolipid breakdown site. Phospholipase D (PLD) catalyzes the hydrolysis of PC to generate choline and PA; phospholipase C (PLC) generates two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), which respectively mediate the activation of protein kinase C and the intracellular release of calcium. Phospholipases A1 (PLA1) and A2 (PLA2) can remove acyl groups by accumulating free fatty acids. During the pathogen-host interaction, PLD and PLA1–2 release free fatty acids deriving from phospholipids. In Arabidopsis thaliana, oleic acid modulates defense responses while trienoic acids (16: 3 hexadecatrienoic acid and 18:3 α-linolenic acid) activate defense responses against bacterial pathogens. Long-chain (LCFA) and very long-chain (VLCFA) fatty acids are present in the endoplasmic reticulum, they are generated by the addition of carbon units to the acyl chain. This function is performed by enzymes called elongases that are distinguished in Elo1,2 and 3. Desaturases (Fad), which also includes Ole1, lead to the formation of mono and polyunsaturated fatty acids (PUFAs, Poly Unsaturated Fatty Acids). The Sct1 enzyme is an Ole1 competitor which acts by reducing the formation of PUFAs. Fatty acids can also be incorporated into complex lipids (phospholipids, acylglycerols and sphingolipids).

Oxidized Fatty Acids: The Oxylipins

Fatty acids also represent the substrates for the formation of oxylipins, a family of compounds with distinct hormonal functions, involved in the host-pathogen interaction. The oxylipins constitute a large family of oxidized fatty acids, they are present in mammals, plants, bacteria and fungi. Oxylipins can regulate growth and mediate biotic and abiotic stress responses in all living organisms. They have been extensively studied in plants; in fact, they are produced by plants in several defense responses to deal with infections caused by different pathogens. Plant oxylipins include hydroperoxides of fatty acids, hydroxyl-, epoxy-, keto and oxy- fatty acids, epoxy alcohols, divinyl ethers, alcohols, volatile aldehydes, jasmonic acid (JA) and its derivatives. All these compounds are synthesized enzymatically or not. In higher plants, 18:2 linoleic acid and 18:3 α-linolenic acid represents the most abundant PUFAs. PUFAs readily react with O_2 which generates hydroperoxides in membrane lipids influencing membrane fluidity. The oxidative burst, or a rapid and transient production of ROS (Reactive oxygen species), is one of the first events activated during the defense mechanisms of plants during plant-pathogen interaction. ROS can oxidize PUFAs and form oxylipins. To limit oxidation reactions, reducing potentially harmful effects, cells have developed a wide range of antioxidant defense systems. In lipids with 18 carbon atoms, the greater the number of $C = C$ double bonds, the greater the reactivity, i.e., the greater the possibility of being oxidized. 18:3 α-linolenic acid is more unstable than 18:2 linoleic acid, which in turn is more unstable than 18:1 oleic acid. The biosynthetic pathway of oxylipins usually begins with a fatty acid peroxidation reaction which is catalyzed by lipoxygenases (LOX). Fatty acid hydroperoxide can be subsequently converted by enzymes belonging to the cytochrome P450 family.

Sphingolipids

Sphingolipids are involved both in the maintenance of cellular structure and in the control of cellular homeostasis. This lipid class has been studied primarily in mammals, since sphingolipids are involved in cell proliferation, in the response to stress and in apoptosis. However, their study has also been deepened in plants, as they are fundamental in some processes such as the development of pollen, and in the response to biotic and abiotic stress. In recent years, the role of sphingolipids has also deserved attention in pathogenic fungi. The main investigations were carried out on the involvement of sphingolipids in signaling processes, growth and virulence (Maor and Shirasu, 2005). The synthesis of sphingolipids in pathogenic fungi is widely conserved between species, it occurs in the endoplasmic reticulum, from which they are transported to other organelles or are directed towards the plasma membrane. Structurally the sphingolipid is composed by a sphingosine, an unsaturated amino-alcohol with a long chain, linked to a fatty acid through an amide bond; this structure is defined as ceramide (Cer). The enzyme that catalyzes the amide bond is Ceramide Synthase (CerS). In mammals there are six isoforms of CerS that can bind different fatty acids to sphingosine. A further determinant of diversification is represented by the degree of hydroxylation (i.e., the presence of hydroxyl groups -OH^+) in the fatty acids and in the sphingoid bases. The heterogeneity in the structure of sphingolipids is also generated by the possibility of binding different components, such as carbohydrates and phosphatidylinositol. Sphingolipids play an important role in controlling the host-pathogen interaction. Pathogens can produce sphingolipids that are not present in the host, they can be considered infection markers. However, the plant has developed a defense system that involves the production of antimicrobial proteins, called defensins, which are released upon recognition of these sphingolipids. In fact, certain sphingolipids, such as glycosylated ones, elicit the defense responses of the plant.

Sphingolipids Main Component of Lipid Rafts

Sphingolipids are essential components of lipid rafts which together with sterols generate these highly dynamic membrane microdomains. In mammals the main sterol is cholesterol, while in fungal lipid rafts the main components are glycosphingolipids and ergosterol. Lipid rafts are involved in dynamic membrane processes, such as protein sorting, polarized growth, and secretion processes, however they are also essential in terms of cell recognition and signaling. There are several evidence of the detection of lipid rafts, in *Saccharomyces cerevisiae* yeast they were detected through selective staining for sterols, which were detected on the hyphal extremities in the presence of pheromone. Lipid rafts have also been identified in fungal pathogens *Cryptococcus neoformans* and *Aspergillus nidulans* during polarized morphogenesis processes. The composition of lipid rafts is fundamental, in fact the presence of ceramides stabilizes the structure and has important physiological consequences, such as involvement in cell signaling processes. Lipid rafts are characterized by reduced lateral mobility, a change in membrane thickness and a composition different from other parts of the membrane. This latter feature seems particularly important in allowing these domains to play a role in protein sorting. In fact, several studies indicate that a change in the composition of lipid rafts generates the reorganization of membrane proteins, therefore these domains could be the preferential sites of interaction between host and pathogen.

How Fungus-Host Communication can Influence the Virulence and the Secondary Metabolism

Mycotoxins are toxic fungal products that are produced when fungi grow in human and animal foods. For plant pathology, mycotoxins are pathogenicity or virulence factors, i.e., they play a role in causing or increasing the plant disease (Mengiste, 2012). On the other hand, there is relatively little evidence that mycotoxins enhance the ability of fungi to grow in vertebrate hosts. *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium* and *Penicillium* are the main mycotoxigenic fungi. Exist several mycotoxins (about 300–400 different mycotoxins have been recognized) that differ from each other structurally and in their toxicity. Most are not of significant dangerous in terms of human health, although aflatoxin B1 (AFB1) is well known for its health impact.

Several fungal pathogens can produce toxic secondary metabolites, mycotoxins. Some of them interfere with the host's sphingolipid metabolism, as shown below. Necrotrophic pathogens can control the activity of CerS through mycotoxins, as does *Alternaria alternata* f. sp. *lycopersici*, a tomato pathogen, which produces the toxin AAL structurally like sphingosine. This inhibition leads to a metabolic imbalance. ALL causes accumulation of sphingoid bases that induces programmed cell death (PCD). Similarly, to the AAL toxin, fumonisins (FBs) produced by the genus *Fusarium* can inhibit the activity of CerS in mammals and plants by inducing cell death. Therefore, the toxins AAL and FB can induce PCD by inhibiting CerS; this mechanism is a virulence strategy of some pathogens suggesting a close link between sphingolipid metabolism and plant PCD. The toxin AAL, produced by *Alternaria alternata* f. sp. *lycopersici* and fumonisins, produced by several *Fusarium* species belonging to necrotroph fungi. These toxins inhibit the ceramide synthase, causing the increase of long chain bases, essential for the ceramide's formation. That increase conduct the cell towards PCD.

The lipid-based communication between the plant and fungus depicts a scenario where the pathogen hijack the plant lipid metabolism to adapt to plant defenses and re-shape the host cell "landscape" to growth pathogenically and eventually kill the plant (Jones and Dangl, 2006). This phenomenon occurs in the widely studied ear rot of maize caused by *A. flavus* or *F. verticillioides*. Notably, *F. verticillioides* overrides defenses of susceptible maize lines by using oxylipin effectors to hijack host oxylipins pathway; essentially shutting down the most effective defense against this pathogen that is JA-based cit "JA-deficient opr7opr8 double mutant displayed extreme susceptibility to *F. verticillioides*. Notoriously, JA orchestrate plant defenses mainly

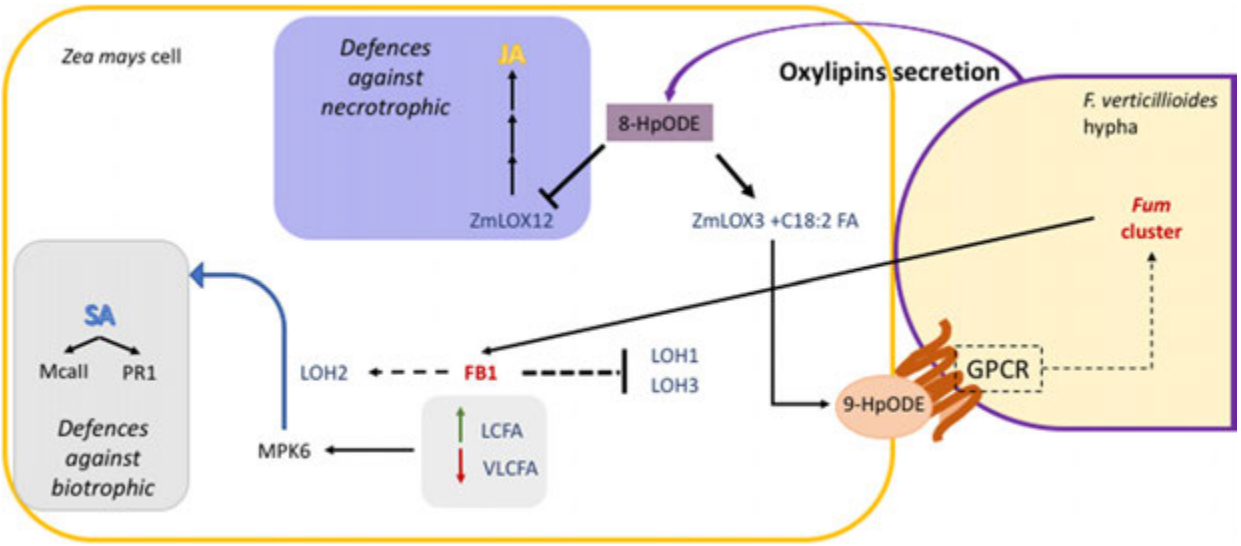

Fig. 2 *Fusarium verticillioides* produces 8-HpODE from LDS1. This fungal 8-oxylipin, likely by mimicking maize oxylipins, repress the JA-producing ZmLOX12 whilst activating the 9-oxylypin producing ZmLOX3. 9-oxylipins may induce FB synthesis. Thus, *F. verticillioides* infects maize and produces fumonisins (FBs). FBs disrupt sphingolipid biosynthesis in the host (*A. thaliana*) by inhibiting the enzyme ceramide synthase. In maize, this disruption increases the concentration of LCFA ceramides and decreases the concentration of VLCFA ceramides that, in turn, may activate Mitogen-activated Protein Kinase 6 (MPK6) a molecular controller of salicylic acid (SA) synthesis. SA turns on defense and programmed cell death (PCD) responses in the maize cell such as PR1 and McaII (type II metacaspase). This cell landscape may support the necrotrophic growth of *F. verticillioides* into maize tissues. Reproduced from Saucedo-García, M., Guevara-García, A., González-Solís, A., *et al.*, 2011. MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in Arabidopsis. New Phytologist 191, 943–957.

upon necrotrophic pathogen insults. After avoiding host defenses through 8-HpODE, *F. verticillioides* induces the host to produce 9-oxylipins (Mengiste, 2012).

It has been recently discussed the role of oxylipins as signal molecules: fungi – i.e., *Aspergillus* spp.- recognize host 9-H(P)ODE through a GPCR mediated cascade pathway (Niu *et al.*, 2020). Downstream this, the global regulon of secondary metabolism (i.e., by acting through LaeA) is switched on. In *F. verticillioides*, 9-H(P)ODE trigger the synthesis of fumonisins (Rispail *et al.*, 2009) that as stated above can – acting, differentially, on ceramide synthases (notably activating LOH2 and repressing LOH1–3) –re-shape the sphingolipids of maize kernels by enhancing S-LCFA and depressing S-VLCFA: these perturbations enhance SA biosynthesis that in conditions of JA-repression could lead to the PCD observed during necrotrophic colonization of host tissues (**Fig. 2**).

Fungi such as *Parastagonospora nodorum* use effectors such as SnToxA for eliciting PCD in wheat leaves which likely aid the switch from an epiphytic to endonecrotrophic growth. Here we suggest FBs as factors supporting *F. verticillioides* virulence allowing it to switch from an endophytic to a necrotrophic lifestyle. This probably is needed for infection of adult plant tissues (e.g., roots) and possibly for the movement of the fungus through the plant into kernels.

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Genome Evolution of Fungal Plant Pathogens

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Introduction to Filamentous Plant Pathogens

The importance of fungi is well acknowledged, since they play important ecological roles, as recycling carbon and mobilizing nitrogen, phosphorus and other bio-elements, support (endophytes, mycorrhizal fungi) or threaten (plant pathogens) plant life (Naranjo-Ortiz and Gabaldón, 2019). Besides the paramount importance of ecological features of fungi, the negative impact of many fungal species on cultivated plants has been recognized starting from the ancient centuries until recent period, when fungal epidemics have had significant social and economic impact. Fungal taxonomy has deeply evolved since the first classification of Linnaeus as belonging to the "Regnum Vegetabile". Phylogenetic relationships of the Kingdom Fungi can be inferred by means of several characters (fossils, comparative morphology, and biochemistry), although most modern phylogenetic trees (evolutionary trees, or cladograms) depend on molecular data coupled with these traditional forms of data. The main and most accepted features defining the Kingdom Fungi are represented by chitin as the major component of fungal walls; ergosterol in cell membranes; synthesis of lysine by a unique pathway and others reviewed in Webster and Weber (2007), Gould (2017). Based on these assumptions, recently the Oomycota are no more recognized as true fungi (Fry and Grünwald, 2010). Oomycetes grow filamentously and feed osmotrophically, so they look and behave like fungi and for these reasons have been thought to be part of the Kingdom Fungi. However, phylogenetic analysis has shown that they are part of the Chromista (Stramenopile) phylum, which includes a range of different forms such as diatoms and kelps. The class Oomycota encompasses a wide diversity of microbial forms, from free-living saprobes to parasites of plants and animals. The class contains some of the most important agricultural parasites of plants (like *Phytophthora*) that cause a range of pathologies, including blights, cankers, wilts, rusts, lesions, and rots.

The assumption that Fungal Kingdom is monophyletic (meaning that all modern fungi can be traced back to a single ancestral organism) has been recently debated; the synapomorphy that separates them from other groups sometimes is not accepted since new common characters are found in different phylogenetic taxa, and additional evidences are recently available after the release of whole genome sequences of many fungi (Richards *et al.*, 2017). As widely recognized, the Kingdom Fungi includes four-phylum (Ascomycota, Basidiomycota, Zygomycota, and Chytridiomycota). The first two are grouped into the monophyletic lineage Dikaria, while the latter were found to be polyphyletic (Tanabe *et al.*, 2005). Even if subject of recent revisions, the classification of fungi based on their pathogenic lifestyle and the way they feed on the host lifestyle is widely accepted (Doehlemann *et al.*, 2017). On one side are positioned the biotrophic, including obligate (rusts and powdery), and non-obligate (smuts and some Claviceps) that depend on their host for completion of their life cycle. To the other side, the necrotrophs are divided into true necrotrophic pathogens, which attack and kill healthy plants, and secondary necrotrophic-like pathogens, which are saprophytic in nature but may occasionally infect plants that have been previously weakened, e.g., by abiotic stresses or other pathogens (Agrios, 2009).

As pathogens of plants, fungi and oomycetes behave similarly and present the same problems so they are usually referred as filamentous plant pathogens.

There is no general agreement about what species are, and some currents of thoughts casts doubt on the existence of a species category in nature, at least as prokaryote are concerned, suggesting that the several proposed Species Concepts should be considered as species criteria and that species correspond to segments of evolutionary lineages that evolve independently from one another (de Queiroz, 2007; Giraud *et al.*, 2008; Ereshefsky, 2010). One of the most important character at the base of speciation in sexually reproducing fungi is the emergence of barriers to gene flow, i.e. mechanisms that prevent mating from spreading genes from one group of organisms into another, both in different geographic areas (i.e., "allopatric divergence") but also without geographic separation (i.e., "sympatric divergence"). In this case, fungi could mate only on their host plants or within their specialized substrate (Giraud *et al.*, 2008). In fungi lacking apparent sexual stage, other strategies are used in order to exchange genetic material (recombination, whether sexual, parasexual, or hybridization) (Taylor *et al.*, 1999; Stukenbrock, 2016; Drenth *et al.*, 2019).

The deep changes in an agricultural ecosystem (denser and genetically more uniform hosts, cultural practices, rapid exchanges of vegetable materials, climate changes), greatly affect the rate of evolutionary changes in fungal populations as compared to a natural ecosystem (McDonald and Stukenbrock, 2016). Knowledge of the reproductive biology of pathogens and of the mechanisms by which populations acquire genes and increase genetic diversity are of paramount importance in defining the genetic structure of a fungal population, intended as the amount and distribution of genetic variation within and among populations. These studies, based on the use of genetic "neutral" markers, allow for understand the role of the forces that shape the genetic structure of a population and, most interesting, consent to obtain insight into the future evolutionary potential of pathogen populations (McDonald and Linde, 2002). In recent years, population genomic studies based on whole genomic data of filamentous phytopathogenic fungi, through genotyping a very large number of loci or re-sequencing of many isolates, are gaining deeper insights into the biology, genetics, and evolution of plant pathogens, deciphering the adaptation or the divergence of closely related taxa, or the genetic basis of phenotypic variation and evolutionary change (Grünwald *et al.*, 2016; Stajich, 2017).

Table 1 Comparison of genome size and no. of genes in Ascomycota, Basidiomycota and Oomycota

Fungal division	*Average Genome size (Mb)*	*Average No. Of Genes*
Ascomycota	36.91	11,000
Basidiomycota	46.48	15,000
Oomycota	74.85	24,000
Mucoromycotina	38.77	13,000

Note: Mohanta, T.K., Bae, H., 2015. The diversity of fungal genome. Biol. Proced. Online 17, 8.

Large-Scale Genome Dynamics: Evolution of Size

Filamentous plant pathogen genomes vary greatly in their organization and composition. In fact, compared to the size of animal genomes, and in particular to plants, fungal genome size may be quite smaller (Tunlid and Talbot, 2002). The yeast *Saccharomyces cerevisiae* genome, has been the first fully sequenced eukaryotic genome in 1996 (Goffeau *et al.*, 1996), with a genome slightly larger than 12 Mb. Indeed, genome size of filamentous plant pathogens can vary by almost 3 orders of magnitude: sequenced genome range in size from ~2 mega-bases (Mb) in Microsporidia to 2 giga bases (Gb) in Pucciniales, with a median of 35 Mb (**Table 1**).

Interesting, the larger fungal known genome belongs to *Entomophaga aulicae;* the genome size is estimated to be as large as 8 Gb based on estimates using nuclear staining approaches, although genome sequencing has not yet been attempted (Stajich, 2017).

The variation in the size and contents of genome has been considered to emerge as a consequence of the differential degree of genetic drift and therefore the efficacy of selection, acting on genomes. Under this "mutation-hazard" (MH) model, species with small effective population size have larger genomes because they can tolerate the slightly deleterious accumulation of extra DNA in the form of transposable elements, multiple introns and gene duplication (Kelkar and Ochman, 2012). The negative association between genome size and population size seems to hold across a wide range of eukaryotes, including fungi (with some exception, Microsporidia). It has been hypothesized that some genome expansions observed in fungi result as an adaptation to their parasitic lifestyle, with obligate parasites, necessarily with low population size, having greatly expanded genomes. Exactly the opposite of what occur in bacteria, for which obligate symbiosis/pathogenicity leads to genome reduction.

Despite the causes that underline the process, mechanisms that bring to expansions or reduction of genetic repertoire can be very different, and can occur at different levels, i.e., modifications can occur at a genome-wide level or involve limited regions of the genome or even particular gene family or single genes. Genetic variation includes single nucleotide substitutions, insertion/deletions, or larger genetic changes like chromosomal rearrangements, that have low impact on genome size.

Genome Contraction

The reduction of whole genome is not a common event in fungi. Genome reduction has been observed in lower fungi, like in some obligate parasitic fungi of the genera Microsporidia, that have little genomes within the range of 2–6 Mb (Stajich, 2017). Gene family contraction and gene loss have contributed to the evolution of fungal genomes. Gene family contractions have been observed for example in the plant pathogen *Colletotrichum* (Glomerellales; Sordariomycetes) and are associated with host range contractions suggesting that host specificity may be a result of gene losses (Stajich, 2017). Also, in case of host jumps to distant species a subset of the effector repertoire could become obsolete, indeed in the genome of the smut fungus *Melanopsichium pennsylvanicum* that evolved from a grass pathogen to a dicot one, effector gene deletion appears to be rampant (Sharma *et al.*, 2014).

Genome Expansion

Genome expansion can occur for:

(1) Dispersal and increased number of transposable elements (TE);
(2) Duplication (Whole Genome Duplication, WGD);
(3) Acquisition of new genetic material via hybridization or Horizontal Gene Transfer (HGT).

Transposable elements (TEs) and effector evolution

The transposable elements (TE) are mobile genetic units able to move in a genome. In 2007 Wicker *et al.* (2007), proposed a unified system for the eukaryotic TEs classification: two main classes depending on their transposition intermediate, class I (retrotransposons), that transpose using RNA intermediate, with a "copy and paste" mechanism, and class II that transpose directly from DNA to DNA. The class I group is split into 5 orders based on their insertion mechanism: (1) LTRs (Long Terminal Repeats), involved in retrotranscription and integration into new genomic location; (2) LINEs (Long Interspersed Nuclear Elements) including genes for RNA packaging and retrotranscription; (3) SINEs (Short interspersed Nuclear Elements), non-autonomous elements, their transposition often depends on the LINEs; (4) DIRS (*Dictyostelium* Intermediate Repeat Sequence), whose

mechanism of replication is not yet well known; (5) PLEs (PENELOPE like elements), coding for reverse transcriptases and endonucleases. The class II group is divided in the Subclass I, to which belong transposons containing a transposase gene flanked by two Terminal Inverted Repeats (TIRs order) using a "cut-and-paste" mechanism, and the Subclass II, using other mechanisms of transposition, not well known yet (Helitrons and Maverick orders). TEs vary in number and in size, and comprise 3%–20% of the sequenced genome of most fungi. In the phytopathogen *Blumeria graminis*, 85% of the genome, estimated at 174 Mb, includes TEs (Parlange *et al.*, 2011). In the last years the massive genome sequencing of an increasing number of species together with comparative genomics and transcriptomic analysis have led to better understand the mobilome impact on gene expression and genome evolution. In 2019 a systematic, Kingdom wide study concerning mobile elements was carried out analyzing genomic location of TEs in 625 publicly available fungal genomes showing that animal-related and pathogenic fungi have more TEs inserted into genes than fungi with other lifestyles (Muszewska *et al.*, 2019). The recent genomic data of fungal plant pathogens indicate that some of them have drastically expanded genomes, mostly due to massive invasion of TEs. In phytopathogens, TEs represents the major contributor of genetic variability, through chromosomal rearrangements, altered gene expression, gene mutation, deletion and expansion. Thanks to the recent advances in third generation sequencing technology, allowing long-reads sequences and good genome assembly, it was possible to better understand the role of transposable elements in the fungal pathogen genome evolution. It is now well known that they contribute in the evolution of genes involved in plant/pathogen interaction (Seidl and Thomma, 2017). The continuous co-evolution of host and pathogen requires a strong genome plasticity enabling a fast adaptation potential. It has been observed that many TEs are located in genomic compartments enriched in repetitive sequences and transposable elements (Razali *et al.*, 2019; Raffaele and Kamoun, 2012; Seidl and Thomma, 2017) and they are often physically associated to effector genes of many plant pathogens. These observations led to the formulation of the two-speed genome theory according to which many filamentous plant pathogens have a bipartite genomes architecture (Dong *et al.*, 2015), which will be discussed later in this chapter. The physical association between TEs and effectors clearly shows that transposable elements contribute to microbial adaptation in response to plant by establishing genetic variability in effector-rich regions (Raffaele and Kamoun, 2012). A lot of studies support these findings. In *Magnaporthe grisea*-rice pathosystem, the *Avr-Pita* effector of the fungus, recognized by the *Pita* immune receptor in rice, shows a gene-for-gene relationship with the resistance genes in its host. This effector resides in a sub-telomeric region and is flanked by transposable elements. This location enables a fast diversification of the gene, by point mutations and genomic instability (deletion or insertion, gene translocation), mediated by TEs, allowing the fungus to overcome *Pita*-mediated resistance. The insertion of a transposable element (POT3) in the *Avr-Pita* promoter region results in gain of virulence of the pathogen (Kang *et al.*, 2001; Okagaki *et al.*, 2016).

Similar TE-mediated evolution of effector genes was observed in different well studied plant/fungi pathogenic interactions; in the wheat pathogen *Zymoseptoria tritici*, a population genomic analysis showed that an effector gene located close to a transposable element cluster was lost multiple times from the population, due to extensive chromosomal rearrangements driven by TEs, to avoid recognition by the plant immune receptor (Hartmann *et al.*, 2017). The genome analysis of the phytopathogenic ascomycete *Leptosphaeria maculans* "brassicae" (Lmb) has shown the presence adenine and thymine -rich blocks, (referred as AT isochors), enriched in TEs and effector genes: these particular regions are subject to repeat-induced point mutation (RIP), a fungal defense mechanism against transposable elements that actively induces point mutations in duplicated sequences, leading to high levels of AT (Rouxel *et al.*, 2011). The RIP mechanism can occasionally overrun the repeated region into adjacent effector genes, resulting in a rapid diversification of them. Comparative genomics between *Leptosphaeria* species have revealed a recent TE expansion in the genome of the pathotype *brassicae* that has promoted chromosomal rearrangements, resulting in the fungus speciation, and the active role of TEs to translocate effector genes within AT isochores of the gen*ome*, leading to a faster adaptation to selection pressure (Grandaubert *et al.*, 2014).

The evidence of avirulence effector genes in L. *maculans* in the plastic genomic regions represents an outstanding example of how diversification and evolution of effector genes in fungal plant pathogens allow a faster adaptation to host resistance genes. However, escaping resistance gene recognition could also have a cost in terms of loss of individual effectors, which is overcome by the presence of a large plethora of candidate effector genes, as revealed by the genome sequencing of more and more filamentous plant pathogens. The picture in the model phytopathogen L. *maculans* is complicated by the recent identification of some effector genes in L. *maculans* core genome, which were involved in systemic host colonization. Only the release of R genes in commercial plant varieties and the following population analysis will contribute the understanding of effector role in L. *maculans* (Rouxel and Balesdent, 2017).

The structural rearrangements of the genome mediated by TEs was deeply analyzed also in the soil-borne vascular wilt pathogen *Verticillium dahliae*: these chromosomal reshuffling establish highly dynamic lineage-specific (LS) genomic regions that act as a source for genetic variation (de Jonge *et al.*, 2013). Such LS regions are enriched in retrotransposons and other repetitive sequence elements, and *in planta*-expressed effector genes. A comparative genomic analysis between two *V. dahliae* strains has shown that TEs are enriched near rearrangement breakpoints and play a role in chromosomal rearrangements through homology-based recombination and erroneous double-strand repair (Faino *et al.*, 2016). Moreover, while the majority of TEs in *V. dahliae* is silent, the transcriptional activity of TEs, assessed by in vitro RNA-seq data analysis, was observed in LS regions. The relative age of TEs was estimated based on sequence divergence from a consensus sequence and a big amount of "younger" TEs resides in LS regions, whereas the majority of "older" TEs is localized in the core genome. This means that younger TEs, contrary to the older ones, tend to be transcriptionally active (Faino *et al.*, 2016).

The evolution potential mediated by TEs has been proposed also for mechanisms of recombination alternative to sexual reproduction, in the fungal pathogen *Pyrenochaeta lycopersici* (Dal Molin *et al.*, 2018): the genome assembly of this soilborne

pathogen, obtained by using long-read Single Molecule Real-Time sequencing technology (PacBio), allowed to read a fraction of repetitive sequences representing 30% of the total bases in which 15 TE super families were identified. The association of putative protein-coding genes with TEs was investigated, based on the physical association on the genomic sequence: 42 predicted heterokaryon incompatibility (HET) protein-coding genes were found in proximity of predicted TEs, suggesting the putative need for this fungus, which lost the ability of sexual reproduction, to increase the evolution of these genes which contribute to genetic recombination.

The control of the activity and the spread of TEs represents a key factor in the evolution of a fungal microbe. Only thanks to fine regulation mechanisms each species developed an evolutionary compromise in which the fitness deficit due to the deleterious effects of a strong TEs expansion (genome "obesity", deleterious genic and structural mutations) is counterbalanced by the selective advantage conferred by enhanced genome plasticity. Generally, the transposable element activity is regulated through epigenetic control mechanisms such as DNA methylation or histone modifications, targeted mutagenesis (RIP), and small RNA interference. The epigenetic regulation within a species population was reported to be variable and environmentally influenced; in particular environmental stresses have been associated to epigenetic de-repression of TEs (Fouché *et al.*, 2020). The plant pathogens represent an excellent model of study to explore TEs regulation dynamics as in the infection process the pathogen must face up a number of severe stresses; the expression of effectors is often regulated by de-repression of facultative heterochromatin (Soyer *et al.*, 2015; Soyer *et al.*, 2019). As discussed above, these regions of heterochromatin encoding effectors, overlap with TEs and consequently de-repression of TEs is associated with the expression of effectors.

Genome evolution in *Pyrenophora teres*, a necrotrophic pathogen of barley, is also featured by the insertion and expansion of TEs, the process is particularly evident in *P. teres f. teres* (PTT) which is characterized by a higher fraction of repetitive elements and a younger transposon mobility than *P. teres f. maculata* (PTM). Virulence of isolates is associated to fungal growth and different effector production and expression, about two hundred effectors were estimated to be produced by PTT isolates and most of the coding genes were localized in subtelomeric regions rich in repetitive elements which confer higher polymorphic trait development. Moreover, the fusion between chromosome 1 and 2 has been detected in one PTT isolate, this is supposed to be a recent event and was also detected in one *Pyrenophora tritici-repentis* isolate, leading to the hypothesis that chromosome fusion could be a common trait of Pyrenophora genus (Syme *et al.*, 2018; Wyatt *et al.*, 2020; Clare *et al.*, 2020).

In a recent transcriptomic study, TEs expression pathways were analyzed in four strains of the wheat pathogen *Zymoseptoria tritici* (Fouché *et al.*, 2020). After exposing the strains to nutrient starvation and host infection stress, surprisingly the two distinct conditions induced different expression profiles of TEs. In particular, the most highly expressed TEs, including TIR and Gypsy elements, show highly distinct de-repression across stress conditions. The genomic location of TEs was a major predictor of de-repression mechanisms during stress. Gene expression profiles under stress varied significantly depending on the proximity to the closest TEs: in other words, effector genes co-localized with TEs are subjected to the epigenetic upregulation during the period of infection.

Whole genome duplication (WGD) and polyploidy

Generally, duplication can favor evolution of new genes, allowing the original gene to maintain continuity in the gene product and in cell function, while providing a paralog that could "experiment" diverging in product and function (Kohn, 2005). Duplication can occur by unbalanced meiotic recombination and TE-mediated.

Analysis of filamentous plant pathogen genomes showed that different species undergo or have undergone to Whole Genome Duplication (WGD). A WGD event can occur as autopolyploidy by doubling the copy number of each chromosome (for example by an asymmetric meiosis) or as allopolyploidy by hybridization of two different species. (We will afford the former here and the latter later on in the chapter). WGD is usually followed by diploidization because polyploid organisms show a general instability with different meiotic defects such as abnormal chromosomal disjunction or atypical meiotic timing and topology (genome instability of polyploids can also be observed during mitosis) (Albertin and Marullo, 2012). Diploidization is the process by which a polyploid organism returns to a more stable diploid mode of chromosome pairing and can involve different mechanisms. Processes that contribute to diploidization include sub-functionalization and/or neo-functionalization causing differentiation between redundant gene copies and/or large-scale chromosomal rearrangements such as translocations, chromosome loss, telomere-to-telomere fusion between chromosomes.

Transposable elements (TEs) and other repeated sequences are traditionally involved in fungi post-polyploid evolution. Following a WGD event, ectopic recombination between TE elements or other repeated sequences can occur resulting in reciprocal translocations in chromosomes. Other well-known repeated sequences associated with genome restructuring in polyploids are the clusters of ribosomal DNA (rDNA) which are known to undergo to concerted evolution that is partial or complete homogenization of their rDNA. Genetic diversification in polyploids may involve smaller sequences and encompass limited duplication of single genes or gene families and/or gene loss. Hence, also in filamentous plant pathogens as in plants and animals, WGD is associated with long- or short-term structural, functional and phenotypical diversification.

From a functional viewpoint, one plus one does not equal two in polyploids. Many plant- and animal-duplicated genomes transgress the additivity hypothesis (predicting mid-parent relative expression), the fewer cases observed in fungi also display non-additive expression. Functional changes may be related to the structural diversification. Dosage compensation, a process by which genes duplicated by polyploidy or aneuploidy show diploid-like expression, has been observed in the functional evolution of *Saccharomyces pastorianus* (Albertin and Marullo, 2012). Moreover, epigenetic regulation of gene expression is possibly involved in fungi, as it has been observed for plants.

In conclusion, polyploidization triggers several structural and/or functional changes that are assumed to favor phenotypic diversification and thus facilitate further evolution and adaptation in filamentous plant pathogens.

It must be said that variations in genome content may also be associated with life cycle or cellular differentiation, this kind of phenomena are defined as somatic polyploidy (or endopolyploidy) rather than actual polyploidy. Several environmental factors have been shown to induce variation of genome content and chromosomal complement in various fungal species, such as heat shock, saline stress, fungicides treatments, or host–pathogen interactions.

Evidences of recent or ancient WGD events have been observed in Blastocladiomycota, Glomeromycota, Basidiomycota and also in the largest fungi phylum of Ascomycota, in which, beside the well-described *Saccharomyces* genus, there are also pathogenic species like *Botrytis* (reviewed in Albertin and Marullo, 2012).

In oomycetes, both autopolyploids and allopolyploids (see below) are known. For example, *Phytophthora megacarya* has an exceptionally large genome (222 Mbp) due to a recent expansion by whole-genome duplication (WGD) followed by diploidization (paleopolyploids). WGD and a dramatic transposable element associated expansion of a few gene families led to this exceptionally large genome and transcriptome and to the diversification of virulence-related genes including secreted RxLR effectors (characterized by the motif: Arg-X-Leu-Arg, where X is any amino acid). Overall, *P. megacarya* has an exceptional effector content of 1382 RXLR effectors (Morales-Cruz *et al.*, 2020).

Lateral DNA transfer and hybridization: Mechanisms of gene exchange between species

Gene exchange between species, through hybridization and Lateral DNA Transfer (LDT), represents another mechanism for fast evolution of filamentous plant pathogens. LDT includes Horizontal Gene Transfer (HGT) and Horizontal Chromosome Transfer (HCT). Actually, the broad occurrence of gene exchange observed among these organisms challenges our species concepts in the Kingdom Fungi and Oomycetes. Comparative genome studies have shown that fungal and oomycetes genomes can evolve very rapidly, evidencing high levels of genomic variation (distinct genome composition, sequence divergence, distinct chromosome organization). Genomic variation has in particular been studied among pathogenic species for which rapid evolution confers a main challenge for disease control.

Horizontal gene transfer (HGT) and horizontal chromosome transfer (HCT)

In fungi, as well as in bacteria, is well known the involvement of LDT in the genomic evolution process. Referring to pathogenic fungal species, HGT represents a fast and functional source of variability. By introducing exogenous genes, HGT gives selective advantages, such as the ability to become pathogenic to a specific host. HGT is defined as the stable transfer of genetic material between individuals, however, this definition is not that simple because is not due to a specific biological process (Doolittle, 1999). HGT excludes transfer through meiotic recombination or mitotic processes, such as parasexual recombination, which are known as vertical gene transfer (Rosewich and Kistler, 2000). In bacteria there are different specific biological processes as conjugation, transformation, or transduction involved in HGT (Davison, 1999).

An increasing number of genomic studies are highlighting and identifying the exogenous DNA sequences, distinguishing when the DNAs come from a more or less related species (Mehrabi *et al.*, 2011). Indeed, HGT may occurred recently in the evolutionary process of a species.

Despite the genomics era pointed up evidence for HGT occurring among prokaryotes and from prokaryotes to eukaryotes or vice versa, there are few occurrences of documented HGT among eukaryotes or even HGT of an entire cluster of genes (Khaldi *et al.*, 2008). In the last two decades, the number of published works on HGT in fungi has increased significantly (**Fig. 1**). Although there are little experimental evidences indicating HGT as inter or intra-species process, HGT was theorized and then demonstrated in a few examples.

In plant pathogenic fungi, the genes responsible for the synthesis of specific metabolic pathways, such as secondary metabolites involved in host/pathogen interactions or communication between organisms, are usually coordinately regulated and located together within gene clusters. HGT of metabolic pathway clusters increases the likelihood that the co-mobilized genes could give an adaptive advantage in certain ecological niches. These clusters can be maintained by positive selection in those environments (Rosewich and Kistler, 2000).

Many filamentous fungi genus, such as *Fusarium*, are capable to produce toxic secondary metabolites, also known as mycotoxins. One of the most studied family of mycotoxins is that of fumonisins, which can accumulate on kernels in field and during harvest and can be dangerous for animals or for human health when they are found on feed or food (Leslie and Summerell, 2006; Munkvold and Desjardins, 1997; Nelson *et al.*, 1991). Fumonisins are classified into more than 90 analogs divided in A, B, C and P-series (Proctor *et al.*, 2013). Although most of the known Fusaria species able to produce fumonisins, predominantly B fumonisin, are included in the *Fusarium fujikuroi* species complex (FFSC), there are some rare exceptions, such as a C fumonisins producing isolate of *Fusarium oxysporum*, a species closely related to FFSC (Seo *et al.*, 1996; Sewram *et al.*, 2005). Often, fumonisin biosynthetic genes (FUM) can be found in gene clusters. FUM is a linear polyketide-derived backbone type cluster organized in 17 contiguous genes in *Fusarium verticillioides* and *Fusarium proliferatum* genomes (Khaldi and Wolfe, 2011; Proctor *et al.*, 2013). The FUM cluster includes genes such as a polyketide synthase (PKS), a fatty acyl-CoA synthase (FAS) and cytochrome P450 monooxygenase (CYP) that contribute to the fumonisins biosynthesis, as well as two transport proteins, and is regulated by a Zn_2Cys6 DNA-binding protein transcription factor (Susca *et al.*, 2014). FUM genes are apparent duplicates of conserved genes in Sordariomycetes fungal class. Two scenarios could be involved in FUM cluster evolution in *F. verticillioides*: (1) the FUM cluster could be the result of horizontal cluster transfer into an ancestor of *F. verticillioides*, (2) the FUM cluster may have been recently assembled after gene duplication in an ancestor of *F. verticillioides* (Khaldi and Wolfe, 2011). FUM has been described in *F. proliferatum and F. verticillioides*, both species included in the FFSC, in *F. oxysporum* and in the different genus and distantly related

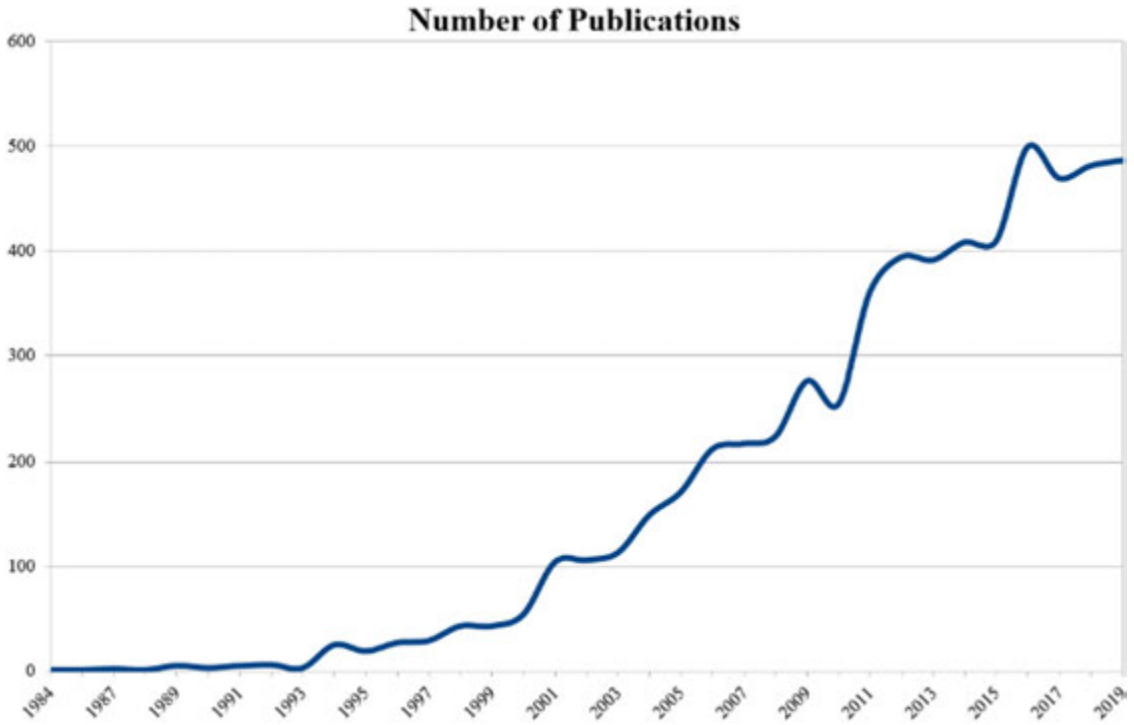

Fig. 1 Publications on Horizontal Gene Transfer in fungi. Number of publications per year, since 1984–2019. https://pubmed.ncbi.nlm.nih.gov/?term=hgt+fungi&sort=date&size=200.

fungus *Aspergillus niger* (Proctor *et al.*, 2013). The *F. oxysporum* strain O-1890 is a known C fumonisins producer, too. Moreover, genomic analyses of FUM cluster in *F. verticillioides*, *F. proliferatum* and *F. oxysporum* showed different genomic locations in the three species, though *F. oxysporum* has different FUM cluster flanking DNA sequences (Proctor *et al.*, 2008).

The homolog of the *Fusarium* FUM cluster present in the *A. niger* genome might represents an interesting example of putative HGT. The *A. niger* cluster includes 11 FUM homologs and also includes a short-chain dehydrogenase gene whose function has not been reported (Baker, 2006). The *A. niger* cluster lacks different FUM genes orthologues (FUM11, FUM12, FUM16, FUM17 and FUM18) and the FUM genes are different; moreover, in the *A. niger* cluster, there are at least three genes that lack in the FUM *Fusarium* cluster. However, no difference in fumonisins structural diversity was reported in *A. niger* fumonisins (Khaldi and Wolfe, 2011). Phylogenetic analysis, comparing different *Fusarium* species, suggests the occurrence of a putative HGT between *A. niger* and a *Fusarium* ancestor prior to the divergence of *F. verticillioides* and *F. oxysporum*. Moreover, the phylogenetic analysis of the FUM clusters, both in *Fusarium* and *Aspergillus*, showed that the HGT event probably occurred from Sordariomycetes to *A. niger*, or his ancestor, rather than the opposite (Khaldi and Wolfe, 2011).

Another very important example of HGT as inter species process, is that occurred between *Stagonospora nodorum* and *P. tritici-repentis*, reported by Friesen *et al.* (2006). They showed evidence of the transfer of a gene encoding a host-selective toxin, ToxA, the gene was transferred from *S. nodorum* to *P. tritici-repentis* conferring to this last pathogen the virulence on wheat and suggesting that this transfer occurred just before 1941. In fact, in that year *P. tritici-repentis* was reported as the cause of the first serious outbreak of leaf spotting on wheat in the USA (Friesen et al., 2006).

Finally, based on sequence homology analysis, in oomycetes some genes related to pathogenicity are hypothesized to have been acquired from donor genomes of fungi by HGT, suggesting a Kingdom-to-Kingdom transfer (Savory *et al.*, 2015).

Another type of horizontal transfer event is represented by the Horizontal Chromosome Transfer (HCT). HCT event assumes the transfer of a "supernumerary chromosome" (SC), a small accessory chromosome, generally less than 2 Mb, characterized by gene density lower than that of the 'core' chromosomes, none or at least few housekeeping genes and rich of repeated elements (Bertazzoni *et al.*, 2018). SCs are often meiotically unstable, i.e., they can be lost during meiotic division processes (Covert, 1998). It has been observed, in different *F. oxysporum* f. sp. *lycopersici* (FOL) isolates, that the size of the "core" chromosomes is well-preserved; nevertheless, the SCs genes content is variable. All FOL analyzed isolates show different profiles of presence/absence of genes encoding virulence effectors secreted in the host xylem (SIX), the presence of these genes can be used to predict host specificity (van Dam *et al.*, 2016; Williams *et al.*, 2016). It has also showed that these SCs may include 'lineage specific' regions (LS) or sequences typical of a single species.

In 2010, Ma and colleagues demonstrated the involvement of HCT by the transfer of pathogenicity chromosomes in *Fusarium*. The mobilization of SCs can transfer an entire set of genes leading to host compatibility to a non-pathogenic strain. Small proteins, secreted by FOL during the interaction with tomato, such as Six1 (Avr3) and Six3 (Avr2), are involved in virulence. These proteins

are coded by genes located on chromosome 14, one of the FOL LS chromosomes. Moreover, genome analysis revealed at least other three small in planta-secreted proteins on chromosome 14 (SIX5, SIX6 and SIX7). These genes are all conserved in pathogenic strains of tomato, but are not present in non-pathogenic strains. Ma and colleagues demonstrated that LS chromosome 14 was responsible for FOL pathogenicity and that HCT of this chromosome between strains explains its presence in several clonal polyphyletic lineages within the *F. oxysporum species complex*, but the absence in other lineages (Ma *et al.*, 2010).

HCT is also known to be involved in the evolutionary process of *Alternaria alternata* pathotypes; indeed, specific toxin coding genes reside on LS chromosomes. Protoplast fusions of two pathotypes yield LS chromosomes from both parental hybrids, supporting the HCT hypothesis (Akagi *et al.*, 2009).

Hybridization through sexual mating and by vegetative fusion of fungal hyphae

Hybridization can result in the formation of new species that occupy new ecological niche, or the hybrid can be a transient stage that can stabilize in different way, for example by backcrosses with the parental species, leaving only fragments of introgressed sequences in the genome (Feurtey and Stukenbrock, 2018). Hybridization in filamentous plant pathogens can occur by sexual mating or by fusion of vegetative cells of individuals belonging to different species.

Sexual mating between heterospecific individuals is prevented by reproductive barriers, but it can occasionally occur and give rise to hybrid individuals. Across all fungal phyla, hybridization is likely the most direct route to sympatric speciation, although there are as yet only a few examples of hybridization adequately supported by evidence. It is more common the emergence of a new lineage by hybridization of different host-specialized lineages or *formae speciales* (f.s.) within the same species, like what it was observed in the cereal pathogen *Blumeria graminis* comprising different f.s. on rye, barley and wheat. Recently, a new powdery mildew lineage emerged on the hybrid crop species triticale (x *Triticosecale*). *Blumeria graminis* f. sp. *triticale* comprises a mosaic of segments with high sequence similarity to either the rye or the wheat pathogen, *B. graminis* f.sp. *secalis* and *B. graminis* f.sp. *tritici* respectively. Detailed analyses of the polymorphism pattern between the three *formae speciales* revealed a very recent origin of *B. graminis* f. sp. *triticale* by a hybridization event of *B. graminis* f. sp. *secalis* and *B. graminis* f. sp. *tritici* followed by a few backcrosses to *B. graminis* f. sp. *tritici* (Feurtey and Stukenbrock, 2018).

Recent allopolyploid hybrids have, however, been identified in diverse genera of non-plant pathogens like several *Neotyphodium* species, symbiotic endophytes of grasses, and several *Saccharomyces* species empirically selected for brewing; but also plant pathogen like *Botrytis allii* the agent of gray mold neck rot of onion and garlic (reviewed in Giraud *et al.*, 2008).

Some hybrids have the ploidy level identical to that of their parents, they are referred to as homoploids, and they are characterized by a broad heterozygosity and by the fact that they are not reproductively isolated from their parents. A well-described case of homoploid speciation is that of the rust *Melampsora columbiana* that emerged from hybridization of *M. medusa*, parasite of *Populus deltoides*, and *M. occidentalis*, parasite of *P. trichocarpa* This hybrid emerged in 1997 when a poplar hybrid resistant to the two parental rust species was widely grown in California, the hybrid rust being able to infect the hybrid poplar. In this case, the homoploid hybrid clearly had a novel ecological niche, a new host (Newcombe *et al.*, 2000).

Unlike fungi, oomycetes are diploid and over the past decade it has become apparent that intraspecific hybridization is a common occurrence in this class. Actually, interspecific hybridizations are increasingly recognized as a driving evolutionary force in the genus *Phytophthora* facilitating adaptation to new environments and expansion of host ranges or host jumps due to accelerated pathogen evolution.

To date, an average of 140 Phytophthora species have been described, but the true global diversity of the genus *Phytophthora* has been estimated as 400 $\pm$ 200 extant species (Brasier, 2007). This is probably due to the huge genome plasticity that allow generation of diversity within this genus. First source of variability of course is sexual recombination: oomycetes can be heterothallic, in which reproduction can occur only between two different but compatible individuals or homothallic, which are capable of sexual reproduction from a single organism. Other sources of variability in oomycetes are based on transposons, gene duplication, asymmetric recombination, horizontal transfer and, in particular, hybridization and polyploidy (common in the genus *Phytophthora*). The lack of heterozygous nucleotides in their maternally inherited mitochondrial genes, a feature common to all known *Phytophthora* hybrids, strongly suggests that they originated from sexual hybridization rather than somatic fusion. Many of these hybrids exhibit polyploidy. The progeny of a single hybridization event can follow different evolutionary paths, in fact hybrid isolates from the same event can show large variations in DNA content indicating different levels of ploidy: polyploid (probably tetraploid) isolates can be present together with diploid isolates or diploid/polyploid heterokaryons. It is now clear that these hybridization events and polyploidy play an important role in host adaptation and speciation of Phytophthora pathogens. For example, the host-specific pathogen *P.* $\times$ *alni* is the product of multiple interspecific hybridization events between unknown parental species that formed *P.* $\times$ *multiformis* which then hybridized with *P. uniformis*. *Phytophthora* $\times$ *alni* is significantly more widespread and aggressive to *Alnus* spp. than its parents, causing epidemic mortality of *Alnus* trees across Europe (Ioos *et al.* 2006). *Phytophthora* $\times$ *alni* and *P.* $\times$ *multiformis* are recent hybrids in a nascent state, characterized by unusually high developmental instabilities and oospore abortion rates. In contrast, the functional heterothallic breeding system and lack of developmental instabilities suggest that *P.* $\times$ *cambivora* already went through the process of stabilization and homogenization and, hence, is not of recent origin. This conclusion is also supported by the fact that the original description as *Blepharospora cambivora* was published one century ago. This means that some hybrids could represent transient hybrid clones but the ones that demonstrated higher fitness could become stable evolutionary lineages.

Most of these new hybrid taxa are apparently a by-product of globalization, with human activities associated with nurseries, ornamental gardens, horticulture and agriculture. These are environments where infected germplasm and other material (such as

soil) from disparate origins are bought together, providing a natural laboratory for the formation and selection of hybrids. Also, natural ecosystems, like tropical ecosystems of Southeast Asia, characterized by the ecological diversity and high degree of plant species endemism have been shown to favor hybridization events.

Despite vegetative incompatibility mechanisms in all filamentous plant pathogens, fusion of hyphae of heterospecific individuals can occasionally occur. Interspecific vegetative fusion can give rise to new lineages that contain the entire genomes of both parental species. This is the case of the ascomycete fungus *Verticillium longisporum* which is diploid but arose from hybridization of haploid ancestors. Population genetic data provide evidence that *V. longisporum* is the product of more than one hybridization event between closely related *Verticillium* species. *Verticillium longisporum* predominantly propagates by asexual reproduction, and hyphal fusion followed by karyogamy is therefore the most plausible hybridization scenario (Feurtey and Stukenbrock, 2018). *Fusarium oxysporum* is another example of the adaptive advantage of hyphal fusion followed by the exchange of genetic material in asexual species. As mentioned above, in this species genomic variability can be achieved by lateral DNA transfer.

Rapid Evolution of Virulence Associated Genes: One, Two, Multi-Speed Genomes

The "two-speed genome" model describes a genome architecture typical of filamentous plant pathogens in which genomes have a bipartite structure, with gene sparse compartments enriched in repetitive sequences and transposable elements showing higher evolutionary rates compared to more conserved gene-dense regions ("core" genome) enriched for housekeeping genes. These repeat-rich regions contain large repertoire of genes coding for effectors involved in virulence and serve as a cradle for adaptive evolution (Dong *et al.*, 2015).

Remarkably, this unusual genome architecture has evolved in independent phylogenetic lineages of filamentous plant pathogens, fungi and oomycetes belonging to different Kingdoms. The distinctive two-speed genome architecture, with the effector genes populating the more rapidly evolving sections of the genome, is associated to the rapid evolutionary *tempo* of effector genes and gives to the lineages that possess it a clear advantage in the antagonistic co-evolutionary conflict with plants. The evolutionary success of filamentous plant pathogens is due to the ability to rapidly overcome plant immunity, adapt to resistant varieties and occasionally jump to new host species. A more adaptable genome prevents them to succumb as their hosts develop full resistance or become extinct.

Mechanisms of mutations

The repeat-rich, gene poor genome compartments show higher evolutionary rates because they represent hot spots for duplication, deletion and symmetric and/or asymmetric recombination; moreover, high transposase activity is observed, altogether resulting in accelerated evolution through increased structural variation. However, also other mechanisms are involved. Beside normal mutations, in some ascomycete fungi, close proximity of effector genes to degenerated transposons can result in increased levels of local mutagenesis through RIP mutations. (No RIP-like mechanism has been demonstrated in Phytophthora species or other oomycetes). Moreover, epigenetic silencing can also affect effector genes, but it still to be shown that these genome compartments accelerated this phenomenon. Proximity to transposons may also facilitate HGT. Of course, shifting or jumping from host to another, is expected to represent a powerful selective pressure on these genetic variants. The change of plant cellular environments dramatically impacts effector evolution, likely the accumulation of mutations can improve or expand effector activity (Dong *et al.*, 2015).

The genome of the oomycete *Phytophthora infestans*, the potato late blight pathogen, and its close relatives *Phytophthora*, represent the prototypes of this two-speed genome compartmentalization. In these pathogens, effector genes can be readily annotated in genome sequences because they contain signal peptides followed by conserved motifs. This is the case of the family effector gene called Crinklers (CRN) or RXLR in *Phytophthora* and downy mildews. In these genomes fast-evolving genes coding for these effectors mainly reside in gene-sparse regions.

However, with more filamentous phytopathogen genomes being sequenced, it is becoming clear that there are rapidly evolving phytopathogens that lack either some or all of the aforementioned characteristics, indicating that there are more modes of genomic organization to obtain different speeds in evolution. In this context, Frantzeskakis *et al.* (2019) recently proposed a one-speed (one-compartment) and a multi-speed (multi-compartment) genome organization, as alternative to the two-speed genome model. In one-speed genome rapid evolution of effector genes is not constraint to certain compartments: these genomes are characterized by over-proliferation of TEs (due to the loss of RIP), equal distribution of TEs and absence of genomic compartmentalization. In these genomes, rapid evolution is supposed to be enhanced by extensive copy-number variation and/or heterozygosity. On the other side, genomes containing accessory chromosomes carrying rapidly evolving effector genes, typical of *F. oxysporum* as an example, could be described as multi-speed genomes, since they seem to possess three speed levels including core, pseudo-core (core chromosomes displaying presence/absence polymorphism) and lineage-specific chromosomes.

Beside further definitions, filamentous plant pathogen genomes surely need rapid evolution of virulence-associated genes to successfully cope in the arm race with the plant and they do not put limit to the different ways to obtain it.

Introduction of Alien Pests in New Environments: *Phytophthora Ramorum*, as Example of Genetic Diversification in Asexually Propagated Filamentous Pathogens

Alien species are often successful invaders in new environments, despite the introduction of a few isolates with a reduced genetic pool. This is called the "genetic paradox" of invasion.

Small populations or clones can reproduce clonally with the advantage to cause a rapid increase and dispersal of a population in a temporal/geographic sense (epidemic), frequently accompanied by the elimination of sexual reproduction due to the absence of one mating type. However, in this way pathogen populations loose genetic diversity and without adaptability and plasticity enabled through genetic recombination invaders will not be able to cope with long-term change in host resistance or environmental changes, and epidemic pathogens will succumb. Successful invasive alien pathogens show instead high genotypic diversity that increase their potential for adaptation, explain their success in invasion and long-term persistence. This genotypic diversity is due to a parasexual life-cycle characterized by mitotic recombination.

Phytophthora ramorum has a wide host range and infects over 100 species of plants, both wild and ornamental, causing leaf lesions and/or branch dieback. It is an invasive pathogen that, despite being limited to asexual reproduction and having a small effective population size, is spreading extensively in Europe and North America, causing heavy mortality on ecologically and economically valuable forest trees such as larch and oak species.

The pathogen comprises four divergent clonal lineages, (NA1, NA2, EU1, EU2) that have spread in the United States, Europe, and Canada. The lineages differ in morphology and aggressiveness and, despite their presumed clonality, exhibit considerable intra-lineage phenotypic (genetic) variation.

Long term studies (reviewed in Dale *et al.*, 2019) have shown that asexual lineages of *P. ramorum* can evolve by mitotic recombination (MR) and rapidly evolving non-core genome. The latter, characterized by rapid evolution of effectors genes in gene-sparse regions enriched in repetitive sequences and transposable elements (TEs), has been already discussed in the chapter on two-speed genomes. MR is one mechanism that can generate genotypic diversity, uncovers beneficial mutations, and increases the potential for adaptation. It is caused by crossing over during mitosis at specific chromosomal breakpoints along the genomes of these *P. ramorum* lineages. All sites after the breakpoint were converted to homozygosity for either the reference or alternate allele relative to the reference genome, resulting in extensive runs of homozygosity (ROH, also known as loss of heterozygosity [LOH]) regions, covering large portions of or entire scaffolds. Conversion of genomic regions to homozygosity may result in the expression of new or recessive alleles and can improve the efficiency of selection on both beneficial and deleterious alleles (Dale *et al.*, 2019). TEs and low gene density are associated with mitotic recombination. The prevalence of MR in *Phytophthora* species suggests it is a salient characteristic of the *Phytophthora* genetic system. MR may therefore be important in enhancing the adaptability of introduced asexual *Phytophthora* species and contribute, along with a rapidly evolving non-core genome and a strong selective pressure, to the initial invasion "success" but also in the long-term adaptation to environmental changes, such as loss of susceptible hosts and the emergence of parasites or competitors.

Conclusions

Evolution of virulence and adaptation in filamentous phytopathogens are ruled by genome plasticity and include different mechanisms: gene duplication, deletions, copy number variation, heterozygosity, loss or acquisition of dispensable chromosomes, chromatin rearrangements. The increasing availability of complete and high-quality genomes, will contribute to understand the frequency of each event of gene evolution, the origin of the genomic architectures observed and whether they are the results of selection pressure or due to chance of genetic drift. The complete genomic datasets available for numerous filamentous pathogens also indicate that closely related species can follow different genomic evolution pathways. This is the case of *F. oxysporum*, where the accessory and pseudo-core chromosomes provide potentially additional genome plasticity, while in *F. graminearum* have not been found dispensable chromosomes, and a small fraction of TEs is present. Another example comes from *Ustilaginales*, where in some species effector genes are clustered in the proximity of TEs, while in other species TE insertion sites are not correlated with effector clusters.

The main tool for increasing the level of genotypic diversity in fungal pathogens is represented by sexual recombination and can influence the virulence spectrum. Knowledge of the mating system of a species is important to be able to differentiate locus-specific effects from genome-wide effects. Several mating-type genes have been cloned from ascomycete fungi and they have proven useful in distinguishing the mating types. Knowledge on the virulence spectrum of a pathogen population and recognition of the risks of changes in the virulence spectrum are essential when breeding crops for disease resistance.

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Further Readings

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Mycoviruses: A Hidden World Within Fungi

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Introduction

There are numerous examples of plant, animal and bacterial viruses that cause severe symptoms of disease (up to death of the host), with considerable socio-economic impacts (Martinelle *et al.*, 2014; Esposito *et al.*, 2005; Vurro *et al.*, 2010). Viruses of fungi, also known as mycoviruses, were described for the first time during 1960s, as pathogens of cultivated mushrooms (Hollings, 1962). Despite their description as disease agents, they often infect their hosts without causing obvious signs of disease and for this reason they are often described as cryptic viruses (Pearson *et al.*, 2009). Like viruses that infect plants, insects and other animals, mycoviruses require the living cells of fungi to replicate (Ikeda *et al.*, 2013). They share some features with other viruses but they also show unique characteristics. Almost all the known mycoviruses lacks an extracellular phase: they are mainly transmitted by hyphal fusion, sexual reproduction or by other mechanisms that require an exchange of cytosolic matter (Chu *et al.*, 2004). Even though, it seems that the restriction of finding a compatible mating isolate for hyphal fusion, and hence virus transmission, occur only during in vitro experiment: in nature the host range of mycoviruses seems to be wider than what expected. For example a virus isolated from *Botrytis porri* was then also found in *B. squamosa* and in *Sclerotinia sclerotiorum* which shared the same ecological niche (Wu *et al.*, 2012). Another similar example was reported for the white rot fungus *Rosellinia necatrix*: in natural environment two incompatible strains were able to exchange viruses between them and also with other fungal species (Yaegashi *et al.*, 2013). The explanation for this phenomenon come from the fact that mycoviruses, as all the other viruses, display a peculiar feature: differently from eukaryotes and prokaryotes which are represented by a single consensus genome sequence, viral genomes are represented by a cloud of closely related mutants termed "viral quasispecies" (Domingo *et al.*, 2012). More in detail, this feature seems to represent an evolutionary advantage, giving to genome variants more chances to adapt to a new environment (which is represented by a new cellular host). The only mycovirus for which an extracellular life-cycle was demonstrated is represented by a DNA mycoviruses which infect *S. sclerotiorum* (Yu *et al.*, 2010). Interestingly, such viruses demonstrated to be infectious when applied extracellularly as purified virus particles. Moreover, the same virus demonstrated also the ability to be transmitted by insects (Liu *et al.*, 2016). In specific, when the mycophagous insect *Lycoriella ingenua* was left feeding on *S. sclerotiorum* infected colonies, it acquired the virus and was then able to infect virus free fungal colonies. In consequence, it seems that transmission in natural environments is more feasible and easily achieved than in axenic condition, also between different species.

Distribution and Phylogeny

Holobiomes is the microbial communities associated with plants or animals, including bacteria, fungi and viruses, that are increasingly recognized as crucial factors influencing host health and fitness (Zilber-Rosenberg and Rosenberg, 2008). Mycoviruses are part of the natural holobiomes and are widespread in all the major taxonomic groups of fungi including pathogenic, saprotrophic and endophytic fungi. For example, one of the most famous case in which a mycovirus play a crucial role for the holobiont adaptation is represented by *Curvularia thermal tolerance virus* (CThTV) (Marquez *et al.*, 2007). In specific, the Marquez and coauthors demonstrated that a mycovirus infecting an endophytic fungus was the keystone which allowed the exploitation of heated soils (up to 65°C) by the tropical panic grass *Dichanthelium lanuginosum*. When they "cured" the fungus, obtaining an isogenic virus-free isolate, its interaction with the plant was unable to induce thermal tolerance. This ground breaking work demonstrated that mycoviruses can play crucial role, providing fundamental ecological services in natural environment. Thanks to such result a new interest for mycoviruses risen up and spurred researchers to look for new mycoviral species in many different environments leading to the description of a number of new viral species. For example, the fungal community inhabiting marine plants (Nerva *et al.*, 2016) and animals (Nerva *et al.*, 2019b) displayed to harbor viruses, as well as endophytic (Marzano and Domier, 2016; Nerva *et al.*, 2019d), pathogenic (Marzano *et al.*, 2016; Donaire *et al.*, 2016; Botella *et al.*, 2015) and symbiotic fungi (e.g., mycorrhizal fungi and fungi associated to lichens) (Neupane *et al.*, 2018; Turina *et al.*, 2018; Petrzik *et al.*, 2019). Thanks to all these works the evolutionary relationship between viral families are now clearer.

The Nobel laureate David Baltimore, in 1971 proposed the modern classification of viruses (Baltimore, 1971): viruses are classified into 7 fundamentally different groups depending on the nature of genome encapsidated into their virions. The central theme of Baltimore system for virus classification is that all viruses must synthesize messenger RNAs (mRNAs) from their genomes, in order to produce proteins and replicate themselves. Despite the presence of 7 distinct groups of viruses, since few years ago the majority of reported mycoviruses displayed a double-stranded RNA (dsRNA) genome, with few reports of positive single-stranded RNA genomes (+ssRNA). This fact was mainly due to the method used, up to that time, for virus discovery, where the crucial step was the enrichment of dsRNA fraction and then the identification through sequencing (Pearson *et al.*, 2009). For these reasons, Next Generation Sequencing (NGS) applied on total RNA extracted from fungi, or directly from complex matrixes (Al Rwahnih *et al.*, 2011; Marzano and Domier, 2016),

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led to the discovery of new classes of mycoviruses. To date, known mycoviruses belong to dsRNA clade, + ssRNA clade, negative single-stranded RNA (-ssRNA) clade and positive circular single-stranded DNA (ssDNA) clade. The latter is represented by *Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1* (SsHADV-1), a member of circular Rep-encoding ssDNA viruses (CRESS DNA) (Zhao *et al.*, 2019), which led to the constitution of a new family named *Genomoviridae* (Krupovic *et al.*, 2016). The genome structure of this family is quite similar to that of *Geminiviradae* (Zerbini *et al.*, 2017), a well-known group of plant viruses, lacking the movement protein indispensable in plant viruses for spreading in their hosts (Deom *et al.*, 1992; Heinlein, 2015).

As previously mentioned, thanks to advent of NGS techniques a number of new viruses were described in both the + ssRNA and the –ssRNA clades. In specific, in the + ssRNA clade there are many viruses belonging to the Tymovirales order which led to the constitution of two new viral families: *Gammaflexiviridae* (Adams *et al.*, 2012) and *Deltaflexiviridae* (Li *et al.*, 2016). In addition to the Tymovirales, many other mycoviruses belonging to the *Virgaviridae* (Adams *et al.*, 2009), *Tombusviridae* (Sit and Lommel, 2001) and other with no obvious phylogenetic placement (Nerva *et al.*, 2016, 2019b; Zhang *et al.*, 2019; Cañizares *et al.*, 2018) were described. Among mycoviral families with a + ssRNA genome, it is worth noting the family *Hypoviridae*. This group of viruses is able to induce a wide fitness reprogramming of the fungal host which lead also to phenotypic and behavioral changes (Rigling *et al.*, 1989; Hillman *et al.*, 1990; Larson *et al.*, 1992; Craven *et al.*, 1993; Zhang *et al.*, 1993; Rigling and Prospero, 2017; Turina and Rostagno, 2007). Finally, the most abundant viruses with + ssRNA genome belongs to *Mitoviridae* and *Narnaviridae* families. These two families host viruses with a very simple genome, which only encode for the RNA-dependent RNA polymerase (RdRP) involved in genome replication. The difference between these two families is that *Mitovirus* species replicate in the mitochondria, as suggested by the name, whereas *Narnavirus* species replicate in the cytosol. A further hint of mitochondrial replication is that in *Mitovirus* genomes tryptophan (Trp or W) is often encoded by the UGA codon (Kitahara *et al.*, 2014). It is worth to note that, in cytosolic ribosomes, UGA is the former codon for translation termination and hence tryptophan is encoded by UGG codon. Further interesting evolutionary theories are also related to these two families as largely discussed in the next paragraph.

Fungal viruses with negative single-stranded genomes are, among mycoviruses, the last discovered group. The first indirect evidence for the presence of this group of viruses was provided in 2013 using a bioinformatics approach (Kondo *et al.*, 2013) on accessible RNAseq experiments in public databases. Then, the following year, it was reported the biological characterization of the first –ssRNA virus replicating in *Sclerotinia sclerotiorum* (Liu *et al.*, 2014). From that moment a number of other viruses belonging to this group were reported, with new member inserted in the Monengavirales, Bunyavirales and Serpentovirales orders (Nerva *et al.*, 2019c). Interestingly, only few examples of multipartite genomes were provided for this group of viruses (Nerva *et al.*, 2019c; Lin *et al.*, 2019; Velasco *et al.*, 2019) whereas for most of them only the RdRP was identified. This last evidence is probably due to the fact that sequences encoding for the nucleocapsid (Nc) and the non-structural protein (Ns1) are not conserved with that of plant and animal viruses, reducing the ability of researchers to identify them through bioinformatics approaches.

Finally, among the dsRNA clade there are many families hosting mycoviruses, whit a variegated genome organization and for which virus particles have been often characterized (**Fig. 1**). For example, the *Totiviridae* family hosts many mycoviruses with a unique genome segment, encoding for two proteins: the RdRP and the coat protein (CP) which build the virus particle (Wickner *et al.*, 2012). This family host a number of viruses infecting not only fungi, but also insects, animals and plants. In addition, one of the most interesting discoveries over the last few years concerns the interaction between a *Totivirus* species and an unrelated + ssRNA mycovirus (Zhang *et al.*, 2016). It has been demonstrated that, a mycovirus belonging to the dsRNA clade is able to share both the capsid protein and the RdRP with an unrelated mycovirus which infect the same fungal strain (Zhang *et al.*, 2016). This research demonstrated that also viruses can be parasitized, or at least that can share indispensable molecular machinery to achieve the host infection and spread into the environment. In addition to the monopartite group, there are also mycoviruses with bipartite genomes. The above mentioned *Curvularia thermal tolerance virus* belongs to a still undefined group of viruses which shows two genome segments. In detail, the latter viruses display an RNA 1 encoding for the RdRP and an RNA 2 which encodes for two proteins: the capsid and a protein with a still undefined function (Marquez *et al.*, 2007; Nerva *et al.*, 2016). A second independent group of bipartite viruses is the family *Partitiviridae*. Also for this group of viruses RNA 1 encodes for the RdRP while the RNA 2 encodes only for the capsid protein (Nibert *et al.*, 2014). In addition, some partitiviruses have also a third genome segment which encodes for a protein with an undefined function that is not indispensable for virus replication (Deng *et al.*, 2017; Jiang *et al.*, 2019; Kotta-Loizou and Coutts, 2017a). Finally, another important viral family hosting mycoviruses is the *Chrysoviridae* family (Ghabrial and Castón, 2011). This group of viruses have a quadripartite genome structure, with the RNA1 encoding for the RdRP, the RNA2 encoding for the coat protein while RNA 3 and RNA 4 encode for two protein with unknown functions (Jiang and Ghabrial, 2004).

It is conceivable that in the next years, thanks to the wide distribution of NGS techniques, the number of viral families hosting mycoviruses will increase considerably.

Evolutionary Theories

Up to few years ago, the most accepted theory about mycoviral evolution was the long coevolution theory. The latter proposed that, due to their intracellular mode of transmission and the ability to infect only one or few hosts, over the years mycoviruses coevolved together with their hosts without interaction between them and also with other class of viruses (e.g., plant and animal viruses). Later on, thanks to the NGS techniques and new experimental evidences, this theory has been completely revised.

One of the observations that brought to a new theory is that many viral families (e.g., *Totiviridae*, *Partitiviridae* and *Chrysoviridae*) host species infecting both fungi and plants (Nerva *et al.*, 2017b; Nibert *et al.*, 2014). Among these families, there are species able

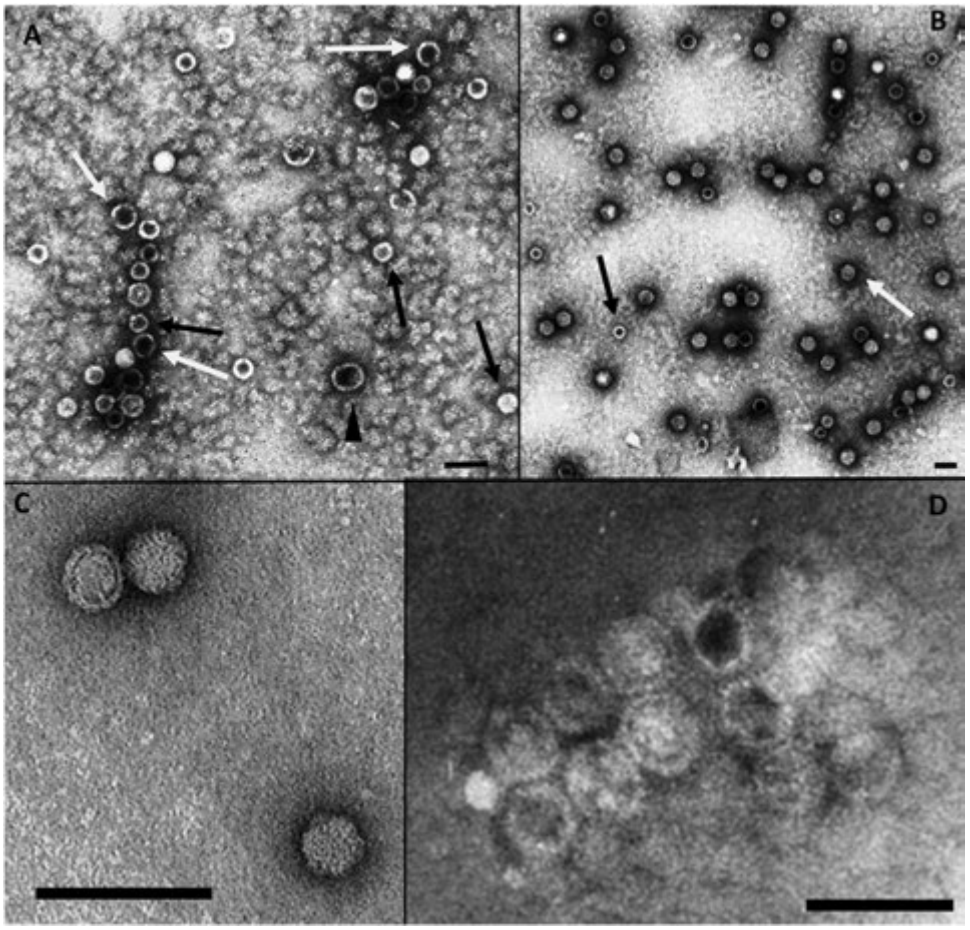

Fig. 1 Examples of virus particles from partially purified fungal extracts photographed at Transmission Electron Microscope (TEM). (A) Virus particles from a *Penicillium aurantiogriseum* isolate carrying multiple viral infection. At least three morphologies are visible. Particles of 32–35 nm in diameter, rounded in profile, either penetrated or not by the stain (black arrows). Particles of 39–42 nm in diameter are hexagonal in profile (white arrows). Particles of about 50 nm diameter, rounded in profile (arrowhead). (B) Virus particles from a *Penicillium* spp. carrying a multiple infection. Two distinct type of particles were observed. Particles with 29–32 nm in diameter, rounded in profile and always penetrated by stain (Black arrow). Particles with 40–44 nm in diameter and never penetrated by stain (white arrow). (C) Virus particles from an isolate of *Aspergillus flavus* infected by a partitivirus. (D) Virus particle from an isolate of *Penicillium* spp. infected by a chrysovirus. Bars in each panel represent 50 nm. Credits: Dr. Marta Vallino, IPSP-CNR (Italy).

to infect plants and other able to infect fungi, but no one is able to infect both plants and fungi (Roossinck, 2015). A further link between plant and fungal viruses was proposed for the first time for the *Hypoviridae* family: analysis of protein sequences revealed that they share a common ancestor with the plant-infecting Potyviruses (Koonin *et al.*, 1991). Moreover, many other families showed a mosaic of hosts or peculiar phylogenetic origins which further suggest that exchanges of viruses between plants and fungi can occur (Donaire *et al.*, 2016; Rastgou *et al.*, 2009; Turina *et al.*, 2017; Plyusnin *et al.*, 2012; Attoui *et al.*, 2012). In consequence to these works, to date, the most supported theory suggest that, (1) thanks to the *quasispecies* nature of viruses (Domingo *et al.*, 2012; Roossinck, 1997) and to (2) the close physical relationship with other organisms (e.g., fungal-plant interface), viruses can easily move from the original host to new distant and taxonomically unrelated hosts. This latter hypothesis was demonstrated for both plant and fungal viruses, which are able to replicate in fungi (Janda and Ahlquist, 1993; Panavas and Nagy, 2003; Mascia *et al.*, 2019), and for mycoviruses which are able to replicate in plants cells (Nerva *et al.*, 2017b). In addition, to further support this hypothesis a clear example of viral cross-species transmission between different taxa was proposed for viruses of several plants and arthropods (Shi *et al.*, 2016).

Another interesting case of mycoviral evolution is linked to the mitoviruses group. Intriguingly, species belonging to *Mitovirus* genus are closely related to the *Leviviridae* family, the only well-defined group of +ssRNA bacterial phages (Turina *et al.*, 2017; Dolja and Koonin, 2018). Considering the theory of mitochondria derived from an ancestral alphaproteobacterial endosymbiont (Raven, 1970), this evidence suggests that mitoviruses are derived from a mitochondrial phage by losing the capsid protein (Koonin *et al.*, 2015), which is unnecessary due to the lack of an extracellular stage (Koonin and Dolja, 2014). The main objection to this theory was that, up to 2018, no evidence of mitovirus sequences was found out from fungi. Then, using bioinformatics

approaches (Nibert *et al.*, 2018) followed by biological characterization of a true replicating virus, mitoviruses were reported also in plants (Nerva *et al.*, 2019e).

All these reports highlighted the importance of viral evolution studies: since fungi are ubiquitous in the environment and often infected by viruses they can play a very important role in defining the virosphere of a specific ecological niche.

Impact on Fungal Behavior

Although mycoviruses are common among fungi, they often remain latent without inducing obvious symptoms in their host, at least in axenic condition (Pearson *et al.*, 2009). In some case, however, they can cause dramatic alteration in their inhabited fungi, including irregular growth, changes in pigmentation, cytological alterations of cellular organelles, changes in enzymatic activities and altered sexual and asexual reproduction (Ghabrial *et al.*, 2015; Jiang *et al.*, 2013; Nuss, 2005; Newhouse *et al.*, 1983; Rigling and Van Alfen, 1993). In addition, the biological and ecological role(s) that mycoviruses play over the multitrophic interactions is largely unknown and further complicated by frequent multiple viral infections (Khalifa and Pearson, 2013; Picarelli *et al.*, 2019; Nerva *et al.*, 2019c).

One of the most important and earliest research on mycoviruses concerned the interaction between the hypovirus *Cryphonectria hypovirus 1* (CHV1) and the chestnut blight fungus *Cryphonectria parasitica*. Virus-infected isolates displayed lower production of spores, an altered pigmentation, a reduced growth in axenic conditions and a reduced virulence in the host plant (*Castanea* spp.) which led to the definition of hypovirulence (Sotirovski *et al.*, 2006; Rigling *et al.*, 1989; Hillman *et al.*, 1990; Hillman and Suzuki, 2004; Turina *et al.*, 2006). As cited before, the latter was only the first report, followed by many other hypovirulence-associated mycoviruses. To date, viruses able to confer the hypovirulent trait have dsRNA, +ssRNA, and circular ssDNA genomes and include representatives of the *Totiviridae*, *Narnaviridae*, *Chrysoviridae*, *Reoviridae* and *Genomoviridae* which infect both Ascomycota and Basidiomycota fungi (Liu *et al.*, 2015b; Khalifa and Pearson, 2013; Hu *et al.*, 2014; Yu *et al.*, 2015; Xie *et al.*, 2016; Du *et al.*, 2014).

In contrast to hypovirulence, the other interesting phenomenon associated to a mycoviral infections is the hypervirulence, the opposite trait. This last feature is less known than the previous one and it was described only in few reports. So far, the first reported case was with the identification of dsRNAs molecules associated to an hypervirulent isolates of *Nectria radicicola* (Ahn and Lee, 2001) which attack the ginseng root. Some years later, the simultaneous description of two partitiviruses inducing the hypervirulence phenotype on *Trichoderma harzianum* and *Talaromyces marneffei* have been noticed (Lau *et al.*, 2018; Chun *et al.*, 2018). The latter example is of particular interest for human health since *T. marneffei* is a pathogen able to cause systemic mycosis which can lead to death of immunocompromised patients (Lau *et al.*, 2018).

The alteration of the fungal behavior above described is probably associated to an altered fungal metabolism. Mycoviruses are obligate intracellular parasites which reprogram the entire host metabolism in order to replicate within host cells avoiding antiviral responses (Collum *et al.*, 2016; Hurwitz *et al.*, 2013). The most studied fungal model system to deepen the metabolic changes in consequence to mycoviral infection is the largely studied *C. parasitica*. In fact, it was demonstrated that viral infection induces an extensive modulation of metabolites belonging to classes of amino acids, carbohydrates, lipids, nucleotides and polyamines (Dawe *et al.*, 2009). In addition, it was also demonstrated that infection of *C. parasitica* with a virus from an unrelated fungus led to an enhanced resilience to osmotic stress (Nerva *et al.*, 2017a), suggesting the possibility of an overlapping between the fungal responses to biotic and abiotic stresses. Finally, it was also demonstrated that secondary metabolites can be modulated by the mycoviral infection as reported for the AK-toxin of *Alternaria alternata* or the ochratoxin A (OTA) in *Aspergillus ochraceus* (Nerva *et al.*, 2019a; Okada *et al.*, 2018).

Over the last decades, the intriguing concept of "good viruses" (Roossinck, 2011; Virgin, 2014) has been proposed to highlight that virus-host relationship are often mutualistic. This concept seems to well-fit with most of the case observed for mycovirus-fungus interaction, which often induce an enhanced fitness through the expression of genes and metabolites that partially overlap those activated during abiotic stresses (Bostock, 2005). Also in this case many researches are needed to uncover the potentiality of mycoviruses and to better elucidate their effects at cellular level.

Mycoviruses as Triggers and Target of RNA Silencing Machinery

RNA silencing is an evolutionarily conserved pathway which was first described in *Caenorhabditis elegans* as a phenomenon called RNA interference (RNAi) (Fire *et al.*, 1998). A similar pathway was also described in plants (Napoli *et al.*, 1990) and fungi (Romano and Macino, 1992). This silencing system is activated in response to invasive nucleic acids, and in specific against dsRNAs. It induces a specific defense response against the invasive agent (e.g., viruses, retrotransposon, transgene, etc.) by cutting the dsRNAs into small RNAs (Ding and Voinnet, 2007; Chapman and Carrington, 2007). The latter lead to the degradation of target RNAs and for this reason are also known as small interfering RNA (siRNA). In plants and fungi, replicating RNA viruses initiate the RNA silencing response by forming replicative double-stranded RNAs (dsRNAs) which are recognized by specific ribonucleases, the dicer proteins (dcl) which lead to the production of 21– to 24–nucleotides virus-derived small RNAs (vsiRNAs) (Ding and Voinnet, 2007).

Although vsiRNAs have been extensively profiled in many plant and animal species, little is known about composition and role (s) of such RNAs in fugal cells while a mycovirus infection is ongoing. The antiviral role of RNA silencing in fungi was first described in *Cryphonectria parasitica* infected by the mycovirus CHV1 (Nuss, 2011) and then many other researches demonstrated the activity of an RNAi pathway in fungi (Himeno *et al.*, 2010; Wang *et al.*, 2016; Vainio *et al.*, 2015). Although fungi confirmed to

have an active antiviral defense, some species displayed peculiar characteristics. For example, length of vsiRNAs is not conserved among fungi: some species display a production of 21 nucleotides long vsiRNAs, consistent with that of plants, whereas some others display a completely different size distributions which range from 21 up to 30 nucleotides (Himeno *et al.*, 2010; Nakayashiki and Nguyen, 2008; Mochama *et al.*, 2018; Nerva *et al.*, 2016). In addition, some fungal species, such as *Saccharomyces cerevisiae* and *Ustilago maydis*, have completely lost the silencing machinery (Nakayashiki and Nguyen, 2008), suggesting the existence of different strategies to counteract viral infections.

Biotechnological Applications and Future Perspectives

To date, there are only two existing example of mycovirus biotechnologically exploited. The first is represented by the case of CHV1. The plant pathogen *Cryphonectria parasitica*, imported from Asia in the United State of America at the end of nineteen centuries, is responsible for the near-disappearance of the American chestnut, *Castanea dentata*, from its natural environment (Anagnostakis, 1987; Nuss, 2005). The fungus was then imported from USA to Europe, described for the first time in Italy on sweet chestnut (*Castanea sativa*) in 1938 (Biraghi, 1946) and then spread to the most of the European countries. Thanks to many efforts, during 1950s it was discovered the biocontrol potential of CHV1 (Grente, 1965; Elliston, 1985) which was then routinely used for the disease control (Elliston, 1985; Choi and Nuss, 1992; Nuss, 1992). Thanks to the exploitation of CHV1, in Europe, chestnut blight is no more threatening the survival of *Castanea* spp. demonstrating the potential of mycoviruses in modulating virulence of plant pathogens.

The second case of a biotechnologically exploited mycovirus is the so called "killer yeast" system for toxin production. Many yeast isolates secrete killer toxins that inhibit growth of other species and/or strains (Marquina *et al.*, 2002). These toxins have no activity against microorganisms other than yeasts, and the killer strains are insensitive to their own toxins (Liu *et al.*, 2015a). Studies on the nature of the killing phenomenon showed the involvement of double-stranded RNAs elements associated with virus-like particles (VLPs) which were then identified as mycoviruses (Bostian *et al.*, 1980; Herring and Bevan, 1977). From that moment, killer isolates have been used to control contaminating yeasts in food commodities and to control pathogenic fungi in plants (Schmitt and Breinig, 2002). In details, killer yeasts have been used to control contamination in the winemaking and brewing processes (Schmitt and Breinig, 2002) as well as to develop novel antifungal drugs for the treatment of human and animal mycoses (Magliani *et al.*, 2008).

Despite CHV1 remains the only example of large scale application, a number of other reports suggest the possibility of exploiting mycoviruses as sustainable tool in plant defense strategies. For example, the potentiality of *Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1* (SsHADV-1) as natural fungicide was proved in *Arabidopsis thaliana* and *Brassica napus*. SsHADV-1 is a circular ssDNA virus which displayed the ability to be transmitted extracellularly when applied to the fungus both in vitro and in vivo (Yu *et al.*, 2013). Interestingly its virus particle displayed a good stability when applied on leaves of plants, remaining detectable up to 15 days after application. Moreover, the crude preparation of virus particles applied on plants inhibited the expansion of fungal pathogen and cured the lesion (Yu *et al.*, 2013). Finally, the same virus can infect a mycophagous insect and use it as transmission vector (Liu *et al.*, 2016). All together these results demonstrated once again the potentiality of mycoviruses as biological control agents for a sustainable plant protection and are pushing the researchers to identify new viral species.

In addition to the hypovirulence traits for plant protection purposes there are other scientific works that suggested the biotechnological potential of mycoviruses. For example, their use in enhancing the host virulence can be exploited to improve action of fungi as biological control agents against other pathogens. This was the case of viruses associated to *Trichoderma* spp., a widely used mycoparasitic fungus, which displayed an enhanced antifungal activity when infected by a mycovirus (Chun *et al.*, 2018). On the contrary, it was demonstrated that infection by a specific mycovirus induce hypovirulence in *Beauveria bassiana* (Kotta-Loizou and Coutts, 2017b), an entomopathogenic fungus with a wide host range and used as a biocontrol agent against arthropod pests.

Finally, the use of mycoviruses as biological control agents can be a suitable alternative also for treatment of systemic mycoses in human patients. In the last few years a growing number of human pathogens displayed resistance against antifungal compounds (McCarthy *et al.*, 2017), highlighting the urgent need for new therapeutic strategies. In this line, one hypothetical therapeutic strategy might involve the use of mycoviruses (van de Sande *et al.*, 2010), which selectively infect one or few fungal species preserving the human holobiome. All together these findings displayed the importance of looking at the virological state of fungi, especially when used in biotechnological purposes. In addition, considering the mycological collections, knowing the viral state of a fungal isolate is very important since isogenic virus-free and virus-infected isolates can display different characteristics and behavior. In conclusion, the potential use of mycoviruses may have significant ecological and economic implications and for these reasons deserve more attention over the next future.

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Transposable Elements in Fungi: Coevolution With the Host Genome Shapes, Genome Architecture, Plasticity and Adaptation

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Introduction: The Extensive Diversity of Repetitive DNA in Fungal Genomes

Repetitive elements comprise both DNA sequences that are present in multicopies in genomes and ubiquitous in eukaryotic genomes (Biscotti *et al.*, 2015). Major contributors of these repetitive sequences are the tandem repeats including microsatellites and transposable elements (TEs). In humans and other mammals, TEs represent up to 50% of the total DNA (Zamudio and Bourc'His, 2010) while, in plants it can reach more than 80% (Schnable *et al.*, 2009). In fungal genomes the range of repeat content is extremely variable. For instance, TEs represent approximatively 3% of the yeast *Saccharomyces cerevisiae* genome (Kim *et al.*, 1998), up to 58% in the black truffle *Tuber melanosporum* (Martin *et al.*, 2010) and covers more than 90% in the soybean pathogen *Phakopsora pachyrhizi* (Sébastien Duplessis, personal communication). For decades, TEs were described as "selfish" DNA elements of host genomes after first being described in maize by McClintock (1984) and Biémont (2010). TEs can replicate autonomously and thereby can be regarded as "self-existing", independent of the host genome, and were eventually considered as "junk" DNA. However, growing evidence from genomics, population genomics and functional analysis studies have demonstrated that TEs can also have a major role for genome evolution (Dubin *et al.*, 2018). In maize for example, TEs associate in maize development such as flowering time (Castelletti *et al.*, 2014), specific TEs exhibit signatures of selection (Studer *et al.*, 2011) and drought tolerance adaptation (Mao *et al.*, 2015). TEs can have considerable consequences on host genome evolution, architecture and plasticity (Castanera *et al.*, 2016). TEs evolutionary success relies not only in their ability to replicate faster than the host genome, but also because TEs can be transmitted both vertically and horizontally (Gilbert and Feschotte, 2018).

A systematic classification system was established to categorize the extensive diversity of TEs based on their function and origin (Wicker *et al.*, 2007). To help TE identification and annotation, a hierarchical classification has been defined, based on transposition mechanisms that distinguish two classes of TEs: retrotransposons and DNA transposons (**Fig. 1**) (see (Wicker *et al.*, 2007) for detailed classification of TEs). The two classes of TEs divide into subclasses, orders and superfamilies depending on respectively, proteins or non-coding domain structures, target site duplication length and DNA sequence conservation (**Fig. 1**) (Wicker *et al.*, 2007). TEs further divide into families that are mostly species-specific or shared only by closely related sister species. Retrotransposons (or Class I) require a RNA intermediate reverse-transcribed into cDNA that integrates in a new position in the genome. The class I transposition mechanism is commonly referred to as a "copy-and-paste" mechanism (Boeke *et al.*, 1985). Each transposition event of a retrotransposon produces an additional copy which explains that retrotransposons often contribute to the main fraction of repeat content in many fungal genomes (**Fig. 1(A)**) (Martin *et al.*, 2008, 2010; Duplessis *et al.*, 2011; Grandaubert *et al.*, 2014; Grandaubert *et al.*, 2015). Retrotransposons comprise five orders namely: Long terminal repeats (LTR) elements, *Dictyostelium* intermediate repeat sequence (DIRS), Penelope-like elements (PLEs), Long INterspersed Elements (LINEs) and non-autonomous Short INterspersed Elements (SINEs). DNA TEs (Class II) consist of two subclasses. Elements of Subclass 1 use a "cut-and-paste" transposition mechanism, where both strands of the DNA are excised. Elements of Subclass 2 replicate by single strand excision and a rolling-circle mechanism (Helitron) or an unknown mechanism (Maverick or Pollinton) (Greenblatt and Brink, 1963; Rubin *et al.*, 1982; Kapitonov and Jurka, 2007). DNA transposons are divided in five orders: Terminal inverted repeat (TIR), Crypton, Helitron and Maverick (Wicker *et al.*, 2007). In additioning, class II contains the sub-family of Miniature inverted-repeat transposable element (MITEs) that are the non-autonomous counterpart of TIR (**Fig. 1(A)**). Non-autonomous TEs including SINEs, TRIM, LARD, MITEs and some Helitrons use the replication machinery of other TEs or genes to transpose. Both MITEs and SINEs are small elements of less than 1000 bp and rely respectively on TIR transposases and LINEs retro-transcriptase to transpose (González and Petrov, 2009; Kramerov and Vassetzky, 2011). The autonomous or non-autonomous status of the different TEs families that colonize fungal genomes have dramatically different impacts on the host genome architecture, notably by inducing structural variation within populations (Badet *et al.*, 2020). In addition, TEs uncontrolled expansions have been demonstrated to induce genome instability even leading to decrease of host fitness (Pasyukova *et al.*, 2004; Zamudio and Bourc'His, 2010).

New TE insertions can have different consequences on host fitness depending on their insertion sites (**Fig. 1(B)**). Overall, the fitness cost of TE insertions has been estimated as deleterious (Horváth *et al.*, 2017). TE insertions into coding or regulatory regions can lead to exonization, truncation, alternative splicing or modification of gene expression (**Fig. 1(B)**) (Bourque *et al.*, 2018).

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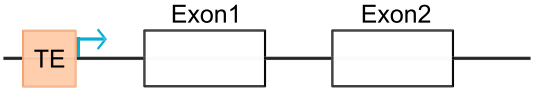
A

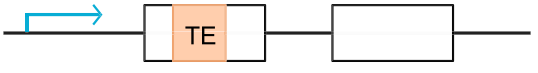
Class	Order	Structure
Class I Retrotransposons	LTR	LTR, gag, pol, LTR
	LINE	ORF1, EN, RT, AAA
	SINE	AAA
	DIRS	TIR, gag, pol, TIR
	Penelope	EN, RT
Class II DNA transposons	TIR	TIR, Transposase, TIR
	Crypton	TR
	Helitron	RPA, Helicase
	Maverick	TIR, INT, pol, TIR
	MITE	TIR, TIR

B

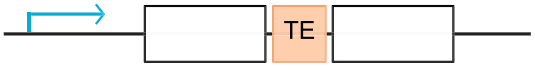
i) Promoter disruption



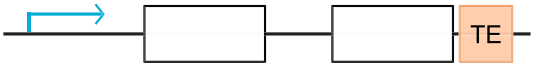
ii) Exonization/Transcript truncation



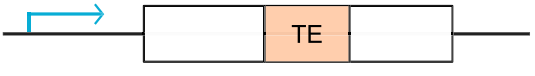
iii) Alternative splicing
Transcript truncation



iv) Add small RNA binding sites,
polyadenilation sites



v) Chimeric genes

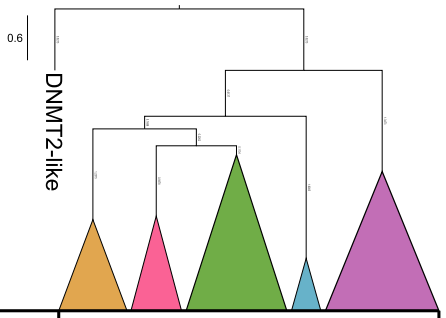


However, gene and transcription modifications are not the only consequences of TEs activity in fungal genomes. At a genome organization level, TEs are involved in telomere maintenance, chromosomal rearrangements, genome compartmentalization and genome size expansions (Möller and Stukenbrock, 2017). However as TEs accumulate independently in their host genome, differences in TEs content have been observed between lineages (Oggenfuss *et al.*, 2020). TEs content variation between two lineages was documented to have prevented gene flow between fungal populations (Giraud *et al.*, 2008). Chromosomal rearrangements can be associated with suppression of recombination and thus represent a barrier to gene flow (Serrato-Capuchina and Matute, 2018). Hence, TEs represent a source of speciation agents (Serrato-Capuchina and Matute, 2018). Sexual reproduction in fungi is based on mating systems whereby different mating types control sexual reproduction outcome (Nielsen and Heitman, 2007). Two mating system strategies can be found in closely related species: homothallism (i.e. self-compatibility) and heterothallism (i.e., self-incompatibility) (Wilson *et al.*, 2015). In several fungal clades, homothallic/heterothallic transitions among closely related species associate with TE activity within or nearby mating-type loci (Rusche and Rine, 2010; Gioti *et al.*, 2012). Finally, it has been hypothesized that the variation in TE content of fungal genomes could be linked to their life-style (Muszewska *et al.*, 2019). Comparative genomics studies demonstrated that TE content and composition depend on the history of TE invasions and expansions in the host species rather than phylogenetic proximity (Hess *et al.*, 2014; Amselem *et al.*, 2015b). Interestingly, by comparing TE content across the fungal kingdom it appears that symbiotic ectomycorrhizal fungi or obligate biotrophic pathogens exhibit a high TE coverage compared to saprophytes (**Fig. 2**). For instance, the tree mutualist *Coenococcum geophilum* (Peter *et al.*, 2016; de Freitas Pereira *et al.*, 2018) and the powdery mildew pathogen *Blumeria graminis* (Frantzeskakis *et al.*, 2018) exhibit 75% and 90% of TE content in their genomes, respectively. The various impacts on host genome evolution reflect the long-term coevolution between TEs and host genomes in fungi.

TEs are known to be deleterious to the host, and fungal species have evolved a variety of defense mechanisms to counterbalance negative impacts on fitness due to TEs invasions and expansions. These mechanisms are largely conserved across animal, plant and fungal genomes and mostly involve epigenetic silencing through DNA methylation, small RNA silencing and histone modification mediated silencing (Feng *et al.*, 2010; Gladyshev, 2017; Möller and Stukenbrock, 2017). A particular and highly efficient defense mechanism in fungi is called Repeat-Induced Point (RIP) mutation (for detailed review see (Gladyshev, 2017)). The RIP mechanism was first described when high level of cytosine-to-thymine mutations and cytosine DNA methylation were observed in duplicated genes of the model system *Neurospora crassa* (Selker and Stevens, 1985). RIP mechanism is currently understood as a two-step process involving cytosine methylation followed by deamination (Gladyshev and Kleckner, 2017). RIP specifically targets duplicated sequences independently of their expression and can spill over to neighboring sequences (Irelan *et al.*, 1994). TE insertions can also be counter-selected in fungal populations at variable strengths depending on their negative effect and the effective population size. Advantageous insertions can potentially be favored by selection and rise in frequency. The genomes of some fungal species are organized in specific TE rich compartments, whereby TEs are associated with rapidly evolving genes and signatures of recent adaptation, often referred as "two-speed" genomes (Dean *et al.*, 2005; Rouxel *et al.*, 2011; de Jonge *et al.*, 2013; Grandaubert *et al.*, 2014; Dong *et al.*, 2015). In addition, silenced TEs can be de-repressed under various stress and developmental stages. Thereby in specific conditions, TEs mobilization may help rapid adaptation to diverse stress conditions (Fouché *et al.*, 2020). Understanding how genomes regulate TEs activity is crucial to understand the diversity of TEs dynamics and impacts across fungi.

Major progress has recently been made to comprehend TEs dynamics in fungal genomes. However, the evolutionary interplay between a host genome and TE invaders is yet to be fully understood. Our current understanding of TE impact on fungal genome evolution is mainly based on comparative genomic studies and few functional analyses in model systems. Most studies agree that TE activity has overall a negative impact on host fitness, notably because TE insertions can induce phenotypic changes either directly (i.e., through gene disruption or expression modification) or indirectly (i.e., by triggering epigenetic changes). Thereby, TE activity can also represent a key mechanism for rapid adaptation. Understanding the impact of TEs on the adaptation of the host requires to deeply study TE dynamics in fungal populations including how effectively genomic defenses prevent proliferation. This chapter provides an overview of how various host genome defense mechanisms regulate TE activity in fungi. Furthermore, we provide insights into how the host population demography impacts TE dynamics. Finally, we summarize the compartment and locus-specific effect of TEs on fungal genome architecture and plasticity.

Fig. 1 *Transposable element (TE) structure and effects on genes.* (A) class and order of transposable elements based on classification in Wicker *et al.* Class I groups retrotransposons using an RNA transposition intermediate. Class I comprises five orders depending on reverse transcriptase phylogeny and TE structure: LTR, long terminal repeats; LINE, long interspersed nuclear element; SINE, short interspersed nuclear element; DIRS, *Dictyostelium* intermediate repeat sequence; Penelope, *Penelope*-like elements. Class II groups DNA TEs and lack RNA intermediate. Class II comprise five orders depending on the number of DNA strands cut during transposition: TIR, terminal inverted repeats; Crypton; Helitron; Maverick; MITE, miniature inverted-repeat transposable element. Pol, DNA polymerase; gag, capsid protein; ORF, open reading frame of unknown function; EN, endonuclease; RT, reverse transcriptase; TR, tyrosine recombinase; RPA, replication protein A; INT, integrase. (B) Effect of TE insertion on gene transcription and regulation. *MITEs are not considered as an order but as the non-autonomous counterpart of TIR. (i) TE insertion upstream of gene-coding regions can lead to promoter disruption either directly or indirectly through epigenetic silencing; (ii) exonic and (iii) intronic TE insertions can lead to truncated transcripts, alternative splicing or exonization; (iv) downstream insertions potentially add small RNA binding or polyadenylation sites and (v) TE insertion can integrate into coding regions leading to the generation of chimeric genes also known as TE domestication. TE, transposable element. Reproduced from Wicker, T., *et al.*, 2007. A unified classification system for eukaryotic transposable elements. Nat. Rev. Genet. 8 (12), 973–982. doi:10.1038/nrg2165.



		Genome size (Mb)	Repeat content (%)
Basidiomycota	*Puccinia striiformis* f. sp. *tritici*	83	60
	Puccinia graminis f. sp. tritici	89	45
	Melampsora larici-populina	110	42
	Rhodosporidium toruloides	21	ND
	Microbotryum lychnidis-dioicae	27	14
	Pleurotus ostreatus	35	12
	Pisolithus tinctorius	71	ND
	Piriformospora indica	30	5
	Phanerochaete chrysosporium	35	<1
	Paxillus involutus	58	ND
	Laccaria bicolor	65	24
	Heterobasidion annosum	33	16
	Hebeloma cylindrosporum	38	ND
	Agaricus bisporus	30	11
Ascomycota	*Tuber melanosporum*	125	58
	Aspergillus flavus	37	<1
	Zymoseptoria tritici	40	17
	Pyrenophora triciti-repentis	38	17
	Pseudocercospora fijiensis	74	4
	Parastagonospora nodorum	37	8
	Leptosphaeria maculans	45	34
	Ceonococcum geophilum	178	81
	Chalara longipes	53	ND
	Blumeria graminis	125	90
	Verticilium dahliae	34	<1
	Neurospora crassa	40	<1
	Magnaporthe oryzae	40	7
	Fusarium graminearum	37	2
	Colletotrichum graminicola	52	5

The Fine-Tuned Regulation of Transposable Elements Proliferation

Coevolution of host genomes and TEs determines the extent and activity of TEs in a genome. In fungi, the majority of TE copies are transcriptionally inactive except under environmental stress conditions (Fouché *et al.*, 2020). Epigenetic silencing is widespread along the genome, affecting both genes and TE expression (Huda *et al.*, 2010; Freitag, 2017; Borgognone *et al.*, 2018). Epigenetic TE silencing mechanisms occur at three different levels: (1) chromatin structure and histone modifications, (2) DNA methylation and (3) small RNA silencing. The following section describes how epigenetic silencing of TEs varies across the fungal tree of life.

Epigenetic Silencing due to Histone Methylation

Chromatin modifications are essential for genome stability notably regarding silencing of TEs in fungal genomes. The high compaction of heterochromatin lead to inaccessibility of transcription machinery (Möller *et al.*, 2019). In fungal genomes, expressed genes often associates within euchromatin while TEs associate with heterochromatin regions (Lewis *et al.*, 2009; Connolly *et al.*, 2013; Chujo and Scott, 2014; Galazka and Freitag, 2014; Schotanus *et al.*, 2015; Feurtey *et al.*, 2020). Different histone modifications specifically mark euchromatic or heterochromatic regions. Euchromatin is marked by H3 lysine 4 di- or trimethylation (H3K4me2/3) while heterochromatin associates with H3K9me2/3 and H3K27me2/3 (Freitag, 2017). Histone modifications are a reversible epigenetic process. In fungi, change in the histone modification marks, notably due to TE insertions, can provide a source for rapid adaptation under stress conditions, pathogenicity and for the regulation of secondary metabolite gene clusters (Chujo and Scott, 2014; Soyer *et al.*, 2014; Krishnan *et al.*, 2018; Fouché *et al.*, 2020).

DNA Methylation

Similarly to animals and plants, DNA methylation can target cytosines of duplicated sequences including TEs in fungal genomes. Although widespread across eukaryotes, impact of the 5-methylcystosine modified (5mC) base on gene expression, TE silencing and genome integrity varies between taxa (Slotkin and Martienssen, 2007; Jones, 2012; Bewick *et al.*, 2019). DNA methylation is specifically mediated by DNA methyltransferases (DNMTs) that can either establish (*de novo DNMT*s) or maintain (*maintenance DNMTs*) DNA methylation (Lyko, 2018). DNMTs are part of a large protein family and are classified in different clades in function of their conserved domains and activities (Bewick *et al.*, 2019)(**Fig. 2**). In fungi, a recent extensive phylogenetic study of DNMTs across more than 500 fungal species revealed a wide presence/absence variation in DNMTs content between closely related species (Bewick *et al.*, 2019). This study described that fungal DNMTs are part of five superfamilies namely DNMT1, DIM-2, RID, DNMT2 and DNMT5 (Bewick *et al.*, 2019). Bewick *et al.* (2019) also showed that DNMTs duplications and losses shaped DNMT genes evolution in eukaryotes. DNMTs show a remarkable presence/absence variation in closely related species with the highest diversity found in Ascomycota whereby some species exhibit all DNMTs (e.g., *Chalara longipes*; **Fig. 2**) (Bewick *et al.*, 2019). Basidiomycota species mostly carry DNMT1 and DNMT5 while RID and DIM-2 are absent (**Fig. 2**). DIM-2 and RID are known to be the key players of RIP mechanism through specific 5mC methylation of duplicated sequences demonstrated in *N. crassa* (Gladyshev and Kleckner, 2014; Mazur and Gladyshev, 2018). DIM-2 and RID can be lost between closely related fungal clades. For instance, Möller and colleagues recently showed an intra-specific presence/absence variation of DNMT proteins in the wheat pathogen *Z. tritici* isolates due to accidental RIP in genes coding DIM-2 (Möller *et al.*, personal communication)(Dhillon *et al.*, 2010). *Z. tritici* isolates sampled at the species center of origin possess active DIM-2 and DNMT5 while *Z. tritici* isolates sampled outside the center of origin possess only DNMT5. Interestingly, the loss of DIM-2 and thereby the reduction of 5mC in *Z. tritici* isolates associates with a reduction in RIP signatures on TEs (Lorrain *et al.*, personal communication). Understanding TE invasion dynamics in fungal genomes requires to evaluate how TEs co-evoled with genome defense mechanisms. Fungal species deploy a variety of mechanisms including the irreversible mutation accumulation of TEs mediated by the RIP mechanism. Mutations induced by RIP in fungi challenges the dating of specific TE family invasions and the identification of ancestral sequences (Grandaubert *et al.*, 2014). The RIP mechanism has been demonstrated to be costly to maintain and can be lost between closely related species (Galagan and Selker, 2004). Interestingly, a modeling approach conducted in the model plant *Arabidopsis thaliana* showed that the retention of methylated TEs could be more efficient to prevent transposition than elimination (Roessler *et al.*, 2018). It would be interesting to investigate if TE regulation converges on similar mechanisms in fungal species with highly repetitive genomes such as in the Pucciniales (Basidiomycetes).

Fig. 2 *Repertoires of DNA methyltransferases genes in Ascomycetes and Basidiomycetes*. Phylogenetic representation of 5-methylcytosine methyltransferases in 29 fungal genomes including 15 Ascomycetes (*Tuber melanosporum*; *Aspergillus flavus*; *Zymoseptoria tritici*; *Pyrenophora tricitirepentis*; *Pseudocercospora fijiensis*; *Parastagonospora nodorum*; *Leptosphaeria maculans*; *Ceonococcum geophilum*; *Chalara longipes*; *Blumeria graminis*; *Verticilium dahlia*; *Neurospora crassa*; *Magnaporthe oryzae*; *Fusarium graminearum*; *Colletotrichum graminicola)* and 14 Basidiomycetes (*Agaricus bisporus*; *Hebeloma cylindrosporum*; *Heterobasidion annosum*; *Laccaria bicolor*; *Paxillus involutus*; *Phanerochaete chrysosporium*; *Piriformospora indica*; *Pisolithus tinctorius*; *Pleurotus ostreatus*; *Microbotryum lychnidis-dioicae*; *Rhodosporidium toruloides*; *Melampsora larici-populina*; *Puccinia graminis* f. sp. *tritici*; *Puccinia striiformis* f. sp. *tritici*). Fungal 5-methylcytosine methyltransferases were extracted from (Bewick *et al.*) and comprise DNMT1; DNMT2a; DNMT5; DIM-2; RID. DIM-2 and RID are involved in the Repeat-induced point (RIP) mutation mechanism. DIM-2 and RID were lost by a majority of Basidiomycetes while showing a patchy phylogenetic distribution among ascomycetes. Reproduced from Bewick, A.J., *et al.*, 2019. Diversity of cytosine methylation across the fungal tree of life. Nat. Ecol. Evol. 3 (3), 479–490. doi:10.1038/s41559-019-0810-9. Gladyshev, E., 2017. Repeat-induced point mutation and other genome defense mechanisms in fungi. Microbiol. Spectr. 5 (4). doi:10.1128/microbiolspec.FUNK-0042-2017.

Small RNA Silencing

Another level of TEs activity regulation deployed by the host genome uses interfering RNAs (RNAi). In animals, TE epigenetic silencing can be mediated by small-interfering RNAs (siRNA) and piwi-interacting RNAs (piRNAs) (Luo and Lu, 2017). In *Arabidopsis*, small amounts of RNAi were shown to be sufficient to initiate methylation and silencing. Growing evidence suggests that RNAi-mediated TE regulation in fungal genomes is important, however the molecular interplay of RNAi-mediated TE silencing remains largely unknown in most fungal species. RNAi-mediated TE silencing has been described mostly in model organisms such as the fission yeast *Schizosaccharomyces pombe*, the filamentous ascomycete *N. crassa* and the basidiomycete *Cryptococcus neoformans* (Alper *et al.*, 2012; Gladyshev, 2017). In *S. pombe*, RNAi are required for heterochromatin assembly (Alper *et al.*, 2012). RNAi-mediated silencing of TEs was demonstrated in *Cryptococcus neoformans* to be mediated by Spliceosome-Coupled And Nuclear RNAi complex (SCANR) (Dumesic *et al.*, 2013). This mechanism uses the RNAi response to silence pre-mRNAs of retrotransposons in vegetative cells and induces stalling of spliceosomes. Interestingly, TE activity also produces TE-derived small RNAs that are involved in TE silencing in *M. oryzae* (Nunes *et al.*, 2011). Finally, in a recent study of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici* authors observed a wave of small RNA targeting TEs during late stages of the host plant infection, suggesting epigenetic TE silencing (Sperschneider *et al.*, 2018). Further analyses of TE silencing by small RNAs is a particularly interesting field to understand TE control in genomes that lack the RIP mechanism such as in Basidiomycetes of the order Pucciniales.

Host Population Dynamics Impact Transposable Element Landscapes in Fungi

Investigations of TEs landscapes and dynamics in fungal genomes should not only need to be addressed in regards to their interplay with host genome regulation pathways but also in regards to host genome evolution at the population level. Despite the efforts to unravel molecular mechanisms of TE activity and fungal defense systems, very few studies have investigated TE evolutionary dynamics at the population level in fungal genomics. In the following section, we describe how the demographic history, natural selection and host defense mechanisms modulate TE landscapes in fungal genomes.

The TE Burst and Decay Model

The burst and decay model is used to describe TE evolution (Arkhipova, 2018). Traditionally, the model of TE transposition activity describes the maintenance of an equilibrium between TE insertions (i.e., birth) and TE excisions (i.e., death). In opposition, the burst and decay model assumes activity of TEs only over short periods (i.e., burst), that are counter-balanced by host genome defenses more or less rapibly (i.e., decay).

Overall, number of TE copies increase during short bursts of activity rather than at a constant transposition rate (Vitte and Panaud, 2005; Hua-van *et al.*, 2011; Castanera *et al.*, 2016). Indeed, TEs can sometimes be de-repressed when the host is stressed and thus be expressed and/or mobilized (Horváth *et al.*, 2017; Fouché *et al.*, 2020). The mechanistic basis of de-repression under stress is poorly understood. De-repression may happen due to a trade-off for resource allocation under stress, or de-repression may be an active strategy of the host genome to increase the TE-driven mutation rate. The latter is generally considered as an unlikely scenario, since active TEs are mostly deleterious (Horváth *et al.*, 2017). As above-mentioned, many fungal species have compartmentalized genomes with TE-rich compartments exhibiting relaxed selection (Croll and McDonald, 2012; Raffaele and Kamoun, 2012; Grandaubert *et al.*, 2019; Zhang *et al.*, 2020). De-repressed TEs can cause expansion of the genome size until they undergo decay by being silenced, mutated or truncated by host defenses (Le Rouzic and Capy, 2005).

The estimation of insertion rate and age are the core parameters to model TE population dynamics (Arkhipova, 2018). Recent insertions show an excess of low frequencies in population while ancient insertions are more likely to show inconsistencies in frequency spectra depending on the efficacy of host genome defense mechanisms. (**Fig. 3(A)**). Over time, most TE copies in a genome can lose function either through mutation accumulation, partial deletions by illegitimate recombination (Devos *et al.*, 2002), or sequence disruption by nested insertions of other TEs (SanMiguel *et al.*, 1996). The age of a TE insertion can be estimated from sequence similarity with other copies in the genome (Kapitonov and Jurka, 1996; Blumenstiel *et al.*, 2014) or the degree of linkage disequilibrium at the insertion site (González *et al.*, 2008). More specific analyses can be performed e.g., for LTRs with the two long terminal repeats flanking the insertion showing divergence over time (SanMiguel *et al.*, 1998). Age estimation can also be done by comparing the coding sequences of the TE (Bowen and McDonald, 2001; Bergman and Bensasson, 2007). Accumulated mutations in TEs can be calibrated with the genome-wide mutation rate to obtain an age estimation (Bowen and McDonald, 2001). In fungal genomes with active RIP, accumulation of mutation can be excessive which can lead to an overestimation of the age that needs to be taken into account for understanding TE dynamics in RIP-proficient fungal species.

New Transposable Element Insertions are Influenced by Selection and Genetic Drift

As a consequence of TE de-repression and TE bursts, new TE insertions can accumulate in the genome. However, the fate of a new TE insertion in the population is based on a combination of selection and genetic drift. Selection acts on TE insertions when there is an impact on host fitness. Insertions into coding or regulatory regions are mostly deleterious or even lethal for the host. Such TE insertions will be rapidly eliminated from populations by purifying selection leading to an excess of low frequency insertions

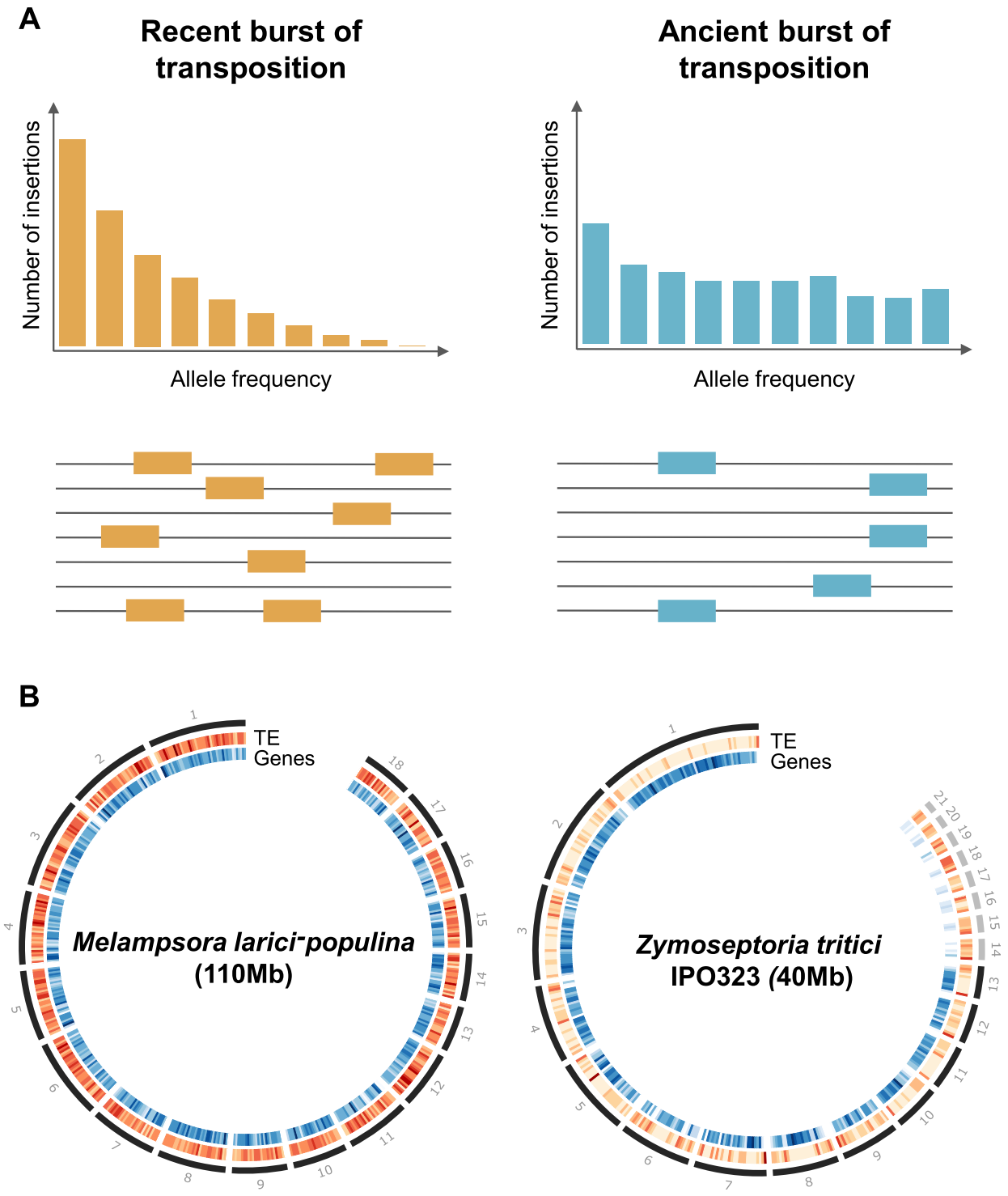


Fig. 3 *Insertion frequency spectra of transposable elements (TEs) depending on their transposition activity and effect on genome architecture.* (A) Recent bursts of transposition (orange) should lead to an excess of singletons. Ancient expansions (blue) should lead to inconsistencies in frequency spectra depending on the efficacy of host genome defense mechanisms. (B) Circos plots of non-compartmentalized (*Melampsora larici-populina* – 110 Mb – no accessory chromosomes) versus compartmentalized (*Zymoseptoria tritici* – 40 Mb – up to eight accessory chromosomes) fungal genomes. TEs (orange) and gene (blue) densities are counted along 100 kb windows per chromosome/linkage group. Core and accessory chromosomes/linkage groups are shown in black and gray, respectively.

(**Fig. 3(A)**). Strong purifying selection has been shown across plants (*Brachypodium distachyon*) and animals (*Drosophila melanogaster*) as well as in the fungal wheat pathogen *Z. tritici* (Cridland *et al.*, 2013; Stritt *et al.*, 2017; Oggenfuss *et al.*, 2020). The impact of TE insertions on regulatory regions or alteration of gene expression profiles has been shown in fungal populations (Chuong *et al.*, 2017; Omrane *et al.*, 2017). Recent insertions can also rearrange chromosomal regions (Badet *et al.*, 2020; Fouché *et al.*, 2020), duplicate genes (Morgante *et al.*, 2005; Yang and Bennetzen, 2009; Devos, 2010), or delete sequences encoding fungal pathogen effectors (i.e., secreted molecules involved in host colonization establishment and maintenance) recognized by the host plant (Hartmann *et al.*, 2017). While most of these effects are deleterious, TE-induced impacts can also be beneficial and lead to local adaptation (van't Hof *et al.*, 2016) or even to extreme examples of co-option whereby TEs are "domesticated" and evolved to a new host function (Feschotte, 2008).

At the population level, changes in the allele frequency of TE insertions can be heavily influenced by genetic drift (Charlesworth and Langley, 1989; Le Rouzic and Capy, 2005). Bottlenecks in the population size, e.g., during the colonization of new habitat, can reduce genetic diversity. As a consequence of genetic drift, allele frequencies in the population are randomly affected. Hence, genetic drift has also consequences on the TE composition in the genome. Genetic drift can lead to the loss of a newly inserted TE or to an increase in frequency in the population. Drift effects are mostly expected in small effective population sizes and for TE insertions that are nearly neutral. When a fungal pathogen colonizes a new territory (e.g., with infected plant material or by spore dispersal), only a subset of all TE insertions of the original population will be present. Genetic drift can also increase the average TE load in a newly established population if the founder genotypes had unusually high TE loads. The application of fungicides in agriculture and other stressors could cause bottlenecks on plant pathogen populations (Lucas *et al.*, 2015). The expansion of *Z. tritici* across the world induced a series of colonization bottlenecks. A population closest to the center of origin was found to have the lowest amount of TEs in the genome and more recently established populations had substantially higher TE contents (Badet *et al.*, 2020; Oggenfuss *et al.*, 2020).

How can Population Dynamics Lead to Differences in TE Content Among Species?

TE bursts and insertion dynamics in populations determine the overall TE load. De-repression, TE bursts and population bottlenecks may occur following major stresses such as host jumps, domestication events or colonization of new environments (Naito *et al.*, 2006; González *et al.*, 2008; Yoshida *et al.*, 2016). Domestication of fungi happened for culinary or medicinal reasons (Payne *et al.*, 2006). In addition, in a broader definition of domestication, crop pathogens were domesticated to the agricultural landscape in parallel to their hosts (Stukenbrock *et al.*, 2011). In *Aspergillus oryzae* and *Z. tritici*, the domestication was associated with an increase in TE content. Over larger time scales, closely related species can evolve distinct TE content in the genome (Lorrain *et al.*, personal communication). Also, genome size expansions resulting from distinct TE accumulations in closely related species were described in the rubber tree pathogen *Pseudocercospora ulei*, among *Fusarium* and *Puccinia* species (Sandra Sayer, personal communication) (Raffaele and Kamoun, 2012; Schwessinger *et al.*, 2018; Zhang *et al.*, 2020). Rebollo *et al.* (2010) hypothesized that TE activity could ultimately favor speciation by creating distinct phenotypes that become locally adapted. Evidence for major TE-mediated adaptations suggests that TEs could have a profound impact on speciation process in fungi as well.

Are Transposable Elements Essential for Rapid Adaptation?

Genome plasticity provides a source of adaptation to new environments for organisms. Host genome plasticity mediated by TEs suggests that the recruitment of TEs could benefit the host by inducing for example gene expression modifications, translocations and even horizontal gene transfer. The following section illustrates how TE-induced host genome plasticity plays a central role in adaptation to ecological niches.

Transposable Element and Host Genome Compartmentalization

TE activity creates rapidly evolving (i.e., rearranging) compartments accompanied by changes in GC content or epigenetic signatures. In fungi, how TEs have shaped host genome architecture is illustrated by TE clusters, compartments and even entire TE-rich chromosomes (**Fig. 3(B)**) (Duplessis *et al.*, 2011; Bertazzoni *et al.*, 2018; Chen *et al.*, 2018; Frantzeskakis *et al.*, 2018; Stam *et al.*, 2018). In the grass symbiont *Epichloë festucae* TE-rich regions shape the 3D-structure of the host genome (Winter *et al.*, 2018). TE-rich and gene-poor chromosomes can show presence/absence polymorphisms in populations and are referred to as accessory chromosomes (Goodwin *et al.*, 2011; Croll *et al.*, 2013; Bertazzoni *et al.*, 2018). Interestingly, accessory chromosomes are observed in a growing number of fungal species (for review see (Bertazzoni *et al.*, 2018)). In some species such as the pathogen *F. oxysporum* these TE-rich accessory chromosomes also present signatures of rapid evolution (Fokkens *et al.*, 2018). In two-speed genomes, TE-rich compartments represent reservoirs for rapidly evolving genes involved in specific ecological niche adaptations and are often conditionally dispensible (Dong *et al.*, 2015; Faino *et al.*, 2016; Fouché *et al.*, 2018). For instance, lineage-specific regions enriched in TEs drive plasticity and adaptation in *M. oryzae* and in the wilt pathogen *Verticillium dahliae* (Thon *et al.*, 2006; Faino *et al.*, 2016). The potential role that TEs play in genome plasticity represents a source for rapid adaptation by generating phenotypic variation which is particularly important in a the context of rapid host plant-fungal pathogen co-evolution.

Transposable Elements are Activated Under Stress

TEs bursts or de-repression in response to stress are observed in various eukaryotes in response to environmental stress conditions or during different life-cycle stages (Voronova *et al.*, 2014; Romero-Soriano and Garcia Guerreiro, 2016; Hummel *et al.*, 2017; Fouché *et al.*, 2020; Pargana *et al.*, 2020). In plants and animals, TE-induced environmental adaptation is well described (Casacuberta and González, 2013), in fungi however little is known about the factors that can activate TEs. In the pathogen species *Ophiostoma ulmi* and in *A. oryzae*, transposition of *OPHIO* and *Crawler* elements are activated during heat shock stress (Bouvet *et al.*, 2008; Ogasawara *et al.*, 2009). During the early stages of fungal pathogens plant colonization, stress-dependent de-repression can lead to TE activity (Chadha and Sharma, 2014; Fouché *et al.*, 2020). In *Z. tritici* for instance, a recent study compared TE activity under stress conditions including nutrient deprivation and during the early stages of wheat infection (Fouché *et al.*, 2020). Specific *Gypsy* TE families were upregulated during early stages of wheat infection (Fouché *et al.*, 2020). However, the overall decrease of TE expression *in planta* over-time and the different expression profiles observed between isolates suggested a fine-tuned lineage-specific epigenetic regulation of TE activity during wheat infection (Soyer *et al.*, 2019; Fouché *et al.*, 2020). The impact of TE activity in plant-associated fungi during host colonization still remains largely unexplored but should be taken into account to decipher the molecular interplay during plant infection.

Locus-Specific Effects of Transposable Elements Insertions

Numerous studies have demonstrated that TE insertions modify gene transcription (**Fig. 1(B)**). Locus-specific modifications by TEs may impact phenotypic traits and confer a selective advantage to the host (**Table 1**). The phenotypic effects of TE insertions are highly diverse depending on insertions as described in **Table 1**. For instance, the evolution of carbohydrate-active enzymes (CAZymes) of saprophytes such as Ceratocystidaceae species is matched by concurrent TE expansions (Van Der Nest *et al.*, 2015). The ligninolytic fungal species *Pleutorus ostreatus* and *Phanerochaete chrysosporium* also exhibit TE-induced variation in ligninolytic genes expansion and expression (Gaskell *et al.*, 1995; Castanera *et al.*, 2016). Secondary metabolite gene clusters of plant-pathogenic fungi such as the broad necrotrophic pathogen *Sclerotinia sclerotiorum* are tightly associated with the presence of TEs

Table 1 Locus-specific TE mediated or TE-associated adaptation in fungi

Species	*Common name*	*Function/Gene affected*	*TE*	*Direct impact*	*Reference*
Blumeria graminis f.sp. hordei	Powdery mildew pathogen	EKA avirulence effector family	LINE retrotransposons domestication	Multigene family expansion	(Amselem *et al.*, 2015a)
Ceratocystidaceae	Plant pathogens and saprophytes	Glycoside Hydrolase GH32 family	Retrotransposon and Fot5 pogo-like element proximity	Multigene family expansion	(Van Der Nest *et al.*, 2015)
Cochliobolus heterostrophus	Corn pathogen	Cluster of polyketide synthase encoding genes	Proximity of TEs	Production of T-toxin in race T	(Santana *et al.*, 2014)
Epichloë festucae	Grass species endophyte	Alkaloids synthesis pathways	MITE insertions	Abolition of alkaloids synthesis	(Fleetwood *et al.*, 2011)
Fusarium oxysporum f. sp. lycopersisci	Tomato pathogen	SIX effectors	MITE insertions flanking genes	Specific association of MITE TE with effector genes	(Schmidt *et al.*, 2013)
Magnaporthe oryzae	Rice pathogen	Avr-Pita avirulence effector	Retrotransposon insertion upstream of Avr-Pita genes	Virulence gain	(Chuma *et al.*, 2011)
Phanerochaete chrysosporium	Wood decaying fungi	Lignin peroxidase gene lip1	Insertion of TE Pce1 upstream	Ligninolytic variation between strains	(Gaskell *et al.*, 1995)
Pleutorus ostreatus	Saprophyte	Ligninolytic and cell-degrading enzymes	Proximity of TEs	Ligninolytic and cell-degrading enzyme genes expansion	(Castanera *et al.*, 2016)
Pyrenophora tritici-repentis *Parastagnospora nodorum* *Bipolaris sorokiniana*	Wheat pathogens	Host specific toxin ToxA	LTR retrotransposon	Horizontal transfer enabling virulence acquisition	(McDonald *et al.*, 2019)
Ustilago hordei	Barley smut pathogen	Avirulence effector UHOR_10022	TE insertion in promoter region	Expression modification of UhAVR1p	(Ali *et al.*, 2014)
Verticilium dahliae	Vascular wilt pathogen	Avirulence effector Ave1	Repeat-rich region highly RIP	Horizontal transfer from host plant	(De Jonge *et al.*, 2012)
Venturia inaequalis	Apple tree pathogen	CYP51A1 fungicide resistance gene	TE insertion in promoter region	Overexpression of CYP51A1 gene leading to fungicide resistance polymorphisms	(Villani *et al.*, 2016)
Zymoseptoria tritici	Wheat pathogen	Multidrug efflux resistance gene MFS1	LTR retrotransposon insertion upstream	Overexpression of MSF1 and detoxification higher efficacy	(Omrane *et al.*, 2017)
Zymoseptoria tritici	Wheat pathogen	Transcription factor Zmr1 gene	LTR retrotransposon insertion upstream	Melanin accumulation variation	(Krishnan *et al.*, 2018)

close by (Graham-Taylor *et al.*, 2020). For plant-associated fungi, there is growing evidence that effector gene diversification is associated either directly or indirectly with TE activity (Fouché *et al.*, 2018). Indeed, several studies reported close physical associations of effector genes and TEs (Lorrain *et al.*, personal communication)(Rouxel *et al.*, 2011; Grandaubert *et al.*, 2014; Soyer *et al.*, 2014; Dong *et al.*, 2015; Fokkens *et al.*, 2018). Horizontal gene transfers are also mediated by TEs as demonstrated for the acquisition of the toxin-coding gene ToxA in the three wheat pathogens *Pyrenophora tritici-repentis*, *Parastagnospora nodorum* and *Bipolaris sorokiniana* (McDonald *et al.*, 2019). Some effectors directly derived from domesticated TEs such as the EKA avirulence effector family of *B. graminis* f.sp. *hordei* that originated from LINE retrotransposons (Amselem *et al.*, 2015a). TEs are key agents in fungal genome evolution and can generate raw material for rapid adaptation in pathogens involved in evolutionary arm's races with their host (Möller and Stukenbrock, 2017). However, we still lack direct evidence of TE recruitment and domestication by the host genome for specific adaptations.

Perspectives

Coevolution between TEs and the host genome have shaped fungal genome evolution. Decades of research aimed at unraveling the complex interplay between TE activity and defense mechanisms of the hosting fungal genome, including the fungal-specific RIP mutation mechanism. Yet, beyond few model system species such as *N. crassa*, the impact and coevolution of TEs and fungal genomes remain largely unexplored. Comparative genomics studies have described the extreme variability of TE content in fungal genomes (Raffaele and Kamoun, 2012; Amselem *et al.*, 2015b; Muszewska *et al.*, 2019). Nevertheless, the annotation of TEs for population-level analyses of polymorphisms requires high quality genome assemblies which remain challenging for numerous fungal species (e.g., Pucciniales) (Aime *et al.*, 2017). In fungi, studies of TE dynamics at the population level are particularly scarce. Oggenfuss *et al.* investigated TEs insertion dynamics in population of the wheat pathogen *Z. tritici* (Oggenfuss *et al.*, 2020). This study highlights that the detection of TE insertions in large dataset of short reads sequencing remains challenging but is indispensable to unravel TE-driven adaptation.

Experimental evidence of specific TE de-repression under stress conditions is very limited in fungi. Several approaches are possible to analyze TE activity under stress. First, transcriptional profiles of transposable elements in various conditions can be used as a proxy for transposition activation (Fouché *et al.*, 2020). Recent method development for TE-associated RNA-seq data analysis has allowed differential expression analysis of TEs (Jin and Hammell, 2018). Second, sequencing genomes resulting from experimental evolution represents a particularly useful approach to study TE activity. Various stressors such as temperature variation can be applied to different isolates from natural populations. Evolved populations can then be sequenced to assess TE structural variants by comparison to the ancestral genome. Further, TE-driven phenotypic variation among the evolved strains could be identified. Such experimental evolution approaches can also be extended to study the potential fitness costs of transposition regulation mechanisms. Mutants lacking genes involved in TE-regulation mechanisms such as RIP or TE-targeted histone modifications can be used to assess (1) efficacy of the different host defense mechanisms as done in (Möller *et al.*, personal communication) and (2) the fitness cost of highly efficient host defense mechanisms (Galagan and Selker, 2004). Future research should address the impact of TE activity in rapid adaptation of fungi, which is particularly relevant in the context of evolutionary arm races established between fungal pathogen species with their host plants.

An important goal of evolutionary biology is to understand how genotypes and phenotypes are linked. In the post-genomics era, studies have focused on variation at the single nucleotide polymorphism (SNPs) level to explain phenotypic variation. However, SNPs alone can only explain a fraction of the phenotypic variations. Structural variants and transposable element have proven to significantly contribute to phenotypic variation in fungi (de Jonge *et al.*, 2013; Oggenfuss *et al.*, 2020). Both genomics and functional analyses of TE-driven phenotypic effects underpin the importance of TE dynamics in generating phenotypic variability and rapid adaptation to new environments, response to stress conditions or artificial selection pressure (e.g., fungicides; **Table 1**). Thereby, high-throughput methods are needed to investigate the biological impact of TE insertions at a larger scale. (Akakpo *et al.*, 2020) proposed to use a TE genome-wide association (TE-GWAS) approach to detect TE-associated genetic factors associated with variation in life-history traits. For this, TE-GWAS methods and validation criteria need to be adapted to the low allele frequency of TE insertions. In rice, such an approach revealed that specific low-frequency LTR-retrotransposon insertions are associated with grain width variation (Akakpo *et al.*, 2020). TE-GWAS approaches could be particularly relevant for the high-throughput identification of TE-driven phenotypes in fungi.

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Aspergilli, More Than Just Fungi: Shaping the Last Decades of Model Systems

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Introduction

According to the Nobel Prize winning physiologist August Krogh's famous admonition, "A convenient organism exists for every biological problem"; however, since we are warned that we are unlikely to ever know everything about every organism, we should agree on some convenient organism(s) to study in depth. This promoted, over centuries of research, the construction of a body of knowledge in that 'model system' that allows us to design appropriate studies of non-model systems to address important questions about their biology. The *raison d'être* of model organisms is generally thought for the study of a specific area of investigation: biological systems that are simple to employ and control obviously facilitate the experimental work, being accessible and, therefore, "convenient". That's why, traditionally, only a few organisms have been widely used with this ambition: a simplified, manageable system that could be used to study a larger theme of biology, revealing not so much a feature of the system itself. The decision to focus on a particular species (or strain, or variant) for biological research is commonly guided by features focused on the interrelation of various criteria, that in 2019 have been efficiently investigated and clustered in the paper "How to choose your research organism" (Dietrich *et al.*, 2020): this process of selection involves a high degree of variability in terms of how such criteria are combined in every instance of system choice, which implications to take into consideration embrace a range of factors including those that are more material, such as access and tractability (in terms of ease of supply and ethical considerations, standardization and responsiveness efficacy), socio-political concerns (such as various economies in terms of financial and institutional support), available resourcing (including epistemic resources such as previous theories), and promise for the future (as novelty and translational potential).

Nonetheless, the modern world poses modern questions, for which the modern science needs modern, advanced tools, encouraging researchers to extend the set of model organisms to include less-studied and more unusual systems: it's easy to argue that appeals to "convenience" are not sufficient to capture reasoning about organism choice. The transition from a model to non-model organism might not be so immediate, for researchers who intend to face the challenge: the ease in using model systems over years led to the development of specific tools and resources for these organisms (techniques and methods, databases and strain collections, molecular toolkits), that in turn made them even more convenient to be used, discouraging others to work on everything else. It was suggested that the gap in methodology and resources between the selected model organisms and other systems resulted in a linguistic shift in how the term "model organism" was understood, from its original sense to "an organism for which a wealth of tools and resources exist" (Russell *et al.*, 2017). In any case, arriving at a certain point it was necessary to admit that, even if undoubtedly suitable for studying various and fundamental aspects of biology, the major model organisms weren't automatically the best systems for all possible questions of general importance. Exploration of organisms (in some cases, different species) as potential new model systems, to ask central questions about biology that have still remained unanswered, is crucial for the progress in knowledge.

The short life cycle, the small size and the easy handling of some microorganisms make them inexpensive subjects for a wide range of studies; additionally, the opportunity of genetic manipulations offers scientists ample prospects to test their theories, unraveling molecular and metabolic mechanisms. Despite indubitable advantages, the downside of using a model is that it is nearly impossible to predict whether this particular system could be representative of another organism; thus, one major difficulty in choosing the adequate model for a specific biological question is the extrapolation of valid results for the real target organism. Being increasingly supported by scientific evidence, it has been argued that while some mechanisms are unique, others similarly operate across different species. This implies that since the basic blocks of life are common to the most of known species, common processes might involve same pathways, that in turn might share same cellular/molecular knots.

Recently spotlighted by Sheldrake's (2020) book "Entangled Life", that explores its potentialities in changing our understanding of life mechanisms the Kingdom Fungi represents an unparalleled source of model organisms for all eukaryotes: although microbial and unicellular systems, and therefore rather easy to manipulate and modify genetically, fungi are eukaryotic cells, sharing a high degree of similarity with animal cells; additionally, the relevant databases are extended and contain the most complete genetic information to date. Of all its divisions, ascomycetes have had the biggest impact on biology, and, in particular, the genus *Aspergillus*, that includes more than 350 known species of filamentous fungi displaying great differences in habitat, pathogenicity, and metabolic properties. For all the *Aspergillus* species, shared data resources have grown exponentially, now including an increasing amount of contents at fungiDB (Stajich *et al.*, 2012); additionally, resource collections like the American Type Culture Collection (ATCC), the Centraalbureau voor Schimmelcultures (CBS) and the USDA NRRL collection, provide open access to well-characterized research materials. Over 1600 projects using *Aspergillus* (containing transcriptome and genome sequences of diverse species, as well as multiple projects with the reference genome strain FGSC A4) are found in the NCBI Short Read Archive (Pontecorvo *et al.*, 1953), while, due to their industrial role, there is a significant amount of data regarding applied use of these organisms: over 11,500 patents include "Aspergillus", with over 1300 solely in 2013.

Various and detailed are the reasons that made *Aspergillus* spp. an ideal model system for studying the mechanisms controlling development and cell differentiation in multicellular eukaryotes, being also well suited for analyzing biochemical events that characterize the cell cycle and metabolism: after all, the word "fungus" shares the root with the Latin verb "*fungor*" (-, *fungĕris*, *functus*

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sum, - , *fungi*), which meaning, "to exercise an office or fulfill a function on behalf of someone", perfectly fits with the use that we are here to discuss. Therefore, we'll try to shape a comprehensive and likely exhaustive survey about the progress of this success story.

What *Aspergillus* Taught us About Eukaryotic Genome Regulation and Maintenance

Probably the most critical process for the genome maintenance is the DNA repair: interlinking with replication, gene transcription and cell cycle checkpoints to promote cell survival and normal regulation of cellular functions after DNA damage, the repair system represents a protective mechanism that ensures the maintenance of genomic integrity during the cell life; when unrepaired or incorrectly repaired, DNA damage can cause a loss of genetic information, or, due to an uncontrolled accumulation of mutations, a genomic instability that results in the cell cycle arrest and cell death. Amongst fungal species, *Aspergillus nidulans* has been extensively studied with respect to metabolism, cellular development, and regulation, since the ability to genetically manipulate this organism, and combine genetic traits by sexual crossing, has been crucial for its pronounced role as genetic model organism. In 2004, Goldman and Kafer reviewed the history of *Aspergillus nidulans* as the object of studies focused on three different cellular processes now identified as essential components of the complex phenomenon of DNA damage response (Goldman and Kafer, 2004). Comparative tests conducted in *Aspergillus* mutants and the analysis of genetic control of their cell cycle, revealed key checkpoints that regulate every step to guarantee the completion of DNA replication and its repair before entry in mitosis. Overall, the possibility to visually distinguish specific aneuploids and other aberrations, identifiable by the segregation of heterozygous markers, made *Aspergillus* exceptionally suited for analysis of genetically unstable types, allowing for example the meiotic mapping of translocation breaks with recognition of the four types of partially aneuploid progeny being crucial, or malsegregation producing trisomics and other aneuploids (Kafer, 1977).

A stress-induced genome instability has been recently underlined, in lead studies focused on the adaptive mechanisms of fungal pathogens, as a key event during the onset of drug resistance: this probably allows the pathogen to promptly manage the continuous changes in the environment it encounters in the host, determining in many cases an increase of the microorganism surviving chance against the fungistatic or fungicidal activity of anti-fungal drugs (Galhardo *et al.*, 2007). In this sense, investigations on heterochromatin structure and function in fungal model systems could serve as a road map to elucidate the role of chromatin in regulating genome plasticity in plant and animal fungal pathogens. Studies performed in various *Aspergillus* species revealed that genetic diversity associated with subtelomeric regions might be important for driving adaptation to different host niche environments, mainly due to its modulatory effect on secondary metabolites gene clusters; these findings are considered to be valid also in *A. fumigatus*, the major etiological agent of invasive pulmonary and cutaneous aspergillosis, whose successful infection largely depends on the ability of the fungus to overcome the cellular response in the surroundings of the infection site (in particular the activation of phagocytes), achieving to adapt to a multiplicity of host-niches environments (Buscaino, 2019 and reference therein).

Besides virulence characteristics, many aspects of the fungal morphogenesis have been proven to be regulated by chromatin remodeling: according to evidences obtained in *A. flavus*, in fact, the production of reproductive, dispersal or resistance structures (such as conidia and sclerotia) were demonstrated to be influenced by the methylation of specific sequences in the genomic DNA (Liu *et al.*, 2012; Yang *et al.*, 2016), as well as the regulation of small bioactive molecules, whose biosynthesis depends on specific gene clusters located throughout the genome and that are critical for the interaction of Aspergilli with other organisms, enabling filamentous fungi to successfully exploit environmental resources by modifying chemical diversity (Shwab *et al.*, 2007).

Drug Resistance Runs Rampant

Genus *Aspergillus* contains a large number of both pathogenic and opportunistic species, that potentially cause mycosis in humans and animals: for example, *A. fumigatus* is responsible for the 90% of human fungal infections, followed by *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans* species. Due to the mortality rate of Aspergillus-related allergic syndromes and invasive aspergillosis (that can range up to 90%), the escalation of fungal drug resistance is a growing concern in the human health context (Morgan *et al.*, 2005). To achieve insight into the mechanism of action of antifungals resistance in *Aspergillus* species remains quite simple, as the occurrence of drug-resistant isolates is frequent and they can straightforwardly be studied. Besides the classical mechanisms that mediate the development of drug resistance phenomena, typically based on the modification of drug targets (owing to mutations which lessen drug-target interactions), the loss of drug effectiveness (due to increase in drug efflux or overexpression of drug targets) or metabolic bypasses (through the activation of compensatory mechanisms which abolishes the drug toxic effect), new mechanisms were recently observed in Aspergillus spp., including the emergence of simultaneous resistance to more than one class of drugs (Sanglard, 2016). Reviewing the literature concerning proteome changes in *Aspergillus* isolates exposed to antifungals and phytochemicals, it could be noticed that factors responsible to provide resistance seem to be mainly enzymes involved in cell wall remodeling, oxidative stress response and energy metabolism (Shishodia *et al.*, 2019); over 70 proteins have been found up-regulated during interaction with different antifungal agents, while at least 26 showed to be affected, in their expression level, by more than two antifungal agents, suggesting a wider response. Globally, available data suggest that alterations in drug targets is the most common strategy for resistance against fungicides in Aspergilli; however, the role of extracellular matrix has been recently associated to the development of resistance in biofilm-forming fungi: the formation of matrix in biofilm structure has been hypothesized to sequester antifungal molecules and reduce the drug susceptibility *via* the activation of multidrug resistance protein that pumps out noxious compounds (as observed for specific efflux-pump in azole resistance and described by Rajendran *et al.* (2011)) and Rajendran *et al.* (2011), disallowing their diffusion

through fungal hyphae. This unexpected cue do not only offers possible reasons for treatment failure in aspergillosis cases, but, showing interesting absorption properties of fungal biofilm, might also suggest new applications for them.

Antifungal agents are also used in crop protection, to defend plants from pathogens attack and to preserve food/feed commodities from fungal decay. In particular, the control of mycotoxigenic fungi diffusion by the use of synthetic fungicides is still the most effective among the good agronomical practices, whose positive effects are appreciable both in field and during storage (Rose *et al.*, 2018). The problem of mycotoxins exposure of consumers raises a serious concern for animal and human health, worsted by the carry-over of certain toxins along the food chain (International Agency for Research on Cancer, 1993; Fanelli *et al.*, 2004). The global warming will impact on disease and pest invasion of staple crops; therefore, a dramatic increase of mycotoxins contamination driven by the climate change is a realistic scenario (Medina *et al.*, 2015). On the other hand, in order to limit the emergence of acquired resistance in relevant fungal species is imperative to reduce the use of antifungals in the environment, identifying at the same time new generation fungicides effective against resistant isolates. Screening based on the use of *Aspergillus flavus* for the assessment of the fungistatic or fungicidal activity of newly synthesized compounds have been developed (Zani *et al.*, 2015; see "Relevant Websites section"): through the development of a simple and high-throughput procedure for the evaluation of mycelium growth and mycotoxin production, associated with toxicity, genotoxicity and *epi*-genotoxicity assays in human cell and plant model systems, a battery of over 200 new compounds was tested; data were organized in a Q-SAR database, that correlating chemical structures and biological/toxicological activities is projected as a tool for users searching for specific bioactives, or to simply predict the potential effect of similar compounds.

What About the Contribution of *Aspergillus* in the Comprehension of Cell Biology and Eukaryotes Development?

Fungi have been long time used to study the progression and regulation of mitosis, meiosis or organelle movement, revealing valuable insights into these evolutionarily conserved processes and emphasizing the correlation between cell biology and differentiation processes in eukaryotes. Aspergillus development and morphogenesis studied at a molecular level endorsed the individuation of genes specifically involved in the regulation of basic cellular events: using temperature sensitive *nimA* mutants of *A. nidulans*, Lies and co-workers suggested the presence of a novel, late G_2 checkpoint, that prevent the inappropriate entry into mitosis by the control of the activation of BIMAAPC3, an anaphase promoting complex/cyclosome (APC/C) component (Lies *et al.*, 1998). *A. nidulans* also provided important cues about dynamics of actin during cytokinesis, since its septation process superficially resembles cytokinesis in animal cells; studies performed in the filamentous fungus demonstrated that an intact mitotic spindle is required for actin ring formation, and that a mitotic checkpoint may prevent further progression of cytokinesis in the absence of an intact mitotic spindle (Bruno *et al.*, 2001; Momany Morrell-Falvey *et al.*, 2011).

The life of eukaryotes is, with few exceptions, characterized by developmental processes more or less involved in specific pathways of secondary metabolism, where the term "secondary", introduced by Kossel in 1891, implies that primary metabolites are present in every living cell capable of dividing, while secondary metabolites are present only incidentally and without any central significance for the organism. Actually we know that's not true, or, at least, it could be true with this restricted interpretation: a large number of physiological, morphological and developmental processes have been demonstrated to be linked to (when not depending on) specific secondary metabolic pathways. Compared with primary metabolism reactions, which are highly conserved over highly different organisms, a huge diversity is observed in secondary metabolism pathways at the level of species, organs, tissues, and single cell, and even at different developmental stages; the environmental advantages represented by the availability of a wide range of extraordinarily different compounds from secondary origin and its high level of catalytic promiscuity are thought to be due to its recent divergence from primary metabolism and to a weaker selection pressure than to primary metabolic enzymes (Tokuriki *et al.*, 2012). A modern conception of "secondary metabolites" defines them as bioactive molecules of low molecular weight that are not specifically required for growth of the organism, but aid survival in harsh conditions, providing resistance against desiccation and UV-derived stress (Nguyen *et al.*, 2013) and improving competition with other organisms: namely, an arsenal of small and versatile compounds that, impacting the environmental fitness, necessarily interact with primary processes.

In this sense, *Aspergillus* spp. embody one of the major examples of variety and diversity (Romsdahl and Wang, 2019), representing excellent model systems for research in metabolomics and comparative phylometabolomics, as comparative studies of conserved and unique metabolic pathways will help in the annotation of metabolites as well as provide important new targets of investigation in biology, biomedicine and industry. For example, the genomic analysis on *A. oryzae*, an important industrial mold used for fermentation purposes, indicated in this specie a large number of putative biosynthetic genes for secondary metabolites, even if many of such compounds have not been identified yet: through the screening of a gene-disruption library of transcription factors including chromatin-remodeling agents, two genes were find to be involved in similarly altered patterns, contributing to a better understanding of secondary metabolites production not only in *A. oryzae*, but also in other fungal species (Shinohara *et al.*, 2016).

Some secondary metabolites are known to overcome the host immune system by affecting immune cell function or by shielding the pathogenic fungus against host defense, or being useful to acquire essential, scarce cofactors, as well demonstrated by the plethora of studies on the human pathogen *A. fumigatus* (Raffa and Keller, 2019). Consistently, the increased interest in secondary metabolites has triggered a number of genome sequencing projects to investigate as well as elucidate their biosynthetic pathways, leading to the conclusion that the number of secondary metabolite gene clusters (SMGCs) greatly outnumbers characterized compounds, challenging current methods to dereplicate and categorize this amount of gene clusters on a larger scale. A detailed analysis has been conducted on *Aspergillus* section *Nigri* to investigate species similarities on the SMGC content level and genetically dereplicate gene clusters: using secondary metabolite gene cluster networks, SMGCs were categorized across genomes

into SMGC families, allowing to highlight, through the dynamics of secondary metabolism in section *Nigri*, that SMGC diversity within the section has the same magnitude as within the genus; when validated to find gene clusters for analogous compounds in newly sequenced genomes, this particular genome analysis resulted successful to predict of the gene cluster responsible for biosynthesis of malformin, an enhancer of anti-cancer drugs, in 18 strains (Theobald *et al.*, 2018).

To date, despite the importance of the metabolomic investigation, there are few databases that provide complete, species-specific information, and even fewer on secondary metabolism and secondary metabolites of biological relevance; with this goal, the open resource A2MDB Aspergillus metabolome repository (Aspergillus Secondary Metabolites Database; see "Relevant Websites section") has been recently created: it collects experimental metabolomic data, cataloged and annotated with literature information, and reports over 800 unique non-redundant secondary metabolites (including mycotoxins) derived from 675 *Aspergillus* species (Vadlapudi *et al.*, 2017). Together with a phylogenetic representation of over 2500 strains and 150 microscopy images of several *Aspergillus* species, the database provides a compilation of 44 secondary metabolic pathways and 100 cellular targets of secondary metabolites, along with all the available molecular docking models of metabolite-target protein interactions.

The Evolutionary Dynamics Explained

Countless are aspects of cell biology under the magnification lens of the evolutionary investigation, but phosphorylation is probably one of the most intriguing, since approximately the 30% of cellular proteins are phosphorylated (Cohen, 2000). Potentially interesting either the DNA or the protein level, phosphorylation may exert different physiological implications for the cell: phosphate groups can change the structural backbone of the DNA, serving as regulators for epigenetic control through the transient activation of defined phases of gene transcription such as initiation, elongation and termination; contextually, as for other post-translational modifications, protein phosphorylation is commonly required for the correct carrying out specific function, whether structural or catalytic. Intra- and intercellular signaling pathways also are affected by the phosphorylation status of the cell, without considering a huge amount of biochemical and biophysical aspects occurring in the cell cycle and involved in the development and reproduction. Hence, questions about the role of phosphorylation/dephosphorylation as a possible trigger for the evolution of eukaryote organisms reasonably would arise: did the natural selection force specific phosphosites over others? How this selection could have been determined, among all the available protein motifs potentially suitable as sites for inserting a phosphorus atom? Could the phosphorylation patterns be used for phylogenetic purposes, aimed for example at clarifying the species origin? While to date we are still lacking a real phylogenetic understanding of phosphosite evolution, at the same time the question could be faced from another side of the coin, that is represented by the analysis of the enzymatic ensemble responsible for the kinase cascades inside the cell environment, since phosphoregulatory networks evolve by the gain or loss of protein-protein interactions, either by changes to substrates, or by changes to kinase specificity. A functional characterization of the kinome and its control was conducted, in 2011, in the plant pathogen *Fusarium graminearum*: authors generated deletion mutants for 96 protein kinase genes, assaying all the resulting knockout individuals for changes in 17 phenotypes (including growth, reproduction, stress responses, and plant infection). At least half of the mutants resulted non-pathogenic or significantly reduced in virulence, while 26 were blocked in ascospore production, that represent the primary inoculum for wheat scab induced by Fusarium species. A number of kinase genes that proved to be unessential for hyphal growth were instead suggested to encode novel fungal virulence factors. (Wang *et al.*, 2011). De Souza *et al.* reviewed and extended in *A. nidulans* the characterization of filamentous fungal kinases from a phylogenetic perspective; their final report underlined kinases in regulating ribosomal biogenesis, mRNA splicing and unfolded protein response, as well as revealing previously unknown roles for kinases in the regulation of hyphae elongation and septation, polarized growth, cell cycle, development, secondary metabolism, DNA damage response, and the cellular response to osmotic stress (De Souza *et al.*, 2013).

Once more in *A. nidulans* it was possible to identify both direct and indirect targets of protein kinase A (PKA) phosphorylation, as well as genes whose expression relies on its functionality; besides providing a broad overview of the PKA regulatory network in a model filamentous fungus, these results would facilitate detailed functional investigation of PKA phosphorylation events and their role in growth and development (Ribeiro *et al.*, 2019).

The human pathogen *Aspergillus fumigatus*, leading infectious killer in immunocompromised patients, served to define an evolutionarily conserved novel mode of regulation of calcineurin, a calmodulin CaM-dependent protein phosphatase, by phosphorylation, thanks to the study of a serine-proline rich region (SPRR) located in a region absent in humans; findings suggest the possibility of harnessing this unique SPRR for innovative antifungal drug design to combat invasive aspergillosis (Juvvadi *et al.*, 2013). The phosphorylation status of calmodulin was also demonstrated to be critical for the secondary metabolism of Aspergilli: the first study on the phosphoproteome of *A. flavus* demonstrated that phosphorylation is tightly connected with the biosynthesis of aflatoxin through the regulation of the acetyl-CoA flux, and provided the first evidence linking the phosphorylation of MAP kinase with growth, conidial production and sclerotial formation in this phytopathogenic species (Ren *et al.*, 2016).

Spilling the Beans on the Antioxidant Potential of Bioactives

Oxygen, the essential element for life as we know on Earth, triggered the evolution of organisms from single entities to specific cells within multicellular systems; studies show how organisms containing the most different cell types evolved following the increase of atmospheric oxygen, giving rise to the complex life we can witness today (Hedges *et al.*, 2004). But the price for an aerobic life is

the inevitable oxidation of molecules: by offering an incredible advantage for cellular energetics by supporting high-efficiency production of ATP, the oxygen-based respiration forces in turn to deal with reactive forms (Reactive Oxygen Species) such as superoxide, hydroperoxides, hydroxyl radicals, that promote the progression of oxidative stress at a cellular level. In eukaryotic cells, oxidation and associated events are contributing-cause of aging in general, and in particular of many human diseases; atherosclerosis, Parkinson´s and Alzheimer´s diseases, skin disorders and cancer are outstanding examples. Hence is not surprising the rampant use of antioxidants in order to achieve and preserve optimal health: nutraceuticals and food supplements are frequently fortified with antioxidants compounds from both synthetic and natural origin, and cosmetic Companies compete in claiming, in their formulation, the presence of bioactives which promise to protect the consumers from undesirable effects of ROS. By definition, "antioxidant" is every substance that, at low concentrations, prevent or retard the oxidation of oxidizable biomolecules, such as lipids, proteins and DNA (Halliwell and Gutteridge, 2007). Actually, plants are the main source of naturally-derived molecules used with these purposes, partially due to the biological ability of secondary metabolites (such as phenolic compounds, vitamins C and E, and carotenoids) to be potent free radical scavengers: anti-inflammatory, antimicrobial, antiviral, antiallergic activities, as well as inhibition of lipid peroxidation, have been proven (Ndhlala *et al.*, 2010 and references therein).

Driven by the need for more effective, less toxic and cost affordable antioxidants, a great number of *in vitro* methods have been developed to measure the efficiency of natural compounds either as pure compounds or as plant extracts. As these bioactive are predominantly reducing agents, singlet oxygen quenchers or metal chelators, *in vitro* classical techniques are chemical-based methods, characterized by high speed and sensitivity, and historically grouped according to the mechanism of action: Oxygen Radical Absorbance Capacity (ORAC), Total radical trapping antioxidant potential (TRAP) and β-carotene bleaching methods rely on hydrogen atom transfer reactions, while Trolox Equivalent Antioxidant Capacity (TEAC), Ferric Reducing Antioxidant Power (FRAP), α, α-diphenyl-β-picryl-hydrazyl Radical Scavenging Assay (DPPH), Total Phenol Assay and others are based on electron transfer reactions.

However, the complex nature of phytochemicals, which effect often seem to be a result of synergism/cooperation with other compounds, the objection that *in vitro* chemical assays are based on free radicals usually not found in living cells, and the comprehension that the complexity and heterogeneity of biological systems do not allow anything to work in isolation (if a redox reaction takes place, then something else must occur to balance electrons in the cell environment) (Shen *et al.*, 2007), supported the ever increasing criticism about the real predictability of such inferred antioxidant capacity. Overall the dichotomic behavior of antioxidants in terms of incoherence between *in vitro* and *in vivo* activity, that was tentatively explained with the heterogeneity of biological systems and the interactions between scavengers and surrounding molecules in the cell environment, has had important implications for updating the current strategies for screening and designing antioxidant drugs. Additionally, not all methods and antioxidant species are compatible: as a consequence, the same compound can yield dissimilar results in different assays. The complementary use of methods employing different mechanisms of action could be recommended to evaluate plant extracts or pure compounds, but eventually, for bioactive purposes, *in vivo* models remain extremely desirable: in fact, even though authors usually find a good degree of correlation between *in vitro* scavenging activity and the total phenolic content, the association between *in vitro* and *in vivo* methods is still debatable; the huge amount of data obtained by merging *in vitro* and *in vivo* results is dotted by often controversial results, since some research outcomes might led to the persuasion that the majority of so-called antioxidants exist as legendary biomolecules.

Whenever possible to perform, *in vivo* investigations are undoubtedly more reliable to assess both potential for interaction as well as mechanisms by which various natural products may interact, for example, with cellular enzymes, membrane transporters or metabolic pathways (Granato *et al.*, 2018). To date, the existing knowledge about the responsible phytochemicals for the biological activity, their mechanisms of action and even the occurrence of biochemical inter-relations, is substantially scarce, while one of the main important cues ofstudies on oxidative stress is to define – at the molecular level – the cellular processes involved in cell sensing, signaling, transduction and the adaptive responses.

Compounds with antioxidant properties frequently display also interesting biological activity, such as antifungal potential and modulatory effects on fungal secondary metabolism (Palumbo *et al.*, 2007; Gacem *et al.*, 2019), so that, an easy-to-handle specie which developmental and metabolic features remain well known is a good candidate to become an excellent model system, as well as the possibility of using high-throughput small-scale *in vivo* model systems to predict a possible antioxidant-related biological activity of compounds (pure or in mixture) and natural extracts such as botanicals before their medical application is therefore desirable (Reddy *et al.*, 2010). Studies comparing antioxidant potential and mycelium growth and/or development in *Aspergillus* spp. date back to decades. Reactive oxygen species (ROS) in particular, and their reactive products such as peroxidized lipids, have been long time associated with fungal stress responsive signaling and secondary metabolite production, stimulating *in vitro* the accumulation of aflatoxin by *Aspergillus flavus* mycotoxigenic strains; conversely, many compounds with scavenging properties exert a containment effect on the biosynthesis of the toxin (Jayashree and Subramanyam, 2000; Passone *et al.*, 2005), mainly through the activity of enzymes involved in oxidative stress alleviation (catalase, glutathione peroxidase, superoxide dismutase); additionally, antioxidant compounds inhibiting aflatoxins have been demonstrated to frequently impair also sclerotia development, probably due to the presence of several, shared regulatory steps (Fountain *et al.*, 2016). The response to redox balance alterations of sclerotia metabolism and aflatoxin production, that was suggested to have been evolved to cope with an excess of intracellular ROS produced in the late phase of growth during morphological and metabolic transitions, have been profitably exploited in recent years to evaluate the antioxidant capacity of both single molecules and natural extracts with the attempt to predict, through a microbial-based *in vivo* assay, biological activities with further potential application in different fields. In this regard, *A. flavus* has been used to investigate a wide number of plant extracts (Reddy *et al.*, 2009; Degola *et al.*, 2019) and newly synthesized molecules (Rogolino *et al.*, 2017), and the development of a high-throughput methodology, based on micro-cultures in multiwell plates and a fluorescence-based, real-time detection of aflatoxins directly in the culture medium have significantly

increased the performance of *A. flavus* (and related species) as model system for such purposes (Degola *et al.*, 2017; Bisceglie *et al.*, 2020; Orsoni *et al.*, 2020).

A Stairway to System Biology

We previously disserted about the great contribution that research on *Aspergillus* is providing to our understanding of eukaryotic primary and secondary metabolism, control of gene regulation by external environmental factors, cell cycle and development; from a clinical perspective, one of the most relevant examples of the value of *Aspergillus* as a model could be found in the identification of the genetic basis of the human metabolic disease alkaptonuria, determined by genetic errors in tyrosine/phenylalanine metabolism: taking advantage of the ability of *Aspergillus* to grow in presence of any single aminoacid as sole carbon source, researchers succeeded in identifying the fungal enzyme and, by using its amino acid sequence, to isolate its human, disease responsible counterpart (Fernández-Cañón *et al.*, 1996). Besides infinite, single examples, the availability of such enormous amount of data obtained in *Aspergillus* species through omics technologies (like genomics, transcriptomics and proteomics) will allow a more comprehensive understanding of genetic traits governing desirable properties, from enzymes production to the pathogenic potential, easily converging in an advanced tool for the systems biology research; in this sense, CRISPR/Cas9 technology will accelerate data generation, speeding up genetic research in the Aspergilli field, and, in turn, an increase in systems biology targeted exploration (Nødvig *et al.*, 2015). The efficiency of targeted genetic manipulation has already proved to enable the assessment of a large number of modifications in a high-throughput manner; therefore, by exploiting this gain in strain development will speed up the elucidation of pathways both in primary and secondary metabolism, as well as enabling the development of genome-wide screens (*e.g.*, knockout libraries) as has been performed in other model systems (Fuller *et al.*, 2015; Katayama *et al.*, 2016).

The increase in throughput of data generation will lead to future challenges in the data handling, integration and interpretation, but today it certainly offers the possibility to acquire a paramount overview of biological knowledge as never before: the consciousness that no element of a cell is an island, but everything is necessarily connected to the rest, has carried systems biology to a holistic approach, in the attempt to conceive (and thus comprehend) life as multilevel systems that have co-evolved, instead of the simple sum of its parts. The recent publication of the genome sequences of several *Aspergillus* species, has, alongside the increase of reductionist studies, been a catalyst for the application of systems biology approaches to this group of fungi; more than 50 laboratories around the world are currently devoted to Aspergillus research, as well as various are the websites dedicated (see "Relevant Websites section"): this new analytical and computational tools have been designed and a systems biology approach has been applied to a wide range of issues.

Over all species belonging to its genus, *Aspergillus niger* probably possesses the most impressive variety of high-yield products, holding the potential to become an even more versatile cell factory platform for industrial microbial purposes and bio-based economy in the future; thanks to the availability of its genome sequence, a high potential for the mining of new products has been suggested, but only an integration of a full genome-scale metabolic network with genomic annotation will allow system biology applications. With this ambition an accurate model has been recently created on the basis of a complete bibliomic survey of the literature on *A. niger*: a complete catalog of all reported intracellular enzymatic activities was used to build a comprehensive database of the entire metabolic network, providing a link between reactions, genes and scientific literature and, hence, being useful for several uses, such as identification of targets for metabolic engineering, interpretation of transcription data and identification of regulatory features and metabolic flux analysis, for the systemwide examination of data in a metabolic context (Andersen *et al.*, 2008). To date, achievements in the field has definitely highlighted the impact of Aspergillus systems biology on industrial biotechnology, making possible to implement systems biology tools to advance metabolic engineering.

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University of Brescia and University of Parma, Italy.

Proteomics in Mycorrhizal and Plant Pathogenic Fungi

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Introduction

Protein represents the molecular product of the genes as the final step of the translation process, whose expression changes according to cellular and environmental conditions. The term "Proteomics" was first coined in 1997 (James, 1997) because of its assonance with genomics, the study of genes, and it was initially defined as a technique for global characterization of all the components of the proteome, which in turn represents the entire set of proteins expressed by an organism.

A better definition of the term Proteomics includes the large scale study of the complete protein asset of a cell, tissue, or organ (Tyers and Mann, 2006; Pandey and Mann, 2000), which is expressed in a particular moment under different environmental conditions (Graves and Haystead, 2002), together with its cataloging (descriptive proteomics), its abundance (quantitative proteomics), genotype-dependent variations (population proteomics), the implication in the development and environmental responses changes (differential or comparative proteomics), post-translational modifications (PTMs), and interactions with other proteins and molecular entities (interactomics) (González-Fernandez and Jorrín-Novo, 2012).

The proteome of an organism represents a dynamic system, with significant variations among different cells and tissues, and development time. During the translation process, not all the mRNA is usually converted into proteins, and the mRNA levels are not always correlated to protein content (Rogers *et al.*, 2008; Zhang *et al.*, 2010, Vogel and Marcotte, 2012). As a result of the differential gene expression among cells and tissues, proteomics could offer a fingerprint of cell, tissue and organ state in a particular time frame.

Proteomics could be used to complement other functional approaches (Tyers and Mann, 2006), including transcriptomics (the study of mRNA), genomics (the study of genes) and metabolomics (the study of metabolites); the integration of all these approaches is called *system biology* (Horgan and Kenny, 2011), representing the study of biological systems whose behavior cannot be reduced to the linear sum of their parts' functions. In this view, proteomics represents a reliable technique due to several aspects since proteins are directly related to function or phenotype of biological systems (Bhadauria *et al.*, 2007). They go through a series of modifications, also called post-translational modification (PTM), which affects their functions. Over 200 forms of PTM are currently known (Zhang *et al.*, 2010; Virág *et al.*, 2020), including phosphorylation, methylation, glycosylation, and sulfation. Historically, single proteins could be separated by a wide range of techniques, like chromatographic technique enzymatically characterized and subjected to N-terminal or partial amino acid sequence analysis (Doyle, 2011).

Quantitative proteomic analysis can be classified into two groups, either gel-based or gel-free methods as well as "label-free" or "label-based" (**Fig. 1**); the latter can be further subdivided into several labeling approaches such as chemical and metabolic labeling (Abdallah *et al.*, 2012).

The gel-based approach is mainly based on the use of SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis) or the 2-DE (two-dimensional electrophoresis). SDS-PAGE is a common technique used for protein separation based on their molecular weight, which was firstly developed by Laemmli (1970).

The 2-DE technique allows the separation of proteins based on their molecular weight and isoelectric point (pI) to resolve complex protein leading to high-resolution fingerprint. Since the publication of O'Farrell (1975), the 2-DE technique represents a powerful tool for the analysis and detection of proteins from complex biological sources. The introduction of immobilized pH gradient (IPGs) instead of the carrier ampholytes (CA) for the first dimension has been allowed to overcome most of the limitation of the 2-DE technique, including reproducibility, resolution, and ability to separate very acidic/basic proteins (Görg *et al.*, 2004). The 2-DE gel images are then acquired and used for quantitative analysis through imaging software for measuring spot intensities (Dowsey *et al.*, 2003).

Nevertheless, some shortcomings still affect the use of this technique, limiting its application. These issues include a limited dynamic range of detention, the exclusion of some classes of proteins (e.g., too acid or basic proteins), as well as the limitations to soluble and abundant proteins (Görg *et al.*, 2009; Chevalier, 2010). 2-DE also represents labor-intensive, which is challenging to automatize (Wang *et al.*, 2005).

In this view, the gel-free approach has progressively replaced the gel-based approach in many research areas due to several advantages, including reproducibility and the ability to study specific classes of proteins (Abdallah *et al.*, 2012; Jorrín-Novo *et al.*, 2015). Initially, these approaches were used as a replacement for 2-DE (Abdallah *et al.*, 2012). However, most recent proteomic papers were only based on gel-free analysis or used both approaches as complementary (Jorrín-Novo *et al.*, 2019). Gel-free studies consist of two main steps peptide separation and MS identification; moreover, also intact proteins can be analyzed using a gel-free approach (Tran *et al.*, 2011; Toby *et al.*, 2016).

The first step includes a wide range of techniques, like the Ion-exchange chromatography (IEC), the reverse phase chromatography (RP), the two-dimensional liquid chromatography (2D-LC), and the off-gel electrophoresis (OGE) (Abdallah *et al.*, 2012). After separation, proteins could be tagged using chemical and metabolic labeling, which represented a significant development for large

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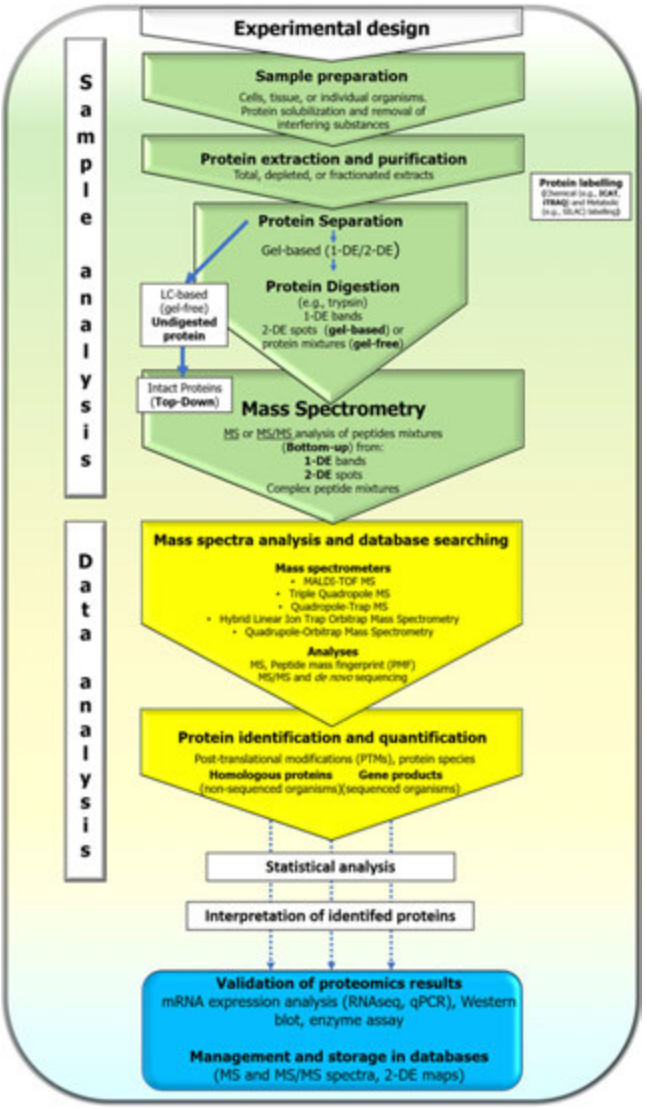

Fig. 1 Schematic overview of a proteomic workflow basing on different approaches (gel-based and gel-free) and MS analysis of intact protein (top-down) or peptides (bottom-up). Image modified from the original one Jorrín-Novo, J.V., Pascual, J., Sánchez-Lucas, R., *et al.*, 2015. Fourteen years of plant proteomics reflected in proteomics: Moving from model species and 2DE-based approaches to orphan species and gel-free platforms. Proteomics 15, 1089–1112. doi:10.1002/pmic.201400349.

scale proteomics. Chemical labeling includes some techniques like the isotope-coded affinity tag (ICAT) (Gygi *et al.*, 1999) and the Isobaric Tags for Relative and Absolute Quantification (iTRAQ) (Abdallah *et al.*, 2012). The ICAT represents one of the first developed labels (Wiese *et al.*, 2007). It consists of marker probes composed by three elements, a chemically reactive group that binds the side chain of specific amino acid in the sample (e.g., iodoacetamide binds the cysteine residues), an isotopically coded region (heavy and light), and the tag (e.g., biotin) for the affinity-based purification (Gygi *et al.*, 1999; Colangelo and Williams, 2006). The iTRAQ is a newer chemical labeling method more suitable for labeling peptides rather than proteins. It consists of isobaric tags, that bind peptide in protein digest via free amines on N terminus and the side chain of lysine residues. The protein pool can be labeled and analyzed simultaneously. The use of isobaric tags does not shift the mass under Mass Spectrometry (MS) analysis leading to an increased sensitivity due to the cumulative signal on the same peptide from different samples (Unwin, 2010). Metabolic labeling is based on two main techniques: stable isotopic labeling with amino Acids in cell culture (SILAC) and N isotopic labeling. Both approaches are based on the incorporation of isotopes in vivo, with some differences. In SILAC, isotopically labeled amino acid is added to the culture medium to differentiate two populations (one labeled and one non-labeled). The efficiency of SILAC techniques depends on the organism's autotrophy, so it is less suitable for plants, cell cultures, and some types of unicellular organisms. However, it can be used for the analysis of fungal pathogen species (Jang and Kim, 2018). N isotope labeling uses 15N-labeled inorganic salt and provides a better incorporation efficiency respect to SILAC (98% respect to 70%–80% in cell cultures), and it is more suitable for quantitative proteomics in plant sciences (Abdallah *et al.*, 2012).

Mass spectrometers are instruments able to analyze ionized analytes in the gas phase. Three main parts compose them, an ion source that produces ion analytes, a mass analyzer that separates them based on their mass-to-charge ratio (m/z), and a detector that records the number of ions at each m/z value (Aebersold and Mann, 2003). The development of ion sources was catalyzed by two technical breakthroughs in the late 1980s: the two-ionization methods Electrospray Ionization (ESI) and Matrix-Assisted Laser Desorption/Ionization (MALDI). Because of the lack or minimal extent of analyte fragmentation during the ESI and MALDI processes, they are also referred to as "soft" ionization methods (Aebersold and Goodlett, 2001).

The analyzers are composed of several types, which can also be combined to form tandem mass spectrometers. The most commonly mass spectrometers used in Proteomic analysis used are the ion Trap, the Time-of-Flight (TOF), the quadrupole mass spectrometer, and the Orbitrap, where the latter represents the newest mass spectrometer. It is also possible to combine different analyzers, forming the so-called hybrid instruments. These high-resolution instruments become available in the last years, with high-resolution mass analyzers such as the LTQ/FT, LTQ-Orbitrap and quadrupole-TOF (Q-TOF) allowing to substantially increased the number of proteomics applications in biological research (Gregorich *et al.*, 2014; Yates *et al.*, 2009; Zhang *et al.*, 2010).

The MS-based proteomics strategies could also be grouped into two main groups based on protein status, namely "bottom-up" and "top-down" each of which presents advantages and disadvantages (Gregorich *et al.*, 2014). In the bottom-up approach, MS analysis is performed on peptides generated by proteolytic digestion. A classic bottom-up approach provides that extracted are subjected to enzyme-based proteolytic digestion (e.g., using a serine protease, trypsin) or are first separated by polyacrylamide gel (2-DE or SDS-PAGE) followed by in-gel digestion (Aebersold and Mann, 2003; Gregorich *et al.*, 2014). In the latter case, gel bands are excised, digested, and separated by reverse-phase LC.

When gel separation does not occur, the peptide mixture can be separated into two dimensions, on the base of the charge (e.g., using cation exchange column) and then on their hydrophobicity (Steen and Mann, 2004). Shortcomings of the bottom-up approach are mostly related to the significant number of peptides that entering the mass spectrometer at the same time, making it difficult to achieve full protein identification and increasing the LC-MS variability (Zhang *et al.*, 2010; Cargile *et al.*, 2004).

In the top-down approach, proteins are analyzed without any proteolytic digestion (Siuti and Kelleher, 2007), leading a characterization of intact proteins from complex biological systems. Intact proteins are then directly introduced into the mass spectrometer, where both their intact and fragmented ions masses are measured (Catherman *et al.*, 2014).

This approach preserves all the information on the native status of protein, with the potential to identify a more significant fraction of protein sequences, including the ability to locate and characterize PTMs (Zhang *et al.*, 2010) or the identification of protein-protein interactions (Aebersold and Mann, 2003; Catherman *et al.*, 2014). Furthermore, when using top-down approaches, time-consuming steps like protein digestion are no longer necessary (Konijnenberg *et al.*, 2015). The disadvantages linked to top-down approaches are based on the fact that this approach is very demanding in terms of the instrumental setup (Schneider and Hall, 2005; Armirotti and Damonte, 2010), both in terms of cost and resolving power and sensitivity; other limitations including the difficulty to detect highly charged proteins and the coelution of intact proteins after LC separation that also complicates MS spectra (Cupp-Sutton and Wu, 2020).

Despite these limitations, the late developments have expanded the field of application of top-down proteomics, mostly lead to capable of proteoform profiling across multiple samples with high-throughput approaches (Toby *et al.*, 2016) through the successful development of new high-resolution mass spectrometers (e.g., quadrupole time-of-flight, Q-TOF) (Chen *et al.*, 2018).

Therefore, also considering the broad range of proteomic studies in fungal research, this chapter will mainly focus on two research fields, mycorrhizal and pathogenic fungi, which are growing in importance due to many economic and environmental aspects.

Ectomycorrhizal and Endomycorrhizal Fungi

In nature, organisms belonging to different Kingdoms may develop intimate relations with beneficial partners. Such kind of relationships are usually referred to as a 'mutualistic symbiosis'. Among them, the association of plants with nitrogen-fixing rhizobia and with mycorrhizal fungi are well known (Markmann and Parniske, 2009; Smith and Read, 2008). Different types of mycorrhizal symbioses can be distinguished through the interaction method developed by the fungal partner (**Fig. 2**): in the so-called 'ectomycorrhizae' (ECM), the fungal hyphae remain outside the plant root cell, while in the "endomycorrhizae" – including orchid, ericoid and arbuscular mycorrhiza (AM) – the fungal hyphae can enter into the plant cells (Balestrini and Lumini, 2018).

ECM represents a resource from many points of view; their role in the forest ecosystem was mainly related to the fact that they played the central role in the nutrient cycle ad reforestation process. It is undoubtedly essential, but the economic value of their fruiting bodies is equally relevant. The economic and agronomic relevance of some ECM species like those belonging to the *Tuber* genus has, over the years, led to the creation of numerous scientific works focusing on the analysis of the protein structure. These works, evaluating the proteomic profile in one or more stages of the development cycle, aimed to clarify the complex biochemistry linked to this genus. Most of the studies concerning the species belonging to the genus *Tuber* focused on the mycelial phase and in the fruiting body, known as truffles, using a wide range of techniques for the purification and analysis of proteins. Many of the studies published to date almost concern three species belonging to the genus *Tuber*: *Tuber borchii*, *T. melanosporum*, and *T. magnatum*.

In *T. borchii*, the fruiting body proteome was firstly analyzed by SDS-PAGE and compared with other species of the same genus, *T. magnatum*, *T. dryophilum*, and *T. puberulum* and highlighting the presence of differences in electrophoretic patterns (De Bellis *et al.*, 1998). In the same work, a protein linked to a very abundant electrophoretic band weighing 12 kDa was identified. Mass spectrometry analysis led to identifying this band as the TBF-1 protein, absent in the other species analyzed, but subsequently also

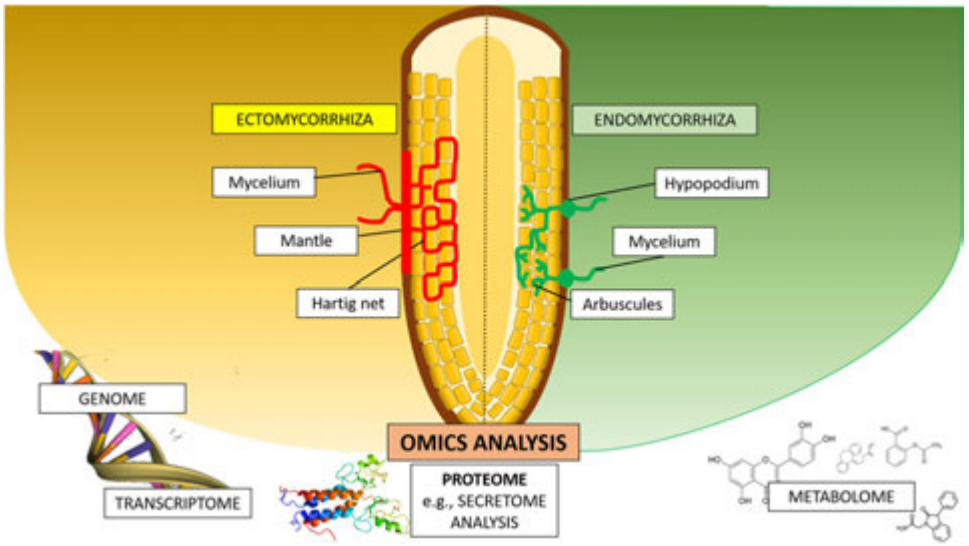

Fig. 2 Representative illustration of root colonization and related structures in ectomycorrhiza and endomycorrhiza (arbuscular mycorrhiza). Image modified from the original one Bonfante, P., Genre, A., 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. Nat. Commun. 1, 1–11.

identified in *T. dryophilum* (Agostini *et al.*, 2000). Subsequent studies based on hemagglutination and inhibition assays have shown how this protein, present in the unripe and ripe fruit bodies but not in ectomycorrhizal roots, represents an essential lectin in bacterial colonization process as it can selectively bind the exopolysaccharides produced by ascoma-associated bacteria like *Rhizobium* spp. (Cerigini *et al.*, 2008).

T. borchii was then analyzed to evaluate changes in the protein profile concerning the maturation stage of the carpophore (Pierleoni *et al.*, 2004), while the mycelial phase was analyzed extensively by evaluating the variations after thirty days of growth in a liquid substrate (Vallorani *et al.*, 2000) through analysis of two-dimensional electrophoresis (2-DE). In a subsequent work by the same working group (Vallorani *et al.*, 2002) the 2-DE analysis approach was used to characterize the NADP-glutamate dehydrogenase protein, reporting that the sequence of this protein was very similar to others found in different species of fungi, such as *Neurospora crassa* and *Penicillium chrysogenum*, and that was also expressed in the symbiotic phase. A recent publication of the genome of several Pezizomycetes (Murat *et al.*, 2018), where *T. magnatum* and *T. aestivum* genomes were released, has undoubtedly brought new information useful for defining the *in silico* proteome of these species. In the case of *T. magnatum*, proteomic studies have mainly dealt with identifying specific proteins related to fruiting bodies collected from different environments to prevent fraud related to the high commercial value of the product (Vita *et al.*, 2013, 2020).

The release of the *T. melanosporum* genome dates back in 2010 (Martin *et al.*, 2010), and the information obtained was useful to improve the level of protein identification for proteomic related studies. A detailed proteomic analysis of *T. melanosporum* fruiting bodies (Islam *et al.*, 2013) was performed using a combined 1D and LC-MS/MS approach leading to the proteomic characterization of 836 truffle proteins.

The relationship of mutual symbiosis between the host plant and the fungal partner was also addressed in other ECM fungi, like those belonging to the genus *Terfezia*. Proteomic analysis *Terfezia claveryi* leading to the identification of a catalase protein, the *TcCat-1*, which is involved in the mycorrhizal colonization process (Marqués-Gálvez *et al.*, 2019). The authors identified high levels of this protein only in mature mycorrhiza, suggesting as its production seems necessary for fungi to elude host defense, including the production of ROS compounds as in some specific stress conditions. Protein profile *T. claveryi* was also analyzed by SDS-PAGE by identifying the presence of a prevalent protein band at 18–20 kDa (Ammarellou, 2007), as previously identified in *T. borchii* fruiting body analysis (De Bellis *et al.*, 1998).

Proteomic studies were also conducted on other ECM, like those belonging to Basidiomycota. Three isolates with *Pisolithus tinctorius* different degrees of ability to induce mycorrhiza (null, moderate, and high) were inoculated on *Eucalyptus grandis* plants using a 2-DE approach. The isolate not able to induce mycorrhizal interaction do not affect the root protein pattern, whereas aggressive mycobionts induce severe alteration in protein expression (Burgess *et al.*, 1995). The same fungal species was also employed to identify cell surface proteins involved in the early stage of ectomycorrhiza symbiosis. After inoculation in *Eucalyptus globulus*, the biosynthesis of cell wall proteins was analyzed using a 2-de approach, identifying both in host plant and mycobiont a novel class of protein of 31–32 kDa named SRAPs (symbiosis-regulated acidic polypeptides) involved in the primary ECM colonization step (Laurent *et al.*, 1999).

Further study of *P. tinctorius* was assessed between mycorrhizal and nonmycorrhizal cork oak plants (Sebastiana *et al.*, 2017) using a 2D-Differential in-gel electrophoresis (DIGE) coupled with MALDI-TOF/TOF tandem mass spectrometry approach. Results

showed as no difference was detected at a foliar level, whereas 42 spots were identified as differentially regulated in roots. The MS analysis allows to identify 34 unique oak proteins, where those related to carbohydrate and energy metabolism, protein folding/assembling, cell wall re-enforcement, defense, cytoskeleton biogenesis, and soil phosphorus transport acquisition were down-regulated following mycorrhiza formation and, conversely, proteins related to the antioxidant defense system, nitrogen assimilation, membrane lipid transport/metabolism, and phosphorus transfer seems as upregulated.

Recently, several studies were focused on the analysis of extracellular proteins, also called secretome, which can be produced by ECM fungi during the early stage of the mycorrhizal symbiosis process.

Proteomic methods have also been applied in *Laccaria bicolor* to assess the analysis of extracellular proteome, also called secretome, produced in a growth medium by free-living mycelium (Vincent *et al.*, 2012). The analysis was performed using a combined approach based on 2-DE, and nLC-ESI MS-MS (tandem mass spectrometry) led to the identification of 224 proteins, among which enzymes involved in cell wall remodeling, as well as those involved in soil organic digestion, were founded. Secretome analysis was also performed on Brown-rot (*Gloeophyllum trabeum*), and white-rot (*Pleurotus ostreatus*) growth in a sugarcane base medium, where an SDS-PAGE approach coupled with LC-MS/MS allows to identify 199 and 63 extracellular proteins, respectively, including a wide range of cellulases and carbohydrate auxiliary proteins involved in lignocellulose degradation (Valadares *et al.*, 2019). Response to abiotic stress was also evaluated according to a proteomic approach. *Boletus edulis* proteome was analyzed under salt stress conditions using a 2-DE ESI Q-TOF MS/MS mass spectrometry approach. Twenty-two spots were selected based on bioinformatical analysis, and the MS analysis leads to the identification of sixteen proteins belonging to different metabolisms, including the biosynthesis of methionine and DNA repair (Liang *et al.*, 2007).

A multidisciplinary approach was applied to the basidiomycete fungus *Hebeloma cylindrosporum* for the study of the fungal exoproteome as well as the effect of the establishment of mycorrhizal symbiosis (Doré *et al.*, 2015). In this paper, transcriptomic data from RNA-seq experiments were performed by comparing transcription abundances in free-living mycelia and mycorrhizas with *Pinus pinaster* seedlings obtained under two different environmental conditions, glasshouse and in vitro.

Proteomic analysis was mainly used to study the exoproteome, and proteins released by fungal mycelia in growth media were extracted, separated through SDS-PAGE, and analyzed via LC-MS/MS.

Computational analysis was used to complement the experimental approach to clarify the consistency of *H. cylindrosporum* exoproteome, which includes at least 869 potential proteins, 641 predicted in silico, and 228 not predicted but identified experimentally. The functional classification of protein identified three major groups, each of which accounts for 17% of the total identified proteins; these three groups are represented by small secreted proteins (SSPs), carbohydrate-active enzymes (CAZzymes), and peptidases. When only 10% of the most abundant proteins were considered, results based on the emPAI score highlights as the SSP represents the most representing group that accounts for 29% of the total proteins. Most SSPs proteins reported an unknown function; a possible explanation regards the role of these proteins that could mediate interaction with host plants and other biotic and abiotic environmental factors.

Transcriptomics data confirm the effect of both symbiosis and environment on the development of fungal transcriptome, with a prevalence of upregulated genes due to mycorrhizal formation and in vitro growth.

Further study was also conducted on the establishment of mycorrhizal symbiosis, aiming to detect the impact of these processes on plant leaf proteome. In the work of Szuba *et al.* (2019), proteomic analysis was performed using *Populus* × *canescens* plants inoculated with two isolates of *Paxillus involutus* that differ on their root colonization rates. These isolated were called H (high mycorrhization level) and L (low mycorrhization level); uninoculated plants were used to deepen the effect of ECM fungus. The proteomic approach was also integrated with biometrical and biochemical analysis to assess the rate of root colonization and to detect any variation in nonstructural soluble carbohydrates (SC), starch, and leaf pigments as a consequence of ECM colonization on the host plant. Results of 2DE and MALDI TOF/TOF mass spectrometry analysis detect a significant difference in terms of protein profiles between leaves of inoculated and those related to non-inoculated poplars. The leaf proteome of plants inoculated showed differences of 9% and 7% for those treated with isolate H and L, respectively. Despite that, increased carbohydrate biosynthesis was not detected between inoculated and non-inoculated plants, with carbohydrate, carbon, and leaf pigment levels and plant biomass that does not change. The MS analysis of differentially regulated spots identified alterations in photosynthesis, with plant treated with isolate H that positively affect carbohydrate biosynthesis and photosynthesis, more specifically light-dependent protein. This data represents the major finding of this work, where root colonization rates were positively correlated with the global efficiency of the photosynthetic apparatus.

Therefore, the analysis of extracellular proteins and their role in mycorrhizal symbiosis still represents an active research field. A different approach to analyzed fungal secretome was applied by Pellegrin *et al.* (2015), where a broad set of fungi, including eleven ECM species among the forty-nine analyzed species. In detail, forty-nine Basidiomycota, six Ascomycota, one Zygomycota, and one Chytridiomycota were tested to determine if differences may be occurred in the secretome of ECM, saprotrophic and pathogenic fungi, mainly regarding SSPs protein abundance. Using the genomic sequences currently available on JGI (Joint Genome Institute), the authors set a specific bioinformatics pipeline to identify and localize the secretome composition of each species. Results showed as the number of predicted proteins did not change on the base of their lifestyle. However, ECM species showed an SSPs enrichment if compared with the other fungal species characterized by a different lifestyle. The presence of an enriched secretome in ECM fungi could be possibly explained through the conservation of SSPs from saprotrophic ancestors, and the expansion of symbiosis-specific SSPs dedicated to the molecular crosstalk between partners, the accommodation of hyphae in plant or the establishment and functioning of the symbiosis (Garcia *et al.*, 2015).

The 2-DE and mass spectrometry approach was also used to analyze the effect of the inoculation of *Lactarius isnuslu*s in *Pinus massoniana* plants (Xu and Wu, 2016). L. *insulus*, also known as L. *zonarius*, is a pine ectomycorrhiza belonging to the family of Russulaceae representing one of the most studied pine ECM in china that was inoculated in *P. massoniana* seedlings and analyzed in drought stress conditions. Mychorrized and non-mychorrized plants were then compared, and results showed as abiotic stress affect the fungal proteome through the regulation of 22 protein spots. MS analysis leads to the identification of differentially expressed proteins, including a 1,4-benzoquinone reductase, an enzyme that prevents aligning degradation. According to the authors, this enzyme decreases in stress conditions as a possible response to drought stress, thus enhancing, as well as the other identified proteins, the drought resistance of *P. massoniana*.

Proteomic analysis was also employed for the study of arbuscular mycorrhizal (AM) symbiosis. Starting from the work of Dumas *et al.* (1990), a native PAGE gel-based approach was applied to detect qualitative protein modifications occurring during AM symbiosis establishment, and proteomics was used to disclose the key players involved in the plant–fungus association establishment.

The dissection of the root colonization process by AM fungi has led to the identification of four main phases (Bonfante and Genre, 2010): during an early stage, the communication between plant and fungus occurs: plant roots exude strigolactones and induce hyphal branching, while the fungus releases LCOs, perceived by the plant, where they trigger calcium spiking through the activation of the common SYM pathway; in the second stage, after that signal transduction has lead to the activation of cellular and transcriptional responses, the AM fungus contacts the root surface and forms hyphopodia; the contact with the root triggers the assembly of a broad but transient aggregation of cytoplasm, named the PrePenetration Apparatus (PPA) in the contacted epidermal cell and underlying outer cortical cell, that defines the path followed by the fungal hyphae toward the inner root layers; in the last stage, the fungal hypha branches and follows the path created by the PPA until the root cortex, where it starts to form a tree-like structure called 'arbuscule', which is the core of a functional AM symbiosis. The contact surface between the plant and the AM fungus greatly increases around the arbuscule, increasing the area where the exchanges of nutrients occur.

Proteomic studies have focused on the intracellular accommodation of unbranched hyphae (during the early stage) and arbuscules (at a later stage) to disentangle this coordinated developmental process between the plant and the fungal cells. The early stage of the interaction is essential to understand the crosstalk between the association actors and how the proteomes of the two organisms rearrange during the colonization events. Pioneering studies focused on the identification of fungal proteins involved in the early stages of the plant-fungus interaction: the identification of fungal proteins is more challenging, in respect proteomic of plant proteins, due to the impossibility to grow AM fungi in axenic cultures, to the lower amount of fungal biomass and to the more scanty sequence information. The first report of arbuscular mycorrhizal fungal protein identifications by using a proteomic approach is ascribable to Dumas-Gaudot *et al.* (2004): they used root-inducing transferred-DNA transformed roots of *Daucus carota* L., in vitro inoculated with *Glomus intraradices* (current name *Rhizophagus irregularis*), to enrich for proteins expressed in the extra-radical mycelium of the fungus. After producing the first 2DE reference map for the extra-radical proteome of an AM fungus with 438 spots, they were able to identify by mass spectrometry four protein: NmrA-like protein, an oxidoreductase, a heat-shock protein, and an ATP synthase beta mitochondrial precursor. The same experiment was repeated five years later by the same group (Recorbet *et al.*, 2009) but using a shotgun proteomic approach named GeLC-MS/M, and they were able to identify 92 different fungal proteins. GeLC-MS/MS method combines a mono-dimensional gel (1D-PAGE) and a nano-scale capillary liquid chromatography-MS/MS. Using the MetaCyc collection of more than a thousand metabolic pathways as reference (Caspi and Karp, 2007), the authors grouped those proteins in 11 pathways, comprising energy, metabolism, and cell rescue processes. Together with previous identifications of putative homologs of the cell-cycle gene in *Glomus mosseae* (current name *Funneliformis mosseae*) and *G. intraradices* (Jun *et al.*, 2002; Requena *et al.*, 2000), these results suggest that signaling pathways described in model species may also operate in AM fungi. Another milestone study on the early stage of root colonization is the one by Amiour *et al.* (2006), aimed at investigating the modifications of the *Medicago truncatula* root proteome elicited in response to appressorium formation by *G. intraradices*. In this study, using two-dimensional electrophoresis, the authors compared the root proteome from non-inoculated roots and from roots synchronized for appressorium formation. This approach was conducted in wild-type (J5), mycorrhiza-defective (TRV25, dmi3), and autoregulation-defective (TR122, sunn) *M. truncatula* genotypes. The work showed that proteins responding to appressorium formation were differentially expressed in different genotypes: the accumulation of proteins related to plant defense reactions, cytoskeleton rearrangements, and auxin signaling upon symbiont contact was recorded between wild-type and hypermycorrhizal genotypes.

In nature, the plant-fungus association is a very dynamic and complex event because, while arbuscules are forming and/or collapsing, new penetration events occur. Due to this, the proteomic analysis of the late stage of the AM symbiosis has never been an easy task; therefore, many studies on the late stage of the symbiosis focused their attention on a specific sub-cellular compartment on the effect of the established symbiosis on the proteome of the partners.

The first mass spectrometry (MS)-based identification of mycorrhiza-related proteins was reported by Bestel-Corre *et al.* (2002). In their work, a time-course analysis of root protein profiles was performed in the model plant *M. truncatula*, inoculated with the fungus *G. mosseae* or with the nitrogen-fixing bacterium *Sinorhizobium meliloti*. The proteins were separated by 2DE-PAGE and image analyses, followed by spots volume quantification to identify differentially expressed protein spots. Those spots were then excised from the gels and analyzed by mass spectrometry. In mycorrhizal roots, analyzed proteins included proteins related to defense responses, root physiology and respiratory pathway, e.g., a glutathione-S-transferase, a fucosidase, a myosin-like protein, a serine hydroxymethyltransferase and a cytochrome-c-oxidase.

In mycorrhizal symbioses, membrane proteins are essential because they likely include the receptors that control the plant-fungus crosstalk and the transporters that facilitate nutrients exchange. For these reasons, many papers have focused on membrane proteins using enrichment protocols.

Benabdellah *et al.* (1998) conducted the first study on the sub-cellular enrichment of membrane proteins. They analyzed the effect of AM colonization on tomato gene expression by comparing 2D-PAGE patterns of crude extracts, soluble and membrane proteins of tomato roots, either mycorrhizal with *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe or non-mycorrhizal, finding that 44 polypeptides were differentially displayed. In 2000, the same group identified for the first time a protein related to mycorrhizal symbiosis by Edman N-terminal sequencing, after plasma membrane enrichment and 2DE-PAGE protein separation (Benabdellah *et al.*, 2000). In 2005, Valot *et al.* (2005) also used a sub-cellular proteomic approach to record membrane-associated protein modifications during the plant-fungus interaction. Membrane proteins were extracted from *M. truncatula* roots either inoculated or not with the AM fungus *G. intraradices*. Comparative 2DE-PAGE revealed that 36 spots were differentially displayed in response to the mycorrhizal colonization, including 15 proteins induced, 3 up-regulated, and 18 down-regulated. 25 out of 36 regulated proteins were then identified using the MALDI-TOF, and none of them resulted in having been previously reported as being regulated during AM symbiosis. To investigate whether AM symbiosis-specific proteins accumulate when the plant plasma membrane (PM) invaginates around the fungal arbuscular structures resulting in the periarbuscular membrane formation, two complementary MS approaches targeting the root PM from *M. truncatula* were used by the same group a year later (Valot *et al.*, 2006). They enriched samples for PM using a discontinuous sucrose gradient method, and the resulting fractions were analyzed with two complementary proteomics methodologies: (1) an automated 2D liquid chromatography-tandem mass chromatography (LC-MS/MS) using a strong cation exchange and reverse phase chromatography, and (2) SDS-PAGE combined with a systematic LC-MS/MS analysis. Only proteins consistently retrieved with the two methodologies were taken into account, resulting in the identification of 78 proteins: of those, 56% were predicted to contain one or more transmembrane domains, while 30% were already known to be localized on the plasma membrane. Comparison between non-inoculated and *G. intraradices*-inoculated roots revealed two proteins that differed in the mycorrhizal root PM fraction: a proton-efflux P-type ATPase (Mtha1) and a predicted glycosylphosphatidylinositol-anchored blue copper-binding protein (MtBcp1). The correct localization of MtBcp1 in the plasma membrane and the periarbuscular membrane around the arbuscular trunk was confirmed a few years later by Pumplin and Harrison (2009), while Wang *et al.* (2014) showed that $H+$-ATPases are required for enhanced proton pumping activity in membrane vesicles. Recently, Aloui *et al.* (2018) performed a comparative protein profiling of PM fractions from *M. truncatula* roots either inoculated or not with the *Rhizophagus irregularis* to obtain a global overview at the proteome level of the host PM proteins as modified by the symbiosis. The analysis of PM proteins isolated from root microsomes using an optimized discontinuous sucrose gradient, by liquid chromatography followed by mass spectrometry, identified 674 proteins. In their work, the analytic downstream workflow allowed for identifying a set of 82 mycorrhiza-responsive proteins that provided insights into the plant PM response to mycorrhizal symbiosis. The PM-associated proteins responsive to mycorrhization also supported host plant control of sugar uptake to limit fungal colonization and lipid turnover events in the PM fraction of symbiotic roots.

Gaude *et al.* (2012), investigating cell type-specific proteome changes in arbuscule-containing cells, provided a solid evidence that the PM acts as important carbon sink in the mycorrhizal symbiosis. They combined Laser Capture Microdissection and LC-MS/MS to determine the specific protein composition in arbuscule-containing cortical cells (arb) and cortical cells of non-mycorrhizal *M. truncatula* roots (cor). From both cell types, they identified 401 *M. truncatula* proteins with similarities to annotated proteins: 188 were exclusively detected in arb cells, whereas 53 proteins were exclusively found in cor cells. The proteome of arb cells showed a strongly increased number of proteins involved in lipid metabolism, most likely related to the synthesis of the PM, such as a ACC-carboxylase, a biotin carboxylase and a β-ketoacyl synthase, while transcriptome data of cor cells suggest mobilization of carbon resources and their symplastic transport toward arbuscule-containing cells for the synthesis of PMs.

Investigating the membrane proteome of *M. truncatula* roots in response to arbuscular mycorrhizal symbiosis with *R. irregularis*, Abdallah *et al.* (2014) were able to identify 1226 root membrane proteins and to report for the first time the proteomic identification of previously functionally characterized symbiosis marker genes (MtPt4, GmAMT4.1, STR half-ABC transporters and VAMP721d/e). In this work, the sample pre-fractionation was performed using 1D SDS-PAGE, and the proteins were quantified by label-free counting: peptide peak intensity and spectral count were measured for individual LC-MS/MS or LC/LC-MS/MS runs, and changes in protein abundance were calculated via a direct comparison between different analyses. With this approach, the authors were able to speculate that accommodation of AM fungi within cortical root cells implies both a dynamic reorganization of the root membrane proteome and the de novo synthesis of AM-related proteins. The effects of arbuscular mycorrhizal symbiosis have also been explored in other plant tissues. Peinado-Guevara *et al.* (2017) performed a comparative proteomic study to analyze the differential accumulation of leaf proteins in response to the symbiosis between *Solanum lycopersicum* and the *R. irregularis*. 2D-SDS-PAGE revealed that 27 spots were differentially accumulated in tomato plants colonized with AMF (M) vs. non-colonized plants fertilized with low phosphate (NM-LP), 23 of which were successfully identified by mass spectrometry. Two of these proteins, 2-methylene-furan-3-one reductase, and auxin-binding protein ABP19a, were up-regulated in M plants. Superoxide dismutase, harpin binding protein, and thioredoxin peroxidase were also down-accumulated in leaves of M tomato plants when compared to NM-LP and to non-colonized plants fertilized with regular phosphate Hoagland's solution, indicating that these proteins are responsive to AMF colonization independently of the phosphate regime under which they were grown.

AM mycorrhizal symbioses have also been investigated for changes in their protein profiles when exposed to abiotic or biotic stresses. Mycorrhizal symbioses contribute to plant growth and plant protection against various environmental stresses or combination of stresses that include drought, salinity, temperature, nutrients, and heavy metals (Begum *et al.*, 2019; Lenoir *et al.*, 2016). Many studies

have been carried out to demonstrate the benefits of the mycorrhizal plant–fungus interaction against environmental stresses. The proteomic approach has been employed to identify the key proteins potentially involved in mycorrhiza-mediated stress tolerance. One of the most investigated protective features of mycorrhizal symbioses is the capability to reduce heavy metal toxicity to the host in soils with potentially toxic amounts of soluble and insoluble metals. Aloui *et al.* (2009) report on the protective effect conferred by AM symbiosis to *M. truncatula* grown in the presence of Cd; they further used a 2-D-based proteomic approach, followed by MS/MS, to compare the proteomes of *M. truncatula* roots either colonized or not with *G. intraradices* in Cd-free and Cd-contaminated substrates. They identified six mycorrhiza-related proteins that displayed changes in abundance upon Cd exposure: a cyclophilin, a guanine nucleotide-binding protein, a ubiquitin carboxyl-terminal hydrolase, a thiazole biosynthetic enzyme, an annexin, a glutathione S-transferase (GST)-like protein, and an S-adenosylmethionine (SAM) synthase. These results suggested that part of the symbiotic program, which displays low sensitivity to Cd, might be recruited to counteract Cd toxicity through the mycorrhiza-dependent synthesis of proteins having functions putatively involved in alleviating oxidative damages, including. Bona *et al.* (2010) studied the leaf proteome of the arsenic hyperaccumulator fern *Pteris vittata* inoculated with *G. mosseae* and *Gigaspora margarita*, with and without arsenic treatment. The downregulation of oxidative damage-related proteins indicated a protective role for AM fungi colonization in the absence of arsenic. The up-regulation of multiple forms of glyceraldehyde-3–phosphate dehydrogenase, phosphoglycerate kinase, and enolase, mostly in *G. mosseae*-inoculated plants, suggests a central role for glycolytic enzymes in arsenic metabolism. Cangahuala-Inocente *et al.* (2011) analyzed the root proteome of grapevine rootstock Selection Oppenheim 4 (SO4) upon colonization with two AMF, *Glomus irregulare* (current name *Rhizophagus irregularis*), and *G. mosseae*. At the proteome level, changes in protein amounts due to AMF colonization resulted in 39 differentially accumulated 2D-PAGE spots in AMF roots relative to control. Out of them, 25 were co-identified in SO4 roots upon AMF colonization, supporting the existence of conserved plant responses to AM symbiosis in a woody perennial species.

In a long time-course experiment, Lingua *et al.* (2012) demonstrated the success of phytoremediation by mycorrhizal poplars, as both copper and zinc concentrations in soil were significantly reduced. In this work, leaf samples of a clone of *Populus alba* L., previously selected for its tolerance to copper and zinc and pre-inoculated or not with *G. intraradices*, were collected from plants maintained in a glasshouse on polluted (copper and zinc contaminated) or unpolluted soil, after four, six and sixteen months of growth. At each time point, about 450 proteins were constantly separated on 2-DE maps, and protein identification was performed by nanoLC Coupled with Q-TOF MS/MS. At the first harvest, the most relevant effect on protein modulation was exerted by the AM fungi, at the second one by the metals, and at the last one by both treatments. In a similar experiment, Guarino *et al.* (2014) demonstrated the effectiveness of the application of an AMs/PGPRs cocktail in promoting growth (both in terms of stem diameter and height values) and ameliorating metal toxicity in *Eucalyptus camaldulensis* plants, even if they are grown on a contaminated site. Using 2-DE coupled with nanoLC-ESI-LIT-MS/MS equipped with an LTQ XL mass spectrometer, they showed how the addition of AMs/PGPRs mixture to the soil increased the activation of enzymes involved in photosynthesis and the Calvin cycle, suggesting the existence of signaling mechanisms that address the energy/reductive power requirement associated with augmented growth performances. Moreover, since proteins guaranteeing elevated glutathione levels were constantly over-represented in the presence of AMs/PGPRs or in plants exposed to HMs for prolonged periods, the Authors suggested that glutathione (and related phytochelatins) could act as key molecules for ensuring the effective formation of HMs-chelating complexes, which are responsible for the observed plant tolerance to metal stresses. More recently, by means of the Isobaric Tag for Relative and Absolute Quantification (iTRAQ) system, Wu *et al.* (2020) evaluated differentially expressed proteins (DEPs) in *Phragmites australis* under metal-stressed conditions and inoculated with *R. irregularis*. They reported that under copper stress, which not only reduces plant biomass but also inhibits photosynthesis, the inoculation of *R. irregularis* induces a total of 459 differently expressed proteins (200 up-regulated and 259 down-regulated) in root buds. The photosynthetic changes triggered by AMF inoculation mainly involve the up-regulated expression of transmembrane protein–pigment complexes CP43 (photosystem II) and FNR (ferredoxin-NADP + oxidoreductase related to photosynthetic electron transport). Salt stress is one of the most important abiotic stresses and limiting factors for plant growth and agricultural production. The mechanism of salinity stress alleviation by AM fungi has also been investigated with proteomic approaches. Jia *et al.* (2019) studied how the inoculum of *R. irregularis* could alleviate NaCl salinity stress in *Elaeagnus angustifolia*. The experimental design comprised four different treatments: *E. angustifolia* inoculated with *R. irregularis* without salt stress; *E. angustifolia* inoculated with *R. irregularis* under salt stress (300 mmol/L NaCl), *E. angustifolia* non-inoculated without salt stress, and *E. angustifolia* non-inoculated under salt stress (300 mmol/L NaCl). The proteomic analyses indicated that inoculated *E. angustifolia* seedlings increased the secondary metabolism level of phenylpropane metabolism, enhanced the signal transduction of Ca^{2+} and ROS scavenging ability, promoted the biosynthesis of protein, accelerated the protein folding, and inhibited the degradation of protein under salt stress. The study by Wang *et al.* (2019) was also aimed to provide new insights into the positive role of AMF in the alkali tolerance of *Puccinellia tenuiflora* and clarify how AMF colonization could affect the physiological adaptation strategies and molecular regulation network of this species in alkali-degraded soil. iTRAQ were used to identify the differentially abundant proteins in plant seedlings inoculated or not inoculated with *Rhizophagus intraradices*, under alkalinity stresses (0, 100, 200, and 300 mM of $NaHCO_3$). The Authors reported that a total of 598 proteins were significantly differentially regulated in *P. tenuiflora* seedlings after AMF inoculation under alkalinity stress compared with under alkalinity stress alone. Moreover, *R. intraradices* inoculation significantly improved protein synthesis, reactive oxygen species scavenging, and nitrogen metabolism to promote the biosynthesis of osmotic substances in response to alkalinity stress. Drought is another constraint that plants must face in some areas of the world, hugely aggravated by climate changes, which are recognized to affect the worldwide plant productivity negatively. AMF inoculations, with their bio-fertilization features, may represent a natural and sustainable way to reduce the negative effects due to drought in the crop, enhancing plant growth and development. Bernardo *et al.* (2017) have

investigated the modulation of wheat root proteome induced by *G. mosseae* inoculation under drought stress, using bread and durum wheat cultivar. Proteomic analyses were performed with SDS-PAGE followed by a shotgun tandem MS approach, using nanoscale liquid chromatography coupled to a hybrid quadrupole-time-of-flight (Q-TOF) mass spectrometer. The Authors identified 50 statistically differential proteins in the bread wheat cultivar and 66 differential proteins in the durum wheat cultivar. The results showed a regulation of proteins involved in sugar metabolism, cell wall rearrangement, cytoskeletal organization, and plant stress responses. Both cultivars showed a down regulation of the sucrose: fructan 6-fructosyltransferase, but a specific response upon AMF interaction was evidenced in durum wheat: two proteins related to the biosynthesis of jasmonic acid (12-oxo-phytodienoic acid reductase and a jasmonate-induced protein) was found to be regulated. These results indicate that the AMF association helps wheat roots reducing the osmotic stress and maintaining cellular integrity. Olalde-Portugal *et al.* (2020) used a consortium formed by *Septoglomus constrictum*, *Funneliformis geosporum*, *Rhizophagus fasciculatum*, *Glomus tortuosum* (current name *Sieverdingia tortuosa*), *G. margarita* and *Acaulospora scrobiculata* to inoculate plants of *Sorghum bicolor* L. Moench (a drought-tolerant crop model) in order to understand the water deficit stress mechanism in colonized and non-colonized plants. 2-DE profiles revealed 51 differentially regulated proteins in response to water deficit, 49 of which were successfully identified by HPLC/MS. They found that, in mycorrhizal plants, proteins related to energy metabolism (ATP synthase-24 kDa, ATP synthase β), carbon metabolism (malate dehydrogenase, triosephosphate isomerase, sucrose-phosphatase), oxidative phosphorylation (mitochondrial-processing peptidase), and sulfur metabolism (thiosulfate/3-mercaptopyruvate sulfurtransferase) were found to be regulated and putatively involved in protection against water deficit. A further confirmation that AM fungi can substantially contribute to plant drought tolerance comes from the Gui *et al.* (2020). They used an iTRAQ-based proteomics approach to evaluate the inoculation effects of *Funneliformis mosseae* on blueberry (*Vaccinium corymbosum*) 'O'Neal' cultivated under well-watered or drought-stressed conditions. The proteomic analysis identified 501 differentially abundant proteins (DAPs), including 127, 30, 236, and 108 DAPs in drought-stressed plants vs. well-watered, drought-stressed plants with AMF inoculation vs. well-watered plants with AMF inoculation, AMF-inoculated well-watered plants vs. non-inoculated well-watered plants, and AMF-inoculated plants under drought stress vs. non-inoculated plants under drought stress paired comparisons, respectively. Specifically, AMF-inoculated plants drought-stressed showed a greater abundance of DAPs involved in amino acid metabolism, antioxidant system, signal transduction, and photosynthesis, including carbon fixation in photosynthetic organisms, porphyrin and chlorophyll metabolism, and carotenoid biosynthesis.

As highlighted, AM fungi promote plant growth and alleviate various stresses, but it remained poorly unexplained how the AMF influence resistance against the pathogen. Turetschek *et al.* (2017) analyzed the infection response of two *Pisum sativum* cultivars with varying resistance levels towards *Didymella pinodes* comprehensively at proteomic and metabolomic levels. Proteomic analyses were performed by means of GC–MS and nanoESI LC-MS/MS. The results showed that symbionts (*G. mosseae* and *Rhizobium leguminosarum* bv. *viceae*) induced variation of the host's infection response, which, however, was overruled by genotypic resistance-associated traits of the tolerant cultivar such as maintenance of photosynthesis and provision of sugars and carbon backbones to fuel secondary metabolism. The Authors remark that resistance appears to be linked to sulfur metabolism, a functional glutathione-ascorbate hub, and fine adjustment of jasmonate and ethylene synthesis, essential traits for sustainment of cell vitality and thus, a more efficient infection response. In addition, Sistani *et al.* (2020) focused on the protective effects of a commercial mycorrhizal inoculum (containing *Claroideoglomus etunicatum*, *C. claroideum*, *R. irregularis*, *F. geosporus*, and *F. mosseae*) against *D. pinodes* on two different *P. sativum* cultivars. Regarding the soybean (*Glycine max*), this plant is susceptible to root rot when subjected to continuous cropping, and this disease can seriously diminish the crop yield. The primary root rot agents include several *Fusarium* species, *Rhizoctonia solani*, *Phytophthora* sp., as well as other fungi. Bai *et al.* (2019) used iTRAQ labeling and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to perform proteomic analysis of continuously cropped soybean inoculated with the AM fungus *F. mosseae*. They showed that the AMF inoculum could reduce the incidence of root rot and increase plant height, biomass index in 1, 2, and 4 years of continuous cropping. At the 1-year time point, 131 DEPs were identified in *F. mosseae*-treated samples, of which 49 and 82 were up- and down-regulated, respectively. The up-regulated levels of glucan 1,3beta-glucosidase, chalcone isomerase, calcium-dependent phospholipid binding, and other defense-related proteins triggered by *F. mosseae*, suggested that inoculation promotes the growth and development of soybean and increases disease resistance.

More recently, proteomic analyses have been used in support of and to validate findings obtained by High Throughput Sequencing technologies and to complement genomic studies. For example, when testing the hypothesis that tomato plants grown on native soils may exert different responses to soil microbiotas, Chialva *et al.* (2018) used transcriptomics, proteomics, and direct quantification of biochemical compounds to describe the responses of two tomato genotypes (susceptible or resistant to *Fusarium oxysporum* f. sp. *lycopersici*), grown on an artificial substrate and two native soils conducive and suppressive to *Fusarium*. In order to study the plant transcriptome under the native soils and the control substrate conditions, the root transcriptome of the 'Battito' (resistant) and 'Cuore di Bue' (susceptible) genotypes were sequenced by RNA-seq. To complement the transcriptomic data, proteome profiling was performed by LC-MS/MS on the same material used for RNA-seq but limited to the 'Cuore di Bue' genotype. The researchers found out that the molecular profile of the tomato roots grown in native soils was different from those grown on control substrate, since the native soil modulate pathways involved in responses to oxidative stress, phenol biosynthesis, lignin deposition, and innate immunity, particularly in the suppressive soil. In tomato plants grown on steam-disinfected soils, total phenols and lignin decreased significantly. This led to the conclusion that the tomatoes grown on native soils built up fortified walls and were already trained to use defensive strategies present in their genome.

Moreover, they showed that the wary response of tomato plants could be, in large part, triggered by *F. mosseae* inoculation to the disinfected soils. Murphy *et al.* (2020) examined the extraradical mycelium proteomic profile of *R. irregularis* grown on Ri

T-DNA transformed Chicory roots in a root organ culture setting. With SDS-PAGE and LC-MS/MS analysis, they detected 529 different peptides that mapped to 474 translated proteins in the *R. irregularis* genome. In particular, they found a high proportion of proteins mediating several signal transduction processes, e.g., Rho1 and Bmh2, Ca-signaling (calmodulin, and Ca channel protein), mTOR signaling (MAP3K7, and MAPKAP1), and phosphatidate signaling (phospholipase D1/2) proteins, as well as members of the Ras signaling pathway. Forty-eight proteins were predicted to be associated with surface/membrane, including multiple hypothetical proteins of unknown functions. Finally, they were able to identify a core set of pathways and processes involved in AMF growth comparing the of *R. irregularis* proteome to previously published AMF proteomes.

Finally, it is worth mentioning that endobacteria have been observed to inhabit some mycorrhizal and pathogenic fungi (Bonfante and Anca, 2009). For example, a nitrogen-fixing bacterium was detected inside the pathogenic fungus Ustilago (Ruiz-Herrera *et al.*, 2015), the genome of a beta proteobacterium living inside *Mortierella* (Fujimura *et al.*, 2014), and the genomes of Mollicutes-related endobacteria living inside many arbuscular mycorrhizal fungi have been sequenced (Naito *et al.*, 2015; Torres-Cortés *et al.*, 2015). The AM fungus *G. margarita* hosts an obligate, stable and structurally integrated population of the gram-negative bacterial endosymbiont *Candidatus* Glomeribacter gigasporarum (*Ca.* G. gigasporarum; Bianciotto *et al.*, 2003; Jargeat *et al.*, 2004). The sequencing of this endobacterium revealed genome traits indicating the strict metabolical dependency on the fungal host for nutrients and energy, explaining why the bacterium cannot be cultured outside its host (Ghignone *et al.*, 2012). A *G. margarita* line lacking its endobacteria (named as B- or cured line) was developed and derives from a stable wild-type (named as B+ or wt line) variant that is still able to establish mycorrhizal symbiosis (Lumini *et al.*, 2007). To shed light on the bacterial effect on fungal fitness, Salvioli *et al.* (2016) used NGS to analyze the transcriptional profile of G. margarita in the presence and absence of its endobacterium. To complement this previous study, Vannini *et al.* (2016) employed the iTRAQ approach to analyze the proteomic profile of *G. margarita* and its endobacterium and to improve the coverage of the protein changes associated with endosymbiosis. While RNA-seq analysis of the fungus in the presence and absence of the endobacterium indicated that endobacteria have an important role in the fungal pre-symbiotic phase by enhancing fungal bioenergetic capacity, this proteomic study revealed that endobacteria influenced fungal growth, calcium signaling, and metabolism, with a significant role on fungal primary metabolism and respiration.

Proteomics of Plant Pathogens Fungi

The study of the plant-pathogen interactions has been efforted in the last years by large scale analysis as the proteomics. The development of new mass spectrometry-based methods has significantly increased the possibility of going further in the analysis of the host-pathogen interaction by deciphering most of the aspects related to fungal infection. Pathogenic fungi are classified based on parasitism and infection strategies and consists of three groups: biotrophic (e.g., *Ustilago maydis*) and hemibiotrophic (e.g., *Magnaporthe oryzae*), that requires living plant tissues to survive and complete the life cycle (Koeck *et al.*, 2011), and necrotrophic. This latter group includes fungi (e.g., *Botrytis cinerea*) that kill the host by producing cell wall-degrading enzymes and phytotoxins, aiming to get nutrients for growth and reproduction (Hakeem *et al.*, 2016).

The complicated life cycle of fungal pathogens consists of sexual and asexual reproduction, together with life stages involving the formation of different infective, vegetative, and reproductive structures (Glawe, 2008). The studies of fungal pathogen strategy for plant colonization have performed using a wide range of approaches, including biochemistry and high-throughput -omics techniques (González-Fernández *et al.*, 2010), including proteomics.

The proteomic approach to plant pathogenic fungi was firstly applied in two pioneering works, both related to *Cladosporium fulvum* inoculated on tomato leaves. In the first work (De Wit *et al.*, 1986), a gel-based approach of intercellular fluid analysis was coupled with radioimmunological detection to firstly report the avirulence protein Avr9, whereas, in the second work, the purification ad characterization of the putative peptide elicitor of Avr9 was performed by PAGE separation, reverse phase HPLC purification and sequencing using a gas-phase protein sequencer (Schottens-Toma and de Wit, 1988).

Starting from these works, proteomic analyses were applied to a wide range of plant pathogenic fungi, also thanks to recent advances in modern technology, and most of the papers are mainly focused on species that cause a severe loss of production. This group includes species such as *B. cinerea*, *Sclerotina sclerotiorum*, *Fusarium graminearum* and *M. oryzae* that are considered as some of the most harmful species for crop production (Dean *et al.*, 2012).

Botrytis cinerea

The fungal pathogen *B. cinerea* causes severe loss in crop production on over 200 dicotyledonous crop species. This species is difficult to control because It attacks plants in different ways, uses the different hosts as inoculum source, and can also survive through sclerotia production in crop debris (Williamson *et al.*, 2007).

The first proteomic study about *B. cinerea* was published by Fernández-Acero *et al.* (2006), where 22 protein out of selected 64 spots were identified through a 2-DE approach coupled with MS analysis (MALDI-TOF/TOF and ESI ion trap) using mycelium. The identification of proteins showed that many of them can be ascribed to primary metabolism (malate dehydrogenase, MDH, and glyceraldehyde-3-phosphate dehydrogenase, GADPH) or strain virulence (e.g., cyclophilin). Following this first paper, the same research group published a second chapter aiming to identify proteins involved in virulence (Fernández-Acero *et al.*, 2007). Comparative analyses were carried out using two mycelium strains, which differ in virulence. The 2-DE analysis allows identifying 73 differentially regulated spots, among which only 27 results in protein identification. These proteins were belonging to similar

metabolism as the previous work, but resulting as upregulated in virulent strain, thus suggesting their possible role as pathogenicity factors. These achieved were performed through a third work (Fernández-Acero *et al.*, 2009), where the fungal proteome map was defined under cellulose degradation to define the fungal proteome map during cellulose degradation. This result was achieved by adding carboxymethylcellulose to the growth media of three mycelial cultures.

By using a gel-based approach (2-DE and MALDI TOF/TOF) as before, 303 proteins were identified out of 267 selected spots. Proteins were then functionally classified, and authors mentioned as most of them are involved in the pathogenicity process and, therefore, possible targets of antifungal activity. The same group also published a review on the new potential use of the proteomic approach for the development of proteomics-based fungicides (Fernández Acero *et al.*, 2011). Furthermore, the phosphoproteome of *B. cinerea* was also analyzed to detect changes induced by two different carbon sources used as plant-based solicitors; glucose and deproteinized tomato cell wall (TCW) (Liñeiro *et al.*, 2016). Using a gel-free approach (LC-MS/MS, Orbitrap Velos Pro), 173 and 11 phosphoproteins were identified exclusively in the presence of glucose and TCW, respectively. Gene ontology (GO) analysis reported that most of the unique proteins belong to signaling and transmembrane transport, thus reinforcing the hypothesis that these processes are essential in fungal adaptation to environmental changes. Results were then expanded by analysis of membrane phosphorylated proteins, going more in-depth in the so-called "phospomembranome" analysis (Escobar-Niño *et al.*, 2019). Using different carbon sources, glucose as constitutive stage and tomato cell wall (TCW) as virulence inductor, authors applied a gel-free approach (LC-MS/MS) that allowed define a specific subproteome pattern, were differentially expressed proteins belongs to several metabolisms (e.g., pyruvate metabolism, unfolded protein response, cell death, thus).

The study of *B. cinerea* proteome was also extended to its secretome, which was analyzed in several works under different experimental conditions (Shah *et al.*, 2009a,b; Fernández-Acero *et al.*, 2010; Espino *et al.*, 2010). In the first paper, Shah *et al.* (2009a) used a gel-based approach (1-DE and LC-MS/MS) to detect differences in secreted proteins as a response to the use of mock infecting media supplemented with tomato, strawberry, and Arabidopsis leaf extracts. MS analysis identified 89 proteins among all the analyzed growth conditions, among which transport proteins, peptidases, and pathogenicity factors were identified, providing new insight about the use of *B. cinerea* secreted proteins for plant infection and colonization.

Following the first work, Shah *et al.* in 2009 published a second paper (Shah *et al.*, 2009b) still dealing with the interaction between host and pathogen by analyzing the impact of the degree of esterification of pectin on the fungal secretion. By using three different carbon nutrient sources, authors analyzed changes occurring during fruit ripening and then describing their role as triggers in the activation of *B. cinerea* from the dormant state to an active infection.

The secretion of *B. cinerea* proteins was also analyzed in the third work (Fernández-Acero *et al.*, 2010), where different carbon sources and plant elicitors (glucose, cellulose, starch, pectin, and tomato cell walls, TCW) were used to induced protein production. The analysis of 2-DE profiles indicates that the number of resolved spots increased when specific elicitors (cellulose and TCW) were added to growth media. Mass spectrometry (MALDI-TOF/TOF) analyses have led to the identification of 95 proteins (56 unique) out of 76 selected spots, some of which involved in the pathogenicity process (i.e., xylanases).

Modified growth media was also used in a fourth work (Espino *et al.*, 2010), where *B. cinerea* conidia were germinated in conditions that resembled the plant environment by adding low molecular weight plant compounds (tomato, strawberry, and kiwi-fruit) to analyze the early secretome. Secreted proteins were then collected after 16 h, and 2-DE-MALDI-TOF analyses identified 11 different proteins out of 16 differential expressed spots. These proteins were identified from a broader set of selected spots (86), so the authors did perform an alternative procedure for protein identification based on 1-DE coupled with LC-MS/MS, thus resulting in a higher number of identified proteins (105), mostly proteases able to degrade plant cell wall.

Secretome analysis was also performed on *B. cinerea* samples grown under different pH conditions (pH 4 and pH 6) to identify the role of ambient pH in protein secretion (Li *et al.*, 2012). Comparative analyses were performed using a gel-based approach to identify 21 unique proteins out of 47 differentially expressed spots. Among these proteins, authors indicate an upregulation of proteolysis proteins at pH 4, whereas the cell wall degrading enzymes were upregulated at pH 6. Proteomic data were then coupled with gene expression analysis (qPCR), confirming as expressions of differential proteins were mainly regulated at the transcription level (Li *et al.*, 2012). Further works were then published about *B. cinerea* secretome, by comparing the secretome of different strains (González-Fernández *et al.*, 2014), deepening the secretome composition through in vitro study (González-Fernández *et al.*, 2015), and identifying specific proteins that occurred during virulence pathogenicity process (Brito *et al.*, 2006; González *et al.*, 2014) or in plant defense response (Frías *et al.*, 2011; Frías *et al.*, 2016).

Sclerotinia sclerotiorum

S. sclerotiorum is a necrotrophic fungus that infects over 400 plant species worldwide, including much important crop species (Bolton *et al.*, 2006). The first comprehensive proteomic study of the *S. sclerotiorum* mycelia and secretome was published in 2006 (Yajima and Kav, 2006), getting new information about the life cycle as well as its phytopathogen activity. Combining 2-DE and LC-MS/MS analysis, authors identified 18 secreted and 95 mycelial proteins, including pathogenicity or virulence factors, one of which (alpha-Larabinofuranosidase) was never reported before. Further studies were focused on sclerotia formation (Liang *et al.*, 2010a), which are long-term multihyphal structures that can remain dormant until a favorable opportunity for germination and growth occurs (Willetts and Bullock, 1992). These structures represent a primary inoculum source for many crop species, and their development was analyzed at three representative stages of sclerotia development through a gel base approach (2-DE and LC-MS/MS). Results indicated as 88 protein spots were identified as differentially abundant among the different experimental conditions. These proteins were then classified into functional categories (e.g., metabolism, energy, cell defense, melanogenesis), also indicating the presence of a development-specific protein (SSP), which may have an essential role in the formation and germination of sclerotia (Liang *et al.*, 2010a). Besides that, the

same research group did also applied a proteomic approach to elucidate the protein composition of liquid sclerotial exudates, which are commonly produced during sclerotia formation (Liang *et al.*, 2010b). Fifty-six proteins were identified and then grouped in different functional categories (e.g., amino acid metabolism, carbohydrate metabolism, lipid, and secondary metabolism), giving new insight about the biological role and the composition of these exudates. More recently, the cell wall proteome was analyzed to determine if its structure could be influenced by the presence (using a PDA growth media) or the absence (using a Vogel's glucose medium) of plant-derived signals (Liu and Free, 2016). A bottom-up approach based on gel separation (SDS-PAGE) and MS analysis was employed and results indicated as the hyphae grown in the presence of plant materials contain a number of additional proteins (laccases, oxalate decarboxylase), that might improve the fungal growth on a plant host. Moreover, some of these cell wall proteins (11) were identified as encoded explicitly by Sclerotinia and Botrytis genome. In 2020, the knowledge of *S. sclerotiorum* proteome was greatly improved by the recent release of the paper of Otun and Ntushelo (2020), where a gel-based approach (SDS-PAGE and AB Sciex TripleToF 6600-MS) was applied, leading to the identification of 1471 proteins. This paper represents the most comprehensive work on *S. sclerotiorum*, giving new information useful to elucidate the various physiological processes of *S. sclerotiorum*.

Fusarium graminearum

F. graminearum Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch] is a fungal pathogen that is the causal agent of Fusarium head blight in several grain species (wheat, barley) that causes severe yield and quality losses. Besides, infected grains may contain high levels of trichothecenes mycotoxin (e.g., deoxynivalenol, DON) that inhibit eukaryotic protein biosynthesis causing severe diseases in human and animals (Goswami and Kistler, 2004; Trail, 2009; Yang *et al.*, 2013).

Considering these aspects, proteomic studies on *F. graminearum* have mainly focused on two aspects: secretome analysis and potential impact of mycotoxin production in the host infection process. The first published study on the secretome of this fungus was performed using a fungal culture grown in medium containing glucose or hop cell walls (*Humulus lupulus*, L.). Through a gel-based approach (1D-2D and LC-MS/MS), the authors identified 23 and 84 unique proteins, respectively, with a prevalence of cell degrading enzymes in the cell wall-grown secretome (Phalip *et al.*, 2005). Following this work, the *F. graminearum* secretome was also analyzed by ming the nutritional condition of fungus in the plant, by using wheat or barley flour as a unique nutrient source. The protein secreted in the culture medium was identified using a gel-based approach, resulting in 69 unique fungal proteins. Secretome composition quantitatively differing between different flour substrates, mainly in enzymes involved in the remodeling and degradation of plant cell walls (Yang *et al.*, 2012). Different approaches were also applied to deepening the knowledge of *F. graminearum* secretome. Using a high-throughput LC-MS/MS gel-free approach, 289 secreted proteins were identified using 13 different growth media (in vitro and planta), mostly glycoside hydrolases and proteases (Paper *et al.*, 2007). Secretome composition was also analyzed to describe the role of a subset of proteins that are required to initiate the infection, which removal results in reduced pathogenicity.

Using a label-free quantitative approach, the secretome of wild type and two non-pathogenic mutants of *F. graminearum* were analyzed. The results reveal a subset of 29 proteins differentially regulated proteins, as measured by spectral counting; some of these proteins decreased in abundance, thus representing potential candidates virulence factors (Rampitsch *et al.*, 2013).

The role of mycotoxin production was evaluated using specific fungal strain, where a gel-based approach (2D DIGE coupled with MALDI-TOF/TOF analysis) was applied on a nivalenol (NIV)-producing strain, leading to the identification of 1102 proteins, many of which never reported before (Pasquali *et al.*, 2013).

A gel-free approach based on iTRAQ analyses was employed to perform a complete protein profile of *F. graminerarum* expression under mycotoxin-inducing conditions. Comparative proteomic analysis reveals 130 differentially regulated proteins, with an upregulation in those involved in virulence. In contrast, the cluster of downregulated proteins included enzymes involved in primary metabolism, protein chaperones, or proteins involved with the cellular translational machinery (Taylor *et al.*, 2008).

The proteome of *F. graminerarum* was also assessed in response to viral infection (Kwon *et al.*, 2009), to identify changes triggered by the polyamine agmatine (Pasquali *et al.*, 2016) or to evaluate the polygalacturonase activity (Ortega *et al.*, 2014). In the first work, the *F. graminearum* virus–DK21 was used to infect *F. graminearum* strains. The analysis was carried out using a gel-based approach (2-DE- ESI-MS/MS), leading to 23 proteins out of 19 differentially regulated spots (Kwon *et al.*, 2009). Seven of these proteins resulting as upregulated, whereas the other sixteen, including enolase, saccharopine dehydrogenase, and flavohemoglobin resulted as downregulated. The second work analyzed the effect of an environmental stimulus at a proteomic level (Pasquali *et al.*, 2016). By adding the polyamine agmatine and glutamic acid as the sole nitrogen source, the production was induced in three different genetic chemotypes. A gel-based approach (2-D DIGE) highlights that proteomic profile and phenotype were drastically reshaped by agmatine, which modulated several proteins, including structural and virulence-related ones. The third work described the use of proteomic tools for purification and characterization of polygalacturonase enzyme, which is essential in the process of virulence. Using a gel-based approach (SDS-PAGE and MALDI TOF/TOF) on purified extracts from wheat spikes, the authors isolated and identified two proteins, including an endopolygalacturonase. This protein was then functionally characterized, in terms of optimum pH and temperature as well as metal ions sensitivity, giving new insight about the enzymatic mechanisms and role of polygalacturonase in the establishment of infection on wheat (Ortega *et al.*, 2014). Proteomic analyses in F. *graminearum* were also extended to phosphoproteomic to verify how phosphorylation events are involved in activating the trichothecene pathway under nitrogen-limiting conditions (Rampitsch *et al.*, 2010). The analyses were performed using different proteomic approaches (gel-free and gel-based), leading to the identification of 348 phosphorylation sites localized in 301 peptides from 241 proteins, including ten kinases and seven transcription factors. Following this first report, a second paper (Rampitsch *et al.*, 2012) was released where the phosphoproteome was analyzed without any nutritional limitation. In this case,

2902 putative phosphopeptides with homologous matches to 1496 different proteins were identified, mostly nuclear proteins with ATP-binding function.

Magnaporthe oryzae

M. oryzae is a filamentous ascomycete fungus that represents the causal agent of the most destructive rice disease worldwide, the rice blast disease (Ou, 1980; Dean *et al.*, 2012). Many papers employed the use of proteomic tools to describe the host-pathogen interaction from a plant point of view, which were gathered in a recently published review (Meng *et al.*, 2019). Despite this, some publications have covered the analysis of the fungus proteome, including the analysis of the fungal secretome (Wang *et al.*, 2011; Jung *et al.*, 2012). The first work on *M. oryzae* secretome (Wang *et al.*, 2011) employed a gel-based approach (2-DE and MALDI-TOF-MS, μLC-ESI-MS/MS) was used to identify 89 differentially expressed spots (14 downregulated and 75 upregulated) in response to N starvation. Functional classification of the identified proteins reveals three main groups, cell wall hydrolase enzyme, protein, and lipid hydrolases, and reactive oxygen species, with a prevalence of proteins containing single peptides (67%). In the second work (Jung *et al.*, 2012), a similar approach (2-DE and nESI-LC-tandem mass spectrometry) was employed by mimicking the early stage of infection in vitro, which led to the identification of 53 nonredundant proteins, including novel and previously known proteins. Further studies on nitrogen starvation on *M. oryzae* were performed using a gel-free approach identifying 5498 proteins. Among these proteins, 363 and 266 resulted as significantly induced or uniquely expressed under nitrogen starved or nitrogen supplemented conditions, respectively (Oh *et al.*, 2017). Functional analysis of protein-induced under nitrogen starvation conditions showed as many metabolic pathways were characterized by an extensive alteration, including the induction of several extracellular proteins.

Furthermore, proteomic studies on M. *oryzae* were also focused on different aspects of fungal lifestyle, including the identification of five novel proteins during the appressorium formation (Sun *et al.*, 2004), or the analysis of differentially regulated proteins in fungal mutants subjected to deletion of a conidial morphology regulating gene called Δ*com1* mutant. In the latter case, the comparison between mutant and wild type proteomes leading to the identification of 31 differentially regulated proteins, suggesting as the deleted genes may play a role in several metabolic processes, including the transcriptional reprogramming of genes implicated in melanin biosynthesis (Bhadauria *et al.*, 2010). Both these papers applied a gel-based approach, like the paper of Gokce *et al.*, where a GeLC-MS/MS analysis of conidia allowed the identification of 1500 proteins (Gokce *et al.*, 2011). Afterward, the same research group lately published a second paper on *M. oryzae* conidial proteome, were a gel-free approach was applied for the identification of 2912 proteins (Gokce *et al.*, 2012).

Proteomic studies have also performed on *M. oryzae* proteome to study the effect of plant hormones on mycelium development. In a recently published paper, high inhibitory effects on mycelium development were detected by adding salicylic acid (SA) to mycelium growth media. Authors used a gel-based approach, reporting as a concentration of 100 μM may reprogram many metabolic pathways, including those related to fungal development, signal transition, stress, and pathogenicity (Wang *et al.*, 2018).

Post-translational modification studies, including glycosylation and phosphorylation, were also performed in *M. oryzae* (Chen *et al.*, 2020; Franck *et al.*, 2015). Glycosylated proteins are widely present at different developmental stages of *M. oryzae*; therefore, a large-scale quantitative gel-free proteomic study (LC-MS/MS-based) was performed to achieve new insight about the functional role of these classes of proteins at different infection stages. A total of 355 unique N-glycosylated proteins were identified, not only the cell wall and plasma membrane proteins but also a wide range of proteins from the secretory pathway with crucial functions in protein glycosylation, folding, quality control, and secretion (Chen *et al.*, 2020).

Phosphoproteome analysis was employed in *M. oryzae* during the developmental process leading to the formation of a well-specialized structure called appressorium. This well-specialized structure is essential for plant infection, allowing the penetration of the plant leaf surface (Howard and Valent, 1996).

By using a gel-free approach, the authors identified a total of 1514 phosphoproteins from different tissues (mycelia, conidia, germinated conidia, and appressoria) from wild type and Δ*cpk*A mutant. The functional classification of proteins reveals as multiple changes were observed in the cell wall integrity pathway, thus providing new evidence about the role of this pathway during appressorium formation (Franck *et al.*, 2015).

Concluding Remarks

In conclusion, proteomics, as well as other "omics" tools, represent a robust set of techniques that could actively improve to complement the information of any fungal studies. The recent technical developments allow extending the analysis to the subcellular compartment and post-translational modification, with a focus on the analysis of fungal-environment interaction to addressing important physiological questions related to fungal biology.

New advances in MS-based methods can also represent the basis for develop new probes (antibodies) leading the chance to conjugate proteomics and immunolabelling for protein localization, with the aim to better highlight their biological role in complex biological systems. This is surely a crucial step to decipher the functions and interaction of proteins across different steps of the fungal life cycle, mainly when transformed systems are not applicable.

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Host-Induced Stress Response in Human Pathogenic Fungi

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Introduction

Within the many circumstances in which fungal stress response ability affects human life, the most pressing is when it contributes for their action as human pathogens. Among human pathogenic fungi, *Candida* species, *Cryptococcus neoformans* and *Aspergillus fumigatus* stand out. *Candida* species are the most common cause of invasive fungal infections among hospitalized patients (Wisplinghoff *et al.*, 2004; Perlroth *et al.*, 2007), causing around 750,000 cases of invasive candidiasis each year (Bongomin *et al.*, 2017). *Candida albicans* is the predominant causative agent of invasive candidiasis (Horn *et al.*, 2009; Yapar, 2014), but an increase in the incidence of non-albicans *Candida* species has been observed, especially *Candida glabrata* (Pfaller *et al.*, 2010; Taei *et al.*, 2019). *Cryptococcus neoformans* colonization occurs via inhalation, leading to pulmonary infections which may disseminate to cause meningitis, encephalitis or meningoencephalitis. Cryptococcal meningitis is one of the most frequent form of meningitis, with particular impact among HIV patients (Park *et al.*, 2009), 220,000 cases being estimated to occur each year (Rajasingham *et al.*, 2017). Invasive aspergillosis is a serious opportunistic infection that is mainly caused by *Aspergillus fumigatus* (Webb *et al.*, 2018). More than 300,000 cases of invasive aspergillosis occur each year (Bongomin *et al.*, 2017).

Most pathogenic fungi are commensal colonizers of the human body and typically, only cause infections in immunocompromised patients. Both as commensal and pathogens, fungi need to cope and adapt to different stressful conditions in host microenvironments (da Silva Dantas *et al.*, 2016). Fungi like *C. albicans* can colonize a wide variety of human niches (gut, vagina, oral mucosa, skin) where nutrient availability, temperature, pH, and oxygen (O_2) levels differ widely (da Silva Dantas *et al.*, 2016). Additionally, pathogenic fungi are exposed to a wide range of stress factors (**Fig. 1**). For example: in the oral cavity, kidney and urine they are exposed to osmotic stress (Brown *et al.*, 2014); in the nasopharyngeal environment they face human antimicrobial peptides (AMP), such as histatin-5 (Batoni *et al.*, 2011; Han *et al.*, 2016); in the gastrointestinal and urinary tract pH stress – indeed, the environmental pH inside the human body can be very dynamic: in human blood and tissues the pH is around 7.4, while the gastrointestinal tract can range from pH 2 to pH 8 and in the vagina around pH 4 (Mayer *et al.*, 2013), weak acid stress can also be found in the vaginal fluid, namely, acetic acid thought to be produced by bacteria (Bernardo *et al.*, 2017); once inside a phagocyte, pathogenic fungi are attacked by released reactive oxygen and nitrogen species, therefore, they must have defense mechanisms against those molecules in order to survive (Brown *et al.*, 2014).

In this chapter, the mechanisms of stress response and resistance that enable fungal pathogens to thrive in the human host, and cause deadly infections, are reviewed. Focus is first given to individual stresses faced by these organisms, including oxidative, nitrosative, pH and weak acid stresses, as well as nutrient limitation. Next, the combination of stresses felt by fungal pathogens upon phagocytosis by host immune cells as well as the multifactorial stress protective mechanisms offered by growth as biofilms are discussed.

Response to Nutrient Limitation in Human Host Environments

The ability to tolerate nutrient limitation, especially carbon and nitrogen sources, oxygen and iron, is crucial for successful fungal colonization and infection of the human host.

Carbon Limitation

Upon carbon source limitation, fungal cells rapidly activate a starvation mode, which includes translation downregulation, a shift from glycolysis to gluconeogenesis, and the activation of genes implicated in alternative pathways such as the glyoxylate cycle, and fatty-acid oxidation (Lorenz *et al.*, 2004). In *C. albicans*, when glucose is absent the CaSnf1 serine threonine protein kinase, which is essential for viability, is activated and consequently phosphorylates Mig1 preventing its translocation to the nucleus and stopping the repression of alternative carbon source metabolic genes (Van Ende *et al.*, 2019; Petter *et al.*, 1997; Orlova *et al.*, 2008). This drastic metabolic rearrangement allows cells to overcome carbon source depletion using carboxylic acids, amino acids, peptides, N-acetylglucosamine and fatty acids as carbon sources. In fact, the deletion of genes encoding key enzymes of these pathways, for example, *ICL1* (isocitrate lyase) and *FBP1* (fructose-1,6-bisphosphatase) results in attenuated virulence of *C. albicans* in a mouse model, highlighting the importance of these alternative pathways in the ability to overcome the depletion of glucose during infection (Lorenz and Fink, 2001; Ramírez and Lorenz, 2007). Similar mechanisms are also present in *C. neoformans*, including an ortholog of Snf1, the deletion of *CnSNF1* leading to defects in the utilization of alternative carbon sources, but also in the response to nitrosative stress, melanin production and virulence (Hu *et al.*, 2008a). *A. fumigatus* and *C. glabrata* also express Snf1 orthologs, CgSnf1 and Afu2g01700, the later remaining uncharacterized (Fetter and Kwon-Chung, 1996). Besides undergoing

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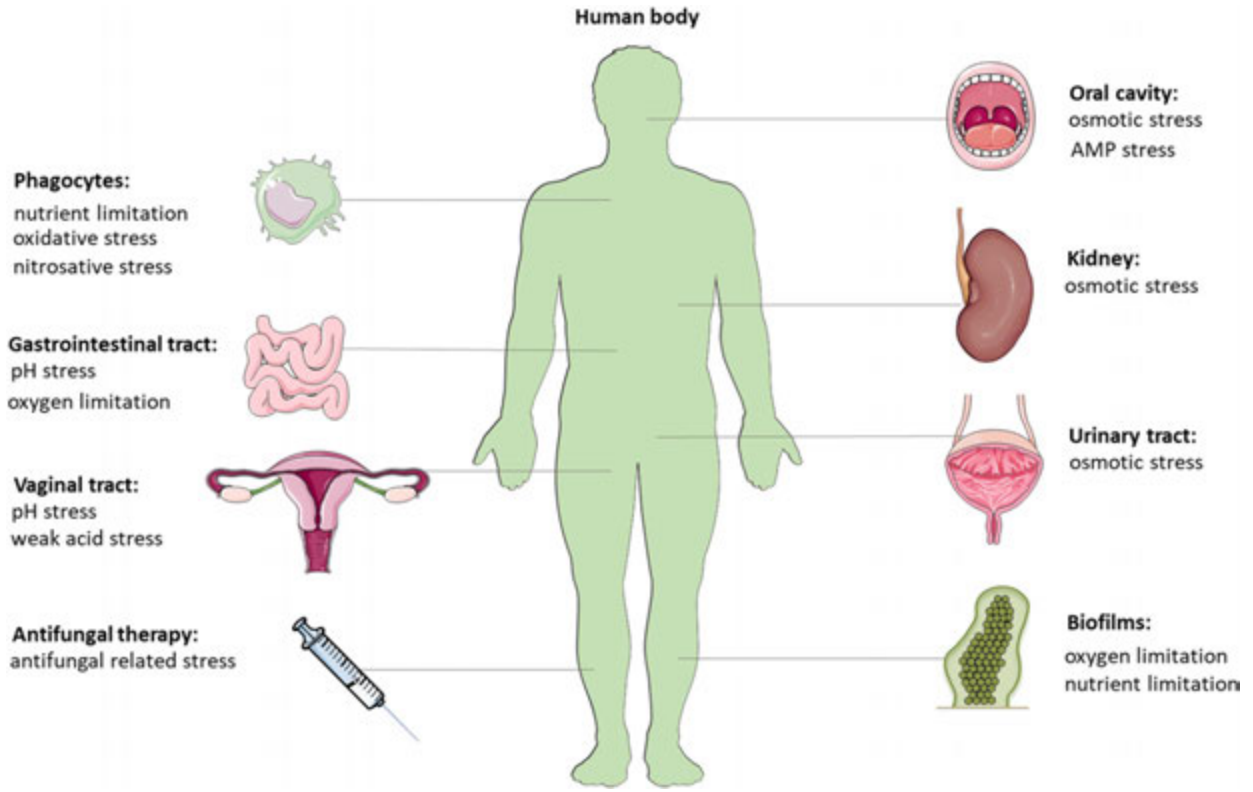

Fig. 1 The multitude of stress sources experienced by fungal pathogens in the human body.

drastic transcriptional remodeling, switching its expression program to genes involved in the utilization of alternative carbon sources, *C. glabrata* is able to use pexophagy, a subtype of autophagy, to recycle intracellular resources and deal with nutrient starvation (Roetzer *et al.*, 2010). Autophagy is a mechanism also used by *C. albicans* (Palmer *et al.*, 2007), *A. fumigatus* (Richie *et al.*, 2007), and *C. neoformans* (Hu *et al.*, 2008b), although it is only a virulence factor for *C. neoformans* and *C. glabrata*.

Beyond metabolic remodeling, nutrient limitation is tackled during tissue invasion by the use of extracellular enzymes to obtain nutrients from the host tissues (Brock, 2009). *C. albicans* secretes 10 aspartic proteases (SAP´s), Sap1–10, which are essential for growth when proteins are the sole nitrogen source (Naglik *et al.*, 2003). Similarly, *C. glabrata* expresses 11 cell surface-associated aspartyl proteases (CgYps1–11), called yapsins, which are key virulence factors (Rasheed *et al.*, 2018). *A. fumigatus* also secretes extracellular aspartic proteases, that play a significant role in invasive aspergillosis and are responsible for the hydrolysis of structural proteins in the lungs (Lee and Kolattukudy, 1995), as well as serine proteases and metalloproteases (Abad *et al.*, 2010). Extracellular protease activity was also reported in *C. neoformans* (Chen *et al.*, 1996). Extracellular lipases and phospholipases hydrolyze host triacylglycerols and glycerophospholipids, respectively, resulting in the release of fatty acids, that can be used as sole carbon source (Park *et al.*, 2013). *C. albicans* expresses at least 10 lipases and several phospholipases, of subclasses A–D (Park *et al.*, 2013). *A. fumigatus* phospholipase A–D activity has been detected (Birch *et al.*, 1996), while *C. neoformans* was mainly seen to display phospholipase B activity (Park *et al.*, 2013). *C. glabrata* is able to produce phospholipases (Fatahinia *et al.*, 2017; Sharma *et al.*, 2017; Pandey *et al.*, 2018) however the production is lower than in *C. albicans*, and in some isolates undetectable (Rossoni *et al.*, 2013; Riceto *et al.*, 2015; Canela *et al.*, 2018).

Iron Limitation

When exposed to an iron-poor environment, microorganisms can synthesize siderophores, high-affinity iron chelators that allow them to scavenge iron from the environment. *A. fumigatus* synthesizes four different siderophores, that are essential for virulence (Schrettl *et al.*, 2007). *C. albicans* and *C. glabrata* do not synthesize siderophores, but have one siderophore transporter each, Sit1/Arn1, which enables the uptake of iron bound to siderophores produced by other organisms (Ardon *et al.*, 2001; Heymann *et al.*, 2002; Hu *et al.*, 2002; Bernier *et al.*, 2005; Nevitt and Thiele, 2011). Similarly, *C. neoformans* does not produce siderophores, but is able to use iron chelators produced by other microorganisms (Tangen *et al.*, 2007).

C. albicans is also able to acquire iron from transferrin (Knight *et al.*, 2005), ferritin (Almeida *et al.*, 2008) and hemoglobin, following erythrocyte hemolysis (Almeida *et al.*, 2009). Hemoglobin is then bound by Rbt5 and internalized via endocytosis (Weissman *et al.*, 2008), followed by iron release from the heme group by the heme oxygenase Hmx1 (Santos *et al.*, 2003). *C. neoformans* also uses heme as iron source, in the dependence of Cig1, an exported mannoprotein, Fre2, a cell surface reductase

(Cadieux *et al.*, 2013; Saikia *et al.*, 2014), and several ESCRT proteins (Hu *et al.*, 2013, 2015). *C. glabrata* has a putative hemolysin, Mam3 (Srivastava *et al.*, 2014), however, high hemoglobin concentrations are required for growth to occur based on this iron source (Weissman and Kornitzer, 2004), while, in *A. fumigatus*, this mechanism does not appear to be present.

C. albicans has also a reductive iron-uptake system to scavenge iron from the host environment composed of CaFre10, a cell-surface ferric reductase, and CaFtr1, a ferric iron permease (Cheng *et al.*, 2013). Similarly, in *C. glabrata* extracellular ferric reductases are responsible for the ferric reductase activity, while the complex Fet3–Ftr1 is responsible for the ferrous oxidation and uptake (Srivastava *et al.*, 2014). *A. fumigatus* can also use a reductive iron assimilation pathway involving reduction of ferric to ferrous iron and the uptake of ferrous iron by the FtrA/FetC complex (Schrettl *et al.*, 2004). *C. neoformans* can also use enzymes such as melanin, reductases, and 3-hydroxyanthranilic acid in the reduction of ferric to ferrous iron, which is then imported through a complex involving the permease Cft1 and ferroxidase Cfo1 (Saikia *et al.*, 2014).

In the four pathogenic fungi, a set of transcription factors (TFs) controls the response to nutrient availability, inducing the expression of iron uptake genes and repressing iron consuming processes when facing iron limitation. These include Sef1, Sfu1 and Hap43 in *C. albicans* (Gerwien *et al.*, 2016), Aft1 and Sef1 in *C. glabrata* (Gerwien *et al.*, 2016), SreA and HapX in *A. fumigatus* (Mulvihill *et al.*, 2017) and Cir1 in *C. neoformans* (Kronstad *et al.*, 2013).

C. glabrata has developed also effective iron acquisition strategies and possesses a unique iron homeostasis mechanism. Aft1 is the main transcription factor regulator of this particular mechanism proposed by Gerwien *et al.* (2016), acting as an activator of iron acquisition genes under iron starvation conditions, such as *FTR1*, *FET3* and *SIT1*, at the same time inducing the expression of Cth2 a mediator of posttranscriptional mRNA degradation that will target iron consumption processes transcripts, namely, *HEM15*, *CCP1*, *ACO1*. The transcription factor Sef1, previously mentioned as an important transcriptional activator in *C. albicans* is also a Cth2 target gene in *C. glabrata* which seems to have a specific impact on the TCA cycle and on iron sulfur cluster-containing proteins assembly (Gerwien *et al.*, 2016).

Oxygen Limitation

Oxygen limitation is also an issue in the survival of fungal pathogens in the host, as it is encountered in anaerobic niches of the GI tract or in the inner sections of biofilms (Dumitru *et al.*, 2004). In lab experiments, most pathogenic fungi are unable to grow under anaerobic conditions in minimal medium, since oxygen is required for sterol and unsaturated fatty acid biosynthesis, and consequently, for the maintenance of plasma membrane and cell homeostasis (Lorenz and Parks, 1991). *C. albicans*, for example, is unable to grow in anaerobic conditions in minimal media, unless the medium is supplemented with oleic acid and nicotinic acid (Dumitru *et al.*, 2004).

When under hypoxia, *C. albicans* displays two responses: a short-term response, controlled mostly by the Sit4, Ccr4 and Sko1 TFs (Sellam *et al.*, 2014); and a long-term response which includes the upregulation of ergosterol biosynthesis by the TF Upc2 (MacPherson *et al.*, 2005), induction of fatty acid biosynthesis regulated by Efg1 (Setiadi *et al.*, 2006) and upregulation of glycolysis regulated by Tye7 (Askew *et al.*, 2009). The induction of hyphal formation, and the activation of genes involved in iron metabolism, heme biosynthesis, glycolysis and fermentation, cell wall and membrane remodeling are also observed. Additionally, the TF Ace2 seems to be required for filamentation under hypoxic conditions (Mulhern *et al.*, 2006). More recently, oxygen-sensitive SWI/SNF chromatin remodeling complex was reported as a regulatory circuit controlling metabolic flexibility, stress, commensalism and virulence in *C. albicans*, mutations in this complex affecting the ability to use alternative carbon sources under hypoxia (Burgain *et al.*, 2019).

C. neoformans is a strict obligate aerobe. Nonetheless, this pathogen causes encephalitis and meningoencephalitis, infections in oxygen limited niches, requiring effective hypoxia responses. These are dependent on the TFs Sre1 (a sterol regulatory element binding protein) and Tco1 (Grahl *et al.*, 2012). Under hypoxia, heme biosynthesis, fatty acid metabolism, ergosterol biosynthesis, and stress response transcripts are increased (Chun *et al.*, 2007), while capsule biosynthesis, melanin production, and phospholipase and urease genes are down-regulated (Kong *et al.*, 2017).

A. fumigatus responds to oxygen limitation by upregulating ergosterol biosynthetic genes, cell wall maintenance and glycolysis (Hartmann *et al.*, 2011; Grahl *et al.*, 2012). A particular response of this pathogen is the induction of GABA (γ-aminobutyrate) biosynthesis (Barker *et al.*, 2012), this molecule contributing to glutamate formation and being involved in the prevention of NADH accumulation in the absence of a terminal electron acceptor such as oxygen (Grahl *et al.*, 2012). *A. fumigatus* also has a sterol regulatory element binding protein, SrbA, that seems to regulate the hypoxia response in this pathogen (Blatzer *et al.*, 2011).

Besides mechanisms similar to those referred before, *C. glabrata* is able to import sterols, including cholesterol, under aerobic and anaerobic conditions (Zavrel *et al.*, 2013; Tsai *et al.*, 2004), through the transporter CgAus1 and the mannoprotein CgTir3, under the control of the TF Upc2 (Nakayama *et al.*, 2007; Inukai *et al.*, 2015).

Oxidative Stress Response

Reactive oxygen species secreted by the phagocytes, such as peroxide, superoxide anions, or hydroxyl radicals, induce an oxidative stress response in pathogenic fungi. Fungi can have different levels of intrinsic resistance to oxidative stress: *C. glabrata*, for example, can tolerate over 30 mM hydrogen peroxide (H_2O_2), while *C. albicans* and *A. fumigatus*, only 5 mM (Nikolaou *et al.*, 2009). When subject to oxidative stress fungal cells undergo transcriptional changes regulated by Yap1/Cap1 and Skn7, TFs

responsible for the expression of key genes encoding enzymes required for ROS detoxification, such as superoxide dismutases, catalases, as well as antioxidant molecules (Zadrąg-Tęcza *et al.*, 2018), this mechanism being present in *C. glabrata* (Roetzer *et al.*, 2011), *C. albicans* (Patterson *et al.*, 2013), *A. fumigatus* (Qiao *et al.*, 2008) and *C. neoformans* (Paul *et al.*, 2015). However, in *A. fumigatus* and *C. neoformans* these TFs are apparently not required for virulence (So *et al.*, 2019).

Superoxide dismutase (SOD) catalyses the conversion of superoxide into H_2O_2 and O_2. There are four known classes of SODs depending on the cofactor (Mn, Fe, Ni, and Cu-Zn) (Missall *et al.*, 2004a). SODs are present in the majority of the pathogenic fungi, including *C. albicans*, *A. fumigatus* (Holdom *et al.*, 2000), *C. neoformans* (Narasipura *et al.*, 2003) and *C. glabrata* (Roetzer *et al.*, 2011). *C. albicans* has five SOD enzymes, 4 cytosolic (Sod1, Sod3, Sod4, Sod5) and 1 mitochondrial (Sod2) (Missall *et al.*, 2004a). *A. fumigatus* has four SOD enzymes, 1 mitochondrial (Sod2) and three cytoplasmic (Sod1, Sod3 and Sod4). *C. glabrata* and *C. neoformans* express 2 SOD enzymes, 1 cytoplasmic (Sod1) and 1 mitochondrial (Sod2), each (Briones-Martin-Del-Campo *et al.*, 2015; Yang *et al.*, 2017).

Catalases (CAT) catalyze the reduction of H_2O_2 into O_2 and H_2O. In yeasts, there are two types of catalases, catalase T (CatT), present in the cytosol, and catalase A (CatA), present in the peroxisomes and mitochondria, to detoxify H_2O_2 generated during peroxisomal fatty acid β-oxidation and through mitochondrial superoxide dismutation (Zadrąg-Tęcza *et al.*, 2018). However, *C. albicans* and *C. glabrata* have only one CAT gene, involved in the protection against peroxide stress (Wysong *et al.*, 1998; Cuéllar-Cruz *et al.*, 2008). *A. fumigatus* expresses three catalase enzymes, one is produced by conidia, and two by mycelia, which confer protection against host oxidative stress (Paris *et al.*, 2003). In *C. neoformans* four CAT enzymes were identified, 2 located in spores (Cat1 and Cat3), 1 peroxisomal (Cat2) and 1 cytosolic (Cat4), but surprisingly, quadruple catalase mutants are not susceptible to oxidative stress (Giles *et al.*, 2006).

Other enzymes such as glutathione peroxidases can replace the function of catalases, reducing H_2O_2 by using glutathione as the electron donor. In this mechanism oxidation of glutathione by glutathione peroxidases results in the formation of disulfide glutathione, that in turn is reduced again by glutathione reductases, using NADPH as the reducing agent (Miramón *et al.*, 2014). In *C. albicans*, three enzymes with glutathione peroxidase activity were reported, gpx31, gpx32, and gpx33 (Miramón *et al.*, 2014). *C. glabrata* has one glutathione peroxidase, Gpx2 (Roetzer *et al.*, 2011). *C. neoformans* contains two glutathione peroxidases, Gpx1 and Gpx2 (Grant, 2001). In *A. fumigatus* this type of enzymes has not been reported.

Finally, glutaredoxins and thioredoxins, are redox scavenging molecules, that together with glutathione, contribute to protect and repair oxidized proteins (Cuéllar-Cruz *et al.*, 2014). *C. albicans* has four glutaredoxin encoding genes (Chaves *et al.*, 2007), while *C. neoformans* expresses one glutaredoxin, Grx4, important in iron sensing and virulence (Attarian *et al.*, 2018). In the remaining pathogenic fungi, the role of glutaredoxins in this response has not been sufficiently studied. Thioredoxin activity relies on the action of thioredoxin peroxidases, such as Tsa1, which, in *C. albicans*, is important for H_2O_2 neutralization (Shin *et al.*, 2005), while in *C. neoformans* it confers oxidative and nitrosative stress resistance (Missall *et al.*, 2004b). *A. fumigatus*, has three thioredoxin peroxidases, one cytosolic, Prx1, and two mitochondrial, PrxB and PrxC, Prx1 conferring oxidative stress resistance (Rocha *et al.*, 2018). *C. glabrata* expresses two thioredoxin reductases, Tsa1 and Tsa2 (Roetzer *et al.*, 2011).

If *C. albicans* (Palmer *et al.*, 2007), *C. glabrata* (Roetzer *et al.*, 2011), *A. fumigatus* (Richie *et al.*, 2007), or *C. neoformans* (Hu *et al.*, 2008b) cells are unable to deal with the imposed oxidative stress, the cell cycle is delayed in order to enable DNA-damage repair and the activation of cellular degradation systems occur (autophagy and ubiquitin-dependent proteasome system) as an attempt to remove damaged or unnecessary proteins and organelles and restore internal homeostasis. If the cell is unable to recover, programmed cell death is activated (Zadrąg-Tęcza *et al.*, 2018).

Nitrosative Stress Response

Reactive nitrogen species (RNS), such as nitric oxide (NO), produced by the innate immune system, can cause molecular damage in DNA, proteins and lipids of an invading microorganism (Hromatka *et al.*, 2005). Flavohemoglobin is one of the most frequent enzymes used by bacteria and eukaryotes to fight nitrosative stress (Hromatka *et al.*, 2005). This enzyme is responsible for the detoxification of NO under aerobic conditions. S-nitrosoglutathione (GSNO) reductase, responsible for the metabolization of the products resulting from the reaction of NO with thiol compounds, is another enzyme often involved in that response. *C. albicans* expresses three flavohemoglobin-related proteins, Yhb1, Yhb4 and Yhb5, and also a GSNO, Fdh3 (Tillmann *et al.*, 2015). *YHB1* is induced in response to NO stress and its deletion results in increased sensitivity to NO (Ullmann *et al.*, 2004). In *C. glabrata* nitrosative response lacks further studies. No GSNO reductase has been identified, however this yeast has an ortholog of CaYHB1 described as a putative flavohemoglobin (Merhej *et al.*, 2015). *C. neoformans* has both enzymes, GSNO reductase encoded by *GNO1* and flavohemoglobin encoded by *FHB1*, both required for the pathogenicity of this organism (De Jesús-Berríos *et al.*, 2003). In *A. fumigatus*, two genes encoding flavohemoglobins and one encoding a GSNO reductase were identified, however, they seem not to be involved in the nitrosative response (Lapp *et al.*, 2014); this pathogen is likely to have other yet unknown mechanisms to deal with NO stress.

pH Stress Response

The different niches in the human body have very variable pH values, ranging from pH 2 to pH 8 (Mayer *et al.*, 2013) thus being important for microorganisms to have mechanisms to deal with this variability.

Under pH stress, cytosolic pH neutrality is maintained through the activity of plasma membrane H^+:ATPase, encoded by *PMA1* gene in *A. fumigatus* (Burghoorn *et al.*, 2002), *C. albicans* (Monk *et al.*, 1991), *C. glabrata* (Bairwa and Kaur, 2011), and *C. neoformans* (Farnoud *et al.*, 2014), and vacuolar H^+:ATPase (vATPase) complex, also present in all species. When cell energy reserves are depleted, the cell loses ability to maintain its intracellular pH leading to protein misfolding and eventually cell death (Skoneczny and Skoneczna, 2018). Alternative mechanisms to overcome pH stress include, in *C. glabrata*, the remodeling of plasma membrane fatty acid and sterol composition, affecting H^+ ATPase activity, under the control of CgMed15B and CgMed3 mediator complex components (Qi *et al.*, 2017; Lin *et al.*, 2017). The TF CgRds2 is also involved in acidic pH stress response, its deletion resulting in decreased intracellular ATP content, membrane permeability and cell survival (Wu *et al.*, 2020), while the yapsin CgYps1 was also found to be essential for survival in an acidic environment, being required for cell wall remodeling, reducing the total β-glucan content (Bairwa and Kaur, 2011). *C. albicans* has a remarkable ability to modulate extracellular pH, being capable to change an acidic or alkaline extracellular pH to neutral pH in just 12 h (Vylkova and Lorenz, 2014). In such conditions, *C. albicans* is able to use amino acid or carboxylic acids like pyruvate, α-ketoglutarate, or lactate as carbon source and generate ammonia as a byproduct, neutralizing acidic environments (Vylkova *et al.*, 2011; Danhof *et al.*, 2016).

Alkaline pH medium, on the other hand, destroys the proton gradient across the plasma membrane, inhibiting important nutrient exchanges, such as glucose, phosphate and metals. In fungi, response to alkaline pH is generally mediated by the Rim101/PacC signaling pathway, controlling Na^+ and Li^+ homeostasis and vATPase activity, while Rim9, Rim21 and Rim8 are involved in the external pH sensing (Garnaud *et al.*, 2018; Bertuzzi *et al.*, 2014; Ost *et al.*, 2015). In *C. albicans* Rim101 signaling pathway seems to act in integration with the calcineurin/Crz1 pathway, since upon alkaline stress an immediate calcium influx occurs in this yeast (Wang *et al.*, 2011). *C. glabrata* has a *RIM101* ortholog, however, this pathway remains uncharacterized.

Weak Acid Stress Response

Weak organic acids are frequently used in the food industry as preservatives, but are also used as herbicides, antimalarial, anticancer and immunosuppressive drugs (Mira *et al.*, 2010). In low pH environments, these compounds are typically found in their undissociated state allowing them to diffuse through the fungal plasma membrane. After entering the cells, these acids dissociate, leading to the release of protons, which will result in cytosolic acidification. Additionally, acid dissociation, leads to the accumulation of the corresponding anionic conjugated bases, which are charged and therefore cannot simply diffuse back out (Skoneczny and Skoneczna, 2018). Cell responses to various weak organic acids seems to be different (Mira *et al.*, 2010). Response to sorbic or benzoic acids involves the plasma membrane H^+-ATPase, but also the up-regulation of the ATP-binding cassette (ABC) anion efflux pump Pdr12 (Ullah *et al.*, 2012), under the control of the TF War1 in *S. cerevisiae* (Schüller *et al.*, 2004), *C. albicans* (Lebel *et al.*, 2006), and *C. glabrata*. Interestingly, in *C. glabrata* sorbic acid stress activates the MAP kinase Hog1, which is required for *PDR12* overexpression (Jandric *et al.*, 2013). There are not many studies on this type of response in *A. fumigatus*, however, very recently, WarA (similar to War1) was characterized in *A. fumigatus*, and was shown to be required for resistance to weak-acid (Geoghegan *et al.*, 2020). No War1 homologs were found in *C. neoformans*. The response to short-chain weak acids, such as acetic acid, on the other hand, is mostly controlled by the TF Haa1. In *C. glabrata*, this TF controls the expression of 75% of the genes activated under acetic acid stress, including *PMA1* and the proposed acetate exporter gene of the Drug: H^+ Antiporter (DHA) family, *TPO3* (Bernardo *et al.*, 2017). Additional DHA proteins found to play a role in acetic acid resistance include Aqr1 (Costa *et al.*, 2013) and CgDtr1, the later contributing to *C. glabrata* proliferation upon phagocytosis and virulence (Romão *et al.*, 2017). In *C. albicans* the regulator of this response seems to be the TF Mnl1, which regulates weak acid stress responses acting antagonistically with the Nrg1 TF. Indeed, it was shown that the deletion of *MNL1* prevents the long-term adaptation of *C. albicans* cells to weak acid stress and compromises their global transcriptional response under these conditions (Ramsdale *et al.*, 2008).

Response to Stress During Phagocytosis

Upon phagocytosis, pathogens are subjected to a panoply of antimicrobial mechanisms in the phagosome environment. Genome-wide and phenotypic screenings from engulfed pathogens have provided a sneak peek into the antimicrobial mechanisms applied by phagocytes. Major stresses associated with phagocytosis include nutrient limitation (carbon, nitrogen, iron), oxidative and nitrosative stress and low pH stress (Flannagan *et al.*, 2012; Pais *et al.*, 2019a,b). Besides adapting their metabolic responses to the phagosome environment, successful pathogens have developed sophisticated strategies to escape immune cells (**Fig. 2**).

A comprehensive response to low nutrient availability typically results in reprogrammed metabolic settings focused on energy generation from alternative carbon sources. Phagocyted *Candida* display changes in carbon/energy metabolism which are focused on the activation of gluconeogenesis, alternative energy production pathways (e.g., glyoxylate cycle) and degradation of fatty acids for utilization as carbon source (Lorenz and Fink, 2001; Lorenz *et al.*, 2004; Fradin *et al.*, 2005; Kaur *et al.*, 2007; Rai *et al.*, 2012; Muñoz *et al.*, 2019; Williams and Lorenz, 2020). This is generally accompanied by downregulation of other pathways, such as plasma membrane and cell wall biosynthesis due to shutdown of sterol metabolism and mannan or glucan metabolism, respectively (Roetzer *et al.*, 2008; Fukuda *et al.*, 2013; Muñoz *et al.*, 2019). *C. neoformans* activates the cAMP/protein kinase A pathway, peroxisome function, lipid metabolism and membrane transport (Fan *et al.*, 2005). Similarly, *A. fumigatus* displays activation of fatty acid oxidation, transport functions and nutritional response genes, while genes involved in fermentation are

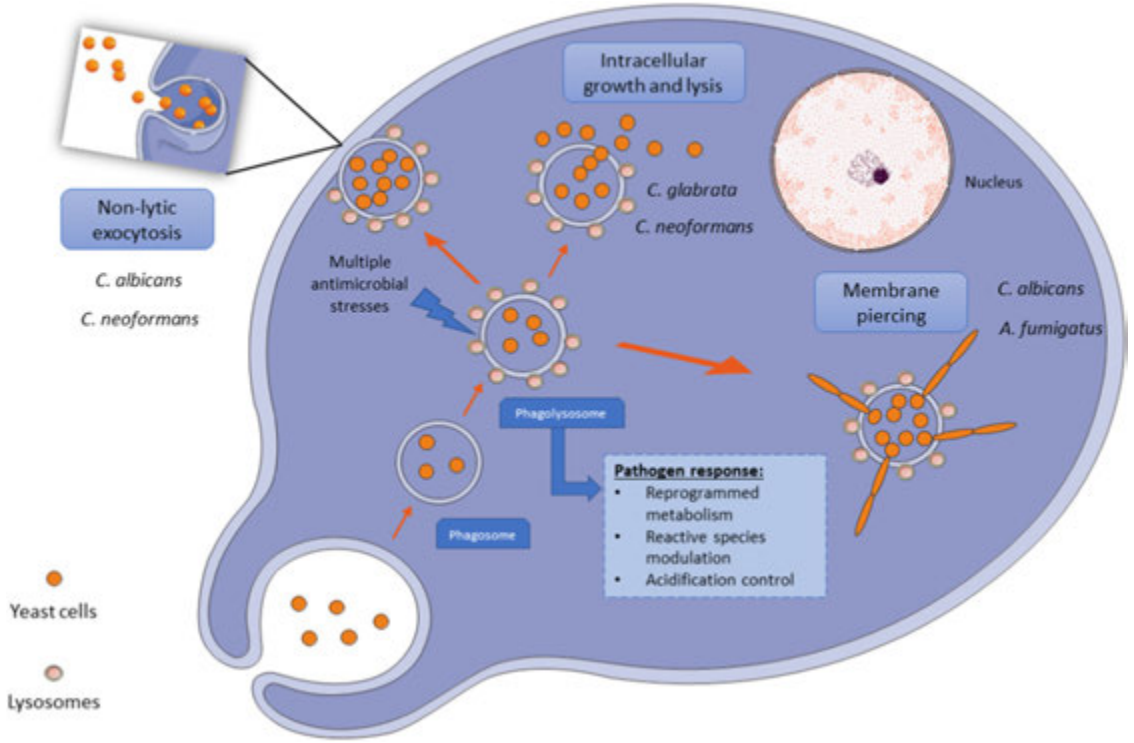

Fig. 2 Fungal pathogens response and escape mechanisms upon phagocytosis. Pathogens are engulfed by phagocytes into early phagosomes that undergo a maturation process accompanied by a decreased pH in the late phagolysosome. Pathogens are submitted to multiple stresses and respond in a concerted action. Common responses across fungal pathogens include reprogrammed energy metabolism (response to nutrient limitation), modulation of reactive species production (response to ROS/RNS stress) and inhibition of phagolysosome acidification (response to low pH). After surviving the harsh phagolysosome environment, fungal pathogens typically apply passive escape mechanisms based on overgrowth inside the phagocyte (*C. glabrata*, *C. neoformans*) or non-lytic exocytosis (*C. albicans*, *C. neoformans*); or active escape mechanisms based on filamentous growth (*C. albicans*, *A. fumigatus*).

down-regulated (Morton *et al.*, 2011). As for nitrogen metabolism, the same response pattern is adopted by fungal pathogens. Phagocytosis usually results in the induction of protein degradation pathways and amino acid biosynthesis (Kaur *et al.*, 2007; Morton *et al.*, 2011; Fukuda *et al.*, 2013; Williams and Lorenz, 2020). Recently, biotin metabolism was also correlated with *Candida* proliferation and virulence (Sprenger *et al.*, 2020). Additional strategies also include the activation of mechanisms to capture extracellular nutrients. A hallmark example is iron acquisition mechanisms, as iron is sequestered from pathogens by host scavengers and transporters (Cellier *et al.*, 2007; Almeida *et al.*, 2009; Haas, 2014). Iron limitation results in the activation of iron acquisition pathways and shutdown of iron consuming ones (Srivastava *et al.*, 2015; Gerwien *et al.*, 2016) and correlates with iron homeostasis genes that are required for survival after phagocytosis (Seider *et al.*, 2014). Furthermore, autophagy and pexophagy upon phagocytosis are yet additional mechanisms applied by *Candida* and *C. neoformans* to sequester resources in a poor-nutrient environment (Fan *et al.*, 2005; Oku and Sakai, 2010; Roetzer *et al.*, 2010, 2011; Till *et al.*, 2012; Fukuda *et al.*, 2013).

Response to oxidative stress includes detoxification of the correspondent reactive species (Goldman *et al.*, 2000; Shao *et al.*, 2005; Fukuda *et al.*, 2013). Besides reactive species detoxification, pathogenic response also includes additional mechanisms, such as cell wall composition and cytokine production, which have been associated with oxidative stress resistance and macrophage survival in *C. glabrata* (Seider *et al.*, 2014). Another crucial response to reactive species is the modulation of their production, which has been observed in *Candida*, *C. neoformans* and *A. fumigatus*, and includes both ROS and RNS production modulation (Goldman *et al.*, 2000; Orciuolo *et al.*, 2007; Seider *et al.*, 2014; Salvatori *et al.*, 2018; Schmidt *et al.*, 2018). In some pathogens, interference with reactive species production has been associated with particular structures or metabolites. The capsule of *C. neoformans* plays a role in response to reactive species, as it helps to quench free radical bursts (DeLeon-Rodriguez and Casadevall, 2016), while the melanin pigment and the toxin gliotoxin produced by *A. fumigatus* contribute to the modulation of ROS production (Orciuolo *et al.*, 2007; Schmidt *et al.*, 2018).

Response to low pH occurs as a reaction to phagosome acidification. After pathogen phagocytosis, intracellular compartments known as phagosomes are created. The phagosome goes through a maturation process accompanied by acidification ranging from pH 6.0 in the initial endosomes to pH 4.5 in the final phagolysosomes. This contributes for a stressful environment, especially when combined with the activity of hydrolytic enzymes (Vieira *et al.*, 2002; Seider *et al.*, 2011). Distinct approaches to cope with this antimicrobial mechanism have been described. In *C. glabrata* response to low pH includes the activation of stress responses, as well as carbohydrate and amino acid metabolic pathways whereas glucose metabolism and cell wall proteins are downregulated

(Bairwa and Kaur, 2011; Wu *et al.*, 2015; Lin *et al.*, 2017). Likewise, *C. albicans* uses catabolism of amino and carboxylic acids to neutralize acidic environments and also resorts to N-Acetylglucosamine (GlcNAc) metabolism to combat phagolysosome acidification (Vylkova and Lorenz, 2014; Danhof *et al.*, 2016; Miramón and Lorenz, 2016; Vesely *et al.*, 2017). The acidic pH in the phagolysosome further favors the filamentation of *C. albicans* (Káposzta *et al.*, 1999), leading to macrophage evasion (Lorenz *et al.*, 2004). The inhibition of phagolysosome maturation and acidification is also a strategy reported in *C. glabrata*, *C. neoformans* and *A. fumigatus* (Seider *et al.*, 2011; DeLeon-Rodriguez and Casadevall, 2016; Schmidt *et al.*, 2018). This is consistent with findings showing an acidic pH is key for *A. fumigatus* killing (Ibrahim-Granet *et al.*, 2003). As for *C. neoformans*, seemingly contradictory results have been described. One study describes inhibition of phagosome acidification (Smith *et al.*, 2015), while another study found a lack of it (Levitz *et al.*, 1999).

The aforementioned strategies mainly represent metabolic responses to counteract stresses exerted in the phagosome and maintain pathogen cell viability. In addition to intracellular survival, fungal pathogens also employ strategies aimed at phagocyte escape. This can be typically achieved by two avenues: hyphal growth leading to phagocyte piercing, or intracellular growth of the pathogen leading to phagocyte lysis. The strategy used depends partially on the ability of the pathogen to display filamentous growth. *C. albicans* enters the host in yeast form but can produce hyphae in response to host physiological conditions. Using a similar approach upon phagocytosis, *C. albicans* induces pyroptotic cell lysis of macrophages (Uwamahoro *et al.*, 2014; Wellington *et al.*, 2014) and at later stages undergoes a morphogenic transformation from yeast to filamentous form, coupled with cell wall remodeling (Muñoz *et al.*, 2019), that mediates phagosome damage and membrane piercing (Jiménez-López and Lorenz, 2013; Westman *et al.*, 2018). Moreover, the cytolytic peptide Candidalysin further contributes to phagocyte cell death (Kasper *et al.*, 2018). Other than damaging the phagocyte structure, *C. albicans* can also escape via non-lytic exocytosis (Bain *et al.*, 2012). As a filamentous fungus, *A. fumigatus* shares much of these traits, entering the host as conidia that subsequently germinate to establish an infection. Upon phagocytosis, conidia are able to germinate in the late phagosome leading to phagocyte necrosis (Wasylnka *et al.*, 2005; Shah *et al.*, 2016). *A. fumigatus* also produces toxins that induce immune cell death (Morton *et al.*, 2012) and due to the production of melanin, *A. fumigatus* conidia can avoid killing by phagocytes, deregulate immune homeostasis and interfere with apoptosis (Chamilos *et al.*, 2016; Mohebbi *et al.*, 2016; Sprenkeler *et al.*, 2016; Kyrmizi *et al.*, 2018).

On the other hand, species like *C. glabrata* or *C. neoformans* do not form hyphae and exist only in yeast form. As a consequence, immune evasion is based on cell surface alterations, such as cell wall remodeling in *Candida* (Kaur *et al.*, 2007) or capsule antiphagocytic properties in *C. neoformans* (Del Poeta, 2004; Chrisman *et al.*, 2011; Bojarczuk *et al.*, 2016). However, once phagocyted, these species employ an escape strategy based on intracellular growth and consequent lysis of the phagocyte (Seider *et al.*, 2011; Dementhon *et al.*, 2012; García-Rodas and Zaragoza, 2012; Coelho *et al.*, 2014). *C. neoformans* can also escape by non-lytic exocytosis (Alvarez and Casadevall, 2006; Ma *et al.*, 2006; Nicola *et al.*, 2011). This strategy implies the ability not only to survive the harsh environment of phagosome, but also to replicate inside it (Tucker and Casadevall, 2002; Seider *et al.*, 2011). In fact, both species were also proposed to use immune cells as "Trojan horses" for dissemination (Charlier *et al.*, 2009; Brunke and Hube, 2013; Duggan *et al.*, 2015).

Biofilm Formation as a Stress Response

Biofilm is a structure defined by an agglomerate of microorganisms attached to a given surface and protected by an extracellular matrix (Costa-Orlandi *et al.*, 2017). The formation of biofilms is a complex and multistage process that both bacterial and fungal microorganisms employ for their survival (Acker *et al.*, 2014), being their most common type of growth (Martinez and Fries, 2010). This process starts with the adhesion of the microorganisms to the chosen surface, following the production of extracellular polymeric substances, such as polysaccharides, proteins, lipids and DNA, which are secreted for the formation of the extracellular matrix, a gel like structure that allows firmer attachment and a consolidated architecture (Flemming and Wingender, 2010).

Fungi are able to develop yeast or filamentous biofilms. *Candida albicans* is able to form a filamentous biofilm with water channels that allow the flux of nutrients, while *Candida glabrata* forms a compact yeast-cell biofilm (Silva *et al.*, 2009), with half of the thickness of *C. albicans* biofilms (Kucharíková *et al.*, 2014). *Candida* species infections in the human host are usually linked to biofilm formation in preferable niches, like the vaginal tract (Harriott *et al.*, 2010; Nett, 2016). In turn, *C. neoformans* biofilm formation consists in the development of microcolonies of clustered cells, combined with capsular polysaccharide fibers and extracellular material (Martinez and Casadevall, 2006a,b), which can be developed after the fungus cross the blood brain barrier, being called cryptococcoma (Aslanyan *et al.*, 2017). The biofilm formed by *A. fumigatus* in the human host is denominated as aspergilloma, a spherical mass of hyphae that can be developed in an immune competent host, but requires a pre-existing cavity, generally from a previous tuberculosis in the respiratory tract. The filamentous biofilm of *A. fumigatus* has some similarities to the one developed by *C. albicans*, with channels between hyphae for the passage of fluids and nutrients (Ramage *et al.*, 2011). All these species are able to form biofilms on abiotic surfaces used on medical-devices (Walsh *et al.*, 1986; Hawser and Douglas, 1994; Ramage *et al.*, 2011; Susewind *et al.*, 2015), and yeast cells/spores from these biofilms are able to detach themselves and enter the blood stream, causing disseminated fungal infections (Costa-Orlandi *et al.*, 2017). Biofilms are thus a microbial strategy to invade and persist within the human host, being approximately 65% of all infections believed to be related to biofilms (Martinez and Fries, 2010; Acker *et al.*, 2014).

Interestingly, biofilms are also used as a stress response by pathogenic fungi (**Fig. 3**). This protective structure has been observed to confer resistance to different host-imposed stresses. For instance, *C. albicans* biofilm formation is stimulated by the presence of

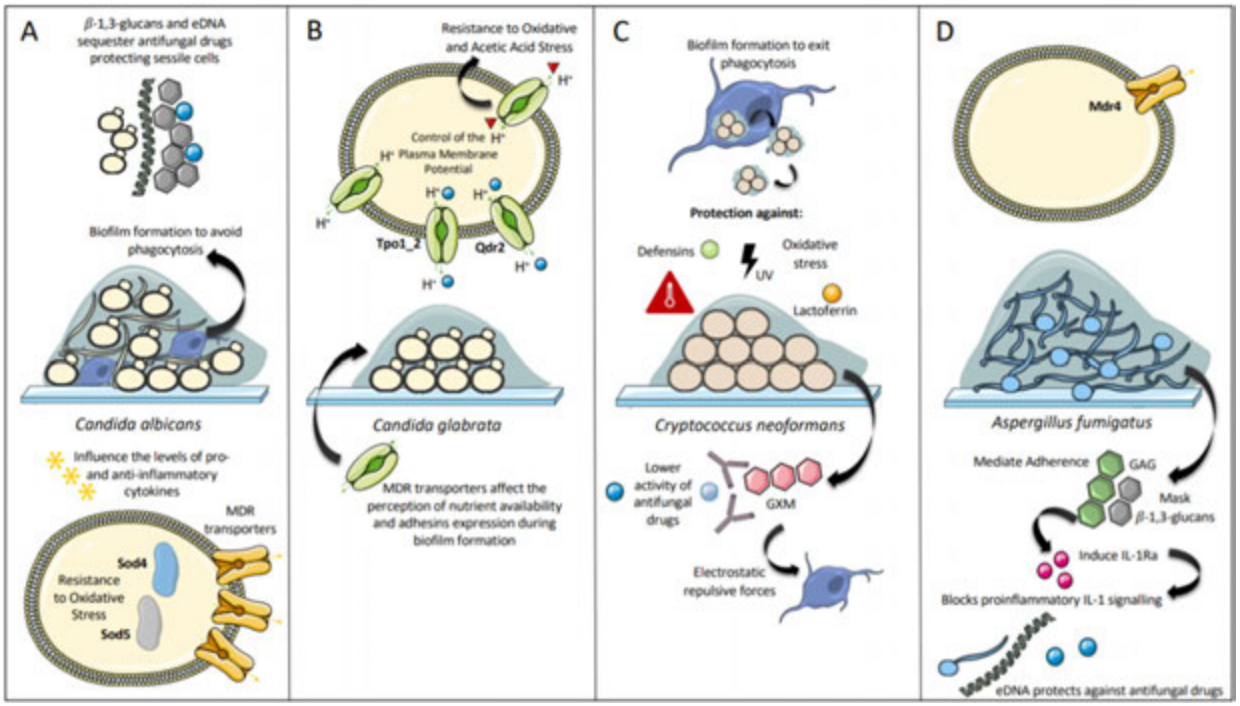

Fig. 3 Implications of biofilm formation in response to stress in (A) *Candida albicans*, (B) *Candida glabrata*, (C) *Cryptococcus neoformans* and (D) *Aspergillus fumigatus*.

peripheral blood mononuclear cells (PBMCs), enriched in monocytes and macrophages. Biofilm formation at such conditions leads to the accumulation of PBMCs in the basal and middle layers of the biofilm, without phagocytosis events, but with the release of differential levels of pro- and anti-inflammatory cytokines (Chandra *et al.*, 2007), allowing a clear modulation of the host immune response. *C. neoformans* biofilms are also linked to a strategy to evade phagocytosis. *C. neoformans* cells exit macrophages in biofilm-like microcolonies, being aggregated by a polysaccharide matrix (Alvarez *et al.*, 2008), suggesting that the formation of this biofilm-like structure allows this fungus to break free from macrophages.

Moreover, *C. neoformans* biofilms have been shown to increase the tolerance of this fungal pathogen to thermal stress (either higher or colder temperatures), to UV irradiation and to the presence of lactoferrin, an effector molecule of the innate immune system (Martinez and Casadevall, 2005, 2007). In addition, cryptococcal biofilm cells are more resistant against oxidative stress and defensins than planktonic cells (Martinez and Casadevall, 2006a,b), exhibiting increased expression of several oxidative stress response genes (Santi *et al.*, 2014). Using the *Galleria mellonella* model of infection, *C. neoformans* cells in biofilms were shown to be more virulent than planktonic cells, being more equipped against the larvae innate immune system (Benaducci *et al.*, 2016).

The tolerance fungal biofilms present to different stresses may rely on specific characteristics of this protective structure. One specific part of fungal biofilms that is intrinsically related to the response to external stresses is the extracellular matrix. Although the composition of the extracellular matrix may differ between *Candida* species, *C. neoformans* and *A. fumigatus* (Gibbons *et al.*, 2011), its protective role is unmistakable. One extracellular matrix component important for stress resistance is the galactosaminogalactan (GAG) of *A. fumigatus* biofilms. GAG is a component found not only to mediate adherence, but also to mask β-1,3-glucans (avoiding recognition by the host immune system) and induce the interleukin-1 receptor antagonist (IL-1Ra) that blocks proinflammatory IL-1 signaling (Loussert *et al.*, 2010; Gravelat *et al.*, 2013; Gresnigt *et al.*, 2014). Similarly, *C. neoformans* produces glucuronoxylomannan (GXM), an essential component of the extracellular matrix (Martinez and Casadevall, 2005). Interestingly, certain specific antibodies against GXM have been found to decrease the pharmacological activity of antifungal drugs against *C. neoformans* biofilms (Martinez *et al.*, 2006). This component also present in the capsule of *C. neoformans* has been linked to the creation of electrostatic repulsive forces to avoid phagocytosis (Aslanyan *et al.*, 2017).

Moreover, several studies show how different antifungal drugs have less effect upon biofilms thanks to the presence of this gel-like matrix (Martinez and Casadevall, 2006a,b). For instance, *C. albicans* and *C. glabrata* biofilms have shown to rely on β-1,3-glucans of the extracellular matrix to sequester antifungal drugs, such as fluconazole (Nett *et al.*, 2007, 2010a,b; Mitchell *et al.*, 2013). *C. albicans* biofilms also count with extracellular DNA from the extracellular matrix, which has been linked to higher resistance towards antifungal drugs (Martins *et al.*, 2012). Likewise, in *A. fumigatus* biofilms, the extracellular matrix has also been identified as the cause of polyenes resistance, thanks to its extracellular DNA, which is also essential for the integrity of the biofilm (Beauvais *et al.*, 2007; Rajendran *et al.*, 2013). Nevertheless, it is important to notice that the extracellular matrix may not be the only aspect of biofilms allowing antifungal resistance. The upregulation of multidrug resistance transports in *C. albicans*, *C. glabrata* and *A. fumigatus* biofilms (*CaCDR1*, *CaCDR2*, *CaMDR1*, *CgTPO1_2*, *CgQDR2*, *AfMDR4*), without exposure to antifungal drugs

(Ramage *et al.*, 2002; Nett *et al.*, 2009; Gibbons *et al.*, 2011; Santos *et al.*, 2020), may be the reason for the intrinsic antifungal resistance of biofilm cells. Such upregulation indicates that these transporters are needed for the normal development of fungal biofilms. In fact, *C. glabrata* found to rely on CgDtr1, CgTpo4, CgTpo1_2 and CgQdr2 Drug: H^+ antiporters for maximum formation of biofilms (Santos *et al.*, 2020). These transporters were found to alter the plasma membrane potential, as well as the expression of certain adhesin-encoding genes, being believed to control the nutrient availability influencing biofilm formation (Santos *et al.*, 2020). In addition, CgDtr1 has been linked to the resistance against oxidative and acetic acid stresses, being necessary for the full virulence of *C. glabrata* in the *G. mellonella* model of infection (Romão *et al.*, 2017).

Other effectors within *C. albicans* biofilms are responsible for the response to oxidative stress, like the superoxide dismutase's Sod4 and Sod5, which fight ROS generated by the presence of antifungal drugs, like miconazole (Bink *et al.*, 2011). In addition, fungal biofilms harbor the so called persister cells, a clonal subpopulation of cells that exhibit high tolerance to antimicrobial drugs. This subpopulation does not appear in planktonic conditions, contrary to what happens in bacterial populations. In fungi, persister cells seem to emerge due to the attachment of fungi to a surface, in the first step of biofilm formation (Lafleur *et al.*, 2006).

Conclusions

Pathogenic fungi are exposed to host microenvironments and need to cope and adapt to different stressful conditions therein. Response to nutrient limitation, including carbon, nitrogen, metals, and oxygen, is a prerequisite for successful fungal colonization and infection of the human host. The response to these starvation conditions is generally conserved among fungi, however, some pathogens have acquired specific mechanisms, for example, the production of their own siderophores by *A. fumigatus*, instead of importing them from the environment, or the import of sterols under anaerobic conditions by *C. glabrata*.

Multiple stress combinations, such as those felt after engulfment by phagocytes, are even more challenging that the sum of individual stresses. Response to these multi-stress environments include adaptation to nutrient limitation, to ROS and RNS and to acidification, but also, if possible, deployment of evasion strategies. Evasion mechanisms differ between fungal pathogens, *C. albicans* and *A. fumigatus* relying on filamentous growth, while *C. albicans* and *C. neoformans* using non-lytic exocytosis.

Another key protection mechanism used by fungi is biofilm formation. This feature is clearly used by the four pathogens considered in this chapter, although the structure and composition of the biofilm and its matrix varies from species to species. Despite their differences, fungi use biofilms as a strategy to evade phagocytosis, while providing protection against host stress factors or even antifungal drugs.

Although, generally speaking, fungal defense mechanisms against individual host stresses are reasonably characterized, some species-specific mechanisms, especially for multi-stress responses, remain unknown. A better understanding of, for example, the alkaline pH signaling pathway of *C. glabrata*; weak acid stress response in *C. neoformans* and *A. fumigatus*; nitrosative stress response in *C. glabrata* and A. *fumigatus*, are still required. Additionally, further studies on the use of alternative nutrient sources by pathogens may be necessary to fully understand their mechanisms of survival within phagocytes. All this knowledge will be crucial to design new and more effective strategies to fight life-threatening infections caused by very well adapted, stress resistant, fungal pathogens.

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Biodegradation of Aromatic Toxic Pollutants by White Rot Fungi

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Introduction

The world population is growing at exponential rate. Consequently, a number of anthropogenic activities have adverse effects on ecosystems and life on the planet. Industrialization, overuse of agrochemicals, and untreated industrial and municipal waste, as well as discharge of toxic effluents to the environment, are causing continuing damage to soil and water. Hence there is an urgent need to reverse and cure the hazardous pollutants negative impacts. One approach thereto is bioremediation of toxic organic chemicals.

Bioremediation involves exploitation of biological systems – plants, algae, bacteria, or fungi – for the purpose of removing or degrading and detoxifying pollutants (Alexander, 1994; Arora, 2018; Pande *et al.*, 2020). As vigorous decomposers, fungi hold promise for the removal of numerous xenobiotics from contaminated soil and water in an efficient, environmentally acceptable, and cost-effective manner, and thus could function as mycoremediation agents. Filamentous fungi have several distinctive properties that lend them advantages in bioremediation: fungal mycelial network and hyphal tips directional growth enable them to search out and penetrate new yet non-colonized, non-soluble substrates as sources of nutrients and energy; the multicellular fungal colony can function as an organism, recycle dead hyphae, and translocate resources; the mycelium of several fungi form rhizomorphs are able to grow through large distances of unfriendly environment, and thus establish in new niches relatively far from the colony source; fungi can survive, grow, and develop under toxic and stressful conditions (Baldrian, 2008; Sardrood *et al.*, 2013; Treu and Falandysz, 2017; Akhtar and Mannan, 2020; Ferreira *et al.*, 2020; Põlme *et al.*, 2020). This chapter focuses on a specific group of wood decay fungi that cause white rot.

White Rot Fungi: The Lignin-Degrading Fungi

White-rot fungi belong to the *Basidiomycetes*, mostly saprotrophic in the wild, where they grow readily on woody substrates. However, they do not form a taxonomic, but rather a physiological group based on their unique ability to efficiently degrade all plant cell wall components, including the most recalcitrant component: lignin. This property is the result of their producing several types of extracellular oxidizing enzymes that comprise the lignin degradation system (Kirk and Farrell, 1987; Hatakka, 1994; Hammel and Cullen, 2008). These fungi are termed "white rot" due to the removal of the brown lignin, giving the residual decayed wood a white appearance. Another group of wood decay fungi are the brown rots, which specialize in degradation of cellulose despite the presence of lignin. However, based on genomic analyzes of 22 wood decay fungi, Riley *et al.* (2014) demonstrated a continuum rather than a dichotomy between these two processes of wood decay. Riley's findings were further confirmed by phenotypic and genomic analyzes of many additional wood decay fungi, citing the complexity of the WRF ligninolytic system and its evolution (Nagy *et al.*, 2017; Schilling *et al.*, 2020). It has been suggested that these fungi originated in the Paleozoic area, evolved about 300 million years ago, and probably overlapped with the end of the Carboniferous period (Floudas *et al.*, 2012).

The biodegradation of the recalcitrant lignin is a central process in the global carbon cycle that enables further utilization of the resulting carbohydrates. The lignin role in woody plants is to provide rigidity as well as physical and chemical shield, thus providing passive defense from attack by most microorganisms. The lignin polymer is derived mainly from three aromatic alcohol monomers that differ in their degree of methoxylation: p-coumaryl, coniferlyl, and sinapyl alcohols. These units are linked together into a three-dimensional polymer via radical coupling reactions resulting in a complex and heterogeneous network of aromatic polymers containing ether and C-C and C-O linkages (Boerjan *et al.*, 2003; Vanholme *et al.*, 2010).

In accordance with the lignin nonspecific structure, the WRF enzymatic degrading system consists of nonspecific oxidative reactions leading to its depolymerization and mineralization. Its capability of degradation of a wide range of toxic aromatic pollutants, the subject of the current review, originates from these enzymes activities. Their non-specific nature and exceptional oxidation potential likely force the fungi to rapidly secrete oxidizing enzymes to avoid damage to the cells. Indeed, these enzymes are known to function in the extracellular environment. The ligninolytic system includes several families of enzymes: Lignin peroxidases (LiP), Manganese peroxidases (MnP), Versatile peroxidases, (VP), Dyeperoxidase (DyP), Phenol oxidases, and Laccases. Also of importance are H_2O_2-producing enzymes, as they provide the peroxide to the peroxidases. These are Glyoxal oxidase (GLOX), Aryl alcohol oxidase (AAO), Vanillyl alcohol oxidase, and Cellobiose dehydrogenase (CDH). These enzymes biochemistry, physiology, genomics, and genetics have been studied and reviewed in detail over the years (Kirk and Farrell, 1987; Hatakka, 1994; Martínez *et al.*, 2005; Hofrichter *et al.*, 2010; Hadar and Cullen, 2013; Knop *et al.*, 2015; Kües, 2015). Besides the ligninolytic system, additional physiological functions are involved in growing on wood, such as intracellular transport and detoxification of aromatic toxic products released during wood utilization, i.e., families of transporters, cytochrome p450 monooxygenases (CYP450), and glutathione-S-transferases (Syed and Yadav, 2012; Morel *et al.*, 2013; Deroy *et al.*, 2015;

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Nagy *et al.*, 2017; Daly *et al.*, 2020). These enzymes are very common in WRF and play an important and central role in detoxification of numerous organic pollutants. More recently, developments in the study of lignin degrading and modifying enzymes as well as barriers and challenges to practical use of these enzymes were reviewed (Kües, 2015; Biko *et al.*, 2020; Chan *et al.*, 2020; Asemoloye *et al.*, 2021).

Among the wide variety of WRF species in nature, only a few have received in-depth attention from the scientific community. Some of their characteristics have been studied in more detail, including enzymatic mechanisms of wood degradation, the ligninolytic system, and biotechnological applications such as biofuel production, as well as pollutant degradation. Here are three representative examples:

Phanerochaete chrysosporium Burdsall

This WRF is the first and most studied organism owing to its lignin biodegradation and mineralization capabilities, and is considered a model fungus. *P. chrysosporium* is a crust fungus, which forms flat, fused reproductive fruiting bodies on woody surfaces. It is thermotolerant and grows relatively fast at 42°C. It was isolated and described in the Sonoran Desert in 1971 (Burdsall and Eslyn, 1974). The enzymes lignin peroxidase and Mn-peroxidase were discovered while investigating lignin degradation mechanisms in *P. chrysosporium* (Glenn *et al.*, 1983; Tien and Kirk, 1983) and it was suggested to be used for organic pollutants bioremediation (Bumpus *et al.*, 1985). *P. chrysosporium* was the first WRF to be sequenced (Martinez *et al.*, 2004).

Trametes (Coriolus) versicolor (L.) Lloyd ("Turkey Tail")

This WRF has distinct morphological features, including concentric multicolored zones on the upper side of the cap. It grows on many deciduous trees (oaks) and some conifers (firs and pines), with the fruiting body appearing on stubs and trunks year round in temperate Asia, North America, and Europe. It is known for its medicinal value as used in Chinese traditional medicine, and contains general health-promoting effects (Hiscox *et al.*, 2010; Habtemariam, 2020).

Pleurotus ostreatus ("Fries") Kummer ("Oyster Mushroom")

The genus *Pleurotus* was defined by Paul Kummer in 1871. Pleurotus species are considered saprophytes, and frequently grow on dead wood and decaying trees (Raman *et al.*, 2021). Unlike many of the well-studied WRFs, several *Pleurotus* species are edible and commercially cultivated in large-scale facilities. *Pleurotus* spp.'s production is significant for the food industry (Deepalakshmi and Mirunalini, 2014), comprising around 25% of cultivated mushrooms worldwide. Some *Pleurotus* species are known for their medicinal properties. *Pleurotus* are grown on a variety of lignocellulosic substrates available locally, such as a wide array of forest and agricultural byproducts (Cohen *et al.*, 2002; Raman *et al.*, 2021).

Degradation of Major Groups of Organic Pollutants

Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a group of chlorinated aromatic compounds that are considered one of the most toxic and persistent organic pollutants. Their properties include longevity and heat absorbance, and they form an oily liquid at room temperature that is useful for electrical utilities and in other industrial applications. In the 1960s, the toxic effects from exposure to PCB began to be apparent (Alharbi *et al.*, 2018). Consequently, the production and use of these compounds is banned or strictly controlled in many countries, which bans and restrictions were adopted internationally under the Stockholm Convention on Persistent Organic Pollutants (Hung *et al.*, 2016).

The degradation of various PCBs by several WRFs has been reported over the years, including *P. chrysosporium* (Eaton, 1985), *T. versicolor* (Cloete and Celliers, 1999), *P. ostreatus* (Chun *et al.*, 2019), *P. sajor-caju* (Sadañoski *et al.*, 2019), and *Irpex lacteus* (Stella *et al.*, 2016). WRFs were reported to have degraded and treated soil and wood from a disused sawmill area contaminated for decades with chlorinated phenols, dibenzo-p-dioxins, and furans (Valentín *et al.*, 2013).

A technical mixture of PCBs' degradation pathway was studied by following the metabolites produced by *P. ostreatus*, wherein 41 PCB degradation products were detected and profiled. Accordingly, it was concluded that both intracellular enzymes (cytochrome P-450 monooxygenase, aryl-alcohol dehydrogenase, and aryl-aldehyde dehydrogenase) and the extracellular ligninolytic systems participate in the process (Čvančarová *et al.*, 2012). *P. pulmonarius*'s proteome was studied in the presence of transformer oil containing a mixture of Aroclors 1242, 1254, and 1260 (Sebastian *et al.*, 2021). A total of 705 proteins were identified, of which 111 were only found in the PCBs exposed fungus, and 390 were only found in the absence of PCBs. Upregulation was found for glyceraldehyde-3-phosphate dehydrogenase and transcription factor as well as reductases and oxidases such as laccases and versatile peroxidases; on the other hand, proteins related to carbohydrate and proteolytic metabolism were repressed, suggesting that the fungus faced oxidative stress during the degradation process (Sebastian *et al.*, 2021).

Possibilities and advances in the remediation of PCBs have been reviewed (Chun *et al.*, 2019; Italy *et al.*, 2020). An interesting, likely cost-effective approach was the use of *P. ostreatus* spent substrate in a trickle-bed bioreactor (Šrédlová *et al.*, 2020). This pilot-scale

bioreactor (working volume 500 L) was able to remove to various levels di-, tri-, tetra-, and pentachlorinated PCB congeners. Overall, this experiment proved the suitability of spent oyster mushroom substrate's use in degradation and detoxification of recalcitrant PCBs under non-sterile conditions, even in the presence of bacterial and fungal contaminations.

Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds comprised of two or more fused aromatic rings originated mostly from anthropogenic activities resulting in inadequate combustion of materials such as coal, oil, or wood. They pose a serious concern, as they are resistant to biodegradation, bioaccumulate, and may be mutagenic, carcinogenic, teratogenic, and immunotoxicogenic to many organisms, including humans. In addition, these compounds are widely distributed in the environment (Patel *et al.*, 2020). Several approaches for remediation of PAHs have been proposed, including the use of various bacteria and fungi (Cerniglia, 1992; Gramss *et al.*, 1999; Bamforth and Singleton, 2005; Kuppusamy *et al.*, 2017).

PAHs' degradation by WRF was reviewed by Kadri *et al.* (2017), who described culture conditions, kinetics, degradation pathways, and the enzymatic degradation mechanisms. Bumpus *et al.* (1985) was the first to suggest the potential of PAHs bioremediation by *P. chrysosporium*. They demonstrated that the fungus partly mineralized benzo[a]pyrene and phenanthrene (Bumpus, 1989) to carbon dioxide. Hence, *P. chrysosporium* has been the focus of many studies on PAHs degradation under ligninolytic, nutrient-rich, and other culture conditions by ligninolytic extracellular as well as intracellular enzymes (Hammel *et al.*, 1986; Syed and Yadav, 2012; Gu *et al.*, 2016; Bhattacharya *et al.*, 2013; Bogan and Lamar, 1996). *P. ostreatus* metabolized a wide variety of PAHs with little correlation between PAH degradation and extracellular laccases, manganese peroxidases, or manganese-independent peroxidases activities (Bezalel *et al.*, 1996). *Ganoderma lucidum* and *Trametes polyzona*, among other WRFs, were also able to remove various PAHs (Teerapatsakul *et al.*, 2014; Torres-Farradá *et al.*, 2017; Agrawal *et al.*, 2018; Torres-Farradá *et al.*, 2019).

Bezalel *et al.* (1996) demonstrated that *P. ostreatus* is able to metabolize phenanthrene to phenanthrene trans-9, 10-dihydrodiol, and 2,2′-diphenic acid, as well as partially mineralizing it to CO_2. The formation of phenanthrene trans-9R,10R-dihydrodiol, wherein only one atom of oxygen originates from molecular oxygen, suggests that *P. ostreatus* initially oxidizes phenanthrene via a cytochrome P-450 monooxygenase and an epoxide hydrolase (Bezalel *et al.*, 1997). While Park *et al.* (2019) showed that *Dentipellis* sp. KUC8613 was capable of removing PAHs without upregulating ligninolytic genes, out of 154 identified P450 genes in the *Dentipellis* genome, transcription of 15 genes was induced by one or more PAHs. They also found that 1922 genes were upregulated during four PAH removal processes, many of which involving unknown molecular functions. They suggested a pathway that includes initial ring oxidation and a transformation step by glycosyltransferase and glutathione S-transferase, as well as transporters for the translocation of PAHs and their metabolites, lipases for the enhanced solubility of PAHs, and antioxidant enzymes for reduced oxidative stress. This is consistent with Bezalel *et al.* (1997) findings based on metabolite analyzes and physiological and biochemical experiments. Genomic and transcriptomic approaches are suggested as the focus for the next decade of research (Park and Choi, 2020).

Textile Dyes

The textile industry is one of the main contributors to pollution via discharge of untreated effluents containing biologically stable dyes into water bodies. Approximately 15% of dyes utilized during various dyeing processes in various industries, pollute water bodies, threaten our aquatic ecosystems, and may affect human health (Vikrant *et al.*, 2018; Lellis *et al.*, 2019; Tkaczyk *et al.*, 2020). Due to the emerging pollution caused by textile dyes during recent decades, microbial degradation became an option for remediation (Chaurasia and Bharati, 2019; Ihsanullah *et al.*, 2020; Varjani *et al.*, 2020), including the study of several WRFs and their enzymes (Heinfling *et al.*, 1998; Wesenberg *et al.*, 2003; Vikrant *et al.*, 2018; Kalia and Singh, 2020). Eichlerová and Baldrian (2020) studied the decolorization of Orange G and Remazol Brilliant Blue R by 150 saprophytes belonging to 77 *Basidiomycetes* of various ecotypes, WRF, brown rots, and litter decomposers. WRFs were the most efficient at decolorizing both dyes.

Besides being studied for their remediation, textile dyes can also serve as an interesting tool for WRF research, due to the dual role that they play in the study of ligninolytic fungi and their oxidative systems. On one hand, dyes are targets for bioremediation, as aforementioned. On the other hand, they can be used not only as a screening tool, but also as a means of elucidating catalytic mechanisms of biodegradation. The use of dyes as research tools offers a number of advantages, as they are chemically stable, soluble, and inexpensive; and have high molar absorption coefficients. Decolorization of dyes can be tracked in simple, quick, and quantitative spectrophotometric measurements, thereby serving as a basis for easy and accurate phenotypic-based assays for WRFs' efficiency and their ligninolytic system's functionality (Glenn and Gold, 1983; Platt *et al.*, 1985; Salame *et al.*, 2010). For example, the ability to follow azo-dye Orange II decolorization was exploited for screening of RNAi silenced mutants of *P. ostreatus* (Salame *et al.*, 2010). Knockdown of mnp3, using RNAi silencing, resulted in the reduction of Orange II decolorization, which was consistent with marked reduction of both *mnp*3 and *mnp*9 genes' expression levels. Knop *et al.* (2014, 2016) used the azo dyes Orange II and Reactive Black 5 to study versatile peroxidase 1 (VP1) reaction mechanism and also to verify its role in vivo by growing *P. ostreatus* wild type under Mn-deficient conditions, as well as showing that Δ*vp1*'s knockdown mutant had negligible decolorization capability.

Lucas *et al.* (2008) screened more than 325 WRFs belonging to 76 genera for their ability to decolorize five azo and two anthraquinone dyes. More recently, screening was employed to study a large population of *Schizophyllum commune*'s genetic and physiological diversity and dye-decolorizing spectrum (Choi *et al.*, 2020). They identified 5 excellent strains that strongly decolorized Crystal Violet, Congo Red, and Methylene Blue.

These type of studies can identify the most efficient strains for practical applications and also provide data on their physiology required for designing environmentally friendly processes (Sekan *et al.*, 2019; Kalia and Singh, 2020).

Pharmaceuticals and Personal Care Products

Pharmaceuticals and personal care products may retain their bioactivity in the environment, either as the original molecule or as transformation products. These compounds can be found in trace concentrations planet-wide in freshwater bodies and soils (Kondor *et al.*, 2020; Yadav *et al.*, 2021). The sources of the pharmaceuticals in the environment could be effluents from hospitals, animal husbandry, as well as wastewater treatment plants, as some of these compounds are stable and are not removed during the sewage purification process or even after incubation in treated wastewater-irrigated soil (Fu *et al.*, 2019; Grossberger *et al.*, 2014). Such compounds can find their way back to humans through the food chain (Paltiel *et al.*, 2016).

Degradation of several of the most recalcitrant pharmaceutical compounds by WRFs has been reported, as they have been recognized as an emerging environmental problem and potential risk. Much attention has been given to carbamazepine (CBZ) modification by various white-rot fungi such as *T. versicolor* (Kang *et al.*, 2008; Rodriguez-Rodriguez *et al.*, 2010) and *P. ostreatus* strains F6, N001 (dikaryons) and PC9 (monokaryon) (Golan-Rozen *et al.*, 2011). In a follow-up study, Golan-Rozen *et al.* (2015) followed CBZ's transformation pathways and identified in liquid culture 10,11-epoxy carbamazepine and 10,11-dihydroxy carbamazepine as a dead-end product. However, in solid-state culture based on lignocellulosic substrate, an additional 22 transformation products have been identified. While Lamotrigine (LTG), another persistence drug, was also removed by *P. ostreatus* (Chefetz *et al.*, 2019), most identified transformation products were conjugates of the original compound. *T. versicolor* was able to remove several other pharmaceuticals such as diclofenac, naproxen, and ketoprofen (Marco-Urrea *et al.*, 2010). *P.ostreatus* metabolized clomipramine, mianserin, paroxetine, and sertraline (Kózka *et al.*, 2020). WRF enzymes could also effectively remove diclofenac, antibacterial compounds such as tetracycline oxytetracycline, and triclosan, (Wen *et al.*, 2009; Inoue *et al.*, 2010; Zhang and Geissen, 2010; Maadani Mallak *et al.*, 2020), as well as various cytotoxic anticancer drugs (Yadav *et al.*, 2021). Sulfonamides' biodegradation mechanisms via *P. chrysosporium* have been studied at the transcriptomic level (Zhang *et al.*, 2021).

Degradation of pharmaceuticals in pure culture is only a first step in designing a practical degradation process. Thus, it was important to develop WRF-based reactors that work under more realistic conditions. CBZ was removed when *P. chrysosporium* was grown on polyether foam under non-sterile conditions (Zhang and Geissen, 2012). In this case, nutrients were added to the culture. In a batch fluidized bed bioreactor, Cruz-Morató *et al.* (2013) demonstrated the complete removal of seven pharmaceuticals in Madrid's municipal wastewater under non-sterile conditions. The same type of reactor was used for pre-treatment of hospital wastewater with *T. versicolor* pellets. It was shown that 46 of the 51 detected compounds were partially or completely removed (Cruz-Morató *et al.*, 2014). An air-pulsed fluidized bed bioreactor was used for the continuous treatment of non-sterile real hospital wastewater by *T. versicolor* (Mir-Tutusaus *et al.*, 2019). The team was able to keep the fungus active and predominant throughout the process despite the bacterial contaminations. Suspensions prepared from mushroom substrate colonized by *P. ostreatus* removed several pharmaceuticals, including diclofenac and lamotrigine (Hultberg *et al.*, 2020). In another study, two bioreactor types were used to treat both synthetic and real hospital wastewater by *T. versicolor*: Stirred tank bioreactor was compared to trickle-bed bioreactor, based on rice husks, which was found to be more efficient at the task (Tormo-Budowski *et al.*, 2021). The topic of real wastewater treatment systems for the removal of various micropollutants, including pharmaceuticals, was reviewed in detail by Mir-Tutusaus *et al.* (2018), who analyzed the advantages, difficulties, challenges, and possible solutions for developing an industrial-scale operation.

Can the Knowledge Accumulated on Biodegradation Lead to Mycoremediation?

Despite the need, interest, and vast research invested in the role of fungi in removing organic pollutants from contaminated environments, to our knowledge, no full-scale WRF-based commercial operation for removal of micropollutants from wastewaters or effluents, as well as terrestrial ecosystems, has been reported thus far.

Many authors have deliberated the advantages and future, but also limitations and bottlenecks, for WRF's application in mycoremediation (Pointing, 2001; Harms *et al.*, 2011; Kulshreshtha *et al.*, 2014; Tortella *et al.*, 2015; Mir-Tutusaus *et al.*, 2018; Chun *et al.*, 2019; Ferreira *et al.*, 2020; Akhtar and Mannan, 2020), some of whose arguments are summarized here:

(1) Mir-Tutusaus *et al.* (2018) described several continuous WRF operations at various scales and times of operation, treating a gamut of micropollutants. They concluded that WRF-based reactors could be an efficient technology for removing micropollutants from specific wastewater streams (Mir-Tutusaus *et al.*, 2018).
(2) Treu and Falandysz (2017) demonstrated the need for more studies under local field conditions with indigenous species. They contended that results from specific sites and species may not be applicable for other locations with differing soil types, climates, ecologies, and indigenous microbiomes.

(3) WRFs' ecology and their constraints and advantages for soil bioremediation were analyzed by Baldrian (2008), who cited the requirement for vigorous fungal inoculum applied with nutrient supplement as a food base. For example, *P. ostreatus* colonizing the spent mushroom substrate (Chung at al., 2019) and *P. chrysosporium* colonizing wood chips (Lamar, 1990) or alginate-immobilized spore suspensions (Lestan and Lamar, 1996).

(4) A major bottleneck, not often discussed, is the long time required by WRF-based processes. Strain improvement, genetic modification, or search for new species appears to be a challenge. Construction of synthetic microbial communities could be useful (Sharma and Shukla, 2020). In addition, employing means for controlling mycelium morphology, optimizing nutrient supplementation, and designing improved reactors for scaled-up ex situ processes of contamination removal from water and effluents, are of importance.

(5) Micropollutants are typically found at trace concentrations. Affordable and accurate technologies and instrumentation for micropollutants' extraction and detection and their transformation products at environmental levels should be further developed. This is important not only for the evaluation of the mother compound fate and potential residual toxicity, but also to elucidate degradation pathways.

(6) Transcriptomic and proteomics information is available for WRF, but mostly from studies related to lignocellulosic biomass degradation (Vanden Wymelenberg *et al.*, 2009, 2010; Alfaro *et al.*, 2016 and, 2020; Fernández-Fueyo *et al.*, 2016; Rytioja *et al.*, 2017; Zhangand and Yamaura, 2020; Wu *et al.*, 2021) or as a response to lignin-related mixtures of aromatic compounds resembling some pollutants (Daly *et al.*, 2020). While only a few omics studies have been conducted thus far in relation to elucidation of pollutant degradation by WRFs' mechanisms, they hold potential for better and deeper understanding of WRF bioremediation processes, and will likely be more common in the near future. Moreover, omics studies will probably assist in discovering new gene functions, so far annotated as "unknown", and gene regulation. Metagenomics is useful for studying mycoremediation at the community level to learn about interactions with other microorganisms under non-sterile environments (Wang *et al.*, 2017; Park and Choi, 2020).

(7) While employing relatively new approaches, such as synthetic biology, systems biology, and metabolic engineering, for bioremediation (Dangi *et al.*, 2019; Jaiswal and Shukla, 2020; Sharma and Shukla, 2020; Zaccaria *et al.*, 2020; Asemoloye *et al.*, 2021) has also been suggested, most data accumulated so far concern bacteria and yeast, and relatively little related to WRFs and their enzymes (Park *et al.*, 2019; Zhang *et al.*, 2021).

In summary, a question that arises in much of the literature reviewed here is: Can the massive knowledge accumulated thus far on the chemistry, biochemistry, physiology, ecology, genomics, and genetics; as well as engineering aspects of aromatic organic pollutant biodegradation by fungi in general and by WRF in particular, be translated into and lead to large-scale, cost-effective, commercial mycoremediation processes? The responses range from skepticism to enthusiasm.

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Fungal Chitin and Chitosan

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Introduction

Around the world, the annual synthetic polymers production is approximately 140×10^6 tons. However, these synthetic polymers are somewhat stable and their biodegradation is limited. This necessitates the need for replacement of the synthetic with biodegradable polymers that are compatible with the ecosystem. Among the biodegradable polymers, chitin and chitosan have attracted the attention of the workers in both scientific and industrial field due to their numerous potential applications in biomedicine, agriculture, paper making, food industry, and textile industry (Akila, 2014). The wider applications of chitin and chitosan are not only due to their abundance but also due to their non-toxicity and biodegradability (Islam *et al.*, 2017).

Chitin is the second abundant biopolymer after cellulose with annual biosynthesis of about 100 billion tons (Tharanathan and Kittur, 2003). Chitin is found in exoskeletons of insects and mollusks and also a main component in the shell wastes of crustaceans and in the cell walls of some fungi (Bhuiyan *et al.*, 2013; Yeul and Rayalu, 2013).

Historically, was first isolated by Braconnot (1811) from the cell walls of some types of fungi, i.e., *Agaricus volvaceus*, *Agaricus acris*, *Agaricus cantarellus*, *Agaricus piperatus*, *Hydnum repandum*, *Hydnum hybridum*, and *Boletus viscidus*, where it was named "fungine." In 1823, fungine was renamed as chitin by Odier (1823), who succeeded in production and isolation of chitin and chitosan from the cuticle of insects almost three decades before the isolation of cellulose (Knorr, 1984). Chitosan was first discovered in 1859 by Rouget (Novak *et al.*, 2003). After its discovery to present, it has become a source of numerous studies due to its versatile biological, chemical, and physical properties and wide range of applications (Bhuiyan *et al.*, 2013). Although it has been found in some types of fungi, chitosan is, mostly, obtained from chitin deacetylation.

Initially, chitin and chitosan were obtained from the shell wastes of shrimps and crabs. However, their discovery in the fungal cell walls opened up the horizon for more research and biotechnological applications due to the absence of allergenic substances and less waste production (Chien *et al.*, 2016). Moreover, fungal chitin and chitosan would offer non-seasonable and stable sources of raw material and consistent characters of the product.

Fungal Cell Wall

The fungal cell wall is a dynamic structure surrounds the fungal cell structures and composed of glycoproteins and polysaccharides. It can be tough, flexible or rigid (Peberdy, 1990; Rouiz-Herrera, 1992). It is essential for cell viability and pathogenesis. It, also, determines the shape of the cell, protects it from osmotic pressure, environmental stress and mechanical injury and acts as a filtering mechanism (Latgé and Calderone, 2002). The fungal cell wall is much more than the outer layer of the fungus; its composition greatly influences the ecology of the fungus and can change in response to environmental conditions and imposed stresses (Latgé, 2010).

Physiological Function of Fungal Chitin and Chitosan

Generally, the fungal cell walls are composed of chitin, chitosan, neutral polysaccharides, and glycoproteins in addition to minor amounts of polyuronides, galactosamine polymers, lipids, and melanin (Wu *et al.*, 2004). Chitin exists in the spores and hyphal cell walls in conjunction with glucan molecules forming microfibrils. These microfibrils are embedded in an amorphous matrix to provide the framework and cell wall morphology and rigidity. Chitosan is not considered as native to animal sources; meanwhile, it presents in some fungal species such as *Mucor*, *Absidia*, and *Rhizopus* as one of the structural components in their cell wall (Rouiz-Herrera, 1992). Chitin and chitosan are thought to increase the cell wall integrity and strength and provide protection against foreign materials, e.g., cell inhibitors and higher temperatures to which fungal cells may be subjected (Adams, 2004; Banks *et al.*, 2005; Baker *et al.*, 2007). Chitin and chitosan represented other functions as revealed by mutants bearing a defect in the complex machinery of chitin biosynthesis, deposition of the polysaccharide in cell walls, or intracellular trafficking of chitin synthases (Specht *et al.*, 1996).

Fungal Cell Wall Composition and Architecture

The fungal cell wall is essential for its survival. The cell wall composition is unique to each fungus, and therefore it forms an ideal target for the development of novel antifungal drugs (Tryphon *et al.*, 2016). Most of the fungal cell walls consist of five major components: β- (1,6) glucan, (1,3) β-glucan, α-(1,3) glucan, glycoproteins, and chitin (un-branched chains of β-(1,4)-linked-N-Acetylglucosamine) (**Fig. 1**). True fungi do not have cellulose in their cell walls (Webster and Weber, 2007).

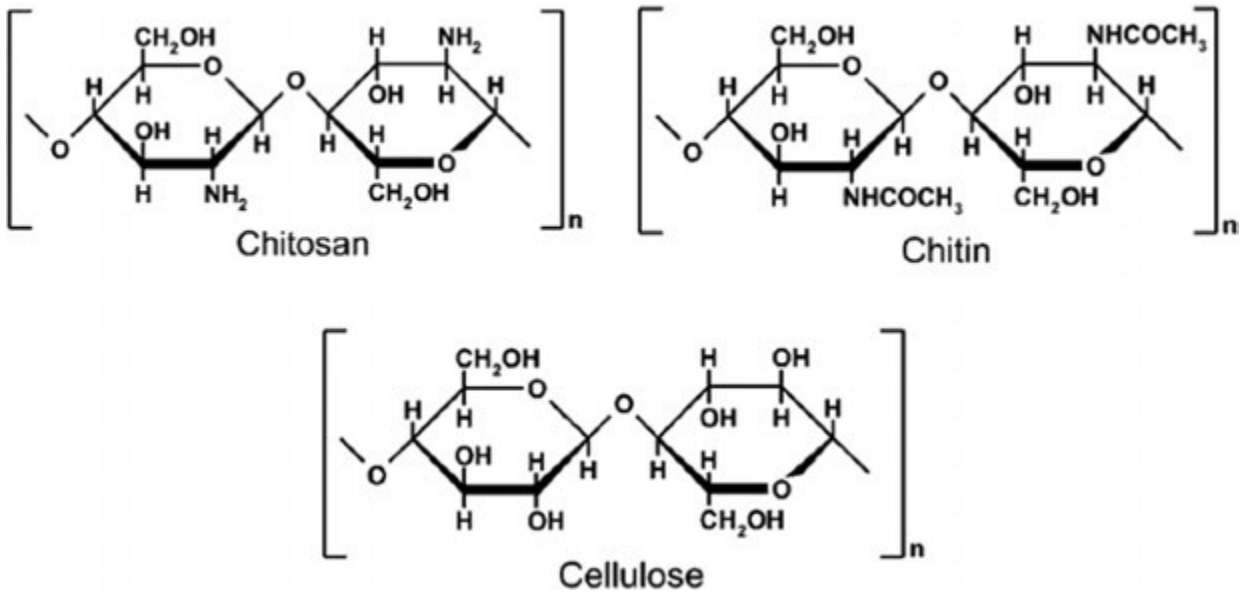

Fig. 1 Structural formula and differences between chitin, chitosan and cellulose.

The fungal cell walls are all organized in a somewhat similar way. The structure of the wall, directly, affects wall function and interactions with the environment (Gow *et al.*, 2017). These interactions include immune recognition by animals and plants. Most cell walls are layered. The innermost layer comprises a relatively conserved structural skeletal layer, whereas the outer layers are more heterogeneous and tailored to adopt to the physiological conditions of fungi. The inner cell wall, in most fungal species, consists of a core of covalently attached branched β-(1, 3) glucan with 3%–4% interchain and chitin (Fleet, 1991; Latgé, 2007). Hydrogen bonds are formed between β-(1, 3) glucan and chitin and can be assembled into microfibrils to form a basket-like scaffold around the cell membrane. These branched β-(1,3): β-(1,6) glucans are getting bound to other components i.e., proteins and/or other polysaccharides, which their type and/or amount may vary with the fungal species.

The outer layers of fungal cell wall vary much more than the inner skeletal layer. In the yeasts cells of *Candida* and *Saccharomyces* species and the human pathogen *Pneumocystis jiroveci*, the outer cell wall comprising highly mannosylated glycoproteins that covers the inner wall. In *Aspergillus* and many human pathogens, α-(1, 3) glucan plays a prominent role in the organization of the cell wall, but is absent from the *Candida* and *Saccharomyces* cell walls. The presence of α-(1,3) glucan prevents dectin-1-mediated immune recognition of the underlying β-(1,3) glucan by immune cells (Rappleye *et al.*, 2007). The chemical composition of the cell wall of some fungal phyla is represented in (**Table 1**).

The cell wall of the yeast *Cryptococcus* is enveloped by a gelatinous capsule composed mainly of glucuronoxylomannan and galactoxylomannan which are attached to the cell wall via α-(1,3) glucan. Worth mentioning, the capsular polysaccharides are synthesized intracellularly and then secreted via exocytosis through the cell wall (Yoneda and Doering, 2006).

The structure and biosynthesis of the budding yeast *S. cerevisiae* cell wall has well been studied. It has been used as model organism to study cell division and cell morphogenesis in eukaryotic cells (Botstein *et al.*, 1997; Hayles and Nurse, 2001). It is, mainly, composed of β-(1,3) glucan, that forms an alkali-soluble fraction or a chitin-linked, alkali-insoluble fraction. Furthermore, it contains β-(1,6) glucan, chitin, and mannoproteins (Klis *et al.*, 1997). *S. cerevisiae* lacks α-glucans. Electron microscopy examination of *S. cerevisiae* showed that the cell-wall is organized in a layered structure where β-(1,3) glucan forms densely interwoven microfibrils present as the innermost layer, followed by β-(1,6) glucan and mannoproteins (Osumi, 1998; Cabib *et al.*, 1982). Most chitin is found in the region of the bud scars which remain after the cell separation, whereas the remainder of the chitin is dispersed over the lateral walls. Similar, with some modifications, layered cell-wall architecture was found in other fungi such as fission yeast *S. pombe* (Kopecká *et al.*, 1995), *C. albicans* (Chaffin *et al.*, 1998), and *A. fumigatus* (Fontaine *et al.*, 2000).

Biosynthesis of Cell Wall Polysaccharides

Polysaccharides of the fungal cell wall such as chitin and glucans are synthesized, in an inactive form, at the plasma membrane of the cell by transmembrane enzymatic complexes via secretory vesicles and then activated after insertion into the plasma membrane (Gow *et al.*, 2017). In contrast to mannans and other glycoconjugates, where they are synthesized in the endoplasmic reticulum and Golgi apparatus then may be conjugated to cell wall proteins, and brought to the cell wall by the classical secretory route via secretory vesicles (Gow *et al.*, 2017). All synthases enzymes use nucleotide diphosphate-sugars as substrates for polysaccharide synthesis, so enzymes of the metabolic pathways responsible for the synthesis of nucleotide sugars are rate-limiting and essential for the construction of the cell wall (Gow *et al.*, 2017).

Table 1 The chemical composition of the cell walls of some fungal phyla (in % dry weight of total cell wall fraction)

Phylum	*Example*	*Chitin*	*Cellulose*	*Glucans*	*Protein*	*Lipid*
Oomycota	Phytophthora	0	25	65	4	2
Chytridiomycota	Allomyces	58	0	16	10	?
Zygomycota	Mucor	9[a]	0	44	6	8
Ascomycota	Saccharomyces	1	0	60	1	8
	Fusarium	39	0	29	7	6
Basidiomycota	Schizophyllum	5	0	81	2	?
	Coprinus	33	0	50	10	?

[a]Mainly chitosan.
Note: Webster, J., Weber, R.W., 2007. Introduction to Fungi. New York, NY: Cambridge University Press, pp. 5–7.

Chitin Synthases and Synthesis

Chitin, a natural mucobiopolymer, is a hard, white, inelastic, and nitrogenous compound, composed of randomly distributed N-acetyl-D-glucosamine (N-GlcNAc) monomers (Islam *et al.*, 2017). Following cellulose, chitin is considered as the second most abundant biopolymer on earth, with annual biosynthesis of more than 100 billion tons (Tharanathan and Kittur, 2003). In nature, chitin polysaccharide is, enzymatically, synthesized by the transfer of a glycosyl of N-GlcNAc from uridinediphosphate-N-acetyl-D-glucosamine to chitodextrin acceptor (Sitanggang *et al.*, 2012). Chitin is the most ancestral structural polysaccharide in the fungal cell wall (up to 60%) and composed of linear chains of β-(1,4) N-acetylglucosamine (Bartnicki-Garcia, 1968). In *Saccharomyces cerevisiae*, chitin represents about 2% (w/w) of the total cell wall and mainly present at the bud scars (Klis *et al.*, 2002). Wessels (1997) suggested that hyphal growth occurs as the result of a continuously replenished supply of soft wall material at the apex, but there is good evidence that the softness of the apical cell wall is also influenced by the activity of wall-lytic enzymes such as chitinases or glucanases (Fontaine *et al.*, 1997; Horsch *et al.*, 1997). The formation of chitin is very similar to cellulose, which is consistent with their role as a structural polysaccharide (Chandrasekaran, 1997). For chitin, the chemical structure is similar to that of cellulose, which consists of a polymer of several hundred units of β-(1-4) linked D-glucose (Bhuiyan *et al.*, 2014). In chitin, the hydroxyl group at position C-2 of cellulose has been replaced by an acetamide group (**Fig. 1**).

Unlike cellulose, the nitrogen content of chitin and chitosan is 5%–8% which makes them suitable for typical amines reactions (Kurita, 2001). The synthesis of chitin is mediated by specialized organelles termed chitosomes (Bartnicki-Garcia *et al.*, 1979; Sentandreu *et al.*, 1994) in which inactive chitin synthases are delivered to the apical plasma membrane and become activated upon contact with the lipid bilayer (Montgomery and Gooday, 1985). The major synthases that are responsible for chitin production reside in the cell plasma membrane and utilize UDP-N-acetylglucosamine as the substrate for the chitin formation that is extruded into the cell wall (**Fig. 2(A)**). In the cell wall, the polysaccharides can be crosslinked or branched together through hydrogen-bonds by the enzymes that reside in the cell wall (**Fig. 2(B, C)**).

The biochemical functions of many chitin synthases are not exactly known (Bowen *et al.*, 1992; Munro and Gow, 2001; Cabib *et al.*, 2001; Roncero, 2002). Some chitin synthase isoforms may have redundant functions. Although, a homopolymer with only one linkage is the product of all chitin synthase enzymes, these enzymes can synthesize chitin fibrils of differing architecture due to the differences in folding degree and intra-chitin hydrogen bonding of the primary chain (Lenardon *et al.*, 2007).

The families of chitin synthases have been identified bioinformatically, and have molecular weights of 100–130 kDa (Garcia-Rubio *et al.*, 2020). Two families of fungal chitin synthases (**Fig. 3**) have been identified based on amino acid sequence (Morozov and Likhoshway, 2016).

The role of each of the seven chitin synthase classes is not well understood. The four chitin synthase classes (III, V, VI, VII) are specific to filamentous fungi (Bowen *et al.*, 1992; Fernandes *et al.*, 2016).

Some genes, named CHS, are not associated with the activity of chitin synthase but are involved in its regulation and/or localization. Some chitin synthases are zymogens that become active only after proteolysis and some others are regulated by phosphorylation (Schorr *et al.*, 2001; Valdivia and Schekman, 2003). Some fungi have more than 20 CHS genes, and some have only one (Lenardon *et al.*, 2010).

The chitin synthases (class V and class VII) in filamentous fungi have unconventional myosin motor-like domains (MMD) (Steinberg, 2011). These enzymes are essential for morphogenesis, growth, virulence and stress tolerance. The presence of myosin motor-like domains (MMD) is not required for the motility of some fungal species, e.g., *Ustilago maydis* and *Aspergillus nidulans* (Treitschke *et al.*, 2010). However, its presence may function in tethering vesicles in the apical dome by increasing the residence time at that location favoring vesicle fusion with plasma membrane (Schuster *et al.*, 2012).

Biosynthesis of Chitin and Chitosan

Glycogen is the starting material for chitin synthesis. The first step is the catalysis of glycogen by phosphorylase enzyme where glycogen is converted to glucose-1-phosphate. In the presence of phosphomutase, glucose-6-P is formed and further converted to

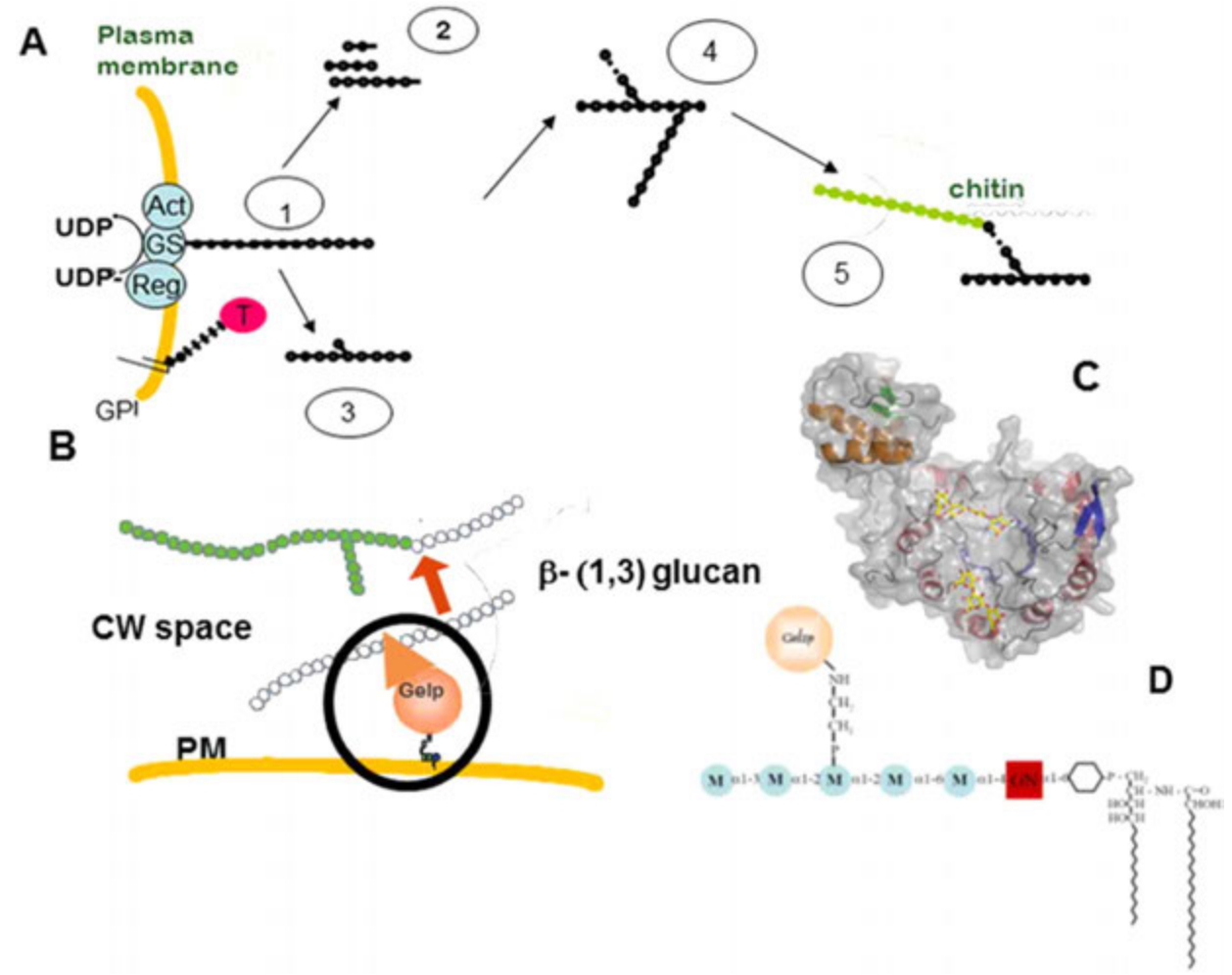

Fig. 2 Synthesis and remodeling of β-(1,3) glucan. (A) Putative sequential or concomitant events in the synthesis and remodeling of β-(1,3) glucan. (1) Synthesis of linear glucan chains (glucan synthase complex composed of a catalytic [GS], activating [Act], and regulating [Reg] subunits). (2) Hydrolysis of glucans. (3) Branching of β-(1,3) glucan. (4) Elongation of β-(1,3) glucan side chains. (5) Cross-linking with branched [β-(1,3)] glucan. GPI-anchored transglycosidase or hydrolases (T) bound to the membrane can act on the polysaccharides in the cell wall space. Panel A provides example. (B) An example of GPI-anchored Gel1 protein involved in the elongation of β-(1,3) glucan inside the cell wall space. (C) Crystal structure of the *S. cerevisiae* Gel1 orthologue, Gas2 complex with acceptor and donor oligosaccharides. The enzyme is shown as a ribbon, the glucan binding domain with green strands and orange helices, and the catalytic domain with blue strands and red helices. A gray transparent molecular surface is shown, revealing an elongated groove on the catalytic domain, in which the laminarioligosaccharides (shown as sticks, with yellow carbon atoms) bind. (D) Biochemical organization of a GPI-anchored protein in *A. fumigatus*. The three domains of the GPI anchor are (1) a phosphoethanolamine linker covalently bound to the protein, (2) a mannan-glucosaminemyo-inositol oligosaccharide, and (3) a ceramide tail attaching the GPI anchor to the cell membrane. Reproduced from Gow, N.A.R., Latge, J.-P., Munro, C.A., 2017. The fungal cell wall: Structure, biosynthesis, and function. Microbiology Spectrum 5 (3). doi:10.1128/microbiolspec.FUNK-0035-2016.

Fig. 3 Classification of fungal chitin synthases based on amino acid sequence.

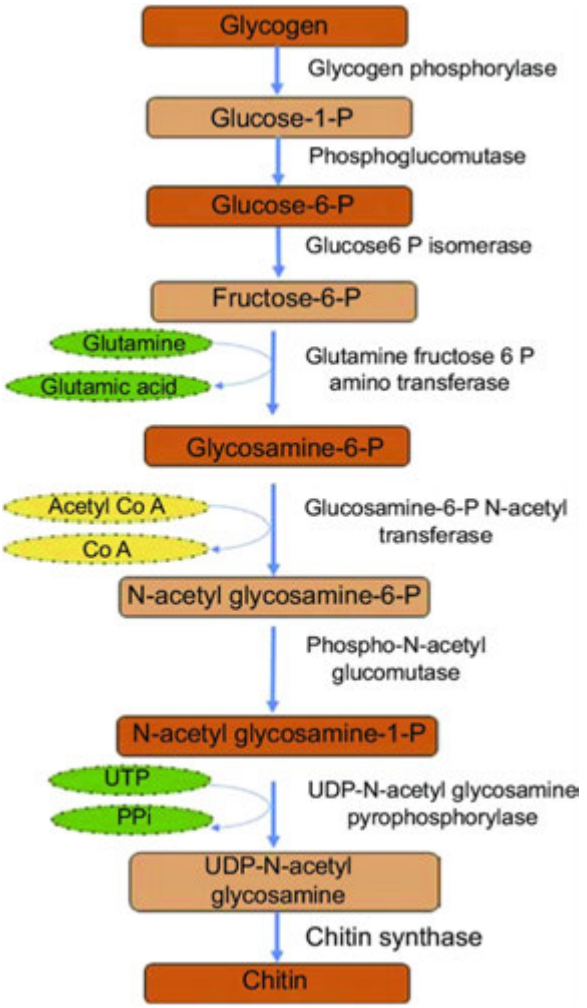

Fig. 4 Biosynthetic pathway of chitin. Reproduced from Balaji, A.B., Pakalapati, H., Khalid, M., Walvekar, R., Siddiqui, H., 2018. Natural and synthetic biocompatible and biodegradable polymers. In: Shimpi, N.G. (Ed.), Biodegradable and Biocompatible Polymer Composites Processing, Properties and Applications. Ohio: Woodhead Publishing, pp. 3–32.

fructose-6-P by hexokinase. Fructose-6-phosphate is then converted to N-acetyl glucosamine which involves amination (glutamine to glutamic acid) and acetylation (acetyl CoA to CoA). Isomerization step is as follows (phosphate transfer to C6 to C1 catalyzed by a phospho-N-acetyl glucosamine mutase). Further inter-conversion, uridine diphosphate (UDP) N-acetyl glucosamine is formed by utilization of uridine triphosphate (UTP). Finally, chitin is formed from UDP N-acetyl glucosamine in the presence of chitin synthase. Chitin deacetylation results in chitosan. Chitin deacetylation in the cell wall of fungi is catalyzed by the chitin deacetylase enzyme (EC 3.5.1.41) (Batista *et al.*, 2018). Biosynthetic pathway of chitin is graphically represented in **Fig. 4**.

Physicochemical Properties

Solubility

Because of it is insolubility in most solvents, chitin has very few applications. In contrast, chitosan has many applications because it is, readily, soluble in dilute acidic solutions such as formic, acetic, lactic acids and most solvents at pH < 6.0. However, 1% acetic acid at pH 4.0 is the widely used solvent of chitosan (Zamani *et al.*, 2008).

Molecular Weight

Chitin and chitosan are high molecular weight biopolymers that vary with the variation of the source material, preparation and extraction methods. The suitable applications of chitin and chitosan are determined according to their molecular weight, e.g., it has

been reported that chitosan with low molecular weight has greater antimicrobial activity (Amorim *et al.*, 2003; Franco *et al.*, 2004; Tajdini *et al.*, 2010; Moussa *et al.*, 2013; Oliveira *et al.*, 2014).

The molecular weight of chitin is as high as many Daltons. However, due to the use of harsh chemical treatments, the molecular weight of chitosan may range from 100 to 1500 kDa. Chitosan of low molecular weight can be obtained either by chemical or enzymatic methods (Cai *et al.*, 2006). Although, fungal chitin has unknown molecular mass, it has been proposed that *Saccharomyces cerevisiae* synthesizes a uniform chain of GlcNAc containing 120–170 monomer units which may correspond to 24,000–34,500 Da (Valdivieso *et al.*, 1999).

Degree of Deacetylation

The degree of deacetylation (DD) is one of the most important parameters that affect the chemical and physical characteristics, activity as well as the applications of chitin and chitosan (copolymers) (Akila, 2014). It can be defined as the molar fraction of D-glucosamine (GlcN) in chitosan and represented by the following equation:

$$DD = 100\frac{nGlcN}{nGlcN + nGlcNAc}$$

where (nGlcN) represents the average number of D-glucosamine units and (nGlcNAc) represents the average number of N-acetyl glucosamine units.

For instance, only chitin samples with degree of deacetylation of about 50% are soluble in water, whereas those with lower or higher degree of deacetylation were insoluble (Wu, 2004). Chitosan molecules with high degree of deacetylation ($>85\%$) have strong positive charge in the aqueous solutions with pH < 6 (Sorlier *et al.*, 2001). Different methods have been proposed by scientists for the determination of chitin and chitosan degree of deacetylation. These techniques include the following:

(1) Infrared spectroscopy (IR) (Brugnerotto *et al.*, 2001; Duarte *et al.*, 2002): Although this technique requires precise calibration, it is the most popular technique because it is very simple and requires minimal sample preparation.
(2) ^{13}C solid-state NMR (Duarte *et al.*, 2001): This technique is not available at most laboratories due to the elevated cost; however, it appears to be the most reliable method and is often used as a reference.
(3) High-pressure liquid chromatography (HPLC) (Han *et al.*, 2015): This method depends on the hydrolysis of chitin and chitosan and analysis of the produced acetic acid.
(4) Potentiometric titration (Ke and Chen, 1990).
(5) Ultraviolet spectrometry (Liu *et al.*, 2006).

Potentiometric titration and ultraviolet spectrometry require dissolved samples and therefore not suitable for chitosan with (DD $< 50\%$) and chitin.

Occurrence and Biological Functions in Nature

Chitin and chitosan are main components in the shells of crustaceans such as crab, shrimp, and lobster, and are also exoskeleton components of insects and mollusks. They compose a main part of the cell wall of many fungi (Yeul and Rayalu, 2013). However, chitin and chitosan are not present in higher plants and higher animals. It has been reported that chitin represents an average of 14% and 21% of the dry weight of crab and shrimp processing wastes, respectively (Ashford *et al.*, 1977), therefore, currently, shrimp and crab shell wastes are the main industrial sources for the large-scale production of chitin and chitosan. Processing of shrimp and crab shell wastes from marine food factories helps recycling and useful use of these wastes in other fields. Crustacean shell wastes are composed of protein, lipids and inorganic salts, in addition to chitin and chitosan, subsequently, the extraction of chitin and chitosan requires stepwise chemical methods (Kim and Rajapakse, 2005).

Chitin is widely distributed in many fungal phyla (division) including: *Ascomycetes*, *Basidiomycetes*, and *Phycomycetes* (Pacheco-Arjona and Ramirez-Prado, 2014). Fungal chitin is a component of the structural membranes and cell walls of stalks, mycelia, and spores. However, chitin is not found in all fungi and may be absent in one species that is closely related to another as the chitin content within fungal species can vary depending on the method of fungal cultivation and fermentation system (Abo Elsoud and El Kady, 2019).

Chitin was reported in *Saccharomyces cerevisiae* as a major component in primary septa between mother and daughter cells (Sbrana *et al.*, 1995).

Industrial Production of Chitin and Chitosan

The crustacean shells annual production has been estimated as 1.2×10^6 tons worldwide (Synowiecki and Al-Khateeb, 2003). As it has, already, mentioned, crab and shrimp shells are the main industrial source for the large-scale production of chitin and chitosan. These crustacean shell wastes contain, in addition to chitin, proteins, inorganic salts, pigments, and lipids as main

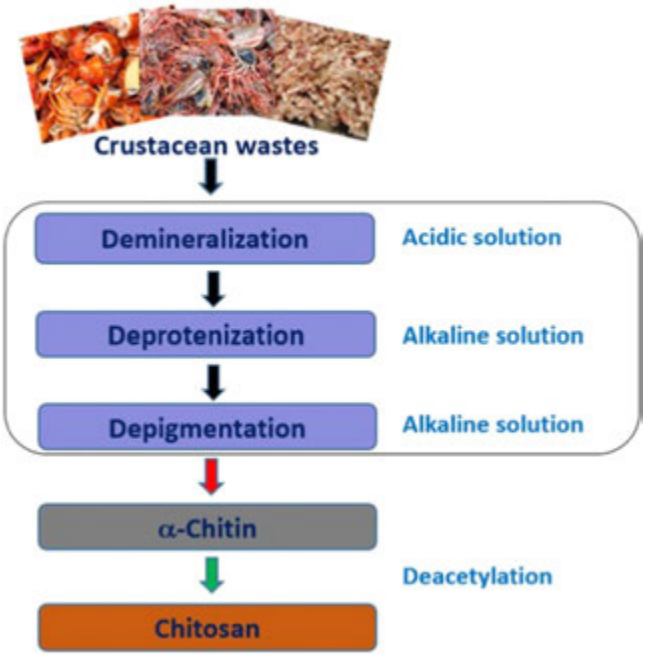

Fig. 5 Process of chitin and chitosan production. Reproduced from Kumar, S., Ye, F., Dobretsov, S., Dutta, J., 2019. Review: Chitosan nanocomposite coatings for food, paints, and water treatment applications. Applied Sciences 9 (12), 2409. doi:10.3390/app9122409.

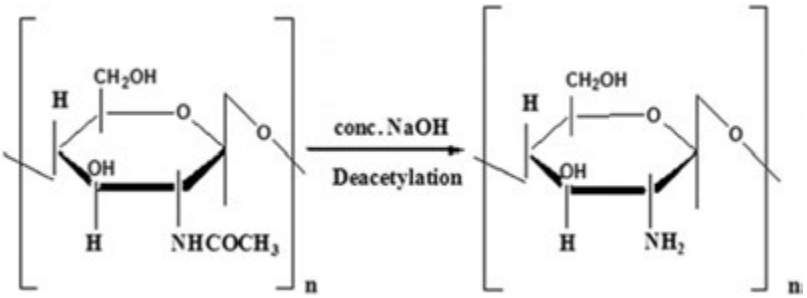

Fig. 6 Deacetylation of chitin to chitosan.

structural components. Therefore, chemical extraction of chitin and chitosan should be employed to obtain the pure product. Briefly, the following steps should be performed (**Fig. 5**):

(1) Demineralization/decalcification:
The shell wastes are washed, dried, and ground to smaller sizes where minerals such as calcium carbonate are removed with good mixing with dilute hydrochloric acid at ambient temperature.

(2) Discoloration:
This step aims to remove pigments from the produced chitin.

(3) Deproteinization:
The pure chitin and chitosan should be deproteinized to remove the protein contamination. The residual materials are treated with dilute aqueous solution of sodium hydroxide to remove proteins, glycoproteins and branched polysaccharides.

(4) Acid reflux:
For the separation of chitin and chitosan, the treated material is acidified using 2%–10% hydrochloric acid or acetic at 95°C for 3–14 h.

(5) Deacetylation:
The resulting chitin is treated with 40%–45% sodium hydroxide, for production of chitosan, in the absence of oxygen, at 160°C for 1–3 h in a process called deacetylation in which the acetyl group is removed from chitin molecules (**Fig. 6**) (Roberts, 1992). Repetition of this process can give a degree of deacetylation (DD) up to 98%, but the complete deacetylation can never be achieved (Mima *et al.*, 1983).

Table 2 Comparison between chitin and chitosan production from fungal and crustacean sources

Basis for comparison	*Fungal mycelia*	*Crustacean sources*
Availability	Does not have seasonal or geographic limitations.	Limited by sites of fishing industry and seasons.
Inorganic materials	Low levels	High levels
Extraction process	Simpler	Requires harsh solvents
Cost of waste management	Low	High
Demineralization treatment	Not required	Required
Physico-chemical properties	Consistent	Vary
Induction of the plant immune response	More effective	Less effective

Note: Abo Elsoud, M.M., El Kady, E.M., 2019. Current trends in fungal biosynthesis of chitin and chitosan. Bulletin of the National Research Centre 43, 59. doi:10.1186/s42269-019-0105-y.

The degree of chitin deacetylation depends on the concentration of NaOH, reaction temperature, and time (Kasaai, 2009). However, at least 85% deacetylation should be achieved for a good solubility of chitosan (No and Meyers, 1995). In contrary to crustacean shell wastes, the crude fungal chitin has a lower level of inorganic matters and thus the step of demineralization is not required during the processing (Teng *et al.*, 2001).

Features of Fungal Chitin and Chitosan Production

During the few last years, the industrial production of fungal chitin and chitosan has attracted the attention of scientists and business men due to their significant advantages over the currently applied processes. These advantages are summarized in (**Table 2**; Knorr *et al.*, 1989; Rane and Hoover, 1993; Teng *et al.*, 2001; Adams, 2004).

In addition to the above-mentioned differences between chitin and chitosan production from fungal mycelia and crustacean sources, it has been reported that the degree of acetylation, molecular weight, and distribution of charged groups in fungal chitin and chitosan are potentially different from that of crustacean source which enhances bioactivity and promotes their functional properties (Wu *et al.*, 2004). However, further researches are required to get the most economical way for obtaining the chitinous material from fungal mycelia due to the relative higher cost of production. The production of chitin and chitosan from fungi has a significant role in lowering the expenses involved in managing fungal-based waste materials in parallel with production of value-added products which may provide a profitable solution to the biotechnological industries (Wu *et al.*, 2005). The worldwide production of the mushroom, *Agaricus bisporus*, results in about 50,000 metric tons/year of useless waste material. On the other hand, the production of citric acid from *Aspergillus niger* results in approximately 80,000 tons/year of mycelial waste materials (Ali *et al.*, 2002). These fungal wastes and others represent a free natural source of chitin and chitosan.

Chitin and Chitosan-Producing Fungi

The cell walls and septa of many fungal species belong to the classes Basidiomycetes, Ascomycetes, Zygomycetes, and Deuteromycetes contain, mainly, chitin to maintain their strength, shape, and integrity of cell structure (Kirk *et al.*, 2008). Chitin is considered as the second most abundant biopolymer in the fungal cell wall. Also, chitosan can be easily recovered from these microorganisms (Synowiecki and Al-Khateeb, 1997; Yokoi *et al.*, 1998). These fungal classes include: *Absidia coerulea*, *Absidia glauca*, *Absidia blakesleeana*, *Aspergillus niger*, *Colletotrichum lindemuthianum*, *Gongronella butleri*, *Lentinus edodes*, *Mucor rouxii*, *Phycomyces blakesleeanus*, *Pleurotus sajo-caju*, *Rhizopus oryzae*, and *Trichoderma reesei* which have been investigated for chitin and chitosan production (Hu *et al.*, 1999; Teng *et al.*, 2001; Chatterjee *et al.*, 2005) and suggested as promising alternative sources to crustaceans (Pochanavanich and Suntornsuk, 2002; Nwe *et al.*, 2002; Suntornsuk *et al.*, 2002). The amount of chitin in the fungal cell wall is specific to environmental conditions and age as well as species. The chitin content in the dry fungal cell wall may vary from 2% to 42% in yeast and Euascomycetes, respectively. Chitosan is also one of the structural components in the fungal cell walls which differ depending on fungal taxonomy. Thus, the cell wall in Zygomycetes contains chitosan-glucan complex, while contains chitin-glucan in Euascomycetes, Homobasidiomycetes, and Deuteromycetes (Rouiz-Herrera, 1992).

Among the investigated species, *M. rouxii* of the Zygomycetes class was the most researched and studied since the quantities of chitin and chitosan in its cell wall reached 35% of the dry weight (Wu, 2004). Chitosan has been mostly studied, produced, and characterized in Absidia and Mucor (Pochanavanich and Suntornsuk, 2002). However, *A. niger* (produced from citric acid industry) and *Agaricus bisporus*, as waste materials, provide a plenty source of raw materials for chitin and chitosan production (Wu, 2004). The chitin content in *Agaricus* cell walls has been reported to be 13.3%–17.3%, 35%, 20%–38%, and 43%. This broad range of chitin concentrations can be attributed to the fact that chitin content varies, significantly, during the mushroom life-cycle as well as during postharvest storage (Wu *et al.*, 2004).

Applications of Chitin and Chitosan

Biological Properties

Chitin and chitosan have many intrinsic characteristics that make them suitable for versatile applications in various fields. These characteristics include biocompatibility, biodegradability, strong antibacterial effect, non-toxicity, and high humidity absorption (Aranaz *et al.*, 2009). Furthermore, other biological properties such as antitumor, analgesic, hemostatic, antitumor, hypocholesterolemic, antimicrobial, and antioxidant properties have also been reported by researchers (Islam *et al.*, 2017).

Most of the chitin and chitosan biological properties are directly related to their physicochemical characteristics. These characteristics include degree of deacetylation, molecular mass, and the amount of moisture content (Aranaz *et al.*, 2009). For example, chitosan-mediated inhibition of fungal and bacterial growth relies on the functional groups and molecular weight of chitosan. Two theories have been proposed for chitosan antimicrobial activity. The first theory relays on molecular weight; the smaller oligomeric chitosan can easily penetrate the cellular membrane and prevents the cell growth by inhibiting RNA transcription (Klaykruayat *et al.*, 2010). The second relays on the polycationic nature of chitosan which changes the cellular permeability and induce the leakage of intracellular components due to interaction with anionic components of the cell membrane leading to cell death (Lim and Hudson, 2004). Moreover, chitosan may disrupt the microbial cell physiological activities by absorbing the electronegative substrate, e.g., proteins, and finally death of cells (Zheng and Zhu, 2003). The distribution of acetyl groups and the length of polymer chain in chitin and chitosan affect their biodegradation kinetics (Zhang and Neau, 2001). It has been reported that chitosan with a high degree of deacetylation (97.5%) has a higher positive charge density compared to the moderate DD (83.7%), thus confers a stronger antibacterial activity (Kong *et al.*, 2010). The chitin poor solubility is the main limiting factor in its utilization. On the other hand, chitosan is considered as a potential biopolymer due to its free amino groups that contribute polycationic, chelating activity, and dispersion forming properties along with its solubility in dilute acetic acid (Akila, 2014). Due to its exceptional versatile biological, chemical, and physical properties, chitosan can be used in a wide variety of applications including industrial, agricultural, medical and pharmaceutical.

Industrial Applications

In industry, chitosan found many applications in various areas including biotechnology, e.g., enzyme immobilization, cosmetics, paper production, textile, semi-permeable membranes, wastewater treatment, and food processing (Bashar and Khan, 2013).

Agricultural Applications

Chitin and chitosan are potent agents that inhibit the growth of bacterial and fungal plant pathogens, consequently, elicit defense reactions in higher plants (Shibuya and Mimami, 2001). In bell pepper fruit pathogen, chitosan has the ability to effectively reduce *Botrytis cinerea* polygalacturonases causing severe cytological damages to the invading hyphae (Glaouth *et al.*, 1997). Chitin was reported to be eco-friendly and safe for controlling the plant parasitic nematodes (Spiegel *et al.*, 1986)Similarly, spraying chitosan or chitin on the surface of cucumber plants before the inoculation with the pathogen *B. cinerea*, the activity of peroxidase and chitosanase increased causing inhibition of *B. cinerea* (Ben-Shalom *et al.*, 2003). Furthermore, a Brazilian patent by Stamford *et al.* (2015) was published describing the successful application of fungal chitosan as a biofertilizer (Batista *et al.*, 2018).

Biomedical and Pharmaceutical Applications

Chitin and chitosan biopolymers are considered as useful non-toxic and biocompatible materials to be used in various medical devices to treat, augment, or replace tissues, organs, or function of the body. Furthermore, chitosan and its derivatives are promising materials for supporting in tissue engineering applications (Islam *et al.*, 2017). Chitosan serves as a potential material for nerve regeneration, wound-healing management products and wound dressing, burn treatment, cancer treatment, artificial kidney membrane, bioartificial liver (BAL), artificial skin, artificial tendon, articular cartilage, drug delivery systems, e.g., carrier in case of vaccine delivery or gene therapy, blood anticoagulation, bone damage, and antimicrobial applications. Moreover, chitosan has antioxidant, antitumor, anti-diabetic, and antiulcer activities. It is provided as dietary supplements, under several proprietary names, in combination with other substances for weight loss (Candlish, 1999). It has been sold to inhibit fat absorption in Japan and Europe as a nonprescription product (Akila, 2014). Glucosamine (GlcN), chitosan hydrolysate, is an amino monosaccharide that has been reported to have the potential to prevent changes in the joint structure in patients with osteoarthritis (Reginster *et al.*, 2001; Richy *et al.*, 2003). Chitin is considered as a promising treatment for umbilical hernia (Islam *et al.*, 2017).

Fermentation Systems of Fungal Chitin and Chitosan

The content of chitin and chitosan within fungal species can vary depending on the method of fungal cultivation and fermentation system. Two types of fermentation systems can be differentiated: solid-state fermentation (SSF) and submerged fermentation

(SmF), both of which can be used for production of chitin and chitosan from fungi. In solid-state fermentation, the microbial growth takes place on moist solid substrates where no free water flowing (Pandey *et al.*, 2000; Gabiatti *et al.*, 2006). On the other hand, submerged fermentation takes place in liquid medium.

Generally, the production of chitin and chitosan in the cell walls of fungal systems are associated with the fungus biomass concentration. Focusing on biomass concentration difference, and subsequently chitin and chitosan concentration, due to the different cultivations technique, it was shown that SSF of the fungus, *Lentinus edodes*, yielded a greater biomass concentration (up to 50 times), after 12 days of incubation, than that of SmF (Crestini *et al.*, 1996).

There are many reports that confirm that SSF is more efficient in fungal biomass production compared with SmF. However, there are some cases in which the biomass production in SmF exceeds that of SSF. Biomass production by *Pleurotus ostreatus* was compared through SSF and SmF in a study by Mazumder *et al.* (2009). The results showed no significant difference between biomass concentrations in both techniques during the initial growth period. However, after 2 days of growth, SmF produced a higher biomass concentration of *P. ostreatus*. From these studies of fermentation systems, it can be concluded that it is difficult to derive a solid comparison between solid-state and submerged fermentations in terms of biomass concentration. In other way, the suitable fermentation system for chitin and chitosan production is specific to species. The main variation in chitin and chitosan productivity arises due to changes in substrate, environmental conditions and medium composition, etc. (Bhargav *et al.*, 2008). The substrate in case of SSF can be homogenous (e.g., polyurethane foam (PUF)) or heterogeneous (e.g., rice bran, wheat straw, soybean, or other agricultural byproducts). The use of homogenous substrate is advantageous as it allows the control of oxygen transfer, feed rate, medium composition, and facilitates the quantification of fungal biomass. On the other hand, the difficulty of fungal biomass measurement is a major hindrance of using SSF or more specifically the heterogeneous substrate (Zhu *et al.*, 1994; Sparringa and Owens, 1999). In brief, the characterization, optimization, and standardization of SSF process is very difficult. The substrate's physical properties, strongly, affect the rate of oxygen and nutrients diffusion, water activity, and consequently metabolic activity. The difficulty of recovering fungal biomass in case of SSF affects the extraction process of chitin and chitosan. These conditions push toward the use of SmF instead of SSF (Sitanggang *et al.*, 2012). The simple step for determination of the fungal biomass in SmF makes this fermentation system preferred for production of chitin and chitosan. However, the higher probability of contamination, if the aseptic conditions are not maintained, the production of much waste water after biomass recovery, and high energy expenditure during the agitation for adequate mixing are the major obstructions in the use of SmF (Sitanggang *et al.*, 2012).

Conclusion

Chitin and chitosan biopolymers exist in the shells of crustaceans, exoskeletons of insects and mollusks, as well as in the cell walls of fungi. Industrially, they are widely produced from the shells of crustaceans and found many applications in many industries, agriculture, and biomedicine. However, due to the inconsistent structure of chitin and chitosan from crustacean origin, the fungal origins represent an ideal alternative, especially, for biomedical and pharmaceutical applications. Chitin and chitosan production from fungal sources relieves the world from a great amount of biological wastes. The quantity and/or quality of chitin and chitosan in the fungal cell wall may change due to environmental and nutritional conditions.

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Chitin Synthases in Fungi

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Introduction

Chitin is a linear homopolymer of N-acetyl-β-D-glucosamine (GlcNAc) residues linked by β-1,4 glycosidic bond, and it is an essential cell wall component of fungi. The general chitin synthesis pathway is divided into sequential reactions; the final step is catalyzed by chitin synthases (CHS), which are specifically associated with chitin biosynthesis (Dorfmueller *et al.*, 2014). CHSs belong to the Glycosyltransferase Family 2. The chitin synthase reaction occurs in specialized regions of the plasma membrane. Chitin synthase add single GlcNAc units, which are derived from the nucleotide sugar donor UDP-N-acetylglucosamine (UDP-GlcNAc), to the non-reducing end of the extending chain (Orlean and Funai, 2018). The linear polymers of chitin then assemble into microfibrils of varying diameter and length. Chitin is integrated into the cell wall by crosslinking via $\beta-1,4$ glycosidic bonds with the fibrillar β-1,3-glucan, which serves as a backbone in fungal cell wall. Chitin also links with glycoproteins and pigments in the cell wall.

Classification of Fungal Chitin Synthases

Fungal CHSs are membrane integrated enzymes with multiple domains responsible for subcellular localization and activation. All CHSs possess the conserved region CON1, which contains the conserved motifs QxxEY, (E/D)Dx, and Q(R/Q)xRW. These three motifs are essential for catalytic activity (Dorfmueller *et al.*, 2014). The CON1 region also exists in CHSs in non-fungi eukaryotes and in the bacterial chitooligosaccharide synthases NodCs. According to their domain structures and phylogeny based on the conserved CON1 region, fungal CHSs are grouped into seven classes: CHSI-VII. CHSI, II and III contain the chitin_synth_1N (PF08407) and CS1 domain (PF01644) followed by the CON1 region. CHSIV has the CS2 (PF03142) and Cyt-b5 domain (PF00173). In addition to the CS2 and Cyt-b5 domain, CHSV and CHSVII also contain the myosin head (PF00063) and DEK-C (PF08766) domain. CHSVI only has a CS2 domain. In Class CHSIV, CHSV, CHSVI and CHSVII, the CON1 region is within the CS2 domain. There are other CHSs that cannot be grouped into any of the seven classes because they are phylogenetically distant from the classified ones though they may have the same domain structure as the members in one of the seven classes (Liu *et al.*, 2017).

Evolution of Fungal Chitin Synthases

The fungal kingdom is the eukaryotic group with the most complex CHS gene family, which is attributed to a complex evolutionary history of this gene family. Fungal CHSs could have three different CON1 region-containing ancestors, which in turn could share an ancestor with bacterial NodC proteins. The ancestor of CHSI, II and III CHSs could be shared by the CHSs from the kingdoms Alveolata and Stramenopiles, whereas Class IV, V and VII CHSs could share an ancestor with the CHSs from the kingdoms Choanoflaggelida, Metazoa, Amoebozoa or Stramenopiles. CHSVI could have its own distinct ancestor. Within the fungal kingdom, two major mechanisms are known to drive the evolution of the CHS gene family. One mechanism is gene duplication and gene loss. The second mechanism is domain recombination and domain accretion. Contraction of the CHS gene family is morphology-specific, with significant loss in unicellular fungi such as baker's yeast *Saccharomyces cerevisiae*, whereas family expansion is lineage-specific with obvious expansion in early-diverging fungi. This complex evolutionary history of CHSs results in a diverse CHS distribution in different fungal taxa. The average number of CHSs in the Ascomycota phylum varies greatly among the subphyla; for example, Pezizomycota, Saccharomycotina and Taphrinomycotina fungi have 6.9, 3.9 and 0.6 CHSs per species, respectively. The number of CHSs is relatively invariable in the Basidiomycota phylum, which ranges from 6 to 8 per species in 9 classes in the Basidiomycota fungi, while only the class Malasseziomycetes has 4.5 CHSs per species. CHSVI has been rarely found in the Basidiomycota fungi. Many more CHSs exist in the early-diverging fungi (Glomeromycota, Zygomycota, Blastocladiomycota, Chytridiomycota and Neocallimastigomycota), and they averagely have $\sim$16 CHSs per species, which is almost 3 times greater than the Dikarya fungi (Liu *et al.*, 2017).

Functions of Chitin Synthases

Chitin provides cell rigidity and determines cell shape. It also confers protection against abiotic stresses and is important for full virulence of pathogenic fungi with diverse host ranges (Liu *et al.*, 2017). For example, CHSV and CHSVII are involved in the formation of the infection structure (appressorium) in the insect pathogenic fungus *Metarhizium robertsii*, and in the plant pathogenic fungi *Magnaporthe oryzae*, *Colletotrichum graminicola* and *Fusarium verticillioides*. These two CHSs are also important for

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full virulence of the opportunistic human pathogenic fungus *Aspergillus fumigatus*. Chitin synthases are absent in plants and mammals, and are consequently considered as promising targets for antifungal drugs (Ruiz-Herrera and San-Blas, 2003).

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Glucose Metabolism and Use of Alternative Carbon Sources in Medically-Important Fungi

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Introduction

Nearly 150 million of human populations are affected by potentially life-threatening mycoses worldwide, and millions of them are killed by these fungal infections despite advancement in medical interventions (Bongomin *et al.*, 2017). However, these fungal diseases are as yet a disregarded issue by public health authorities even though most deaths caused by fungal infections are preventable. Although the global species richness of fungi is estimated to be around 1.5–5.1 million, only a hundred of these identified fungi are medically-important and are able to cause serious mycoses in human (O'Brien *et al.*, 2005; Köhler *et al.*, 2015). In contrast to ectothermic vertebrates like insects and plants, endothermic and homeothermic vertebrates such as humans and mammals are generally resistant to fungal infections owing to their evolved antifungal immunity (Robert and Casadevall, 2009; Bergman and Casadevall, 2010). In order to thrive as a successful fungal pathogen in human host, these fungi must be able to grow at human body temperature (37°C and above), breach the host tissue barriers, scavenge and assimilate nutrients from the host tissues and most importantly, also withstand the host immune onslaught (Köhler *et al.*, 2015). Therefore, it is sensible that only a relatively small subset of these identified fungi can cause critical fungal infections in humans.

The growth of fungal pathogens in human host depends primarily on their ability to exploit and assimilate the nutrients efficiently. Although the host niches contain a rich and diverse variety of nutrients, it is still challenging for a fungal pathogen to scavenge for these food resources as the host constantly imposes restriction of the essential nutrients available to the pathogens, a mechanism termed "nutritional immunity" (Rohmer *et al.*, 2011). Therefore, the ability to counteract the limited nutrient condition is particularly crucial for intracellular fungal pathogens. The microenvironments within the host cells often lack of major nutrients (e.g., carbon and nitrogen sources) and trace elements that are readily usable by these pathogens, as most of these nutrients are sequestered in complex forms (Steele *et al.*, 2015). To survive inside the host cells, these fungal pathogens must be equipped with high degree of metabolic flexibility and possess unique metabolic adaptation strategies that are required for effective nutrient acquisition, as pathogenic fungi require high amount of energy for proliferation and dissemination (Ene *et al.*, 2014). Furthermore, it is undeniable that the linkages between nutrients assimilation and fungal pathogenicity as well as their efficacy in combating host defenses are also inextricable (Brown *et al.*, 2014). For instance, multiple fitness and pathogenic attributes of *Candida* species such as biofilm formation, morphogenesis, antifungal resistance as well as immune evasion have been reported to be drastically influenced by the host nutrients, and many of these changes also significantly confer an edge for fungal pathogenicity (Ene *et al.*, 2012, 2013; Brown *et al.*, 2014; Chew *et al.*, 2019a).

All major medically-important fungi such as *Candida* and *Pneumocystis* (exclusively found in warm-blooded mammals), *Aspergillus*, *Cryptococcus* and *Histoplasma* (also found in environment niches) display very distinct lifestyles, and, these differences have exerted evolutionary pressures that shifted or streamlined the fungal metabolic profiles and flexibility (Ene *et al.*, 2014). Carbon sources represent one of the most essential nutrients and the main source of energy required for biosynthesis processes (e.g., biomass generation), and a considerable quantity of these carbon sources are usually needed to sustain the survival of fungal pathogens in the host (Ene *et al.*, 2014; Ramachandra *et al.*, 2014). Similar to most organisms, fungi have preference for certain carbon sources. Glucose is generally the most favorable carbon source for most pathogenic fungi, as metabolism of glucose via glycolysis and respiration are rapid and energetically favorable. However, when the host niches are under glucose deprivation, these fungal pathogens can also utilize a wide range of fermentable sugars and alternative carbon sources, although the mechanisms involved are energetically demanding. Nevertheless, the alternative carbon utilization profiles vary greatly among medically-important fungi, presumably reflect their long-term co-evolution with multiple host niches in human. In the following sections, we review the current knowledge on the glucose and alternative carbon metabolism in fungal pathogens and their importance in the fungal survival and pathogenicity with occasional reference to *Saccharomyces cerevisiae*. Here, we decided to pivot on the metabolism of glucose and alternative carbon sources in major fungal pathogens that are commonly found in human diseases, with the main focus on *Candida*, *Aspergillus* and *Cryptococcus* species.

Glucose Metabolism in Medically-Important Fungi

Glucose is the primary carbon source for most fungi. It is the most preferred source for metabolism in comparison to other carbon sources such as acetate, lactate and fatty acids. Other than metabolism, glucose also plays role in the pathogenicity of many human fungal pathogens. It affects the adhesion property, oxidative stress resistance, biofilm formation, morphogenesis, invasion, and antifungal drug tolerance (Rodaki *et al.*, 2009; Sabina and Brown, 2009; Buu and Chen, 2014; Mandal *et al.*, 2014; Ng *et al.*, 2016; Mikamo *et al.*, 2018; Chew *et al.*, 2019a). Being the most important carbon source, glucose is found in varying concentrations in various host niches and fungi have evolved to adapt to these conditions as they make human as their host. Various organs and host

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niches have different concentration of glucose (Ehrström *et al.*, 2006); hence each fungus has evolved to ensure their survivability in these niches such as the mouth, gut, skin, vagina and blood. The median concentration of glucose in the human host hovers around 5.0–5.2 mM in plasma and vagina, respectively (Ehrström *et al.*, 2006). To enable them to utilize glucose, these fungi are equipped with glucose sensors which are found on their cellular plasma membrane.

Most of glucose metabolism mechanisms are discovered mainly through the study of *S. cerevisiae* as the model organism. Glucose metabolism in fungi begins with its acquisition from their surroundings. For this acquisition to take place, fungi develop glucose sensing and transport mechanism. To date, there are three glucose sensing mechanisms which have been identified (Ozcan and Johnston, 1995; Rolland *et al.*, 2000; Thevelein *et al.*, 2000; Kayikci and Nielsen, 2015). The first mechanism involves sugar receptor-repressor (SRR) or hexose transporter (HXT) induction pathway where it involves transporter-like proteins known as Snf3 and Rgt2. The protein orthologues of both the proteins in medically important fungi *Candida albicans* and *Candida glabrata* are Hgt4 and Snf3/Rgt2, respectively. However, for *Cryptococcus neoformans* and *Aspergillus*, attempts so far have not been able to identify them (Liu *et al.*, 2013) except in *Neurospora crassa* where *RCO-3* gene was identified (Madi *et al.*, 1997). Besides, Hxs1 and Hxs2 of *C. neoformans* are homologs of Snf3 and Rgt2 in *S. cerevisiae* but both do not act as glucose sensors but as transporters (Liu *et al.*, 2013). The second sensing mechanism is through G-protein coupled receptor Gpr1 and the third involves the glucose repression pathway. All three pathways are interconnected in regulating the level of glucose according to the demand and its availability in the surrounding.

In the SRR pathway, glucose sensors are divided into two groups, namely the high and low affinity glucose sensors. The availability and concentration of glucose influence the activation of these glucose sensors. When glucose concentration is low, the high affinity glucose sensor Snf3 is activated and likewise when glucose concentration is high, the low affinity glucose sensor Rgt2 is activated (Ozcan and Johnston, 1999; Ng *et al.*, 2015). Upon their activation, downstream signals are detected by casein kinase I Yck1/2 which then phosphorylate two transcriptional regulators, Mth1 and Std1 (Moriya and Johnston, 2004). The phosphorylation of both the regulators results in their ubiquitination with Grr1 that ends with their degradation by proteasome (Moriya and Johnston, 2004). Without the presence of Mth1/Std1 coupled Rgt1 complex, the expression of glucose transporters is unimpeded and hence the process of glucose uptake ensues.

In the glucose sensing mechanism by G-protein coupled receptor Gpr1, the presence of glucose results in the activation of adenylate cyclase Cyr1 which increases the level of cAMP levels and activation of protein kinase A (PKA) (Rolland *et al.*, 2000). Coupled together with the presence of sugar-phosphate activation of Cdc25 and Ras, the uptake of glucose takes place. The last mechanism which is known as glucose repression pathway begins with dephosphorylation of Snf1 and Mig1. The dephosphorylation as a result of the presence of glucose enables Mig1 to gain entry in nucleus and acts as a transcriptional repressor of the *GAL* and *MTH1* genes. Activation of both the G-protein coupled receptor and glucose repression pathways consequently leads to the preference of fermentation process over respiration and represses the utilization of alternative carbons for metabolism in *S. cerevisiae*. Following glucose sensing, the glucose sensors genes send signals to glucose transporters. Further in depth reading on all three glucose sensing mechanisms in *S. cerevisiae*, *C. albicans* and *C. glabrata* can be found in a review chapter by Van Ende *et al.* (2019).

Glycolysis is an essential central metabolic pathway that is responsible for catabolism of glucose in order to produce energy and key intermediates for use in the subsequent metabolic pathways (Dashty, 2013). Upon uptake by the cells, glucose enters glycolysis after the first phosphorylation step catalyzed by hexokinases and turns into glucose-6-phosphate. Following a series of enzymatic reactions, catabolism of glucose via this glycolytic pathway ultimately converts this sugar phosphate into the key metabolite pyruvate (Askew *et al.*, 2009). Other than the two pyruvates, two adenosine triphosphate (ATP) and two nicotinamide adenine dinucleotide (NADH) molecules are also generated in the process. At the end of glycolysis, fungi employ two distinct approaches in liberating the remaining energy within pyruvate, i.e., via respiration and fermentation. In general, fungi can be classified as either Crabtree-positive or Crabtree-negative based on their preference in carbon utilization. Crabtree-positive yeasts like *S. cerevisiae* prefer fermentation over cellular respiration even in the presence of oxygen. Fast consumption of glucose and ethanol production are believed to assist Crabtree-positive yeasts to thrive and outcompete other microbes within a polymicrobial ecosystem (Ata *et al.*, 2018). In comparison, Crabtree-negative fungi like *C. albicans* prefer cellular respiration in the presence of glucose (or both respiration and fermentation simultaneously), and generate significantly more energy in the form of ATPs via tricarboxylic cycle (TCA cycle) and oxidative phosphorylation.

Pyruvate oxidation, a decarboxylation process catalyzed by pyruvate dehydrogenase complex, subsequently converts pyruvate molecules into acetyl coenzyme A (acetyl-CoA). Acetyl-CoA is an important fuel for the TCA cycle in cellular respiration for energy production. In comparison to fermentation, cellular respiration generates significantly more energy (18 ATPs per glucose molecule) via oxidative phosphorylation as it can generate additional ATPs from the products of the TCA cycle (mainly NADH and $FADH_2$) (Askew *et al.*, 2009; Pfeiffer and Morley, 2014). In contrast, the fermentation process does not involve the electron transport chain system, thus no additional ATPs is generated due to lack of oxygen (two ATPs per glucose molecule) (Pfeiffer and Morley, 2014).

Availability of glucose affects the fungi's decision to undergo cellular respiration using glucose or to utilize alternative carbon sources. Generally, *S. cerevisiae* and many other yeast species repress metabolic pathways dedicated for alternative carbon utilization when glucose availability is not a limiting factor (Crabtree-positive), thus suggesting that they prioritize sugar assimilation over other alternative carbon sources (Flores *et al.*, 2000). On the contrary, human fungal pathogen like *C. albicans* simultaneously uses both glucose and other carbon sources even in the presence of glucose (Crabtree-negative). It is perhaps owing to the reasons that *C. albicans* has adapted to not solely dependent on glucose unlike its distant cousins *S. cerevisiae* and *C. glabrata* which all three

share common ancestors 200 million years ago (Dujon, 2005). This is in anticipation that *C. albicans* faces host niche environment that are mostly hostile where glucose level is commonly found to be in low concentration (Sandai *et al.*, 2012).

Multiple studies have shown that key enzymes for alternative carbon utilization in *S. cerevisiae* were subjected to ubiquitin-mediated degradation in the presence of glucose (Schork *et al.*, 1995; Schüle *et al.*, 2000; Horak *et al.*, 2002; Regelmann *et al.*, 2003; Sandai *et al.*, 2012). However, Sandai *et al.* (2012) have demonstrated that differential evolutionary rewiring of ubiquitination sites in the glyoxylate cycle enzyme isocitrate lyase (Icl1) has bestowed *C. albicans* the ability to retain its key metabolic functions, greatly enhances the fungal metabolic flexibility, and permits concurrent assimilation of glucose and alternative carbon sources. This trait is extremely crucial for a successful fungal pathogen like *C. albicans* as its prime directive involves rapid colonization in various host niches (Sandai *et al.*, 2012). The improved metabolic flexibility in *C. albicans* attributed to the insensitivity to ubiquitin-mediated catabolite inactivation is a virulent determinant. Childers *et al.* (2016) have reported that *S. cerevisiae* that lack glucose-mediated degradation system (similar to *C. albicans*) were less susceptible to macrophage killing and showed enhanced virulence in murine model, further suggesting the importance of post-transcriptional modification in metabolic flexibility and pathogenesis of medically-important fungi.

Alternative Carbon Metabolism in Medically-Important Fungi

In most conditions, glucose is the preferred carbon source for medically-important fungi, and it is crucial for maximum fungal growth and development. For many microorganisms, glucose is not always available and the only accessible carbon sources are simpler carbon compounds (Lorenz and Fink, 2002). Therefore, when the availability of glucose is scarce, survival of these fungal pathogens depends on the efficient uptake, utilization and metabolism of alternative carbon sources (Ene *et al.*, 2014). Medically-important fungi have been reported to be able to utilize other fermentable sugars (e.g., pentoses and hexoses) and non-fermentable carbon sources, including but not limited to long- and short-chain fatty acids, organic acids, alcohol and amino acids (Lorenz and Fink, 2001; Rude *et al.*, 2002; Hynes *et al.*, 2006; Ramírez and Lorenz, 2007; Turcotte *et al.*, 2010; Chew *et al.*, 2019b). However, most human fungal pathogens have streamlined their carbon utilization profile and prioritize glucose over other carbon sources, mainly because the metabolic processes involving glucose metabolism are energetically favorable (Ene *et al.*, 2014).

In human body, alternative carbon sources can be found in different host niches. Lactate and acetate, two of the most common carboxylates, are commonly produced by the lactic acid bacteria (LAB) in the gastrointestinal and vaginal tract, via breakdown of complex polycarbohydrates (Yamaguchi *et al.*, 2005; Turcotte *et al.*, 2010; Miramón and Lorenz, 2017). While most tissues in human body also produce lactate in excess, highest amount of lactate production usually occurs in the muscle tissue (Andersen *et al.*, 2013). Under normal circumstances, lactate concentration is below 2.0 mmol/L, as approximately 70% of the excessive lactate is rapidly cleared by the liver and also to the lesser extent by the kidneys (Phypers and Pierce, 2006; Andersen *et al.*, 2013). Similarly, low concentration of acetate is generally present in human blood, ranging from 0.05 to 0.2 mmol/L, and subjected to further increase following intake of ethanol (Richards *et al.*, 1976; Tollinger *et al.*, 1979; Pomare *et al.*, 1985; Davies *et al.*, 2011).

Fatty acids are important components that serve many essential roles in human body, and generally can be found as free circulating fatty acids or in esterified forms such as glycolipids, phospholipids and triacylglycerols (Nagy and Tiuca, 2017). The amounts of fatty acids in plasma and human tissue varies considerably in response to various factors, such as differences in gastrointestinal absorption rate, metabolic activity and dietary intake (Risé *et al.*, 2007). Nitrogen sources like amino acids can also be exploited as alternative carbon sources to support fungal growth during glucose-limited condition. In the midst of glucose starvation, endogenous alternative carbon sources can also be generated by fungal pathogens via mobilization of intracellular resources through autophagy. In fact, the ability to subvert nutrient deprivation through recycling of intracellular component is extremely vital for many fungi like *Aspergillus fumigatus*, *C. glabrata* and *C. neoformans* (Richie *et al.*, 2007; Hu *et al.*, 2008; Roetzer *et al.*, 2010; Shimamura *et al.*, 2019). This observation further strengthens the importance of alternative carbon sources in maintaining cellular function and survival of intracellular fungi.

Alternative carbon metabolism is initiated when glucose availability is scarce, and the first step of the metabolism involves efficient uptake of extracellular carbon sources. The modes of transportation are unique to each types of carbon sources, and this has been extensively reviewed (Turcotte *et al.*, 2010; Chew *et al.*, 2019c). Many fungal pathogens including *Candida*, *Aspergillus* and *Cryptococcus* species are able to utilize glycerol as the sole carbon source (Ramírez and Lorenz, 2007; Price *et al.*, 2011; De Assis *et al.*, 2015; Chew *et al.*, 2019b). Glycerol is first converted into glycerol-3-phosphate (G3P) by glycerol kinase (EC 2.7.1.30), subsequently into dihydroxyacetone phosphate (DHAP) by glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and finally reversibly converted into glyceraldehyde 3- phosphate (GA3P) by triose phosphate isomerase (EC 5.3.1.1). On the other hand, uptake of lactate involves short chain carboxylates transporters such as Jen1 and Jen2 in *S. cerevisiae* and *C. albicans* (Casal *et al.*, 1999; Soares-Silva *et al.*, 2004), Jen4 in *C. neoformans* (Niedźwiecka *et al.*, 2019) and JenB in *Aspergillus nidulans* (Sá-Pessoa *et al.*, 2015). Lactate is ultimately converted to pyruvate by oxidoreductase (EC 1.1.2.3). Both GA3P and pyruvate serve as intermediates to fuel gluconeogenesis for fungal growth in the absence of glucose.

Metabolism of fatty acids in fungal pathogens requires a functional beta-oxidation, and this pathway is essential for the breakdown of fatty acids to obtain acetyl-CoA as an important carbon and energy intermediates. Similarly, acetyl-CoA is also generated from utilization of ethanol and acetate. Ethanol is transported via passive diffusion, whilst acetate is believed to be transported via the acetate uptake transporter (AceTr) family, including Ady2 and Ato1 orthologues in *Candida* species (Casal *et al.*, 2008; Alves *et al.*, 2020), and AcpA in *A. nidulans* (Robellet *et al.*, 2008). Net oxaloacetate derived from acetyl-CoA can be used in

fungi via the glyoxylate cycle to replenish the tricarboxylic acid (TCA) cycle for energy production, or to fuel gluconeogenesis for cellular building blocks generation.

Pathways for Alternative Carbon Metabolism and Their Roles in Fungal Pathogenicity

Beta Oxidation of Fatty Acids

It is undeniable that catabolism of fatty acids via beta-oxidation is imperative for growth and development of many fungi (Hynes *et al.*, 2006). In mammals, beta-oxidation of long-chain fatty acids occurs in peroxisomes, whilst shorter fatty acids are metabolized by the mitochondria (Strijbis and Distel, 2010). Although beta-oxidation of fatty acids is believed to be exclusively peroxisomal in certain fungal species such as *S. cerevisiae*, *C. albicans*, *Yarrowia lipolytica* (Smith *et al.*, 2000; Strijbis and Distel, 2010), multiple studies have suggested that many fungal species harbored both peroxisomal and mitochondrial beta-oxidation pathways (Cornell *et al.*, 2007; Shen and Burger, 2009). In fact, mitochondrial beta-oxidation of fatty acids has been reported in *A. nidulans*, *C. neoformans* and *Candida lusitaniae* (Maggio-Hall and Keller, 2004; Kretschmer *et al.*, 2012; Gabriel *et al.*, 2014). Activation of fatty acids generates their corresponding acyl-coenzyme A (CoA), followed by oxidation steps catalyzed by three distinct enzymes, namely acyl-CoA oxidase (EC 1.3.3.6), multifunctional enzyme [enoyl-CoA hydratase (EC 4.2.1.17) and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)] and 3-ketoacyl-CoA thiolase (EC 2.3.1.16). At the end of the reactions, acetyl-CoA and an acyl-CoA that is two carbons shorter are generated, with the latter substrate subjected to further beta-oxidation (Hynes *et al.*, 2006; Schulz, 2008; Strijbis and Distel, 2010). Different fungi encode different isoforms of these beta-oxidation enzymes that possess varying affinities toward long-, medium- and short-chain fatty acids. For instance, it has been reported that human fungal pathogen *C. albicans* possess two acyl-CoA oxidases, one multifunctional enzyme and three 3-ketoacyl-CoA thiolase. Interestingly, *C. albicans* has a higher number of enzyme isoforms compared to the baker's yeast, which possess only one acyl-CoA oxidase, one multifunctional enzyme and one 3-ketoacyl-CoA thiolase (Strijbis and Distel, 2010). The increased number of isoforms in *C. albicans* suggests the enhance capability of human fungal pathogen in fatty acids utilization. The resulting acetyl-CoA from catabolism of fatty acids can be further processed by the glyoxylate cycle.

Previous study conducted by Lorenz *et al.* (2004) has demonstrated that almost all enzymes encoding genes in the beta-oxidation pathway (with the exception of genes encode for three isoforms of 3-ketoacyl-CoA thiolase) were significantly up-regulated in *C. albicans* phagocytosed by macrophages, indicating intracellular fatty acids utilization. In addition, genes involved in the beta-oxidation of fatty acids (e.g., *POX1-3*, *PXP2*, *ECN1*, *FOX2*, *FOX3* and *POT1*) were also similarly induced in *C. parapsilosis* and *C. tropicalis* in response to human whole-blood (Kämmer *et al.*, 2020). Disruption of *FOX2* severely reduce the capability of *C. albicans* in metabolism of oleic acid, while complementation of the mutant strain with the wild-type gene fully restored beta-oxidation activity (Piekarska *et al.*, 2006). Moreover, *C. albicans* mutant strain lacks *FOX2* also demonstrated reduced virulence in a murine model of systemic candidiasis (Piekarska *et al.*, 2006; Ramírez and Lorenz, 2007). In *A. nidulans*, deletion of a putative multifunctional enzyme (*foxA*), homolog of *S. cerevisiae* and *C. albicans FOX2* responsible for peroxisomal beta-oxidation inhibited growth on very long-chain fatty acids (VLCFAs, C_{22} and above), severely hampered the growth on long-chain fatty acids (LCFAs, C_{13}- C_{21}), but has no effect on the growth on short-chain fatty acids (SCFAs, C_6 and below) (Maggio-Hall and Keller, 2004). Indeed, *A. nidulans* possess mitochondrial enoyl-CoA hydratase (*echA*), an enzyme involved mitochondrial beta-oxidation of short-chain fatty acids (Maggio-Hall and Keller, 2004). It is reported that disruption of *echA* significantly reduced *A. nidulans* growth on VLCFAs, LCFAs and completely abolished the growth on SCFAs. In *A. fumigatus*, putative genes involved in beta-oxidation such as *mfp* (*foxA*), *echA*, *farb1*, *farb2*, *scdA*, and *aoxA* have been reported to be highly induced in response to human neutrophils, signifying the importance of this pathway in human fungal pathogen (Sugui *et al.*, 2008).

In *C. neoformans*, both peroxisomal (*MFE2*) and mitochondrial (*HAD1* and *HAD2*) beta oxidation have been shown to be highly induced following exposure to fatty acids, and in response to lung pulmonary cryptococcosis in mice (Kretschmer *et al.*, 2012). Defect in *MFE2* and *MFE2* plus *HAD1* (but not *HAD1* alone) significantly attenuated the virulence of *C. neoformans* in a mouse model of cryptococcosis, firmly suggesting that peroxisomal beta-oxidation plays a more prominent role in the pathogenicity of this fungal pathogen, as compared to the mitochondrial pathway (Kretschmer *et al.*, 2012). The same study also showed that a non-functional peroxisomal beta-oxidation also affect the phenotypes of *C. neoformans* at multiple levels, this includes reduction of melanin and capsule formation, increased resistance to cell wall inhibitory and antimycotic agents, and enhanced proteases activity. Ironically, another major human fungal pathogen *Histoplasma capsulatum* does not seem to favor fatty acids utilization in order to propagate within macrophages (Shen *et al.*, 2020). It is reported that *H. capsulatum* is unable to utilize both SCFAs and LCFAs *in vitro*, and down-regulates beta-oxidation pathway in response to macrophage engulfment. Furthermore, disruption of *FOX1* involved in beta-oxidation does not render the strain avirulent, confirming the insignificant role of fatty acid metabolism in pathogenicity of *H. capsulatum* (Shen *et al.*, 2020). Also, beta-oxidation is not found in all three species of *Pneumocystis*, namely *Pneumocystis jirovecii*, *Pneumocystis carinii* and *Pneumocystis murina* (Ma *et al.*, 2016).

The Glyoxylate Cycle

The glyoxylate cycle is a special variant of the tricarboxylic cycle (TCA) that allows utilization of two carbons compounds in the absence of glucose. The glyoxylate cycle is generally not present in human and animal tissue, and can only be found in plants,

bacteria, fungi and protists (Chew *et al.*, 2019c). As a shunt in the TCA cycle, the glyoxylate cycle shares three out of five metabolic enzymes with the cycle: malate dehydrogenase (EC 1.1.1.37), citrate synthase (EC 2.3.3.1) and aconitase (EC 4.2.1.3), by-passing the two decarboxylation steps catalyzed by isocitrate dehydrogenase (EC 1.1.1.41) and α-ketoglutarate dehydrogenase complex (EC 1.2.4.2, EC 2.3.1.61, EC 1.8.1.4) (Kondrashov *et al.*, 2006; Kunze *et al.*, 2006). Instead of converting isocitrate to α-ketoglutarate in TCA cycle, the glyoxylate cycle enzyme isocitrate lyase (EC 4.1.3.1) catalyzes the conversion of isocitrate (C_6) into glyoxylate (C_2) and succinate (C_4). Subsequently, malate synthase (EC 2.3.3.9) catalyzes the condensation of glyoxylate with acetyl-CoA (C_2) to produce malate (C_4) and a free CoA molecule (Chew *et al.*, 2019c). In this way, malate can be further oxidized into oxaloacetate, an important precursor for gluconeogenic biosynthesis of glucose and other sugars. Alternatively, malate that is generated from the alternative carbon utilization (e.g., fatty acids, ethanol and acetate, via acetyl-CoA) also replenishes the energy-producing TCA cycle (Dunn *et al.*, 2009). In short, the glyoxylate cycle intermediate serve as a linkage between anabolic and catabolic fungal metabolism in the midst of glucose deprivation and enables assimilation of alternative carbon sources. As both isocitrate lyase and malate synthase are the exclusive enzymes required for a functional glyoxylate cycle, they are often considered to be the hallmark for this anaplerotic pathway (Kondrashov *et al.*, 2006). These key enzymes of the glyoxylate cycle are also highly conserved among plants, bacteria and fungi (Kondrashov *et al.*, 2006; Dunn *et al.*, 2009). With the only remarkable exception of *S. cerevisiae* cytosolic isocitrate lyase, both key enzymes are generally peroxisomal in fungi (Taylor *et al.*, 1996; Kunze *et al.*, 2006; Hynes, 2010; Strijbis and Distel, 2010).

Both key enzyme genes from the glyoxylate cycle *ICL1* (isocitrate lyase) and *MLS1* (malate synthase) are highly up-regulated in phagocytosed *C. albicans* subjected to human blood (Lorenz and Fink, 2001; Fradin *et al.*, 2003; Lorenz *et al.*, 2004), and *ICL1* is proven to be required for the full virulence of this intracellular pathogen (Lorenz and Fink, 2001; Ramírez and Lorenz, 2007). Similar observation has also been reported in non-*C. albicans Candida* (NCAC) species like *C. glabrata*. It has been reported that the glyoxylate cycle is essential for the metabolic flexibility and virulence of *C. glabrata* in a murine model of invasive candidiasis (Chew *et al.*, 2019b). In comparison to *C. glabrata*, loss of *ICL1* engendered much more defects in *C. albicans*, as it also affects the growth on glycerol, in addition to other carbon sources such as ethanol, acetate and fatty acids (Ramírez and Lorenz, 2007; Chew *et al.*, 2019b). In both *Candida* species, the utilization of alternative carbon sources via the glyoxylate cycle also induced multiple physiological changes associated with the pathogenicity of the fungi. For instance, alternative carbon sources greatly influenced the cell wall properties of *C. albicans*, resistance to environmental stresses and antimycotic agents, immune recognition, fitness and virulence of *C. albicans* in vivo (Ene *et al.*, 2012; Ene *et al.*, 2013). For *C. glabrata*, growth on various carbon sources, i.e., glucose, acetate, lactate, ethanol, glycerol and oleic acids altered the fungal cell wall (*β*-glucan and chitin), biofilm growth and resistance to antifungal and oxidizing agent (Mota *et al.*, 2015; Chew *et al.*, 2019a). Furthermore, assimilation of acetic acid as alternative carbon source also significantly affects the interaction of *C. glabrata* with macrophages (Mota *et al.*, 2015).

In *Aspergillus* species, the homologs of *ICL1* and *MLS1* genes are known as *acuD* and *acuE*, respectively (Ebel *et al.*, 2006; Hynes *et al.*, 2006). In comparison to *Candida* species like *C. albicans* and *C. glabrata*, whereby the glyoxylate shunt is essential; *acuD* and *acute*, and hence the glyoxylate cycle is not required for the full virulence of *A. fumigatus* (Schöbel *et al.*, 2007; Olivas *et al.*, 2008). Schöbel *et al.* (2007) speculated that *A. fumigatus* does not depend on fatty acids and acetate as one of the major carbon sources for survival and invasive growth, as the *acuD* mutant is still capable of strong hyphal formation. Potentially, *A. fumigatus* could rely on other carbohydrates and proteins during a short period of time upon phagocytosis, and prioritize on aggressive hyphal elongation to assist in macrophage killing and escape from the immune cells (Waldorf, 1989; Schöbel *et al.*, 2007). Therefore, it is possible that various carbon utilization pathways (e.g., methylcitrate cycle) could work together and compensate for the loss of a functional glyoxylate cycle (Ibrahim-Granet *et al.*, 2008; Dagenais and Keller, 2009). Removal of the glyoxylate cycle key enzyme genes also renders *C. neoformans* inability in utilization of certain carbon sources such as ethanol and acetate. Similar to *A. fumigatus*, deletion of *ICL1* and *MLS1* does not appear to affect the virulence of *C. neoformans* (Rude *et al.*, 2002; Idnurm *et al.*, 2007). The glyoxylate cycle is only indispensable for the virulence of *Candida* species, and this could be attributed to the long history of association and evolution of this fungal pathogen with the host. While both *Aspergillus* and *Cryptococcus* species can be found in the environment and within a host, *Candida* species lacks any apparent environmental reservoir and can only be found in the mammalian hosts (Kadosh and Lopez-Ribot, 2013), which may explain the different approaches employed by these fungal pathogens in alternative carbon utilization.

Gluconeogenesis

Gluconeogenesis is typically regarded as a reversal of glycolysis, albeit with multiple variations that distinguish the two pathways. Gluconeogenesis is a metabolic process responsible for the biosynthesis of glucose from non-carbohydrate precursors. These precursors include fatty acids, lactate, acetate, oxaloacetate, ethanol, glycerol, and glucogenic amino acids. Like the TCA and glyoxylate cycles, a large number of metabolic enzymes are identical to both glycolysis and gluconeogenesis (Turcotte *et al.*, 2010), while only two enzymes, phosphoenolpyruvate carboxykinase (EC 4.1.1.32) and fructose-1,6-bisphosphatase (EC 3.1.3.11) are exclusive to gluconeogenesis (Eschrich *et al.*, 2002). In fungi, both glycolytic and gluconeogenic pathways are regulated so that only one of the pathways is active at a time while the other remains relatively idle to prevent futile cycle, and these regulations are essentially controlled by the availability of glucose (activation of glycolysis) or non-fermentable alternative carbon sources (activation of gluconeogenesis) (Berg *et al.*, 2002). Following the flux through the glyoxylate cycle, oxaloacetate produced from alternative carbon utilization can be converted into phosphoenolpyruvate by the enzyme phosphoenolpyruvate carboxykinase (Lorenz *et al.*, 2004; Hynes *et al.*, 2007). Alternative carbon sources such as glycerol (in the form of GA3P), lactate (in the form of pyruvate), acetate, ethanol and fatty acids (in the form of oxaloacetate) all serve as potential precursors to fuel gluconeogenesis. Fructose-1,6-

bisphosphatase catalyzes the conversion of fructose-1,6-biphosphate to fructose-6-phosphate at the final step in formation of hexose monophosphate (Lorenz *et al.*, 2004; Hynes *et al.*, 2007). In summary, the three interrelated metabolic networks (beta-oxidation, the glyoxylate cycle and gluconeogenesis) join forces to enable alternative carbon metabolism in medically-important fungi. This greatly enhances the survival of these fungi in many host niches in human body that have limited glucose supply.

Disruption of the gluconeogenic-specific enzyme gene, *PCK1* (phosphoenolpyruvate carboxykinase) attenuated the virulence of *C. albicans* in mice, while re-introduction of the wild-type *PCK1* restored the fungal virulence (Barelle *et al.*, 2006). On the other hand, Ramírez and Lorenz (2007) reported that disruption of *FBP1* (fructose-1,6-bisphosphatase) greatly affected the growth of *C. albicans* on glycerol, lactate, acetate, ethanol and oleic acid. Furthermore, loss of *FBP1* significantly reduced the virulence of *C. albicans* in vivo, as up to 30% of mice still survived after 20 days of infection, while all mice infected with the wild-type *C. albicans* succumbed to death after 10 days (Ramírez and Lorenz, 2007). Similar to *C. albicans*, deletion of *PCK1* in *H. capsulatum* impaired the ability of this fungi to grow within macrophages and in vivo virulence (Shen *et al.*, 2020). Being a nearly exclusive intracellular fungal pathogen, *H. capsulatum* required utilization of gluconeogenic intermediates via gluconeogenesis for survival and proliferation in the host (Deepe *et al.*, 2008; Shen *et al.*, 2020). Targeted knockout of *PCK1* also considerably reduced the virulence of *C. neoformans* mutant as compared to the wild-type strain in a mouse inhalation model of cryptococcosis, and complementation of wild-type *PCK1* restored its full virulence (Panepinto *et al.*, 2005). However, Price *et al.* (2011) reported that although *PCK1* is highly up-regulated in *Cryptococcus* infection, this gluconeogenic enzyme gene is not required for the persistence and viability of this fungal pathogen in rabbit cerebrospinal fluid model of cryptococcosis. As for *A. fumigatus*, phosphoenolpyruvate carboxykinase-encoding gene *acuF* has been reported to be up-regulated in response to stress conditions such as heat-shock and hypoxia (Do *et al.*, 2009; Barker *et al.*, 2012). However, the role of this gluconeogenic gene in the virulence of *A. fumigatus* still remains to be determined.

Glucose and Alternative Carbon Metabolism: A Prospective Therapeutic Target for Fungal Diseases

It is undeniable that carbon metabolism is integral to the pathogenicity of human fungal pathogen, as changes in the metabolic adaptation greatly impact the fungal pathogenicity at multiple levels (Brown *et al.*, 2014). Lately, research have been focusing on targeting these metabolic pathways in searching for potential antifungal targets (Chen *et al.*, 2020). Although the development of effective antifungals is often hindered by the eukaryotic biology these fungi share with human, there are still a number of fungal-exclusive metabolic pathways that can be targeted for drug development (Zhang *et al.*, 2012; Kaltdorf *et al.*, 2016). One of the major pathways essential to fungal pathogens like *C. albicans* and *C. glabrata* that is not found in human is the glyoxylate cycle (Icl1 and Mls1) (Lorenz and Fink, 2001; Ramírez and Lorenz, 2007; Chew *et al.*, 2019b). Multiple studies have demonstrated that potential inhibitors such as cadiolides E and G-1, caffeic acid, apigenin, rosmarinic acid, mohangamides A and B, suvanine sesterterpenes and monoterpenoid perillyl alcohol (PA) are able to inhibit *C. albicans* isocitrate lyase activity (Ahn *et al.*, 2013; Cheah *et al.*, 2014; Lee *et al.*, 2014; Bae *et al.*, 2015; Ansari *et al.*, 2018). In addition, Ansari *et al.* (2018) showed that PA inhibited *C. albicans* malate synthase activity and mRNA expression. Furthermore, it has been reported that four β-carboline alkaloid compounds also inhibited malate synthase from *Paracoccidioides* (Costa *et al.*, 2015). Several synthetic isocitrate lyase inhibitors such as itaconate, 3-nitropropionate and 3-bromopyrvuate have been previously described (McFadden and Purohit, 1977; Schloss and Cleland, 1982; Ko and McFadden, 1990). These inhibitors serve as structural analogs for intermediates of the glyoxylate cycle – succinate and glyoxylate. However, these potential inhibitors have not been introduced as antimicrobials for human use, mainly due to their toxicity and detrimental effects to other metabolic enzymes in vivo (Lee *et al.*, 2015).

Other carbon metabolic pathways required for glucose and alternative carbon metabolism also harbored many potential therapeutic targets for fungal diseases. Among the components that are involved in these pathways are glucose sensors and transporters (e.g., Snf3, Rgt2, Hgt4), glycolytic enzymes (e.g., pyruvate kinase, enolase, hexokinases), gluconeogenic enzymes (e.g., Fbp1 and Pck1), and enzymes for beta-oxidation of fatty acid (Eschrich *et al.*, 2002; Barelle *et al.*, 2006; Brown *et al.*, 2006; Piekarska *et al.*, 2006; Ramírez and Lorenz, 2007; Ko *et al.*, 2013; Ng *et al.*, 2015; Laurian *et al.*, 2019). The potential antifungal targets based on these metabolic pathways of human fungal pathogen (*C. albicans*) has been discussed and summarized previously (Chen *et al.*, 2020). Nevertheless, the suitability and feasibility of most of these potential targets or inhibitors to be developed into full-fledged antifungals still remain to be determined, as fungi are more closely-related to human which represent a key fundamental challenge in drug development (Roemer and Krysan, 2014). Many inhibitors that are toxic to fungi are most probably also toxic to human. Currently, fungal-specific structures are the prime target for most of the major classes of antifungals (polyenes, azoles and echinocandins) commonly used (Roemer and Krysan, 2014). After identification of a potential fungal-specific drug target, it is crucial that the strategies used for antifungal development should focus on maximizing the drug effectiveness and minimizing the drug toxicity and adverse effects to the host. Also, it is also important that the novel antifungal agents targeting carbon metabolism can overcome the issue of fungal resistance for a better management of patients affected by fungal diseases.

Conclusion and Future Prospects

The knowledge and understanding of glucose and alternative carbon sources metabolism in medically important fungi have increased and deepened over the years. Appreciating these physiological processes which confer metabolic fitness to these fungi have partially lent to opening up of finding novel approaches in tackling the impending slow discovery and development of new

antifungal drugs. Though much has been discovered about these metabolic processes, it is an ongoing task as we are experiencing increasing number of novel fungal species that cause infections among us with unique and distinct types of metabolism. Needless to mention, with the growing advancements in medical knowledge and technologies, i.e., cancer treatments, organ and tissue transplantation, and etc. which render human population to have a longer life span, the number of immunocompromised and immunosuppressed individuals will also increase in tandem with it. The recent three to four decades have seen such trend but unfortunately, the awareness and research funding on fungal infections especially on invasive types have not caught up with it. In addition, the urbanization and industrialization activities and opening up of forest areas for human habitation that affect climate change has accelerated human encounter with novel fungal species which are generally known to be highly resistant to existing antifungal drugs. In our race to acquire more information, mountainous tasks await us. One of the challenges that we need to overcome is to be able to speed up and translate all these information and data into impactful applications. Till to date, there has been little or no significant outcome from these investigations to reduce the burden of mortality and morbidity due to fungal infections. Looking from that perspectives, there are questions that need further deliberation. The utilization of glucose and other alternative carbon sources by fungal pathogens is "more than meets the eye". It goes beyond meeting the energy demand of the pathogens. Most studies on carbon metabolism related genes are based on investigating their monogenic effects and a handful of them looked into two and more genes. As expounded in the earlier paragraphs, many metabolism-related genes have yet to be fully discovered and functionally characterized. There are also instances where gene homologs of various fungal pathogens perform different functions. On another note, there are more and more accumulating transcriptomics data that are made available in the primary and secondary bioinformatics databases. One strategy is to combine and perform deeper analysis of these data utilizing advanced software where the interconnectedness of the various carbon metabolic pathways can be better appreciated and manipulated. Last but not least, again on bioinformatics front in addition to wet lab experiments, *in silico* experimentation on testing various carbon sources (not just one at a time) with varying ratio that mimic host niches will definitely spur further understanding of glucose and alternative carbon metabolism in medically important fungal pathogens.

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Ergosterol Synthesis

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Introduction

Sterols are important components of eukaryotic cell and mitochondrial membranes maintaining membrane fluidity, structure, function, and permeability. According to their origin, sterols are broadly classified into three types. First, the animal sterols, most important of which is cholesterol. Cholesterol aids in synthesis of cell membranes, bile acids, vitamin D and steroid hormones. Second, phytosterols, which are plant sterols, most important of which are stigmasterol, sitosterol and campesterol (Jorda and Puig, 2020; Hu *et al.*, 2017). These phytosterols help in maintaining proper plant growth and development. Plants also contain cholesterols especially in seeds, roots, stems, and leaves. Third kind of sterol is fungisterol, which is present in fungi. Ergosterol is a unique fungisterol present in the cell membrane within microdomains called lipid rafts. These lipid rafts are synthesized by fusion of sphingolipids and sterols. Lipid rafts are enriched with many biologically important molecules that include ion pumps (sodium and potassium pumps), multi-drug resistance (MDR) pumps, and nutrient transporters (Bhattacharya *et al.*, 2018). These rafts are critical for proper functioning of the cells, and abnormal cellular ergosterol levels cause severe growth defects in response to different environmental stressors (Bhattacharya *et al.*, 2018). Recent studies demonstrated that ergosterol can induce host cell pyroptosis. Pyroptosis is a necrotic and inflammatory programmed cell death induced by inflammatory caspases (Jorda and Puig, 2020; Koselny *et al.*, 2018; Rodrigues, 2018). Besides cell and mitochondrial membrane, ergosterols are also present in vacuoles, peroxisomes, and endoplasmic reticulum (ER). The physiological functions of ergosterol are described below:

Physiological Function of Ergosterol

As mentioned above, ergosterol is unique to fungi and is an integral part of the fungal cell and the mitochondrial membranes. In the cell membrane, ergosterol stabilizes the membrane by interacting with sphingolipids and regulating membrane structure, fluidity, and permeability (Bhattacharya *et al.*, 2018). Ergosterol fuses with sphingolipids to produce lipid rafts, which host important metabolic enzymes and membrane transporters. These transporters are crucial for exporting xenobiotics, toxic compounds, ions and drugs from the cell thereby preventing cellular damage. Additionally, ergosterol can alter cell membrane mobility affecting phospholipase transport. Besides transport, ergosterol can disrupt nutrient absorption by regulating the activities of membrane bound ATPase (Hu *et al.*, 2017). Hence, maintenance of cellular ergosterol levels is crucial for proper cellular functions. Ergosterol levels are maintained by sterol pools, which are composed of lipid droplets that store ergosterol. Proper ergosterol levels are also important for stress response. For example, during ethanol treatment, baker's yeast, *Saccharomyces cerevisiae* elevates cellular ergosterol levels and protects its cell membrane from any damages. Conversely, when antifungal drugs inhibit ergosterol biosynthesis, the yeast cells become significantly more sensitive to osmotic and salt stresses (Hu *et al.*, 2017; Bhattacharya *et al.*, 2018). Addition of ergosterol in the growth media significantly increases yeast cells' tolerance to osmotic stress (Hu *et al.*, 2017). Further, cellular ergosterol levels are also critical for hypoxic and temperature stress response. For example, *S. cerevisiae* cells with disrupted ergosterol biosynthesis were temperature sensitive (Hu *et al.*, 2017). Additionally, cellular ergosterol levels can elevate in response to hypoxic conditions causing yeast cells to grow and proliferate properly (Hu *et al.*, 2017). A recent study has linked ergosterol with host macrophage pyroptosis. Pyroptosis is a proinflammatory form of programmed cell death triggered by inflammasome-mediated activation (Koselny *et al.*, 2018; Rodrigues, 2018). The study identified several genes in the ergosterol biosynthesis pathway that participate in the induction of pyroptosis-related macrophage lysis (Koselny *et al.*, 2018).

Ergosterol Biosynthesis

Ergosterol is biosynthesized in ER by a cascade of 25 biosynthesis enzymes (Bhattacharya *et al.*, 2018). The pathway is conserved in different pathogenic and non-pathogenic fungi. For simplicity, this chapter describes ergosterol biosynthesis in model organism *S. cerevisiae*. Ergosterol biosynthesis is extensive studied in *S. cerevisiae*. This biosynthesis pathway is broadly divided into two parts: (1) Mevalonate pathway (MVP) and (2) Late pathway (**Fig. 1**). The MVP uses acetyl CoA as a substrate to synthesize farnesyl pyrophosphate (FPP) (Bhattacharya *et al.*, 2018). The first step of the pathway involves condensation of two acetyl-CoA groups to form acetoacetyl-CoA (**Fig. 1**). This step is catalyzed by acetoacetyl-CoA thiolase, which is encoded by *ERG10* in baker's yeast *Saccharomyces cerevisiae* (**Fig. 1**). The enzyme is also present in various fungi including *Candida sp*, *Aspergillus sp*, *Cryptococcus sp*, *Schizosaccharomyces sp*, and several other pathogenic and non-pathogenic fungi. The next step in MVP involves the synthesis of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). This step involves condensation of another acetyl-CoA molecule with

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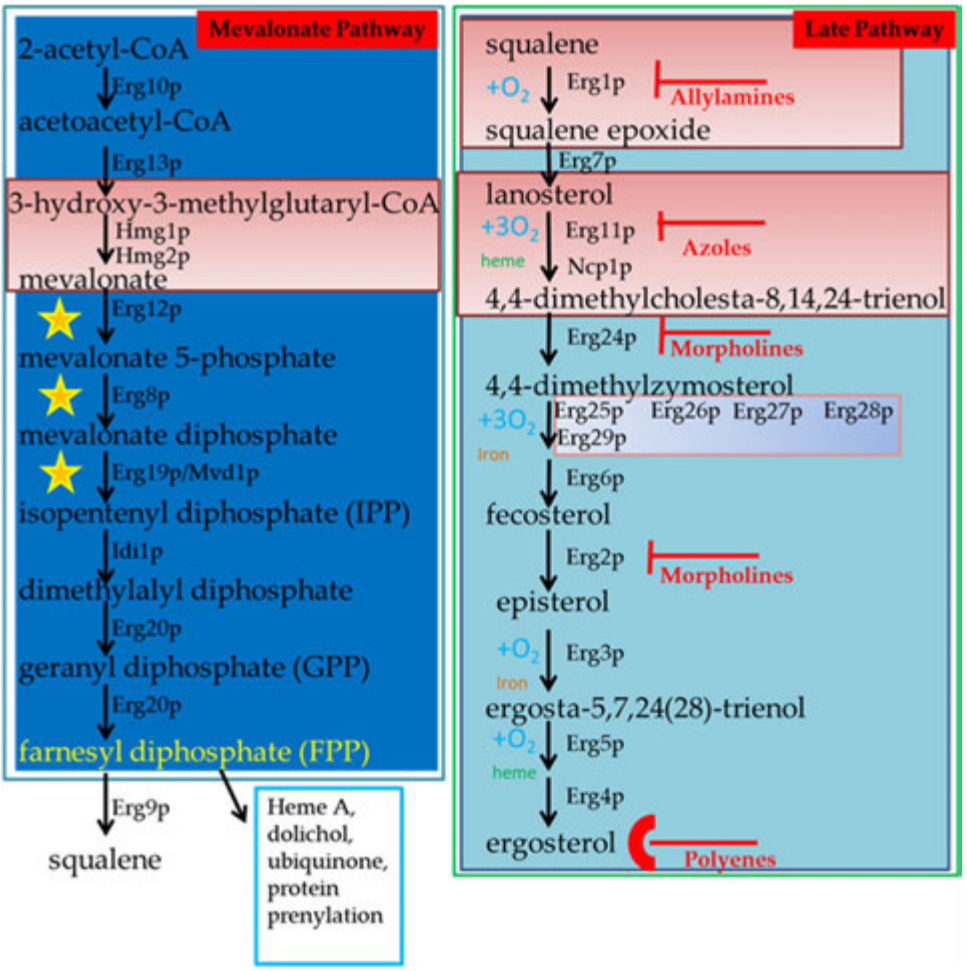

Fig. 1 Ergosterol Biosynthesis Pathway: A cascade of 25 enzymes are responsible for synthesis of ergosterol. The pathway is divided into two parts; (a) Mevalonate Pathway (MVP) and (b) Late Pathway. MVP uses Acetyl-CoA as the substrate to synthesize FPP (yellow text). FPP is the precursor for many metabolic processes represented in the blue box. Late pathway uses FPP to synthesize ergosterol. The rate limiting steps in the ergosterol synthesis pathway is depicted with red shaded box. Yellow stars signify that these reactions require ATP. Different ergosterol inhibitors are listed with red texts. The enzyme complex is depicted by blue shaded box. The steps in the ergosterol synthesis affected by heme, oxygen and iron are also marked.

acetoacetyl-CoA in an enzymatic reaction catalyzed by HMG-CoA synthase. The enzyme is encoded by *ERG13* in *S. cerevisiae* (**Fig. 1**) and is present in different fungi.

The next step involves an important rate-limiting step in MVP (Veen *et al.*, 2003). In this step, HMG-CoA reductase uses 2 molecules of NADPH to reduce substrate HMG-CoA to mevalonate (**Fig. 1**). The reductase enzyme has two paralogs in *S. cerevisiae* namely Hmg1p and Hmg2p. These paralogs are synthetic lethal, which means that deletion of both of the genes encoding these paralogs result in cell death (Bhattacharya *et al.*, 2018). Besides *S. cerevisiae*, this enzyme is also present in other pathogenic and non-pathogenic fungi. The enzyme is an important target for sterol synthesis inhibitory drugs, the statins (Bhattacharya *et al.*, 2018). In the next steps, mevalonate is first converted to mevalonate 5-phosphate and then to mevalonate diphosphate by two phosphorylation reactions catalyzed by enzymes mevalonate kinase and phosphomevalonate kinase respectively. These enzymes are encoded by *ERG8* and *ERG12* genes in *S. cerevisiae* respectively (**Fig. 1**). Both the enzymatic reactions are ATP-dependent. Mevalonate diphosphate then undergoes dehydration-decarboxylation in an ATP-dependent enzymatic reaction catalyzed by mevalonate pyrophosphate decarboxylase. The resulting product is isopentenyl pyrophosphate (IPP). The carboxylase is encoded by *ERG19/MVD1* in *S. cerevisiae*.

Next, IPP-dimethylallyl diphosphate isomerase converts IPP to dimethylallyl diphosphate (DMAPP). The enzyme is encoded by *IDI1* in *S. cerevisiae* (**Fig. 1**). DMAPP serves as the precursor of geranyl diphosphate (GPP) with the help of GPP-synthase enzyme. In this reaction, coupling of IPP and DMAPP occurs to produce GPP. GPP is further elongated by FPP-synthase to produce FPP, the final product of the MVP. GPP-synthase and FPP-synthase are encoded by *ERG20* in *S. cerevisiae*.

FPP is an important metabolic intermediate that aids in biosynthesis of heme, ubiquinone, dolichol, sterols and prenylated proteins (**Fig. 1**). These products are used as intermediates for many metabolic processes. For example, ubiquinones play a central role in mitochondrial respiration, while dolichols are inserted in ER and serve as glycosyl carrier during protein glycosylation.

In *S. cerevisiae* and related yeasts, all the enzymes in the mevalonate pathway are essential for cell growth and development (Bhattacharya *et al.*, 2018). These enzymes are distributed in several cellular compartments that include cytoplasm, ER, mitochondria and glycosomes. Most of these enzymes require oxygen and hence fungi cannot survive anaerobically without ergosterol supplementation (Zavrel *et al.*, 2013).

After MVP, ergosterol biosynthesis enters the late pathway (LP, **Fig. 1**). This pathway commences with the conversion of FPP to squalene. This is an enzymatic reaction catalyzed by squalene synthase. The enzyme aids in coupling of two FPP moieties to synthesize squalene. Squalene synthase is encoded by *ERG9* gene in *S. cerevisiae* and is present in wide varieties of fungi. The gene is essential for fungal growth and fungi cannot survive in absence of *ERG9* unless supplemented with ergosterol. Next step in LP is an important rate-limiting step in the pathway during which squalene epoxide is synthesized (Veen *et al.*, 2003). The enzyme that catalyzes this reaction is squalene epoxidase, which is encoded by *ERG1* in *S. cerevisiae*. The enzyme causes epoxidation of squalene to 2–3 oxidosqualene and is a target of antifungals, allylamines (terbinafine) (Bhattacharya *et al.*, 2020b). *ERG1* is an essential gene and cells lacking *ERG1* cannot survive unless supplemented with ergosterol. Repression of this enzyme causes aberrant mitochondrial morphology but increased competitive fitness. The enzyme requires oxygen and is localized in ER and lipid particles (Leber *et al.*, 1998). Following epoxidation, lanosterol is synthesized with the help of lanosterol synthase enzyme. The enzyme is localized in lipid bodies and is encoded by *ERG7* in *S. cerevisiae* and catalyzes the cyclization of 2–3 oxidosqualene to produce lanosterol. *ERG7* is an essential gene and required for fungal growth.

Lanosterol is an important precursor in biosynthesis of ergosterol. Lanosterol is then converted to 4,4-dimethylcholesta 8,14,24 trienol in an important enzymatic reaction catalyzed by 14α demethylase enzyme. The enzyme belongs to cytochrome p450 family and requires heme and oxygen for proper functioning. The enzyme catalyzes C14 demethylation of lanosterol to produce 4,4-dimethylcholesta 8,14,24 trienol. This step is another important rate-limiting step and the demethylase enzyme is the target for most common class of antifungals, the azoles (Bhattacharya *et al.*, 2018; Veen *et al.*, 2003; Bhattacharya *et al.*, 2020b). The demethylase enzyme is localized in ER and is encoded by *ERG11* in *S. cerevisiae*. The enzyme functions in association with another enzyme, NADP-cytochrome 450 reductase. The reductase is encoded by *NCP1* in *S. cerevisiae* and is regulated by the demethylase enzyme (Bhattacharya *et al.*, 2018).

In the next step, the trienol is reduced to 4,4-dimethyl zymosterol by C-14 sterol reductase enzyme. This enzyme is encoded by *ERG24* in *S. cerevisiae* and is a target for antifungals morpholines (Bhattacharya *et al.*, 2020b). The cells lacking this enzyme produces an abnormal sterol intermediate, ignosterol and cannot grow in nutrient rich media unless supplemented with calcium (Crowley *et al.*, 1996). Dimethyl zymosterol is then processed by a complex of 5 enzymes to produce zymosterol. These enzymes include C-4 methyl sterol oxidase, C-3 sterol dehydrogenase, 3 keto sterol reductase, and ergosterol biosynthesis protein 28. In *S. cerevisiae*, ergosterol biosynthetic protein 28 is encoded by *ERG28* and aid in protein-protein interactions between C-3 sterol dehydrogenase and 3 keto sterol reductase (Gachotte *et al.*, 2001). The dehydrogenase and reductase are encoded by *ERG26* and *ERG27* respectively and are localized in ER.

Zymosterol is then further processed to synthesize fecosterol. Delta(24)-sterol C methyltransferase enzyme catalyzes the production of fecosterol from zymosterol. The enzyme is encoded by *ERG6* in *S. cerevisiae*. The transferase enzyme is localized in several regions that include lipid particles, mitochondrial outer membrane, and plasma membrane associated ER (Parks *et al.*, 1995). Fungi can survive in absence of this enzyme but have severe growth defects in response to various environmental stressors (Bhattacharya *et al.*, 2018).

Fecosterol is then converted to episterol by C-8 sterol isomerase encoded by *ERG2* in *S. cerevisiae*. The enzyme catalyzes the isomerization of delta 8 double bond to delta 7 position to produce episterol. Fungal cells can survive in absence of this enzyme but accumulate aberrant sterol intermediates and show severe growth defects against different environmental stressors (Bhattacharya *et al.*, 2018). Episterol is then processed to produce ergosta 5,7,24(28) trienol in an enzymatic reaction catalyzed by C5-sterol desaturase. The enzyme is encoded by *ERG3* in *S. cerevisiae*. The desaturase is a glycoprotein that introduces C5(6) double bond in episterol. Fungal cells can survive in absence of this enzyme but show severe growth defects to different environmental stressors (Bhattacharya *et al.*, 2018).

Finally, the trienol is further processed by two enzymes, C-22 sterol desaturase and C24(28) sterol reductase to produce ergosterol. The two enzymes are encoded by *ERG5* and *ERG4* respectively in *S. cerevisiae*. The desaturase enzyme is a cytochrome p450 enzyme that catalyzes the formation of C22(23) double bond in the side chain during ergosterol biosynthesis. The reductase catalyzes the final step in the ergosterol biosynthesis. The fungal cells can survive in absence of these enzymes.

Subcellular Localization of Ergosterol Enzymes

Though ergosterol biosynthesis occurs in ER, the biosynthesis enzymes are localized in several cellular compartments that include ER, cytoplasm, glycosomes, lipid particles and mitochondria (Jorda and Puig, 2020). For example, ergosterol enzymes Erg1p, Erg7p, Erg27p and Erg6p are localized in both lipid particles and ER (Kristan and Rizner, 2012; Mullner *et al.*, 2004). The subcellular localization of the ergosterol enzymes determine their functions. For example, Erg1p is localized in both ER and lipid particles, however, the enzyme is only active when localized in ER (Leber *et al.*, 1998). When ergosterol levels are low due to iron

deficiency, Erg1p is exclusively localized in ER, where it is activated (Shakoury-Elizeh *et al.*, 2010). Erg7p and Erg27p are also located in two subcellular regions, ER and lipid particles. Erg27p binds with Erg7p in lipid particles and is rapidly migrated to ER when mitochondrial respiration is blocked (Cirigliano *et al.*, 2019). Erg11p and Erg24p are localized in ER. Erg28p tethers the C-4 demethylation enzyme complex of Erg25p-Erg26p-Erg27p to ER and also acts as a bridge to Erg6p enzyme for synthesis of ergosterol (Mo *et al.*, 2004). Erg6p is localized in both lipid particles, ER, cytoplasm and mitochondria. The ergosterol enzymes in the later part of the pathway, Erg2p. Erg3p, Erg4p and Erg5p are all localized in the ER (Jorda and Puig, 2020).

Regulation of Ergosterol Synthesis

Appropriate ergosterol content is required for the maintenance of lipid bi-layer and also for adequate environmental stress response (Jorda and Puig, 2020). Hence, ergosterol biosynthesis is tightly regulated. The different ways by which fungal cells regulate ergosterol synthesis are described below:

Post-Translational Feedback Regulation

As described above, the ergosterol biosynthesis pathway comprises of three rate-limiting steps. The first rate-limiting step is the reduction of HMG-CoA to mevalonate by HMG-CoA reductase enzymes, Hmg1p and Hmg2p. Regulation of this step is extremely important since this step is not only essential for the synthesis of ergosterol but also critical for mevalonate derived metabolites (Jorda and Puig, 2020; Bhattacharya *et al.*, 2018). In *S. cerevisiae*, increased oxysterol triggers degradation of Hmg2 enzyme through ubiquitination. The process is mediated by E3 ligase Hrd1p and E2 ubiquitin conjugating enzyme Ubc7p. Hmg2p is ER localized protein and upon initiation of ubiquitination, are released from ER to cytoplasm, where they are degraded by proteosomes (Hampton *et al.*, 1996; Hampton and Bhakta, 1997).

The second rate-limiting step in the pathway is the step producing lanosterol. The step is catalyzed by squalene epoxidase enzyme encoded by *ERG1* in *S. cerevisiae*. This enzyme is also degraded in presence of excess lanosterol via ubiquitin ligase Doa10p. Regulation of this step is crucial to prevent the accumulation of toxic sterol intermediates (Jorda and Puig, 2020; Bhattacharya *et al.*, 2018; Foresti *et al.*, 2013).

Transcriptional Regulation

- *Regulation by sterols*: Sterol availability regulates the transcription of genes encoding the ergosterol biosynthesis enzymes. Majority of the enzymes in the pathway are controlled by transcription factors that bind to 7-base pair DNA motifs known as sterol regulatory element (SRE), located in the promoter regions of their respective genes. In mammals, these transcription factors are called sterol regulatory element binding proteins (SREBP). SREBP is cleaved in the ER by SREBP cleavage activating protein (SCAP), which is activated when sterol is depleted. Most fungi contain homologs for SREBP and SCAP except *Saccharomyces sp.*, and *Candida sp*. They instead have Zn_2–Cys_6 binuclear cluster family of transcription factor called Upc2p. Upc2p has a paralog Ecm22p that arose due to whole genome duplication in *S. cerevisiae* (Zavrel *et al.*, 2013; Bhattacharya *et al.*, 2020b; Hoot *et al.*, 2010). N- terminal domain of Upc2p is the DNA binding domain that binds to the SRE regions in the promoter regions of ergosterol biosynthesis genes (**Fig. 2**). The c-terminal end of Upc2p binds to ergosterol and acts as an ergosterol sensor. In presence of ergosterol the transcription factor is switched off and retained in the cytosol. During ergosterol depletion, Upc2p undergoes structural changes exposing its nuclear localization signal (NLS). This causes the transport of Upc2p into nucleus, where it binds to the SRE regions of the promoters of ergosterol synthesis genes, resulting in their transcriptional activation. The c-terminus of Upc2p also contains the activator domain. During ergosterol dissociation, Upc2p undergoes confirmational changes to expose the activator domain that can recruit downstream transcriptional Upc2p co-activators. A previous study showed that SAGA co-activator complex is recruited to the promoter of ergosterol synthesis genes to cause transcriptional activation (**Fig. 2**) (Dewhurst-Maridor *et al.*, 2017). This complex may act as a co-activator that Upc2p recruits to enhance the expression of ergosterol biosynthesis genes during sterol depletion.

- *Regulation by oxygen:* Ergosterol biosynthesis requires heme and oxygen as cofactors. Hap1p is a heme and oxygen sensing transcription factor that regulate several ergosterol biosynthesis genes in *S. cerevisiae*. Under normal aerobic conditions (normoxia), Upc2p transcription factor requires the action of heme- bound Hap1p for basal level production of ergosterol. Heme bound Hap1p not only activates ergosterol biosynthesis genes but also activates DNA binding protein Rox1p. Rox1p along with Mot3p transcription factor serve as repressors for hypoxic genes, which are genes activated under low oxygen levels. These hypoxia-regulated genes allow proper utilization of oxygen under limited oxygen levels. A drop in oxygen levels causes decrease in heme and ergosterol levels that triggers the signaling pathways to induce the expression of ergosterol biosynthesis genes. Upc2p and Ecm22p are activated under hypoxic conditions causing increased expression of the genes in the ergosterol synthesis pathways. Besides, Upc2p is also responsible for sterol transport. Under hypoxic condition, upregulation of Upc2p leads to upregulation of sterol transporters Aus1p and Pdr11p. This results in uptake of sterols from the environment. This is true in fungi *S. cerevisiae* and *C. glabrata*, however, in *C. albicans* no sterol uptake was observed under hypoxic conditions (Zavrel *et al.*, 2013).

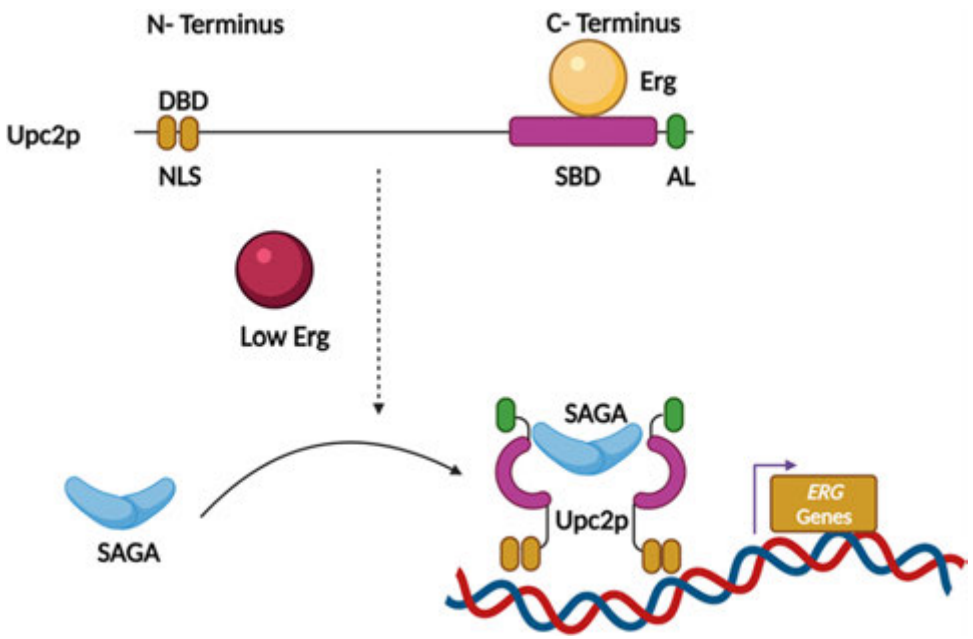

Fig. 2 Regulation of Ergosterol Synthesis under sterol-limiting condition: Upc2p contains a DNA-binding domain (DBD) with a nuclear localization signal (NLS) at its N-terminus. The C-terminus region contains sterol binding domain (SBD) to which ergosterol (Erg) binds and an activation loop (AL). Under sterol limiting condition, ergosterol is released from SBD, and SAGA complex is activated that binds to Upc2p resulting in confirmational changes in Upc2p exposing the DBD of Upc2p. This causes transcription of ergosterol biosynthesis genes (*ERG* genes). The image was drawn with the help of BiorRender.com.

- *Regulation by osmotic stress:* High osmolarity glycerol (HOG) pathway regulates hyperosmotic stress response in yeast (Jorda and Puig, 2020). Under osmotic stress such as in presence of high salt, Hog1p induces the transcription of Mot3p that negatively regulates ergosterol biosynthesis genes. This results in rapid decrease in cellular ergosterol levels in response to osmotic stress that is necessary for stress adaptation. In presence of extracellular ergosterol, the cells become highly sensitive to osmotic stress while on the other hand when drugs inhibit ergosterol biosynthesis, the cells become more tolerant to osmotic stress (Jorda and Puig, 2020).
- *Regulation by iron availability:* Four steps during the ergosterol biosynthesis requires the availability of iron. These include the steps catalyzed by Erg11p, Erg5p, Erg25p and Erg3p. Iron deficiency causes decrease in ergosterol and zymosterol levels resulting in accumulation of squalene and lanosterol (Shakoury-Elizeh *et al.*, 2010). Squalene and lanosterol are substrates of Erg1p and Erg11p respectively. Erg11p activity decreases in response to scarce iron due to decrease in heme levels resulting in accumulation of lanosterol. This inhibits the function of Erg1p causing accumulation of squalene (Shakoury-Elizeh *et al.*, 2010). Transcriptomics in iron deprived conditions showed altered expression of several ergosterol biosynthesis genes (Puig *et al.*, 2005).

Different factors that regulate ergosterol synthesis are summarized in **Fig. 3**.

Inhibitors of Ergosterol Synthesis and Ergosterol Target

Due to uniqueness of ergosterol in fungi, several enzymes in the biosynthesis pathway are targets of many antifungals. These antifungals inhibit ergosterol biosynthesis and can produce toxic sterol intermediates. Different inhibitors of ergosterol synthesis are described below:

- *Azoles*: Azoles are the most common antifungal class that inhibits 14α demethylase enzyme (Erg11p) in the ergosterol biosynthesis pathway (**Fig. 4**). The enzyme is the third rate-limiting step in the ergosterol biosynthesis pathway that converts lanosterol to 4,4-dimethylcholesta 8,14,24 trienol. The azoles inhibit the Erg11p causing other ergosterol biosynthesis enzymes to process lanosterol. The enzyme Delta(24)-sterol C methyltransferase (Erg6p) converts lanosterol to sterol intermediate eburicol. Eburciol is then converted to 14α methyl fecosterol with the help of enzyme complex encoded by genes *ERG25*, *ERG26*, and *ERG27*. Next, C5-sterol desaturase enzyme (Erg3p) converts 14α methyl fecosterol to toxic sterol intermediate, 14α methylergosta 8, 24(28) dienol. The accumulation of toxic intermediate causes inhibition of fungal growth (**Fig. 4**) (Bhattacharya *et al.*, 2018; Bhattacharya *et al.*, 2020b). Besides toxic sterol production, azoles lower the ergosterol content of the cell thereby preventing the proper functioning of the cell membrane. Azoles also cause elevated mitochondrial reactive oxygen species (mROS) that can damage mitochondria eventually leading to mitophagy or autophagy (Delattin *et al.*, 2014).

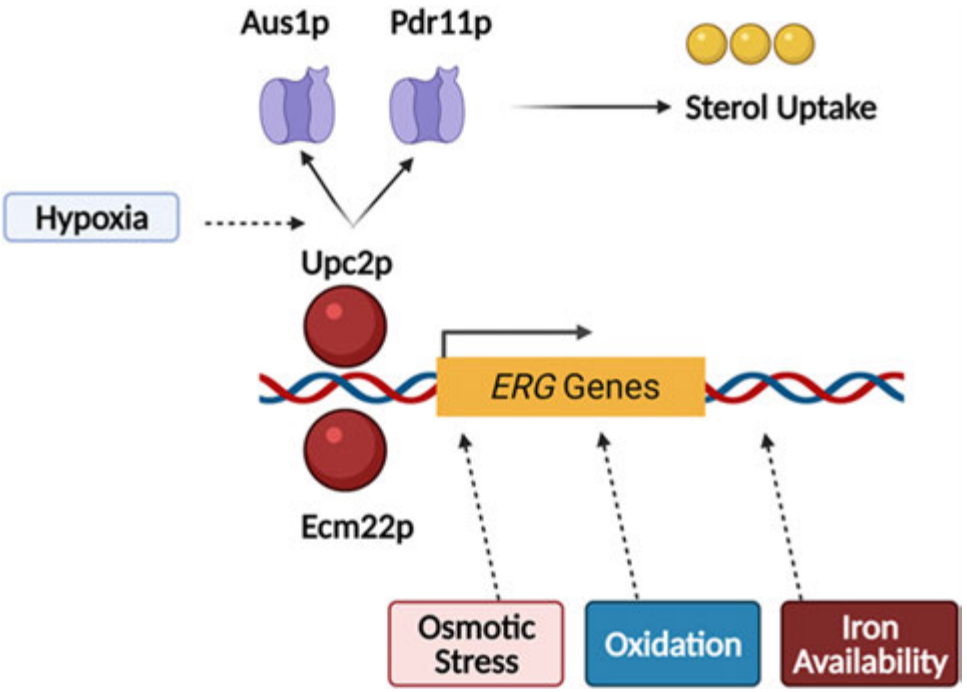

Fig. 3 Regulation of Ergosterol synthesis by different environmental factors. The image was drawn with the help of BioRender.com.

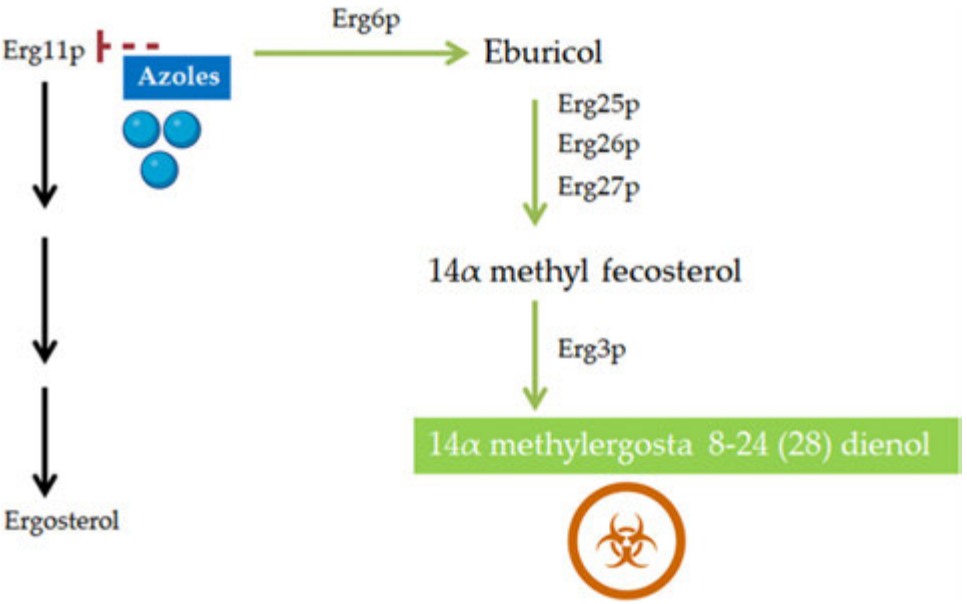

Fig. 4 Mode of Action of Azoles: Azoles inhibit Erg11p in the ergosterol biosynthesis pathway causing production of fungistatic toxic sterol. The image was drawn with BioRender.com.

Azoles are broadly classified into two subclasses: (1) triazoles and (2) imidazoles. Triazoles contain three nitrogen group and includes azoles such as fluconazole, itraconazole, voriconazole, isavuconazole, and posaconazole (Bhattacharya *et al.*, 2020b). Fluconazole is the most common antifungal used in treating fungal infections. Imidazoles are azole subclass that contain two nitrogen rings and include azoles such as ketoconazole, clotrimazole, and miconazole (Bhattacharya *et al.*, 2020b).

Azoles are fungistatic drugs i.e., they can only inhibit fungal growth without killing, causing fungal cells to evolve resistance. Over several decades, extensive studies in different pathogenic fungi have been done to identify the different molecular mechanisms behind azole resistance. Most common azole resistance mechanism observed in *Candida sp.*, *Aspergillus* sp.,

Cryptococcus sp., and *S. cerevisiae* is overexpression of genes encoding membrane transporters (Bhattacharya *et al*., 2020b, 2019, 2016; Chang *et al*., 2018; Esquivel *et al*., 2020; Bhattacharya and Fries, 2018). These membrane transporters are either ATP-dependent (ABC-transporters) or proton gradient dependent (MFS-transporters). These transporters export azoles from the cells thereby preventing cellular damage. These increased expression of both classes of efflux pumps are attributed to gain of function (GOF) mutations in their respective transcription factors, Tac1p and Mrr1p in *Candida sp*. (Bhattacharya *et al*., 2020b; Whaley *et al*., 2016; Morschhauser *et al*., 2007; Kalkandelen *et al*., 2015). These are zinc cluster (Zn_2-Cys_6) transcription factors in which several GOFs are associated with increased azole resistance. For example, GOF mutations in *C. albicans TAC1* that include T225A, V736A, N972D, N977D, G980E, and G980W were observed in fluconazole resistant clinical isolates (Bhattacharya *et al*., 2020b; Coste *et al*., 2006). GOF mutations in *C. albicans MRR1* that include P683S and P683H were also reported in fluconazole resistant clinical isolates (Bhattacharya *et al*., 2020b; Kalkandelen *et al*., 2015; Dunkel *et al*., 2008). Additionally, chromosomal alterations are also associated with azole resistance. For example, chromosomal alterations due to segmental aneuploidy causing two copies of the left arm of chromosome 5 was observed in azole resistant *C. albicans* clinical isolates. The left arm of chromosome 5 contains azole drug target encoding gene *ERG11* and transcription factor encoding gene *TAC1* (Selmecki *et al*., 2006). Chromosomal alterations were also observed in fluconazole resistant *C. neoformans* (Stone *et al*., 2019). Besides, increased copy number of genes *CDR1 (*encoding ABC-transporter Cdr1p) and *ERG11* were observed in azole resistant generationally aged old cells of *C. auris* (Bhattacharya *et al*., 2019, 2020a).

Azole resistance is also associated with altered ergosterol biosynthesis. One of the important resistance mechanisms is the over expression of drug target encoding gene *ERG11* (Bhattacharya *et al*., 2020b, 2016). As described above, Erg11p catalyzes an important rate-limiting step in the ergosterol biosynthesis pathway. Besides overexpression, point mutations in *ERG11* were also reported in azole resistant clinical isolates of *Candida sp*. For example, *ERG11* point mutations that include A61V, A114S, Y132F, Y132H, K143Q, K143R, Y257H, S405F, G448E, F449S, G464S, R467K, and I471T were reported in azole resistant *C. albicans* clinical isolates (Xiang *et al*., 2013). Besides *ERG11* point mutations, mutations in *ERG3* are also associated with azole resistance. *ERG3* encodes C5 sterol desaturase as described above during ergosterol biosynthesis. This enzyme is also responsible for producing toxic sterol intermediate in presence of azoles. Mutations causing disruption of Erg3p function in *C. albicans* and point mutation, Q139A in *C. glabrata* are associated with azole resistance (Bhattacharya *et al*., 2020b; Sanglard *et al*., 2003; Yoo *et al*., 2010). Mutations in *ERG6* also contributes to azole resistance. *ERG6* encodes Δ24 sterol C-methyl transferase, which is responsible for producing toxic sterol intermediate in presence of azoles. Disruption of the transferase activity causes azole resistance, increased membrane permeability and inefficient efflux pump activities (Bhattacharya *et al*., 2020b; Anderson *et al*., 2003; Akins, 2005; Xu *et al*., 2007; Kodedova and Sychrova, 2015).

The GOF mutations in Upc2p, the transcription factor regulating majority of the ergosterol biosynthesis genes are also associated with azole resistance. These mutations include G648D, G648S, A643T, A643V, Y642F, G304R, A646V, and W478C were reported in azole resistant *C. albicans* clinical isolates (Flowers *et al*., 2012). Majority of these mutations trigger overexpression of *ERG11*.

- *Allylamines*: These are the class of antifungals that target squalene epoxidase (Erg1p) in the ergosterol biosynthesis pathway. Erg1p catalyzes a rate limiting of the synthesis pathway step as described above. The most common antifungals of this class are terbinafine, flunarizine and naftifine of which terbinafine is commonly used in treating dermatophyte infections (Sanglard *et al*., 2009). Terbinafine resistance has also been observed in dermatophytes, which resulted from point mutations in the gene encoding the squalene epoxidase enzyme. For example, in clinical isolates of dermatophyte, *Trichophyton mentagrophytes*, point mutations leading to amino-acid substitutions at positions Leu^{393}, Phe^{397}, Phe^{415}, and His^{440} in squalene epoxidase enzyme showed increased terbinafine resistance (Yamada *et al*., 2017).
- *Morpholines*: These are the class of drugs that inhibit the ergosterol biosynthesis enzymes C-14 sterol reductase (Erg24p) and C-8 sterol isomerase (Erg2p). Morpholines include fenpropimorph, tridemorph, and morpholine, and are commonly used in agriculture. They exhibit toxicity to humans and are used in treating nail dermatophyte infections (Bhattacharya *et al*., 2020b; Sanglard *et al*., 2009; Shirwaikar *et al*., 2008).
- *Polyenes:* This class of drugs include amphotericin B and nystatin. They bind to ergosterol in the membrane and form pores through which monovalent ions leak causing cell death (Efimova *et al*., 2014). Amphotericin B is commonly used antifungal to treat systemic infections. Amphotericin B has more affinity towards Ergosterol than cholesterol (Bhattacharya *et al*., 2020b). However, the drug has toxic affects affecting kidney. Disruption of ergosterol synthesis are associated with polyene resistance in *Candida sp*. For example, disruption of *ERG3* and *ERG6* lowered ergosterol levels and increased amphotericin B resistance in *Candida sp*. (Vandeputte *et al*., 2012).

Conclusion

Ergosterol is a sterol unique to fungi and is important for fungal growth, proliferation, stress adaptations, and cellular detoxification. Thus, studying ergosterol biosynthesis is of great importance to understand fungal biology. Additionally, ergosterol is used as precursor for synthesizing steroidal drugs (Hu *et al*., 2017). Yeast fermentation is the main source for production of ergosterol. Many molecular biology-based strategies have been used to increase the yield of ergosterol. These strategies include optimization of fermentation conditions, genetic manipulation of the ergosterol biosynthesis pathway, and screening for mutants

that yield high levels of ergosterol (Nahlik *et al.*, 2017). Besides ergosterol, zymosterol and lanosterol, the two important intermediates of ergosterol biosynthesis are used as emulsifiers and precursors of cholesterol lowering substances (Wriessnegger and Pichler, 2013). Artificial production of zymosterol and lanosterol are also carried out using genetic manipulations (Paramasivan and Mutturi, 2017). Metabolic manipulations of ergosterol biosynthesis pathway also aided in synthesizing terpenoids and steroids. For example, site directed mutagenesis of Upc2p (G888A), Erg20p (K197G), and Hmg2p (K6R) are used to produce terpenes, mono-terpenes, and mono,di-sequesterpenes (Wriessnegger and Pichler, 2013). Additionally, since ergosterol is unique to fungi, the each enzymatic step in the pathway is potential target for novel drug designing (Rodrigues, 2018). Thus, studying fungal ergosterol biosynthesis is very important for better understanding fungal biology, designing novel drug targets, and generating economically important steroids and terpenoid molecules.

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Fungal Volatile Organic Compounds

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Introduction

The term *volatile organic compound (VOCs)* encompasses all carbon-based, low molecular weight compounds that easily enter the gaseous state due to their low boiling point and high vapor pressure. When found in liquid form, they have a propensity to evaporate easily at ambient temperatures and pressures. When found in a solid phase, they can sublimate into the gas phase. Some of the best-known VOCs include synthetics that result from industrial processes such as gasoline combustion, laboratory solvents (e.g., chloroform, toluene), or compounds released from household products such as epoxies and paints. Examples of specific VOCs commonly found in modern buildings include benzene, ethylene glycol, formaldehyde, methylene chloride, tetrachloroethylene, and xylene. At elevated concentrations these synthetic VOCs are toxic.

VOCs in nature are found as mixtures of different compounds and are generated by all living things. The best-known biogenic VOCs are produced by plants and are often perceived by their distinctive odors. Well known plant VOCs include the isoprenoids limonene, pinene and menthol that smell, respectively, citrusy, resinous and minty. Most fungal VOCs also have characteristic odors, and people usually recognize the smell of yeast fermentations or moldy basements even if they know nothing about the organisms producing the odor or the fact that odors are caused by volatile molecules.

The compound term *fungal VOCs* describes a large group of the easily volatilized carbon compounds that have been associated with members of the fungal kingdom. In nature, fungal VOCs are found as heterogeneous blends composed of varying amounts and concentrations of alcohols, aldehydes, esters, ketones, modified amino acids, furans, terpenes, as well as sulfur compounds and their derivatives. The single most famous fungal VOC, ethanol, is produced by yeasts during alcoholic fermentation. The single most common volatile from filamentous fungi, 1-octen-3-ol, is produced by the breakdown of linoleic acid.

The fungal kingdom is distinguished by its distinctive osmotrophic form of nutrition whereby extracellular enzymes are secreted to the environment, followed by the absorption of the externally "digested" nutritional products. Fungi are absorptive heterotrophs, or put more simply, fungi have their stomachs on the outside. Consequently, many of the compounds detected from analyses of fungal VOC are breakdown products of the nutrient source on which the fungus is growing, made by the action of nonspecific extracellular fungal enzymes. This means that detection of a particular volatile compound in a profile sampled from the headspaces of a fungal source does not necessarily imply that the compound is endogenously produced within the fungus. Substrates are an extremely important determinant of the total volatile profile that is detected from a given species. Moreover, many endogenously produced fungal VOCs are by-products of primary metabolism generated during amino acid or fatty acid catabolism. For example, the respective transamination of valine, isoleucine, and leucine leads to the production of 2–methylpropanal, 2–methylbutanal, and 3–methylbutanal, all common members of fungal VOC profiles.

VOC profiles detected from fungi are extremely variable, changing not only with species, but with growth parameters, age of the fungal colony, and the method used for analysis and identification. The enormous difference associated with these variables makes it difficult to compare published research studies. Another important caveat about fungal VOCs is that most of these low molecular weight compounds are not unique to fungi but also are produced by different life forms. An online searchable database that encompasses microbial VOCs, including those from fungi is available from Lemfack and colleagues (see "Relevant Website section").

Volatilome and Volatome

Recently, the jargon words *volatilome* or *volatome* have entered the scientific literature to describe the entire spectrum of volatiles detected during a given analysis from a particular source, as in "fungal volatilome" or "fungal volatome". These synonymous terms usually are used without an accompanying stipulated definition.

What do these terms mean? Are authors correct in thinking that everyone understands their meaning in the same way?

Some biological etymology is pertinent here. The suffix "-ome" is used in two different but overlapping ways by different communities of biologists. Parsing these different usages reveals something of the complexity of studying volatile profiles. Since early in the 20th century, the word *biome* has been used by ecologists to encompass an entire complex community characterized by distinctive animal, microbial and plant species that are maintained under the climatic conditions of a given region and specific climate (e.g., aquatic, forest, grassland, desert, tundra, etc.). The second use of "-ome" finds its roots in genetics. In the early twentieth century, genomes were defined as the complete set of genetic information in an organism. In the late 20th century, the explosion of nucleic acid sequencing methods and other analytical techniques led to a proliferation of "-omics" word such as genomics, transcriptomics, and so on. Many readers, especially molecular biologists, assume that all jargon words containing the suffix "-ome" are an extension of the "-omes" used in molecular geneticists, i.e., *genome* (the entire set of DNA in an organism), *transcriptome* (usually used to refer to the RNA molecules expressed at a certain time under certain environmental conditions); the *metabolome* (the products of a biological reaction, including intermediates), and *exometabalome* (the extracellular metabolites).

However, while the *volatilome/volatome* is the complete collection of volatile metabolites, it is not necessarily the same thing as an exometabalome. The total sum of the volatiles from a fungal sample may include the products of a resident fungal microbiome, incidental degradation products of the substrate, artefacts of the assay process, and other small molecules that are not directly associated with the genome of the producing fungus. Therefore, we suggest that it is best to take a "big umbrella" approach to generating a stipulated definition of "volatome /volatilome". Our stipulated definition encompasses the entire sample of volatile obtained from extracting a fungal source. This definitional frame encapsulates the endogenous products of the fungal metabolome (the "-ome" of genomics), the products of the resident bacterial and yeast microbiomes (the microbiome of ecologists), as well as the substrate degradation products released by fungal exogenous enzymes (the nonspecific products of fungal exoenzymes). Additionally, it can include the unrecognized artefacts of extraction and analytical techniques that may be present. Put simply, a fungal volatome/volatilome is all the VOCs in a particular sample of fungal origin, not just those encoded in some way by the fungal genome.

The word *microbiome* reflects a similar fusion of the two "-ome" meanings. Microbiome research is conducted by isolating nucleic acids from a sample, not by isolating the organisms. Nevertheless, as used in the scientific literature, *microbiome* simultaneously refers to all the microorganisms from a certain environment such as a soil sample or a part of the human body, as well as the combined genetic material of the microorganisms in a particular environment.

Some common VOCs such as 1-octen-3-ol, 3-octanone, and 2-pentylfuran are primarily synthesized by fungal genera. Other compounds such as dimethyl disulfide, acetic acid, 2-nonanone, dimethyl trisulfide, 2-undecanone, isovaleric acid, 2-tridecanone, propanoic acid, tend to be produced by bacteria and when detected in a fungal volatome are likely due to the presence of a resident bacterial microbiome growing on or in the fungus.

Finally, it is important to remember that all published data on fungal volatilomes are "snapshots." They do not capture the dynamic nature of volatile emissions nor do they tell us much about changing populations in communities. Moreover, such volatilomes do not differentiate between the volatiles that may have little or nothing to do with the fungal metabolome, those produced by resident complex bacterial microbiomes and those that are not directly related to the genomes of either the bacteria or the fungi. The sections below highlight some important findings related to fungal volatomes/volatilome – broadly defined – and some individual fungal volatiles.

Chemical Analysis

VOCs are difficult to study. By definition, these small molecules easily enter the gas phase where they rapidly dissipate. As they disperse, they become less concentrated. The dilution effect increases with distance, and other factors associated with the experimental or natural environment. Not only are most VOCs present in only low concentrations, they are found as components of complex mixtures which necessitates separation, and further complicates their identification.

Early research on fungal VOCs depended on steam distillation coupled with liquid-liquid extraction, followed by concentration of individual compounds and chemical identification of the isolated compounds. Technical developments in gas chromatography and mass spectroscopy (GC–MS) have greatly expanded our knowledge of volatile chemistry and most VOCs are now studied by headspace analysis. Because GC–MS links the separation, identification and quantification steps, individual VOCs in complex mixtures can be identified more rapidly. Individual volatiles are identified by comparisons of mass spectra with authentic standards, chemical library spectra, and/or chromatographic retention indices. Examples of specialized versions of this general approach include proton-transfer-reaction mass spectrometry (PTR-MS); selected-ion flow-tube mass spectrometry (SIFT-MS); and time of flight mass spectrometry (GC-TOFMS). Perhaps the most broadly used method is solid phase microextraction (SPME) in which VOCs are concentrated on a portable fiber and later delivered to the input of a detector. Desorption occurs in the GC injector itself. SPME is convenient and combines extraction, concentration, and introduction.

The experimental objectives of the research in question often determine the methods used. The initial step for gas chromatographic analysis is the purging and trapping of the compounds in the volatile headspace. For isolation of individual compounds, absorbance materials with the desired binding qualities are used. The choice of solvent is another important aspect of volatile analysis because the solvent itself can influence the VOC profile. It is important to remember that the method used to analyze VOC profiles can alter the outcomes. Classical studies using exhaustive solvent extraction procedures and heat often yielded VOCs that are solvent degradation products produced during the extraction steps. With more contemporary methods, fewer artifacts are found but nevertheless they still can be introduced by solid sorbents used during headspace analysis and other steps of the analytical process.

In summary, the methods used for isolation and identification affect the results found. Sample preparation and methods used for separating, identifying and quantifying the VOCs have an impact on the kinds and amounts of VOCs detected.

Fungal VOCs as Odorants

In order for an odorant to be detected by the human olfactory system, the compound must be volatile. Macrofungi often emit distinctive odors and many guides for mushroom hunting mention characteristic odors. In particular, stinkhorns are infamous not only for their notable penile shape but also for their foul-smelling, fly-attracting fruiting bodies. Stinkhorns are said to smell like a

powerful combination of decaying meat and dog feces. Dominant VOCs include dimethyl sulfide and trimethyl sulfide, phenol, p-cresol and indole and skatole. Other mushrooms with unpleasant odors include *Gymnopus perforans*, which smells like rotten cabbage and *Cortinarius camphoratus*, which smells like rotten potatoes.

On the other hand, numerous macrofungi are known for their pleasant odors. *Agaricus subrufescens*, also called the royal sun *Agaricus*, has the fragrance of almonds; *Russula laurocerasi* smells like slightly "off" maraschino cherries; *Clitocybe odora* has an anise-like aroma; *Lactarius hibbardae* emits a distinct coconut smell; and the chanterelle *Cantharellus cibarius* smells like apricots. *Lactarius camphoratus* resembles fenugreek, which is a key ingredient in curries; *Inocybe lacera* is said to resemble the odor of human semen. *Cortinarius traganus* has a distinct odor resembling dried apples or pears and *Cortinarius violaceus* smells like cedar wood.

There are often significant differences in the type and concentration of VOCs detected from the same species. For fungi isolated from the natural world, the main variances detected by different studies are due to nutritional, regional and environmental differences in the place the fungi grew, as well as the freshness of the samples. The age of the sample and the way in which samples were stored also can have a big impact on VOCs detected.

Foods and Flavors

Volatile organic compounds play a large role in gastronomy. For example, sulfur-containing volatiles emitted by plants such as onions and garlic in the plant genus *Allium* are important for their aromatic and flavor profiles. These thiols are also utilized in many traditional medicines. Similar organosulfur compounds provide the source of aromas associated with popular edible fungi such as shiitake mushrooms and truffles.

The reputation of truffles as gourmet foods rests upon their singular aroma profiles. The distinctive odors are caused by a blend of volatile molecules, of which the most distinctive contain sulfur atoms. VOCs identified from different truffle species include 3-methyl-4,5-dihydrothiophene; bis(methylthio) methane; dimethyl disulfide and dimethyl sulfide. In addition, the aldehyde 2-octenal, and the alcohol 1-octen-3-ol have been found in both white and black truffles. Additional aromatic compounds from truffles include 2,3-butanedione, ethyl butyrate, 3-methyl-1-butanol and 3-ethyl-5-methylphenol. Some of the words that have been used to describe truffle volatiles compare their odor to a mixture of garlic, horseradish, and cabbage. Others descriptive terms include meaty, fruity, green apple, anise, rose, buttery, cotton candy, malty, wet forest, and cooked potatoes. The aroma also is said to have a slight taste of radish and a hint of hazelnut; The odors can range from mild to intense, and can vary from cheese-like, earthy, garlicky, pungent, to vanilla-like, creamy, and leathery.

Recent findings have shown that it is likely that the sulfur-containing volatiles of the truffle fruit body are made by the biotransformation of non-volatile precursors by the members of the truffle microbiome, i.e., the thiophene volatiles that give truffles their distinctive aroma are biosynthesized by microbiome bacteria that live in and on the truffle, but are not metabolic products of the truffle genome.

Many Asian food fermentations get their distinctive flavors and aromas from fungi. For example, tempeh, a popular vegan dish, is a sliceable loaf of soybeans or grain fermented with *Rhizopus oligospora*. The aroma of tempeh is described as nutty, mushroom-like and earthy. Dominant VOCs emitted by tempeh are ethanol, acetone, ethyl acetate, 2-butanone, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-methyl-1-butanol. When barley is the substrate, acetaldehyde and 2-methyl-propanal are also produced; when soybeans were the substrate, 2-pentanone, methyl acetate, 2-butanol and 3-methyl-3-buten-1-ol were detected. Dominant VOCs from fermented fish miso include 2-methylbutanal, 3-methylbutanal, methional, isoamyl acetate, dimethyl disulfide, dimethyl trisulfide, 2,3-butanedione, 3-methylethyl butanoate, 3-methyl-1-butanol, ethyl hexanoate, 1-octen-3-ol, heptanol, heptanal and 2-undecanone. There is a growing realization that many fermented foods in which a filamentous fungus like *Rhizopus* or *Aspergillus* is a dominant organism are actually complex fermentations involving many species of yeasts and bacteria. The individual species participating in the microbial communities shifts with time, nutrient source, salt concentration and the like. The volatilome detected in the fermentation head space consequently will vary as these parameters change.

Biotechnology

Synthesized liquid versions of natural volatiles are used in the food industry for flavor enhancement as well as for components in cosmetic and pharmaceutical products. The US Food and Drug Administration defines *natural flavors* as flavor compounds extracted from live cells. In many cases volatile compounds are derived using yeasts or filamentous fungi to produce increased quantities of compounds that are produced less abundantly in plants. Such compounds can be utilized in foods and fragrances to duplicate natural plant-derived metabolites. Many fungal VOCs are "bioidentical" to flavors derived from plants. For example, the coconut odor volatile 6-pentyl-α-pyrone, produced by some *Trichoderma* species can be used to substitute for the same compound isolated from coconuts. Utilization of yeast in fermentation aroma cultivation is yet another commonplace example of the importance of VOCs in the food and beverage industry.

Fungal VOCs also show promise as components of bio-renewable fuel ("mycodiesel"). Various compounds made by fungi include the monoterpene 1,8-cineole, an octane derivative that has potential use as a fuel additive. In addition to alkanes and certain long-chain hydrocarbons, many fungal species produce ethylene, ethane, propane and propylene as well as terpenes and

isoprenoids. It is believed that fungi are an excellent platform for exploiting biosynthetic routes to hydrocarbon biofuels and biofuel precursors.

VOCs for Indirect Detection of Fungal Growth

People often become aware of the presence of fungi by their odors, even when they are not visible. The human olfactory system is a sensitive finding system for fungi. Without any specialized equipment, fungal odors frequently can be detected before visible fungal growth. People recognize that food has spoiled because of "off" odors. Water damaged buildings also have distinctive musty odors. In agriculture, sampling of VOCs in storage bins and silos can detect the presence of mycotoxigenic fungi. Modern technologies have been developed as indirect bioassays to detect the presence of "hidden" fungal growth. Thus, VOCs can be used to uncover fungal colonization by potentially harmful species in a variety of different environments. Because VOC detection is noninvasive, it is useful in many different contexts that range from the pharmaceutical and cosmetics industries to military installations. For example, scientists have taken advantage of this capability and use it as a way to detect food spoilage in commercial facilities and various kinds of factories. Potentially harmful fungi are detected by the presence of VOCs produced by the microbes and their interaction with the food source. Such "off" odors in foodstuffs have been used to identify spoilage in bakery products, jam factories, and silos of stored grains. Compounds such as 3-octanone, 1-octanol, and e-methyl-1-butanol have been associated with *Aspergillus* contamination of stored grains. Inside water damaged buildings, VOC detection provides a nondestructive way to find hidden mold growth behind walls and paneling, underneath carpeting, in air conditioning ducts, under wall paper and so forth. VOCs regularly identified from molds growing on building materials include 3-octanone, 1-octen-3-ol, 3-methyl-1-butanol, and 3-methyl furan.

Some plant diseases can also be identified by means of volatile detection such as the powdery mildew fungus *Uncinula necator* and the common bunt pathogen, *Tilletia caries*, the latter of which produces a characteristic fishy odor associated with trimethylamine.

Aside from being indicators of food spoilage, mycotoxin contamination, and indoor fungal growth, another use for VOCs is to identify human diseases. In classical times, physicians used their sense of smell to diagnose certain illnesses. Medical research has shown that exhaled breath or other VOC samples can be utilized to diagnose a variety of human illnesses. For example, some human cancers can be diagnosed by means of identifying VOC profiles unique to tumor cells and breath tests are accepted clinical tools in the diagnosis of certain gastroenterological diseases. Fungi that live on and within humans also emit volatile compounds that can be indicators of disease, and a promising use for VOC detection is in medical mycology. For example, 2-pentylfuran has been found in the breath of patients at early stages of Aspergillus *fumigatus* infection. *A. fumigatus* is the causative agent of invasive aspergillosis; unfortunately, this compound is also present in some foods such as soy milk and peanuts, which may lead to false positives by this detection mechanism. The sesquiterpene farnesene also has been suggested as a biomarker for *Aspergillus fumigatus* infection. It is posited that detection of species-specific volatile sesquiterpene metabolites of fungal origin can be found in the breath of patients with invasive aspergillosis. The challenge will be to identify stable and unique VOC profiles that reflect disease states. Studies have shown that the profile of volatile production depends on the nutritional environment. *A. fumigatus* produces a multiplicity of different VOCs. To date, scientists have been unable to identify key diagnostic VOCs by breath analysis but it remains a very promising area of research (Li *et al.*, 2016).

Artificial olfaction sensory technology is a method of VOC detection in which artificial or electronic noses ("e-noses") are used in agricultural science, building science, and biomedical healthcare settings for this type of diagnostic approach. An electronic nose system typically contains a multisensor array with an information-processing unit, software with pattern-recognition algorithms, and reference-library databases. The sensor array is designed to respond to a wide assortment of chemical classes and to discriminate diverse mixtures of possible analytes. Identification and classification of individual mixtures of compounds is accomplished through recognition of the resultant unique aroma signature or "electronic fingerprint". Future research is expected to make VOC sensing a more common approach to VOC testing.

Chemical Ecology

Many fungal VOCs mediate interspecific biotic interactions. Such bioactive VOCs have been called "infochemicals". They influence growth, morphology, and population dynamics. Some serve specific ecological functions that include growth stimulation, or antibiosis, or as communication molecules for insects and other arthropods. Many fungal VOCs serve as signaling molecules for insects. They attract or repel arthropods, serve as aggregation agents, and/or are important in host location and attraction to food resources. Volatile compounds released from beneficial microbes present in the rhizosphere communicate with plants present in the ecosystem by increasing plant growth, influencing root morphology, inhibiting seed germination, and inducing systemic pathogen resistance. In addition to interacting with plants and arthropods, fungal VOCs also mediate interactions between various microbial populations (Hung *et al.*, 2015).

Plant VOCs are known to function to protect against herbivores, pathogens and competitors. They also are used to attract pollinators, dispense seeds, and attract mutualistic microbes. Fungal VOCs have not been the subject of as much research as plant VOCs, however, it is hypothesized that they play cognate roles in fungal life cycles. Like plants, fungi are not mobile. Therefore, it is theorized that members of both the plant and fungal kingdoms use volatiles to solve some of the ecological challenges that result from their immobility. These challenges include reproduction, dispersal, cooperation from mutualists, and protection from

competitors. Volatiles are useful in below-ground and above-ground signaling. In ecosystems containing a variety of microbes, the VOC profile is influenced by the various constituent members of the ecological community. Thus, the volatomes that arise during microbial growth influence the establishment and allocation of organisms within the ecological system. This arrangement of organisms is important in environments such as soil ecosystems where they play a role in competition, defense and recognition signaling. Volatile signaling takes place within an organism, between organisms, and across species of different kingdoms over narrow and wide ranges of communication.

Animals can detect a wide variety of volatile organic compounds. In particular, entomologists were among the first scientists to appreciate the power of volatile compound signaling and have developed a specialized vocabulary to describe molecules used in signaling. *Semiochemical* is the general term for signaling molecules. More specifically, *pheromone*s are defined as volatiles involved in mediating interactions between the same species while *allelochemicals* mediate interactions between different species. Depending on concentrations, some fungal VOCs act as semiochemicals that mediate antagonistic and beneficial interactions between fungi and other life forms. Examples of volatiles known to function as semiochemicals include hexanal,(E) – 2-hexanal, hexanoic acid, b-pinene, limonene, and 2-butyl-2-octenal which function as arthropod pheromones for uses such as signaling alarm and trial marking. Often the role of a volatile as a signaling molecule is context dependent. For example, mushroom alcohol (1-octen-3-ol) has been characterized as a semiochemical, a pheromone, a hormone and/or an allelochemical due to its varied roles in diverse interactions.

Blood-feeding insects have olfactory receptors present that serve as an integral part of the olfactory nervous system. These receptors bind to volatile compounds following their entry through the antennae. Many of the compounds that function as attractant and repellent semiochemicals are eight-carbon alcohol and ketone fungal volatiles. The same volatile compound can have diverse effects on different species. For example, VOCs released by decay fungi simultaneously attract bark beetles and are anti-attractant or "bypass" trophic signals for bark beetle predators. Interactions between insects and fungi include odor-mediated attraction and repulsion of insects to fungi and fungal substrates. Insect oviposition is also influenced by volatile compounds such as phenylacetaldehyde, 2-phenylethanol, dimethyl disulfide, citronellal, and norphytone. VOCs produced by fungi on pine weevil frass protect the insect's eggs and influence odor-based detection of the host. Specifically, *Debaryomyces hansenii* produced methyl salicylate as a major compound which strongly reduced pine weevil's attraction to the *Pinus sylvestris*. Artificial pheromones can be synthesized from known natural compounds and utilized in insect traps and potentially in the attraction of beneficial insects.

In nature, VOCs are found as composites containing multiple compounds at once, rather than a single, isolated volatile compound. For this reason, it is challenging to study the nature of volatile interactions because numerous variables within the experimental design may be difficult to control or compare.

VOCs as Antimicrobial Agents

Numerous fungal VOCs have antibacterial, antifungal, biostatic and/or fungistatic properties. *Mycofumigation* is the use of fungal VOCs to eradicate or inhibit other microbes, especially pathogenic species. The volatiles released by the cinnamon tree endophyte *Muscodor albus* can kill several different pathogens. Similarly, the VOCs emitted by different *Trichoderma* species emit volatiles that inhibit growth of a variety of plant pathogens including *Alternaria brassicicola*, *Fusarium oxysporum*, *Phytophthora* spp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and others.

In some instances, gas-phase molecules are involved in microbial quorum sensing. Quorum sensing is a type of cellular communication that bacteria use to modulate population responses to cell density. The volatile compound 3-hydroxyl palmitic acid methyl ester has been observed to function as a regulator of infection and virulence for the pathogenic bacteria *Ralstonia (Pseudomonas) solanacearum*, the causal agent of bacterial wilt in a variety of crops. Due to the limited opportunities of aqueous phase signaling on plant tissue surfaces and in soil environments, it is likely that additional examples of microbial volatile signaling are yet to be discovered.

Eight Carbon VOCs

Eight carbon VOCs are particularly characteristic of fungi (Combet *et al.*, 2006). Mushroom alcohol, also known as matsutake alcohol, is the eight carbon alcohol 1-octen-3-ol, first identified many decades ago from *Tricholoma matsutake*. It is the single most frequently reported fungal volatile compound from filamentous species and is produced by the enzymatic oxidation and cleavage of linoleic acid. Other related fatty acid degradation products include 3-octanone, 3-octanol, octanol, and 1-octen-3-one, which are responsible for generating a typical mushroom smell. The human olfactory system can detect 1-octen-3-ol at concentrations as low as 10 $\mu g/m^3$. The chirality of 1-octen-3-ol can influence its odor, as is also true with other VOCs. Its S configuration has grassier and moldier characteristics than the R form. The R enantiomer, sometimes called "Roctenol" is the most common form of the compound in nature and is used commercially as an insect attractant.

Mushroom alcohol has a myriad of physiological properties and a wide range of effects and interactions in various systems. It has been shown to participate in fungal spore induction, production, and inhibition; plant development regulation; plant defense gene induction, and reactive oxygen species production. It is a self-inhibitor of spore germination in *Lecanicillium fungicola*, *Aspergillus nidulans and Penicillium paneum* and can be used as a treatment for "dry bubble disease" caused by the mycoparasite *Lecanicillium fungicola* which is widespread on cultivated button mushrooms.

When plants are treated with 1-octen-3-ol they show heightened expression of defense genes and changes in ethylene and jasmonic acid signaling and enhanced resistance to the plant pathogen, *Botrytis*. In addition, volatile phase 1-octen-3-ol is effective in reducing the growth of *Pseudogymnoascus destructans*, the causative agent of white nose syndrome in bats.

When found in human sweat, where it is likely a product of the human skin mycobiome, 1-octen-3-ol serves as a mosquito attractant. In addition to attracting insects, 1-octen-3-ol has also been observed to repel insects or act in mediating other entomological behaviors. For example, 1-octen-3-ol disrupts aggregation hormone responses in beetles. In combination with geosmin, 1-octen-3-ol functions as a millipede allomone used in defense signaling. In addition to its signaling properties, at higher concentrations 1-octen-3-ol functions as a toxin and has potentially harmful health effects. This has been demonstrated as respiratory rate depression in rats and toxicity to human embryonic cells in vitro. Furthermore, 1-octen-3-ol is among the compounds that may contribute what is known as "sick building syndrome" a phenomenon where symptoms such as fatigue, eye and skin irritation and respiratory issues arise from spending time indoors, specifically in mold contaminated buildings. Both mycotoxins and fungal VOCs are hypothesized to be among the causative agents of sick building syndrome. This syndrome is hypothesized to be a consequence of chronic exposure to the molds, mildews and other microbes inhabiting aging and damp buildings. Furthermore, exposure of *Drosophila melanogaster* to 1-octen-3-ol caused Parkinson-like symptoms including loss of dopamine neurons and disruption of vesicular monoamine transporter (VMAT) dopamine uptake in a fly model system.

Summary

Fungal volatile organic compounds (VOCs) are fungal associated, carbon-based, low molecular weight compounds that easily enter the gaseous state due to their low boiling point and high vapor pressure. In particular, 1-octen-3-ol, also known as mushroom alcohol or matsutake alcohol, is the single most common VOC isolated from filamentous fungi. Fungal volatiles from nature are isolated as mixtures of different compounds that change dynamically and vary with substrate, temperature, and other parameters of growth. The terms volatome and volatilome are used to describe all the VOCs found in a given chemical analysis of fungal VOCs, and encompass the reality that macrofungi and microfungi possess their own characteristic microbiomes of yeasts and bacteria.

Fungi often are detected by their smell because most of their VOCs are detected by the human olfactory system. When direct sampling techniques are not available, odors can identify the presence of mold contaminated foods and feeds, as well as buildings with hidden mold growth. Testing for volatile biomarkers in clinical samples promises a rapid, inexpensive and non-invasive disease screening tool in medical mycology.

Many macrofungi such as truffles produce blends of VOCs that are considered desirable in epicurean cuisine. Microscopic fungi including molds and yeasts also have distinctive odors profiles and some species have been cultivated and valued for the aromas they contribute to fermented food products such as mold ripened cheese, miso, soy sauce, and so forth. Certain fungal VOCs are used to imitate natural plant-derived scents. Others are used as indirect monitors to detect the presence of hidden fungal growth. Human breath analysis of key volatiles may provide a noninvasive early diagnostic test for invasive aspergillosis and other lung disease caused by fungi. Many fungal VOCs play important ecological roles in communication where they are an integral part of interspecies symbiotic interactions. Much is still to be discovered about the physiological function of VOCs and their involvement in intracellular and intercellular signaling. Moreover, many fungal VOCs remain uncharacterized, so there is considerable potential for new discoveries. From reproduction and defense within microbial communities – to disease detection, flavor enhancement and perhaps aromatherapy for humans – fungal VOCs are a near ubiquitous aspect of life with a wide array of possible future biotechnological functions.

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Outline of Ascomycota

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Introduction

Fungi are one of the most diverse and ubiquitous living groups, thus, its diversity, taxonomy, classification and applications are popular topics among interested groups. Nevertheless, 'what are the real fungi' is one of the debatable questions among mycological community. Currently, Tedersoo *et al.* (2018) and Wijayawardene *et al.* (2020a) accepted 19 phyla viz., *Aphelidiomycota, Ascomycota, Basidiobolomycota, Basidiomycota, Blastocladiomycota, Calcarisporiellomycota, Caulochytriomycota, Chytridiomycota, Entomophthoromycota, Entorrhizomycota, Glomeromycota, Kickxellomycota, Monoblepharomycota, Mortierellomycota, Mucoromycota, Neocallimastigomycota, Olpidiomycota, Rozellomycota* and *Zoopagomycota* in kingdom *Fungi* while excluding *Oomycota*, *Dictyosteliomycetes* and *Myxomycetes*.

In this chapter, we discuss the development of classification of Phylum *Ascomycota*, its current status and future perspectives.

Introduction to Ascomycota

Phylum *Ascomycota* is one of the largest phyla in Kingdom *Fungi*, which comprises ca. 9000 genera (Wijayawardene *et al.*, 2020a,b). Phylum *Ascomycota* has been grouped in Sub Kingdom *Dikarya* with Phylum *Basidiomycota* (Hibbett *et al.* 2007; Tedersoo *et al.*, 2018). Kendrick (2000) listed four main characteristics of dikaryomycotan fungi; 1. The cell wall which comprises chitin; 2. Cell wall with cross walls, i.e., septa, except in yeasts; 3. Assimilative hyphae to fuse with one another (i.e., anastomosis) to exchange nuclei; and 4. Show unique nuclear phenomenon called dikaryon. The main distinguishable feature between Ascomycota and Basidiomycota is the fructification of sexual morphs.

Ascus (a sac like or cylindrical structure) which usually contains eight haploid spores (ascospores) at maturity, is the characteristic feature of sexual *Ascomycota*. However, Kirk *et al.* (2008) regarded that 'the presence of lamellate hyphal walls with a thin electron-dense outer layer and a relatively thick electron-transparent inner layer also appears diagnostic'. This feature is regarded as a feature to recognize asexual ascomycetous taxa even in the absence of asci. Mainly, there are two morphological groups of asexual fungi based on fructification or conidiomata i.e. coelomycetes (taxa which produce conidia within a cavity lined by either fungal tissue, host tissue, or a combination of both *fide* Sutton, 1980) and hyphomycetes (which produce conidia from aggregated or separated modified hyphae or conidiogenous cells) borne on the exterior face of substrates and not enclosed by additional fungal or host tissue *fide* (Sutton, 1980).

Kendrick (1989, 2000) used the term 'holomorph' to express whole genome of a taxon. Holomorph can be referred to; 1. only asexual morph (i.e., no sexual morph is reported during the life cycle); 2. only sexual morph (i.e. no asexual morph is reported during the life cycle); and 3. both sexual morph and asexual morph are reported during the life cycle (in some cases, more than one asexual morphs are reported i.e., synasexual morphs; e.g., *Teratosphaeria* with *Colletogloeopsis* and *Kirramyces* coelomycetous morphs and Batcheloromyces-like hyphomycetous morph). Third circumstance is called as "pleomorphism" and Saccardo (1904) introduced Dual Nomenclature System of Fungi to name these genera.

In traditional mycology, links between sexual and asexual morphs of pleomorphic genera were established mainly based on co-occurrence of different morphs on same host material and culture-based methods (i.e. asexual morph is produced in the cultures of sexual morph). In modern taxonomy, links are being established mainly on culture-based methods and DNA sequence analyses. Recent taxonomic studies encourage to provide morphological characteristics (i.e. based on holotype), cultures and DNA sequences (i.e., from ex-type cultures) and asexual or sexual morph from cultures or from DNA sequence analyses, when introduces a new taxon.

Life Modes and Habitats

The members of Ascomycota are diverse and occur in different ecosystems. Some of these habitats show extreme environmental conditions such as high water salinity (e.g., saline lakes), low light (e.g., fungi in caves), and low nutrition and organic materials (e.g., rock inhabiting fungi). A large number of species have been reported from dry and cold deserts in the Antarctic or highest mountain peaks worldwide (Selbmann *et al.*, 2005, 2015; Egidi *et al.*, 2014), Arctic glaciers (Perini *et al.*, 2019), geothermal and humid soils in volcanic areas (Connell and Staudigel, 2013), acid mine drainages with sulfuric acid (Baker *et al.*, 2004; Selbmann

 doi:10.1016/B978-0-12-819990-9.00064-0

et al., 2008) or highly alkaline sites (Rojas *et al.*, 2008). Moreover, some species have been reported from deep permafrost soils (Gilichinsky *et al.*, 2007; Zucconi *et al.*, 2012).

Besides extreme environments, ascomycetous taxa are reported in other different ecosystems such as forests, agricultural lands and commercial forests. In these ecosystems, ascomycetous taxa mainly occur as saprobes, symbionts, pathogens, endophytes and epiphytes. Saprobes play a vital role in decomposition of organic materials and recycling of nutrients in ecosystems. Saprobes are reported in different environments such as aquatic (fresh water, marine) or terrestrial. Fungi on algae, salt marsh fungi, marine derived fungi, fungi in deep-sea environments, mangrove and *Pandanus* associated fungi have been extensively studied (Jones *et al.*, 2015).

Pathogenic taxa are important as it directly influences plant health of forest plants, agricultural crops or ornamental plants. Dieback of trees in primary forests and commercial forests are being reported from different countries and it could impact on both esthetic and commercial values (e.g., see Crous *et al.*, 2019 for *Eucalyptus* pathogens and other associated species). Pathogenic and other associated taxa on ornamental plants are also widely reported and essential to study (see Wanasinghe *et al.*, 2018 for fungi on *Rosa*). However, some studies suggest that these taxa play an important role in regulation of species in the natural ecosystem (Hantsch *et al.*, 2014).

Some fungi are reported as pre-harvest and post-harvest pathogens on agricultural crops. Both types of pathogens are widely studied based on DNA sequences based phylogenetic analyses which provide precise species identification (see Hyde *et al.*, 2014). Several studies showed that these pathogenic species have been reported as species complexes thus identification based on molecular tool is essential (e.g., *Colletotrichum gloeosporioides fide* Weir *et al.*, 2012; *Colletotrichum acutatum fide* Damm *et al.*, 2012a; *Colletotrichum boninense fide* Damm *et al.*, 2012b; *Colletotrichum destructivum fide* Damm *et al.*, 2014).

Epiphytic fungi, which live on leaf or other plant material are also an important group which were mostly reported as sooty molds. These species exclusively grow on honeydew excretes by sap-feeding insects. Honeydew is rich in nutrients thus provide a suitable micro habitat for over 200 epifoliar fungal species (Chomnunti *et al.*, 2011). Lichenized ascomycetous species are one of the examples for symbiotic taxa. Lücking *et al.* (2017) accepted more than 19,000 lichenized species which represents 27% of known *Ascomycota*. A large number of studies are being published on taxonomy and classification of lichenized *Ascomycota* and some major and recent studies are Lücking *et al.* (2017), Divakar *et al.* (2017), and Kraichak *et al.* (2018). Ectomycorrhizal *Ascomycota* taxa have also been reported as other examples for symbiosis. For example, Spatafora *et al.* (2012), and Leonardi *et al.* (2013) reported *Cenococcum* in *Gloniaceae* (*Dothideomycetes*) and *Pezizales* taxa respectively.

Among other life modes of environmental fungi, lichenicolous (parasitic on lichens), fungicolous (parasitic on other fungi), and algicolous (parasitic on algae) taxa could be regarded as groups that need more studies. However, studies of taxonomy and classification of lichenicolous taxa are comparatively higher than other groups (e.g., Diederich *et al.*, 2018).

Clinically important ascomycetous taxa are also important group as it directly impacts on human and animal health and other disciplines such as animal husbandry, pharmaceutical industry. These taxa could be morphologically simple taxa (yeasts) or very complex taxa (filamentous fungi).

Species Number

Predicting the number of species in Kingdom Fungi has been a controversial topic for decades and different opinions continue to be expressed based on different approaches such as host-fungi ratio, including modern methods such as next generation sequencing and environmental sequencing (e.g., Blackwell, 2011; Tedersoo *et al.*, 2014; Hawksworth and Lücking, 2017; Wu *et al.*, 2019; Hyde *et al.*, 2020a,b). Hawksworth and Lücking (2017) predicted that the species between 2.2 and 3.8 million of species would be more realistic. However, Wu *et al.* (2019) estimated the number of species as 12 (11.7–13.2) million and this was based on both culture-dependent and -independent survey on same samples.

Currently, number of species of *Ascomycota* stands at ca. 114,000 (84,000 sexually producing taxa and ca. 30,000 asexually producing taxa) (Wijayawardene *et al.*, 2021). Majority of species which were introduced in every year are ascomycetous (see the series of publications, Botanica Marina series, Fungal Diversity notes, Fungal Biodiversity Profiles, Fungal Systematics and Evolution —New and Interesting Fungi, Mycosphere notes and Fungal Planet). According to recent estimations, the estimated sexually producing species would be around one million (1 M) while the number of asexually producing fungi has been predicted as 570,000 (410,000 – 730,000) (Wijayawardene *et al.*, 2021). These numbers were based on the host-fungi ratio, as in Hawksworth and Lücking (2017). However, predicted numbers could be less due to synonymy (e.g. some species which have been introduced based on host and morphological characteristics are regarded as synonyms based on DNA based phylogenetic analyses; Groenewald *et al.* (2013) synonymized 45 species of *Cercospora* under several other *Cercospora* species). Nevertheless, there is a high possibility of discovering novel taxa from less studied habitats and life modes, from biodiversity rich areas and existing collections. Moreover, even there could be a large number of undiscovered species from well-studied environments such as fresh water and marine.

Attempts of Higher-Level Classification of *Ascomycota*

Higher level classification is the most challenged topics as 'it always been contentious and prone to subjectivity' (Hyde *et al.*, 2017). Nevertheless, attempts of accommodating of species in higher ranks were carried out in several historic studies (e.g. 1.

Table 1 Major studies of *Ascomycota* in the last decade (2010–2020)

Study	*Targeted taxonomic group*
Hyde *et al.*, 2014	Class *Dothideomycetes*
Wijayawardene *et al.*, 2014	Class *Dothideomycetes* (nomenclature)
Jaklitsch *et al.*, 2016	Phylum *Ascomycota*
Liu *et al.*, 2017	Class *Dothideomycetes*
Hongsanan *et al.*, 2017	Class *Sordariomycetes*
Wijayawardene *et al.*, 2018a,b	Phylum *Ascomycota* (outline, classification)
Ekanayaka *et al.*, 2019	Class *Pezizomycetes*
Ekanayaka *et al.*, 2019	Class *Leotiomycetes*
Johnston *et al.*, 2019	Class *Leotiomycetes*
Hongsanan *et al.*, 2020a,b	Class *Dothideomycetes*
Hyde *et al.*, 2020a,b	Class *Sordariomycetes*
Wijayawardene *et al.*, 2020a,b	Kingdom *Fungi* (outline, classification)

Saccardo (1884) accepted 6 classes of fungi: Schizomycetes, Myxomycetes, Phycomycetes, Ascomycetes, Basidiomycetes, Deuteromycetes; 2. Sparrow (1943) Accepted 9 classes of fungi: Chytridiomycetes, Oomycetes, Ascomycetes, Basidiomycetes). Subsequent studies of higher ranking of Kingdom *Fungi*, broadly accepted the Phylum *Ascomycota* (e.g., Ainsworth, 1966, 1973; Hawksworth *et al.*, 1983) but Ainsworth (1966) accommodated asexual taxa in a separate subdivision, *Deuteromycotina* which comprises three classes, *Coelomycetes*, *Hyphomycetes* and *Aganomycetes*. This was accepted in Sutton (1980) who proposed suborders in class *Coelomycetes*. Higher level classification of asexual taxa was not according to the evolutionary relationships but according to the morphological characters; such as conidiomata, conidiogenous cells, conidiogenesis and conidia.

However, since White *et al.* (1990), taxonomists implemented using DNA sequence analyses for species identification and in classification thus, the understanding of higher-level classification has been subjecting to rapid progression. Among numerous studies on higher level classification of *Fungi*, Seif *et al.* (2005), James *et al.* (2007), Liu *et al.* (2006), Steenkamp *et al.* (2006), Hibbett *et al.* (2007) and Tedersoo *et al.* (2018) are some studies which accepted subdivision *Dikarya* which comprises *Ascomycota* and *Basidiomycota*.

The long-running series *Outline of the Ascomycota* (1982–1986) and *Notes on Ascomycete Systematics* (1986–2010) were exceptional attempts which aimed to provide a current awareness system of *Ascomycota*, and provided 5113 notes. This includes the contribution of Hawksworth and Eriksson (1997–2007) and later, Lumbsch and Huhndorf (2010) provided the updated outline. Lumbsch and Huhndorf (2010) included only the sexual genera in their outline. However, at that period, several studies were published with multi-gene phylogenetic analyses and they provided classification of asexual genera as well (e.g., Schoch *et al.*, 2006, 2009; Crous *et al.*, 2009). These works encouraged to provide an incorporated outline of *Ascomycota*, thus Hyde *et al.* (2011) published "*Towards incorporating anamorphic fungi in a natural classification – checklist and notes for 2010*". Later, Wijayawardene *et al.* (2021, 2017b) updated this list with taxonomic notes. Nevertheless, during the period of 2010–2020, several important studies were published which address classification of *Ascomycota* (this includes studies which were carried out for different classes as well) (see **Table 1**). Moreover, Wijayawardene *et al.* (2017a, 2018a) provided notes for genera of *Ascomycota* (including their classification, number of species, and important references since Kirk *et al.*, 2008) and it included both sexually and asexually typified genera.

Jaklitsch *et al.* (2016) and Wijayawardene *et al.* (2018b) provided the outline of *Ascomycota* with asexually typified genera. Wijayawardene *et al.* (2020a, b) provided the outline of kingdom *Fungi* and fungus-like taxa and it included *Ascomycota*. In both studies, Wijayawardene *et al.* (2018b, 2020a, b) followed the classification proposed by Tedersoo *et al.* (2018).

Besides the phylogenetic studies, several studies have presented the evolutionary relationships of major taxonomic ranks of *Ascomycota*. Studies by Prieto and Wedin (2013), Samarakoon *et al.* (2016, 2019), Hongsanan *et al.* (2017), Liu *et al.* (2017) and Kraichak *et al.* (2018) are some examples which proposed higher level classification of different taxa of *Ascomycota*, based on divergence times.

One Fungus, One Name

Kirk *et al.* (2008) defined the nomenclature as; "the allocation of scientific names to units which a systematist considers merit formal recognition". During last decade of mycology, numerous changes have been occurring in nomenclature of fungi. These changes could be mainly on pleomorphic fungi, i.e. one taxon shows both sexual and asexual morphs during its life cycle. In some cases, it becomes complicated when one taxon shows two or more asexual morphs, i.e., synanamorphs/synasexual morphs. Saccardo (1904) proposed dual nomenclature system of fungi, i.e., providing different names for different morphs of one taxon (which are morphologically distinct). This was accepted by the International Botanical Congress (IBC) in Vienna, Austria (Briquet, 1905), and became Chapter 59 in more recent editions of the International Code of Botanical Nomenclature (ICBN) (Taylor, 2011). As an example, Samuels and Rogers (1986) introduced the genus *Botryohypoxylon* Samuels & J.D. Rogers and its

Table 2 Major studies proposed to update the name changes of pleomorphic *Ascomycota*.

Taxonomic group	*Reference*
Ascomycota	Rossman *et al.*, 2016, Wijayawardene *et al.*, 2017a,b, 2018a,b
Diaporthales	Rossman *et al.*, 2015b
Dothideomycetes	Wijayawardene *et al.*, 2014, Rossman *et al.*, 2015a, Hongsanan *et al.*, 2020a,b
Leotiomycetes	Johnston *et al.*, 2014
Magnaporthales	Zhang *et al.*, 2016
Orbiliaceae (*Orbiliomycetes*)	Baral *et al.*, 2017, Ekanayaka *et al.*, 2018
Sordariomycetes	Maharachchikumbura *et al.*, 2015, 2016, Réblová *et al.*, 2016, Hyde *et al.*, 2020b
Xylariales	Wendt *et al.*, 2018
Xylariomycetidae	Senanayake *et al.*, 2015
Pezizomycetes	Healy *et al.*, 2016

Note: Adopted from Wijayawardene, N.N., Hyde, K.D., Anand, G., *et al.*, 2021. Towards incorporating asexually reproducing fungi in the natural classification and notes for pleomorphic genera. Mycosphere 12, 238–405.

coelomycetous asexual morph from a culture and named it as *Iledon* Samuels & J.D. Rogers. The link between two morphs is well-established but it is obligated to provide two names for different morphological taxa.

Dual nomenclature system caused confusions in other different disciplines such as plant quarantine and trade in food and fiber, human health, industrial mycology, and plant breeding (Wingfield *et al.*, 2012; Wijayawardene *et al.*, 2014). Moreover, continuation of two different names for one fungus was questioned since holomorph of most of these taxa were revealed by culture-based techniques and DNA based phylogenetic analyses. Hence, dual nomenclature system was ended since 30 July 2011 (McNeill *et al.*, 2012; Hawksworth, 2012).

International Committee on Taxonomy of Fungi (ICTF) appointed sub committees as 'working groups' for different fungal groups (such as *Dothideomycetes*, *Leotiomycetes* and *Sordariomycetes*) to propose one name for pleomorphic fungi. Hence, nomenclature of pleomorphic, ascomycetous fungi has been changed significantly, and several major studies were published to propose the "adopted name" and "suppressed name" (see **Table 2**).

Current Status

The most recent classification of *Ascomycota* (as a whole phylum) was provided in Wijayawardene *et al.* (2020a,b). In this study, Wijayawardene *et al.* (2020a,b) followed Tedersoo *et al.* (2018) who provided the higher-level classification of Kingdom *Fungi* based on phylogenies and divergence time estimates. A combined data set of LSU, SSU, RPB1 and RPB2 genes was used in Wijayawardene *et al.* (2020a,b) and it comprises all orders in kingdom *Fungi*.

Bauer *et al.* (2015) introduced the phylum, *Entorrhizomycota* (which comprises *Entorrhiza* the causal agents of galls on roots of Cyperaceae and Juncaceae) and showed it belongs in subkingdom *Dikarya*. Both analyses of Tedersoo *et al.* (2018) and Wijayawardene *et al.* (2020a,b) showed that *Ascomycota* has grouped with *Basidiomycota* and *Entorrhizomycota*. Hence, phylogenetically and evolutionary, *Ascomycota* has a closer relationship with *Basidiomycota* and *Entorrhizomycota*.

Several important studies were published on different classes of *Ascomycota*, *including Dothideomycetes*, *Leotiomycetes*, *Sordariomycetes* (**Table 1**) and provided comprehensive studies on their life modes, distribution and classification.

Orphaned Genera

In traditional morphology-based classification, phenotypic characteristics are important. However, in natural classification, characteristics of sexual fungi are mainly used (such as type of the ascomata- Apothecial, Perithecial and Pseudothecial; number of layers in ascus- unitunicate operculate and inoperculate, proto-tunicate, bitunicate). In this classification system, asexual genera were not included. However, some asexual typified genera were linked with sexual typified genera as both taxa occur on the host at the same time. Thus, provisionally placed them in the natural classification system (e.g., sooty molds). However, currently, taxonomists use DNA sequences to assign the phylogenetic placements of both sexual and asexual genera.

Currently, 1548 asexually typified genera (which comprise ca. 3850 species) have been treated as *Ascomycota* genera *incertae sedis*. This is mainly due to lack of cultures of older species thus lacking DNA sequences. However, studies which focus epitypification and neotypification of older taxa, re-collected older species and carried out morpho-molecular analyses. Hence, it provides precise morphological boundaries, putative cultures, DNA barcodes and phylogenetic placements.

Compare to orphan asexually typified taxa, number of sexually typified orphaned genera are low in number. Apparently, most of the older sexual taxa possess well preserved micro characteristics compare to asexual taxa. Hence, even after a long period of time, taxonomists could observe the holotype or isotypes to accommodate them in modern classification. The structures produced in conidial fungi are delicate and easily deforms. As an example, hyphomycetous taxa produce conidia from a conidiogenous cells

which are not covered by and structure as in sexual fungi or coelomycetous taxa. Thus, it is highly fragile and easy to destroy its important characteristics (such as conidiogenous cells, conidial characteristics- chains, appendages).

Some recent studies suggest to extract DNA from the older herbarium specimens (e.g., Forin *et al.*, 2018, 2020). This is an interesting attempt since a large number of old species were based only on herbarium materials and cultures were not deposited. Nevertheless, Forin *et al.* (2018) stated that the 'ancient herbarium samples have both time and conservation related DNA damages, besides exogenous DNA contamination, that make nucleic acid extraction and amplification challenging'. Hence, we believe that the epitypification should be prioritized first for barcoding older taxa. (epitypification will be discussed in the last section).

Novel Lineages Within the Phylum

Revealing phylogenetic lineages of ascomycetous taxa is being increased by DNA-based phylogenetic analyses. Wijayawardene *et al.* (2021) highlighted some families with a high number of asexual typified genera such as *Mycosphaerellaceae* (*Capnodiales*, *Dothideomycetes*), *Phaeosphaeriaceae*, *Didymellaceae* (*Pleosporales*, *Dothideomycetes*), *Nectriaceae*, *Stachybotryaceae* (*Hypocreales*, *Sordariomycetes*). Interestingly, most of genera in these particular families have been introduced in the last decade (2010–2020). Polyphasic approach (mainly DNA based molecular techniques and morphological characteristics) was the main tool for species identification, as well to provide a higher-level ranking. Apparently, these families can harbor more taxa, which belong in the same genera or as new genera, in particular families since known taxa show worldwide distribution and are not host specific. As an example, the genus *Allophoma* Qian Chen & L. Cai was introduced by Chen *et al.* (2015) to accommodate six species (including five new combinations and one new species). Subsequent studies by Chen *et al.* (2017), Babaahmadi *et al.* (2018), Valenzuela-Lopez *et al.* (2018), Jayasiri *et al.* (2019), Marin-Felix *et al.* (2019), Hou *et al.* (2020) and Yuan *et al.* (in prep.) introduced eight *Allophoma* species.

Besides lineages in known higher-level ranks, several recent studies recognized new phylogenetic lineages of asexual taxa in *Ascomycota*. *Neocelosporiaceae* Crous, *Strelitzianaceae* Crous & M.J. Wingf. and *Cochlearomycetaceae* Crous are three families that have been introduced recently exclusively based on asexual taxa and DNA based phylogenetic analyses were the main tool to recognize them (Crous *et al.*, 2015, 2017, 2018). Detailed and systematic studies targeting particular hosts and geographic regions (from which known taxa are reported) may reveal numerous new taxa in these families.

However, phylogenetic and evolutionary stability of monospecific families and higher-ranks have to be considered prior to introduce them. Nevertheless, in a "conservation point of view, these families are important as they may represent species-rich relic taxa from early lineages that hold a unique gene pool" (Chethana *et al.*, 2020). It would be foreseeable to discover new taxa in such new and less studied lineages, such as those in *Basidiomycota* that have been less studied compared to *Ascomycota* (Wijayawardene *et al.*, 2021).

Future Perspectives and Conclusion

Current studies of *Ascomycota* could be recognized in several disciplines such as taxonomy (based on morphology, DNA sequence, secondary metabolites), industrial applications, ecological studies and pathological studies. Among these, taxonomic studies are important since its outputs directly impact on other disciplines. For an example, precise species identification of pathogens is a key factor in agriculture, import-export industry, plant breeding. Instead, DNA based techniques impact on species identification, linking sexual-asexual morphs, phylogenetic placements thus it is essential to integrate all these changes.

a. Continuously updating web-based classification system

During preparation of Wijayawardene *et al.* (2017a,b, 2018a,b, 2020a,b), these authors recognized two crucial limitations while compiling the outline.

(1) There can be disagreement and dispute among different research groups on taxonomic boundaries of genera and higher taxa.
(2) As phylogenetic data are still being collected at a very high pace and increasingly being used in taxonomy, there is the necessity of constantly updating classification schemes and incorporate new findings.

Hence, the classification of whole kingdom is provided in see "Relevant Websites Section" (Wijayawardene *et al.*, 2020a,b) which has been targeted to update continuously. Additionally, several other important web sites have been launched recently (e.g., onestopshopfungi.org (see "Relevant Websites Section"), dothideomycetes.org ("See Relevant Websites Section"), fungalgenera.org ("See Relevant Websites Section"), facesoffungi.org ("See Relevant Websites Section"), theyeasts.org (see "Relevant Websites Section") provide information on pathogenic genera, Dothideomycetes genera, typification data, descriptions of species and other taxonomic ranks, and yeast genera, respectively (Jayasiri *et al.*, 2015; Jayawardena *et al.*, 2019; Monkai *et al.*, 2019; Pem *et al.*, 2019). Doctor Fungus (see "Relevant Websites Section"), Mycology Online (see "Relevant Websites Section"), and the *Aspergillus* and Aspergillosis Website (see "Relevant Websites Section") are dedicated websites for clinically important fungi. The website "See Relevant Websites Section" deals with the latest taxonomy of marine fungi (Jones *et al.*, 2019).

(1) Epitypification, culture depositing and barcoding

It is essential to designate a new type "in cases where the type material of a species is an illustration, is lost, or is in poor condition" (Ariyawansa *et al.*, 2014). When an old specimen is not in good condition, an epitype (i.e., a specimen or illustration selected to serve as an interpretative type when the holotype, lectotype, or previously designated neotype, or all original material associated with a validly published name, is demonstrably ambiguous and cannot be critically identified for purposes of the precise application of the name of a taxon. When an epitype is designated, the holotype, lectotype, or neotype that the epitype supports must be explicitly cited *fide* McNeill *et al.*, 2012) can be designated and the new material can be used as the material for precise morphological identification, isolation and obtain living cultures and extract DNA. Ariyawansa *et al.* (2014) discussed the steps and procedures of designating an epitype.

A culture collection is "a library containing examples of the genotypic and phenotypic diversity of plant pathogens" (Abd-Elsalam *et al.*, 2010). Ariyawansa *et al.* (2014) strongly suggested "to deposit at least two collections of fungus cultures, one should be a public culture collection". Currently, most of the publications included taxa with culture details while some studies used dried cultures as herbarium material.

Recent molecular assays used for identification of common pathogens and other fungi. Different gene regions have been using in different studies to precise identification and Schoch *et al.* (2012) regarded ITS gene region as the universal barcode. However, the other gene regions, including protein genes are widely used for species identification and to confirm phylogenetic placement. During the epitypification of old species, it is essential to recognize the best gene regions for the particular species to serve as its barcoding genes.

(2) Looking for missing taxa

Current species number of kingdom *Fungi* stands at ca. 150,000 and approximately, 110,000 species belong in *Ascomycota*. However, this number comprises synonyms (e.g species introduced based on host) thus, number could be lower. Nevertheless, still a large number of species are regarded as "missing species". Resolving species complexes which comprises cryptic species, and extensive studies in existing and reference collections, studying habitats and life modes which have been less-studied, and biodiversity rich areas will be helpful to discover missing species (Hawksworth and Lücking, 2017).

Isolation and cultivation of microbes and their characterization through direct observation investigations of the diversity and functioning of microbial communities have been based on of distinct morphologies. However, recent studies questioned whether we can discover all the missing taxa. Metagenomics emerges as the study of genetic material recovered directly from environmental samples in an untargeted way (Riesenfeld *et al.*, 2004). Based on cutting-edge sequencing techniques and computational methods for genome assembly, nowadays, it is possible to identify full (or nearly full) microbial genomes directly from environmental samples. By obtaining these "uncultured genome sequences", the breadth of our understanding of microbial ecology is undoubtedly enhanced. Moreover, unculturable and missing taxa might be thoroughly characterized at molecular level. Even if some characteristics might be inferred from genome sequence, the lack of living cultures limits the full taxonomical description (e.g., morphologically, physiologically, biochemically) of the microbes corresponding to these "uncultured genome sequences". This genome-guided approach could definitely enhance current isolation and cultivation strategies for these "unculturable" taxa.

(3) Fossil genera and placing them in modern classification

Recent outline of fungi, Wijayawardene *et al.* (2020a,b) included the fossil taxa and provided their classification. As the second major step, Saxena *et al.* (2021) incorporated all the fossil species into one draft. However, apparently, a large number of taxa are related to modern Ascomycetous genera thus we suggest that it is worthy to accommodate these genera in modern genera.

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Structure and Development of Ascomata

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Introduction

Fungi are a diverse group of eukaryotic organisms that have been recovered from numerous habitats (Hyde *et al.*, 2020). The *Ascomycota* is the largest phylum of fungi encompassing over 6500 genera with over 80,000 species (Wijayawardene *et al.*, 2017). The phylum includes yeasts, filamentous fungi and lichenized fungi which occupies a variety of niches. The ascomycetes are of great economic importance as saprobes, pathogens, producers of antibiotics, as control of insect pests and several other industrial applications (Hyde *et al.*, 2019). Fungi can reproduce either sexually or asexually. Sexual reproduction is considered the most prevalent form of reproduction in eukaryotes (Seidl *et al.*, 2009). Sexual reproduction is vital for the long-term population persistence of most eukaryotes, but it requires more time and energy (Heitman *et al.*, 2007). In some cases, sexual reproduction can be less efficient compared to asexual reproduction as it depends on the production of several chemicals for the recognition and attraction of a compatible mate (Martin *et al.*, 2013).

During asexual reproducing, fungi produce genetically identical spores and it is characterized by mitotic divisions which result in the formation of endospores or conidia. During sexual reproduction in fungi, haploid cells fuse during plasmogamy, which is followed by the fusion of two haploid nuclei during karyogamy (Wallen and Perlin, 2018). The diploid cells then undergo meiosis to produce haploid cells. It is hypothesized that sexual reproduction occurs as a means of DNA repair due to exposure to adverse environmental conditions (Neiman, 2011). Sexual reproduction also occurs as a means to generate new combinations of alleles thus increasing the fitness of the offspring (Rodenburg *et al.*, 2018).

In *Ascomycota*, the sexually produced ascospores are contained in the asci, which are enclosed in an aggregation of hyphae called the ascomata or ascocarps (Meyer and Luttrell, 1986). The ascoma can therefore be described as a multicellular structure that protects the ascospores. The ascospores of unicellular fungi such as yeasts are not enclosed in ascomata. The development of ascomata is a complex process which requires the expression of specific genes and it can be affected by environmental factors.

In this chapter, we provide a description of the morphology of ascomata and an overview of the development of ascomata. We also discuss factors that can control the development of ascomata and provide examples of model organisms which have been vital in the study of ascomata formation.

Morphology of Ascomata

The ascomata are usually composed of the peridium, the asci and the hamathecium (Eriksson, 2001). The asci can form a distinct, inner layer in the ascoma called the hymenium (Eriksson, 2001). The different ascomata morphologies were previously used to classify filamentous fungi, but this classification was not fully supported as monophyletic by molecular-based analyzes (Schoch *et al.*, 2009; Ebersberger *et al.*, 2012). There are four main types of ascomata, apothecia, cleistothecia, perithecia and pseudothecia. Apothecia are open to cup-shaped ascomata that have a hymenium which is directly exposed to the environment. The hymenium in apothecia can sometimes be covered by a thin sterile tissue during the early stage whereby the asci and hamathecium are arranged in a palisade-like hymenium (Eriksson, 2001). Apothecia can be found in species including *Ascobolus immersus* and *Pyronema confluens* (Fischer *et al.*, 2004).

Cleistothecia are closed, spherical ascomata that open in a variety of ways to disperse the mature spores as a result of the peridium dissolving or cracking gradually or explosively (Eriksson, 2001). Several *Aspergillus* and *Penicillium* species produce cleistothecia such as *A. fumigatus*, *A. nidulans* and *P. chrysogenum* (Sohn and Yoon, 2002; Yu *et al.*, 2018). Perithecia are ascomata with flask-shaped structures with a preformed opening, the ostiole for spore release. Sterile hyphae can sometimes surround the perithecial cavity that encloses the hymenium. Perithecia can be found in species including *Neurospora crassa* and *Sordaria clematidis* (Bistis *et al.*, 2003; Phukhamsakda *et al.*, 2020). Pseudothecia are spherical ascomata that contain loculi. Spores are released through openings which are formed from the lysis of the peridium. Pseudothecia can usually be found in several families in *Dothideomycetes* including *Anastomitrabeculiaceae*, *Delitschiaceae* and *Didymellaceae* (Barr, 2000; de Gruyter *et al.*, 2009; Hongsanan *et al.*, 2020; Bhunjun *et al.*, 2021).

Ascomata Development in Model Systems

Ascomata development has been studied in several ascomycetous species whose sexual life cycle can be induced in the laboratory. Ascomata are highly complex structures that contain different tissues which protect the internal structures with the envelope and

the dikaryotic hyphae forming part of the integrated structure (Wilson *et al.*, 2019). Ascomata are formed as a result of fertilization which occurs through several highly regulated development processes. Sexual reproduction in fungi involves the differentiation of mating structures and the recognition of mating partners. The mating identity is genetically defined by the mating-type loci (MAT) whereby the identity and mating compatibility is established by the production of sexual pheromones (Jones and Bennett, 2011).

Ascomata formation begins with fertilization which depends on whether the species is genetically self-compatible (homothallic) or genetically self-incompatible (heterothallic) (Wilken *et al.*, 2012). The mating system in heterothallic fungi only occurs between compatible partners with MAT locus which encompasses alternative idiomorphic alleles located at the same genomic position but with different sequence information. The mating system in homothallic fungi is also controlled by the MAT locus, but in these fungi, the locus has adopted an arrangement which allows sexual reproduction of genetically identical cells. Fruiting body development also depends on several other factors including coordinated enzyme activity in cell wall biogenesis and also depends on genes responsible for the cytoskeleton (Peraza-Reyes and Malagnac, 2016).

The model organism *Neurospora crassa* is a heterothallic filamentous species of the *Sordariaceae*. *Neurospora crassa* has been found in tropical, subtropical and temperate regions as well as on the surface of fire-scorched vegetation due to its ability to metabolize cellulose and because the heat activates the sexual spores (Ruger-Herreros and Corrochano, 2020). The fruiting body of *N. crassa* is very complex as it can have up to 15 different cell types (Bistis *et al.*, 2003). It has a complex sexual life cycle as it possesses two alternative versions of the MAT loci (MAT1–1 and MAT1–2) and both versions can produce the male gametangia (macro- and microconidia) and the female gametangia (ascogonia) (Debuchy *et al.*, 2014; Kück *et al.*, 2016). Sexual reproduction starts with the fusion of compatible gametes. The male gametangia, also known as the antheridia or spermatia are small conidia-like cells that are formed directly on the mycelium whereas the ascogonia are large coiled cells. In *N. crassa*, the ascogonia mature into preformed fruiting bodies, called protoperithecia after two to three days while surrounded by protective layers of vegetative hyphae. The maturation of ascogonia to protoperithecia is dependent on metabolic regulation as it only occurs at low nitrogen concentrations. The mature ascogonia await fertilization in this protective envelope and the protoperithecia produce specialized hyphae called trichogynes which fuse with the male haploid nucleus leading to the induction of the dikaryotic phase. After fertilization, the fusion of the two haploid nuclei does not proceed immediately, but instead the male and female haploid nuclei are formed, which migrate into called croziers which are hook-shaped ascogenous hyphae. These dikaryotic tissues grow within the haploid envelope where they obtain nutritional resources. The fruiting bodies also contain sterile hyphae and paraphyses which are usually hyphae from the female parental strain. The dikaryotic cells formed by heterothallic species result in a genetically compatible haploid nucleus of each mating type, which can consequently undergo karyogamy to form a diploid cell. After several rounds of conjugated divisions, the diploid cell undergoes meiosis, resulting in the development of asci which starts with the formation of ascogenous hyphae and crozier cells. There is a post-meiotic mitosis stage prior to spore formation which results in the formation of eight spored asci. A heat shock is required to induce ascospore germination in *N. crassa* (Noguchi *et al.*, 2007).

Podospora anserina is a heterothallic *Sordariomycetes*, which has a similar sexual development pattern to *N. crassa* (Silar, 2013; Lehr *et al.*, 2014). The ascogonia of *P. anserina* also mature into preformed fruiting bodies while surrounded by protective layers of vegetative hyphae. It also has two mating-type strains, but there are some differences as *Podospora anserina* does not generate macroconidia which can germinate under specific environmental conditions. *Podospora anserina* has four spored asci due to specific nuclear mechanisms. Following the post-meiotic mitosis stage, the spore-wall formation contains two genetically distinct nuclei, each with one type of mating strain. The spermatia and the ascogonia in *P. anserina* start to differentiate after growing for three days at an optimal temperature of 27°C. The perithecia mature four days after fertilization whereas the peridium neck and ostiole are formed two days after fertilization. Once fully mature, the fruiting body continuously releases hundreds of ascospores for five days. The perithecia of *P. anserina* can enlarge over 100-fold during the development process, which requires a significant amount of nutrient. Therefore, the nutrient availability of the colony and the ability to use appropriate signaling pathways to detect the nutrient status is vital for sexual development (Wilson *et al.*, 2019). In *P. anserina*, it is hypothesized that the developing peridium sends a signal to the surrounding mycelium through diffusible reactive oxygen species as a result of the PaNox1 NADPH oxidase enzyme and mediated by the Pezizomycotina-specific protein IDC1 (Jamet-Vierny *et al.*, 2007; Nguyen *et al.*, 2018). These proteins are important for the development of the perithecium envelope. The signal is then transduced by the PaMpk1 MAP kinase cascade which results in the mobilization of nutrients for the development of the ascomata (Kicka and Silar, 2004; Lalucque *et al.*, 2017).

Sordaria macrospora is a filamentous ascomycete from *Sordariales*. It has been used extensively as a model system to study ascomata development (Teichert *et al.*, 2020). *Sordaria macrospora* has the advantage of having a short life cycle, which takes only seven days to complete in laboratory conditions. This fungus has a similar life cycle to *N. crassa* and *P. anserina*, but *S. macrospora* differ as it is homothallic and a single strain can complete the life cycle without a mating partner. *Sordaria macrospora* produces only ascospores as it lacks any vegetative spores. Unlike *N. crassa*, the ascospores of *S. macrospora* do not require a heat shock or a resting period for germination (Teichert *et al.*, 2020). The sexual cycle contributes to the propagation of *S. macrospora* and it starts with the formation of ascogonia but the mechanism leading to the formation of the dikaryotic hyphae is poorly understood. Karyogamy of two nuclei in the crozier cells is followed by meiosis to produce ascospores. Meiosis is then followed by postmeiotic mitosis which results in an ascus with eight ascospores (Teichert *et al.*, 2020). Recombination can occur between two strains of homothallic species such as *S. macrospora*. Therefore, they can be used for conventional genetic analysis which usually uses strains of compatible partners from heterothallic species with opposite mating types such as *N. crassa* (Teichert *et al.*, 2020).

Aspergillus nidulans is another model organism which has been used to study genetic recombination and ascomata development (Braus *et al.*, 2002). It is a homothallic ascomycete from *Eurotiomycetes* which is hypothesized to have a similar life cycle to *S. macrospora*. This species has no proper gametes and self-fertilization results in the formation of a small coiled lump of cells

similar to ascogonia. These calls are surrounded by a loose layer of growing hyphae which develop into the cleistothecium envelope. The developing fruiting body of *A. nidulans* is surrounded by a second layer of thick-walled multinucleate hülle cells which protects and provides nutrients to the developing fruiting body. Following karyogamy and meiotic division, the dikaryotic ascogenous hyphae can produce up to 1000 ascospores per cleistothecium (Sohn and Yoon, 2002). All *Aspergillus* species were hypothesized to propagate asexually, but several heterothallic *Aspergillus* species can undergo sexual reproduction including *A. flavus*, *A. fumigatus* and *A. parasiticus* (Kück and Pöggeler, 2009; Dyer and O'Gorman, 2011; Dyer and Kueck, 2017). The sexual lifecycle allows the application of conventional genetic analysis via crossing to investigate fruiting-body development.

Several other model species have recently been used to investigate the development of fruiting bodies including *Botrytis*, *Trichoderma* and *Penicillium* species. *Botrytis cinerea* is a plant pathogen with a wide range of host. It is a member of the *Helotiales* and it is an important model in molecular plant pathology. *Botrytis cinerea* is heterothallic and it possesses two alternative versions of the MAT loci: MAT1–1 and MAT1–2 (Amselem *et al.*, 2011). Asexual reproduction in *B. cinerea* is influenced by light as microconidia are released in the presence of light whereas sclerotia are released in the absence of light. During the early stages of development, the fruiting body of *B. cinerea* grow towards the light source (Schumacher, 2017). The limitation of using *B. cinerea* as a model organism is that it is time-consuming to induce its sexual life cycle which can take up to six months to develop a mature fruiting body (Faretra and Antonacci, 1987).

Trichoderma reesei is one of the most important cellulase and hemi-cellulase producers (Seidl *et al.*, 2009). It is an important model that has been used to study the development of the ascomata and the regulation of plant cell wall degrading enzymes. *Trichoderma* species have been considered to be largely asexual, but crossing experiments of the industrial strain have led to fertilized ascomata and the production of mature ascospores. The industrial strain however is unable to produce female reproductive structures and is therefore female sterile (Seidl *et al.*, 2009). Female sterility in this strain is hypothesized to be due to the collection being maintained in laboratory conditions for a long time. It could also be due to an accumulation of mutations during vegetative reproduction resulting in a loss of meiosis which produces female sterile mutants (Leslie and Klein, 1996). *Penicillium chrysogenum* is a model organism with major biotechnological importance and it has been considered to be asexual for over 100 years (Böhm *et al.*, 2013). Most industrial filamentous fungi lack a sexual cycle, which is problematic for crossing experiments to generate strains with novel combinations. Böhm *et al.* (2013) provided evidence for the presence of a heterothallic sexual cycle in *P. chrysogenum* and Ropars *et al.* (2014) induced the sexual cycle in *P. roqueforti*.

Factors Affecting the Development of Ascomata

The development of ascomata can be affected by several environmental factors including light, nutrients and atmospheric conditions. Each fungus responds differently to environmental factors. Light can influence the phototropic growth of reproductive structures and it can also affect gene expression and secondary metabolism (Casas-Flores and Herrera-Estrella, 2016). In *Aspergillus glaucus* and *A. nidulans*, asexual reproduction usually occurs in the presence of light and sexual reproduction usually occurs in the absence of light (Geiser, 2009; Dyer and O'Gorman, 2012; Debuchy *et al.*, 2014). There are several ascomycetes whose ascomata formation, phototropism of perithecial necks and ascospore discharge are light-dependent (Moore-Landecker, 1992). The apothecia formation in *Ascobolus magnificus*, *Pyronema omphalodes* (= *Pyronema confluens*) and *P. domesticum* are light-dependent (Traeger *et al.*, 2013; Casas-Flores and Herrera-Estrella, 2016). In the model organism *Trichoderma reesei*, stromata formation occurs only in the presence of light (Seidl *et al.*, 2009). In *Botrytis cinerea*, the formation of sclerotia which serves as survival structures and female mating partners is inhibited in the presence of light (Schumacher, 2017). The positioning of the perithecial neck in species such as *Neurospora crassa* is dependent on the presence of light and the action of the nucleoside diphosphate kinase (*NDK-1*) (Harding and Melles, 1983; Ogura *et al.*, 2001).

The development of ascomata is also influenced by the availability of nutrients. Fruiting bodies are usually generated when there are limited nutrients with the exception of fungi such as *A. nidulans* and *S. macrospora* (Dyer and O'Gorman, 2012; Wilson *et al.*, 2019). The development of ascomata can also be affected by temperature. Mycelium growth can usually occur at a range of temperature, but ascomata development requires a restricted range. For example, ascomata formation in *Fusarium solani* (*Nectria haematococca*) usually occurs between 21°C and 24°C (Moore-Landecker, 1992). Sub-optimum temperature can result in sterile ascomata. For example, ascomata formation in *Pyronema domesticum* occurs at the optimum temperature of 20°C, but sterile ascomata without ascogenous hyphae and asci are produced at 30°C (Moore-Landecker, 1992). Another factor that influences the formation of ascomata is the ratio of carbon and nitrogen (Moore-Landecker, 1992). Ascomata formation in some fungi can also be dependent on the availability of additional nutrients such as vitamins or amino acids. For example, ascomata formation in *Sordaria fimicola* and *S. macrospora* is dependent on biotin (Barnett and Lilly, 1947). The completion of the sexual life cycle of *S. macrospora* is dependent on arginine which is vital for several biological processes (Molowitz *et al.*, 1976). Ascomata formation in some species can be increased in the laboratory by adding fatty acids to the medium. Ascomata formation in *Ophiostoma ulmi* (*Ceratocystis ulmi*) and *Fusarium solani* (*Nectria haematococca*) can be increased by adding linoleic acid whereas oleate and linoleate can be used to increase ascomata formation in *N. crassa* (Marshall *et al.*, 1982; Dyer, Ingram and Johnstone, 1993). The development of ascomata in some fungi can also be influenced by the availability of carbon dioxide. Ascomata formation in *Aspergillus nidulans* is promoted during increased pressure of carbon dioxide whereas limitation of carbon dioxide promotes asexual sporulation (Han *et al.*, 2003; Dyer and O'Gorman, 2012). Ascomata formation in *A. nidulans* is also promoted during high concentration of carbon sources such as lactose, galactose and glycerol as well as during high concentration of organic nitrogen sources

such as casein hydrolysate and glycine whereas their limited availability promotes asexual development (Han *et al.*, 2003). High concentration of certain ions such as potassium, sodium or magnesium promotes asexual development in *A. nidulans* (Markina-Iñarrairaegui *et al.*, 2020).

There are several signaling pathways that detect nutrient status during ascomata development. Nutrient sensing usually occurs through plasma membrane proteins especially the G-protein-coupled receptors which are especially important for regression of sexual development (van Dijck *et al.*, 2017). *Fusarium graminearum* strains which have proton-coupled dipeptide transporters formed more ascomata compared to strains lacking the transporters (Droce *et al.*, 2017). Signaling proteins play an important role in nutrient utilization and ascomata formation. The sucrose non-fermenting 1 (*SNF1*) protein kinase is important in the transcription of glucose-repressible genes in response to glucose starvation, thus enabling the utilization of alternative carbon sources. *Fusarium graminearum* strains lacking *SNF1* formed fewer ascomata which matured more slowly and formed asci with abnormally shaped ascospores (Lee *et al.*, 2009). The protein kinase *IME2* (Inducer of meiosis 2) is involved in the regulation of several cellular processes including ascospore formation and ascomata formation (Xie *et al.*, 2020). The first identification of the kinase *IME2* was as a gene which is involved in meiotic control in *Saccharomyces cerevisiae* (Smith and Mitchell, 1989; Yoshida *et al.*, 1990). In *N. crassa*, the *IME2* downregulates the development of the protoperithecia in the presence of nitrogen (Irniger, 2011). Homologs of *IME2* have also been identified which are involved in the repression of fruiting-body formation in response to environmental factors such as limited light and nutrient availability (Irniger, 2011). The protein kinase *Crk1* which is a homolog of *IME2*, is responsible for morphogenesis, mating and virulence in *Ustilago maydis* (Garrido *et al.*, 2004). As the stromata formation of *T. reesei* occurs only in the presence of light, photoreceptors such as *BLR1* and *BLR2* play an important role in the pheromone system and fruiting body development (Seibel *et al.*, 2012). Another important photoreceptor is the *ENV1* (ENVOY1) which is crucial for the proper regulation of the sexual development in *T. reesei* unlike *BLR1* and *BLR2* (Bazafkan *et al.*, 2017). The photoreceptor *ENV1* and *PhLP1* (class I phosducin-like protein) act together as regulators of heterotrimeric G-protein signaling and they are involved in the proper regulation of light responsiveness during long-term exposure. Vacuolar $H+$-ATPases (V-ATPases) are proton pumps which are important for lysosomal and endosomal functions (Parra *et al.*, 2014). Mutation in the *vma-1* gene which encodes for subunits of V-ATPases result in female-sterile *N. crassa* strains which can only grow in an acidic medium (Bowman *et al.*, 2000). The PHOA cyclin-dependent kinase (CDK) modulates differentiation of asexual and sexual cells in response to environmental conditions such as pH and phosphorus concentration in *A. nidulans* (Dou *et al.*, 2003).

The formation of ascomata is also dependent on endogenous substances such as primary and secondary metabolites as well as pheromones which are vital for the completion of the cycle. Mutations in the genes for primary metabolism can interfere with ascomata formation. In *A. nidulans*, loss of the *hisB* gene (histidine biosynthesis) and the *trpB* gene (tryptophan synthase-encoding) inhibit the formation of ascomata during low concentration of histidine and tryptophan (Eckert *et al.*, 2000; Busch *et al.*, 2001). Ascomata formation in *A. nidulans* can be induced by oleic acid, but this induction is prohibited in strains lacking *PexF* genes (peroxin) (Hynes *et al.*, 2008). In *Fusarium graminearum*, loss of the *ICL1* gene (Glyoxylate cycle) which is essential for the utilization of carbon sources, results in defects during ascomata formation (Lee *et al.*, 2009). Nitric oxide can also influence the formation of ascomata formation as it is important in morphogenesis and reproduction (Cánovas *et al.*, 2016). In *A. nidulans*, the concentration of nitric oxide increases sharply after switching from vegetative growth to sexual or asexual growth (Cánovas *et al.*, 2016). Nitric oxide could therefore represent a signal of development in filamentous fungi (Zhao *et al.*, 2020). Ascomata formation can be promoted by citric acid for example in *Aspergillus glaucus* and the loss of the *citA* gene (citrate synthase) inhibits meiosis, which results in ascomata without ascospores (Cai *et al.*, 2010; Murray and Hynes, 2010).

Secondary metabolites can also influence ascomata development. Pigmented ascomata or ascospores in ascomycetes is usually due to the presence of polyketide melanin (Coppin and Silar, 2007). It is hypothesized that the pigments protect ascospores from environmental factors and it also provides structural support to the ascomata (Langfelder *et al.*, 2003). The polyketide synthase (*PKS*) gene is vital in the formation of structures during sexual reproduction and mutation of the gene results in female sterility in *Neurospora crassa* (Noar *et al.*, 2019). The loss of *PKS-4* gene in *S. macrospora* inhibits the formations of ascomata whereas expression of the gene results in large, abnormally shaped ascomata (Schindler and Nowrousian, 2014). Another important class of secondary metabolites are the oxylipins which have important signaling roles in mammals, plants and fungi (Naranjo-Ortiz and Gabaldón, 2020). Oxylipins are vital in several processes including morphological switches and secondary metabolism, but their role in the sexual cycle of ascomycetes remains unclear (Brodhun and Feussner, 2011; Naranjo-Ortiz and Gabaldón, 2020). In *Aspergillus nidulans*, asexual reproduction occurs in the presence of light and sexual reproduction occurs in the absence of light. The *veA* gene (Velvet complex subunit A) is important to maintain this light-dependent balance and the loss of *veA* gene inhibits the formation of ascomata (Mooney and Yager, 1990; Kim *et al.*, 2002). In *T. reesei*, the VEL1 (*veA* orthologue) The *csnD* gene (COP9 signalosome complex) is also another important regulator of the light-dependent balance similar to the *veA* gene, but it is also important for correct sexual development (Dyer and O'Gorman, 2012).

During sexual reproduction, heterothallic ascomycetes produce trichogynes which fuse with the male haploid nucleus leading to the dikaryotic phase. In *Saccharomyces cerevisiae*, diffusible peptide pheromones activate the cognate G-protein-coupled receptors (GPCRs) at the surface, which activates signals that are responsible for chemoattraction and fusion of the male and female cells (Alvaro and Thorner, 2016; Bennett and Turgeon, 2017). In *N. crassa*, diffusible peptide pheromones are responsible for the directional growth of trichogynes towards the male cells, but the directional growth is inhibited in the presence of mutations at the mating-type locus of the male cells. This suggests that these locus control the regulation of pheromones production (Kim and Borkovich, 2006). In heterothallic ascomycetes, pheromones and their receptors are involved in the recognition of opposite mating types (Martin *et al.*, 2011). In heterothallic and homothallic fungi, pheromones and their receptors are involved in the

regulation of post-fertilization events (Jones and Bennett, 2011). In heterothallic ascomycetes, deletion of pheromone genes results in male sterility whereas in homothallic ascomycetes, it does not impair vegetative growth or fruiting-body development (Mayrhofer *et al.*, 2006; Klix *et al.*, 2010; Martin *et al.*, 2011). Pheromone precursor genes are highly expressed under optimum conditions for sexual reproduction in the heterothallic *N. crassa* and the homothallic *P. anserina* (Coppin *et al.*, 2005). This suggests that pheromones and their receptors can adapt to different lifestyles and they can be involved in more complex processes.

Regulatory Networks Involved in the Development of Ascomata

The development of ascomata is a complex process which is controlled by several systems including signaling pathways (Wilson *et al.*, 2019). These signals are important for several processes including mate recognition, induction of ascomata formation and ascomata formation. In *N. crassa*, the mitogen-activated protein kinase (MAPK) cascades are important for sexual development (Lamb *et al.*, 2012). These are highly conserved signaling transduction modules that transmit signals from the cell surface to the nuclei. In ascomycetes, three different MAPK are present which control cell wall integrity (CWI), pheromone signaling (PR) and osmotic stress (HOG) (Manfiolli *et al.*, 2019). The CWI pathway is important in ascomata development and the pathway is mainly activated by cell wall stress (Levin, 2005). Strains lacking the kinases of the CWI pathway are unable to produce mature ascomata and it had an impact on the cell wall integrity (Sanz *et al.*, 2018). The PR pathway is important for early colony development, cell fusion and cell to cell communication (Jonkers *et al.*, 2014). The HOG pathway is vital for adaptation to stress conditions (Rodríguez-Pena *et al.*, 2010). The HOG pathway is activated by a range of stressors including oxidative stress, acid stress, temperature stress and heat stress (Winkler *et al.*, 2002; Bilsland *et al.*, 2004; Mollapour and Piper, 2006; Panadero *et al.*, 2006).

The striatin-interacting phosphatase and kinase (STRIPAK) complex was first discovered in humans and homologous complexes have also been identified in several eukaryotes (Ribeiro *et al.*, 2010; Hwang and Pallas, 2014). In fungi, the STRIPAK regulates ascomata development, vegetative growth, hyphal fusion, asexual development as well as pathogenic and symbiotic interactions with the host (Bloemendal *et al.*, 2012; Märker *et al.*, 2020). The reactive oxygen species produced by NADPH oxidases act as important regulator of cellular processes in cell differentiation and development (Aguirre *et al.*, 2005). The NADPH oxidases are important regulators of ascomata formation, hyphal fusion and ascospore germination (Malagnac *et al.*, 2004). The COP9 signalosome (CSN) complex is conserved from fungi to human and it is involved in post-translational processes, including protein ubiquitination and phosphorylation (Braus *et al.*, 2010). Strains lacking *c sn* gene show defects in sexual fruiting-body development, despite initiating sexual development (Busch *et al.*, 2007).

Concluding Remarks

Fruiting body formation is a complex process and filamentous ascomycetes can generate several different types of ascomata with up to 15 different cell types. Filamentous ascomycetes serve as an important model to study various important processes including the development of ascomata. The complexity of ascomata formation is enhanced due to the effect of different environmental factors and different signaling molecules on the development of ascomata. Ascomata development is also dependent on several developmentally regulated genes.

Sexual reproduction in ascomycetes provides insight regarding the mechanism and regulation of developmental processes. These ascomycetes are ideal model organism due to their relatively small genome size and due to the ability to culture them in the laboratory. Sexual reproduction in fungi reduces the accumulation of deleterious mutations, thus leading to better adaptability to changing environmental conditions. Despite intensive studies of the regulation and development of ascomata, relatively little is known regarding signals involved in the formation of different cell types. The different shapes of ascomata impact the dispersal of spores and only four main types have been described. However, only around one percent of the estimated number of fungi have been described so far. Therefore, future studies could potentially allow the discovery of more types of ascomata. The dikaryotic phase is an important phase in fungal sexual development, but the signaling events responsible for the maintenance of the dikaryon and the regulated entry into karyogamy remain unclear.

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Laboulbeniomycetes, Enigmatic Fungi With a Turbulent Taxonomic History☆

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From Roland Thaxter to the Present: Synergy Among Mycologists, Entomologists, Parasitologists

Laboulbeniales were discovered in the middle of the 19th century, rather late in mycological history (Anonymous, 1849; Rouget, 1850; Robin, 1852, 1853; Mayr, 1853). After their discovery and eventually their recognition as fungi, occasional reports increased species numbers and broadened host ranges and geographical distributions; however, it was not until the fundamental work of Thaxter (1896, 1908, 1924, 1926, 1931), who made numerous collections but also acquired infected insects from correspondents, that the Laboulbeniales became better known among mycologists and entomologists. Thaxter set the stage for progress by describing a remarkable number of taxa: 103 genera and 1260 species.

Fewer than 25 species of *Pyxidiophora* in the Pyxidiophorales are known. Many have been collected rarely, often described from single collections and never encountered again. They probably are more common and diverse than known collections indicate, but their rapid development in hidden habitats and difficulty of cultivation make species of *Pyxidiophora* easily overlooked and, thus, underreported (Blackwell and Malloch, 1989a,b; Malloch and Blackwell, 1993; Jacobs *et al.*, 2005; Gams and Arnold, 2007). The group is crucial, however, because of its taxonomic position within the Laboulbeniomycetes to provide a morphological link between the thallus-forming Herpomycetales and Laboulbeniales (Haelewaters *et al.*, 2021c) and perithecial ascomycetes (**Fig. 1**).

Cooperation among entomologists, botanists, and parasitologists easily goes back as far as the mid-19th century as evidenced by the correspondence of Charles Darwin and Henry Denny, an entomologist and authority on parasites (Darwin, 1865). Likewise, Thaxter's major contributions were possible not only from his own field collections and museum visits, but also from specimens sent to him by a wide network of entomologists. These contacts provided access to many hosts from places never visited by Thaxter, such as the African continent and southeastern Asia, from which he described many taxa. After Thaxter's death, most of the Laboulbeniomycetes literature concerned regional studies and parasite–host lists in different geographical regions, including Belgium and the Netherlands (De Kesel and Rammeloo, 1992; De Kesel *et al.*, 2020; Haelewaters and De Kesel, 2020); Finland (Huldén, 1983); Germany (Scheloske, 1969); Poland (Majewski, 1994, 2003), the Iberian Peninsula (Santamaría, 1998, 2003), and Japan (Sugiyama, 1973). Many species reported by these authors continue to result from hosts provided by entomologists or from museum insect collections (e.g., Santamaría *et al.*, 2016; Haelewaters and Rossi, 2017; Kaishian and Weir, 2018; De Kesel and Haelewaters, 2019; Kaishian *et al.*, 2020; Rossi and Leonardi, 2020). Currently, projects focusing on Laboulbeniales associated with the bat fly parasites of bats, revive the tradition of collaboration among mycologists, entomologists, and even mammalogists (Walker *et al.*, 2018; Haelewaters *et al.*, 2021a).

The major contribution of Benjamin (1971) made the massive work of Thaxter more accessible; he also reviewed his own work and the research of the post-Thaxer half century. Fifty years later, Haelewaters *et al.* (2021c) reviewed the developments in Laboulbeniomycetes of yet another half century.

The Winding Road to Molecular Phylogenetics: Progress in the Study of Laboulbeniomycetes

Advancement in the study of Laboulbeniales was initially slow because of their minute size, limited morphological traits to distinguish them among themselves, inability of most taxa to grow in axenic culture, and absence of comparative traits to place them among other fungi. Microscopes provided early evidence of the existence of Laboulbeniales, previously unknown minute ectoparasites of arthropods, and led to their recognition as fungi. After their fungal character was confirmed, better communication and transportation means enabled the discovery of additional species and broadened geographical and host ranges. Transmission electron microscopy (TEM), although less successfully applied to Laboulbeniomycetes compared to other groups of fungi, brought critical proof of free cell formation involving an ascospore-delimiting membrane in ascosporogenesis, the hallmark of the Ascomycota (Hill, 1977). Even limited success at cultivation has helped to cast light on nutritional requirements of certain Laboulbeniomycetes (Whisler, 1968; Blackwell and Malloch, 1989b; Jacobs *et al.*, 2005).

☆We dedicate this chapter to Dr. Donald H. Pfister, Curator of the Farlow Reference Library and Herbarium of Cryptogamic Botany and Asa Gray Professor of Systematic Botany at Harvard University. For almost half a century, he has been committed to preserving critical research materials and promoting their use while conducting his own extensive research and mentoring students of all ages.

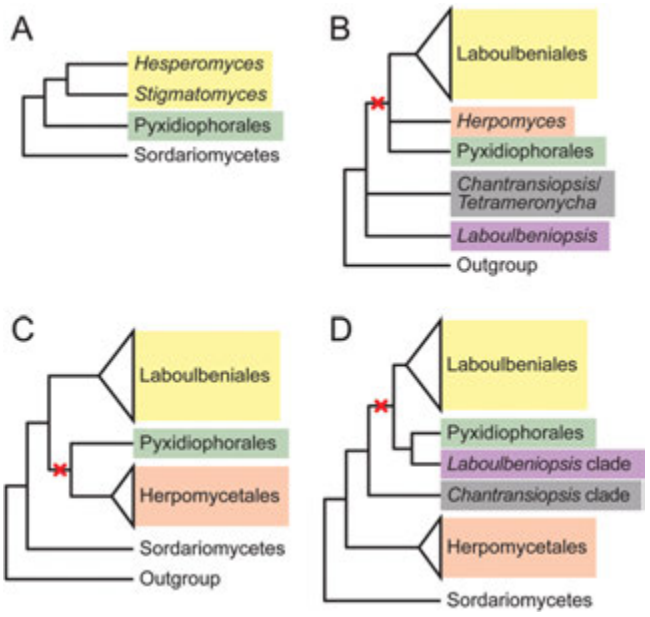

Fig. 1 Evolutionary hypotheses about the relationships between clades within the class Laboulbeniomycetes. **A.** Six-locus phylogeny based on 434 isolates, including 4 Laboulbeniomycetes isolates (modified from Schoch *et al.*, 2009). **B.** SSU rDNA phylogeny based on 65 isolates (modified from Goldmann and Weir, 2018). **C.** Three-locus rDNA phylogeny based on 61 isolates (modified from Haelewaters *et al.*, 2019d). **D.** Two-locus rDNA phylogeny based on 75 isolates (modified from Blackwell et al. 2020). Nodes without support are marked with a red "×".

Despite the discovery of free cell formation, disputes over the taxonomic position of the Laboulbeniales persisted (Cavalier-Smith, 1998; 2000), until the development of the polymerase chain reaction (PCR). Since the mid-1990s, DNA characters and ever-increasing taxon sampling have begun to unravel evolutionary relationships of Laboulbeniomycetes, to gain a better picture of their diversity, and to delineate species with innumerable discrete characters compared to those provided by morphology (e.g., Blackwell, 1994; Weir and Blackwell, 2001b; Goldmann and Weir, 2012; 2018; Haelewaters *et al.*, 2018, 2019d, 2021c; Blackwell *et al.*, 2020). Based on sequences of the small subunit (SSU) of the ribosomal RNA gene (rDNA), Weir and Blackwell (2001b) rejected a Cavalier-Smith (1998, 2000) hypothesis and showed Laboulbeniales + Pyxidiophorales to be a strongly supported single clade, class Laboulbeniomycetes, within the Ascomycota. However, no assessment of the relationship of the class among other ascomycetes was possible due to lack of support primarily due to absence of adequate sampling of ascomycete taxa. Schoch *et al.* (2009) presented the phylogenetic reconstruction of an Ascomycota-wide six-locus dataset and found strong support for the sister relationship of the classes Laboulbeniomycetes and Sordariomycetes, suggestive of a single origin of perithecial fungi.

Based on SSU rDNA, Goldmann and Weir (2018) published the taxonomically broadest molecular phylogeny of the Laboulbeniomycetes to date. Several lineages were supported within the class and the problematic filamentous conidial insect ectoparasites, *Chantransiopsis* and *Tetrameronycha*, were included in the class. In addition, a sequence of *Herpomyces* from Haelewaters *et al.* (2015b) fell outside of the Laboulbeniales in an unresolved position based on Bayesian inference or in an unsupported clade with *Laboulbeniopsis* using maximum likelihood criteria. Goldmann and Weir (2018) considered this analysis to be supportive of the placement of *Herpomyces* in the suborder Herpomycetinae (*fide* Tavares, 1985). A three-locus phylogenetic reconstruction led Haelewaters *et al.* (2019d) to elevate Herpomycetinae to order level. This move resulted in recognition of three major lineages in the class Laboulbeniomycetes: Herpomycetales, Laboulbeniales, and Pyxidiophorales.

Members of Laboulbeniales and Herpomycetales, arthropod biotrophic ectoparasites, are characterized by the formation of a non-hyphal, three-dimensional thallus of up to a few thousand cells. In contrast, species of arthropod-dispersed Pyxidiophorales are dependent on other fungi for enhanced growth or as hosts for mycoparasites; they develop hyphae and produce perithecia. It is interesting to note that because Herpomycetales and Laboulbeniales do not form a monophyletic lineage, the thallus may have originated independently in these two orders (**Fig. 1**; Blackwell *et al.*, 2020; Haelewaters *et al.*, 2021c). There are, however, some species of *Pyxidiophora* known to produce 3-dimensional cell divisions resulting in limited parenchymatous areas in parts of the mycelium and in older ascospore-derived conidial states attached to mites in moist chambers (Blackwell and Malloch, 1989b). The three formally described orders and two informal clades supported by DNA analysis are discussed below.

Thallus-Forming Ectoparasites

Laboulbeniales

The order Laboulbeniales with about 2325 described species in 145 genera (Kirk, 2019; Haelewaters *et al.*, 2020a) forms the most diverse fungal lineage associated with Arthropoda, predominantly insects (subphylum Hexapoda). These fungi occur selectively on the following insects: ants (order Hymenoptera: family Formicidae), beetles (order Coleoptera), cockroaches and termites (order Blattodea), crickets and allies (order Orthoptera), earwigs (order Dermaptera), flies (order Diptera), lice (order Psocodea), thrips (order Thysanoptera), and true bugs (order Hemiptera). Numerous other arthropods are also known to host Laboulbeniales, including millipedes (subphylum Myriapoda: class Diplopoda), harvestmen (subphylum Chelicerata: order Opiliones), and mites (subphylum Chelicerata: subclass Acari) (Weir and Hammond, 1997; Santamaría *et al.*, 2017; Haelewaters *et al.*, 2019d). Although beetles are parasitized by approximately 80% of described species (Weir and Hammond, 1997), they host a relatively small number of genera of Laboulbeniales (Haelewaters *et al.*, 2019b). Fewer species have been described from other groups, such as the Hemiptera (true bugs), with only 96 described species occurring across the suborder Heteroptera (Benjamin, 1967; Santamaría, 2008; Lee and Na, 2009; Kaishian and Weir, 2018; Kaishian *et al.*, 2020).

First sightings

The study of Laboulbeniales started with observations of *thalli* (multicellular units with determinate cell number) of *Laboulbenia* on carabid beetles in the 1840s and early 1850s (Anonymous, 1849; Rouget, 1850; Mayr, 1853). Some authors thought that the structures they observed were insect parts (Mayr, 1853) whereas others recognized them as living organisms. In those days, researchers referred to Laboulbeniales as "parasitic plants" (Anonymous, 1849) or even acanthocephalan worms (Kolenati, 1857). Robin (1852) was the first to recognize them as fungi, and de Bary (1884) listed the family Laboulbeniaceae as ascomycetes with doubt. The first to use the name "Laboulbeniaceae" was Peyritsch (1873). Five genera were recognized at that time – *Chitonomyces*, *Heimatomyces*, *Helmintophana* [= *Arthrorhynchus*], *Laboulbenia*, and *Stigmatomyces* – and twelve species had been described, eight of which were in the genus *Laboulbenia* (Benjamin, 1971). By the time Thaxter published his first *Contribution towards a monograph of the Laboulbeniaceae* (Thaxter, 1896), there were 28 genera and 152 species, most of which had been published in a series of preliminary papers (Thaxter, 1891, 1892, 1893, 1894, 1895).

Current versus estimated species diversity

While only 2325 species have been described thus far, many more species are expected to be discovered in years to come. A formal estimation of the species richness of Laboulbeniales was based on the results of a beetle survey in Sulawesi, Indonesia, in which 80,000 beetles were screened for the presence of Laboulbeniales to yield an overall prevalence of 0.6% (Weir and Hammond, 1997). The principal study area was a 500-ha patch of lowland rainforest. Of a total of 4026 beetle species, 127 were hosts of Laboulbeniales fungi (3.15%). Based on their data and comparisons with other studies (Huldén, 1983; Lee, 1986; Santamaría *et al.*, 1991; Majewski, 1994; Weir, 1996), the authors asserted that greater Laboulbeniales diversity is associated with moist tropical areas and beetle hosts. Provided that the number of beetle species is estimated to be 2 million and that the prevalence of Laboulbeniales on beetles is about 3%, Weir and Hammond (1997) inferred that the number of Laboulbeniales species on beetles would be 60,000. If the ratio of host species to parasite species is 2:1 (meaning that some species of Laboulbeniales are associated with multiple host species), this results in an estimated 30,000 Laboulbeniales species on beetles. Given that at least 75% of infections occur on beetles, one could assume that the total estimate of species in the order is 40,000 [15,000 – 75,000].

There have been few other detailed studies of Laboulbeniales at a given site. Huldén (1983) surveyed the Laboulbeniales of Finland and adjacent regions of what was formerly the U.S.S.R. based on the study of museum collections. About 160,000 insects representing 1100 species were screened for the presence of Laboulbeniales. A total of 166 insect species (beetles and flies) were found to be host to 88 species of Laboulbeniales, 24 of which were newly described. The overall prevalence for this study was approximately 1%, which is thought to be low. For example, in Central Europe, the rate of infection ranges from 10% to 35% (Huldén, 1983). This striking difference may be attributed to changing climatic conditions leading to higher winter mortality of potential hosts, in addition to smaller, more isolated host populations that overlap less frequently. Wide-scale studies of neotropical Laboubeniales diversity are lacking compared to studies from temperate regions, and no comprehensive site-based study of the group has been published from the Neotropics, although one long-term inventory project has been initiated in Cusuco National Park, Honduras (Haelewaters *et al.*, 2021b). Such studies will result in a substantial addition of new biodiversity data.

The advent of molecular studies has revolutionized our understanding of organismal relationships. These techniques are of particular value when investigating cryptic species and could help sharpen estimates of species numbers within the group. However, the isolation of DNA from thalli of Laboulbeniales was an early problem to overcome, due to their inability to grow in artificial culture, minute size, and melanized tissue (Weir and Blackwell, 2001a; Haelewaters *et al.*, 2015b; Sundberg *et al.*, 2018a). It does not come as a surprise that only 12 of 174 species of Laboulbeniomycetes described between 2010 and 2020 were accompanied by sequences (**Fig. 2**), mostly of nuclear rDNA regions, but also mitochondrial small subunit rDNA and translation elongation factor 1α gene. As integrative taxonomy practices become commonplace in species delimitation of many groups of fungi, Laboulbeniales researchers have struggled to keep pace. However, molecular phylogenetic data have successfully clarified relationships at lower taxonomic ranks (e.g., Weir and Hughes, 2002; Goldmann and Weir, 2012; Goldmann *et al.*, 2013; Sundberg *et al.*, 2018b; Haelewaters *et al.*, 2019a; Liu *et al.*, 2020) and revealed the existence of cryptic diversity in the

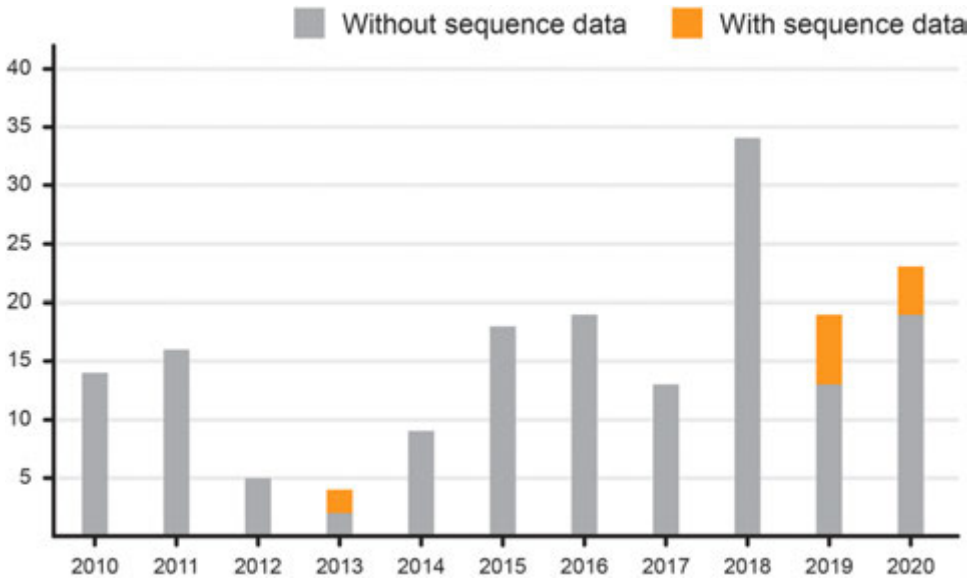

Fig. 2 Number of described Laboulbeniomycetes species – including *formae* – since 2010, based on morphology alone (without sequence data) versus using an integrative taxonomy approach (with sequence data).

Laboulbeniales, with *Arthrorhynchus eucampsipodae*, *Hesperomyces virescens*, and *Laboulbenia flagellata* having been recognized as species complexes (Haelewaters *et al.*, 2018, 2019b, 2020a; De Weggheleire, 2019). These findings make the 40,000-figure from Weir and Hammond (1997) a conservative estimate for species richness in the order.

Still, generating sequences for Laboulbeniales remains a challenge, especially for material from dried museum collections or specimens collected >10 years ago. This dilemma necessitates consideration of the value of alpha-taxonomy. While researchers of certain groups, such as the fleshy mushrooms, may challenge the validity of species descriptions made in the absence of molecular characters, morphological descriptions have been critical in building foundational knowledge of Laboulbeniales. And while integrated taxonomy – the use of combined morphological, ecological, geographical, and molecular data – is an ideal to which researchers strive. Hibbett *et al.* (2016) pointed out that many researchers lack access to sufficient funding or equipment to generate molecular data. These researchers are often based in tropical areas where most of the world's undescribed species reside. Because millions of fungal taxa remain undiscovered, it seems prudent for taxonomists to continue working using available resources with the understanding that future molecular phylogenetic work may confirm or shift species limits. Concurrently, collaboration between fungal molecular systematists and classically trained taxonomists should be the end goal.

Museum collections, citizen science projects, and social media

There is a large discrepancy between the number of described species of fungi (138,000, Kirk, 2019) and the number of estimated species (2.2–6 million, e.g., Hawksworth and Lücking, 2017). A common inquiry asks, *where are the millions of missing fungi?* Wijayawardene *et al.* (2020) put forward five sources of undescribed fungi: (1) habitats that are naturally diverse but poorly studied, (2) cryptic taxa, (3) fungal collections that might contain cryptic or new species hidden under current names, (4) molecular novelties, and (5) natural history collections including plant herbaria and entomological collections. Indeed, insect collections are a treasure trove for Laboulbeniales researchers. Thalli of Laboulbeniales persist indefinitely on the host body whether the host is preserved in ethanol or dried and pinned. Because museum collections often contain a wide array of taxa from a broad range of localities, researchers utilizing collections can pursue a research scope focusing on specific geographical areas and/or taxa. Making use of museum collections for research as opposed to conducting fieldwork has logistical advantages, saving time and expenses, and circumventing the need to kill hundreds to thousands of insect specimens to find only a few ones hosting Laboulbeniales. Another huge advantage is that the host is often already identified by an expert.

Collections have been used to investigate an array of questions about biodiversity, taxonomy, biogeography, and host usage patterns of Laboulbeniales. Blackwell (1980a,b) screened 2517 nycteribiid bat flies at the Natural History Museum (London) and found thalli of *Arthrorhynchus* on 56 specimens. She used this material along with Thaxter's slide mounts from the Farlow Herbarium to study fungal development and morphology, host associations, and within-species phenotypic plasticity. Haelewaters *et al.* (2015c, 2019b) reported nine new country records of Laboulbeniales (Canada, USA, Croatia, Slovenia, Ukraine, DR Congo) based on dried collections of Carabidae, Coccinellidae, and Staphylinidae from the Harvard Museum of Comparative Zoology, the American Museum of Natural History (New York), Tupper Center of the Smithsonian Tropical Research Institute (Ancon, Panama), and the Collection d'insectes du Québec (Canada). Kaishian and Weir (2018) and Kaishian *et al.* (2020) described eight new species of the genera *Laboulbenia* and *Prolixandromyces* based on material from the collection of Dr. John T. Polhemus, Department of Entomology, Smithsonian National Museum of Natural History (Washington, D.C.). Santamaría *et al.* (2016)

utilized the millipede collection preserved at the Natural History Museum of Denmark (Copenhagen) to describe nine new species of *Rickia*. Haelewaters *et al.* (2017) proposed that a lag time occurred between establishment of the invasive alien ladybird *Harmonia axyridis* in nature and acquisition of *Hesperomyces virescens* by this host. The authors based their findings on the study of 7404 ladybirds collected in 1991–2015, of which 521 were from eleven museum collections in North America and Asia.

Contributions from citizen scientists have been of great importance to biodiversity research since the 19th century. Citizen science projects have gained more traction in recent years and examples of large-scale projects dependent on input from non-professionals, are commonplace (e.g., Douglas, 2016). Haelewaters *et al.* (2019c) downloaded and curated North American occurrences of *Hesperomyces virescens* associated with the invasive alien species *Harmonia axyridis* from citizen science platforms Bugguide and iNaturalist. All records were used to build a map at Beetlehangers.org (see "Relevant Websites section"), the primary aim of which is to track the distributional range of the ladybird–parasite association through time. As another example, Báthori *et al.* (2017) reported a new country record of *Rickia wasmannii* from Greece, which was found and identified using the digital image collection at AntWeb (see "Relevant Websites section"). Finally, Santamaría *et al.* (2020b) described a new species of *Troglomyces*, initially discovered in a photograph of a millipede shared on Twitter (see "Relevant Websites section") and then found on millipedes at the Natural History Museum of Denmark and the Muséum National d'Histoire Naturelle in Paris. These recent studies also speak to the timelessness of natural history collections and their continued value in modern research.

Classification of the order

Roland Thaxter not only contributed invaluable taxonomic additions, he was also the first – and for a long time the only one – to propose a classification system for the order. At the time of the first volume of his *Contribution towards a monograph of the Laboulbeniaceae* (Thaxter, 1896), what we currently refer to as the Laboulbeniales was designated a family, Laboulbeniaceae. Thaxter (1896) split up the Laboulbeniaceae into two "groups", the Exogenae and Endogenae. Development of spermatia, gametes produced on the appendages, was the sole criterion for grouping of taxa. The Exogenae included genera with species that form spermatia exogeneously. They are borne on intercalary cells or terminally on short branchlets. Only the genera *Ceratomyces* and *Zodiomyces* were part of this Exogenae group. The Endogenae comprised taxa in which spermatia are formed within antheridia. This group included two "orders": Laboulbenieae (with simple antheridia, 15 genera) and Peyritschielleae (with compound antheridia, 11 genera).

Thaxter (1908) accepted the ordinal name Laboulbeniales, which included the recently described genus *Herpomyces* (Thaxter, 1902), and replaced the terms Exogenae and Endogenae by subordinal names Laboulbeniinae and Ceratomycetinae. The two subdivisions of the original "group" Endogenae were replaced by families Laboulbeniaceae and Peyritschiellaceae. Thaxter did not recognize a family within the Ceratomycetinae. The name Ceratomycetaceae, now widely accepted, was introduced by Maire (1916) as a *nomen nudum*, later validly published by Colla (1934). This scheme of organizing taxa was widely accepted until Tavares (1967, 1985) introduced new characters for classification of the Laboulbeniales: perithecial development and perithecial wall structure. Later, Goldmann and Weir (2018) found that the number of perithecial wall cells is phylogenetically informative across the order Laboulbeniales; the authors described a progressive reduction of number of perithecial wall cells in the four vertical rows. Thaxter's (1908) two suborders, two families and twenty tribes were reorganized to two suborders, four families, six subfamilies, 13 tribes and 28 subtribes in Tavares' (1985) classification system.

Tavares (1985) recognized three families in the suborder Laboulbeniinae: Ceratomycetaceae, Euceratomycetaceae, and Laboulbeniaceae (Majewski, 1994; Santamaría, 2003). Ceratomycetaceae comprises twelve genera: *Autoicomyces*, *Ceratomyces*, *Drepanomyces*, *Eusynaptomyces*, *Helodiomyces*, *Phurmomyces*, *Plectomyces*, *Rhynchophoromyces*, *Synaptomyces*, *Tettigomyces*, *Thaumasiomyces*, and *Thripomyces*. Synapomorphic characters are (1) the primary receptacle consisting of a single series of superposed cells and (2) cells VI and VII being successive, intercalary cells of the primary receptacle (Tavares, 1985). In the Euceratomycetaceae, cells VI and VII are successive cells of the lateral secondary appendage arising from the primary appendage. The lateral appendage extends beyond the base of the perithecium (arising from cell VII). Depending on the genus, there may be a single perithecium or multiple ones. Genera included in the Euceratomycetaceae are *Cochliomyces*, *Colonomyces*, *Euceratomyces*, *Euzodiomyces*, and *Pseudoecteinomyces*.

Taxa in the genus *Euzodiomyces* are exceptional among Laboulbeniales in the construction of their primary receptacle, which is many-celled and parenchymatous (Tavares, 1985; Santamaría, 2003). Other than in *Euzodiomyces*, this feature is only present in the genera *Columnomyces*, *Kainomyces*, *Scepastocarpus*, and *Zodiomyces*, all of which are classified in the Laboulbeniaceae (Rossi *et al.*, 2016). All genera but one (*Tettigomyces*) in Ceratomycetaceae are associated with aquatic hosts, whereas those in Euceratomycetaceae have terrestrial hosts. Finally, the family Laboulbeniaceae is recognized by the tiers of perithecial outer wall cells, which are four or five in number and unequal in height. Although the genus *Zodiomyces* has perithecial outer wall cells that are arranged in eight tiers subequal in height, Tavares (1985) also placed this genus in Laboulbeniaceae, in its own subfamily Zodiomycetoideae. This is in stark contrast to Thaxter (1908) who had placed *Zodiomyces* in the suborder Ceratomycetineae.

Currently, the classification system of Tavares (1985) is still in use, although molecular phylogenetic studies have repeatedly shown that several taxa within this system are polyphyletic. At least three subtribes, two tribes, and two subfamilies are polyphyletic (Goldmann and Weir, 2018; Haelewaters, 2018). In addition, *Herpomyces* was recently removed from the Laboulbeniales and placed in its own order with strong support from multiple sources (Haelewaters *et al.*, 2019d; Blackwell *et al.*, 2020). In the SSU rDNA phylogeny of Haelewaters (2018), four genera with species that are associated with aquatic hosts were placed basally in the Laboulbeniales order. This might be seen as evidence for an ecological viewpoint rather than, or in addition to, a structural-based one. All in all, while the classification of the Laboulbeniales order is in urgent need of complete revision, current taxon and character sampling is far too insufficient to make changes that are taxonomically stable. Continued development of molecular

protocols – optimized for scarce material, e.g., including a whole-genome amplification step prior to PCR – will lead to the progress needed to propose a stable evolutionary hypothesis for the order.

Notes on ecology

Where Laboulbeniales receive their nutrients from is not entirely understood, which can be partly explained by the presence of taxa without a penetrating haustorium (Tragust *et al.*, 2016). In addition, to date, attempts to obtain axenic cultures of Laboulbeniales have failed whereas transmission experiments of Laboulbeniales among hosts have been used to prove specificity and host-related nutritional requirements. Several authors put forward different hypotheses about nutrient uptake—including chitin of the integument, secretions from exocrine glands, substances available at the cuticle (waxy substances, components from plants, substrate, microbiota, host fecal materials), and waxy lipids produced by the epidermal cells. It is most widely accepted that nutrition is obtained through the attachment area. Support for this hypothesis came from Scheloske (1969) who injected Nile sulfate dye into an insect and observed it flowing from elytral tissues to *Laboulbenia* thalli.

Since Laboulbeniales are phenotypically plastic, their morphology can vary dramatically depending on the position on the host's integument as well as the sex of the host. Some authors considered these morphologies as separate taxa (Thaxter, 1926; Benjamin and Shanor, 1952; Benjamin, 1967), whereas others described them as morphotypes of the same biological species (Rossi and Kotrba, 2004; Rossi, 2006; Rossi and Proaño Castro, 2009; Santamaría and Faille, 2009; Haelewaters and Rossi, 2017). Based on molecular analysis, the phenomenon of position specificity was recently confirmed for Laboulbeniales from aquatic hosts (Goldmann and Weir, 2012); speciation was not connected to nutrition of the fungus, but to a highly specific and precise transmission of spores during copulation. However, the understanding of how Laboulbeniales from terrestrial hosts are directly transmitted, mainly by copulation (Scheloske, 1969), made an end to position specificity and sex-of-host specificity.

The need for a living host is clear, but the occasionally reported dramatic host shifts have remained unexplained for a very long time. Examples are *Rickia wasmannii* in ant nests parasitizing both *Myrmica* ants (main host) and co-habitating arthropods, which belonged to a different order (Diptera) and even subphylum (Chelicerata) (Pfliegler *et al.*, 2016), and *Stichomyces conosomatis* in a subterranean cave on *Speonemadus algarvensis* (Leiodidae), which represented the only report of this fungus on a host other than species of *Sepedophilus* (Staphylinidae) (Reboleira *et al.* 2017). Scheloske (1969) and later Majewski (1994) and De Kesel (1997) showed that a single species of *Laboulbenia* can have main, occasional, and accidental hosts. In most cases, these hosts occupy the same habitat. Based on his observations, Scheloske (1969) stated that plurivorous Laboulbeniales need both the host and its habitat and launched the concept of "ecological specificity" – although the term "habitat specificity" is more accurate because host specificity also assumes resource availability and niche specialization.

De Kesel (1996) carried out experiments testing the impact of habitat on growth, development, and transmission. Via a gradient analysis it was found that under certain habitat conditions, *L. slackensis* can be transmitted and will develop successfully on a series of atypical hosts. The potential host range of Laboulbeniales is much wider than initially thought and opportunities for host shifting are therefore entirely governed by the host, its behavior (habitat choice), and life history. Although host shifting of Laboulbeniales is possible under certain conditions, there are some barriers. Species of Laboulbeniales are fairly isolated on their respective host populations. This is because direct transmission of ascospores is mostly intraspecific (among hosts of the same species), in the form of grooming, copulation, and random aggregation contacts (*Herpomyces*: Richards and Smith, 1955; *Hesperomyces*: Nalepa and Weir, 2007; *Laboulbenia*: De Kesel, 1995a,b; *Rickia*: Haelewaters *et al.*, 2015a). Indirect transmission is negligible because of the short lifespan of ascospores (De Kesel, 1995b; Cottrell and Riddick, 2012), and opportunities for interspecific contacts with other hosts are often few because of their separation in time and space—unless they cohabit the same microhabitats.

Most species of Laboulbeniales only develop on adults; transmission can only take place if adult generations overlap. Because of this, suitable hosts that are parasitized by a given species of Laboulbeniales in warmer areas lack the parasite in colder localities (northern, alpine), where they overwinter as larvae (Huldén, 1983). Parasite prevalence, that is, the number of parasitized host specimens in a population, fluctuates greatly throughout the year and is affected by the host life cycle (spring breeders vs. autumn breeders), the emergence period of new generations (Scheloske, 1969), and host population density (De Kesel, 1995a). Thallus density, the number of thalli on a host individual, on the other hand, seems to increase with increasing age of the host (Haelewaters *et al.*, 2015a; De Kesel *et al.*, 2016). This is an important feature because it boosts opportunities for transmission of ascospores between old and new generations.

Thallus morphology

The *thallus* (**Fig. 3**) is a multicellular unit with a restricted number of cells, derived from two-celled ascospores through a defined number of mitotic divisions in multiple planes (Blackwell *et al.*, 2020). A primary septum separates the larger cell of the ascospore from the smaller one. This septum is often visible by its thickness and color, even in mature thalli. The main axis of the thallus is formed by the receptacle, which is the part of the multicellular unit that is connected to the host by means of a foot. The receptacle and foot are derived from the larger cell of the ascospore, which is released first from the perithecium. Additional divisions of particular cells of the receptacle produce the perithecium or perithecia. The perithecium is the only spore-forming structure of the Laboulbeniales; there are no asexual spores. The smaller cell of the ascospore produces the primary appendage system, which carries the spermatia-producing antheridia. The entire ontogeny, from ascospore to mature thallus, was studied for a few *Laboulbenia* species (Tavares, 1985, De Kesel, 1989). In terms of orientation, the anterior side is the one on which the perithecium

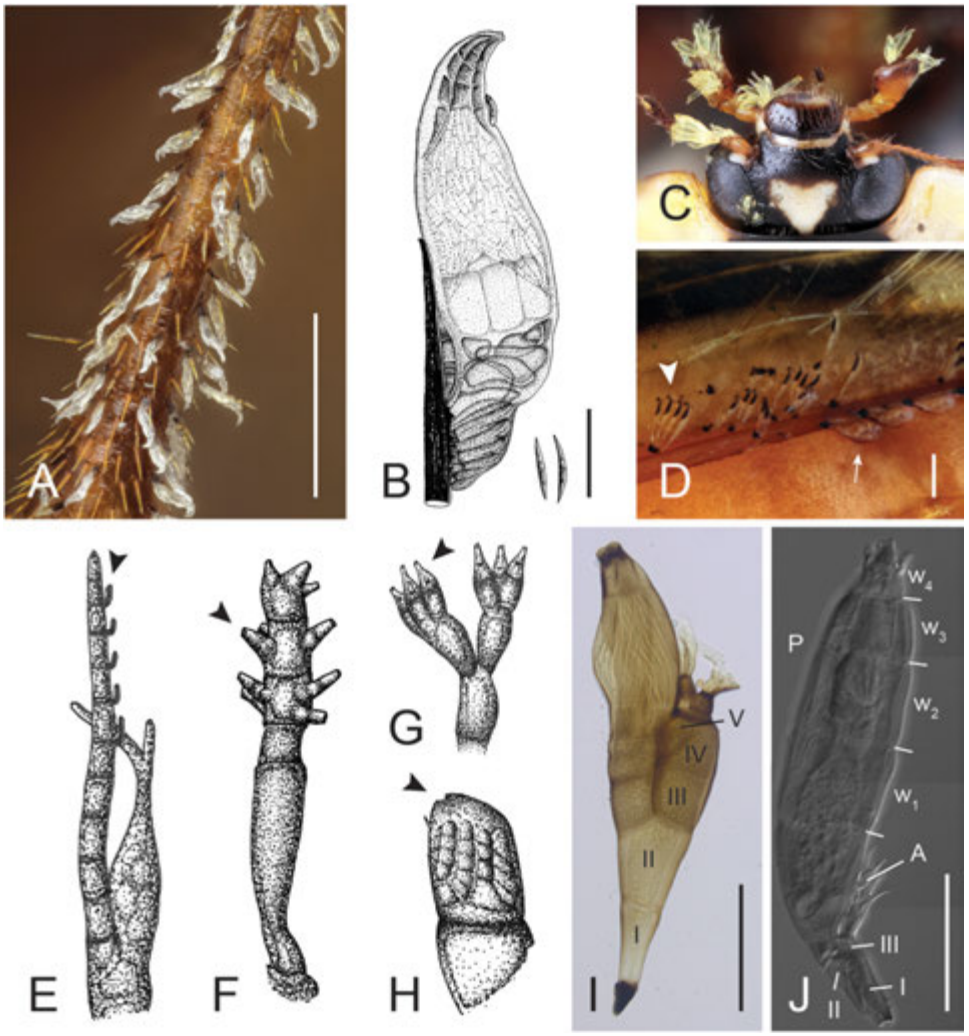

Fig. 3 Herpomycetales and Laboulbeniales. **A–B.** *Herpomyces chaetophilus* on *Periplaneta americana*. **A.** Thalli attached to the setae of an antenna. **B.** Single thallus of *H. chaetophilus* attached to an antennal seta. Pencil drawing by Jingyu Liu. **C**. A *Harmonia axyridis* ladybird with tufts of *Hesperomyces* thalli on its mouthparts and left eye. **D.** Growth positions of *Chitonomyces* thalli on the left elytral margin of *Laccophilus hyalinus*; thalli of *C. melanurus* (arrowhead) are found above and on the epipleuron, whereas *C. paradoxus* thalli (arrow) consistently grow on the lower part of the margin. **E–H.** Spermatia-producing structures. Pencil drawings by Jingyu Liu. **E.** Appendage cells forming spermatia exogeneously (arrowhead) in *Drepanomyces* sp. (Ceratomycetaceae). **F.** Simple antheridia (arrowhead) in *Arthrorhynchus nycteribiae* (Laboulbeniaceae). **G.** Tufts of simple antheridia (arrowhead) in *Laboulbenia disenochi* (Laboulbeniaceae). **H.** Compound antheridium, with spermatia leaving the chamber through a single opening (arrowhead), in *Neohaplomyces medonalis* (Peyritschiellaceae). **I.** Mature thallus of *Laboulbenia fuscata* from *Pterostichus* sp. collected in Argentina; the receptacle is formed by cells I through V (labeled); cells III, IV, and V in *Laboulbenia* are often referred to as the androstichum. From the Roland Thaxter collection of slide mounts at the Farlow Herbarium. **J.** Mature thallus of *Hesperomyces virescens* (sensu lato) from *Harmonia octomaculata* collected in Micronesia; annotated are cells I, II, and III of the receptacle, the appendage (A) with simple antheridia, and the perithecium (P) with four tiers of wall cells (w_1 to w_4). Scale bars A 500 μm; B, D 50 μm; I, J 100 μm.

is located, whereas the posterior side is the side away from the perithecium. Other authors use ventral and dorsal for anterior and posterior, respectively.

Receptacle

The primary receptacle forms the base for all parts of the thallus. Its shape and structure are extremely variable within the order, and this variability is an important criterion in generic delimitation. Apparently, the lower cell of the ascospore generally divides into 3 cells denoted by Roman numerals I, II, and III. Further divisions in different planes may take place, depending on the genus. Many genera, those in the subtribe Stigmatomycetinae, have only those three cells in the receptacle but their positions are variable with respect to one another. Cell I is the basal cell, forming the connection with the host's integument (referred to as the foot). Multiple divisions of cell I can occur, for example in female thalli of *Dimeromyces*. These secondary cells will further give rise to

perithecia or sterile appendages. Cell II, the suprabasal cell, generates the perithecium by successive divisions. Cell II undergoes multiple divisions in many genera, forming an elongate uniseriate receptacle. Examples of genera with this structure are *Chaetomyces*, *Ecteinomyces*, *Filariomyces*, and *Ormomyces*. Secondary divisions of cell III can occur. For example, in the genus *Laboulbenia*, these divisions form cells IV and V. The entire complex of cells III to V is called androstichum (**Fig. 3I**). Some species of *Laboulbenia* have an undivided cell III + IV or cell III + IV + V (e.g., *L. nisotrae*, *L. obesa*, *L. richardiana*).

Perithecium

The perithecium is derived from the receptacle, in species without secondarily divided receptacle cells it arises from divisions of cell II. Benjamin (1971) described three types of perithecial development. In the first type, a single cell arises laterally from the receptacle to divide into a lower and upper cell. The lower cell, by continued divisions, gives rise to the perithecial stalk cell (VI), secondary stalk cell (VII), and basal cells m, n, and n′. The upper cell will give rise to the female sexual organ, which initially comprises three cells: basal carpogenic cell, trichophoric cell, and terminal trichogyne. The trichogyne is a thin appendage-like outgrowth of the young perithecium. It may or may not develop into a multicellular simple or branched structure, depending on the species. Its function is to receive spermatia. Before the perithecium is mature, the trichogyne will deteriorate, often leaving a visible scar. This "carpogonial upgrowth" is enveloped by the perithecial walls, which arise from cells m (forming a single vertical row of wall cells) and n and n′ (forming three rows).

After interception by the trichogyne, the male nucleus from a spermatium will migrate to the carpogenic cell, thus resulting in the formation of an ascogenous cell (or multiple ones by mitotic divisions), in which both the male and female nuclei are present. This is the dikaryotic phase of the Laboulbeniales life cycle. Asci are produced by mitotic divisions of the ascogenous cells in multiple planes. Upon fusion of the two nuclei, the diploid ascus mother cell is formed, which after meiosis gives rise to an ascus with 4 ascospores. This developmental type was described and illustrated by Thaxter (1896) for *Laboulbenia elongata*, *Peyritschiella geminata*, and *Stigmatomyces baeri*. According to Benjamin (1971) there is only one genus of Laboulbeniales that does not follow this type of development; *Coreomyces* forms what Thaxter (1908) named a pseudoperithecium.

The mature perithecium is more or less elongated and narrowed towards the tip (distally). Sometimes there is a clear differentiation into a rounded or ovoidal venter and a narrow neck, terminating in an ostiole. The perithecial wall cells surrounding the ostiole often form distinct lips (e.g., in *Hesperomyces*) or (sub)apical outgrowths (e.g., in *Diphymyces*). The perithecium usually consists of a well-defined number of cells. The perithecial wall cells appear in two layers; the external wall cells are clearly visible and have taxonomic importance (Tavares, 1985; Majewski, 1994). The most ancestral perithecium is the one in which each of the four vertical rows of outer wall cells consists of many cells that are equal in height, as in Ceratomycetaceae. This was stated by Tavares (1985) and supported by the use of sequence data (Goldmann and Weir, 2018; Haelewaters, 2018). Morphological studies of the genera *Nycteromyces* and *Polyandromyces* (Dimorphomyceteae) failed to distinguish perithecial cell walls (Thaxter, 1920, 1924; Haelewaters, 2018). Presumably this represents a highly derived situation (Tavares, 1985).

Appendage and antheridia

The primary appendage usually is a direct continuation of the receptacle axis. It is produced by divisions of the upper, smaller cell of the ascospore. In some genera, the primary appendage is very simple, consisting of one or two cells only. Examples are *Dioicomyces* and *Filariomyces*. In very few species the appendage can even become aborted (Tavares, 1985). Well-developed primary appendage systems exist in many species of, e.g., *Corethromyces* and *Laboulbenia*. Sometimes, the original spore apex remains visible at maturity as a spinose process because the branches are formed at a level below the apex. This process is an important feature to identify species in the genera *Acompsomyces*, *Eucantharomyces*, *Ilyomyces*, and *Rhachomyces* (Santamaría, 2003, 2006; Haelewaters, 2013; Santamaría *et al.*, 2020a). The primary appendage system of *Laboulbenia* deserves extra attention. Its basal cell, called insertion cell or cell e, is flattened and usually obscure and carries the inner and outer appendages. The inner appendage bears flask-shaped, simple antheridia. The outer appendage is usually longer, simple or branched, and almost always sterile.

The primary appendages of *Chitonomyces* and *Hydraeomyces* break off early, right above the constricted black septum (Tavares, 1985). The primary appendages of *Columnomyces* and *Diphymyces* are usually partly or completely broken off (Thaxter, 1918, 1931; Benjamin, 1955; Haelewaters *et al.*, 2014; De Kesel and Haelewaters, 2019; Perreau *et al.*, 2021). This damage has been linked to the behavior of the host insects, Cholevinae (Coleoptera, Leiodidae). Cholevine beetles have evolved a largely underground lifestyle and make extensive use of narrow channels and tunnels in the soil, which may account for breakage of parts of Laboulbeniales thalli on these hosts (Sokolowski, 1942). Also the extensive appendage system of *Laboulbenia clivinalis* regularly breaks off (and regenerates, Majewski, 1994). Similar to cholevines, *Clivina fossor*, the host of *L. clivinalis*, has a partly subterranean lifestyle (De Kesel, 1995a).

When sterile or antheridial branches are derived from the lower cell of the ascospore, they are referred to as secondary appendages. All appendages of *Scepastocarpus* and *Zodiomyces* are secondary in origin. Little is known about the function of sterile appendages, whether primary or secondary. Cavara (1899) speculated that thalli could retrieve nutrients from the environment by means of their sterile appendages. De Kesel (1996) showed experimentally that the successful establishment of *Laboulbenia slackensis* requires not only a suitable host but also favorable environmental conditions, which could be linked to the extensive appendage system of that species. Recently, Tragust *et al.* (2016) found no visible penetration damage at the host integument using light and electron microscopy techniques in four species of Laboulbeniales, revealing the necessity for alternative explanations to the hypothesis that Laboulbeniales may only receive nutrients through a haustorium. Further experimental work might be directed

toward the function of the sterile appendages; this would entain injecting dye in thalli of both haustorial and non-haustorial representatives of Laboulbeniales (see *Notes on ecology*).

Spermatia are produced either exogeneously or endogeneously within simple or compound antheridia (**Fig. 3E–H**). Exogenous spermatial formation has mainly been observed in species that have aquatic hosts (Weir and Blackwell, 2005), such as species of *Ceratomyces* and *Zodiomyces*. In these genera, spermatia may be borne on intercalary cells or terminally on a short branchlet (Majewski, 1994). Simple antheridia are flask-shaped, with the neck serving as a discharge tube. Sometimes, old antheridia can proliferate into sterile branches, this is often seen in members of *Laboulbenia*. In some genera, corner cells or intercalary cells of the appendage serve as antheridia with only the discharge tube being free. Most Laboulbeniales possess simple antheridia. Compound antheridia only occur in taxa of Monoicomycetoideae and Peyritschielloideae. Antheridial cells are structurally united and release their spermatia into a chamber that has a single opening. In the subfamily Monoicomycetoideae, compound antheridia are distally rounded and lack a discharge tube. Compound antheridia with an elongated neck occur in the Peyritschielloideae. This observation led Faull (1911) to suggest that compound antheridia had arisen independently more than once. Almost a century later, this suggestion was confirmed based on molecular phylogenetic data (Goldmann and Weir, 2018; Haelewaters, 2018). Antheridial characters were important to Thaxter's (1896, 1908) – obsolete – classification system.

Ascospores

The ascospores of Laboulbeniales are two-celled, hyaline, elongate, and spindle-shaped. They are typically surrounded by a mucilaginous envelope, which provides adhesiveness. In addition, the foot is usually melanized before release. The ascospores are almost exclusively transferred by the activities of the host (De Kesel, 1995a; Cottrell and Riddick, 2012). Ascospores are produced in perithecia such that their larger cell, which becomes attached to the host, is directed upwards and subsequently released first.

Herpomycetales

Until very recently, discussions of "Laboulbeniales" included the order Herpomycetales, which is now separated based on molecular and morphological evidence. Herpomycetales was erected following the molecular phylogenic analysis of a three-locus rDNA dataset. The order is monotypic, with the dioecious *Herpomyces* as the single genus. The genus currently includes 27 species, all described between 1902 and 1931 except for two recent species, *H. shelfordellae* and *H. spegazzinii* (Haelewaters *et al.*, 2019d; Gutierrez *et al.*, 2020). All species are exclusively associated with cockroaches (Blattodea), both nymphs and adults. Thalli of *Herpomyces* are developmentally and morphologically distinct (Tavares, 1965, 1966, 1980, 1985; Hill, 1977). This evidence provides additional support for the idea that thallus formation in the two orders Herpomycetales and Laboulbeniales has evolved independently (**Fig. 1D**). For example, whereas both orders have double-layered perithecia, the way their perithecial walls are formed is fundamentally different. Ascus formation, number of ascospores per ascus, and positioning of the ascospore septum are also different between Herpomycetales and Laboulbeniales (reviewed in Haelewaters *et al.*, 2019d). *Herpomyces* also perforates its host in multiple places whereas Laboulbeniales perforate their host only at the single point of attachment or not at all (Tragust *et al.*, 2016). Species of *Herpomyces* can vary in their morphology depending on their position on the host (Thaxter, 1908, Tavares, 1985), as is also the case with some members of Laboulbeniales.

The thallus of *Herpomyces* has a differently structured receptacle compared to genera of Laboulbeniales. The primary receptacle of female thalli is small, typically consisting of four cells. The suprabasal cell gives rise to a secondary axis that consists of a series of narrow cells each perforating the integument of the host by small haustoria. Male thalli are similar in that they have a primary axis, usually consisting of four superposed cells, and that the suprabasal cell may produce a secondary axis; both the third and fourth cell may give rise to a single cell or branch carrying antheridia. The perithecium of Herpomycetales has four vertical rows of outer wall cells each consisting of many cells equal in height. This condition, which is also seen in Ceratomycetaceae, has been considered the "ancestral" perithecium but the situation is likely more complex provided the thallus of Herpomycetales and Laboulbeniales may have evolved independently. This evolutionary hypothesis is supported by the development of the perithecium that is different in *Herpomyces* and Laboulbeniales. The entire perithecium of *Herpomyces* develops from an outgrowth of the suprabasal cell of the 4-celled primary receptacle, by subsequent transverse and longitudinal divisions (Tavares, 1965, 1966). The carpogonial upgrowth(s) is initiated by a specific outer wall cell. Finally, in the *Herpomyces* ascus mother cell, mitosis will take place after meiosis, forming an ascus with 8 ascospores, as do most of the other species in Ascomycota. The entire ontogeny, from ascospore to mature thallus, was studied for *Herpomyces ectobiae* (Tavares, 1985).

Contrary to infections with most species of Laboulbeniales, *Herpomyces* infections often display a high parasite prevalence. For example, Richards and Smith (1955) mentioned a 100% prevalence of *H. stylopygae* on 50 *Blatta orientalis* cockroaches. Similarly, Wang *et al.* (2016) reported a 96.8% prevalence of *H. chaetophilus* on 31 *P. americana* roaches. This can be explained by the fact that populations of cockroaches are often densely packed, they occur in moist, damp environments, and they are in constant contact with each other, for example by their grooming behavior. Pfliegler *et al.* (2018) studied eleven populations of cockroaches, originating from either pet stores, biological supply companies, or laboratory colonies. In eight populations, infections with *Herpomyces* spp. were detected, and parasite prevalence ranged between 8.77% and 86.36%. The authors suggested that at least some species of *Herpomyces* are spread by globally invasive host species as well as through the international pet and pet food trade.

Pyxidiophorales, Hyphal Mycoparasites

The order Pyxidiophorales contains the perithecial genus *Pyxidiophora* and *Mycorhynchidium*, reported to vary by its cleistothecial form. *Pyxidiophora* and close relatives are contact mycoparasites varying in their reliance on a host fungus from greatly improved growth to obligate fungal biotrophy (Kirschner, 2003; Jacobs *et al.*, 2005). The life cycle of most species of *Pyxidiophora* is complicated, consisting of three different morphs: (1) a dispersal morph (*Thaxteriola* state) derived from an ascospore that delivers conidia to a fresh substrate, (2) a conidial morph developed on hyphae, and (3) an ascospore-producing perithecial morph to come full circle (**Fig. 5**). Only 21 species of *Pyxidiophora* have been formally described although it is unclear whether they are all distinct (Lundqvist, 1980; Doveri and Coué, 2006). At least five undescribed species are known (M. Blackwell and M. Gorczak, unpublished) and more are certain to be discovered. Names listed as synonyms of *Pyxidiophora* in MycoBank (2020) are based on all three life cycle states of *Pyxidiophora* (see below).

Taxonomy and Phylogeny

Pyxidiophora was described by Brefeld and Von Tavel (1891), and its complicated nomenclatural history discussed by Lundqvist (1980) resulted in typification with *Pyxidiophora asterophora*. Both MycoBank (2020) and Index Fungorum (2020), however, list *P. nyctalidis* as the type species, likely because of recent retroactive changes in the *International Code of Nomenclature for algae, fungi, and plants* (Turland *et al.*, 2018). The gradual maturation of the perithecium and its ephemeral nature, slow ascospore maturation, difficulty of cultivation, and the complex three-morph life history of these fungi, hampered the use of morphological characters. For example, the early deliquescent asci lead some researchers to describe *Pyxidiophora* perithecia as pycnidia (e.g., *Mycorhynchus)* (Petch, 1936). These problems have caused creation of many synonyms based on the three different morphs (perithecial, hyphal phialidic conidial, and ascospore-derived dispersal morphs) in the life cycle.

Genera based on the perithecial morph are considered synonyms of *Pyxidiophora*, often based on immature specimens: *Ascolanthanus*, *Coprophilous*, *Mycorhynchus*, *Rhynchonectria*, *Treleasia* (Hawksworth and Webster, 1977; Lundqvist, 1980), and perhaps the cleistothecial *Mycorhynchidium*. The type of *Treleasia* is no longer intact, but Spegazzini's drawing of the ascospores and perithecial neck (Arambarri *et al.*, 2007) leave no doubt of the identity of a species from an unusual habitat: rolled, deteriorating leaves of sugar cane. Named phialidic conidial morphs derived from hyphae include *Chalara*-like, *Gabarnaudia*-like, *Gliocephalis*, and *Pleurocatena* (Arnaud, 1952, 1953; Blackwell and Malloch, 1989b; Jacobs *et al.*, 2005; Gams and Arnold, 2007). No perithecial state is known for *Gliocephalis hyalina* (Jacobs *et al.* 2005), but an SSU rDNA sequence places it in *Pyxidiophora*, and culture attempts of this fungus were successful only when co-inoculated with *Fusarium* as expected of a mycoparasite (Corlett, 1986). Other synonyms are based on the dispersal morph developed from the ascospore: *Acariniola*, *Thaxteriola*, and perhaps other ascospore-derived forms (Blackwell, 1994). The presumptive ascospore derived morphs including *Amphoropsis*, *Endosporella*, *Entomocosma*, *Myriapodophila*, and *Thaxteriola* spp. were placed in an informal group, "Thaxteriolae," although this name has not been defined consistently (Thaxter, 1914, 1920; Spegazzini, 1918; Majewski and Wiśniewski, 1978; Blackwell, 1994; Blackwell *et al.*, 2020). Gäumann (in Gäumann and Dodge, 1928) "regarded" some of these forms as male thalli of Laboulbeniales, an idea rejected by Thaxter (Gäumann and Dodge, 1928).

The ordinal placement of *Pyxidiophora* was uncertain until DNA analyses became available. Most often it was placed in the order Hypocreales (e.g., Lundqvist, 1980; Barr, 1990) or considered a member of the order Ophiostomatales (von Arx and van der Walt, 1987; Eriksson and Hawksworth, 1989, Blackwell and Spatafora, 1994). Based on a primarily speculative review (Blackwell and Malloch, 1989b), Eriksson and Hawksworth (1993), placed *Pyxidiophora* in the Laboulbeniales. Soon after, with increased taxon sampling, the higher-level classification of Pyxidiophorales (P.F. Cannon in Kirk *et al.*, 2001) was formally established in the family Pyxidiophoraceae (Arnold, 1971). If they exist, closer relatives of the class Laboulbeniomycetes other than Sordariomycetes among Ascomycota have not been discovered (Blackwell *et al.*, 2020).

Morphology of Pyxidiophorales Life Cycle States

The three-morph life cycle of *Pyxidiophora* described above with its specialized dispersal morph, is unique among Ascomycota (**Fig. 5**). It has been compared to the life cycles of plant-parasitic rust fungi (Basidiomycota) with respect to Tranzschel's law in its host associations (Malloch, 1995; Blackwell *et al.*, 2020).

Perithecial morphs

Ascocarps of *Pyxidiophora* are perithecia (**Fig. 4C,D**). One species, *Mycorhynchidium saccatum*, was described as cleistothecial from a moist chamber development (Malloch and Cain, 1971). The pseudoparenchymatous perithecia usually have a distinct bulbous base with irregularly angular to globose cells and tapers slowly to an elongating neck composed of parallel, closely packed cells (Hawksworth and Webster, 1977; Lundqvist, 1980; Kirschner, 2003; Doveri and Coué, 2006). A unique feature of the mature perithecial peridium is that it is composed of a single layer of cells. This unusual character is known only in species of *Kathistes* among other perithecial ascomycetes (Malloch and Blackwell, 1990). Interascal tissues (e.g., paraphyses) have not been observed in any species of *Pyxidiophora*. The presence of an apical ring may be ephemeral and Lundqvist (1980) does not consider it a generic character. Perithecia can be single or grouped, free on the substrate or developed on a stroma, hairy or naked, and short to very long necked. Unlike thallus-forming Laboulbeniomycetes,

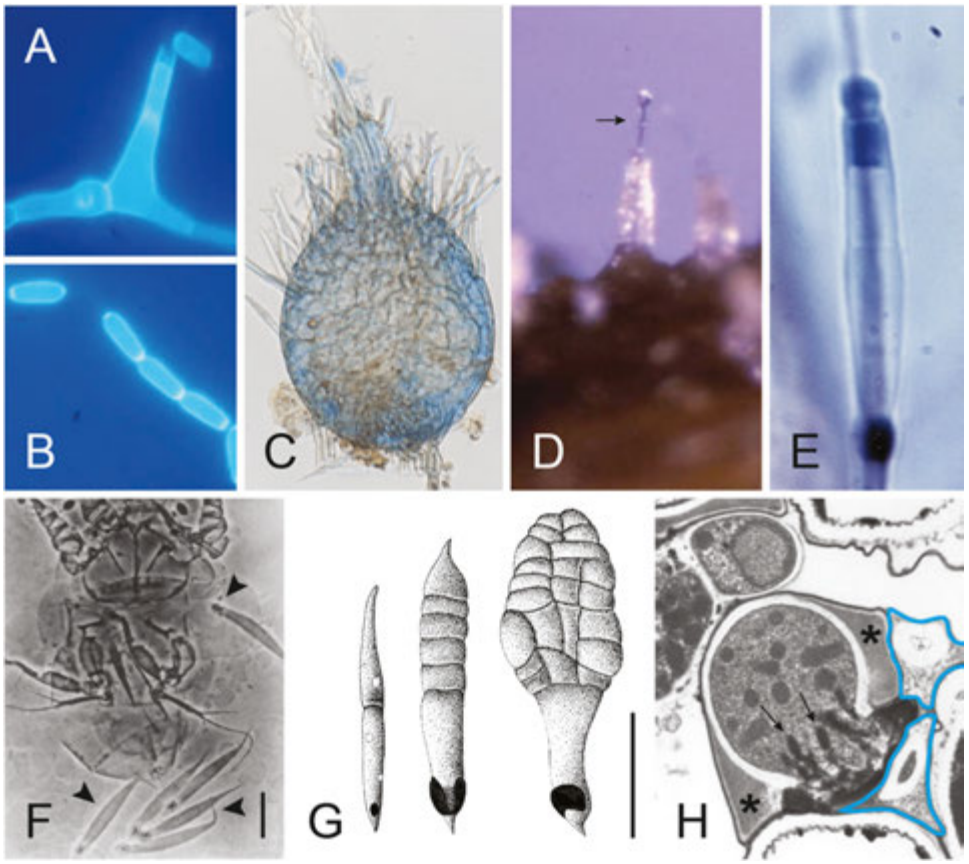

Fig. 4 Pyxidiophorales. **A–B.** *Chalara*-like conidial hyphal morph from a two-membered culture on Leonian's agar. Fluorescence microscopy using calcofluor white stain. **A.** Conidial succession and second conidium differentiated within a phialide. **B.** Chain of conidia showing contact pads; note the similarity with conidia of *Gliocephalis hyalina* (Jacobs *et al.*, 2005). **C.** Perithecium of *Pyxidiophora corallisetosa*, Białowieża Primeval Forest, Poland. **D.** Perithecium of *Pyxidiophora* sp. in moist chamber of moose dung, New Brunswick, Canada; the base is partially embedded in the dung; ascospores at the perithecial tip (arrow) are ready to attach to a disperser. **E.** Ascospore-derived conidial morph (*Thaxteriola*) with darkened attachment region, developed in moose dung moist chamber, Ontario, Canada. Lactophenol-cotton blue stain. **F.** *Tarsonemus ips* mite with six *Thaxteriola* sp. thalli (arrowheads). Picture by John C. Moser. **G.** Developing thallus of *Thaxteriola* sp. The parenchymatous thalli are not common until after the mites are held in moist chambers past the time they would become phoretic. Pencil drawing after David Malloch by Jingyu Liu. **H.** Attachment region of the ascospore-derived conidial morph; note the thickened spore wall (*) and secretory channels (arrows) with electron dense material that are similar to those in *Coreomycetopsis* and *Laboulbeniopsis*. Basal cells of other ascospores are encircled in blue. Transmission electron micrograph. **D–F, H.** Reprinted with permission from Mycologia. © The Mycological Society of America. Scale bars F, G 25 μm. Reproduced from Blackwell, M., 1994. Minute mycological mysteries: The influence of arthropods on the lives of fungi. Mycologia 86 (1), 1–17. Blackwell, M., Perry, T.J., Bridges, J.R., Moser, J.C., 1986. A new species of *Pyxidiophora* and its *Thaxteriola* anamorph. Mycologia 78 (4), 605–612.

cell-by-cell development of fruiting bodies of Pyxidiophorales is not known in detail but likely is not fixed as in Herpomycetales and Laboulbeniales. Asci are not reported for all species because of their early evanescence (Hawksworth and Webster, 1977; Lundqvist, 1980). Production of ascocarps in pure culture was reported for *Pleurocatena acicularis* (Gams and Arnold, 2007) and in mixed cultures for *Pyxidiophora* [as *Ascolanthanus*] *trisporus* (Cailleux, 1967), *Pyxidiophora asterophora* (Brefeld and Von Tavel, 1891), *P. corallisetosa* (Kirschner, 2003), and others (M. Blackwell, unpublished).

Asci of *Pyxidiophora* are unitunicate, thin-walled, non-amyloid and fusiform, clavate or obovoid. The number of ascospores per ascus is 3–8. The common condition of three ascospores per ascus in this genus is unusual among ascomycetes. Viewed with transmission electron microscopy, 3-spored asci appear to be due to exclusion of one of four post-meiotic nuclei from the enveloping membrane system during ascosporogenesis (M. Blackwell and E.A. Richardson, unpublished); details are not known. Asci mature sequentially and the ascospores are released passively in a sticky droplet hanging at the tip of the perithecium, awaiting an arthropod for dispersal (Breton and Faurel, 1967; Blackwell, 1994). Ascospores are two-celled, single-septate, usually symmetrical, and enveloped in a mucilaginous sheath before maturity (Lundqvist, 1980; Blackwell and Malloch, 1989a; Blackwell *et al.*, 1989). At maturity, ascospores of *Pyxidiophora* are single-septate, elongated, and fusiform to subclavate. For all species that have been observed at maturity, ascospores have a darkened attachment apparatus at their exiting ends by the time they exit the

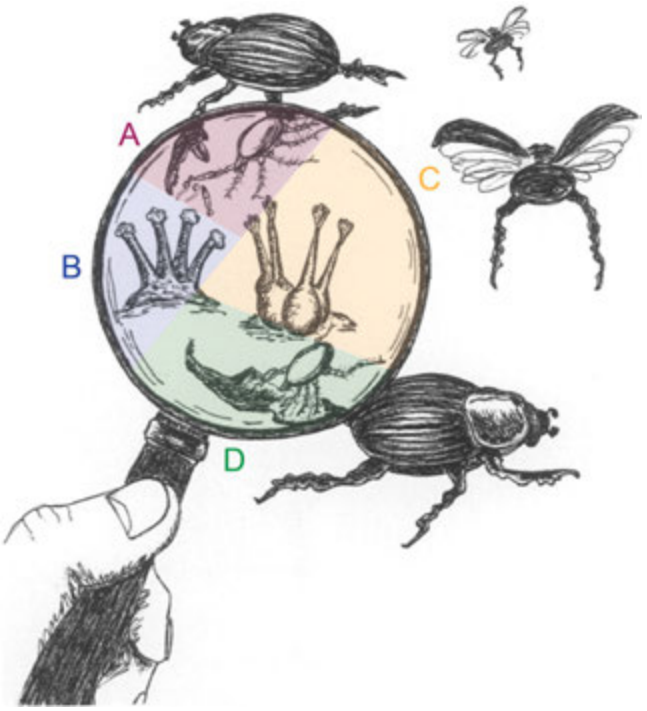

Fig. 5 Cartoon illustrating the life history of *Pyxidiophora* sp. **A.** A beetle and phoretic mite carrying an ascospore-derived dispersal morph arrive at a substrate where a suitable host fungus is growing. **B.** Conidial morph develops nourished by the host fungus in the substrate and produces conidia, which are spread locally on the surface of the dung by immature mites and nematodes. **C.** The next event is the development of perithecia, followed by evanescence of the asci and release of sticky ascospores to the tip of the perithecium neck. **D.** The phoretic mite bearing ascospores attaches to a beetle disperser. The attached ascospores develop into the dispersal morphs to reinitiate the cycle in another targeted substrate. Pencil drawing by Ty Keller. Reprinted with permission from Mycologia. © The Mycological Society of America. Reproduced from Blackwell, M., 1994. Minute mycological mysteries: The influence of arthropods on the lives of fungi. Mycologia 86 (1), 1–17.

perithecium. This is one of the inconsistent features in species descriptions, resulting from the observation of immature material (Blackwell, 1994).

Phialidic conidial hyphal morphs

A number of different conidial states arise from the hyphae, and these were described in connection with *Pyxidiophora* (Lundqvist, 1980; Gams and Arnold, 2007). The conidia of *Pyxidiophora* are blunt-ended or bullet-shaped conidia, often in chains. Arthric anamorphs probably were inaccurately mistaken for chains of conidia (Cailleux, 1967). The holoblastic conidia are produced in phialides on the mycelium. Most have been described as *Chalara*-like (e.g., in *P. asterophora*, *P. spinuliformis*) or *Gabarnaudia*-like (e.g., in *P. corallisetosa*, *P. cuniculicola*). *Pleurocatena*, first observed in the early 1950s (Arnaud, 1952, 1953; Arambarri *et al.*, 1981), was studied again in culture by Gams and Arnold (2007) with the new strains described as *Gabardnaudia*-like, although living cultures were not maintained. The authors distinguished the conidial state on the presence of setae mixed among the phialides. They connected the conidial state to *Pyxidiophora* sp. when perithecia were developed in a culture of *Pleurocatena acicularis* after several months. Other hyphal forms, probably also with phialidic conidia have been described, including *Gliocephalis hyalina* for which no perithecial state is known (Jacobs *et al.*, 2005). *Pyxidiophora spinulorostrata* was described as having heavily walled outgrowths near the perithecial necks that produced conidia (Webster and Hawksworth, 1986). Perithecia of the same species from the type locality (River Teign, Devon), however, did not produce such conidiophores; instead, a *Gabarnaudia*-like anamorph was observed before appearance of perithecia (M. Blackwell, unpublished).

Ascospore-derived conidial morphs

The second type of conidial morph in the three-morph life cycle develops from an ascospore (**Fig. 4E–H**). Although the entire life cycle has not been studied in all species, the ascospore-derived form occurs consistently in the best studied species of *Pyxidiophora*. Germination by germ tube has been observed on agar but rarely. Most of the ascospore-developed morphs have been referred to as *Thaxteriola* or *Acariniola*. The ascospores have gelatinous mucus sheaths early in ascospore maturation. The dark-pigmented attachment apparatus develops at the exiting end of the ascospores by the time they are released in a sticky droplet at the tip of the perithecial until disperser contact. In at least five observed species of *Pyxidiophora* the primary dispersers were phoretic mites. TEM revealed the basal cell apparatus had a system of channels with an electron-dense material that were proposed to secrete a presumptive "glue" to attach the cell to the phoretic mite (**Fig. 4H**; Blackwell, 1994). After attachment of an ascospore to the

phoretic mite disperser, conidium development began (**Fig. 4E**). Ascospores of one strain were examined for up to a week under glass cover slips on agar. Yeast cells budded from the conidia, and within 24 h, several rounds of yeast cells had been produced and the yeast cells developed germ tubes several days later (Blackwell and Malloch, 1989b). Unless a fungal host was available, there was no further development in that strain.

As mentioned above, a number of minute fungi described from insects have been suggested to be conidial morphs of *Pyxidiophora* (Blackwell, 1994). The genera *Amphoropsis*, *Endosporella*, *Entomocosma*, *Myriapodophila*, and *Endosporella*, were described by Spegazzini (1918) and Thaxter (1920). Spegazzini suggested grouping a number of these minute fungi in the "Thaxteriolae" but Thaxter (1920: 15) doubted whether "these uninteresting little plants" were closely related to each other (see Blackwell *et al.*, 1986b, 2020; Blackwell, 1994). After *Acariniola* was described (Majewski and Wiśniewski, 1978), Lundqvist (1980) placed that genus in synonomy with *Pyxidiophora*.

Fine-Tuned Life Histories

The life history of *Pyxidiophora* spp. is a fine-tuned association of a variety of organisms. Accumulating evidence from axenic culture attempts has led to the conclusion that most species of *Pyxidiophora* are mycoparasitic or at least they grow better in two-membered culture with appropriate fungi (Corlett, 1986; Webster and Hawksworth, 1986; Blackwell and Malloch, 1989b; Kirschner, 2003; Jacobs *et al.*, 2005). Some species of *Pyxidiophora* were once considered saprotrophs (Lundqvist, 1980), and difficulties in obtaining axenic cultures led to earlier assumptions of nematophagous or bacteria-dependent modes of nutrition (Cailleux, 1967). The superb light and transmission electron micrographs of *Gliocephalis hyalina* (Jacobs *et al.*, 2005) provided strong evidence of contact mycoparasitism. It should be noted that two strains of *Pyxidiophora arvernensis* (CBS 657.82, CBS 253.81) isolated from *Rhizoctonia solani* baits in soil, have been used for DNA sequencing, because they do grow, albeit poorly, in axenic culture (Schoch *et al.*, 2012).

Timing is essential to the closely associated assemblage of organisms. For example, species on dung substrates use fungal hosts that arrive with the dung substrate by gut passage (Blackwell and Malloch, 1989b). Potential fungal hosts for *Pyxidiophora* spp. on seaweed probably arrive on the beach already growing in the seaweed, ready to nourish *Pyxidiophora* conidial morphs dispersed by arthropods on the beach (M. Blackwell and D. Malloch, unpublished). Kirschner (2003) pointed out that *P. corallisetosa* and *P. cuniculicola* and their fungal hosts were dispersed together by arthropods. *Pleurocatena acicularis*, a *Pyxidiophora* hyphal conidial state, was baited with fungi from soil (Gams and Arnold, 2007). The actual host of *P. asterophora*, observed on the mushroom of *Asterophora lycoperdoides* (Agaricales, Agaricomycetes), is unclear. *Asterophora* spp. are parasites of other mushrooms (*Lactarius* and *Russula* species), and an assemblage of organisms is present on the *Asterophora* mushrooms, including a tremellaceous yeast (Prillinger *et al.*, 2007) and fungi and bacteria developed in age (M. Blackwell, unpublished). The actual host of *Pyxidiophora asterophora*, therefore, remains unknown, but it may not be the mushroom. Buller (1924) suggested air dispersal of *Asterophora* chlamydospores and, although scarce in some of the mushrooms, basidiospores. Because the timing of arrival and dispersal is essential in the life history, it would be informative to know if these events occur consistently.

Documented fungal hosts used by *Pyxidiophora* include *Ascobolus* sp. (Pezizales, Pezizomycetes) growing on moose dung for *Pyxidiophora* sp. (**Fig. 6A**; Haelewaters, 2014), *Clonostachys rosea* (Hypocreales) for *P. corallisetosa*, *Esteya vermicola* (Pezizomycotina *incertae sedis*) for *P. cuniculicola* (Kirschner, 2003), *Fusarium poae* (Hypocreales) on seeds of *Triticum aestivum* for *P. lundqvistii* (Corlett, 1986), *Neonectria lugdunensis* (Hypocreales, Sordariomycetes) for *Pyxidiophora spinulorostrata* (Webster and

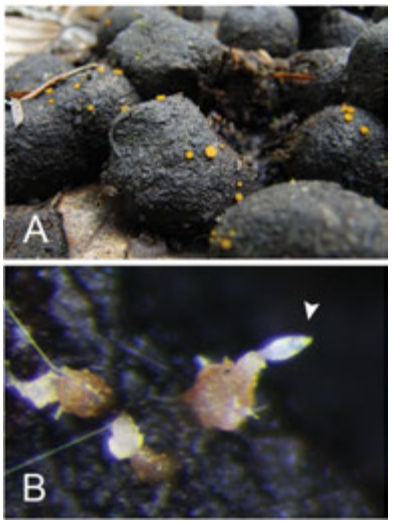

Fig. 6 Substrates for *Pyxidiophora*. **A.** Moose dung with apothecia of *Ascobolus* sp., from which perithecia of *Pyxidiophora* were isolated (White Mountain National Forest, Maine, USA). **B.** Perithecium of *P. corallisetosa* with package of ascospores at the tip (arrowhead), from a gallery of *Ips typographus*, the European spruce bark beetle, in Norway spruce (Białowieża Primeval Forest, Poland).

Hawksworth, 1986), and apothecia of coprophilous Pezizales for *Pyxidiophora* sp. and *P. spinuliformis* (Blackwell and Malloch, 1989b). Specificity of the fungal hosts of *Pyxidiophora* has not been studied in detail but has been noted (Corlett, 1986; M. Blackwell and D. Malloch, unpublished).

Blackwell and Malloch (1989b) studied the life histories of *Pyxidiophora* sp. and *Pyxidiophora spinuliformis* from moose dung in Algonquin Park, Ontario, Canada, and gathered data on timing of those species. The study was conducted by leaving the major part of a dung pile in the field for daily comparison with portions of the same substrate in moist chamber in the laboratory. On the day of deposition, the dung had no surface growth noticeable to the eye. The first appearance of *Pyxidiophora* sp. occurred after 5–7 days when synnemata of the hyaline *Chalara*-like conidial state (**Fig. 4A,B**) developed, clustered on the apothecium of a host fungus. Maximum conidium viability at seven days corresponded with rapid movements by immature nematodes and fungus-feeding and predaceous mites that apparently spread the conidia on the dung surface; the nematodes may be food for the mites. A week after dung deposition, perithecia developed in the vicinity of the synnemata, often growing though the synnemata. Ascospores matured rather slowly beginning within the ascus and continuing after ascus evanescence and ascospores were released passively to the perithecium tip. Ascospores were transported by predaceous mites 1–3 days after ascospore release (**Fig. 4F**). The second species, *P. spinuliformis*, developed more slowly compared to *Pyxidiophora* sp., with the hyphal conidial state of *P. spinuliformis* appearing 12–15 days after appearance of *Pyxidiophora* sp., and perithecia developing 3–4 days later on the same apothecia. Passive ascospore release to the perithecium tip corresponded in time with maturation of a different mite. Ascospore maturation coincided precisely with mite maturation judged by the number of attached ascospores compared.

The timing of life cycles of species on substrates other than dung is not well known. One example, *P. spinulorostrata*, has a longer incubation period than species growing on ephemeral substrates. With the help of John Webster, twigs were collected from the River Teign in Devon, England, the type locality of the species, and mailed to Baton Rouge, Louisiana, USA, where they were incubated in plastic containers at room temperature for observation (M. Blackwell, unpublished). About a month after collection, *Gabardnaudia*-like conidia developed, and about 7 days later, *P. spinulorostrata* perithecia appeared in association with *Heliscus lugdunensis*. No additional information, especially on potential vectors, is available. Conidia were not observed on the perithecial outgrowths as had been described (Webster and Hawksworth, 1986).

In addition to the need for a host, a second requirement for *Pyxidiophora* life cycle completion is escape from a depleted substrate. Insects, often beetles, are essential for targeted dispersal of the ascospore-derived morphs. Mites, however, may be of vital importance (Blackwell *et al.*, 1986a, 1986b, 1989). For example, *Pyxidiophora corallisetosa* and *P. kimbroughi* grow in the secondary phloem in bark beetle galleries (**Fig. 6B**). Bark beetles, however, mature in the outer bark of the tree requiring phoretic mites to transport the ascospores to the beetle vector. On substrates that can be observed, mites are more plentiful than beetles and they become very active, almost frantic in human terms, in moist chamber cultures coinciding with time of ascospore maturation (Blackwell *et al.*, 1986a; Blackwell and Malloch, 1989b).

Expanding Diversity

Easily discovered species of *Pyxidiophora* perithecial morphs have come from exposed substrates with abundant flying insects and phoretic mites. These include herbivore dung, fleshy fungi, beetle galleries in wood, decaying plant material, and beached seaweed known as wrack (Blackwell and Malloch, 1989a). Species occurring in bark beetle galleries, sometimes have been detected by observing the ascospore-derived conidial states on mites (Blackwell *et al.*, 1989). In fact, the wrack habitat was discovered by finding the ascospore-derived conidial state (*Thaxteriola*) attached to mites from wrack in the Natural History Museum, London. Unlike looking for a needle in a haystack, once a fertile habitat is discovered, *Pyxidiophora* species usually can be recollected. Some species have been discovered in BLAST searches of nucleotide databases. For example, the ITS sequence of *Pyxidiophora arvernensis* (CBS:657.82, GenBank accession number MH861535) was a close match with NCBI GenBank (accession number MF484620), "an uncultured Glomerales soil isolate." Is the sequence that of an arbuscular mycorrhizal (AM) fungus or is it more likely that of a *Pyxidiophora* using the AM fungus as a host? Several environmental ITS sequences from GenBank (see "Relevant Websites section"), the UNITE database (Abarenkov *et al.*, 2010), and Tedersoo *et al.* (2014) share $\geq$95% identity with sequences of known Pyxidiophorales (**Fig. 7**).

Laboulbeniopsis Clade

Laboulbeniopsis termitaria is an ectoparasite of termites, described from specimens collected in Grenada (Thaxter, 1920). The species was re-discovered fifty years later and is now known from Florida (Kimbrough and Gouger, 1970; Blackwell and Kimbrough, 1976b), Georgia (Blackwell, 1980a,b), Louisiana (Henk *et al.*, 2003), and Japan (Guswenrivo *et al.*, 2018). The minute thalli are under 150 μm in size and usually comprised of four superposed cells. The basal cell has a system of channels that secrete what is apparently a glue-like material attaching the thallus to the termite. This basal cell also contains a darkened pad, which seems diagnostic for *Laboulbeniopsis*. The elongated, and often brown in age distal-most cell produces spores at its base, which have been suggested to be ascospores. In fact, the attachment apparatus is almost identical with that of *Pyxidiophora* (see above) and *Coreomycetopsis* (see below). The presumptive asci evanesce and the presumptive ascospores are released through an apical ring at the thallus tip (Blackwell and Kimbrough, 1976b).

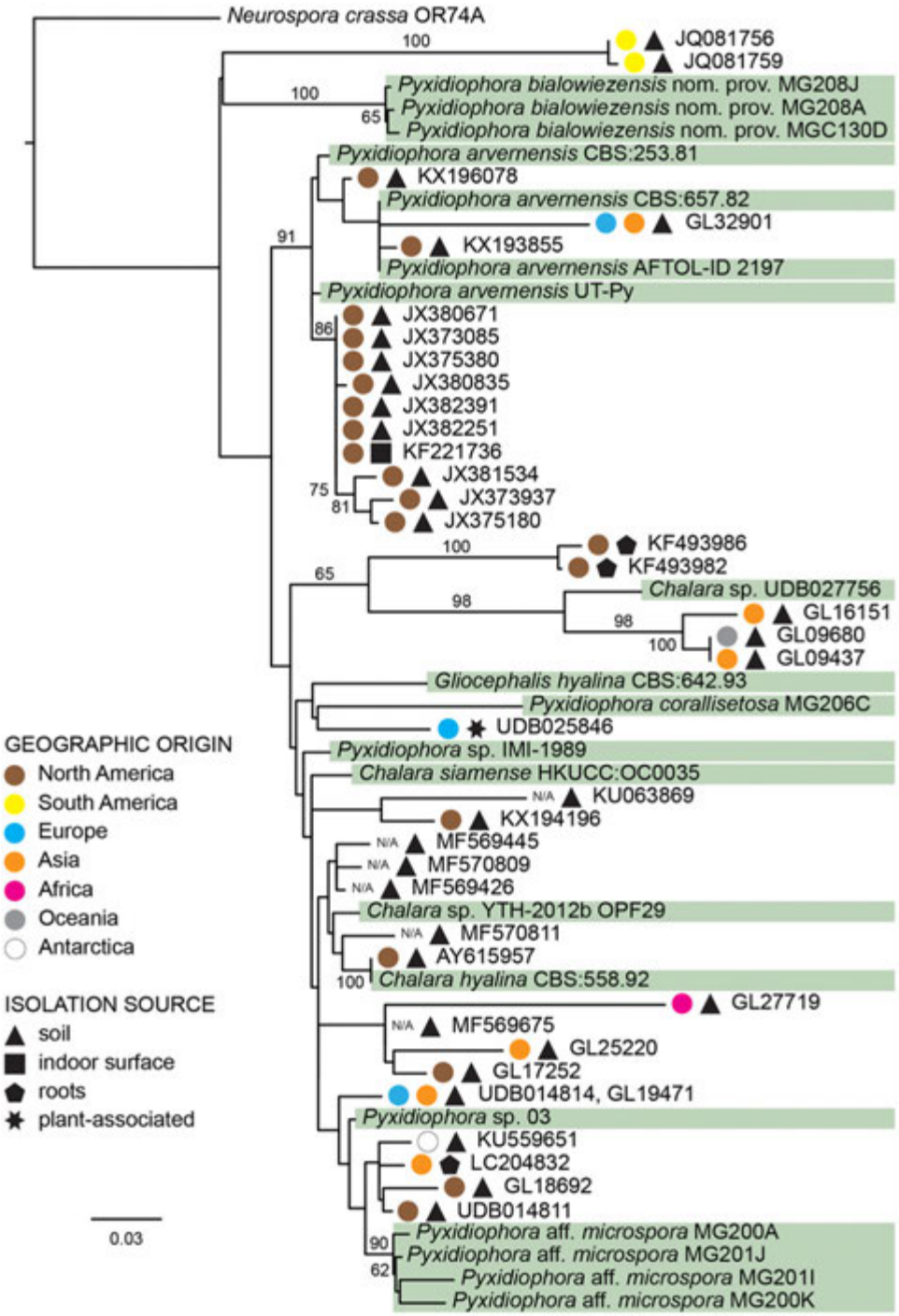

Fig. 7 Diversity of environmental ITS sequences of Pyxidiophorales. The topology is the result of maximum likelihood inference using RAxML (Stamatakis, 2014). Colored circles indicate geographic origin, black symbols indicate isolation source, sequences highlighted in green are known representatives of the order Pyxidiophorales.

An SSU rDNA phylogeny resulted in placed *Laboulbeniopsis* in the Laboulbeniomycetes with strong support (Henk *et al.*, 2003). Its placement was previously unknown even though it had been suggested to be associated with Laboulbeniomycetes based on morphology. More recently, Blackwell *et al.* (2020) found strong support for the sister relationship of *Laboulbeniopsis* and Pyxidiophorales in a two-locus rDNA phylogeny. The phylogeny revealed high support for five clades in the Laboulbeniomycetes: the orders Herpomycetales, Laboulbeniales, and Pyxidiophorales, and in addition the *Laboulbeniopsis* clade and the *Chantransiopsis* clade (**Fig. 1D**).

Coreomycetopsis oedipus, another minute fungus known only from termites, is placed in this clade based upon its morphological similarity to *Laboulbeniopsis*. The typical thallus of *C. oedipus* is under 150 μm in size and consists of fewer than fifteen superposed cells. Based on a report by Blackwell and Kimbrough (1976a), eight of the distal-most cells are obliterated by upward growth of thin filaments in the development of a "sporogonium." Some of the filaments developed as phialides and produced elongated phialospores. *Coreomycetopsis oedipus* is less often collected than *Laboulbeniopsis termitaria*, and although it may be less common, it is more difficult to discern against the pale termites because it has no dark pigments. It was described from Grenada (Thaxter, 1920) and since then recorded, in Florida (Blackwell and Kimbrough, 1976a), Georgia and Louisiana (M. Blackwell, unpublished), and Panama (D. Haelewaters, unpublished). Several mycologists suggested that *Coreomycetopsis* and *Laboulbeniopsis* may be different conditions or states of the same fungus, but morphological data are not supportive of this hypothesis (Blackwell and Kimbrough, 1976a,b). As already noted, at the ultrastructural level, the attachment region of *Laboulbeniopsis termitaria* and *C. oedipus* and *Pyxidiophora* are identical, though *Coreomycetopsis* lacks the darkened pad present in *Laboulbeniopsis*. Additionally, the terminal cells of the *Coreomycetopsis* thallus are thought to form a cavity where phialospores develop; a similar cavity in *Laboulbeniopsis* thalli is reported to produce ascospores in an evanescent ascus (Blackwell and Kimbrough, 1976b). The ultrastructural studies describing reproductive structures in these two termite-associated fungi need confirmation.

Chantransiopsis Clade

The *Chantransiopsis* clade comprises two conidial fungi known only as insect ectoparasites, *Chantransiopsis* and *Tetrameronycha* (**Fig. 1D**; Thaxter, 1914, 1920; Spegazzini, 1918; Rossi and Blackwell, 1990). Not much is known about these genera, other than their sparse filamentous growth occurs on insects. The other member of this clade is *Subbaromyces splendens*, a perithecial ascomycete with a distinctive collar-like structure at the base of the long perithecium neck, giving the perithecium the look of an injection syringe. *Subbaromyces splendens* was discovered on rocks in trickling sewage filter beds in New York, USA (Hesseltine, 1953). A second morphologically and ecologically similar species, *S. aquaticus*, was isolated from an open drain in Hyderabad, India (Manoharachary and Ramarao, 1974). Both species have limited growth in culture but produce conidia; production of perithecium and ascospores occurs only in mixed cultures. As with *Pyxidiophora*, these related fungi appear to have some dependence on other fungi, and perhaps are mycoparasites.

The genera *Chantransiopsis* and *Tetrameronycha* were for the first time included in the Laboulbeniomycetes by Goldmann and Weir (2018) in their SSU rDNA phylogenetic reconstruction of class members (**Fig. 1B**). Both genera were placed in a maximally supported clade in the phylogenetic reconstruction by Blackwell *et al.* (2020), presenting evidence for at least two clades with conidial states (*Chantransiopsis* clade, Pyxidiophorales) (**Fig. 1D**). Blackwell *et al.* (2020) generated sequences of *S. splendens* from a Hesseltine (1953) culture (CBS:357.53) and found high support for a placement among *Chantransiopsis* and *Tetrameronycha*. The inclusion of *Subbaromyces* in Laboulbeniomycetes was perhaps surprising, but the long-necked perithecia, evanescent asci, and accumulation of ascospores at the tip of perithecia are characteristic of other members of the class and suggests dispersal by arthropods.

Filling Knowledge Gaps

We close our discussion of the 170-year story of the Laboulbeniomycetes by pointing out missing chapters and pages. The basic outline for telling the complete story is a stable, well-resolved phylogeny that places these fungi within the rest of the ascomycetes; while we know that the Laboulbeniomycetes and Sordariomycetes form a monophyletic clade in the *Ascomycota Tree of Life*, the direct sister group of the Laboulbeniomycetes remains to be discovered. Progress in developing evolutionary hypotheses of the group will depend on (1) sampling new specimens, (2) studying the ecology, and (3) using multiple molecular markers and phylogenomic approaches.

Sampling new specimens of fungi associated as ectoparasites of arthropods but also free-living arthropod-dispersed fungi is crucial to obtain a complete picture of the diversity that is present in the class. Known host groups should continue to be investigated for the presence of Laboulbeniomycetes by both collecting in the field and screening museum collections. Existing literature will remain essential to point out hosts and localities in these endeavors. Priority should be given to those host species from which types have been described. Once infected specimens are found, sequences must be generated to define species limits and describe within-species phenotypic plasticity. In fact, not understanding the extent of morphological variation, obscures our conclusions concerning host shifting, specificity, and cryptic diversity.

Ecological aspects of Laboulbeniomycetes remain understudied. Climate effects on the distribution and survival of Laboulbeniomycetes and their hosts are poorly known. Focusing on ant- and bat fly-associated Laboulbeniales across Europe, Szentiványi *et al.* (2019) found that localities with low annual mean temperature and humidity, the likelihood of Laboulbeniales presence is higher. Welch *et al.* (2001) reported a prevalence of *Hesperomyces virescens* on *Adalia bipunctata* ladybirds as high as 54.7% in London, whereas prevalence had decreased to 0% at 25 km outside of the city. This extreme, short-range variation in prevalence was attributed to host phenology; the "urban heat island effect" shortens winters and may promote aphid growth – as do car pollution and urbanization. These factors increase host survival and enhance inter-generational contacts between ladybirds, providing ample opportunities for transmission of *H. virescens* (Welch *et al.*, 2001). Although these two examples seem to be

contradictory at first sight (lower versus higher temperature promoting development of Laboulbeniales), this illustrates that different Laboulbeniales may have different environmental preferences. More data are necessary from more parasite–host systems and larger geographical areas to pinpoint general patterns. A genomics approach has been used in a somewhat similar situation to look at fungal range extensions by detecting genome-wide differences in strains of the fungi involved that were associated with certain temperature and precipitation conditions across the range (Galagan *et al.*, 2005; Smith *et al.*, 2019).

Multiple molecular markers are already being applied to increase phylogenetic resolution and to discover additional members of the class among newly collected specimens. A significant milestone in Laboulbeniomycetes research, however, has been the acquisition of a 15-Mb draft genome sequence assembly of *Herpomyces periplanetae* (Haelewaters *et al.*, 2020b). An increasing number of reviews and research papers describe the use of genomics and proteomics applied to fungal mutualists (Biedermann and Vega, 2020) and pathogens of insects (Wang and Wang, 2017). Genome-scale data offer a short-cut to answering many questions, essentially turning any of the Laboulbeniomycetes into model fungi well suited for studies of symbiosis tracking interactions of fungi and their arthropod hosts.

Genomics will soon be used to predict reciprocal interactions of Laboulbeniomycetes and their associates. For example, basic questions about the *mode of nutrition*, can be addressed by an approach referred to as reverse ecology (Ellison *et al.*, 2011). In a study of endophytes of rubber trees, enzyme profiles were evidence of an unexpected insect-association rather than the expected plant-associated profile similar to that of the Xylariales (Gazis *et al.*, 2016). Correlations of variation in nutrition over parts of geographic ranges of broadly distributed species with genome changes (e.g., gene family expansions and enzyme family diversification) (Qian and Zhang, 2014), identification of genes unique to fungus–insect symbiosis (Wang *et al.*, 2018), and discovery of recurrent symbiont replacements by entomopathogenic fungi (Matsuura *et al.*, 2018), are the type of studies that will inform interactions among Laboulbeniomycetes and their associates. Are there other interactions, for example, signaling among Laboulbeniomycetes and loosely associated organisms (Becher *et al.*, 2018)? The tight associations suggest the possibility of horizontal gene transfer of siderophores found in microbe-packed insect guts (Tabima *et al.*, 2020).

Several species of Laboulbeniales and Herpomycetales occur on a *diverse variety of hosts* and have extremely *wide distribution ranges*, from subboreal to tropical areas. Molecular phylogenetic analysis and sequence-based species delimitation methods are necessary to evaluate whether these taxa are effectively ubiquitous, that is, capable of thriving on a wide range of hosts in many different microhabitats and climates, or whether they belong to separate cryptic or near-cryptic species. Examples are a number of taxa in *Laboulbenia*. One of the most widespread and most commonly sampled species is *L. flagellata*. Since its description (Peyritsch, 1873), it has been reported from more than 80 genera of Carabidae in many countries on all continents except Antarctica (Santamaría *et al.*, 1991; Majewski, 1994). Based on a limited LSU rDNA dataset, Haelewaters and De Kesel (2020) revealed three clades of *L. flagellata* sensu lato. It goes without saying that a lot more work will be needed to resolve the taxonomy of this and other species complexes. Genome comparisons have shown that variation occurs across taxa with broad distributions and have helped to identify genomic factors involved in adaptation in parts of the range (Galagan *et al.*, 2005; Smith *et al.*, 2019; Mei *et al.*, 2020).

Epilogue

Harkening back to the beginning of this chapter, the section "From Roland Thaxter to the Present: Synergy Among Mycologists, Entomologists, Parasitologists", and the mention of reviews at fifty-year intervals (Benjamin, 1971; Haelewaters *et al.*, 2021c), we anticipate a next comprehensive review – but likely many years before 2071. Major achievements in the taxonomy and systematics of Herpomycetales, Laboulbeniales, Pyxidiophorales, and related arthropod-associated fungi will be accomplished, but so much else can be discovered. We expect that significant progress based on molecular phylogenetic and phylogenomic studies, but also new collections, will speed the effort. But, who will there be write it? Perhaps a young student who is reading this chapter now.

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Phylogenetic Advances in Leotiomycetes, an Understudied Clade of Taxonomically and Ecologically Diverse Fungi

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Introduction

The class Leotiomycetes represents a large, diverse group of Pezizomycotina, Ascomycota (LoBuglio and Pfister, 2010; Johnston *et al.*, 2019) encompassing 6440 described species across 53 families and 630 genera (**Table 1**). Comprising, among other morphologies, the inoperculate discomycetes, Leotiomycetes fungi represent an enormous amount of ecological diversity – including mutualists and pathogens of plants, saprotrophs, animal pathogens, et cetera. Owing at least partially to their small size or the absence of a fruiting body, Leotiomycetes fungi are often overlooked in the field, in the mycology classroom, and in community ecology studies. The major challenges that currently exist in studying Leotiomycetes include a lack of understanding about (1) the subclass-level relationships within this clade, (2) the diversity of taxa that are exclusively detected by environmental DNA studies, and (3) the functional roles of such undescribed taxa in the environment.

Morphological and Ecological Diversity of Leotiomycetes

Morphology

Many Leotiomycetes that form sexual fruiting bodies have various forms of small apothecia with exposed hymenia as well as inoperculate asci – hence the historical grouping to which many Leotiomycetes belonged, the *inoperculate discomycetes* (Eriksson, 2005). The asci of Leotiomycetes taxa do not possess a lid-like structure (operculum); ascospores are extruded through an amyloid apical ring or by the asci splitting open apically (in which case they are inamyloid). Variations of the typical apothecium morphology exist within the class including those that remain closed until maturity as seen in several orders including Lahmiales and Rhytismatales. Other types of sexual fruiting bodies include the permanently closed cleistothecia, produced by *Cleistothelebolus*, *Leptokalpion*, *Thelebolus* (Thelebolales); *Bicornispora* (Rutstroemiaceae), *Connersia*, *Pleuroascus* (Helotiaceae), and members of Amorphothecaceae, Erysiphaceae, and Myxotrichaceae (Helotiales).

Several lineages are amenable to growth in axenic culture. Asexual reproduction is commonly observed in Leotiomycetes, with some taxa that are exclusively known from their asexual forms (e.g., Castañeda-Ruiz and Kendrick, 1990; Palmer *et al.*, 2014; Ashrafi *et al.*, 2018). Johnston *et al.* (2014) made nomenclatural recommendations to reconcile genera where both sexual and asexual morphs were independently named.

Ecology

Known ecologies from this clade are highly diverse, but Leotiomycetes are most commonly known in association with plants either as saprotrophs on already dead material (Baral and Haelewaters, 2015; Hernández-Restrepo *et al.*, 2017; Haelewaters *et al.*, 2018b), endophytes of roots, leaves, bark (Griffith and Boddy 1990; Rodriguez *et al.*, 2009; Grünig *et al.*, 2011), mycorrhizae (Cairney, 2006), or pathogens of roots, shoots, and leaves (Glawe, 2008; Saharan and Mehta, 2008). The plant pathogens in this class are of considerable economic importance – including the powdery mildews of cucurbits and other food crops and the white mold, *Sclerotinia*, that can infect at least 408 species of plants at any stage of development and any tissue type. Bryophilous (or bryosymbiotic, moss-associated) taxa exist as well (Döbbeler, 1997; Stenroos *et al.*, 2010). Examples are members of *Bryoscyphus* and *Mniaecia*, which are biotrophic parasites. Many Leotiomycetes are ecologically classified as aquatic hyphomycetes, which decay various plant material in freshwater ecosystems (Baschien *et al.*, 2013).

Taxa not associated with plants are also well represented throughout the class. These include the recently described *Polyphilus*, a genus associated with nematodes, truffle fungi, and plant roots (Ashrafi *et al.*, 2018). Other species associated with animals are species of *Pseudogymnoascus* such as *P. destructans* (the causal agent of white-nose syndrome in bats) (Gargas *et al.*, 2009) and *P. pannorum* (a pathogen of humans) (Gianni *et al.*, 2003). There are many so-called lichenicolous taxa (e.g., *Epicladonia*), which fruit epiphytically on lichens, and the newly described lichen-forming leotiomycete, *Lichinodium* (Prieto *et al.*, 2019). In addition, one species of *Trochila* is a potential mycoparasite on rusts (Gómez-Zapata *et al.*, 2021). Like many fungi in Ascomycota, Leotiomycetes are important producers of secondary metabolites (Vaca and Chavez, 2019), including *Glarea*, which makes pneumocandin B – the precursor to one of the most potent antifungal drugs, Caspofungin B (Chen *et al.*, 2013). Yet many isolated Leotiomycetes, such as *Glarea*, still have unknown ecological roles.

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Table 1 Current classification of the class Leotiomycetes with numbers of genera and species per family[a]

	Order, Family	*Genera*	*Species*
	Order Chaetomellales		
1	Family Chaetomellaceae	4	75
	Order Cyttariales		
2	Family Cyttariaceae	1	13
	Order Helotiales		
3	Family Amorphothecaceae	1	21
4	Family Arachnopezizaceae	4	65
5	Family Ascocorticiaceae	3	4
6	Family Ascodichaenaceae	2	4
7	Family Bloxamiaceae	1	19
8	Family Bryoglossaceae	5	8
9	Family Calloriaceae	14	152
10	Family Cenangiaceae	11	156
11	Family Chlorociboriaceae	1	23
12	Family Chlorospleniaceae	1	17
13	Family Chrysodiscaceae	1	1
14	Family Cordieritidaceae	18	117
15	Family Dermateaceae	12	227
16	Family Discinellaceae	12	75
17	Family Drepanopezizaceae	8	48
18	Family Erysiphaceae	20	976
19	Family Gelatinodiscaceae	9	50
20	Family Godroniaceae	5	43
21	Family Helotiaceae (including Roesleriaceae)	31	483
22	Family Heterosphaeriaceae	1	7
23	Family Hyaloscyphaceae	38	219
24	Family Lachnaceae	17	237
25	Family Leptodontidiaceae	1	11
26	Family Loramycetaceae	2	4
27	Family Mitrulaceae	1	16
28	Family Mollisiaceae	19	382
29	Family Myxotrichaceae	4	45
30	Family Neocrinulaceae	1	2
31	Family Neolauriomycetaceae	3	8
32	Family Pezizellaceae	24	277
33	Family Ploettnerulaceae	12	245
34	Family Rutstroemiaceae	7	115
35	Family Sclerotiniaceae	31	278
36	Family Vibrisseaceae	5	42
37	*Hysteropezizella* lineage	1	26
38	*Stamnaria* lineage	8	119
39	*Strossmayeria* lineage	2	42
40	Helotiales genera *incertae sedis*	136	516
	Order Lahmiales		
41	Family Lahmiaceae	1	2
	Order Lauriomycetales		
42	Family Lauriomycetaceae	1	11
	Order Leotiales		
43	Family Cochlearomycetaceae	2	5
44	Family Leotiaceae	4	51
45	Family Mniaeciaceae	2	10
46	Family Tympanidaceae	7	123
47	Leotiales genera *incertae sedis*	4	12
	Order Lichinodiales		
48	Family Lichinodiaceae	1	4
	Order Marthamycetales		
49	Family Marthamycetaceae	9	60
	Order Medeolariales		
50	Family Medeolariaceae	1	1
	Order Micraspidales		
51	Family Micraspidaceae	1	3

(*Continued*)

Table 1 Continued

	Order, Family	*Genera*	*Species*
	Order Phacidiales		
52	Family Helicogoniaceae	7	33
53	Family Phacidiaceae	9	82
54	Phacidiales genera *incertae sedis*	1	1
	Order Rhytismatales		
55	Family Cudoniaceae	2	30
56	Family Rhytismataceae	52	607
57	Family Triblidiaceae	2	15
58	Rhytismatales genera *incertae sedis*	9	12
	Order Thelebolales		
59	Family Pseudeurotiaceae	8	44
60	Family Thelebolaceae	10	90
	Leotiomycetes genera incertae sedis	20	76

[a]References: Minnis and Lindner (2013), Karakehian *et al.* (2014, 2019), Baral (2016), Crous and Groenewald (2016), Guatimosim *et al.* (2016), Prasher *et al.* (2016), Crous *et al.* (2017, 2018), Pärtel *et al.* (2017), Ashrafi *et al.* (2018), Marmolejo *et al.* (2018), Quijada *et al.* (2018, 2020), Baral (2019), Baral and Polhorský (2019), Ekanayaka *et al.* (2019), Fryar *et al.* (2019), Johnston and Park (2019), Johnston *et al.* (2019), Prieto *et al.* (2019), Wijayawardene *et al.* (2020), Species Fungorum (2020).

Current Understanding of Evolutionary Relationships

Challenges of Leotiomycetes Systematics

Ascomycota is the largest phylum of fungi and among the best studied ones. As a consequence, one might assume that the diversity and systematics within this large and ubiquitous clade is well understood, and for most of its classes this is largely true. However, Leotiomycetes have suffered from several issues that have impeded systematics of the class, such that its classification is one of the most poorly understood of any fungal clade. Marker loci designated for the large-scale Assembling Fungal Tree of Life project (AFToL) (Spatafora *et al.*, 2006), which have been successful for most fungal lineages, result in conflicting and unsupported relationships within Leotiomycetes. These markers also suggest that traditional morphological characteristics are uninformative in discerning even family-level relationships (Wang *et al.*, 2006b; LoBuglio and Pfister, 2010; Zhang and Wang, 2015; Baral, 2016). Indeed, since molecular characters have become available, the classification of Leotiomycetes has undergone multiple drastic updates. However, if there is something that has been consistent among all phylogenetic reconstructions of the class thus far, it is the presence of polytomies, polyphyletic higher taxa, and long branches. Even today, evolutionary hypotheses about family- and order-level relationships are being inferred using a single locus or few uninformative loci, and many taxa have no molecular data at all.

Whole-genome sequences for plant-pathogenic Leotiomycetes such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* were first published nearly a decade ago (Amselem *et al.*, 2011), but it was not until very recently that genomes were employed in Leotiomycetes systematics. Johnston *et al.* (2019) provided the first evidence that genome-scale data have the potential to resolve relationships within the class, especially within the hyper-diverse order Helotiales. The authors also presented a 5–15 locus phylogeny, which still seems to conflict with the topology of genome-scale sampling (**Fig. 1**). Genome sampling for many clades was completely lacking, especially outside of Helotiales, which resulted in a lack of support at all deep nodes within the class (Johnston *et al.*, 2019). If genome-scale data are required for resolving the systematics of Leotiomycetes, then much work is left to be done (**Fig. 2**).

The classification by Kirk *et al.* (2008) in the *Dictionary of Fungi* included six orders: Cyttariales, Erysiphales, Helotiales, Leotiales, Rhytismatales, and Thelebolales (with uncertainty). Baral (2016) accepted ten orders of Leotiomycetes: Cyttariales, Erysiphales, Helotiales, Lahmiales, Leotiales, Medeolariales, Phacidiales, Rhytismatales, Thelebolales, Triblidiales. Since that time, several papers were published describing new orders within Leotiomycetes (Crous *et al.*, 2017; Hernández-Restrepo *et al.*, 2017; Prieto *et al.*, 2019; Johnston *et al.*, 2019; Quijada *et al.*, 2020). On the other hand, Karakehian *et al.* (2019) synonymized Triblidiales under Rhytismatales, and Johnston *et al.* (2019) found support for the powdery mildews (Erysiphaceae) to be part of Helotiales. In addition, several leotiomycetous families have shifted in time from one place to another. For example, Tympanidaceae was placed in Phacidiales (Baral, 2016) but the 5–15 locus tree from Johnston *et al.* (2019) recovered the family in Leotiales with high statistical support. Both families Amorphothecaceae and Myxotrichaceae were previously considered Leotiomycetes *familiae incertae sedis* but multilocus and genomic-scale phylogenetic analyses have shown that they are both placed in Helotiales (Johnston *et al.*, 2019). Myxotrichaceae is paraphyletic based on the ITS region (Seifert *et al.*, 2007). Ekanayaka *et al.* (2019) synonymized Myxotrichaceae under Amorphothecaceae but only had a single isolate of *Amorphotheca* available, which was placed sister to their Myxotrichaceae clade – highlighting the need for improved taxon sampling in addition to increased sequencing efforts.

Chaetomellales

Well-defined and distinct from other families (Baral, 2016), this group was formerly treated as family Chaetomellaceae within Helotiales. The elevation to ordinal level was based on a nuclear ribosomal RNA large subunit (LSU) phylogeny (Crous *et al.*, 2017)

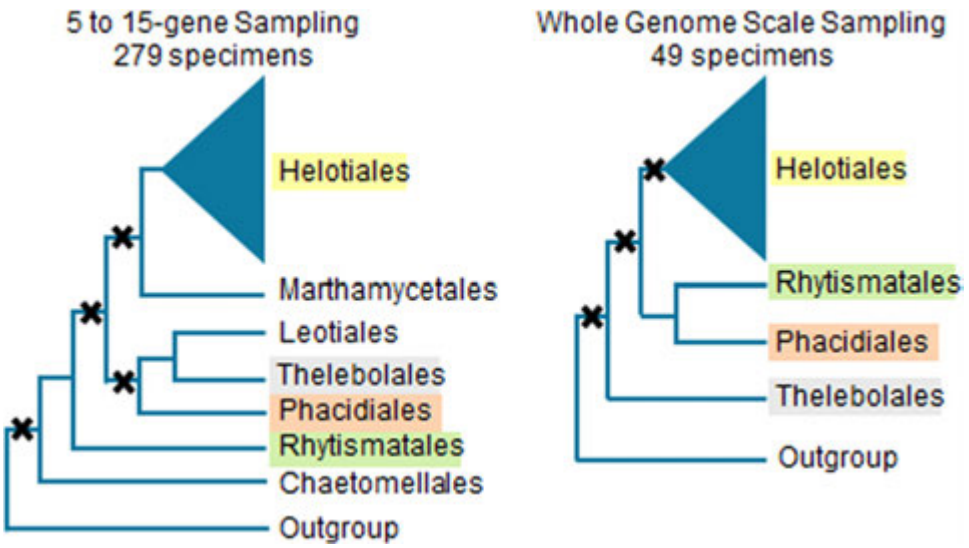

Fig. 1 Current evolutionary hypotheses about interordinal relationships within the class Leotiomycetes, based on Johnston *et al.* (2019). *Left*, 5–15 locus phylogeny based on 279 isolates; *right*, whole-genome scale phylogeny based on 49 isolates. Nodes where support is lacking are marked with a black " × ". Orders that are represented in *both* analyses are highlighted in color (Helotiales in yellow, Phacidiales in peach, Rhytismatales in green, Thelebolales in gray), showing major topological disagreement between the two analyses. Other orders have thus far not been considered in Leotiomycetes-wide multilocus or genome-scale analyses. Modified from Johnston, P.R., Quijada, L., Smith, C.A., *et al.*, 2019. A multigene phylogeny toward a new phylogenetic classification of Leotiomycetes. IMA Fungus 10, 1.

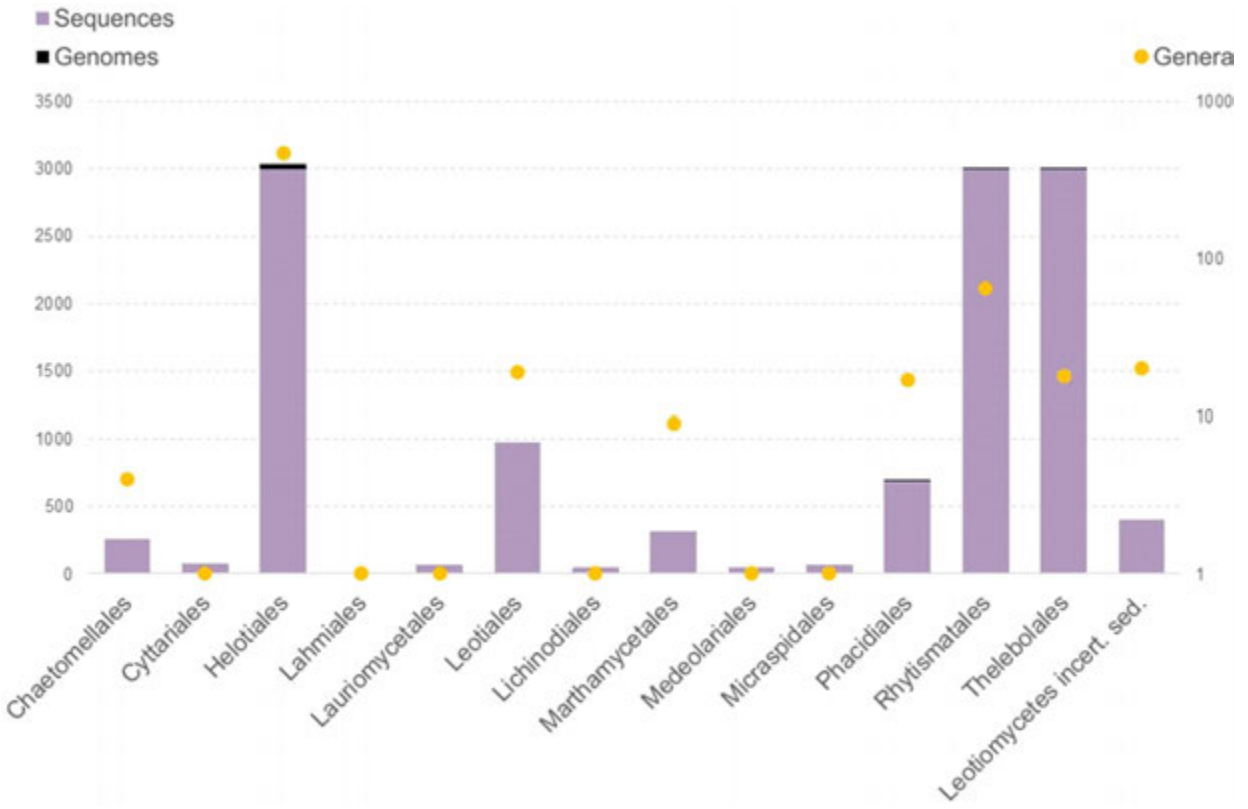

Fig. 2 Current status of sequences submitted to NCBI GenBank and published and/or publically available genomes of class Leotiomycetes. Numbers of sequences were capped at 3000. A logarithmic scale is used for numbers of genera (Y axis, *right*). Note that whole-genome sampling has only been done in four orders: Helotiales (35 genomes), Phacidiales (1), Rhytismatales (6), and Thelebolales (4). Sequences are available for every order with the exception of Lahmiales.

and confirmed by the phylogenetic reconstruction of a 5–15 locus dataset (Johnston *et al.*, 2019). Apothecia of Chaetomellales are 0.2–1.0 mm in diameter, develop beneath the host epidermis, and are hairless (*Pilidium*) or covered with long setae (*Chaetomella*). The asci are 8-spored and have a thick-walled inamyloid apex. There are two distinct anamorphs (synanamorphs): sessile pycnidia that open by fissures in *Sphaerographium* and sessile or long-stalked sporodochia in *Synchaetomella*. These anamorphs can be hairless or have scattered setae. Members of Chaetomellales are parasitic or saprotrophic on leaves, herbaceous stems, and dicot fruits. Some taxa are host-specific, whereas others may have multiple hosts.

Cyttariales

Species of *Cyttaria*, the single genus in this order, are obligate biotrophic associates of *Nothofagus* trees in southern South America and southeastern Australasia (Peterson and Pfister, 2010). They produce trunk and branch cankers on their host trees. During his voyage on the *HMS Beagle*, Charles Darwin collected in Chile the golf ball-shaped fruiting bodies that would serve as type material for the first two described *Cyttaria* species (Berkeley, 1842). *Cyttaria* species are distinct from other leotiomycetous taxa by their spherical fruiting bodies of sterile stroma with numerous apothecial cavities in a honeycomb-like arrangement. Asci have an amyloid apical ring and the anamorph stage is pycnidial. Peterson *et al.* (2010) found high co-phylogenetic structure between *Cyttaria* and *Nothofagus*, even though they did not report simple one-to-one relationships. Cyttariales is apparently closely related to Helotiales. Using a four-locus phylogenetic reconstruction of a Leotiomycetes-wide dataset, Peterson and Pfister (2010) retrieved Cyttariales as sister to Cordieritidaceae, leaving the order Helotiales paraphyletic. In the concept of Helotiales sensu Johnston *et al.* (2019), Cyttariales would be a family-level clade in this mega-order, but multilocus data for *Cyttaria* is still lacking.

Helotiales

The most speciose and best studied order in the class (**Table 1**), initial phylogenetic work discerned that this expansive order was polyphyletic (Wang *et al.*, 2006a,b; Schoch *et al.*, 2009). Currently, a broad concept is maintained based on multilocus and genome-scale phylogenetic analyses; Helotiales sensu Johnston *et al.* (2019) also includes Cyttariaceae (pending multilocus data) and the Erysiphaceae family of powdery mildews (see Section "Helotiales, a Mega-Order in Disarray"). Helotiales fungi are mostly apothecial, with apothecia that are usually < 2 mm in diameter, sessile to long-stalked, dark to bright-colored, superficial or erumpent through the plant tissue. Some representatives form non-apothecial ascomata; *Amorphotheca* (Amorphothecaceae), *Bicornispora* (Rutstroemiaceae), *Connersia*, *Pleuroascus* (Helotiaceae), and members of Erysiphaceae and Myxotrichaceae sensu Johnston *et al.* (2019) are cleistothecial, whereas members of Loramycetaceae and *Unguicularia* (Hyaloscyphaceae) are perithecial. Most members of Helotiales are saprotrophs, decaying dead organic material, but some are associated with living organisms as either parasites, pathogens, or mutualists (Stenroos *et al.*, 2010; Baral, 2016; Haelewaters *et al.*, 2018b; Tanney and Seifert, 2020).

Lahmiales

The order Lahmiales was introduced by Eriksson (1986) to accommodate a single genus with currently two species (Species Fungorum, 2020). In 2007, the order was placed as Pezizomycotina *incertae sedis* by Hibbett *et al.* (2007), along with orders Medeolariales and Triblidiales, which we now know all belong to Leotiomycetes. Raitviir and Spooner (1994) suggested several placements for Lahmiales, in Dothideomycetes and Lecanoromycetes. Currently, still, no sequences exist but Baral (2016) suggested a placement of the order within Leotiomycetes, noting the resemblance of ascomata of *Lahmia* with Rhytismatales except the bitunicate asci and the absence of a clypeate stroma. Ascomata of *Lahmia* measure 0.13–0.30 mm in diameter, they are black, erumpent, and open at maturity by irregular radial splits. These fungi occur on the bark of *Populus* trees in boreal North America and northern Europe. The Lahmiales order is probably the least studied of leotiomycetous orders; the last described species, *Lahmia waghornii*, dates from 1900. (Note that *Lahmia plumbina* was described in 1930, but has since been recombined in the genus *Toninia*, Ramalinaceae, Lecanoromycetes.) Recent collections of Lahmiales are scarce and ambiguous, and no sequences are currently available (**Fig. 2**).

Lauriomycetales

This recently described order (Hernández-Restrepo *et al.*, 2017) consists of a single family with a single asexual genus, *Lauriomyces* (Castañeda-Ruiz and Kendrick, 1990), and includes 11 species (Somrithipol *et al.*, 2017). Characterized by brown conidiophores with acropetal chains of hyaline conidia, these species are only known from leaf litter and have a cosmopolitan distribution. Phylogenetic placement of Lauriomycetales suggests this is an early diverging lineage within Leotiomycetes, possibly sister to Chaetomellales (Hernández-Restrepo *et al.*, 2017; Somrithipol *et al.*, 2017) although so far this relationship is based solely on ribosomal DNA data.

Leotiales

Carpenter (1988) erected Leotiales with genus *Leotia* as its type to accommodate taxa in Helotiales. The most recent conception of Leotiales is based on Johnston *et al.*, 2019, and includes large, stipitate to clavate apothecial members of Leotiaceae (including

Leotia and *Microglossum*), stipitate to sessile apothecial taxa in Tympanidaceae s.s. and *Mniaecia*, and several aquatic hyphomycete genera. Ecological niches in this order vary from plant-pathogenic taxa such as *Tympanis* (Ouellette and Pirozynski, 1974), to the liverwort-parasitic or possibly lichenized *Mniaecia* (Raspé and de Sloover, 1998; McCune and Stone, 2020), and potentially arbutoid mycorrhizal taxa such as *Leotia* (Kühdorf *et al.*, 2015), although some consider this genus to be saprobic.

Lichinodiales

Formerly classified in Lichinomycetes, the lichenized genus *Lichinodium* is now recognized as a member of Leotiomycetes (Prieto *et al.*, 2019). Lichinodiales is possibly allied with Leotiales, although more sampling is needed, especially given the recent report that *Mniaecia* could be lichenized (McCune and Stone, 2020). The four recognized species of *Lichinodium* are filamentous lichens (so called because of the lack of a stratified lichen thallus) with cyanobacterial photobionts in the genus *Rhizonema* (Prieto *et al.*, 2019). They produce gelatinous brown apothecia with indistinct margins, 0.5–1 mm in diameter, and prototunicate asci without amyloid staining. They are found on various substrates in cool, humid environments.

Marthamycetales

Erected by Johnston *et al.* (2019) to accommodate the single family Marthamycetaceae, these taxa are all saprobic on plant material and produce ascomata erumpent through host tissue (Minter, 2003). Asci are thin-walled and amyloid with variation in ascospore septation among the different taxa. Notable genera include *Propolis* (see Minter, 2003 for a review of species traditionally placed in this genus) and *Marthamyces* in which there has been considerable recent work describing new species (Johnston, 2006; Johnston and Park, 2019; Crous *et al.*, 2019).

Medeolariales

This monotypic order is represented by *Medeolaria farlowii*, a pathogen of *Medeola virginiana*, a small tuber-bearing plant found in eastern North America (Thaxter, 1922). Korf (1973), in Eriksson (1982), placed this taxon in its own family and order but with uncertain placement within Ascomycota presumably due to lack of characters (including ascomatal and ascus types) that unite it with other clades. Based on the phylogenetic reconstruction of a nuclear ribosomal DNA dataset, LoBuglio and Pfister (2010) transferred Medeolariales into Leotiomycetes. This enigmatic taxon makes a loosely organized hymenium directly below the leaf whorls of its host. The fungus is present in multiple parts of infected plants, including in seemingly uninfected leaves (Pfister and LoBuglio, 2013). *Medeolaria farlowii* has an inamyloid ascus apex and a thus far unknown method of ascospore release from the ascus (Korf, 1973; LoBuglio and Pfister, 2010). Its occurrence is reported as widespread in the northeastern US (Pfister, 1984; Pfister and LoBuglio, 2013).

Micraspidales

The genus *Micraspis* was described by Darker (1963) to accommodate a fungus that caused a foliar disease resembling *Phacidium* snow-blight of *Picea mariana* in Canada. Ascomata (apothecia) and conidiomata are macroscopically indistinct; they are immersed and become erumpent from the host tissue. The genus was historically placed in either Helotiales (Eriksson, 1999; Lumbsch and Huhndorf, 2009) or Phacidiales (Darker, 1963; Korf, 1973; Baral, 2016). However, based on the combination of unique morphological features and a multilocus phylogenetic reconstruction, Quijada *et al.* (2020) proposed a new family (Micraspidaceae) and order (Micraspidales). Synapomorphic morphological characteristics of *Micraspis* are: the ectal excipulum and covering layers of both ascomata and conidiomata are composed of *textura epidermoidea*, ectal excipulum is covered on the outside by a thick refractive yellowish gel, ascospores germinate at the poles, and conidia are produced directly from germ tubes or ascospore walls (Quijada *et al.*, 2020).

Phacidiales

Quijada *et al.* (2018) referred to Phacidiales as "a good example of the chaotic situation within the class." Since Bessey (1907) described the order, different genera and families have been placed in it. Baral (2016) and Quijada *et al.* (2018) considered four major lineages: Helicogoniaceae, Phacidiaceae, Tympanidaceae, the *Mniaecia* lineage, and *Coma* as Phacidiales *incertae sedis*. However, the 5–15 locus phylogenetic analysis of Johnston *et al.* (2019) retrieved the *Mniaecia* lineage (as family Mniaeciaceae) and Tympanidaceae as highly supported clades within Leotiales. Phacidiales includes both saprobic and parasitic species of plants, fungi, and lichens. Whereas intrahymenial parasitic *Helicogonium* species only form ascogenous hyphae (no ascomata), other members of Phacidiales produce apothecia that open in the prohymenial to mesohymenial phase (*fide* Kimbrough, 1981b), with paraphyses lacking vacuolar bodies, asci with or without amyloid ring, and ascospores with variable lipid content (Baral, 2016).

Rhytismatales

Fungi in Rhytismatales are plant-associated either as pathogens, endophytes, or saprotrophs with a near-global distribution. Fruiting bodies vary from the earth-tongue to club-shaped members of Cudoniaceae to the host-immersed fruiting structures of Rhytismataceae (the largest family in the order), which may or may not include a stromatic layer that splits open to reveal the hymenium at maturity. Some recognize Cudoniaceae as included within Rhytismataceae (Lantz *et al.*, 2011) while others maintain both families. In the 5–15 locus phylogenetic reconstruction of Johnston *et al.* (2019), Cudoniaceae was retrieved as a well-supported clade within a paraphyletic Rhytismataceae. Although no synapomorphy exists for the modern concept of Rhytismatales, filiform ascospores with a gelatinous sheath are characters present in many taxa. Many genera formerly included in Rhytismatales such as *Propolis*, *Marthamyces*, *Pseudophacidium*, and *Ascodichaena* were recognized as belonging to different clades (Lantz *et al.*, 2011) and subsequently reclassified into other orders (Johnston *et al.*, 2019; Karakehian *et al.*, 2019).

Thelebolales

Historically, most coprophilic discomycetes were placed in Pezizales (Pezizomycetes), however as now conceived that order is composed mainly of taxa with operculate asci. Based on morphology and more recently molecular phylogenetic studies, *Thelebolus* and other allied coprophilic taxa with inoperculate asci were recognized as an independent lineage from Pezizales (Kimbrough and Korf, 1967; Landvik *et al.*, 1998). Species of *Thelebolus* have cleistothecioid ascomata with variable numbers of ascospores per ascus, ranging from 8 to more than 1000 spores (Kimbrough, 1981a). An expansive definition of the order now includes Pseudeurotiaceae in addition to Thelebolaceae (Johnston *et al.*, 2019; Batista *et al.*, 2020). Coprophilic and psychrophilic species are found in both of these families (Robinson, 2001; de Hoog *et al.*, 2005) although morphologically they differ significantly, hence their previously unrecognized relationship (Baral, 2016). Species of Pseudeurotiaceae form immersed or superficial cleistothecia on wood and decaying plant material. *Pseudogymnoascus destructans*, the causal agent of white-nose syndrome in bats, is a member of this family but only known from its asexual state. Little work has been done to understand the systematics of the family Pseudeurotiaceae (Minnis and Lindner, 2013).

Biases in Sampling of Leotiomycetes

Distributional Unevenness

Geographically, Leotiomycetes are found on all continents including Antarctica, but their taxonomy has been based primarily on the diversity in the temperate Northern Hemisphere, especially in western Europe and the United States, while tropical locations are underrepresented (**Fig. 3**); Piepenbring *et al.*, 2018. Leotiomycetes and Helotiales in particular include some of the oldest descriptions of mycological taxa (Micheli, 1729). As the cradle for fungal taxonomy, Europe has been well-documented in terms of Leotiomycetes diversity, with early workers such as Pier Antonio Micheli (Italy, 1679–1737), Marie-Anne Libert (Belgium, 1782–1865), Elias Fries (Sweden, 1794–1878), Heinrich Rehm (Germany, 1828–1916), Émile Boudier (France, 1828–1920), and Pier Andrea Saccardo (Italy, 1845–1920). Of the 630 genera that have been placed in the class, 85% are based on species described from temperate Europe and to a lesser extent North America, whereas only 5% are based on species described from the tropics, Asia, or the temperate Southern Hemisphere, with the fewest descriptions from Africa (Haelewaters *et al.*, 2021).

Recent efforts in North America have focused mostly on macrofungi (Bruns, 2011; 2012) but there are some reports of undescribed and rare Leotiomycetes – including taxa that were previously unsequenced. Localities include New Brunswick, Canada (Quijada *et al.*, 2020), the Rocky Mountains in Colorado (C.A. Quandt, unpublished), Boston Harbor Islands National Recreation Area in Massachusetts (Haelewaters *et al.*, 2018a), and Great Smoky Mountains National Park in North Carolina and Tennessee (Hustad and Miller, 2011).

Helotiales, a Mega-Order in Disarray

Since the description of Helotiaceae (Rehm, 1896), this family has been expanded with 100s of species such that it currently is the most speciose family of the order Helotiales. Several taxa within the family, such as *Hymenoscyphus*, turned out to be polyphyletic (Stenroos *et al.*, 2010; Baral *et al.*, 2013; Baral *et al.*, 2015; Johnston *et al.*, 2019). Also higher taxa within Helotiales have been shown to be polyphyletic, although phylogenetic results from different studies heavily depend on taxon sampling and the number of loci used to estimate evolutionary relationships. Baral *et al.* (2015) found that Helotiaceae and Lachnaceae were paraphyletic based on the phylogenetic reconstruction of an ITS–LSU dataset. Johnston *et al.* (2019), on the other hand, presented a 5–15 locus tree in which both Helotiaceae and Lachnaceae were monophyletic. Taxon sampling within Helotiaceae differed between the two studies but mostly overlapped for Lachnaceae, clearly showing the importance of multiple, phylogenetically informative loci.

Baral *et al.* (2015) referred to several helotialean families and subfamilies as wastebaskets. Throughout the years, several taxa were described in these higher lineages based on morphological synapomorphies – traditionally, the morphology of ascomata (Zhang and Wang, 2015). As an example, the subfamily Encoelioideae encompassed taxa with long-lived and desiccation-tolerant apothecia. However, a five-locus phylogenetic analysis revealed that Encoelioideae was highly polyphyletic (Pärtel *et al.*, 2017).

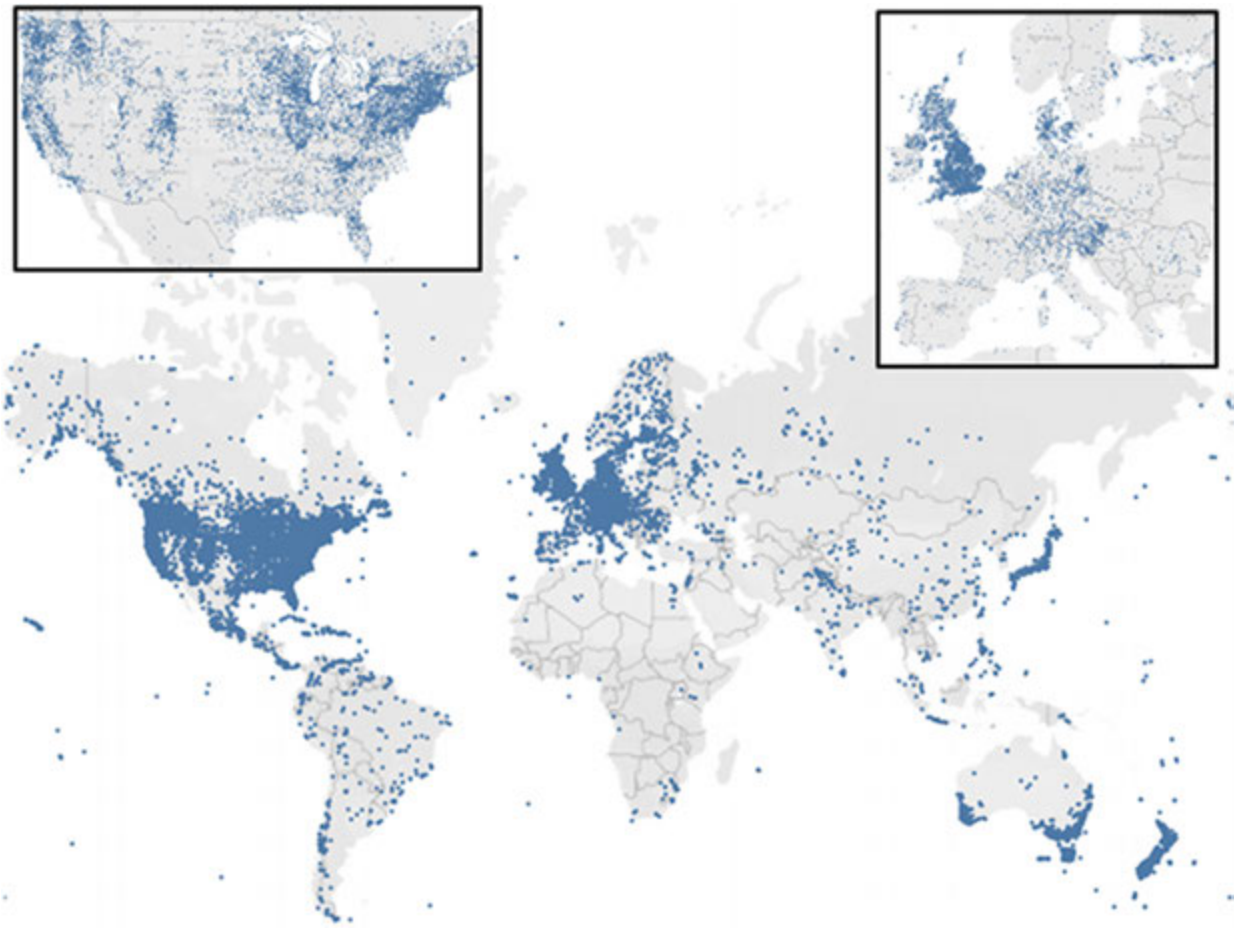

Fig. 3 Global distribution of Leotiomycetes collections deposited in database herbaria, based on a dataset of 217,480 records downloaded from MyCoPortal (2020). Insets show closer views of collections from the continental US and Europe.

Species known or combined as *Encoelia* were retrieved in seven different genera in six families, two of which had to be resurrected (Cenangiaceae and Cordieritidaceae). As more multi-locus sequences and genome-scale data become available, researchers are learning that several of the characters once thought to define a higher taxon have multiple origins in the order.

Evidently, several taxa once considered as Helotiales are now recognized as members of new, distinct orders. In addition, in recent years and often based on molecular phylogenetic studies, several new families have been erected within the order and several more unnamed lineages proposed (Han *et al.*, 2014; Baral, 2016; Crous *et al.*, 2017; 2018; Pärtel *et al.*, 2017; Baral and Polhorský 2019; Johnston and Baschien, 2020). Johnston *et al.* (2019), supported by their 5–15 locus and genome-scale phylogenies, chose to recognize a larger, more inclusive definition of Helotiales in lieu of a more restricted definition, which would have necessitated the creation of several new orders. The highly diverse mega-clade Helotiales sensu Johnston *et al.* (2019) also includes the previously segregated order, Erysiphales, the powdery mildews, which encompasses more than 976 species in 20 genera (Marmolejo *et al.*, 2018; Wijayawardene *et al.*, 2020). However, not all authors agree with this proposal because of the morphological and ecological distinctiveness of powdery mildews (Ekanayaka *et al.*, 2019; A.H. Ekanayaka and K.D. Hyde in Wijayawardene *et al.*, 2020).

Understudied Ecological Niches

Historically, the majority of Leotiomycetes have been described from decaying, terrestrial plant materials. However, based on environmental studies, we know that Leotiomycetes, such as the psychrophilic *Pseudogymnoascus* (Rosa *et al.*, 2019) and the mycorrhizal symbiont of moss, *Rhizoscyphus*, are dominant members of polar environments (de Hoog *et al.*, 2005; Bridge and Spooner, 2012; Rosa *et al.*, 2019). Culture-based studies have isolated Leotiomycetes from marine (Baral and Rämä 2015; Fryar *et al.*, 2019) and a multitude of freshwater aquatic environments (Baschien *et al.*, 2013; Tsui *et al.*, 2016). The so-called Dark Septate Endophytes, most of which are Leotiomycetes, can be dominant in Alpine ecosystems. Studies based purely on DNA barcoding have suggested that Leotiomycetes are dominant in many environments including peat bogs (Lamit *et al.*, 2017), the

arctic tundra, and in tropical montane soils (Tedersoo *et al.*, 2014). These studies, however, are often limited in their geographical scope and many of the detected taxa have no names.

Future Research Perspectives

Much has changed in the field of evolutionary biology since Leotiomycetes has been tackled holistically, in addition to the widespread availability and low cost of whole-genome sequencing. Improved technologies and techniques including amplicon-based sequencing, single-cell genomics, metagenomics, transcriptomics, and high-throughput computing, have the ability to transform our understanding of the diversity and ecology in this class. Examples of both culture-dependent studies and environmental sequencing work suggest that Leotiomycetes diversity is broader than currently understood. It is estimated that only 5–7% of Leotiomycetes diversity has been formally described. This warrants a focus on taxa that are difficult to culture and undersampled geographic areas and habitats that could be diverse in Leotiomycetes. Examples of such areas are tropical and subtropical regions around the world, Africa, and much of the Asian continent. Efforts are being undertaken to fill some of these distributional gaps of leotiomycetous knowledge, with fieldwork planned in southeastern Africa (Mozambique) and northern Asia (Siberia). In addition to targeted sampling of geographic areas, certain taxonomic lineages that are currently lacking molecular phylogenetic studies should be targeted in future research. Taxonomically poorly sampled regions of the class have recently produced many phylogenetically distinct genus-level and family-level clades (e.g., Somrithipol *et al.*, 2017; Quijada *et al.*, 2018), and others that remain unnamed (Johnston *et al.*, 2019). Other higher taxonomic level groups that need taxonomic revision include Lahmiales and Thelebolales, in addition to groups such as Calloriaceae, Hyaloscyphaceae, and the "*Stamnaria* lineage" in Helotiales and Cudoniaceae + Rhytismataceae in Rhytismatales. Finally, any molecular phylogenetic data for the 170 *incertae sedis* genera throughout the class would greatly contribute to our understanding of evolutionary relationships of Leotiomycetes. It is likely that these sampling initiatives will reveal undescribed clades within the class and thereby help to resolve some of the deeper nodes that have not yet received support.

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Pezizomycetes

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Introduction

The Pezizomycetes comprise a single order, Pezizales, with ≥22 families currently recognized. Along with the Orbiliomycetes, the class represents one of the basal lineages among the filamentous Ascomycota (Shen *et al.*, 2020). The class is thought to have originated between 400 and 540 mya (Beimforde *et al.*, 2014; Martin *et al.*, 2010; Murat *et al.*, 2018). The full diversity of the order has yet to be completely documented since previously undetected lineages continue to be found through application of molecular methods. There are approximately 200 genera and perhaps 2000 species. Ascomata are epigeous (above ground), or hypogeous (below ground). The truffles of commerce belong to this latter group. The epigeous ascomata are apothecial, cleistothecial or are highly reduced. The reduced forms are composed of only a few asci in clusters on vegetative hyphae with little or no sterile supporting tissue (excipulum). In the ep,lppigeous lineages, ascospores are generally forcibly released by an opening at the ascus apex resulting in the formation of an operculum, or lid. Hypogeous members occur in several of the families. There are at least 30 independent origins of truffle-like members (Alvarado *et al.*, 2011, 2016; Cabero *et al.*, 2016; Grupe *et al.*, 2019; Hansen *et al.*, 2013; Kraisitudomsook *et al.*, 2019; Kumar *et al.*, 2017; Læssøe and Hansen, 2007; Smith, 2014; Smith and Healy, 2009; Trappe *et al.*, 2010).

Distribution and Ecology

Pezizomycetes can be found around the world but representatives are unevenly distributed. Members of certain families, especially those found in or on soil, show a particularly high diversity in temperate regions (Tedersoo *et al.*, 2014). These include the Pezizaceae, Morchellaceae, Helvellaceae, Rhizinaceae and many of the Pyronemataceae. Others are more abundant in tropical or subtropical regions, as exemplified by the Sarcosomataceae, Wynneaceae and Sarcoscyphaceae. Members of bryophilous Pyronemataceae are particularly common and diverse in the Arctic and Antarctic, but these fungi also occur in other regions (Olech and Mleczko, 2000; Schumacher, 1993). Although fieldwork and discovery in the Pezizomycetes is robust, recent work has shown that even in localities considered well studied, such as the Northern Hemisphere, new species have come to light. In the Southern Hemisphere, much remains to be documented.

Nutritionally Pezizomycetes are saprobic, mutualistic as mycorrhizae (Tedersoo *et al.*, 2006) or as endophytes (Arnold, 2007), or parasitic on bryophytes or vascular plants (Hansen and Pfister, 2006). An additional nutritional niche was recently discovered where some species were documented as bacterial farmers (Pion *et al.*, 2013), or otherwise mutualistic with certain bacteria (Benucci and Bonito, 2016; Giordano *et al.*, 2013; Splivallo *et al.*, 2015).

The plant parasitic species are scattered in various lineages. *Rhizina undulata* is reported as a root parasite of conifers (Ginns, 1968). *Pithya* species may cause branch dieback in conifers. *Caloscypha fulgens* is reported as a seed pathogen of conifers (Paden, 1972; Salt, 1974; Sutherland and Woods, 1978; Paden *et al.*, 1978). *Urnula craterium* has been implicated as the causal agent of strumella canker in oak (Davidson, 1950). *Phymatotrichopsis ominivora* is a serious root pathogen of cotton and other dicotyledonous plants and is known only in its asexual state (Uppalapati *et al.*, 2010). Several Pyronemataceae are parasitic on bryophytes, including species of *Filicupula*, *Lamprospora*, *Moravecia*, *Neottiella*, *Octospora*, *Octosporella*, and *Octosporopsis* (Döbbeler, 1979; Egertová and Sochor, 2017; Hansen and Pfister, 2006; Sochorová *et al.*, 2019; Stenroos *et al.*, 2010). Apothecia occur directly on their moss or liverwort hosts or on surrounding soil. These often parasitize the rhizoids (Döbbeler, 1979; Stenroos *et al.*, 2010). There is evidence that species of *Wynnea* are parasitic on mushroom species in the genus *Armillaria* (Xu *et al.*, 2019).

With the advent of molecular approaches to studying fungal ecology, such as the sequencing of ectomycorrhizal root tips, a number of examples of mycorrhizal Pezizomycetes have been documented. Mycorrhizal Pezizomycetes most commonly form ectomycorrhizae with a broad range of angiosperm and coniferous tree species in boreal, temperate, and Mediterranean forests in the Northern Hemisphere and temperate forests of the Southern Hemisphere (Tedersoo and Smith, 2013; Tedersoo *et al.*, 2010), and with Cistaceae and some Rosaceae in arid shrub lands and deserts (Giovannetti and Fontana, 1982; McDonald *et al.*, 2010), but a few are also tropical (Tedersoo and Nara, 2010). Tedersoo and Smith (2013) hypothesized 20 independent origins of the ectomycorrhizal symbiosis in the Pezizomycetes although evidence is still equivocal for some lineages, such as *Sowerbyella*. *Sphaerosporella* species are ectomycorrhizal, but unusual in that they can grow saprobically as well (Danielson, 1984; Sánchez *et al.*, 2014). Some species of Tuberaceae and Pyronemataceae form orchid mycorrhizae with certain understory terrestrial orchids (Bidartondo and Read, 2008; Těšitelová *et al.*, 2012). Morphologically Pezizomycete ectomycorrhizae have a thin mantle, a well-developed Hartig net and they generally form short-distance or contact-type mycorrhizas without rhizomorphs (Agerer, 2001; Tedersoo *et al.*, 2006; Wei *et al.*, 2010). Most of the mycorrhizal Pezizomycetes fruit in or on soil, particularly in disturbed areas or in soil with a neutral or elevated pH (Tedersoo *et al.*, 2014) and low content of organic matter. Petersen (1985) provides a review of the edaphic factors involved in growth and reproduction.

Truffle or truffle-like taxa are largely found within ectomycorrhizal lineages. However, truffles in the Tarzettaceae, Glaziellaceae, and some in the Pezizaceae such as *Carbomyces, Kalaharituber* and *Eremiomyces* are not known to be mycorrhizal. Their nutritional strategy is presumed to be saprobic (Hansen *et al.*, 2013; Kumar *et al.*, 2017). In some families, such as Chorioactidaceae, Sarcoscyphaceae and Sarcosomataceae, there are no truffle-like species and no mycorrhizal species are known.

Some non-ectomycorrhizal lineages of Pezizomycetes have been detected as a relatively small component of the endophytic community in healthy leaves, bark, branches and/or roots (Arnold, 2007). Lineages of endophytic Pezizomycetes include non-mycorrhizal Pezizaceae (Arnold, 2007), Sarcosomataceae (Wang *et al.*, 2005), Pyronemataceae and Tarzettaceae (Tedersoo *et al.*, 2013). *Sphaerosporella* species (in a complex of species under the name *S. brunnea*) are an exception to this observation as they have been reported as both endophytic and ectendo- or ectomycorrhizal with a broad range of hosts. However, endophytic and mycorrhizal *Sphaerosporella* are not usually detected on the same tree (Hughes *et al.*, 2020). Detected endophytic Pyronemataceae were mostly pyrophilous species (Raudabaugh *et al.*, 2020; Tedersoo *et al.*, 2013). Many lineages have also been found within lichen thalli (Tedersoo *et al.*, 2013).

The majority of Pezizomycetes are saprobic. They occur on organic material of various types – decaying wood, dung, leaf litter and twigs. The diversity of Pezizomycetes on dung is well documented with many studies and keys available (Bell, 1983; Doveri, 2004; Richardson and Watling, 1968). Some clades, such as the Ascobolaceae, are found almost exclusively on dung (Brummelen, 1967). Dung inhabiting species are also found in several clades of the Pseudombrophilaceae, Pyronemataceae and Pezizaceae (Hansen *et al.*, 2001; Hansen and Pfister, 2006; Perry *et al.*, 2007). In most cases little is specifically known of the biology of saprobic species in regard to nutrient requirements and utilization. Sarcoscyphaceae, Sarcosomataceae and Chorioactidaceae are found exclusively on wood, leaves and plant debris and are presumed to be saprobic.

There are a number of pyrophilous taxa for which fruiting is enhanced by fire or they fruit only after a burn. These are part of a succession of fungi colonizing burned areas, and these species are from various families including Morchellaceae, Pezizaceae, Pyronemataceae, Rhizinaceae, and Tarzettaceae. There is evidence that these fungi may persist in soil, the living tissues of bryophytes ferns, lichens and lycopods, woody debris and other substrates. Fire triggers the release of the fungus (Hughes *et al.*, 2020; Raudabaugh *et al.*, 2020). Certain species of the economically important morels (*Morchella* sp.) are stimulated to fruit prolifically after burns (Alexander *et al.*, 2002; Larson *et al.*, 2016; Pilz *et al.*, 2004).

Morphological Features

Ascomata

Apothecia are the basic type of ascomata in the Pezizomycetes. These range in size from several millimeters to about 20 centimeters and they vary from sessile cups of small to large size to stalked cups to the stipitate-piliate structures found in the Helvellaceae, Discinaceae, and Morchellaceae. Some highly reduced members, such as species of *Ascodesmis*, produce little more than small fascicles of asci. More highly reduced forms are found in *Eleutherascus* and *Monascella* (Guarro and Arx, 1986) in which solitary asci are produced on hyphae or in clusters on unspecialized hyphae. Ascomata are often highly pigmented, particularly the hymenium. Carotenoid pigments have been characterized (Arpin, 1968). Other pigments may give the hymenium brown, orange, purple or black colors.

Variation in typical apothecial ascomata are found, some examples are presented in **Fig. 1**. On the one hand there are highly reduced types that are merely asci scattered on hyphae as mentioned above but also small cleistothecial (closed) ascomata are found in a few cases. In the case of *Orbicula parietina*, active discharge has been lost and spores are liberated in a powdery mass (Hansen *et al.*, 2005). *Heydenia alpina* forms stalked cleistothecial fruitbodies and is closely related to *O. parietina* (Leuchtmann and Clémençon, 2011). It is a small fungus that occurs on plant debris and mosses. It is stipitate with a dark stipe that is topped by a pale mass of hyaline spores and radiating hyphae. No asci are present in mature specimens. Molecular evidence confirms that this species belongs in the Pseudombrophilaceae along with *Orbicula*. The two genera share characteristics of a cleistothecial fruitbody and asci that disintegrate early in the development of the ascomata but they are different in important morphological features (Leuchtmann and Clémençon, 2011). These genera were often placed outside of the Pezizomycetes and, in the case of *Orbicula*, confused with Myxomycetes.

Truffle fruitbodies are found in a number of lineages. Following the terminology of Weber *et al.* (1997) these ascomata have been characterized by the position of the asci and the hymenia in relationship to the tissues of the ascomata. Stereothecia are ascomata that lack a hymenium, the asci being scattered individually or in chambers in the mass of sterile tissue. When the hymenium is located on the outside of an ascoma the term exothecium is used. In some cases the hymenium is present as a distinct layer but the ascoma is highly convoluted; this type of ascoma is termed a ptycothecium. All of these specialized types of ascomata are derived from apothecial forms. The literature on many of these taxa as a whole is reviewed in Ekanayaka *et al.* (2018), Læssøe and Hansen (2007) and O'Donnell *et al.* (1997).

Some highly reduced forms are found among the bryophyte parasites. The ascomata are partly closed and have only a few asci and are reminiscent of perithecia.

The Hymenium

This is the layer composed of asci and paraphyses that line the surface of the apothecium. Asci are the structures in which karyogamy and meiosis occur in the life cycle of the Ascomycota. The asci arise from ascogenous hyphae, which in turn have formed after the process of plasmogamy between two receptive structures from compatible individuals. Ascogenous hyphae are

Fig. 1 Examples of ascoma morphologies in families of Pezizomycetes. A. Ascobolaceae: *Ascobolus denudatus*; B. Caloscyphaceae: *Caloscypha fulgens*; C. Chorioactidaceae: *Chorioactis geaster*; D. Chorioactidaceae: *Desmazierella acicola*; E. Discinaceae: *Gyromitra brunnea*; F. Geomoriaceae: *Geomorium furciae*; G. Glaziellaceae: *Glaziella aurantiaca*; H. Helvellaceae: *Helvella stevensii*; I. Morchellaceae: *Morchella americana*; J. Otideaceae: *Otidea concinna*; K. Pezizaceae: *Hydnobolites* sp. nov.; L. Pezizaceae: *Peziza varia*; M. Pyronemataceae: *Humaria hemisphaerica*; N. Pyronemataceae: *Scutellinia pennsylvanica*; O. Pulvinulaceae: *Pulvinula convexella*; P. Sarcoscyphaceae: *Microstoma floccosum*; Q. Sarcosomataceae: *Galiella rufa*; R. Tarzettaceae: *Tarzetta* sp.; S. Tuberaceae: *Tuber mexiusanum*; T. Wynneaceae: *Wynnea americana*. Photo credits: A, E, H-S, R. Healy; B, Arthur Grupe; C, D.H. Pfister; D, Roy Kristiansen, F., Matthew, E., Smith, G., Alan Franck, T., Mark Elliot.

dikaryotic. These dikaryotic cells may proliferate by the production of croziers. Croziers are hook-like structures formed at the base of the ascus. They are formed by an outgrowth of the ascus before karyogamy and allow for the formation of a new dikaryotic cell. This aids in the proliferation of the dikaryotic system and gives rise to additional asci. Although considered a characteristic of the filamentous Ascomycota, not all Ascomycota produce croziers. Along with asci, the hymenium generally has paraphyses. These are located between the asci and arise from the sterile tissues surrounding the developing ascogenous hyphae.

The Ascus

Brummelen (1994,1978) summarized the structural characters of the ascus in the Pezizomycetes. Important characters are the apical apparatus, the operculum in most taxa, its position, the reaction of the ascus wall layers in various staining agents as seen in light microscopy and the ultrastructure of the ascus walls. Asci that stain blue in iodine-containing reagents (said to be amyloid) are found in Pezizaceae and Ascobolaceae (Hansen *et al.*, 2001) but this character has been lost several times in members of the Pezizaceae, most notably in some of the hypogeous species. Amyloid asci are not found outside these families. The bluing material is a long-chain polysaccharide. The function of this polysaccharide is unknown but it seems to be located on the outer surface of the wall and is sometimes seen in the gelatin matrix of the hymenium (Samuelson, 1978).

The layering of the wall as seen in TEM helps delimit groups, particularly the taxa with thick lateral walls in the Chorioactidaceae, Sarcoscyphaceae and Sarcosomataceae. The asci of these fungi were termed "suboperculate" by Le Gal (1953, 1946) because of their thick, sometimes eccentrically placed operculum. Later Eckblad (1968) and Samuelson (1975) discounted the term finding that the opercula were variously placed and wall construction conformed with that of other Pezizomycetes. In these groups the operculum is often eccentrically positioned at the ascus tip.

Asci are typically cylindrical in shape, and there are usually eight ascospores per ascus. Ascus shape can vary and spore number is often reduced in some truffle-like species, and spores may be more numerous in certain coprophilous species. In most epigeous members ascospores are actively discharged from the ascus by the build-up of osmotic pressure within the ascus. This causes a rupture at thinner areas at the apex of the ascus which creates the operculum. The releasing pressure causes the wall to give way and for the operculum to fold back allowing the ascospores and the cytoplasm of the ascus to be expelled. In some cases the asci deliquesce or are mechanically broken down and dry powdery masses of spores are produced, as in *Orbicula*. In these cases the spores are passively liberated and spread by air currents. Not all members of the Pezizomycetes have opercula or forcible discharge of ascospores. Hypogeous taxa (Læssøe and Hansen, 2007) and some cleistothecial forms (Hansen *et al.*, 2005) have lost the operculum and spores remain either within asci until the fruitbody is eaten or broken down, or the asci disintegrate and a powdery mass is formed. Most hypogeous species produce odors that attract animals and the fruiting bodies are eaten. Ascospore liberation and dispersal for these truffle-like fungi occurs through the process of digestion and excretion.

Ascospores

No member of the Pezizomycetes has septate ascospores. Septa may develop as spores germinate but only at the time a germ tube has been established. The spores may take on a variety of shapes from globose to naviculate. Spore surfaces may be smooth or with various types of surface ornamentations. Generally the ornamentations are derived from deposition of secondary wall material although a few examples of spore ornamentation without a contribution of secondary walls are known. The spore walls are multilayered and the thickness of each wall is highly variable (Merkus, 1976). Most ascospores are hyaline but purple, brown or yellowish ascospores are known. The number of nuclei per spore varies from 1, 2, 4 to many and such variation has been shown to have some taxonomic value (Berthet, 1964; Korf, 1972, 1973). Ascospores also may contain an oil guttule, which is seen under the light microscope as a refractive body within the spore. The number of guttules can also vary; some spores have one guttule, some have two guttules and in some species there are many small guttules in each spore. In some taxa there are no oil droplets. Spore guttulation may provide an important taxonomic character in identification at the genus and species level. It is important to understand that guttulation may be altered when specimens are dried, under such conditions oil droplets may fuse into a single large inclusion.

Paraphyses

Hymenium color is attributed to pigments found in the paraphyses. Yellow, red and orange carotenoid pigments are distinctive features of some of these fungi. These pigments are often present in lipid inclusions in the paraphyses. There are a variety of carotenoid pigments known to occur in these fungi as summarized by Arpin (1968). Other pigments are present either within the cytoplasm of the cells of the paraphyses or encrusted on the cell surface. The chemical composition of these other pigments is largely unknown; they give the hymenium brown, purple or black coloration. Paraphyses are generally septate. The individual cells in the paraphyses may be either uninucleate or multinucleate and this can be useful for classification (Berthet, 1964; Pfister *et al.*, 2008). In some species paraphyses are interspersed with hyaline or darkly pigmented, often thick-walled, elements or setae. Examples of such setae are found in members of the Rhizinaceae, Sarcosomataceae, Chorioactidaceae, and Sarcoscyphaceae. Paraphyses may be simple (unbranched) or they may branch either at the base or along their length. They may also anastomose (fuse) with a cell of a neighboring paraphysis. The apical cell may be the same width as subtending cells or it may be swollen, curved or bent. In some hypogeous species (e.g., *Genea* spp.), paraphyses that have elongated substantially beyond ascus length may interweave to form an epithecium over the asci.

Septal Structures

Ultrastructural characters of septal pore plugs and Woronin bodies correlate to some degree with families and lineages in the class. Kimbrough (1994) summarizes much of the information. Septal pores in vegetative hyphae generally have a lamella embedded in a matrix. Woronin bodies are generally associated with septa. The Woronin bodies take on several morphologies – globular, hexagonal or cylindrical, ranging from globose to considerably elongate. The form of the Woronin bodies is an important taxonomic and phylogenetic character. The septa at the base of the asci also provide important characters. The simplest septal pore structure is the uni- or biconvex band found in the Pezizaceae. Other families exhibit asci with basal septa that are occluded by dome-shaped, pyramidal, or dumbbell-shaped structures. These plugging structures show electron dense bands, or lamellate structures that in some species produce ray-like extensions. In general, Woronin bodies are not found in the asci of Pezizales (but see *Leucangium* in Li, 1997).

Anamorphic States

Anamorphic states have been reported across many families, though they are not known in Glaziellaceae, Helvellaceae, Karstenellaceae, Otideaceae, or Pseudombrophilaceae. **Fig. 2** provides some examples of the conidia and modes of conidial formation of members of the class. Conidia are holoblastic (spores are blown out from, and produced with both inner and outer wall of the conidiogenous cell) in development and are hyphomycetous (occurring in cushions or mats). In cases where the mitotic spores germinate to produce hyphae they are considered true conidia (e.g., Paden, 1967; Sánchez *et al.*, 2014). In many cases, however,

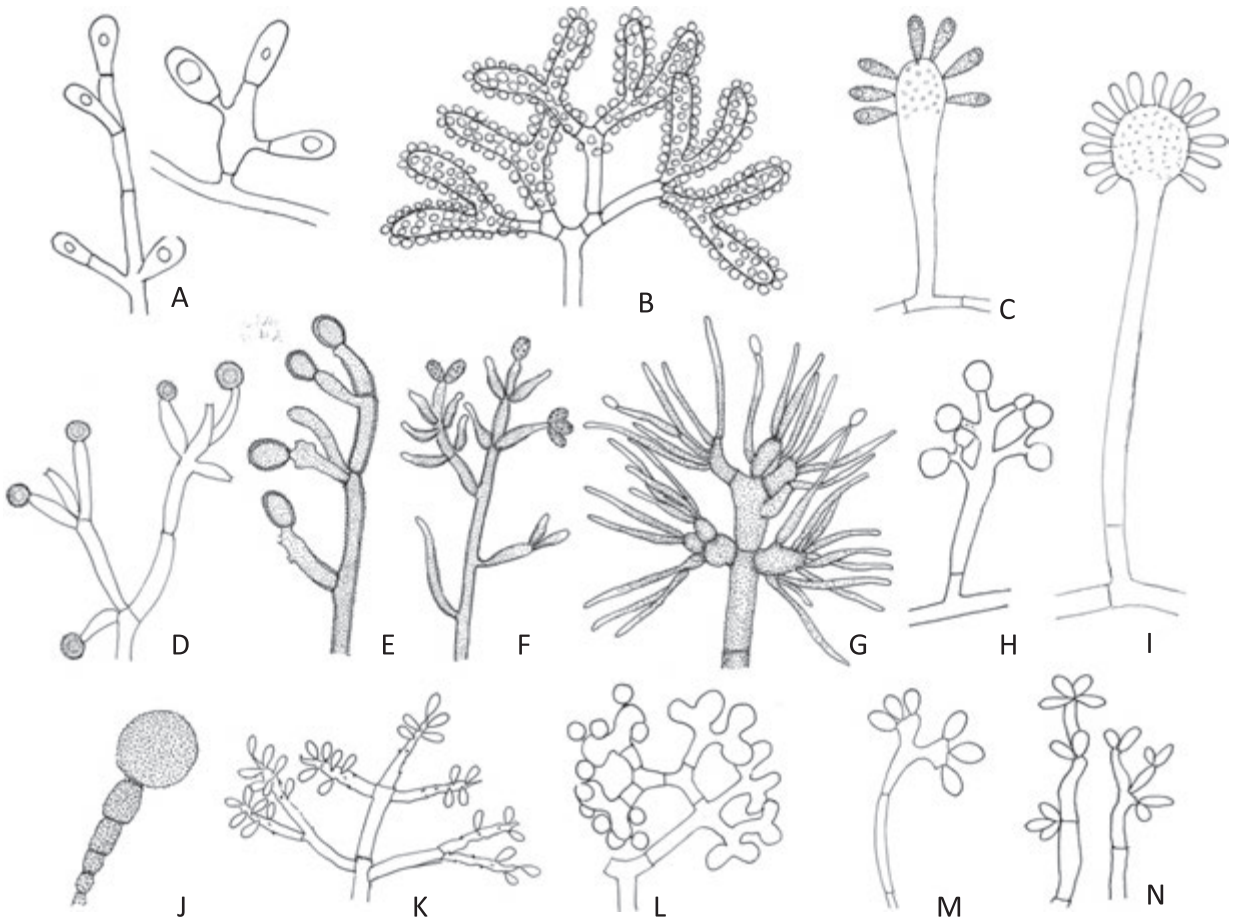

Fig. 2 Schematic representations of some conidial states of Pezizomycetes. A. *Adelphella babingtonii* (Pezizaceae), redrawn from Harrington and Pfister (2001); B. Chromelosporium-like state (Pezizaceae) found in *Peziza* species, redrawn from Seifert *et al.* (2011) C. Reduced Ostracoderma-like state (Pezizaceae), redrawn from Berthet (1964); D. Conidial state of *Hydnocystis transitoria* (Tarzettaceae), Kumar *et al.* (2017); E. Conoplea-like state of a *Plectania* species (Sarcosomataceae), redrawn from Ellis (1971); F. Conidial state of Chorioactis geaster, redrawn from Peterson *et al.* (2004); G. *Verticicladium*-like state of *Desmazierella acicula* (Chorioactidaceae), redrawn from Ellis (1971); H. *Tuber* species (Tuberaceae), redrawn from unpublished images, R.A. Healy; I. *Ostracoderma* state of *Peziza varia* (Pezizaceae), redrawn from unpublished images, R.A. Healy; J. *Complexipes* species (Pyronemataceae) clamydospore, redrawn from Yang and Korf (1985); K. *Geniculodendron* state of *Caloscypha fulgens* (Caloscyphaceae), redrawn from Paden (1978); L. *Dichobotrys*-like state of *Trichophaea abundans* (Pyronemataceae), redrawn from Carmichael *et al.* (1980), M. *Dichobotrys*-like state of *Pyropyxis rubra* (Pyronemataceae), redrawn from Egger (1984); N. *Mollardiomyes* state of *Nanoscypha tetraspora* (Sarcoscyphaceae) redrawn from Pfister (1973).

germination of mitotic spores has not been observed and this suggests that mitotic spores may act as spermatia and are thus involved in the establishment of the dikaryon. Although sexual reproduction with spermatia in saprobic Pezizomycetes has long been documented, (e.g., Bistis and Raper, 1963; Harper, 1900) it is unverified in Pezizomycetes of other nutritional modes. However, sexual outcrossing is known in *Tuber* (Murat and Martin, 2008) and suggests a possible role of mitospores in plasmogamy. The investigations by Healy *et al.* (2013) identified spore mats in several clades in the Pezizaceae and Tuberaceae that are composed of masses of mitospores. Several anamorphic states with no known teleomorphs within the Pezizomycetes have been discovered through molecular phylogenetic studies. Most notable among these is *Phymatotrichopsis omnivora*, the cotton root pathogen. This falls within the Rhizinaceae based on molecular studies but despite intensive study of this important pathogen, no evidence of a teleomorph has been discovered (Duggar, 1916; Marek *et al.*, 2009; Uppalapati *et al.*, 2010). Another example is *Cephaliophora*, a soil and dung inhabiting fungus, and one that has been implicated in causing keratitis (Goos, 1964; Hoog *et al.*, 2000).

Genomes, Microbiomes, and Model Organisms

A few Pezizomycetes have been used as model organisms. These include species in the genera *Ascobolus* where classic genetic information has been collected (Decaris *et al.*, 1974) and *Pyronema confluens*, which has been used in research on ascocarp development as well as in studies of light detection and circadian rhythms (Traeger *et al.*, 2013; Traeger and Nowrousian, 2015; Nowrousian and Kück, 2006). Comparative fungal genomics has paved the way to a broader and deeper understanding of fungal evolution, phylogenetic structure, sexual reproduction, pathogenicity and epidemiology of plant pathogens, symbiosis and other fungal biology topics (e.g., Gow, 2005; Grigoriev *et al.*, 2014; Howlett *et al.*, 2015). Despite the systematic position of the Pezizomycetes as an early derived lineage of the Pezizomycotina, along with the Orbiliomycetes, there has been relatively limited whole genome sequencing within the class. There are currently 36 complete genomes of Pezizomycetes, out of 775 genomes known for the entire subphylum Pezizomycotina (Shen *et al.*, 2020). Much of the work has focused on edible members of economic importance, such as the morels, desert truffles and "true" truffles of the Morchellaceae, Pezizaceae, and Tuberaceae (Martin *et al.*, 2010; Murat and Martin, 2008, Murat *et al.*, 2018). Analyses of the *Tuber melanosporum* genome unveiled some of the genes involved in mycorrhizal symbiosis (Martin *et al.*, 2010), and revealed the heterothallic nature of this species as well as predicting some of the genes involved in mating (Martin *et al.*, 2010, 2013).

Complete genomes of Pezizomycetes include the following: *Ascobolus immersus*, *Ascodesmis nigricans*, *Caloscypha fulgens*, *Choiromyces venosus*, *Geopyxis carbonaria*, *Gyromitra esculenta*, *Kalaharituber pfeilii*, *Leucangium carthusianum*, *Morchella* (9 species), *Peziza echinospora*, *Plectania melastoma*, *Pyronema confluens*, *Rhizina undulata*, *Sarcoscypha coccinea*, *Sphaerosporella brunnea*, *Terfezia boudieri*, *Terfezia claveryi*, *Tirmania nivea*, *Tricharina praecox*, *Trichophaea hybrida*, *Tuber* (6 species), *Verpa conica*, and *Wilcoxina mikolae*.

The average genome size in the subphylum Pezizomycotina, the filamentous Ascomycota, is around 42 Mb, which is larger than in the other two subphyla of the Ascomycota. The Pezizomycotina have a high number of genes that seem to be derived from prokaryotes whereas this is less common in Saccharomycotina (Shen *et al.*, 2020). The genome sizes of Pezizomycetes can be up to 192 Mb in size, with the genomes of *Tuberaceae* species being significantly larger and more complex than those in other families of Pezizomycetes. For example, genomes of *Morchella importuna* (48 Mb) and *Terfezia boudieri* (63 Mb) are much smaller compared to *Tuber magnatum* (192 Mb). The larger genomes in Tuberaceae species are due to the acquisition of an unusually high number of transposable elements (Murat *et al.*, 2018). Tuberaceae also has a significantly accelerated evolution of its gene repertoire compared to other ascomycetous species, possibly reflecting an increased rate of genomic evolution underlying traits specific to either symbiosis and/or hypogeous ascomatal formation. The higher rate of evolution has been hypothesized to result from the genome rearrangements caused by repositioning of transposable elements (Martin *et al.*, 2010).

Shen *et al.* (2020) used genomes in reconstruction of an Ascomycota phylogeny. This phylogeny attempted to clarify the relationship of the Pezizomycetes and the Orbiliomycetes in relationship to their position as basal taxa in the Pezizomycotina. Debate had centered on which of these classes were the most basal with several multigene phylogenies showing one or the other to have the most basal position. Shen *et al.* (2020) suggest that the Orbiliomycetes and Pezizomycetes together form a clade that is the sister group to the remainder of the Pezizomycetes.

The microbiome of yeasts, bacteria and fungi that characterize the Pezizomycetes has been an active area of study in recent years. A comparative study of bacterial microbiomes within fruiting bodies and mycelia of four genera of truffles revealed that although there are multiple bacterial genera in the microbiome, one *Bradyrhizobium* genotype was dominant in all analyzed *Tuber* species, regardless of geographic location, but not present to the same degree in the other truffles analyzed (Benucci and Bonito, 2016). Bradyrhizobiaceae bacteria have been implicated in the release of sulfur containing compounds from *Tuber borchii* microbiomes. It is thought that a mixture of volatiles produced through interactions among the fungi and bacteria in the microbiome may characterize the odor perceived by humans (Splivallo *et al.*, 2015). These odors help to attract the animals that disperse the spores of truffles, and thus the bacterial-fungal relationship may be symbiotic. Similar studies of other species of epigeous and hypogeous Pezizomycetes may help to understand the extent of such interactions between these fungi and their microbiomes.

Classification and Phylogenetic Studies

The class Pezizomycetes has undergone significant reorganization in recent years due to results from molecular phylogenetic studies. These studies have provided new insights into relationships and have facilitated the evaluation of both morphological and

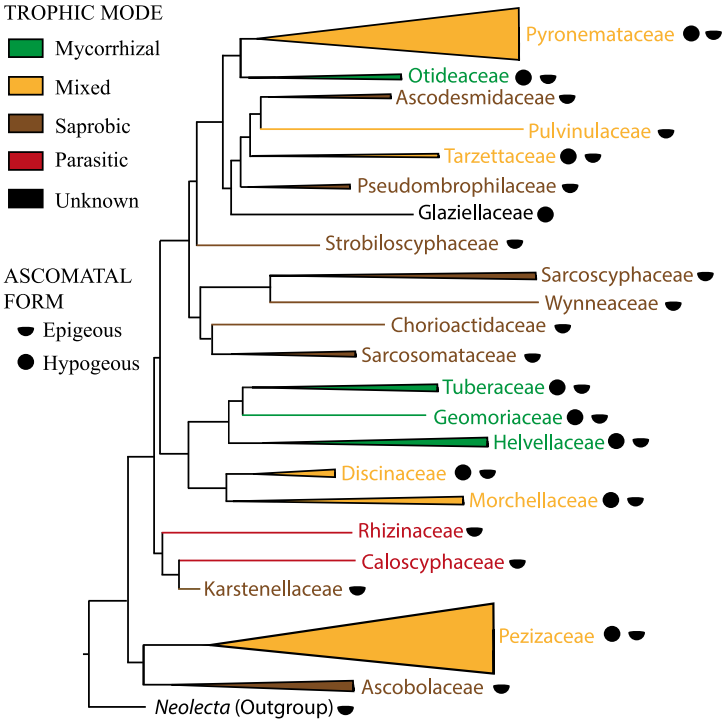

Fig. 3 Current phylogenetic framework for 22 families of Pezizales. Phylogeny based on maximum likelihood analysis of combined DNA loci (SSU, ITS, LSU, TEF, RPB2). Symbols depict known ascomatal form (epigeous and apothecial or hypogeous and truffle-like) and colors indicate ecological roles in each family. "Mixed" refers to a mixture of trophic modes within a family. Sizes of triangles are approximately proportional to numbers of known species in each family. Graphic of phylogenetic framework modified from Ekanayaka *et al.* with additional families approximately placed as determined by Kraisitudomsook *et al.* and Pfister *et al.* Modified from Ekanayaka, A.H., Hyde, K.D., Jones, E.B.G., Zhao, Q., 2018. Taxonomy and phylogeny of operculate discomycetes: Pezizomycetes. Fungal Divers. 90, 161–243. doi:10.1007/s13225-018-0402-z. Kraisitudomsook, N., Healy, R.A., Pfister, D.H., *et al.*, 2019. Resurrecting the genus Geomorium: Systematic study of fungi in the genera Underwoodia and Gymnohydnotrya (Pezizales) with the description of three new South American species. Persoonia 44, 98–112. doi:10.3767/persoonia.2020.44.04. Pfister, D.H., Quijada, L., LoBuglio, K.F., 2020. Geodina (Pezizomycetes: Wynneaceae) has a single widespread species in tropical America. Fungal Syst. Evol. 5, 131–138. doi:10.3114/fuse.2020.05.08.

ecological characters. For example, the hypogeous species of Pezizomycetes were almost all previously treated in the order Tuberales due to their convergent morphological forms. However, recent studies have now placed these hypogeous taxa in at least ten different Pezizomycete families. All of these families with hypogeous members have at least some ectomycorrhizal species and indeed nearly all of the hypogeous Pezizomycetes are ectomycorrhizal, with exceptions in Glaziellaceae, Pezizaceae and Tarzettaceae. **Fig. 3** shows relationships among the Pezizomycetes as we currently recognize them and also indicates growth habit and trophic status.

The 22 families we currently recognize in the class are summarized below. Apothecial morphology varies greatly, particularly in the larger families; **Fig. 1** provides an overview of the patterns of apothecial forms found in this class. **Table 1** is a guide to some critical characters of the families.

Families

(1) Ascobolaceae – These generally small apothecial fungi have amyloid asci (asci that give a blue reaction in iodine solutions). This character is shared with the Pezizaceae listed below. This reaction is diffuse in the Ascobolaceae and is often detected in the gelatinous material in the hymenium. The members of the family are distributed worldwide and are mostly found on dung. A few species are found on soil or burned debris. In *Ascobolus* and *Saccobolus* the spores become purple or brownish at maturity. The genus *Thecotheus*, that occurs on dung but also other substrates, has unpigmented ascospores. Sclerotia are not known in this family. Names used for the anamorphs in *Ascobolus* referred to the form genera *Oidia*, *Papulaspora*, and *Rhizostilbella*.
Genera included: *Ascobolus*, *Cleistoiodophanus*, *Saccobolus*, and *Thecotheus*.

(2) Ascodesmidiaceae – These fungi occur on dung but also on soil and debris. The morphology in the family ranges from a single ascus occurring along hyphae to simple fascicles of a few asci to apothecia with a hymenium and well-developed apothecial tissues. This family is currently placed as the sister group to Pulvinulaceae but there is low statistical support for this placement. Sclerotia are not known in this family. The family Coprotaceae was recently described recognizing a clade previously detected by Hansen *et al.* (2013). The genera included in this family are *Coprotus* and *Boubouvia*. Other genera

Table 1 A guide to salient features of members of the families of the Pezizomycetes. This table is intended to offer guidance for the morphological placement of species within the framework of current families. Note that characters may appear in several columns

Family	*Ascus J+*	*Ascus J−*	*Ascus +/− thick-walled*	*Asci indehiscent*	*Asci opening via an operculum*	*asci produced in naked fascicles or directly on hyphae*	*Saprobic*	*Plant parasitic including mosses*	*Mycorrhizal*	*Mycoparasitic*	*Ascomata epigeous*	*Ascomata hypogeous*	*ascomata minute cleistothecial or perithecial*	*ascomata stipitate*	*ascomata sessile or substipitate*	*Ascospores uninucleate*	*Ascospores multi-nucleate*	*2-4-nucleate*	*Ascospores hyaline*	*ascospores pigmented*
Ascobolaceae	X				X		X				X		X		X	X			X	X
Ascodesmidaceae		X		X	X	X	X				X				X	X			X	X
Caloscyphaceae		X			X		X	X			X				X	X			X	
Chorioactidaceae		X	X		X		X				X				X		X		X	
Discinaceae		X		X	X		X		X		X	X		X	X			X	X	
Glaziellaceae		X	X	X			X ?		?			X			X	?	?	?	X	
Geomoriaceae		X		X	X				X		X	X		X	X	?	?	?	X	
Helvellaceae		X		X	X				X		X	X		X	X			X	X	
Karstenellaceae		X			X		?	?	?		X				X			X	X	
Morchellaceae		X		X	X		X		X		X	X		X	X		X		X	
Otidiaceae		X		X	X	X	X		X		X	X	X	X	X	X			X	
Pezizaceae	X	X		X	X		X		X		X	X		X	X	X			X	X
Pseudombrophilaceae		X		X	X	X	X				X		X		X	X			X	
Pulvinulaceae		X			X		X		X		X				X	X			X	
Pyronemataceae		X		X	X	X	X	X	X	X	X	X	X	X	X	X			X	
Rhizinaceae		X			X		X	X			X				X	?	?	?	X	
Sarcosyphaceae		X	X		X		X	X			X			X	X		X		X	
Sarcomataceae		X	X		X		X				X			X	X		X		X	
Strobiloscyphaceae		X	X		X		X				X				X	?	?		X	
Tarzettaceae		X		X	X		X		X		X	X			X	X			X	
Tuberaceae		X		X	X		X		X		X	X			X	X		X	X	X
Wynneaceae		X	X		X					X	X			X			X?		X	

remaining in the Ascodesmidaceae may also belong here such as *Dictyocoprotus* and *Pseudobobouvia*. Two genera have no known teleomorphs: *Cephaliophora* and *Pulchromyces* (Hennebert, 1973; Tanabe *et al.*, 1999; Thaxter, 1903).
Genera included: *Ascocalathium (?)*, *Ascodesmis*, *Bobouvia*, *Cephaliophora*, *Coprotus*, *Dictyocoprotus*, *Eleutherascus*, *Lasiobolus*, *Ochotrichobolus*, *Pseudoboubovia*, *Pseudocoprotus*, *Pulchromyces (?)* and *Trichobolus*.

(3) Caloscyphaceae – Two apothecial genera are placed in this family: *Caloscypha* and *Kallistoskypha*. *Caloscypha fulgens* is the only species currently recognized in the genus. It is yellow-orange and often colors blue green when handled. *Kallistoskypha* is also a monotypic genus, with *K, incarnata* as the sole member. Ekanayaka *et al.* (2018) placed it as the only genus in a separate family, Kallistoskyphaceae. However, this placement was based on only two genes in a five-gene analysis, resulting in its placement on a long branch. A study by Pfister *et al.* (2013) placed this species in the Caloscyphaceae with strong statistical support. More robust molecular data may better resolve the relationships between *Kallistoskypha* and other Pezizomycetes. The ascomata of *K. incarnata* are cupulate, pinkish and the ascospores are globose and smooth. The species has been collected on soil in the Mediterranean region. Because of its association with *Eucalypus* Pfister *et al.* (2013) suggested that it may represent an introduction of an australasian fungus to the Mediterranean region. *Caloscypha fulgens* is found on soil in north temperate regions. Sclerotia are not known in this family. The conidial state of *C. fulgens*, known as *Geniculodendron pyriforme*, has been implicated as a seed pathogen of conifers (Salt, 1974).
Genera included: *Caloscypha*, *Kallistoskypha*.

(4) Chorioactidaceae – Members of this family are characterized by thick-walled asci with sometimes eccentrically-placed opercula. The apothecia are clothed with brown hairs that are generally encrusted with pigmented granules. The ascospores are ornamented with either pits or ridges. There is a tendency for the asci to mature simultaneously within a single apothecium. Sclerotia are not known in this family. The form genus *Kumanasamuha* was applied to the anamorphs in *Chorioactis* and *Trichaleurina* (Carbone *et al.*, 2013; Nagao *et al.*, 2009), and a *Verticicladium*-like conidial state was described for *Desmazierella acicula* (Gremmen, 1949).
Genera included: *Chorioactis*, *Desmazierella*, *Neuronal*, *Psueodosarcosoma*, *Trichaleurina*, and *Wolfina*.

(5) Discinaceae – There are both epigeous and hypogeous taxa in the family (Læssøe and Hansen, 2007). The hypogeous *Hydnotrya* is ectomycorrhizal; other taxa are saprobic associated with wood, soil with organic matter, or woody debris. The epigeous members are generally large and either stipitate or sessile. Some are distinctive members of the spring funga. Conidial states have been described by Carris *et al.* (2015) and Healy *et al.* (2013) and are similar to the *Costantinella* type observed in the Morchellaceae.
Genera included: *Discina*, *Gyromitra*, and *Hydnotrya*.

(6) Geomoriaceae – The single genus in this family includes both epigeous and hypogeous species. Ascomata are either modified columnar apothecia or exothecia, and vary widely in size from 1 to 40 mm high. Young ascomata are typically white or pale tan but often change to brown, purple, or black as the hymenium matures. The outer excipulum and stipe are only present in the epigeous taxa. Paraphyses may slightly or greatly exceed the asci in length. The asci lack opercula. They are ectomycorrhizal, found in forests or at forest edges, and known only from the Southern Hemisphere. Conidial states and sclerotia are unknown in this genus.
Genus included: *Geomorium*.

(7) Glaziellaceae – Large hollow ascomata of this fungus are generally formed under or on the surface of soil and debris on the forest floor in tropical localities. The family has a single genus and *G. aurantiaca* is the recognized species (Gibson *et al.*, 1986; Hansen and Pfister, 2006). Other species described as *Glaziella* have since been determined to belong to an unrelated lineage (Thaxter, 1922). Prior to electron microscopy and phylogenetic studies, *G. aurantiaca* was variously placed among the Endogonaceae (Mucoromycota) and other lineages of the Ascomycota. There has been limited molecular sampling of collections of *G. aurantiaca*, which appears to be restricted to the new world. Other species have been assigned to the genus from Asia but these are molecularly unverified. Transmission electron microscopy revealed single-spored asci that deliquesce with maturity (Gibson *et al.*, 1986), and molecular methods placed it in the Pezizomycetes (Landvik and Eriksson, 1994). The trophic status is not known; there is no convincing evidence that it forms ectomycorrhizae. The single-spored globose asci are scattered in the rind-like flesh of the ascomata. Conidial states are unknown in this genus.
Genus included: *Glaziella*

(8) Helvellaceae – Ascomata are hypogeous or epigeous, they are ectomycorrhizal. Epigeous members are cupulate, ear-shaped, saddle-shape or sparassoid with or without a stipe. They are generally without carotenoid pigments. The asci are thin walled and the ascospores are smooth or irregularly ornamented. Generally there is a single large guttule and there are four nuclei per spore. *Balsamia* and *Barssia*, hypogeous genera, produce stereothecia or ptychothecia.
Genera included: *Balsamia*, *Barssia*, *Helvella*, *Midotis*, *Pindara*, *Underwoodia*.

(9) Karstenellaceae – The only species in the genus, *Karstenella vernalis*, produces thin apothecia on a resupinate mycelial mat that grows directly on soil (Hansen *et al.*, 2008) or woodland litter (Harmaja, 1969). It is presumed to be saprobic (Ekanayaka *et al.*, 2018) but the ecology of this species is not well-known. Ascospores are bi- or multiguttulate. The species has been collected in widely disjunct geographical regions of Finland and New Mexico, USA (Hansen *et al.*, 2008). There have been no reports of sclerotia or conidial states in this species.
Genus included: *Karstenella*.

(10) Morchellaceae – The morels are choice edible fungi and the family also has hypogeous members. The family includes the genus *Morchella* that is characterized by large stalked ascomata that have a convoluted and ridged fertile region. *Verpa* is

similarly constructed but with a continuous hymenial layer whereas *Disciotis* is cupulate, sessile and folded or convoluted. *Morchella*, *Disciotis* and *Verpa* are epigeous but there are several hypogeous genera in the family. These produce exothecial, ptychothecial or stereothecial ascomata. One, hypogeous species, *Fischerula subcaulis*, has a residual to well-formed stalk In the past some studies suggested that the species in this group are ectomycorrhizal but at least some species of *Morchella* grow without any ectomycorrhizal host plants nearby and they can be grown in culture. Tedersoo and Smith (2013) suggested that *Fischerula*, *Imaia*, *Kalapuya* and *Leucangium* are ectomycorrhizal based on morphological studies of ECM roots and based on the isotopic signatures of fruiting bodies but more evidence is needed to test this hypothesis. Sclerotia are formed by at least some *Morchella* species (Ower, 1982; Volk and Leonard, 1989). *Morchella* species have anamorphic states that were previously placed in the form genus *Costantinella*, and *Disciotis* has a similar anamorph (Carris *et al.*, 2015). An anamorph was also reported for *Fischerula* (Healy *et al.*, 2013).

Genera included: *Disciotis*, *Fischerula*, *Imaia*, *Kalapuya*, *Leucangium*, *Morchella*, *Verpa*.

(11) Otideaceae – The family was formerly used by some authors in a broad sense to include many of the Pyronemataceae. In the restricted sense, this family includes the ectomycorrhizal genus *Otidea* and a few others. At least one hypogeous *Otidea* species is known. Ascomata are apothecial in most species, ptychothecial in *O. subterranea*, or cleistothecial in *Warcupia*. In *Monascella* the asci are produced on hyphae in small clusters. The genus *Acervus* is apothecial but in early development the ascomata are closed. Species of *Otidea* form ectomycorrhizal associations whereas the other species are presumed saprotrophic. There are no sclerotia or anamorphs reported for this family.

Genera included: *Acervus*, *Arpinia (?)*, *Ascosparassis*, *Monascella*, *Otidea*, *Warcupia*, and *Wenyingia*.

(12) Pezizaceae – Members are ectomycorrhizal or saprobic. Some species occur after fire. They range in size from small to large and generally are fragile due to the large cells that often compose the excipulum. Hypogeous species may be ptychothecial, stereothecial, or exothecial. Most members of the family have iodine positive asci (amyloid) but this character is lost in most of the hypogeous taxa and absent in some of the early derived lineages. This family and the Ascobolaceae are the only families with the iodine positive reaction of the asci. The extent, location and strength of the iodine reaction is diagnostic at the clade level. In some species there is a strong wall reaction with or without an apical ring. In other species the reaction is diffuse and often appears to be in the gel surrounding the asci. These differences in reactions in iodine have been discussed by VanVooren (2020), who also established or reinstated several genera to recognize groups within the heterogeneous genus *Peziza*. There are several species-rich hypogeous lineages that are derived within ectomycorrhizal clades (with exceptions noted in previous sections). The desert truffles belong to this family (Kovács and Trappe, 2014). Among the desert truffles, *Terfezia* and *Tirmania*, are economically important, and in some cases, medically important in the Middle East (Hamza *et al.*, 2016; Shavit, 2014; Shavit and Shavit, 2014). Sclerotia are produced by species of *Cheilymenia* (Smith *et al.*, 2015) and *Mattirolomyces* (Kovács *et al.*, 2007). Anamorphic states described in this lineage were historically placed under the names *Chromelosporium*, *Glischroderma*, *Oedocephalum*, *Ostracoderma*, and *Rhinotrichum* (Hennebert, 1973; Norman and Egger, 1999; Paden, 1972), and a different anamorphic state was reported for *Hydnobolites* (Healy *et al.*, 2013). Some of the ectomycorrhizal taxa are only known from their anamorphic states.

Genera included: *Adelphella*, *Ahmadea*, *Amylascus*. *Antrelloides*, *Aquapeziza*, *Babosia*, *Boudiera*, *Calongea*, *Carbomyces*, *Cazia*, *Chromelosporiopsis*, *Cleistoiodophanus*, *Daleomyces*, *Delastria*, *Elaiopezia*, *Elderia*, *Eremiomyces*, *Geoscypha*, *Hansenopezia*, *Hapsidomyces*, *Hydnobolites*, *Hydnoplicata*, *Hydnotryopsis*, *Iodophanus*, *Iodowynnea*, *Ionopezia*, *Kalaharituber*, *Legaliana*, *Lepidotia*, *Luteoamylascus*, *Malvipezia*, *Marcelleina*, *Mattirolomyces*, *Mycoclelandia*, *Pachyella*, *Pachyphlodes*, *Paragalactinia*, *Peziza*, *Phaeopezia*, *Phylloscypha*, *Planamyces*, *Plicaria*, *Plicariella*, *Purpureodiscus*, *Rhodopeziza*, *Ruhlandiella*, *Sarcopeziza*, *Sarcosphaera*, *Sphaerosoma*, *Sphaerozone*, *Stouffera*, *Temperantia*, *Terfezia*, *Tirmania*, *Ulurua*.

(13) Pseudombrophilaceae – Included here are several taxa that are saprobic on dung or decaying plant material. Several of the included taxa, such as *Heydenia* species, *Lasiobolidium orbicularis* and *Orbicula* species, produce cleistothecioid ascomata whereas *Pseudombrophila* species produce apothecia. Sclerotia are formed by some species of *Pseudombrophila* (Pfister, 1984). Anamorphs are not known in this family.

Genera included: *Heydenia*, *Orbicula*, *Pseudombrophila*, *Lasiobolidium sensu lato*

(14) Pulvinulaceae – These fungi produce small, pulvinate apothecia on soil. Many are orange or yellow due to carotenoid pigments. Some species in this group produce paraphyses that are clavate to circinate, which is an important diagnostic feature. Species of *Pulvinula* are ectomycorrhizal whereas taxa in the other genera are saprobic. Anamorphs and sclerotia are not known in this family.

Genera included: *Lazuardia*, *Pseudoboubovia*, *Pulvinula*

(15) Pyronemataceae – Despite a good deal of recent phylogenetic work this family remains difficult to characterize. Some species are saprobic on plant material and dung, others are ectomycorrhizal, and some are endophytic or endolichenic. Several species fruit prolifically in response to fire. Species of several genera are parasitic on bryophytes. Pigmentation ranges from those that are brightly colored with carotenoids to drably colored species lacking these pigments. Although some ascomata are smooth, many members have hairs at the base or hairs or setae that emanate from the excipulum. The family also has some hypogeous and perithecioid species. Sclerotia are produced by some species of *Cheilymenia* (Smith *et al.* 2015) and *Pyronema* (Moore, 1962). Anamorphs have been reported for some lineages, under the form genera names *Actinospora*, *Ascorhizoctonia* (recently separated from *Tricharina* as a holomorphic genus by Van Vooren *et al.* (2017)), *Complexipes*, *Dichobotrys* and *Micronematobotrys* (Egger, 1984; Hennebert, 1973; Sun and Guo, 2010; Yang and Korf, 1985). An anamorph was recently described for *Octospora* (Sochorová *et al.*, 2019). One form genus, *Sphaerosporium*, has no known teleomorph (Song *et al.*, 2019).

Genera included: *Aleuria, Aleurina, Anthracobia, Ascorhizoctonia, Aurantiolachnea, Barlaea, Boudierella, Byssonectria, Chaetotheirsia, Chalazion, Cheilymenia, Cleistothelebolus, Cupulina, Eoaleurina, Filicupula, Galeoscypha, Genabea, Genea, Geopora, Gilkeya, Hiemsia, Hoffmannoscypha, Humaria, Hypotarzetta, Jafnea, Lamprospora, Lasiobolidium sensu stricto, Lasiocupulina, Lathraeodiscus, Leucoscypha, Lotinia, Luciotrichus (?), Melastiza, Miladina, Moravecia, Mycogalopsis, Myrmecocystis, Neottiella, Octospora, Octosporella, Octosporopsis, Oviascoma, Parascutellinia, Paratricharina, Paratrichophaea, Parawilcoxina, Perilachnea, Petchiomyces, Picoa, Pseudaleuria, Pseudotricharina, Pyronema, Pyropyxis, Ramsbottomia, Rhizoblepharia, Rhodoscypha, Rhodotarzetta, Scutellinia, Sepultaria, Sepultariella, Smardaea, Smarodsia, Sowerbyella, Sphaerosoma, Sphaerosporium, Sphaerosporella, Spooneromyces, Terracavicola, Tricharina, Tricharinopsis, Trichophaea, Trichophaeopsis, Wilcoxina*

(16) Rhizinaceae – Three genera are placed in this family. Species of *Rhizina* and *Psilopezia* are known only as teleomorphs; they occur on wood and woody roots. *Rhizina*, which occurs on burned areas, has been implicated as causing a root rot. *Psilopezia* species grow on wet, water-soaked wood. Spores are large and in *Rhizina* ascospores are smooth and have a pronounced apiculus. Ascomata in these two genera expand through indeterminate marginal growth. The third genus in this family, *Phymatotrichopsis omnivora*, is a serious pathogen of cotton and other dicots (Marek *et al.*, 2009; Uppalapati *et al.*, 2010). Sclerotia are formed by *P. omnivorum*, and this taxon is only known from its anamorphic state (Marek *et al.* 2009).
Genera included: *Phymatotrichopsis, Psilopezia, Rhizina*

(17) Sarcoscyphaceae – These include some of the most charismatic members of the class with their sometimes large apothecia that can be bright red to pink and are sometimes fringed with hairs. Some have a prominent stalk. Asci are thick-walled and the operculum is generally eccentrically placed. The bright colors originate in the paraphyses of the hymenia and the spores tend to be laterally asymmetrical. Spores may be smooth or in some species ornamented with ridges and folds. The ornamentation is cyanophobic (they do not take up cotton blue stain). Some species are found in temperate regions but many others are tropical or subtropical. They are assumed to be saprobes or weak plant parasites. The form genus *Molliardiomyces* has been applied to conidial states in species of *Nanoscypha, Pithya, Phillipsia* and *Sarcoscypha* (Paden, 1984; Pfister, 1973), and an anamorph has also been described for *Cookeina* species (Boedijn, 1929, 1932).
Genera included: *Cookeina* (including *Boedijnopeziza*), *Kompsoscypha, Microstoma, Nanoscypha, Phillipsia* (including *Aurophora*), *Pithya, Pseudopithyella, Rickiella, Sarcoscypha, Thindia*

(18) Sarcosomataceae – These fungi are black or darkly pigmented. Like the Sarcoscyphaceae the ascus walls tend to be thick and the operculum is eccentrically placed. The outer layers of the excipulum tend to be encrusted with dark pigments. Spores are thick-walled with or without folds and ornamentation. Often hymenial hairs or setae are present. Species are considered to be saprobic or weakly parasitic on plants. Anamorph states include *Conoplea* and possibly *Strumella* for *Urnula* and *Plectania* (Paden, 1972). At least one species of *Sarcosoma* also produces an anamorph (Paden, 1972).
Genera included: *Donadinia, Galiella, Korfiella, Plectania, Pseudoplectania, Sarcosoma, Selenaspora, Urnula*.

(19) Strobiloscyphaceae – A single genus, *Strobiloscypha*, with two species makes up the family. The species are small, have brown apothecia with olive-toned hymenia, and are presumed to be saprobic, occurring on the dead branches and cones of Cupressaceae (Perić *et al.*, 2013). Even with molecular data, the relationship between Strobiloscyphaceae and other Pezizomycetes is unsettled. Previous placement was speculative, and there is no strong support for its relationships in the most recent treatment (Ekanayaka *et al.*, 2018). A *Verticicladium*-like conidial state was described for one species in this family (Perić *et al.*, 2013).
Genus included: *Strobiloscypha*

(20) Tarzettaceae – This family contains both epigeous and hypogeous members. Kumar *et al.* (2017) treated the hypogeous members. Wang *et al.* (2016) treated the genus *Geopyxis*. *Tarzetta* is ectomycorrhizal but the nutritional strategies of the other genera are non-mycorrhizal and not well understood. At least some species of *Geopyxis* can act as root biotrophs but there is no strong evidence for ectomycorrhizal formation (Vrålstad *et al.*, 1998). Anamorphs are known for three of the five genera and are similar within the family. The form genera *Dicyma, Hansfordia* and *Nodulisporium* have been misapplied to some of these conidial states (Conway, 1975; Egger, 1984; Hennebert, 1973; Paden, 1972; Vrålstad *et al.*, 1998).
Genera included: *Densocarpa, Geopyxis, Hydnocystis, Paurocotylis, Tarzetta*

(21) Tuberaceae – Most species are hypogeous with the exception of two in the Southern Hemisphere genus *Nothojafnea*, which form apothecial ascomata. All other known taxa form ptychothecial or stereothecial ascomata. All known species are ectomycorrhizal and most are considered early successional. The highly prized "true truffles" in the genus *Tuber* belong to this family. Sclerotia are not reported in this family. Conidial states that are similar in morphology have been described for some species in the/maculatum and/puberulum lineages of *Tuber* (Grupe *et al.*, 2018; Healy *et al.*, 2013; Urban *et al.*, 2004).
Genera included: *Choiromyces, Dingleya, Labyrinthomyces, Loculotuber, Nothojafnea, Reddellomyces, Tuber*

(22) Wynneaceae – Recently separated from the Sarcoscyphaceae based on molecular and ecological data (Pfister *et al.*, 2020). The elaborate fruitbodies of *Wynnea* species arise from a mass of tissues that include hyphae and rhizomorphs of *Armillaria* species. It is suggested that *Wynnea* species may parasitize fungi from the genus *Armillaria* and produce large sclerotia-like bodies built-up of hyphae from both *Amillaria* and *Wynnea* (Xu *et al.*, 2019). The biotrophic status of *Geodina* is unknown but the unusual report of this as occurring on soil suggests that, like *Wynnea* species, it may be associated with another fungus. Anamorphs are not known in this family.

Genera included: *Wynnea, Geodina*.

Genera of uncertain position include: *Aparaphysaria, Ascocalathium, Microeurotium*.

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Outline of Basidiomycota

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Brief Introduction of Basidiomycota

Basidiomycota R.T. Moore 1980 is a major lineage of fungi embracing more than 40,000 species which takes nearly 1/3 fungal species (Hawksworth and Lücking, 2017; He *et al.*, 2019). It comprises most of the macrofungi including edible and medicinal mushrooms which is a significant part of human diet. It was estimated that the global mushroom market is at US$36,825.4 million by the end of 2016 and is expected to exhibit a compound annual growth rate (CAGR) of 8.2% from 2016 to 2024, rising to a valuation of US$69,267.9 million by 2024 (Transparency Market Research, 2016). The top three mushrooms in the market are *Agaricus bisporus* (J.E. Lange) Imbach (button mushroom), *Lentinus edodes* (Berk.) Singer (Shiitake Mushroom) and *Pleurotus ostreatus* (Jacq.) P. Kumm. (Oyster Mushroom). Medicinal mushrooms have an established history of use in traditional ancient therapies. They contain biologically active polysaccharides in the basidiomes which were associated in many medicinal studies of antitumor, immunomodulating, antioxidant, radical scavenging, cardiovascular, antihypercholesterolemia, antiviral, antibacterial, antiparasitic, antifungal, detoxication, hepatoprotective, and antidiabetic effects (Gao *et al.*, 2002, 2003, 2004; Wasser and Didukh, 2003; Rowan *et al.*, 2003; Zhang *et al.*, 2007; Dai *et al.*, 2009). Some famous species are Ling Zhi (*Ganoderma lingzhi* Sheng H. Wu, Y. Cao & Y.C. Dai), Sanghuang (*Sanghuangporus* Sheng H. Wu, L.W. Zhou & Y.C. Dai), *Inonotus obliquus* (Pers.: Fr.) Pilát, and *Fomitopsis officinalis* (Vill.: Fr.) Bond. et Singer (Poder, 2005; Cao *et al.*, 2012; Zhou *et al.*, 2016). Meanwhile, many poisonous mushrooms are also well-known due to its lethality, for example, death cap (*Amanita phalloides* (Vaill. ex Fr.) Link), which killed thousands of people throughout the history, was on the world's ten most feared fungi list (Hyde *et al.*, 2018). Besides the macrofungi, plant pathogenetic smut, rust and industrial important yeast are also members of Basidiomycota. Rust fungi as a group are among the most economically important pathogens of many native and cultivated plants, for example, wheat steam rust (*Puccinia graminis* Per.) and coffee leaf rust (*Hemileia vastatrix* Berk. & Broome). Diseases caused by rust fungi are the major concerns and sometimes limiting factors for successful cultivation of such internationally important crops as wheat, corn, coffee and pine (Cummins and Hiratsuka, 2003).

In the ecosystem, species of Basidiomycota are possibly the main contributors to wood and litter degradation, including degradation of the different components of wood, and they play a key process in carbon recycling (Pelaez *et al.*, 1995; Pointing, 2001; Pointing *et al.*, 2005; Oberwinkler, 2012). They form symbiotic relationships with plant species called ectomycorrhizal which play a fundamental role in the nutrient cycle in terrestrial ecosystems, especially in forest systems (Domínguez-Núñez and Albanesi, 2019). They also form symbiotic relationships with insect, for example, species of *Termitomyces* R. Heim, which grow as symbionts in the termite nests. The basidiomes cultivated by termites were considered as delicate in many countries (Wood and Thomas, 1989; Hsieh and Ju, 2018).

Advances on Phylogeny of Basidiomycota

With the evidences from molecular phylogeny, Basidiomycota was shown to be a monophyletic group sister to Ascomycota (James *et al.*, 2006; Hibbett *et al.*, 2007; Zhao *et al.*, 2017), and four major clades were revealed: Agaricomycotina (mushrooms, jelly fungi, bracket fungi and others), Pucciniomycotina (rusts and others), Ustilaginomycotina (smuts and others) (Hibbett *et al.*, 2007) as well as Wallemiomycotina (Matheny *et al.*, 2006; Padamsee *et al.*, 2012; Zhao *et al.*, 2017). Higher-level classification of Basidiomycota was published around ten years ago, based mainly on a series phylogenetic studies, such as the Deep Hypha and AFTOL projects (Lutzoni *et al.*, 2004; Blackwell *et al.*, 2006; James *et al.*, 2006; Hibbett *et al.*, 2007). All of the recently introduced higher-level taxa (hereon refers to orders and above) were studied alongside related taxa. No recent study has combined all high-ranked taxa in a single phylogeny to establish whether they are well-resolved. Furthermore, a large amount of knowledge on Basidiomycota has been published in the past ten years. Numerous new species recognized, which has greatly enriched our knowledge on diversity of Basidiomycota. At the same time, related new taxonomic categories were proposed. For example, in phylogenetic studies of basidiomycetous yeasts, three new classes Malasseziomycetes, Monilielliomycetes, and Spiculogloeomycetes were introduced as well as three new orders, 16 new families, and 47 new genera (Nasr *et al.*, 2014; Wang *et al.*, 2014; Wang *et al.*, 2015a,b; Liu *et al.*, 2015).

In recent years, divergence times have been used as an additional criterion to rank taxa which have been accepted and successfully applied in many fungal systematic studies (Drummond *et al.*, 2012; Zhao *et al.*, 2016; Hongsanan *et al.*, 2017; Liu *et al.*, 2017). The following are criteria applied in these systematic studies to rank taxa above species level: (1) the taxa must be monophyletic and statistically well-supported in multi-gene analyzes; (2) their respective stem ages should be roughly equivalent, and higher taxa stem ages must be older than lower level taxa stem ages; and (3) the taxa should be identifiable phenotypically, whenever possible.

Based on the advances of systematic studies of Basidiomycota in the past ten years, we first revised the phylogenetic relationships of Basidiomycota with multigene and genomic data by a series analyzes: multi-genes phylogenetic analyzes with six genes (LSU,

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SSU, 5.8 s, rpb1, rpb2, ef1) from 539 species representing 18 classes, 3 subclasses, 62 orders, 183 families and 392 genera; phyloproteomics analyzes with 396 orthologous genes from 116 species representing 17 classes and 54 orders (Zhao *et al.*, 2017); molecular dating analyzes including 771 species from 60 orders and 185 families (He *et al.*, 2019). We estimated the divergence times of Basidiomycota and propose to use following time ranges to rank taxa in Basidiomycota: subphyla should be in 406–430 Mya, classes should be in 211–383 Mya, orders should be in 99–323 Mya and families should be in 27–222 Mya (Zhao *et al.*, 2017; He *et al.*, 2019). Based on the revised phylogenetic relationships of key clades of Basidiomycota, the taxonomic status of 3198 genera names in Basidiomycota had been revised (He *et al.*, 2019).

The Taxonomic System of Basidiomycota

Historically, the taxonomic systems of fungi had been gathered in Dictionary of Fungi. The last version was published in 2008, in that version, there are 3 subphyla, 16 classes, 52 orders, 177 families, 1598 genera and 31,315 species in Basidiomycota (Kirk *et al.*, 2008). As mentioned before, numerous new taxa were published and related new taxonomic categories were proposed. Thus, the following outline of Basidiomycota was proposed which associated all these taxonomic changes of Basidiomycota.

All generic names gathered from Index Fungorum (2019) were checked through Kirk *et al.* (2008, 2013) and Species Fungorum (2019). Nomina invalida, nomina rejicienda and synonyms were excluded. There are 1928 legal genera verified with 1263 synonym names and these legal genera are taxonomically arranged. Furthermore, a brief note for each accepted genus was provided including information on their classification, number of accepted species, type species, life mode, habitat, distribution, and sequence information (He *et al.*, 2019). At present, there are 41,270 species in Basidiomycota and the most comprehensive systems of Basidiomycota including four subphyla, 18 classes, 68 orders, 241 families and 1928 genera.

Outline of Basidiomycota

Phylum Basidiomycota R.T. Moore 1980
Subphylum Agaricomycotina Doweld 2001
Class Agaricomycetes Doweld 2001
Order Agaricales Underw. 1899

Family Agaricaceae Chevall. 1826
Family Amanitaceae E.-J. Gilbert 1940
Family Bolbitiaceae Singer 1948
Family Broomeiaceae Zeller 1948
Family Biannulariaceae Jülich 1981
Family Chromocyphellaceae Knudsen 2010
Family Clavariaceae Chevall. 1826
Family Cortinariaceae R. Heim ex Pouzar 1983
Family Crassisporiaceae Vizzini, Consiglio & M. Marchetti 2019
Family Crepidotaceae (S. Imai) Singer 1951
Family Cyphellaceae Lotsy 1907
Family Cystostereaceae Jülich 1982
Family Entolomataceae Kotl. & Pouzar 1972
Family Hemigasteraceae Gäum. & C.W. Dodge 1928
Family Hydnangiaceae Gäum. & C.W. Dodge 1928
Family Hygrophoraceae Lotsy 1907
Family Hymenogastraceae Vittad. 1831
Family Inocybaceae Jülich 1982
Family Limnoperdaceae G.A. Escobar 1976
Family Lycoperdaceae Chevall. 1826
Family Lyophyllaceae Jülich 1982
Family Macrocystidiaceae Kühner 1980
Family Marasmiaceae Roze ex Kühner 1980
Family Mycenaceae Overeem 1926
Family Mythicomycetaceae Vizzini, Consiglio & M. Marchetti 2019
Family Niaceae Jülich 1982
Family Omphalotaceae Bresinsky 1985
Family Physalacriaceae Corner 1970
Family Pleurotaceae Kühner 1980
Family Pluteaceae Kotl. & Pouzar 1972

Family Porotheleaceae Murrill 1916
Family Psathyrellaceae Vilgalys, Moncalvo & Redhead 2001
Family Pseudoclitocybaceae Vizzini, Consiglio, P.-A. Moreau & P. Alvarado 2018
Family Pterulaceae Corner 1970
Family Schizophyllaceae Quél. 1888
Family Stephanosporaceae Oberw. & E. Horak 1979
Family Strophariaceae Singer & A.H. Sm. 1946
Family Tricholomataceae R. Heim ex Pouzar 1983
Family Tubariaceae Vizzini 2008
Family Typhulaceae Jülich 1982

Order Amylocorticiales K.H. Larss., Manfr. Binder & Hibbett 2010

Family Amylocorticiaceae Jülich 1982

Order Atheliales Jülich 1981

Family Atheliaceae Jülich 1982

Order Auriculariales J. Schröt. 1887

Family Auriculariaceae Fr. 1838
Family Hyaloriaceae Lindau 1897

Order Boletales E.-J. Gilbert 1931

Family Boletaceae Chevall. 1826
Family Boletinellaceae P.M. Kirk, P.F. Cannon & J.C. David 2001
Family Calostomataceae E. Fisch. 1900
Family Coniophoraceae Ulbr. 1928
Family Diplocystidiaceae Kreisel 1974
Family Gasterellaceae Zeller 1948
Family Gomphidiaceae Maire ex Jülich 1982
Family Gyroporaceae (Singer) Manfr. Binder & Bresinsky 2002
Family Hygrophoropsidaceae Kühner 1980
Family Paxillaceae Lotsy 1907
Family Protogastraceae Zeller 1934
Family Rhizopogonaceae Gäum. & C.W. Dodge 1928
Family Sclerodermataceae Corda 1842
Family Serpulaceae Jarosch & Bresinsky 2001
Family Suillaceae Besl & Bresinsky 1997
Family Tapinellaceae C. Hahn 1999

Order Cantharellales Gäum. 1926

Family Aphelariaceae Corner 1970
Family Botryobasidiaceae Jülich 1982
Family Ceratobasidiaceae G.W. Martin 1948
Family Hydnaceae Chevall. 1826
Family Oliveoniaceae P. Roberts 1998
Family Tulasnellaceae Juel 1897

Order Corticiales K.H. Larss. 2007

Family Corticiaceae Herter 1910
Family Dendrominiaceae Ghobad-Nejhad 2015
Family Punctulariaceae Donk 1964
Family Vuilleminiaceae Maire ex Lotsy 1902

Order Geastrales K. Hosaka & Castellano 2007

Family Geastraceae Corda 1842
Family Sclerogastraceae Locq. ex P.M. Kirk 2008

Order Gloeophyllales Thorn 2007

Family Gloeophyllaceae Jülich 1982

Order Gomphales Jülich 1981

Family Clavariadelphaceae Corner 1970
Family Gomphaceae Donk 1961
Family Lentariaceae Jülich 1982

Order Hymenochaetales Oberw. 1977

Family Hymenochaetaceae Donk 1948
Family Neoantrodiellaceae Y.C. Dai, B.K. Cui, Jia J. Chen & H.S. Yuan 2015
Family Nigrofomitaceae Jülich 1982
Family Oxyporaceae Zmitr. & V. Malysheva 2014
Family Rickenellaceae Vizzini 2010
Family Schizoporaceae Jülich 1982

Order Hysterangiales K. Hosaka & Castellano 2007

Family Gallaceaceae Locq. ex P.M. Kirk 2008
Family Hysterangiaceae E. Fisch. 1899
Family Mesophelliaceae Jülich 1982
Family Phallogastraceae Locq. 1974
Family Trappeaceae P.M. Kirk 2008

Order Jaapiales Manfr. Binder, K.H. Larss. & Hibbett 2010

Family Jaapiaceae Manfr. Binder, K.H. Larss. & Hibbett 2010

Order Lepidostromatales B.P. Hodk. & Lücking 2014

Family Lepidostromataceae Ertz, Eb. Fisch., Killmann, Sérus. & Lawrey 2008

Order Phallales E. Fisch. 1898

Family Claustulaceae G. Cunn. 1931
Family Gastrosporiaceae Pilát 1934
Family Phallaceae Corda 1842

Order Polyporales Gäum. 1926

Family Cerrenaceae Miettinen, Justo & Hibbett 2017
Family Dacryobolaceae Jülich 1981
Family Fomitopsidaceae Jülich 1982
Family Fragiliporiaceae Y.C. Dai, B.K. Cui & C.L. Zhao 2015
Family Gelatoporiaceae Miettinen, Justo & Hibbett 2017
Family Grifolaceae Jülich 1982
Family Hyphodermataceae Jülich 1981
Family Incrustoporiaceae Jülich 1982
Family Irpicaceae Spirin & Zmitr. 2003
Family Ischnodermataceae Jülich 1981
Family Laetiporaceae Jülich 1981
Family Meripilaceae Jülich 1982
Family Meruliaceae Rea 1922
Family Panaceae Miettinen, Justo & Hibbett 2017
Family Phanerochaetaceae Jülich 1982
Family Podoscyphaceae D.A. Reid 1965
Family Polyporaceae Fr. ex Corda 1839
Family Sparassidaceae Jülich 1981
Family Steccherinaceae Parmasto 1968

Order Russulales Kreisel ex P.M. Kirk, P.F. Cannon & J.C. David 2001

Family Albatrellaceae Nuss 1980
Family Auriscalpiaceae Maas Geest. 1963
Family Bondarzewiaceae Kotl. & Pouzar 1957
Family Echinodontiaceae Donk 1961
Family Hericiaceae Donk 1964

Family Hybogasteraceae Jülich 1982
Family Peniophoraceae Lotsy 1907
Family Russulaceae Lotsy 1907
Family Stereaceae Pilát 1930
Family Xenasmataceae Oberw. 1965

Order Sebacinales M. Weiss, Selosse, Rexer, A. Urb. & Oberw. 2004

Family Sebacinaceae K. Wells & Oberw. 1982
Family Serendipitaceae M. Weiss, Waller, A. Zuccaro & Selosse 2016

Order Stereopsidales Sjökvist, E. Larss., B.E. Pfeil & K.H. Larss. 2013

Family Stereopsidaceae Sjökvist, E. Larss., B.E. Pfeil & K.H. Larss. 2013

Order Thelephorales Corner ex Oberw. 1976

Family Bankeraceae Donk 1961
Family Thelephoraceae Chevall. 1826

Order Trechisporales K.H. Larss. 2007

Family Hydnodontaceae Jülich 1982

Order Tremellodendropsidales Vizzini 2014

Family Tremellodendropsidaceae Jülich 1982

Class Dacrymycetes Doweld 2001
Order Dacrymycetales Henn. 1897

Family Cerinomycetaceae Jülich 1982
Family Dacrymycetaceae J. Schröt. 1888

Order Unilacrymales Shirouzu, Tokum. & Oberw. 2013

Family Unilacrymaceae Shirouzu, Tokum. & Oberw. 2013

Class Tremellomycetes Doweld 2001
Order Cystofilobasidiales Fell, Roeijmans & Boekhout 1999

Family Cystofilobasidiaceae K. Wells & Bandoni 2001
Family Mrakiaceae X.Z. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015

Order Filobasidiales Jülich 1981

Family Filobasidiaceae L.S. Olive 1968
Family Piskurozymaceae X.Z. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015

Order Holtermanniales Libkind, Wuczk., Turchetti & Boekhout 2011

Family Holtermanniaceae Redhead 2015

Order Tremellales Fr. 1821

Family Bulleraceae X. Zh. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015
Family Bulleribasidiaceae X. Z. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015
Family Carcinomycetaceae Oberw. & Bandoni 1982
Family Cryptococcaceae Kütz. ex Castell. & Chalm. 1919
Family Cuniculitremaceae J.P. Samp., R. Kirschner & M. Weiss 2001
Family Naemateliaceae X. Z. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015
Family Phaeotremellaceae A.M. Yurkov & Boekhout 2015
Family Phragmoxenidiaceae Oberw. & R. Bauer 1990
Family Rhynchogastremaceae Oberw. & B. Metzler 1989
Family Sirobasidiaceae Lindau 1897
Family Tremellaceae Fr. 1821
Family Trimorphomycetaceae X. Z. Liu, F.Y. Bai, M. Groenew. & Boekhout 2015

Order Trichosporonales Boekhout & Fell 2001

Family Tetragoniomycetaceae Oberw. & Bandoni 1981
Family Trichosporonaceae Nann. 1934

Subphylum Pucciniomycotina R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Class Agaricostilbomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Agaricostilbales Oberw. & R. Bauer 1989

Family Agaricostilbaceae Oberw. & R. Bauer 1989
Family Chionosphaeraceae Oberw. & Bandoni 1982
Family Kondoaceae R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Family Ruineniaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015

Class Atractiellomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Atractiellales Oberw. & Bandoni 1982

Family Atractogloeaceae Oberw. & R. Bauer 1989
Family Hoehnelomycetaceae Jülich 1982
Family Phleogenaceae Gäum. 1926

Class Classiculomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Classiculales R. Bauer, Begerow, Oberw. & Marvanová 2003

Family Classiculaceae R. Bauer, Begerow, Oberw. & Marvanová 2003

Class Cryptomycocolacomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Cryptomycocolacales Oberw. & R. Bauer 1990

Family Cryptomycocolacaceae Oberw. & R. Bauer 1990

Class Cystobasidiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Buckleyzymales R.L. Zhao & K.D. Hyde 2017

Family Buckleyzymaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015

Order Cystobasidiales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Cystobasidiaceae Gäum. 1926

Order Erythrobasidiales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Erythrobasidiaceae Denchev 2009

Erythrobasidiales genera incertae sedis
Order Naohideales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Naohideaceae Denchev 2009

Order Sakaguchiales R.L. Zhao & K.D. Hyde 2017

Family Sakaguchiaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2017

Cystobasidiomycetes families incertae sedis

Family Microsporomycetaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015
Family Symmetrosporaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015

Class Microbotryomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Heterogastridiales Oberw. & R. Bauer 1990

Family Heterogastridiaceae Oberw. & R. Bauer 1990

Order Kriegeriales Toome & Aime 2013

Family Camptobasidiaceae R.T. Moore 1996
Family Kriegeriaceae Toome & Aime 2013

Order Leucosporidiales Sampaio, M. Weiss & Bauer 2003

Family Leucosporidiaceae Sampaio, M. Weiss & Bauer 2003

Order Microbotryales R. Bauer & Oberw. 1997

Family Microbotryaceae R.T. Moore 1996

Family Ustilentylomataceae R. Bauer & Oberw. 1997

Order Sporidiobolales Doweld 2001

Family Sporidiobolaceae R.T. Moore 1980

Microbotryomycetes families incertae sedis

Family Chrysozymaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015
Family Colacogloeaceae Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015

Class Mixiomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Mixiales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Mixiaceae C.L. Kramer 1987

Class Pucciniomycetes R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006
Order Helicobasidiales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Helicobasidiaceae P.M. Kirk 2008

Order Pachnocybales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Pachnocybaceae Oberw. & R. Bauer 1989

Order Platygloeales R.T. Moore 1990

Family Eocronartiaceae Jülich 1982
Family Platygloeaceae Racib. 1909

Order Pucciniales Clem. & Shear 1931

Family Chaconiaceae Cummins & Y. Hirats. 1983
Family Coleosporiaceae Dietel 1900
Family Cronartiaceae Dietel 1900
Family Melampsoraceae Dietel 1897
Family Mikronegeriaceae Cummins & Y. Hirats. 1983
Family Phakopsoraceae Cummins & Hirats. f. 1983
Family Phragmidiaceae Corda 1837
Family Pileolariaceae Cummins & Y. Hirats. 1983
Family Pucciniaceae Chevall. 1826
Family Pucciniastraceae Gäum. ex Leppik 1972
Family Pucciniosiraceae Cummins & Y. Hirats. 1983
Family Raveneliaceae Leppik 1972
Family Sphaerophragmiaceae Cummins & Y. Hirats. 1983
Family Uncolaceae Buriticá 2000
Family Uropyxidaceae (P. Syd. & Syd.) Cummins & Y. Hirats. 1983

Order Septobasidiales Couch ex Donk 1964

Family Septobasidiaceae Racib. 1909

Class Spiculogloeomycetes Q.M. Wang, F.Y. Bai, M. Groenew. & Boekhout 2015
Order Spiculogloeales R. Bauer, Begerow, J.P. Samp., M. Weiss & Oberw. 2006

Family Spiculogloeaceae Denchev 2009

Class Tritirachiomycetes Aime & Schell 2011
Order Tritirachiales Aime & Schell 2011

Family Tritirachiaceae Aime & Schell 2011

Subphylum Ustilaginomycotina Doweld 2001
Class Exobasidiomycetes Begerow, M. Stoll & R. Bauer 2007
Order Ceraceosorales Begerow, M. Stoll & R. Bauer 2007

Family Ceraceosoraceae Denchev & R.T. Moore 2009

Order Doassansiales R. Bauer & Oberw. 1997

Family Doassansiaceae R.T. Moore ex P.M. Kirk, P.F. Cannon & J.C. David 2001
Family Melaniellaceae R. Bauer, Vánky, Begerow & Oberw. 1999
Family Rhamphosporaceae R. Bauer & Oberw. 1997

Order Entylomatales R. Bauer & Oberw. 1997

Family Entylomataceae R. Bauer & Oberw. 1997

Order Exobasidiales Henn. 1898

Family Brachybasidiaceae Gäum. 1926
Family Cryptobasidiaceae Malençon ex Donk 1956
Family Exobasidiaceae J. Schröt. 1888
Family Graphiolaceae Clem. & Shear 1931
Family Laurobasidiaceae Pinruan, Sommai, Suetrong, Somrith. & E.B.G. Jones 2018

Order Georgefischeriales R. Bauer, Begerow & Oberw. 1997

Family Eballistraceae R. Bauer, Begerow, A. Nagler & Oberw. 2001
Family Georgefischeriaceae R. Bauer, Begerow & Oberw. 1997
Family Gjaerumiaceae R. Bauer, M. Lutz & Oberw. 2005
Family Tilletiariaceae R.T. Moore 1980

Order Golubeviales Q.M. Wang, Begerow, F.Y. Bai & Boekhout 2015

Family Golubeviaceae Q.M. Wang, F.Y. Bai, Begerow & Boekhout 2015

Order Microstromatales R. Bauer & Oberw. 1997

Family Microstromataceae Jülich 1982
Family Quambalariaceae Z.W. de Beer, Begerow & R. Bauer 2006
Family Volvocisporiaceae Begerow, R. Bauer & Oberw. 2001

Order Robbauerales Boekhout, Begerow, Q.M. Wang & F.Y. Bai 2015

Family Robbaueraceae Boekhout, Begerow, Q.M. Wang & F.Y. Bai 2015

Order Tilletiales Kreisel ex R. Bauer & Oberw. 1997

Family Erratomycetaceae Denchev & T. Denchev 2013
Family Tilletiaceae J. Schröt. 1887

Class Malasseziomycetes Q.M. Wang & F.Y. Bai 2014
Order Malasseziales R.T. Moore 1980

Family Malasseziaceae Denchev & R.T. Moore 1980

Class Moniliellomycetes Q.M. Wang, F.Y. Bai & Boekhout 2014
Order Moniliellales Q.M. Wang, F.Y. Bai & Boekhout

Family Moniliellaceae Q.M. Wang, F.Y. Bai & Boekhout

Class Ustilaginomycetes R. Bauer, Oberw. & Vánky 1997
Order Uleiellales Garnica, K. Riess, M. Schön, H. Butin, M. Lutz, Oberw. & R. Bauer 2016

Family Uleiellaceae Vánky 2001

Order Urocystidales R. Bauer & Oberw. 1997

Family Doassansiopsidaceae Begerow, R. Bauer & Oberw. 1998
Family Fereydouniaceae S. Nasr, Soudi, H.D.T. Nguyen, M. Lutz & Piątek 2014
Family Floromycetaceae S. Nasr, Soudi, H.D.T. Nguyen, M. Lutz & Piątek 2014
Family Glomosporiaceae Cif. 1963
Family Mycosyringaceae R. Bauer & Oberw. 1997
Family Urocystidaceae Begerow, R. Bauer & Oberw. 1998

Order Ustilaginales G. Winter 1880

Family Anthracoideaceae Denchev 1997
Family Cintractiellaceae Vánky 2003

Family Clintamraceae Vánky 2001
Family Geminaginaceae Vánky 2001
Family Melanotaeniaceae Begerow, R. Bauer & Oberw. 1998
Family Pericladiaceae Vánky 2011
Family Ustilaginaceae Tul. & C. Tul. 1847
Family Websdaneaceae Vánky 2001

Order Violaceomycetales Albu, Toome & Aime 2015

Family Violaceomycetaceae Albu, Toome & Aime 2015

Subphylum Wallemiomycotina Doweld 2014
Class Wallemiomycetes Zalar, de Hoog & Schroers 2005
Order Geminibasidiales H.D.T. Nguyen, N.L. Nick. & Seifert 2013

Family Geminibasidiaceae H.D.T. Nguyen, N.L. Nick. & Seifert 2013

Order Wallemiales Zalar, de Hoog & Schroers 2005

Family Wallemiaceae R.T. Moore 1996

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Cantharellales Gäum

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Overview

Cantharellales (syn. *Tulasnellales*, *Ceratobasidiales* s. auct. plur.) comprises about 629 recognized species and has a cosmopolitan distribution. Some species are economically important because of their edibility (*Cantharellus*, *Craterellus*, *Hydnum*; Watling, 1997) or being plant pathogens that cause damage in crops (Veldre *et al.*, 2013). Basidioma configuration is very diverse across *Cantharellales* (**Fig. 1**). Resupinate, pileate-stipitate (cantharelloid, hydnoid, polyporoid), and clavarioid basidiomata are predominant, but cyphelloid species do exist. Several bulbil-forming asexual propagules belong also to *Cantharellales*. Nearly all known trophic strategies of fungi are present in *Cantharellales*. Ectomycorrhizal fungi (*Hydnaceae*) and saprotrophs constitute a significant part of the diversity of *Cantharellales*, but orchid-mycorrhizal, mycobionts with liverworts (Preußing *et al.*, 2010), lichenicolous (parasitic), lichenized and endophytic species do exist (Dearnaley *et al.*, 2016). Seemingly, there is no synapomorphic trait unique of *Cantharellales* when it is broadly circumscribed as here, *i.e.*, including *Cejpomycetaceae* and *Tulasnellaceae*. Available knowledge suggests, however, that *Cantharellales* may be split in two better defined groups that share a few, possibly synapomorphic, characters:

(1) *Hydnaceae* and *Botryobasidiaceae*, characterized by the presence of resupinate or more complex basidioma types, and above all, the predominance of stichic basidia, often suburniform or urniform, with more than 4 sterigmata along with spores that are not repetitive. Stichic basidia, *i.e.*, with meiotic spindles parallel to the long basidial axis, appear to be universal in this group,

Fig. 1 Diversity of basidioma configuration in *Cantharellales*. (a) *Cantharellus cibarius* (*Hydnaceae*), cantharelloid basidiomata. (b) *Hydnum ellipsosporum* (*Hydnaceae*), hydnoid basidomata. (c) *Clavulina cinerea* (*Clavulinaceae*), clavarioid basidiomata. (d) *Multiclavula mucida* (*Hydnaceae*). (e) *Sistotrema confluens* (*Hydnaceae*), hydnoid-polyporoid basidiomata. (f) *Sistotrema brinkmannii* (*Hydnaceae*), corticioid basidiomata. (g) undescribed clavarioid *Sistotrema*, close to *S. athelioides* (*Hydnaceae*). (h) bulbil-forming *Burgoa angulosa* (*Hydnaceae*). (i) *Botryobasidium danicum* (*Botryobasidiaceae*), corticioid basidiomata.

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in contrast with the chiastic type, with a transverse orientation of the spindle, present in nearly all other *Agaricomycetes*. Stichic basidia have hitherto been reported in *Cantharellus*, *Clavulina*, *Craterellus*, *Hydnum*, and *Multiclavula* (Juel, 1898; Donk, 1964; Restivo and Petersen, 1976; Hubbard and Petersen, 1979), while information for *Sistotrema* s.l. and *Botryobasidiaceae* is still missing. Stichic basidia are also known to be present in other basal lineages of *Basidiomycota*, such as in *Auriculariales*, *Dacrymycetales*, *Exobasidiales*, *Septobasidiales*, *Uredinales*, and *Ustilaginales* (Zmitrovich and Wasser, 2004). Basidia with more than 4 sterigmata, as opposed to the universal 4-spored condition of basidia in *Basidiomycota* might be correlated with the stichic nuclear behavior (Hibbett *et al.*, 2014). Basidia are typically 6–8-sterigmate in *Sistotrema* s.l. and *Botryobasidium*, while 5- and 6-spored basidia are present and usually predominant in *Cantharellus*, *Craterellus*, *Hydnum* and *Multiclavula*. Exceptions are nearly all species of *Clavulina*, along with a few species of *Sistotrema* and *Craterellus*. The presence of basidia with more than 4 sterigmata is extremely unusual outside *Cantharellales* and might be related with a stichic nuclear division (Hibbett *et al.*, 2014). Most genera possess basidia that are distinctly urniform (*Sistotrema* s.l.) or suburniform – with their broadest part in the middle or on the lower half –, the only exception being *Cantharellus* and *Craterellus* with extremely long – often exceeding 100 μm – claviform basidia. With regard to basidioma configuration, *Botryobasidiaceae* is characterized by resupinate basidiomata, whereas other basidioma types – clavarioid, cyphelloid, hydnoid and cantharelloid – are represented only within *Hydnaceae* among the *Cantharellales*.

(2) *Cejpomycetaceae* and *Tulasnellaceae*, characterized by resupinate basidiomata, and the combination of chiastic, aseptate basidia and spores germinating by repetition. Basidia in *Tulasnella* appear to be chiastic (Rogers, 1932) and possibly also in *Rhizoctonia* (Langer, 2001), unlike in *Hydnaceae* and possibly also in *Botryobasidiaceae*. Repetitive spores are an almost exclusive character of heterobasidiomycetes (Donk, 1964) belonging to the *Agaricomycetes* (*Auriculariales*, *Exidiales*, *Sebacinales*, *Dacrymycetales*) and *Tremellomycetes*. Nevertheless, *Oliveoniaceae* produces also holobasidia and repetitive spores, but available molecular data indicate that it nests outside *Cantharellales* (Roberts, 1998; Olariaga, unpublished). *Pseudotulasnella*, included here in *Cantharellales* and producing basidia with partial cruciate longitudinal septa, if considered phragmobasidiate, is also an exception. *Tulasnellaceae* and *Cejpomycetaceae*, however, differ in many other important respects, like in the morphology of the septal pore (see below). Further, basidia in *Tulasnellaceae* have inflated sterigmata that may be evolutionarily interpreted as phragmobasidia.

The septal pore morphology shows also variation within *Cantharellales* and has been used to infer relationships. While *Botryobasidium* and *Tulasnella* possess imperforate parenthesomes, *Cantharellus* and *Sistotrema*, and at least some species of *Rhizoctonia* have perforate parenthesomes, among which a great diversity in pore openings exists as well (Van Driel *et al.*, 2009). At present, it is uncertain whether septal pore morphologies are useful to depict monophyletic lineages within *Cantharellales*.

Phylogenetic Incongruences, Family Classification and The Polyphyly of *Sistotrema*

Cantharellales was first recognized by Hibbett and Thorn (2001, called then cantharelloid clade) for a morphologically diverse assemblage of fungi recovered as monophyletic in studies based on nuclear ribosomal regions (Bruns *et al.*, 1998; Hibbett *et al.*, 1997; Pine *et al.*, 1999). Moncalvo *et al.* (2006) showed that nuclear ribosomal regions are involved in phylogenetic conflicts due to an accelerated rate of evolution and cause long-branch attraction problems. Although 4 monophyletic lineages – *Hydnaceae*, *Cejpomycetaceae*, *Tulasnellaceae*, *Botryobasidiaceae* – are consistently retrieved in phylogenetic studies, the relationships among these remain unclear and are strongly dependent upon the genetic marker, taxon sampling and the type of phylogenetic analysis implemented for inference (**Fig. 2**). As an example, *Tulasnellaceae* is sister to the *Cantharellus-Craterellus* clade when nuclear ribosomal regions are employed for phylogenetic inference (Moncalvo *et al.*, 2006), but *Tulasnellaceae* is sister to *Botryobasidiaceae* when the mtSSU region is employed (Hibbett and Thorn, 2001). When the RPB2 region is utilized for phylogenetic reconstruction, the position of *Tulasnellaceae* is basal to the rest of the *Cantharellales* (González *et al.*, 2016) or sister to *Hydnaceae*, being *Botryobasidiaceae* and *Cejpomycetaceae* the basal lineages in *Cantharellales* (Matheny *et al.*, 2006). Phylogenetic incongruences also affect relationships within the *Hydnaceae* and the sister groups of *Hydnum*, *Sistotrema confluens* and *Cantharellaceae*, are different depending on the region analysed.

In this scenario, family classification proposals up to present are not founded on robust phylogenetic hypotheses employing an adequate taxon sampling. *Tulasnellaceae* is sometimes treated at the order rank as *Tulasnellales* and placed outside *Cantharellales* (Begerow *et al.*, 2018). The families *Cantharellaceae*, *Sistotremataceae*, *Hydnaceae* and *Clavulinaceae* are sometimes accepted, albeit relationships and delimitation among these are unclear. *Botryobasidiaceae* is currently recognized but it could be merged with *Cejpomycetaceae* if both form a monophyletic group (González *et al.*, 2016). The phylogenomic reconstruction by Nagy *et al.* (2016) placed *Cejpomycetaceae* in a basal position to the clade formed by *Tulasnellaceae* and *Botryobasidiaceae*, by sampling only 3 species of *Cantharellales*. This phylogenetic hypothesis, thus, needs to be tested using a more comprehensive taxon sampling. A conservative approach until phylogenomic analyses that address current phylogenetic incongruences appropriately become available is to recognized the 4 main lineages of *Cantharellales* at family level: *Botryobasidiaceae*, *Hydnaceae* (syn. *Cantharellaceae*, *Clavulinaceae*, *Sistotremataceae*), *Cejpomycetaceae* (*Ceratobasidiaceae* s. auct., see Oberwinkler *et al.* (2013)), and *Tulasnellaceae*, as done by Hibbett *et al.* (2014) and followed here.

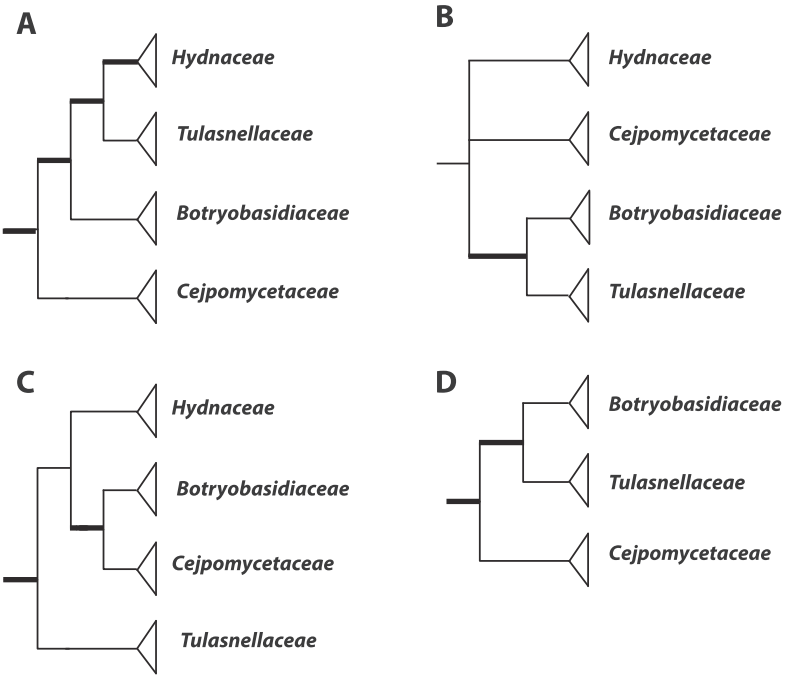

Fig. 2 Summary of the main phylogenetic hypotheses published up to date. A, nrLSU-nrSSU-RPB2-mtSSU. B, mtSSU. C, RPB2. D, analysis based on 623 genes. Reproduced from Moncalvo, J.-M., Nilsson, R.H., Koster, B., *et al.*, 2006. The cantharelloid clade: Dealing with incongruent gene trees and phylogenetic reconstruction methods. Mycologia 98, 937–948. Hibbett, D.S., Thorn, R.G., 2001. *Basidiomycota*: Homobasidiomycetes. In: McLaughin, D.J., Mclaughin, E.G., Lemke, P.A. (Eds.), The Mycota vol. VII, Systematics and Evolution. Berlin: Springer. pp. 121–168. González, D., Rodríguez-Carres, M., Boekhout, T., *et al.*, 2016. Phylogenetic relationships of *Rhizoctonia* fungi within the Cantharellales. Fungal Biology 120, 603–619. Nagy, L.G., Riley, R., Tritt, A., *et al.*, 2016. Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. Molecular Biology and Evolution 33, 959–970.

A further major problem in naming many genera within the core of the cantharelloid clade is that *Sistotrema* s.l. is composed of at least 4 polyphyletic lineages (Moncalvo *et al.*, 2006) and that several monotypic or oligotypic bulbil-forming genera belong to these as well (Lawrey *et al.*, 2007). The type species of *Sistotrema*, *S. confluens*, along with other species with an irpicoid-poroid hymenophore, is ectomycorrhizal and suggested to be closely related to *Hydnum* (Moncalvo *et al.*, 2006), rather than to the rest of *Sistotrema* species, which mostly have similar urniform 4–8-spored basidia, saprotrophic habit and resupinate basidiomata (Bernicchia and Pérez-Gorjón, 2010). Corticioid *Sistotrema* species appear in 5 paraphyletic lineages, the first four recognized by Moncalvo *et al.* (2006): (a) *S. brinkmannii-oblongisporum* group, *Burgoa moriformis, Burgella* and *Rogersiomyces malaysianus* (*Sistotrema* I), apparently close to *Clavulina*, (b) *S. athelioides* (*Sistotrema* II), (c) *S. adnatum*, *Minimedusa* and *Bryoclavula phycophila* (*Sistotrema* III), (d) *S. eximum-octosporum* group (*Sistotrema* IV), and (e) *Sistotrema raduloides* (*Sistotrema* V). As before, a robust phylogenetic hypothesis that elucidates the relationships among all these lineages is lacking, partly due to the fact that only nuclear ribosomal sequences are available for bulbil-forming species (*e.g.*, Lawrey *et al.*, 2007) and newly described species (Purtseva *et al.*, 2016; Masumoto and Degawa, 2020) and that several basal nodes supported in the phylogeny by Moncalvo *et al.* (2006), **Fig. 2(A)** lose support when these are included due to increased missing data.

Diversity, Current Knowledge and Issues in Species Delimitation

Previous species estimates (Hibbett *et al.*, 2014, 260 sp.; Begerow *et al.*, 2018, 541 sp.; He *et al.*, 2019, 589 sp.) were considerably lower than our current estimate of 629 described and recognized species belonging to *Cantharellales*. The much higher amount of OTUs in the UNITE database – 1824 OTUs when most conservative threshold of 3% is applied – suggests that the actual diversity of *Cantharellales* is considerably higher (Nilsson *et al.*, 2018, consulted on 23rd August 2020). This estimate must be, however, interpreted with caution, as the accelerated rate of evolution of nuclear ribosomal markers in some groups of *Cantharellales* (Moncalvo *et al.*, 2006), or possible errors in sequences (Haas *et al.*, 2011; Hughes *et al.*, 2015; Aas *et al.*, 2017), may have caused an overestimation. In contrast, it is known that a low number of described species have been sequenced for the ITS region (Nilsson *et al.*, 2006) and thus, one would expect the number of OTUs to increase as sequences of these are incorporated to public sequence databases. Poorly known groups and other issues are discussed below.

Ectomycorrhizal species with fleshy basidiomata of the core of the cantharelloid clade (*Cantharellus*, *Craterellus*, *Hydnum*, *Clavulina*) account for more than a half of the described diversity (336 accepted species). These genera share a similar taxonomic

intricacy, its species showing a considerable macroscopical plasticity but limited microscopical diversity compared to other fungal groups (Buyck, 2016; Olariaga *et al.*, 2009). Usual taxonomic problems in these genera are that several close species are subsumed under a single name and that European names are often misapplied to taxa occurring in other continents, while many taxa, possibly most, are continental endemics (Buyck *et al.*, 2014; Niskanen *et al.*, 2018; Olariaga *et al.*, 2017; Thacker and Henkel, 2004). Molecular data are being decisive for delineating limits between sibling species. Of these *Hydnum* and *Cantharellus* are better known and in constant state of flux, but the diversity of *Clavulina* and *Craterellus* is not well understood yet, especially in the tropics.

Also, soil-inhabiting and mycorrhizal endophytic *Cejpomycetaceae* and *Tulasnellaceae* associated with orchid roots, often called "Rhizoctonias" (Dearnaley *et al.*, 2016), harbor certainly a considerably higher diversity that known today. The majority of orchids investigated associate exclusively with a few fungal partners – rarely only one (Bougoure *et al.*, 2009) – across its distribution range (Dearnaley *et al.*, 2016). Although many "Rhizoctonias" appear not to be highly host specific (Veldre *et al.*, 2013), at least some endophytic *Tulasnellaceae* are only known from a single orchid genus (Linde *et al.*, 2017). As new orchid hosts are screened for "Rhizoctonias", undescribed species are being continuously detected (*e.g.*, Fujimori *et al.*, 2019; Linde *et al.*, 2017; Silva Freitas *et al.*, 2020) and further sampling, especially in tropical areas, will probably yield the discovery of many novel species (Veldre *et al.*, 2013). An important part of the operational taxonomic units (OTUs) discovered remains unnamed (*e.g.*, Jacquemyn *et al.*, 2012; Oja *et al.*, 2015; Smith *et al.*, 2010) as these studies are mainly based on environmental sequencing techniques and no physical specimens were available for typification of new taxa. Endophytic "Rhizoctonias" associated with liverworts are less diverse than orchid associates but remain likewise largely unexplored (Bidartondo and Duckett, 2010).

The problems in determining how many of the accepted of phytopathogenic species of *Rhizoctonia* s. str. (*Ceratobasidium* s. auct., see Oberwinkler *et al.*, 2013) have been formally described adds uncertainty to the estimation of the known number of species within *Cantharellales*. *Rhizoctonia* s.l. has been conventionally used to accommodate anamorphic states of *Botryobasidium*, *Ceratobasidium* s. auct. and *Tulasnella* (Stalpers and Andersen, 1996). Andersen and Stalpers (1994) revised 117 epithets then available in *Rhizoctonia* and considered that 41 of these belonged to *Rhizoctonia* s. str. based on morphological characters. Nevertheless, morphological characters are of limited value in delineating species boundaries (Roberts, 1999; Vilgalys and Cubeta, 1994). To undertake species delimitation, plant pathologists have developed instead a method based on the number of nuclei per cell and a biological species recognition approach, *i.e.*, "anastomosis group concept" (referred to as AG) (Matsumoto *et al.*, 1932), based on the ability of isolates of the same species to recognize and fuse with each other in co-culture. This approach has also some limitations (Sharon *et al.*, 2008). Based on the AG concept, at least 29 groups have been recognized some of which have been further divided into subgroups (Sharon *et al.*, 2008), but most of these have not been linked to the binomial nomenclature. While most of these AGs have received phylogenetic support (Sharon *et al.*, 2008), other studies have shown that some AGs are polyphyletic, *i.e.*, strains assigned to the same AG do not form monophyletic groups. Veldre *et al.* (2013) recovered 52 OTUs of plant pathogenic species based on the analysis of all ITS sequences available then in public databases but assigned a binomen to only one of these. Meanwhile a serious effort to link sequences obtained from type specimens or ex-type cultures and described AGs is not made, this issue will remain opened.

Based on all of this, it is concluded here that *Cantharellales* may comprise at least 1500 species, more than a half of which is still undescribed.

Trophic Strategies

Cantharellales is an ecologically very diverse order, in which saprotrophic groups and a broad array of symbiotic relationships (mycorrhizal, lichenized, parasitic, endophytic) exist (Hibbett *et al.*, 2014). Although the exact story of nutritional transitions cannot be determined with certainty in the absence of a robust phylogenetic hypothesis, it is likely that the common ancestor was saprotrophic (Martin *et al.*, 2016) and resupinate.

Saprotrophic Cantharellales are usually species with resupinate basidiomata, present in all the families recognized here and can be easily cultured in common agar media (*e.g.*, Langer, 1994; Matsumoto *et al.*, 1932; Warcup, 1985) due to their saprotrophic abilities, sometimes in addition to other lifestyles. Interestingly, no primary wood decayers exist in *Cantharellales* and most saprotrophic species occur in soft wood or plant remnants in advanced stage of decay. Saprotrophic *Cantharellales* have been claimed to cause an ancestral "soft rot" and to have the plesiomorphic decay mode of the Agaricomycetes, characterized by having diverse repertories of gene families involved in cellulose decomposition but lacking key enzimes of lignine decomposition. *Botryobasidium* has been claimed to possess a limited ability to degrade wood and to function as a carbohydrate scavenger in white rot fungal communities or suggested to be a mycorrhizal symbiont (Nagy *et al.*, 2016). Those conclusions have been drawn by analyzing a handful of species.

Ectomycorrhizal Cantharellales usually form complex non-resupinate basidiomata and are almost exclusively found in *Hydnaceae* and belong to presumedly obligate genera of ectomycorrhizal fungi, such as *Cantharellus*, *Clavulina*, *Craterellus*, *Hydnum* and *Sistotrema* s. str. (Tedersoo *et al.*, 2010 and references therein). In vitro cultures have been successfully obtained from basidiospores (Itävaara and Willbeg, 1988) and ectomycorrhizal root tips (Ogawa *et al.*, 2019) in *Cantharellus* and from basidioma tissue in *Hydnum repandum* (Peksen *et al.*, 2013). The notably low ∂^{15}N value found in *Clavulina cristata* suggests that species of *Clavulina* have saprotrophic abilities (Trudell *et al.*, 2004). Basidiomata of fleshy *Hydnaceae*, as it is well documented in *Cantharellus*, are unusually long-lived (Largent and Sime, 1995; Norvell, 1995) and resistant against insect predation (Pilz *et al.*, 2003), probably

due to its insecticidal properties (Masota *et al.*, 2017). Two lineages of *Cejpomycetaceae* (Veldre *et al.*, 2013) and some species of *Tulasnellaceae* are also known to facultatively produce ectomycorrhizae (Tedersoo *et al.*, 2010 and references therein; Tedersoo and Smith, 2013) with ectomycorrhizal plant hosts, whereas they form endomycorrhizae with orchids (Warcup, 1985) or are able to establish symbiotic associations with hepatics by colonizing their rhizoids (Bidartondo *et al.*, 2003). Mycorrhizal associations produced by species of *Cantharellales* show a low host specificity (Pilz *et al.*, 2003) and are able to associate with plants of different genera and families if those are available. Danell (1994) reported that *Cantharellus formosus* can establish ectomycorrhizal associations with 14 plant genera. In Europe, *C. pallens* occurs mainly in association with *Pinaceae* and *Fagaceae*, but also with *Cistaceae* (Vila and Llimona, 2009, as *C.* aff. *cibarius*) and *Ericaceae*, while *C. romagnesianus* has been recorded with *Pinaceae* and *Fagaceae* (Olariaga *et al.*, 2017) but also forms putative ectomycorrhizae with planted *Eucalyptus* trees (*Myrtaceae*; Olariaga, 2009). Well-known species of *Clavulina*, *Craterellus*, and *Hydnum* constitute further examples of broad host ranges (Olariaga, 2009). Species putatively restricted to a tree genus have also mentioned (Redhead *et al.*, 1997), but the confirmation of a strict association requires further experimental evidence. A unique "ectomycorrhiza-like" interaction has been described between *Cantharellus tropicalis* and *Dendrocalamus strictus* (Poaceae) and even synthesized *in vitro* (Sharma *et al.*, 2011).

Other mycorrhizal interactions with orchids occur in "Rhizoctonias" belonging to *Tulasnellaceae* and *Cejpomycetaceae*, as well as in some other fungal groups (Dearnaley *et al.*, 2012). *Tulasnellaceae* appear to be the most frequently recorded orchid associates (Jaquemyn *et al.*, 2017; Yuan *et al.*, 2010). Orchid mycorrhizas are unusual in that, compared to other mycorrhizal associations, orchids protocorms (heterotrophic, achlorophyllous stage after seed germination) are heterotrophic and obtain carbon and other nutrients from the mycobiont (Dearnaley *et al.*, 2012; Smith and Read, 2008). When adult orchids develop photosynthetic capability, they continue to benefit from the associated fungus by obtaining mineral nutrients. Although the nature of this type of mycorrhizal association has been suggested to be mutualistic (Cameron *et al.*, 2006, 2008), the carbon flow is sometimes from orchid to the fungus, whereas evidence supporting that some adult orchids obtain carbon from fungi is also available (Selosse and Roy, 2009; Yagame *et al.*, 2012). Symbiotic "Rhizoctonias" colonize the space between the cell wall and the plasmatic membrane, shaping coiled structures known as pelotons (Dearnaley *et al.*, 2012), where the nutrient exchange is very likely to occur. As orchid mycobionts are easily isolated *in vitro* – most, probably all, have an additional saprotrophic or parasitic lifestyle –, orchid mycobionts could be only linked to teleomorphic genera in the rare occasions in which teleomorphic states are successfully induced in axenic cultures. Thus, most orchid associated *Cantharellales* were assigned to two genera encompassing asexual morphs, *Ceratorhiza* and *Epulorhiza*, which became synonyms of *Rhizoctonia* and *Tulasnella*, respectively, with the end of dual nomenclature in fungi. Currently, the use of molecular data is helping to understand the high diversity of orchid-associated *Cantharellales* (*e.g.*, Fujimori *et al.*, 2019; Linde *et al.*, 2017; Silva Freitas *et al.*, 2020). A mycorrhizal association has been also suggested between *Botryobasidium* and the orchid genus *Apostasia* (Yukawa *et al.*, 2009), but this putative relationship should be explored further. Mycorrhiza-like interactions between *Tulasnellaceae* and liverworts have been also documented in hepatics of the family *Aneuraceae* (Bidartondo *et al.*, 2003; Kottke *et al.*, 2003) and have been defined as a model or early evolved symbiotic associations (Krause *et al.*, 2011).

Lichenization with green unicellular algae has evolved two times in *Cantharellales*, in the genera *Multiclavula* and *Bryoclavula* (Masumoto and Degawa, 2020). Both lineages share minute clavarioid basidiomata and more or less urniform basidia that often produce more than four basidiospores. The thallus can be rather conspicuous, continuous or formed by scale-like or granular units (Oberwinkler, 2012). Globose hyphal-algal associations are characteristic of *Multiclavula* (Oberwinkler, 1980), while in *Bryoclavula* mycelial hyphae surround photobiont cells. Haustoria are not produced (Masumoto and Degawa, 2020; Oberwinkler, 2012). The association with algae seems obligatory in both cases. Although both lineages share striking similarities, analyses of the 28S or the nuclear rDNA suggest that *Multiclavula* is nested in the *Sistotrema* I clade (Moncalvo *et al.*, 2006), whereas *Bryoclavula* is supported in the *Sistotrema* III clade (Masumoto and Degawa, 2020). As discussed above, a more robust phylogeny is needed to further test this phylogenetic hypothesis too. Some species accommodated in *Multiclavula* may belong to *Lepidostromataceae*, in the *Hygrophoraceae*, due to having up to 4-spored claviform basidia (Oberwinkler, 2012), and likely a chiastic nuclear behavior unlike *Multiclavula*.

Although basidiomycetous *endophytes* are underrecorded (Martin *et al.*, 2015), some *Cejpomycetaceae* and *Tulasnellaceae* are known to be widespread endophytes in a broad variety of plants that occur in different environments, such as in leaves (Martin *et al.*, 2015), roots of orchids (Racharin *et al.*, 2018) and other plants (*e.g.*, Cosoveanu and Cabrera, 2018; Sen *et al.*, 1999), even in saline environments (Soares *et al.*, 2016) or ferns (Younginger and Ballhorn, 2017). As with other endophytic fungi, little is known about the frequency or the possible commensalistic or mutualistic nature of endophytic *Cantharellales* (Sen *et al.*, 1999).

A *parasitic* nutrition strategy occurs mainly in *Rhizoctonia*, in which the asexual *R. solani* is a common, problematic pathogen on a broad range of crops (Veldre *et al.*, 2013). *Rhizoctonia* attacks primarily roots and stem bases of herbaceous plants and produces occasionally its sexual stage on infected plants. Asexual bulbilliferous *Cantharellales* – *Adamflackia*, *Burgella*, *Burgoa*, *Minimedusa*, *etc.* – are also considered weak parasites on lichens (Diederich and Lawrey, 2007). As *Sistotrema*-like urniform basidia have been observed in cultures of *Burgoa* species (Weresub and LeClair, 1971), bulbilliferous *Cantharellales* are linked to saprotrophic *Sistotrema* species, and therefore, appear to be facultative parasites.

Synopsis

Botryobasidiaceae Jülich 1982.

Botryobasidium Donk 1931 (syn. *Acladium* Link 1809, *Alysidium* Kunze 1817, *Haplotrichum* Link 1824, *Allescheriella* Henn. 1897, *Botryohypochnus* Donk 1931, *Cyanohypha* Jülich 1982). Basidiomata resupinate, thin. Hyphae cylindrical, without ampullaceous septa. Dolipores with perforate parenthesomes. Clamps present or absent. Leptocystidia seldom present. Basidia broadly claviform to suburniform, usually shorter than 20 μm, 4–8-spored, with a stichic nuclear division. Basidiospores smooth or ornamented, thin or thick-walled, sometimes cyanophilous. Anamorphs of *Haplotrichum*, *Allescheria* or *Alyssidium* type. Saprotrophic, suggested to be mycorrhizal. Lignicolous on very decayed wood, seldom on bare ground. Cosmopolitan. The name *Botryobasidium* would need to be conserved against older priority synonyms for its continued usage.

Suillosporium Pouzar 1958. Basidiomata resupinate, porulose to odontioid. Hyphae short-celled in the subhymenium, without ampullaceous septa. Clamps present. Septocystidia present. Basidia broadly claviform, usually shorter than 20 μm, 4-spored, with unknown nuclear division. Basidiospores smooth, not repetitive. Anamorph unknown. Saprotrophic, lignicolous on very decayed wood. North hemisphere, south America, Mascarenes and Réunion islands. Molecular data unavailable; 4-spored basidia suggest a placement elsewhere.

Cejpomycetaceae Jülich 1982 (syn. *Ceratobasidiaceae* Martin 1948 sensu auct.).

Rhizoctonia DC 1815 (= *Thanatephorus* Donk 1956). Basidiomata resupinate, thin, sometimes producing sclerotia. Hyphae cylindrical, occasionally monilioid, without ampullaceous septa. Dolipores with perforate parenthesomes. Clamps present or absent. Leptocystidia mostly absent. Basidia cylindrical to claviform, usually shorter than 20 μm, 2–4-spored, possibly chiastic. Basidiospores smooth or ornamented, thin or thick-walled, sometimes repetitive. Anamorphs of *Rhizoctonia* type, rarely bulbilliferous. Saprotrophic, parasitic, ectomycorrhizal or orchid mycorrhizal. Basidiomata found on very decayed wood. Cosmopolitan.

Hydnaceae Chevall. 1826 (syn. *Cantharellaceae* J. Schröt. 1888, *Clavulinaceae* Donk 1970, *Sistotremataceae* Jülich 1982).

Adamflackia (Diederich and Lawrey, 2007). Bulbilliferous, basidiomata and conidiomata unknown. Bulbils beige to orange, without hairs, surface cells polyhedral, internal cells roundish to polyhedral. Hyphae thin-walled. Clamps absent. Lichenicolous. South America.

Bryoclavula (Masumoto and Degawa, 2020). Basidiomata clavarioid, minute. Hyphae cylindrical, without ampullaceous septa. Clamps present. Cystidia absent. Basidia clavate to suburniform, mostly 4–6-spored. Basidiospores smooth, narrowly ellipsoid, thin-walled. Lichenized on rocks, associated with green coccoid algae. Japan.

Burgella (Diederich and Lawrey, 2007). Bulbilliferous, basidiomata and conidiomata unknown. Bulbils yellow or honey-colored, without hairs, cells in surface polyhedral. Hyphae thin-walled. Clamps present. Lichenicolous. North and South America.

Burgellopsis (Diederich and Lawrey, 2007). Bulbilliferous, basidiomata and conidiomata unknown. Bulbils white, without hairs, internal cells roundish to polyhedral. Hyphae thin-walled. Clamps absent. Lichenicolous. Europe.

Cantharellus Adans. 1763, *nom. sanct.* (syn. *Goossensia*, *Afrocantharellus*). Basidiomata pileate-stipitate, plane or depressed, usually thick-fleshed, seldom cyphelloid, with bright colors. Hymenophore smooth to veined. Hyphae cylindrical or inflated, without ampullaceous septa. Clamps present or absent. Cystidia absent. Basidia claviform, usually exceeding 100 μm, generally 4–6-spored, with a stichic nuclear division. Basidiospores ellipsoid, smooth. Ectomycorrhizae with unbranched rhizomorphs. Carotenoids bicyclic. Cosmopolitan.

Clavulina Schrot. 1888 (syn. *Membranomyces* 1975). Basidiomata clavarioid, sometimes resupinate, rarely cantharelloid. Hyphae cylindrical to inflated, without ampullaceous septa. Clamps present or absent. Leptocystidia present in a few species, occasionally encrusted. Basidia claviform to suburniform, older basidia with a cross secondary septum, typically 2-spored, 4–6-spored in a few species, stichic. Basidiospores usually ovoid, with a large guttule. Ectomycorrhizal, with saprotrophic ability as suggested by isotope ratios. Cosmopolitan.

Craterellus Pers. 1825, *nom. sanct.* (syn. *Pseudocraterellus* 1958, *Pterygellus* 1966). Basidiomata pileate-stipitate, depressed to infundibuliform, usually thin-fleshed, nearly always with a hollow stipe. Hymenophore smooth to veined. Hyphae cylindrical or inflated, without ampullaceous septa. Clamps absent or present. Cystidia absent. Basidia claviform, usually exceeding 100 μm, 2–6 (8)-spored, stichic. Ectomycorrhizae without rhizomorphs. Carotenoids aliphatic. Cosmopolitan.

Hydnum L. 1753, *nom. sanct.* Basidiomata pileate-stipitate, usually with an ocher or orange-red pileus. Hymenophore odontoid. Hyphae inflated, with ampullaceous septa in the basal mycelium. Clamps present. Cystidia absent. Basidia suburniform, generally 3–7-spored, stichic. Basidiospores smooth, ellipsoid to nearly globose. Ectomycorrhizal. With cytotoxic diepoxides. Cosmopolitan.

Multiclavula Petersen 1967. Basidiomata clavarioid, minute, simple, white to orange. Hyphae cylindrical to slightly inflated, thin-walled to somewhat thick-walled, without ampullaceous septa. Clamps present or absent. Cystidia absent. Basidia suburniform, 2–8-spored, with at least some basidia with more than 4 spores in several species. Basidiospores cylindrical to ovoid. Lichenized on soil or wood, associated with green coccoid algae. Cosmopolitan.

Neoburgoa (Diederich and Lawrey, 2007). Bulbilliferous, basidiomata and conidiomata unknown. Bulbils yellow to orange, without hairs, internal cells roundish to polyhedral. Hyphae thick-walled. Clamps present in culture. Lichenicolous. Europe.

Sistotrema Fr. 1821, *nom. sanct.* (syn. *Hydnotrema* 1833, *Burgoa* 1937, *Urnobasidium* 1968, *Minimedusa* 1971, *Ingoldiella* 1972,). Basidiomata usually resupinate, smooth, odontioid or poroid, seldom stipitate-pileate or clavarioid. Hyphae cylindrical to inflated, sometimes with ampullaceous septa, especially in the rhizomorphs. Cystidia seldom present as leptocystidia or gloeocystidia. Basidia characteristically urniform, 2–8-spored, usually with more than 4 sterigmata, presumedly stichic. Basidiospores usually ellipsoid to allantoid, not repetitive. Anamorphs bulbilliferous or hyphomycetous. Saprotrophic or ectomycorrhizal. Cosmopolitan. Polyphyletic, in need of rearrangement, treated here in a conventional sense.

Tulasnellaceae (Juel, 1898) (syn. *Gloeotulasnella* Höhn and Litsch. 1908).

Pseudotulasnella Lowy, 1964. Basidiomata resupinate, waxy-gelatinous. Hyphae cylindrical, thin-walled. Clamps absent. Cystidia absent, Basidia with longitudinal cruciate partial septa, with inflated sterigmata (epibasidia). Basidiospores repetitive. Saprotrophic. South America.

Tulasnella Schröt, 1888. Basidiomata resupinate, smooth, waxy to subgelatinous, sometimes producing sclerotia. Hyphae cylindrical, thin to thick-walled, sometimes monilioid. Clamps present or absent. Dolipore with continuous parenthesomes. Leptocystidia sometimes present. Basidia holobasidiate, with inflated sterigmata (epibasidia). Basidiospores cylindrical to allantoid, sometimes sigmoid, repetitive. Anamorphs of Rhizoctonia or Epulorhiza type. Saprotrophic, ectomycorrhizal or orchid mycorrhizal. Basidiomata found on decayed wood. Cosmopolitan.

Stilbotulasnella Oberw. and Bandoni, 1982. Basidiomata composed of conidiophores, later forming sporodochia. Conidia blastic, annelidic. Hymenium absent. Hyphae cylindrical thin-walled. Clamps absent. Dolipores without parenthesomes. Basidia holobasidiate, with inflated sterigmata (epibasidia). Basidiospores ellipsoid, repetitive. Saprotrophic, foliicolous. North America.

Families Previously Included in *Cantharellales* and Excluded Here

Aphelariaceae Corner, 1970.

This family encompasses species that produce clavariod basidiomata and that lack any of the typical characters of *Cantharellales*. Basidia in *Aphelariaceae* are short and claviform, 4-spored, ampullaceous septa are absent and spores are not repetitive. Molecular data of the type *Aphelaria*, *A. dendroides*, are needed to propose a better phylogenetic placement. In the meantime, it is excluded here from *Cantharellales*.

Oliveoniaceae Roberts, 1998

Combined analyses of the RPB1-RPB2-EF regions indicate that *Oliveonia* belongs to the *Auriculariales*, outside *Cantharellales* (Olariaga, unpublished) and it is accordingly excluded here.

Genera previously included inCantharellales and excluded here.

Ceratobasidium Rogers 1932.

Corallofungus Kobayasi 1983.

Gloeomucro Petersen 1980.

Odontiochaete Rick 1940.

Parastereopsis Corner 1976.

Paullicorticium Erikss 1958.

Repetobasidiellum Erikss and Hjortstam 1981.

Repetobasidium Erikss 1958.

Radulochaete Rick 1940.

Schildia Franchi and Marchetti 2015.

Waitea Warcup 1985.

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Boletales

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Introduction

As part of the astounding biodiversity of terrestrial macrofungi, the boletes may undoubtedly be considered one of the most fascinating and iconic component of larger mushroom-forming fungi. Commonly known as fleshy pored mushrooms, boletes are globally distributed and belong in the monophyletic order Boletales, an extraordinary rich assemblage of polymorphic, often colorful fungi mostly characterized by stipitate-pileate boletoid and, to a lesser extent, both epigeous and belowground sequestrate, agaricoid and pleurotoid lamellate, corticioid and polyporoid habit (Binder, 1999; Binder and Hibbett, 2006; Kirk *et al.*, 2008; Watling, 2008; Nuhn *et al.*, 2013; Wu *et al.*, 2014, 2016a; He *et al.*, 2019). For over more than two centuries the identification of species of boletoid mushrooms and related groups relied exclusively on morphological, biochemical and ecological criteria. In the last thirty years, however, rapid progress of molecular phylogenetic analysis dramatically revolutionized the approach to mycological investigation through a cross-disciplinary methodology, integrating and implementing traditional research methods based on morphology, ecology and chemotaxonomy with a new generation of advanced genetic tools which played a crucial role in redefining taxonomic expertises and enabled the establishment of a more natural classification of the Boletales and its lower taxonomic ranks. Such an innovation allowed the assessment of historically established taxa and led to the uncovering of several new genera and species, therefore contributing to a better deciphering of their evolutionary affinities and biogeographic patterns worldwide.

Morphological Framework, Chemical and Ecological Features, Taxonomic Limits

Order **Boletales** E.-J. Gilbert.

Les Livres du Mycologue Tome I-IV, Tom. III: Les bolets: 83. 1931.

= Lindtneriales Jülich, Bibliotheca Mycologica 85: 350. 1981.
= Melanogastrales Svrček in Pilát, Čescoslovenská Akademie Věd Praha: 527. 1958.
= Plectobasidiales Gäumann, Vergleichende Morphologie der Pilze: 537. 1926.
= Rhizopogonales Kreisel, Grundzüge eines natürlichen Systems der Pilze: 153. 1969. (nom. inval.)
= Sclerodermatales G. Cunningham, The Gasteromycetes of Australia and New Zealand: 112. 1944.
= Tremellogastrales Zeller, Mycologia 40 (6): 661. 1948.

Typus: Boletaceae Chevallier 1828.

Although a comprehensive sharp definition of the order Boletales currently does not exist due to the prominent plasticity of physical, biochemical and ecological traits, nonetheless it is possible to define a series of common features that are shared by all or at least most of the representatives of this eclectic assemblage. The Boletales includes mushroom-forming fungi with homobasidiomycetous fruiting bodies; homoiomerous trama (hyphal tissues only) as opposed to the heteromerous trama of the Russulaceae; monomitic or occasionally dimitic o trimitic hyphal system; gymnocarpic, angiocarpic, primarily or secondarily hemiangiocarpic (velangiocarpic) or metavelangiocarpic ontogenetic development; homogeneous structure (in pileate-stipitate forms hyphae of the pileus trama confluent with and undifferentiated from those of the stipe trama); terricolous to infrequently lignicolous growth; fructifications annual, ephemeral, short-lived, easily putrescent, seldom tending to mummify in habitat (*Strobilomyces* Berk., *Cupreoboletus* Simonini, Gelardi & Vizzini), solitary, scattered to gregarious or infrequently caespitose; extremely variable polymorphism including mainly orthotropic pileate-stipitate boletoid habit, considerably less frequently orthotropic pileate-stipitate agaricoid or laterally stalked to sessile pileate-stipitate pleurotoid habit, but also secotioid or gasteroid habit, both epigeous (earthballs and earthstars) and tuberoid emergent or (sub)hypogeal indehiscent truffle-like (false truffles) (the sequestrate morphology is the second best represented in the Boletales after the boletoid one), more rarely xylotrophic wood-decay resupinate or effuso-reflexed (corticioid or crust-like) habit and occasionally sessile polyporoid morphology, teratologic, aberrant or aborted forms (carpoforoidsm) extraordinary occurring (clavarioid, coralloid and ramarioid morphologies are not at all represented in the Boletales); hymenophoral structure (spore-bearing tissue) mainly tubular-poroid (restricted to suborders Boletineae, Sclerodermatineae and Suillineae), deeply decurrent to almost free to the stipe, to a far lesser extent lamellate and in this case ± distinctly decurrent (gills attachment to the stipe other than decurrence is not observable in the Boletales) and often anastomosate, in sequestrate taxa gleba enclosed or exposed, convoluted, labyrinthine, lacunose or chambered, in corticioid taxa with a smooth, folded to meruloid or raduloid or even hydnoid hymenophore, in poroid and lamellate forms the hymenophore is located on the underside of the pileus and is generally easily detachable from the pileal context above; variable dimensions of the sporophores ranging from tiny (*Gasterella* Zeller & L.B. Walker) to massive (*Phlebopus* (R.Heim) Singer); sporophores moderately to decidedly fleshy in pileate-stipitate and sequestrate forms, initially firm then softer, otherwise corky or suberous (in corticioid

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genera); context white, yellowish, orange, brownish or grayish, frequently changeable (bluing, reddening, browning, blackening, etc.) by auto-oxidation; odor generally agreeable fungoid or fruity, otherwise indistinct, occasionally peculiar (sweetish, mealy, garlic or shallot-like, chicory or licorice-like, of vinyl glue, iodoform, naphthalene, vanilla, boiled milk, roasted potatoes, rotting meat, etc.); taste mild, less frequently bitterish to bitter, exceptionally peppery or sour; spore sprint usually olive-brown but also white, cream yellowish, yellow ochraceous, flesh pink, brownish pink, reddish, rusty brown, purplish brown, dark sooty brown to blackish; occasional formation of sclerotia (dense aggregation of fungal tissue) reported to occur in nature and cultural studies for a wide array of species belonging in a moltitude of genera across the Boletales.

Anatomically, the Boletales are characterized by: mostly ellipsoid-fusiform but also elongate fusoid to cylindrical, broadly ellipsoid to ovoid or (sub)globose (more rarely with other shapes such as amygdaliform, allantoid, nephroid, etc.), usually somewhat thick-walled, hyaline to more commonly pigmented, uninucleate basidiospores with rounded, pointed to sometimes truncate distal end, with or without germ pore, smooth to less frequently with various eusporial ornamentations (obscurely striate, longitudinally costate or winged, rugulose-verrucose, pustulate-tuberculate, echinulate, cristate, reticulate-alveolate, pitted, bacillate, denticulate, etc.), ballistosporic and axially asymmetric (heterotrophic) in pileate-stipitate forms, statismosporic and axially symmetric (orthotrophic) in sequestrate representatives, with a lengh usually comprised in a range between 10 and 15 μm (medium-sized) but also on average smaller (small-sized, <10 μm) (i.e., *Suillus* Gray), slightly larger (large-sized, >15 μm) (i.e., *Leccinum* Gray) to distinctly larger (very large-sized, >20 μm) (Gomphidiaceae), tiny (i.e. *Hygrophoropsis tapinia* Singer) to massive (i.e. *Aureoboletus projectellus* (Murrill) Halling) basidiospores also occurring, generally cyanophilic, inamyloid, dextrinoid or very rarely amyloid, sometimes with a weak metachromatic reaction; cylindrical-clavate to clavate chiastobasidia predominantly 4-spored but also 1-, 2- or 3-spored, very rarely 6–10-spored (in some gasteroid genera such as *Pisolithus* Alb. & Schwein., *Scleroderma* Pers., *Rhizopogon* Fr., *Alpova* C.W. Dodge, *Marthanella* States & Fogel); hymenial cystidia (cheilo- and pleurocystidia) occurring in most genera, usually ventricose-fusiform or lageniform but also differently shaped (cylindrical, clavate, capitate, mucronate, sphaeropedunculate, etc.), fertile and sterile hymenial cells (culobasidia and caulocystidia) are also present on the stipe surface of several pileate-stipitate genera, pseudocystidia occasional (*Alessioporus* Gelardi, Vizzini & Simonini, *Cupreoboletus*, some *Tylopilus* P. Karst. species, etc.); hymenophoral trama in pileate-stipitate genera $\pm$ distinctly bilaterally divergent ("*Boletus*-type", "*Phylloporus*-type" or intermediate), hardly nearly regular (referred by some European authors to as "*Mariaella*-type"); pileipellis/peridium structure in pileate-stipitate, secotioid and sequestrate forms variously arranged, usually a (ixo)trichoderm or (ixo)cutis, less frequently hymeniform (epithelium), sphaerocytic (inflated or globose cells) or compound (i.e. ixohyphoepithelium). Simple clamp connections absent or extremely rare in most of poroid genera (in the Boletaceae regularly and abundantly present in a handle of species only), usually present in lamellate genera (i.e. *Paxillus* Fr. and Paxillaceae as a whole). Multiple clamp connections and medallion clamp connections occurring in few genera (i.e. *Tapinella* E-J. Gilbert). Generative hyphae frequently slightly restricted at septa, hyaline to variously pigmented (epiparietal, cytoplasmatic, vacuolar pigments), thin- to more rarely thick-walled, smooth or incrusted. Hyphae of the context at the stipe base in pileate-stipitate genera mostly inamyloid, seldom distinctly amyloid (*Suillellus* Murrill, *Caloboletus* Vizzini pro parte, *Chroogomphus* (Singer) O.K. Miller, etc.). Oleipherous (thromboplerous) hyphae usually present. At the ultrastructural level the order exhibits perforate parenthesomes (wall next to the dolipore with holes).

Pigments, bioactive chemical compounds and secondary metabolites extremely heterogeneous. Different derivatives of the pulvinic acid (variegatic acid, xerocomic and isoxerocomic acid, vulpinic acid, variegatorubin, xerocomorubin, sclerocitrin, badione and norbadione, cyclovariegatin, etc.), which are responsible of the presence of yellow, red and brown pigments and also of the enzymatic bluing oxidation phenomenon evident in many boletes, occur in most of the Boletales (including the Coniophoraceae); other chemical compounds such as boviquinones (bovinone, bovilactone, helveticone and amitenone), carboxylic aromatic acids (caffeic acid, gallic acid), terphenyl quinones, atromentinic acid (atromentin, leucomentin-3, -4, -5, -6, orange-yellow flavomentin A-D, violet spiromentin A-D), polyporic acid, gomphidic acid, gomphilacton, cyclopentenones derivatives (chamonixin, involution, gyrocianin and gyroporin), phenolic metabolites (involuton), prenylated phenols (suillin, bolegrevilol and several other pigments producing grayish or brownish colorations and different oxidation reactions), prenylated benzoquinones (diboviquinone, metilenediboviquinone, tridentoquinone, rhizopogone), grevillins (grevillin A-D, anhydrogrevillin D), and many others are sporadically present in a number of different suborders, families or genera such as *Gyroporus* Quél., *Leccinum*, *Chamonixia* Rolland, *Paxillus*, *Gyrodon* Opatowsky, *Melanogaster* Corda, *Rhizopogon*, *Suillus*, *Gomphidius* Fr., *Tapinella*, *Leucogyrophana* Pouzar, *Sutorius* Halling, Nuhn & Fechner, *Tylopilus*, etc. (see, among others, Bresinsky and Orendi, 1970; Schmitt, 1970; Bresinsky and Besl, 1979; Besl and Bresinsky, 1997; Besl *et al.*, 1986; Gill and Steglich, 1987; Gill, 1996, 2003; Liu, 2007; Nelsen, 2010; Zhou and Liu, 2010; Velíšek and Cejpek, 2011).

As far as the nutritional status is concerned, most of the poroid, lamellate and sequestrate genera in the Boletales are mutualists ectotrophically mycorrhizal (ECM) (potentially more than 90% and may represent roughly 18–25% of all ECM fungi) (Halling *et al.*, 2007b, 2008), occasionally ericoid (restricted to the plant family Ericaceae) or tuberculate ectomycorrhizal (TECM) (this latter especially in Suillaceae and Rhizopogonaceae) with roots of a large variety of living green woody plants belonging in conifers (gymnosperms) and broadleaved trees (angiosperms) (Newman and Reddell, 1987; Halling and Mueller, 2003; Binder and Hibbett 2006; Tedersoo *et al.*, 2010; etc.), mainly with members of the Fagaceae (*Castanea*, *Castanopsis*, *Chrysolepis*, *Cyclobalanopsis*, *Fagus*, *Lithocarpus*, *Notholithocarpus*, *Quercus*, *Trigonobalanus*), Pinaceae (*Abies*, *Cedrus*, *Keteleeria*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga*, *Tsuga* and perhaps *Cathaya*), Myrtaceae (*Angophora*, *Chamelaucium*, *Corymbia*, *Eucalyptus*, *Eugenia*, *Gomidesia*, *Kunzea*, *Leptospermum*, *Lophostemon*, *Melaleuca*, *Myrtus*, *Syncarpia*, *Syzygium*, *Tristaniopsis*, *Xanthostemon*), Dipterocarpaceae (*Anisoptera*, *Dipterocarpus*, *Hopea*, *Marquesia*, *Monotes*, *Pakaraimaea*, *Parashorea*, *Pseudomonotes*, *Shorea*, *Vateria*, *Vateropsis*, *Vatica*, etc.), Casuarinaceae (*Casuarina*,

Allocasuarina), Fabaceae, including subfamilies Faboideae (*Ammopiptanthus*, *Andira*, *Millettia*, *Pterocarpus*, *Pultanea*, *Robinia*, *Sesbania*, *Swartzia*), Mimosoideae (*Acacia*, *Enterolobium*, *Inga*, *Lysiloma*, *Mimosa*, *Parkia*), Papilionoideae (*Aldinia*, *Pericopsis*) and Caesalpinoideae (viz. caesalpinoid legumes) (*Afzelia*, *Bauhinia*, *Berlinia*, *Brachystegia*, *Burkea*, *Cassia*, *Delonix*, *Dicymbe*, *Gilbertiodendron*, *Haematoxylum*, *Intsia*, *Isoberlinia*, *Julbernardia*, *Paramacrolobium*), Betulaceae (*Alnus*, *Betula*, *Carpinus*, *Corylus*, *Ostrya*), Salicaceae (*Populus*, *Salix*), Ericaceae (*Andromeda*, *Arbutus*, *Arctostaphylos*, *Comarostaphylis*, *Erica*, *Pieris*, *Rhododendron*), Cistaceae (*Cistus*, *Helianthemum*, *Halimium*, *Hudsonia*), Nothofagaceae (*Nothofagus*), Nyctaginaceae (*Guapira*, *Neea*, *Pisonia*), Polygonaceae (*Coccoloba*, *Gymnopodium*), Uapacaceae (*Uapaca*), just to name a few. Furthermore, the orchid genus *Vanilla* should be added to the list as it has been found for the first time to form symbiosis with a representative of the Boletales (Gonzáles-Chávez *et al.*, 2018). In native and alien naturalized ectotrophic forests, woodlands and scrublands dominated by plants of these families, boletes typically constitute an integral part of the mycota during the rainy season. Additionally, many species of *Suillus*, *Scleroderma*, *Pisolithus*, *Rhizopogon* and other notoriously ectotrophic genera are commonly used as biotechnology tools in pure-culture synthesis and in forestry (mycosilviculture) as optimal candidates for ECM mycelial inoculum to enhance seedlings establishment and plant growth through improved water and nutrient uptake (by enlarging root's effective absorptive area), increased tolerance to drough stress, the ability of linking plant roots for a profitable interplant exchange of substances and therefore to ensure a better establishing of artificial plantations in reforestation programmes or vegetational restoration plans and of outplanted landscape trees both in pristine and disturbed or otherwise depauperate, contaminated or degraded ecosystems (Singer, 1986; Molina and Trappe, 1994; Agerer, 2006; Watling, 2008; etc.). Members of the suborder Suillineae are almost exclusively obligate symbionts of the Pinaceae, however, the Gomphidiaceae can establish, although infrequently, ordinary ECM symbiosis with Pinaceae but they are also able to facultatively parasite rhizomorphs and ectomycorrhizas of the Suillaceae and Rhizopogonaceae, as already noted by Miller (1964) and Smith and Zeller (1966) and subsequently confirmed by several authors (Singer, 1986; Agerer, 1990, 1991, 1996, 2002, 2006; Agerer *et al.*, 1996; Olsson *et al.*, 2000; Binder and Hibbett, 2006; Kirk *et al.*, 2008; Watling, 2008; Li *et al.*, 2009; McLaughlin and Spatafora, 2014; etc.). It is therefore possible to define the Gomphidiaceae as "episymbionts" engaging a three-(four-)way interaction between the tree hosts and other representatives of the Suillineae (Olsson *et al.*, 2000; Knudsen and Taylor, 2008). Nonetheless, the exact biological nature, myco-parasitism dynamics and the degree of specificity among these families have not yet been clarified (Li *et al.*, 2009; Scambler *et al.*, 2018) and further studies are urgently required to assess their multipartite relationships (Agerer, 2006).

A few genera in the Boletaceae are selective species-specific mycoparasites (*Chalciporus* Bataille, *Pseudoboletus* Šutara, *Buchwaldoboletus* Pilát pro parte) of some ECM genera in the Boletales or other Basidiomycetes (*Amanita* Pers. spp., *Phaeolus Schweinitzii* (Fr.) Pat., etc.). Some species belonging in *Phlebopus* (Boletinellaceae) appear to be entomosymbiotic or symbiotic/parasitic in relation to the root infection of numerous trees, such as in the case of the plant genus *Citrus* (Rutaceae) in Brazil, provoked by the insect *Pseudococcus comstocki* and by at least 13 other soil mealy bugs (belonging mostly in Pseudoccocidae but also in Monophlebidae and Eriococcidae) brought by ant species (i.e., *Solenopsis moelleri* Forel). *Phlebopus* species establish a unique mutually beneficial tripartite nutritional relationship through the formation of fungus-insect galls (also called "crypta") on the roots of the host plants (Singer, 1946, 1986; Singer and Digilio, 1957; Singer *et al.*, 1983; Binder and Bresinsky, 2002b; Watling, 2008; Tedersoo *et al.*, 2010; Pham *et al.*, 2012; Zhang *et al.*, 2015; Fang *et al.*, 2020; Yu *et al.*, 2020; Raghoonundon *et al.*, 2021). Fang *et al.* (2020) stressed that *P. portentosus* (Berkeley & Broome) Boedijn forms a symbiotic association with mealy bugs, whose galls afford a safe and confortable shelter for the insects, which in turn provide essential nutrients for the mycelial growth of fungal organism with the secreted honeydew (rich in amino acids and sugars) and, according to Mei *et al.* (2021), possibly also other metabolites or necromass. In line with the trophic behavior of congeneric species, the recently described *Phlebopus roseus* M. Yang, C.-Y. Liu & Y. Wang has been observed to form a symbiotic association with the insect *Coccus hesperidum* L., developing fungus-insect galls on roots of an agriculturally exploited loquat trees orchard (*Eryobotria japonica* (Thunb.) Lindl.) in south-western China (Mei *et al.*, 2021). Similarly, in the closely related genus *Boletinellus* Murrill (Boletinellaceae), the so-called "ash bolete" *B. merulioides* (Schwein.) Murrill, is known to be able to establish a three-way association with *Fraxinus* spp. (Oleaceae) and an arthropod, *Prociphilus fraxinifolii* Riley (aphids), which is nested in the sclerotia (or fungal-insect galls) produced by the mushroom itself, receiving protection from ants and centipedes and probably returning to fungal organism nutrients in form of honeydew (Brundrett and Kendrick, 1987; Gruhn *et al.*, 1992; Nagasawa, 2001; Binder and Bresinsky, 2002b; Wang and Qiu, 2006; Tedersoo *et al.*, 2010; Wilson *et al.*, 2012; Nuhn, 2016). The nature of ecological and physiological interactions among these organisms certainly deserves further investigation. Anyway, based on available knowledge there would appear to be no parasitic member of the Boletales being strictly pathogen of arboreous plants.

A relatively low percentage of saprotrophic taxa are found either on soil or on woody substrates. All saproxylic wood-rotting members of the Boletales inhabit decayed stumps, debris, fallen twigs or branches and dead standing trees preferably of conifers and exclusively produce Coniophoraceae-type brown rot, dry rot or brown cubical heart rot (named from the typical fracturing of wood in orthogonal structures) (white rot and soft rot lignicolous decomposers are not at all present in the Boletales), leading to the rapid oxidation of cellulose through the Fenton's mechanism. Corticioid genera (*Coniophora* DC., *Serpula* (Pers.) Gray, *Leucogyrophana* Pouzar, etc.), which are among the most dangerous wood-destroying fungi, can also develop and extend upon handmade building structures such as wooden timber, plaster, porous bricks and stones (Gilbertson, 1981; Kämmerer *et al.*, 1985; Hibbett and Binder, 2002; Binder *et al.*, 2005; Binder and Hibbett, 2006; Watling, 2008; Hyde *et al.*, 2018; Zmitrovich *et al.*, 2019b). However, as a general rule the occurrence of basidiomes in elevated positions on woody substrata is not necessarily a prerequisite of saprotrophic nutritional mode (Rayner *et al.*, 1985; Henkel *et al.*, 2000). Examples of lignicolous growth of ECM pileate-stipitate Boletaceae have been reported from all around the world and this fruiting behavior could be explained as a

strategy for enhancing spore dispersal, for selective foraging of N in rotting wood, or otherwise as a behavior to preserve basidiomata from becoming water-soaked under wet conditions (Weber and Sundman, 1986; Jurgensen *et al.*, 1987; Henkel *et al.*, 2000, 2012; Lindahl and Tunlid, 2015; Farid *et al.*, 2018).

Ectomycorrhizal families Boletaceae, Gyroporaceae, Melanogastraceae, Paxillaceae, Rhizopogonaceae, Sclerodermataceae and Suillaceae share the most advanced type of rhizomorphs (boletoid rhizomorphs) and the ECM mantle is plectenchymatous (consisting of interwoven filamentous hyphae), frequently with a ring-like pattern; genera in the Gomphidiaceae exhibit amyloid ECM (Agerer, 1999, 2006).

Last but not the least, another unifying trait of the Boletales is indirectly represented by pathogen organisms; members of this order are often infected and colonized by some species of the ascomycete genus *Hypomyces* (Fr.) Tul. (teleomorph) (anamorph *Sepedonium* Link), especially *H. chrysospermus* Tulasne & C. Tulasne and related taxa, suggesting a certain degree of co-evolution and specialization between parasite and host species (Rogerson and Samuels, 1989; Sahr *et al.*, 1999; Douhan and Rizzo, 2003; McLaughlin and Spatafora, 2014).

Brief Historical Overview and Current State of Knowledge

The order Boletales, corresponding to the tribe Bolétés as circumscribed by Patouillard (1900), was first formally settled by Gilbert (1931), whose work laid the foundation for the modern study of boletoid mushrooms. Two suborders, namely Boletineae (including 9 genera with smooth spores, viz. *Boletinellus*, *Boletinus* Kalchbr. (= *Suillus*), *Boletus*, *Gyrodon*, *Ixocomus* Quél. (= *Suillus*), *Krombholzia* P. Karst. (= *Leccinum*), *Phylloporus* Quél., *Porphyrellus* E.-J. Gilbert and *Xerocomus* Quél.) and Strobilomycetineae (including taxa with ornamented spores, viz. *Strobilomyces* and *Boletellus* Murrill) were also established by the French author to place European and American boletes. For a long time, however, the classification of boletes and allied groups has been particularly instable, the order Boletales was largely disregarded (Horak, 1968) or not unanimously conceived by posterior authors and its circumscribing boundaries as well as those of lower taxonomic ranks (suborders, families, genera, etc.) remained controversial and unclear due to strongly divergent opinions as to how delimiting lines ought to be drawn for this group. Kühner and Romagnesi (1953) divided the European boletes into two informal groups, namely "bolétacées lamellées" (lamellate boletes) (encompassing *Phylloporus*, *Gomphidius* and *Paxillus*) and "bolétacées porées" (poroid boletes) and assigned the poroid boletes to a single genus *Boletus* divided into ten subgenera (*Strobilomyces*, *Gyroporus*, *Krombholzia*, *Tylopilus*, *Tubiporus* P. Karst. (= *Boletus*), *Porphyrellus*, *Xerocomus*, *Ixocomus*, *Boletinus*, *Gyrodon*). Hongo (1960) accounted four families (Paxillaceae, Gomphidiaceae, Boletaceae and Strobilomycetaceae) in the order Agaricales to accommodate Japanese boletes and closely related taxa. No mention was done by Watling (1970) about the order they belong in, but he grouped British boletoid mushrooms and allies into three families by recognizing only a limited number of genera: Boletaceae (including *Aureoboletus* Pouzar, *Boletinus*, *Boletus*, *Gyroporus*, *Leccinum*, *Porphyrellus*, *Strobilomyces*, *Suillus*, *Tylopilus* and *Uloporus* Quél. (= *Gyrodon* Opat.)), Gomphidiaceae (*Chroogomphus* and *Gomphidius*) and Paxillaceae (*Paxillus* and *Phylloporus*). Shortly after Smith and Thiers (1971), following Watling's perspective, proposed a single family Boletaceae for the fleshy pored mushrooms of Michigan (USA). Corner (1972b), based on the enormous diversity of southeastern Asian boletes (with a special focus on the Indo-Malayan region), preferred to maintain a taxonomic conservative approach and recognized only four genera, specifically *Boletus* (with a number of discrete subgenera), *Gyroporus*, *Heimiella* Boedijn (now *Heimioporus* E. Horak) and *Strobilomyces*. Two years later Pilát and Dermek (1974), in their monographic treatment on central European boletes, disposed poroid mushrooms and relatives into a family Gomphidiaceae (including *Gomphidius*) and a widely delimited family Boletaceae (including 16 boletoid genera and the lamellate *Phylloporus*), which was in turn subdivided into two subfamilies, Strobilomycetoideae (including *Strobilomyces* and *Porphyrellus*, i.e. taxa with ornamented spores or with a purplish brown spore print) and Boletoideae (including all remaining genera with smooth basidiospores). Rolf Singer, in his numerous publications and especially in his fourth and last edition of a comprehensive mycological milestone such as "The Agaricales in modern taxonomy" (Singer, 1986), did not recognize the Boletales as an autonomous consortium standing by its own, instead he assigned 34 genera worldwide to the suborder Boletineae within the order Agaricales, sorting them out in three different families: Paxillaceae, Gomphidiaceae and a broadly conceived family Boletaceae (this latter divided in turn into subfamilies Gyroporoideae, Gyrodontoideae, Suilloideae, Strobilomycetoideae, Boletoideae and Xerocomoideae), which subsumed genera that are presently accommodated into five different families belonging in three separate suborders (see below). Prior to Singer and conversely to his systematic framework, Kühner (1977) recognized the order Boletales and allocated the genera *Strobilomyces*, *Gyroporus*, *Boletus* (divided into subgenera *Gyrodon*, *Phylloporus*, *Xerocomus*, *Boletus*, *Tylopilus*, *Porphyrellus* and *Leccinum*), *Suillus* (including *Suillus* and *Boletinus*) and *Gomphidius* (including subgenera *Gomphidius* and *Chroogomphus*) to a sole family Boletaceae and the genera *Hygrophoropsis* (J. Schröter) Maire ex Martin-Sans and *Paxillus* (including subgenera *Paxillus* and *Tapinella*) to the family Paxillaceae. Pegler and Young (1981) maintained and rearranged the Boletales on a global scale based primarily on basidiospore morphology and distributed 35 genera within six families, namely Boletaceae, Gomphidiaceae, Gyrodontaceae, Paxillaceae, Strobilomycetaceae and Xerocomaceae. Likewise Moser (1983) accepted the order Boletales as distinct from the Agaricales and listed 21 European genera distributed in four different families (Strobilomycetaceae, Boletaceae, Paxillaceae and Gomphidiaceae).

A large number of regional mycological floras, comprehensive monographic treatises, color atlas, reference books, checklists and popular field guides have dealt with fleshy pored mushrooms and related lamellate, sequestrate and corticioid taxa over the past two centuries (Fries, 1821, 1838; Frost, 1874; Quélet, 1888; Peck, 1889; Bataille, 1908; Smotlacha, 1912; Murrill, 1914; Coker and Couch, 1928; Coker and Beers, 1943; Singer, 1945, 1946, 1947, 1965, 1967, 1986; Chiu, 1948, 1957; Heinemann, 1951;

Imazeki, 1952; Singer and Digilio, 1957, 1964; Pilát, 1958; Hongo, 1960, 1973; McNabb, 1967, 1968, 1969; Horak, 1968, 2011; Corner, 1971a,b, 1972b; Snell and Dick, 1970; Smith and Thiers, 1971; Pilát and Dermek, 1974; Kühner, 1977; Vasiljeva, 1978; Tai, 1979; Wolfe, 1980; Pegler and Young, 1981; Moser, 1983; Singer *et al.*, 1983, 1990, 1991, 1992; Alessio, 1985; Garrido, 1988; Imazeki *et al.*, 1988; Watling and Hollands, 1990; Watling and Turnbull, 1992, 1994; Both, 1993; Lakhanpal, 1996; Nagasawa, 1997; Watling and de Meijer, 1997; Ginns, 1998; Watling and Li, 1999; Bessette *et al.*, 2000; Li and Song, 2000; Montecchi and Sarasini, 2000; Lannoy and Estadès, 2001; Ladurner and Simonini, 2003; Muñoz, 2005; Šutara, 2005, 2008; Zang, 2006; Klofac, 2007; Ortiz-Santana *et al.*, 2007; Knudsen and Taylor, 2008; Bernicchia and Gorjón, 2010; Zang *et al.*, 2013; etc.), however, the approach to the study of bolete taxonomy has radically changed with the increasing availability of molecular genetic investigation applied to the study of basidiomycetes, casting doubts on the validity of relying solely on visible features for identification and highlighting the necessity to clarify the discrepancies between genetic and morphological criteria.

The order Boletales is one of the most diverse and widespread groups of Basidiomycetes. Unlike R. Heim's hypothesis indicating a polyphyletic origin of the boletes (Singer, 1981), the order Boletales is definitely a monophyletic lineage recovered within the subclass Agaricomycetidae, superorder Agaricanae, alongside and in a close proximity to five other orders namely Agaricales, Amylocorticiales, Stereopsidales, Atheliales and Lepidostromatales, the latter two being sister and closer to the Boletales, and together being sister to the remaining aforementioned orders (Bruns *et al.*, 1998; Kretzer and Bruns, 1999; Jarosch, 2001; Binder and Bresinsky, 2002b; Larsson *et al.*, 2004; Binder *et al.*, 2005, 2010; Binder and Hibbett, 2006; Matheny *et al.*, 2007; He *et al.*, 2019; Varga *et al.*, 2019; Sánchez-García *et al.*, 2020).

At present, according to the most recent molecular phylogenetic inference, the order Boletales is subdivided into 5 suborders, 18 families and some 150 accepted genera (including the sclerodermatoid fossil genus *Palaeogaster* Poinar, Alfredo & Baseia, see Poinar *et al.*, 2014), containing approximately 2194 species worldwide (this number accounts officially described species as well as those molecularly detected but not yet formally named) (Pers. Obs.), far beyond the 1316 species estimated by Kirk *et al.* (2008) but roughly similar to the number (2022) indicated by He *et al.* (2019). The backbone suprageneric arrangement of the Boletales is summarized up as follows:

- suborder Tapinellineae Agerer

a. family Tapinellaceae C. Hahn

- suborder Coniophorineae Agerer & C. Hahn

a. family Coniophoraceae Ulbrich
b. family Hygrophoropsidaceae Kühner

- suborder Suillineae Besl & Bresinsky

a. family Gomphidiaceae R. Maire ex Jülich
b. family Rhizopogonaceae Gäumann & C.W. Dodge
c. family Suillaceae (Singer) Besl & Bresinsky
d. family Truncocolumellaceae Agerer

- suborder Sclerodermatineae Manfr. Binder & Bresinsky

a. family Boletinellaceae P.M. Kirk, P.F. Cannon & J.C. David
b. family Calostomataceae E. Fischer
c. family Diplocystidiaceae Kreisel
d. family Gyroporaceae (Singer) Manfr. Binder & Bresinsky
e. family Pisolithaceae Ulbrich
f. family Sclerodermataceae Corda

- suborder Boletineae Rea emend. E.-J. Gilbert

a. family Paxillaceae Lotsy
b. family Boletaceae Chevallier

- *incertae sedis*

a. family Gasterellaceae Zeller
b. family Serpulaceae Jarosch & Bresinsky

Kirk *et al.* (2008) and He *et al.* (2019) merged the families Pisolithaceae and Truncocolumellaceae with the Sclerodermataceae and Suillaceae, respectively. In the present account, however, we retain these two families at their autonomous hierarchic rank following Binder and Hibbett (2006) and Wu *et al.* (2020). Moreover, the suborder Sclerodermatineae has been differently interpreted by Moreau *et al.* (2013a). Based on the assumption that gasteroid genera in the Sclerodermatineae formed a unique evolutionary line based on similar anatomical features, they proposed, following Agerer (1999), to replace a systematic arrangement of suborder

Sclerodematineae including multiple families with a single family Sclerodermataceae to be subdivided into four different subfamilies namely Boletinelloideae, Gyroporoideae, Sclerodermoideae and Calostomatoideae.

Suborders Tapinellineae and Coniophorineae occupy the basal position in the Boletales, representing the earliest evolutionary diverging lineages; they mostly consist of lignicolous corticioid, pleurotoid and polyporoid genera. In addition, the Tapinellineae form a sister group to all remaining suborders (Binder *et al.*, 2010). In the suborder Suillineae boletoid and agaricoid taxa are found in the Suillaceae and Gomphidiaceae, respectively, while the Rhizopogonaceae and Truncocolumellaceae harbor sequestrate species. The suborder Sclerodermatineae mostly comprises epigeous gasteroid genera distributed among Calostomataceae, Diplocystidiaceae, Pisolithaceae and Sclerodermataceae, with boletoid genera restricted to Boletinellaceae and Gyroporaceae. However, this suborder has been resolved as paraphyletic by Sato and Toju (2019) being formed by the sister lineages Sclerodermatineae s.str. and Boletinellaceae. In addition the Sclerodermatineae are strongly supported as sister of the clade including Boletinellaceae, Paxillaceae and Boletaceae (Sato and Toju, 2019). Within suborder Boletineae, the Paxillaceae contains a mixed assemblage of boletoid, agaricoid, hypogeous sequestrate, polyporoid and perhaps corticioid taxa (although the phylogenetic placement of *Hydnomerulius* Jarosch & Besl currently remains uncertain), whereas the hyperdiverse Boletaceae represents the core lineage in the Boletales and contains the bulk of boletoid mushrooms, embracing at present roughly 1427 species distributed in 95 different genera. Sequestrate members are well-represented in this family but it is mainly consisting of boletoid genera with a few additional lamellate representatives. Based on multilocus phylogenetic analysis, Wu *et al.* (2014) recovered five major subclades in the Boletaceae, four out of which have been formally introduced as monophyletic subfamilies Boletoideae, Xerocomoideae, Leccinoideae, Zangioideae and Chalciporoideae, this latter along with the genus *Pseudoboletus* occupying the basal position in the family and being sister to all other infrafamiliar groups; in particular the *Chalciporus*/*Rubinoboletus* and *Buchwaldoboletus* lineages represent the primeval, most archaic branch in the Boletaceae and have a position even more basal than that of *Pseudoboletus* (Binder and Hibbett, 2006; Nuhn *et al.*, 2013; Wu *et al.*, 2014, 2016a,b; Sato and Toju, 2019). The fifth subclade, that still remains unresolved, has provisionally been named "Pulveroboletus group" (Wu *et al.*, 2014).

The number of newly defined (or resurrected) genera and species recognized in the Boletaceae has steadily increased over the last two decades and are still being discovered as a result of the advancement of molecular techniques (Binder and Bresinsky, 2002a; Bresinsky and Besl, 2003; Halling *et al.*, 2007a, 2012a,b; Desjardin *et al.*, 2008, 2009; Šutara, 2008; Orihara *et al.*, 2010, 2016b; Li *et al.*, 2011b, 2014b; Lebel *et al.*, 2012; Zeng *et al.*, 2012, 2014; Hosen *et al.*, 2013; Trappe *et al.*, 2013; Arora and Frank, 2014; Gelardi *et al.*, 2014, 2015a, b; Vizzini, 2014a,b,c,d,e,f, 2015; Zhao *et al.*, 2014; Zhu *et al.*, 2014, 2015; Assyov *et al.*, 2015; Smith *et al.*, 2015; Castellano *et al.*, 2016; Henkel *et al.*, 2016; Wu *et al.*, 2016a,b, 2018; Orihara and Smith, 2017; Crous *et al.*, 2018, 2020; Farid *et al.*, 2018; Parihar *et al.*, 2018; Zhang and Li, 2018; Chai *et al.*, 2019; Khmelnitsky *et al.*, 2019; Vadthanarat *et al.*, 2019a; Sulzbacher *et al.*, 2020) and several traditionally established genera which were inferred to be unwieldy and polyphyletic (*Boletellus*, *Boletus* Fr., *Leccinum*, *Pulveroboletus* Murrill, *Tylopilus*, *Xerocomus*, etc.) have been thoroughly re-organized by means of morphological, ecological and extensive DNA sequencing (Peintner *et al.*, 2003; den Bakker and Noordeloos, 2005; Taylor *et al.*, 2006; Dentinger *et al.*, 2010; Feng *et al.*, 2012; Cui *et al.*, 2015; Halling *et al.*, 2015; Wu *et al.*, 2016a; Gelardi *et al.*, 2019) (**Table 1**). However, as opposed to the current trend of proliferation of new genera, an alternative, much broader and conservative approach to the systematic of the Boletaceae has most recently been proposed by Kuo and Ortiz-Santana (2020). They have suggested to re-arrange this family by lumping single taxonomic units into larger groupings in order to facilitate the understanding of their respective phylogenetic relationships. Notably, they collapsed all genera of the core leccinoid clade into a very broadly circumscribed genus *Leccinum* based on a purported paraphyly of its representatives as previously suggested by den Bakker and Noordeloos (2005), Lebel *et al.* (2012) and Wu *et al.* (2014).

Anyway, as a result of the ongoing discoveries, the most recently compiled monographic works, dichotomous keys and mycological divulgative books are gradually starting to metabolize the lately proposed molecular phylogenetic novelties (Bessette *et al.*, 2016, 2019; Mikšík 2017; Noordeloos *et al.*, 2018; Klofac and Krisai-Greilhuber, 2020).

Origins, Diversification and Historical Evolutionary Dynamics

The monophyly of the order Boletales is strongly supported by molecular phylogenetic analysis based on sequences of barcode genes, consequently all taxa belonging in this order share a single, common origin (Binder *et al.*, 1997; Bruns *et al.*, 1998; Kretzer and Bruns, 1999; Jarosch, 2001; Binder and Bresinsky, 2002b; Eberhardt and Taylor, 2005; Binder and Hibbett, 2006; Zhao *et al.*, 2017; Sato and Toju, 2019). The accuracy of calibrated molecular clock models allowed to infer the origin of the Boletales in the Mesozoic Era, Upper Permian to Lower Cretaceous, more or less overlapping in age with the Pinaceae or a little later but pre-dating the formation of rosids (Malloch *et al.*, 1980; LePage, 2003; Hibbett and Matheny, 2009; Dentinger *et al.*, 2010; García-Sandoval *et al.*, 2011; Wilson *et al.*, 2012; Han *et al.*, 2018). However, the earliest diversification of the Boletales from their close relatives in the Agaricomycetes has been differently estimated in terms of million years ago (mya) by various authors (**Table 2**).

The most recent estimate using six fossil calibration dates the origin of the Boletales back to 151 mya (Sánchez-García *et al.*, 2020). The same authors estimated the origin of the Boletales to be older than that of the Agaricales (134 mya), roughly contemporaneous to the Amylocorticiales (144 mya) and Atheliales (144 mya) but younger than Polyporales (193 mya) and Phallales (203 mya) and much younger than Russulales (236 mya) and Cantharellales (309 mya) (Sánchez-García *et al.*, 2020). In sharp contrast, Varga *et al.* (2019) inferred the Agaricales to be significantly older (173 mya) than the Boletales and this latter to be slightly older than the Russulales.

Table 1 Genera of the Boletales. ▲Boletoid genera; △Agaricoid genera; ◇Pleurotoid genera; ○Epigeal gasteroid genera; ●Hypogeous gasteroid genera; @Secotioid genera or genera containing secotioid species; #Corticioid genera; □Polyporoid genera; †Fossil genera; *Genera not yet molecularly investigated

Boletales *E.-J. Gilbert*

Suborder **Boletineae** Rea

Family **Boletaceae** Chevallier

Afroboletus Pegler & T.W.K. Young▲@
Afrocastellanoa M.E. Smith & Orihara●
Alessioporus Gelardi, Vizzini & Simonini▲
Aureoboletus Pouzar▲
Australopilus Halling & Fechner▲
Austroboletus (Corner) Wolfe▲
Baorangia G. Wu & Zhu L. Yang▲
Binderoboletus T.W. Henkel & M.E. Smith▲
Boletellus Murrill▲
Boletochaete Singer▲*
Boletus Fries▲@
Borofutus Hosen & Zhu L. Yang▲
Bothia Halling, T.J. Baroni & Manfr. Binder▲
Buchwaldoboletus Pilát▲
Butyriboletus D. Arora & J.L. Frank▲
Cacaoporus Raspé & Vadthanarat▲
Caloboletus Vizzini▲
Carolinigaster M.E. Smith & S. Cruz●
Castellanea T.W. Henkel & M.E. Smith●
Chalciporus Bataille▲
Chamonixia Rolland●
Chiua Yan C. Li & Zhu L. Yang▲
Corneroboletus N.K. Zeng & Zhu L. Yang▲
Costatisporus T.W. Henkel & M.E. Smith●
Crocinoboletus N.K. Zeng, Zhu L. Yang & G. Wu▲
Cupreoboletus Simonini, Gelardi & Vizzini▲
Cyanoboletus Gelardi, Vizzini & Simonini▲
Durianella Desjardin, A.W. Wilson & Manfr. Binder○
Erythrophylloporus Ming Zhang & T.H. Li△
Exsudoporus Vizzini, Simonini & Gelardi▲
Fistulinella Hennings▲@
Octaviania Vittadini emend. Orihara●
Parvixerocomus G. Wu & Zhu L. Yang▲
Phylloboletellus Singer in Singer & Digilio△
Phylloporopsis Angelini, A. Farid, Gelardi, M.E. Smith, Costanzo & Vizzini△
Phylloporus Quélet△
Porphyrellus E.-J. Gilbert▲
Pseudoaustroboletus Yan C. Li & Zhu L. Yang▲
Pseudoboletus Šutara▲
Pulchroboletus Gelardi, Vizzini & Simonini▲
Pulveroboletus Murrill▲
Retiboletus Manfr. Binder & Bresinsky▲
Rheubarbariboletus Vizzini, Simonini & Gelardi▲
Rhodactina Pegler & T.W.K. Young●
Rossbeevera T. Lebel & Orihara●
Royoungia Castellano, Trappe & Malajczuk●
Rubinoboletus Pilát & Dermek▲
Rubroboletus Kuan Zhao & Zhu L. Yang▲
Rugiboletus G. Wu & Zhu L. Yang▲
Setogyroporus Heinemann & Rammeloo▲*
Gastroboletus Lohwag@
Gastroleccinum Thiers@*
Guyanaporus T.W. Henkel & M.E. Smith▲
Gymnogaster J.W. Cribb@
Harrya Halling, Nuhn & Osmundson▲
Heimioporus E. Horak▲
Heliogaster Orihara & Iwase●
Hemileccinum Šutara▲
Hortiboletus Simonini, Vizzini & Gelardi▲
Hourangia Xue T. Zhu & Zhu L. Yang▲
Hymenoboletus Yan C. Li & Zhu L. Yang▲
Imleria Vizzini▲
Imperator G. Koller, Assyov, Bellanger, Bertéa, Loizides, G. Marques, P.-A. Moreau, J. A. Muñoz, Oppicelli, Puddu & F. Richard▲
Indoporus A. Parihar, K. Das, Hembrom & Vizzini▲
Ionosporus O. Khmelnitsky▲
Ixechinus R. Heim▲*
Jimtrappea T.W. Henkel, M.E. Smith & Aime●
Kombocles Castellano, T.W. Henkel & Dentinger●
Lanmaoa G. Wu, Zhu L. Yang & Halling▲
Leccinellum Bresinsky & Manfr. Binder▲@
Leccinum Gray emend. Snell▲
Longistriata Sulzbacher, Orihara, Grebenc, M.P. Martín & Baseia●
Mackintoshia Pacioni & Sharp●
Mucilopilus Wolfe▲
Mycoamaranthus Castellano, Trappe & Malajczuk●
Neoboletus Gelardi, Simonini & Vizzini▲@
Nigroboletus Gelardi, Vizzini, E. Horak, T.H. Li & Ming Zhang▲
Singerocomus T.W. Henkel & M.E. Smith▲
Solioccasus Trappe, Osmundson, Manfr. Binder, Castellano & Halling●
Spongiforma Desjardin, Manfr. Binder, Roekring & Flegel○
Spongispora G. Wu, S.M.L. Lee, E. Horak & Zhu L. Yang▲
Strobilomyces Berkeley▲
Suillellus Murrill▲
Sutorius Halling, Nuhn & Fechner▲
Tengioboletus G. Wu & Zhu L. Yang▲
Tubosaeta E. Horak▲
Turmalinea Orihara & N. Maekawa●
Tylocinum Yan C. Li & Zhu L. Yang▲
Tylopilus P. Karsten▲
Veloboletus Fechner & Halling▲
Veloporphyrellus L.D. Gómez & Singer▲
Xanthoconium Singer▲
Xerocomellus Šutara▲@
Xerocomus Quélet▲
Zangia Yan C. Li & Zhu L. Yang▲

Family **Paxillaceae** Lotsy

Alpova C.W. Dodge●
Gyrodon Opatowski▲
Meiorganum R. Heim□*
Melanogaster Corda●
Neoalpova Vizzini●
Paragyrodon (Singer) Singer▲
Paxillus Fries△
Phyllobolites Singer△*

Incertae sedis (family rank)

Hydnomerulius Jarosch & Besl#

Suborder ***Sclerodermatineae*** Manfr. Binder & Bresinsky
Family ***Boletinellaceae*** P.M. Kirk, P.F. Cannon & J.C. David
Boletinellus Murrill▲*
Pseudogyrodon Heinemann & Rammeloo▲*
Phlebopus (R. Heim) Singer▲
Family ***Calostomataceae*** E. Fischer
Calostoma Desvaux○
Family ***Diplocystidiaceae*** Kreisel
Astraeus Morgan○*Diplocystis* Berkeley & M.A. Curtis○
Tremellogaster E. Fischer○
Family ***Gyroporaceae*** (Singer) Manfr. Binder & Bresinsky
Gyroporus Quélet▲
Family ***Pisolithaceae*** Ulbrich
Pisolithus Albertini & Schweinitz○
Family ***Sclerodermataceae*** Corda
Chlorogaster Læssøe & Jalink○*
Horakiella Castellano & Trappe●*
Corditubera Hennings●
Scleroderma Persoon emend. Guzmán○
Incertae sedis (family rank)
Palaeogaster Poinar, Alfredo & Baseia†*
Suborder ***Suillineae*** Besl & Bresinsky
Family ***Suillaceae*** (Singer) Besl & Bresinsky
Psiloboletinus Singer▲
Suillus Gray▲@
Rhopalogaster J.R. Johnston○
Family ***Gomphidiaceae*** R. Maire
Chroogomphus (Singer) O.K. Miller△@
Gomphidius Fries△
Cystogomphus Singer△*
Gomphogaster O.K. Miller@*
Family ***Rhizopogonaceae*** Gäumann & C.W. Dodge
Rhizopogon Fries●
Family ***Truncocolumellaceae*** Agerer
Truncocolumella Zeller○
Suborder ***Coniophorineae*** Agerer & C. Hahn
Family ***Coniophoraceae*** Ulbrich
Chrysoconia McCabe & G.A. Escobar○*
Gyrodontium Patouillard#
Coniophora De Candolle#
Penttilamyces Zmitrovich, Kalinovskaya & Myasnikov#
Coniophoropsis Hjortstam & Ryvarden#*
Sedecula Zeller●
Corneromyces Ginns#*
Family ***Hygrophoropsidaceae*** Kühner
Hygrophoropsis (J. Schröter) Maire ex Martin-Sans△
Leucogyrophana Pouzar#
Suborder ***Tapinellineae*** Agerer
Family ***Tapinellaceae*** C. Hahn
Bondarcevomyces Parmasto□
Tapinella E.-J. Gilbert◇
Pseudomerulius Jülich#
Incertae sedis (suborder rank)
Family ***Gasterellaceae*** Zeller
Gasterella Zeller & L.B. Walker○*
Family ***Serpulaceae*** Jarosch & Bresinsky
Austrogaster Singer@*
Paxillogaster E. Horak in E. Horak & M.M. Moser@
Austropaxillus Bresinsky & Jarosch△
Serpula (Persoon 1801) Gray#
Gymnopaxillus E. Horak in E. Horak & M.M. Moser emend. Claridge, Trappe & Castellano@
Singeromyces M.M. Moser in E. Horak & M.M. Moser@*
Meruliporia Murrill#*
Incertae sedis (suborder and family rank)
Phaeoradulum Patouillard#*
Doubtful placement in the Boletales
Hoehnelogaster Lohwag●*
Marthanella States & Fogel●*
Lamyxis Rafinesque-Schmaltz nom. prov. ▲*
Family ***Protogastraceae*** Zeller
Protogaster Taxter○*

Within the Boletales, the suborder Boletineae presently concentrates the greatest number of boletoid genera and likely originated in the Upper Cretaceous, around 91–71 mya (range comprised between 133 and 41 mya) but was supposed to radiate rapidly, leaving few genetic signatures at the time of its divergence from a common ancestor (Dentinger *et al.*, 2010). The Boletineae are monophyletic and strongly supported by molecular analysis, however, their taxonomic and phylogenetic backbone still remains scarcely resolved (Binder and Hibbett, 2006; Halling *et al.*, 2007a, 2012a, 2015; Nuhn *et al.*, 2013; Wu *et al.*, 2014,

Table 2 Origin of the order Boletales as inferred in recently published works. Extreme values in parenthesis indicate the time range whereas average values are in bold face. Dating estimations are expressed in million years ago (mya)

Estimated divergence dates (mya)	Reference(s)
(192) *140* (92)	Dentinger *et al.*, 2010 (based on calibrations by Taylor and Berbee, 2007)
(247) *179* (114)	Dentinger *et al.*, 2010 (based on calibrations by Lucking *et al.*, 2009)
(254) *189* (128)	Feng *et al.*, 2012
(178) *128* (91)	Wilson *et al.*, 2012
146	Zhao *et al.*, 2017
(279) *218* (160)	Han *et al.*, 2018
(153) *142* (133)	Varga *et al.*, 2019
259	He *et al.*, 2019
151	Sánchez-García *et al.*, 2020

2016a; Sato and Toju, 2019). Similarly, the suborder Sclerodermatinae originated in eastern and south-eastern Asia and North America in the Upper Cretaceous, in a period comprised between (115) 82–80 (54) mya, while the diversification of the "core Sclerodermatineae" (including major gasteroid genera such as *Scleroderma*, *Pisolithus* and *Astraeus* Morgan) was inferred between the middle and Early Cenozoic (Wilson *et al.*, 2012). According to Wilson *et al.* (2012), the suborder Suillineae originated in the Early Cenozoic, some (64) 56–53 (50) mya. Recent molecular studies placed the family Serpulaceae close to the suborder Tapinellinae which constitutes its sister group and from which it separated during the Upper Cretaceous around 64 mya, diverging from a common ancestor established in the temperate belt of western North America (Skrede *et al.*, 2011). In the Boletaceae, the diversification of porcini mushrooms (*Boletus* s.str.) from allied taxa has been estimated in the Late Eocene, at the beginning of Cenozoic or Tertiary Era, orientatively between 44 and 34 mya (Dentinger *et al.*, 2010), or even earlier in Late Cretaceous (109) 77 (46) mya (Feng *et al.*, 2012).

From an evolutionary viewpoint based on ancestral state reconstruction, advanced pileate-stipitate forms in the Agaricomycetes and particularly in the Agaricomycetidae would have arisen in the Lower Jurassic (180–170 mya). They are correlated with increased diversification rates when compared to non-pileate forms and likely derived from plesiomorphic saprotrophic resupinate ancestors producing a white rot (Hibbett and Matheny, 2009; Binder *et al.*, 2010; Varga *et al.*, 2019; Sánchez-García *et al.*, 2020). Cifelloid, clavarioid/ramarioid and sequestrate (including secotioid) forms would appear to be even more recent (Varga *et al.*, 2019). Likewise the order Boletales, conversely to earlier assumptions by Hibbett *et al.* (1997) would not descend from a boletoid ancestor but rather from a saprotrophic resupinate or more probably polyporoid mushroom-forming fungus producing a brown rot on conifer decayed wood (Binder and Hibbett, 2001, 2006; Larsson *et al.*, 2004; Eberhardt and Taylor, 2005; Watling, 2008). As stated above, suborders Tapinellineae and Coniophorineae occupy the basal position and represent the ancestral state in the Boletales, harboring taxa morphologically most similar to the shape of the purported common ancestor of the Boletales (Eberhardt and Taylor, 2005; Sato and Toju, 2019). Therefore, Petersen's theory (Petersen, 1971a,b) appears to be wrong in suggesting a gomphoid mushroom as the forefather of the Boletales based on similarities in basidiospore ornamentation and some other morphological features of *Gomphus* Pers. with certain boletoid genera such as *Austroboletus* (Corner) Wolfe and *Strobilomyces* (see also Pegler and Young, 1981).

Resupinate lignicolous forms would appear to represent the most recent common ancestors of the families Serpulaceae, Hygrophoropsidiaceae and the suborder Coniophorineae. Moreover pileate-stipitate fungi with lamellate hymenophore in the basal lineages such as *Tapinella* and *Hygrophoropsis* would have evolved various times from resupinate ancestors whereas the most recent precursor of the suborders Sclerodermatineae and particularly Boletineae should be a pileate-stipitate form with tubular hymenophore and smooth basidiospores from which both lamellate and gasteroid forms might have later derived (Hibbett *et al.*, 1997; Binder *et al.*, 2005, 2010; Binder and Hibbett, 2006; McLaughlin and Spatafora, 2014; Wu *et al.*, 2014). As a demonstrative example, *Sedecula pulvinata* Zeller is the only known sequestrate hypogeous species in the Coniophoraceae and might have evolved from resupinate forms in this same family. Furthermore, this monotypic genus constitutes one of the earliest non-resupinate taxa in the evolutionary history of the Boletales (Trappe *et al.*, 2015) and would appear to be the only sequestrate member of the boletales with a confirmed non-ectomycorrhizal trophic mode.

Beside pileate-stipitate genera, both epigeous and hypogeous sequestrate taxa constitute a major presence within the order Boletales and their spore discharge, being non-active, involves weathering (air, rainfall) or animal consumption (mammals, reptiles, birds and invertebrates such as worms, centipedes, slugs, etc.) for dispersal in the environment. Far before the development of molecular phylogenetic analysis several authors had already speculated on strict evolutionary relationships between gasteroid and boletoid genera (Malençon, 1931; Smith and Zeller, 1966; Heim, 1971; Thiers, 1971, 1984), but it was generally supposed that resupinate taxa represented a valid taxonomic unit (Gasteromycetes) based on the assumption that forcible spore discharge was rarely lost during the evolutionary history of basidiomycetes (Coker and Couch, 1928), or that the secotioid/gasteroid state was ancestral and therefore primitive with respect to the pileate-stipitate morphology (Singer, 1981). By converse, modern phylogenetic approaches have definitely rejected these scenarios and reinforced theories based on some previous morphological and chemo-taxonomical studies (Heim, 1971; Moore, 1998; Reijnders, 2000), that sequestrate fungi (including hypogeous taxa) are polyphyletic, generally young (50–4 mya) and evolved independently several times in numerous fungal

lineages in the Agaricomycetes starting from different morphologies and following a process defined as gasteromycetation (Bruns *et al.*, 1989; Kendrick, 1992; Bougher *et al.*, 1993; Hibbett *et al.*, 1994, 1997; Mueller and Pine, 1994; Miller *et al.*, 2000; Peintner *et al.*, 2001; Giachini *et al.*, 2006; Trappe *et al.*, 2009; Gube and Dorfelt, 2012; Lebel and Syme, 2012; Lebel *et al.*, 2015; Ge and Smith, 2013; Han *et al.*, 2017; Sánchez-García *et al.*, 2020). The order Boletales makes no exception and gasteroid genera have been inferred to have derived from both boletoid and agaricoid forms (Kretzer and Bruns, 1997; Bruns *et al.*, 1998; Miller and Aime, 2001; Binder and Hibbett, 2006; Nuhn *et al.*, 2013; Wu *et al.*, 2014; Smith *et al.*, 2015). The sequestrate hypogeous *Chamonixia*, *Octaviania* Vittadini, *Rossbeevera* T. Lebel & Orihara and *Turmalinea* Orihara & N. Maekawa would have transformed independently from different boletoid ancestors in the leccinoid clade (subfamily Leccinoideae) (Binder, 1999; den Bakker and Noordeloos, 2005; Orihara *et al.*, 2016b). Moreover, subfamily Leccinoideae appears to be the richest in number of gasteroid representatives within the Boletaceae (Orihara *et al.*, 2016b). These results are consistent with the surmise that the common ancestor of the suborders Boletineae and Sclerodermatineae must have been a boletoid form (Binder and Hibbett, 2006). In the Boletales only few genera are entirely secotioid, such as the monospecific *Singeromyces* M.M. Moser and *Gymnogaster* J.W. Cribb and the polyphyletic *Gastroboletus* Lohwag, whereas a number of secotioid or even understorey sequestrate species are nested in predominantly boletoid genera, namely *Afroboletus* Pegler & T.W.K. Young, *Boletus* s. str., *Fistulinella* Hennings, *Leccinellum* Bresinsky & Manfr. Binder, *Neoboletus* Gelardi, Simonini & Vizzini and *Xerocomellus* Šutara) (Trappe *et al.*, 2003; Dentinger *et al.*, 2010; Wu *et al.*, 2016a,b; Han *et al.*, 2017; Smith *et al.*, 2018; Frank *et al.*, 2020). Similarly, secotioid taxa are also occurring in the genera *Suillus* (Suillaceae) and *Chroogomphus* (Gomphidiaceae) (Baura *et al.*, 1992; Kretzer and Bruns, 1997; Miller and Aime, 2001; Miller, 2003; Nguyen *et al.*, 2017). Assuming Thier's hypothesis (Thiers, 1984) as true, in the family Paxillaceae (in relation to the presence of thromboplerous hyphae in the agaricoid genus *Paxillus* and their seemingly gradual disappearance in the sequestrate *Alpova*) and in the suborder Sclerodermatineae, gasteroid taxa would have evolved from a complex of pileate-stipitate taxa with a tubulate hymenophore consisting of *Boletinellus*, *Phlebopus* and *Gyroporus*, loosing at first their ability to forcible discharge spores in the environment in favor of a non-active spore dispersal and subsequently reducing their stipe progressively (Reijnders, 2000; Binder and Bresinsky, 2002b; Moreau *et al.*, 2013b). Such an evolutionary process thus entails a hypothetical reversal from gasteroid to non-gasteroid forms as highly unlikely (Sánchez-García *et al.*, 2020) since it would involve a return to the complex mechanism of forcible spore discharge of ballistosporic fungi, ultimately supporting the general assumption that the loss of ballistospory was irreversible (Savile, 1955; Thiers, 1984; Bruns *et al.*, 1989; Mueller and Pine, 1994; Hibbett *et al.*, 1997; Hibbett, 2004; Wilson *et al.*, 2011; Halling *et al.*, 2012b). A similar scenario may be virtually extended to all secotioid/gasteroid fungi (Lebel *et al.*, 2012), such as the polyphyletic *Gastroboletus* (Smith and Zeller, 1966), *Royoungia* Castellano, Trappe & Malajczuk and *Truncocolumella* Zeller (Castellano *et al.*, 1992) which would not at all represent the ancestral archetypes of boletoid mushrooms but rather aberrant phenotypes (or mutations) of these latter, characterized by limited environmental adaptation and reduced or absent reproductive ability, therefore exposed to disadvantages and vulnerability in natural selection and more susceptible to an elevated risk for extinction (Baura *et al.*, 1992; Wilson *et al.*, 2011). This hypothesis would also be confirmed by their rarity and restricted geographical distribution (Baura *et al.*, 1992) which could finally drive them towards an evolutionary dead-end. Miller argued that secotioid forms evolved in many genera (predominantly in the family Gomphidiaceae) as a response to adverse and stressful climatic conditions (Miller, 2003), especially moisture shortage, which might represent the principal selective factor (States and Fogel, 1999). These outcomes have been corroborated and supported by several other authors (Bruns *et al.*, 1989; Besl and Bresinsky, 1997; Hibbett, 2007; Yang, 2011; Wu *et al.*, 2014). On the other hand in the suborder Suillineae, despite it might appear much unlikely, a gasteroid form was strongly supported as the ancestral state from which both tubulate/poroid and lamellate morphologies (Suillaceae and Gomphidiacae) could have evolved in parallel, implying an improbable although possible restoration of ballistospory via reversals of gasteromycetation and therefore a return to ballistospory from statismospory (Binder and Hibbett, 2006; Hibbett, 2007; Sánchez-García *et al.*, 2020). Recently developed evolutionary models forecast that sequestrate forms will eventually come to predominate over non-sequestrate ones in most of the clades in which they have arisen regardless the purported irreversibility of the gasteroid state, especially considering that the sequestrate condition does not preclude morphological diversification rate, rather it tends to diversify faster in gasteroid taxa than in their non-gasteroid relatives (Wilson *et al.*, 2011; Yang, 2011).

The tubulate-poroid hymenophore evolved separately at least five times in the eight lineages retrieved in the class Agaricomycetes (= Homobasidiomycetes) (Hibbett and Thorn, 2001; Kirk *et al.*, 2008). The lamellate hymenophore would have in turn arisen independently at least five times in the Boletaceae (genera *Phylloporus*, *Phylloboletellus* Singer, *Erythrophylloporus* Ming Zhang & T.H. Li and *Phylloporopsis* Angelini, A. Farid, Gelardi, M.E. Smith, Costanzo & Vizzini) and Paxillaceae (*Paxillus*) (Drehmel *et al.*, 2008; Farid *et al.*, 2018; Zhang and Li, 2018) as a deriving structure from a tubulate-poroid configuration (Pegler and Young, 1981; Neves *et al.*, 2012) and not a primeval form as previously conjectured by E.J.H. Corner (Corner, 1972a) and subsequently by R. Kühner, this latter author claiming that "…les *Paxillus* sont sans doute ceux qui rappellent le plus ce que furent les types qui ont donnè naissance aux Boletaceae" (Kühner, 1977: 106). Nevertheless, the evolutionary dynamics of the hymenophore in the family Boletaceae still remain somewhat obscure (Neves *et al.*, 2012). It is proof that in the suborder Suillineae the tubulate-poroid structure would appear to derive from gills, moreover the boletinoid hymenophore (which is typical of the species formerly assigned to the genus *Boletinus* and consisting of angular and radially strongly stretched pores) would represent the intermediate evolutive step between the two extremes (Bruns and Szaro, 1992; Kretzer *et al.*, 1996).

In the family Boletaceae the convergent evolution is evident, for example, in the basidiospores ornamentation which did not take origin from a single evolutionary event but instead developed independently multiple times in several subfamilies (Binder and Hibbett, 2006; Nuhn *et al.*, 2013; Wu *et al.*, 2014). As a matter of fact, basidiospores with variously ornamented walls are

found in five subfamilies (Austroboletoideae, Boletoideae, Leccinoideae, Xerocomoideae and Zangioideae) with a predominance in the Xerocomoideae (Wu *et al.*, 2014; Sulzbacher *et al.*, 2020). The eusporial ornamentation evolved independently more than ten times in the Boletaceae from a common smooth-spored ancestor (Wu *et al.*, 2014). Specifically, in three out of the five subfamilies mentioned above smooth-walled basidiospores represented the ancestral state, the exception being the subfamily Xerocomoideae in which the ornamented spores would appear to be the ancestral state (Wu *et al.*, 2014). Such a conclusion is in contrast with Corner's theory (Corner, 1972b), who believed that ornamented spores had to be considered more primitive than smooth ones (Corner, 1972b; Pegler and Young, 1981). No information is currently available on the spore wall appearance of the common ancestor of the Zangioideae, especially in the light of the most recently described sequestrate truffle-like genus *Longistriata* Sulzbacher, Orihara, Grebenc, M.P. Martín & Baseia, which possesses longitudinally ribbed basidiospores and occupy the basal position in this subfamily (Sulzbacher *et al.*, 2020). Anyway, the results obtained by Wu *et al.* (2014) are consistent with those by Halling *et al.* (2015), who postulated that longitudinally ribbed basidiospores may alternatively represent a convergent evolution or the ancestral state of the common ancestor of *Boletellus* s.l., *Heimioporus* E. Horak and allied taxa.

Pigments and oxidation phenomena (bluing, reddening, browning and blackening) are phenotypical characters that also arose repeatedly in the Boletaceae, with the only exclusion of the subfamily Austroboletoideae where all taxa exhibit constantly unchanging sporophore tissues (Wu *et al.*, 2014). In the subfamily Zangioideae the presence of bright yellow pigments at least in some parts of the sporophores represents a unifying feature. In *Strobilomyces* the few species showing golden yellow (*S. echinatus* Beeli and *S. mirandus* Corner) or reddish brown colors (*S. brunneolepidotus* Har. Takah. & Taneyama, *S. atrosquamosus* J.Z. Ying & H. A. Wen and *S. glabellus* J.Z. Ying) are not strictly related to each other, as well as phylogenetically distant appear the species with a context directly turning blackish on exposure, consequently these morphological characters evolved several times separately in *Strobilomyces* (Han *et al.*, 2018, 2020).

As far as the TECM formation is concerned, Smith and Pfister (2009) proposed an evolutionary scenario in which, given their occurrence in the Suillineae and Boletineae and the phylogenetic distance between these two suborders, they postulated that their presence could be explained either as an ancient feature of a common ancestor of the Suillineae and Boletineae or with a convergent evolution in two separate clades of the Boletales. In any case, there have been a large number of independent losses of TECM over time in several taxa of the Boletales, remaining sporadically as a synapomorphic trait in a few genera (Smith and Pfister, 2009).

Symbiotic Partners, Mutualistic Interactions, Paleoecological Processes and Evolutionary Ecology

Ectomycorrhizal fungi are an integral part of all forest ecosystems as they mediate the interaction between plants and soil and constitute one of the most diverse and ecologically important groups of vegetation partners. It is nowadays established that more than 80% of extant plant species form symbiosis with fungi (Selosse *et al.*, 2015; Strullu-Derrien *et al.*, 2018). The number of ECM fungal species worldwide is estimated to be around 25,000, mostly belonging in the phyla Basidiomycota and Ascomycota (Rinaldi *et al.*, 2008; Tedersoo *et al.*, 2010). The acquisition of mutualistic host-symbiont associations is considered one of the key innovations promoting evolutionary diversification (Lutzoni *et al.*, 2018; Sato and Toju, 2019). As stated above, ectomycorrhizal-forming genera constitute the vast majority in the Boletales and the ECM trophic status appeared to have evolved from saprotrophism (Hibbett and Matheny, 2009; Sato and Toju, 2019; Sánchez-García *et al.*, 2020). By converse, reversal from ECM to saprotrophy is generally considered unlikely (Sánchez-García *et al.*, 2020). Recently published molecular studies suggest that ECM arose independently and asynchronously at least five times in the stem positions of the Suillineae, Sclerodermatineae s.str., Boletaceae, Paxillaceae and in the monophyletic group *Austropaxillus* Bresinsky & Jarosch/*Gymnopaxillus* E. Horak (Serpulaceae) (Hibbett and Matheny, 2009; Skrede *et al.*, 2011; Sato and Toju, 2019; Sánchez-García *et al.*, 2020). The earliest acquisition of the ECM symbiosis likely occurred in the Suillineae, followed shortly after by an evolution in the Sclerodermatineae s.str., then it more recently originated in the Boletaceae and Paxillaceae (Sato and Toju, 2019) and finally the most recent ECM evolutionary time point was observed in *Austropaxillus* (Skrede *et al.*, 2011). A single transition from a saprotrophic nutritional mode of *Serpula* to the ECM trophism of *Austropaxillus* and *Gymnopaxillus*, both being nested within *Serpula*, happened some 50–22 mya (Skrede *et al.*, 2011). As far as the Boletaceae are concerned, it is interesting to note that taxa occupying the most basal position (*Pseudoboletus*, *Buchwaldoboletus* and *Chalciporus*/*Rubinoboletus*) all have either an apparently saprotrophic or mycoparasitic trophic status, indicating that the ancestral state in the family might have been non-ectomycorrhizal (Pers. Obs.).

However, the evolution of ECM nutritional mode does not necessarily promote rapid diversification rates, particularly in the early-diverging ECM fungal lineages, as in the case of the Boletales (Sato and Toju, 2019) as well as in other groups of agaricoid fungi (Sánchez-García and Matheny, 2017; Sánchez-García *et al.*, 2020). Accordingly, the highest diversification rate in the Boletales would not be correlated to the most primeval ECM lineages, rather it appears to have occurred at a local phylogenetic scale and linked to the acquisition of mutualistic symbiosis in the more recent clade of the Boletaceae, probably contingent on the key innovation dominant pileate-stipitate morphology in this family or in combination with other biotic and abiotic factors (Sato and Toju, 2019; Sánchez-García *et al.*, 2020).

Despite the fact that some authors (Ducousso *et al.*, 2004; Alexander, 2006) speculated on a Gondwanian origin of ECM, the most ancient group of extant ECM host plants were probably the Pinaceae (Hibbett and Matheny, 2009; Tedersoo *et al.*, 2010), which were naturally distributed in Laurasia. It is not a coincidence that the genesis of Pinaceae is dated back to the Mesozoic Era, between the Lower Jurassic and Lower Cretaceous (180–130 mya), roughly the same period or slightly earlier than the origin of the

order Boletales (Malloch *et al.*, 1980; LePage, 2003; Hibbett and Matheny, 2009; Dentinger *et al.*, 2010; Lin *et al.*, 2010; García-Sandoval *et al.*, 2011; Lutzoni *et al.*, 2018), although the most primitive mycorrhizal association (endomycorrhizal) between vascular plant and fungal organisms was traced back by Honrubia (2009) to 400 mya. Based on fossil records, the ancestor of *Pinus* subgenus *Pinus* originated in the Lower Cretaceous (130–125 mya) whereas the diversification of the subgenus *Strobus* was dated to a later period, in the Paleocene, around 60 mya (Axelrod, 1986; Wu *et al.*, 2000). The most important ECM angiosperm families (Fagaceae, Betulaceae, Salicaceae, Dipterocarpaceae, Casuarinaceae, Myrtaceae, Fabaceae) originated later than Pinaceae, during the Cretaceous Period (Malloch *et al.*, 1980; Ding, 1995; Manos and Stanford, 2001; Wikström *et al.*, 2001; Thornhill *et al.*, 2015; Lutzoni *et al.*, 2018). More recent studies, however, would be inclined to date the origin of gymnosperms and angiosperms to a much more ancient time, between Carboniferous and Permian (around 300 mya) and between Late Permian (Lopingian Epoch) and Jurassic (260–180 mya), respectively (Krah *et al.*, 2018). As pointed out by Sato and Toju (2019), fossil findings indicate that Pinaceae were sporadically distributed in cool montane environments in the northern hemisphere during Jurassic and Cretaceous, conversely the major genera of Fagaceae became widespread and abundant in warm temperate and subtropical areas of the northern hemisphere from Upper Cretaceous to Late Eocene (LePage, 2003; Taggart and Cross, 2009; Tedersoo and Brundrett, 2017). Divergences in terms of distribution and habitat between Pinaceae and Fagaceae would reflect differences in fungal diversification rate and may have enabled host switches from Pinaceae to Fagaceae in the early ECM lineages. Moreover, this scenario would explain the limited diversification rate in the Suillineae and Sclerodermatineae due to a shortage of partners in the Pinaceae during Jurassic and Cretaceous, whereas the acquisition of ECM symbiosis in co-evolution with angiosperms (mainly Fagaceae) between the Upper Cretaceous and Late Eocene may have triggered a consistent and rapid, explosive evolutionary diversification of the mycobionts with a resulting acceleration of speciation in the Boletaceae (Sato and Toju, 2019; Varga *et al.*, 2019; Sánchez-García *et al.*, 2020).

Bolete genera leaning to have a more specialized trophism tend to be restricted to circumboreal geographical distribution (Pegler and Young, 1981). On a more regional scale, in eastern Mediterranean biome it seems to exist a degree of specialization between vegetational symbiotic partners and boletes, most of which appear to be associated with evergreen oaks of the ilicoid group (*Quercus alnifolia* Poech, *Q. coccifera* L.) rather than deciduous oaks (Binyamini and Avizohar-Hershenzon, 1973; Loizides *et al.*, 2019).

Some of the most specialized genera are found in the Suillineae. The genus *Suillus* is associated with at least two of the four subfamilies of the Pinaceae: Pinoideae (*Pinus*), Laricoideae (*Larix*, *Pseudotsuga*) and perhaps Piceoideae (*Picea*, *Cathaya*) but not Abietoideae (*Abies*, *Cedrus*, *Keteleeria*, *Tsuga*) (Nguyen *et al.*, 2017), exceptionally it is found with Betulaceae (*Betula*), Salicaceae (*Populus*) and Fagaceae (*Quercus rubra* L.), with respect to the North american species *S. subaureus* (Peck) Snell (Smith and Thiers, 1964, 1971; Singer, 1981, 1986; Bessette *et al.*, 2000, 2016; Wu *et al.*, 2000; Nguyen *et al.*, 2017). Ecologically, the host-specificity association of *Suillus* with *Larix* appears to be primeval when compared to that with *Pinus*, *Pseudotsuga* and with broadleaved trees, these latters being thus considered as derivative (Kretzer *et al.*, 1996; Wu *et al.*, 2000). Likewise, the family Gomphidiaceae co-evolved with the Pinaceae (Miller, 2003; Watling, 2004). Mujic *et al.* (2019) investigated on the evolutionary dynamics correlated to the symbiosis *Pseudotsuga-Rhizopogon*, noticing that this relationship evolved just once in *Rhizopogon*. In general, from an ecological viewpoint suilloid fungi play a crucial role as primary drivers in processes of co-invasion with Pinaceae of new geographic areas outside their natural distribution range (Vellinga *et al.*, 2009; Policelli *et al.*, 2019).

The suborder Sclerodermatinae originated in mesophitic forests in ancestral association with angiosperms (rosids) and Pinaceae and only later the symbiotic linkage extended to ECM partners belonging in the Dipterocarpaceae, Ericaceae, Myrtaceae, Nothofagaceae, Sapindaceae and Fabaceae (Wilson *et al.*, 2012). It is interesting to note that the fossil genus *Palaeogaster* recently described from Birmania and preserved in amber from an auraucarian tree source (possibly *Agathis*), dated back to the Lower-Mid Cretaceous, Upper Albian (110–97 mya), has been assigned to the Sclerodermatineae (Poinar *et al.*, 2014). It is the only fossil genus ascribable to this suborder and to the Boletales as a whole and the most ancient gasteroid genus known to date (Poinar *et al.*, 2014).

In the Boletineae, the most recent common ancestor of the sister genera *Afroboletus* and *Strobilomyces* was originally associated with members of the Cesalpinoideae, Monotoideae, Detarioideae, Phyllantaceae as the earliest symbiotic partners in Africa. Subsequently *Strobilomyces* made a host shift to the Dipterocarpaceae in tropical Asia, then switched to the Fagaceae and Pinaceae in subtropical and temperate environments in Laurasia and even more recently to the Nothofagaceae and *Eucalyptus* in the southern hemisphere (Han *et al.*, 2018). Additionally, in four lineages associated with Fagaceae/Pinaceae it has been inferred an increase of the net diversification rate respectively of four and two times higher than that of the lineages associated with the remaining symbiotic partners, testifying that host shifts provide ECM fungi ecological opportunities to enhance their distribution range, resulting in an acceleration of diversification (Sato *et al.*, 2017; Han *et al.*, 2018). In *Leccinum* the plant host *Betula* (and probably also *Populus*) represents the common ancestral symbiotic partner from which all other ECM associations derived over time (den Bakker *et al.*, 2004). It also appears that the high number of current symbiotic partners of *L. quercinum* Pilát may derive from an older monospecific association with *Populus* spp. (den Bakker *et al.*, 2004). In addition, all European members of *Leccinum* forming obligate ECM association with *Betula* spp. (*Leccinum* sect. *Scabra* A.H. Smith, Thiers & Watling) are indifferently found in temperate, boreal, subalpine and arctic habitats with the only exclusion of L. *rotundifoliae* (Singer) A.H. Smith, Thiers & Watling which is restricted to cold climates and never occurs in temperate areas (den Bakker *et al.*, 2007).

Based on the available knowledge it does not seem to be a clear evidence for a possible reversal towards a non-ECM nutritional status in the evolutionary trends of ECM lineages, although such an eventuality could not be excluded with certainty in the order Boletales (Tedersoo *et al.*, 2010). Mycoparasitism in the Boletales likely derived from an ECM nutritional mode (Binder and Hibbett, 2006; Sánchez-García *et al.*, 2020) and would have evolved at least twice independently (McLaughlin and Spatafora, 2014), in the Suillineae (Gomphidiaceae) and Boletaceae (*Pseudoboletus*) (Sánchez-García *et al.*, 2020).

Historical Biogeography, Paleo-biogeographical Dynamics and Distribution Patterns

Fungi are found in all terrestrial and marine ecosystems and represent a key component for interactions among living organisms in natural environments. Taking into consideration the kingdom Fungi in its entirety, fungal biodiversity progressively increases towards pantropical areas and the equatorial belt where average annual precipitations are more conspicuous and the degree of endemism of fungal species appears particularly pronounced with numerous taxa being confined to restricted ecological niches (Tedersoo *et al.*, 2014). However, ECM fungi appear to be more diversified in temperate and boreal ecosystems (albeit in tropical habitats the abundance of ECM species is much higher than it was generally assumed in the past, see Corrales *et al.*, 2018) and their geographic range expands towards the poles (Tedersoo *et al.*, 2014).

Fungal species belonging in the order Boletales are cosmopolitan, they occur from the equatorial belt to the arctic zones, from arid or semiarid environments to wetlands and from the sea level to the timberline or even above, up to 4000–5000 m alt. in certain ecological-climatic ecosystems (Zang, 1986, 2006), have a worldwide distribution and are found in most terrestrial biomes and in all continents with the exception of Antarctica (but they were probably also present in the latter continent during the Cretaceous in co-habitation with trees of the genus *Nothofagus*, which is not accidentally considered a sort of "living fossil"). However they are significantly more abundant in temperate habitats and most of all in the (sub)pantropical belt of the northern hemisphere with biodiversity hotspots located in North America (especially north-eastern North America), in neotropical montane cloud forests of Central America and the Caribbean and even more in eastern and south-eastern Asia, with peninsular and insular Malaysia (Borneo) being renowned as one of the world's most important biodiversity hotspots for boletes, since this region laids at the intersection of several migration routes (Singer, 1965; Smith and Thiers, 1971; Corner, 1972b, 1993; Horak, 1983; Binder and Bresinsky, 2002b; Wu *et al.*, 2014, 2016a). By converse, members of the Boletales appear to be far less represented in the southern hemisphere, for instance in southern South America (Watling, 2001b; Palfner, 2005; Nouhra *et al.*, 2012; Truong *et al.*, 2017; Horak, 1983, pers. comm.), South Africa (Coetzee, 2011) and New Zealand (Horak, 1983) albeit decidedly more numerous in Australia (Watling, 2001b; May *et al.*, 2006).

As a demonstrative example of the biodiversity of the order Boletales in the northern hemisphere, around 500 species belonging in more than 55 genera have been reported from the sole China (Li and Song, 2000; Yang, 2005; Li and Yang, 2011; Wu *et al.*, 2016a; Pers. Obs.) with an obvious preponderance in Yunnan Province (south-western China), where the easternmost peaks of the Himalayan range and the Hengduan Mountains, owing to lighter impact of Quaternary glaciations, favorable climatic conditions of monsoon type and particularly fertile soil, presently represent one of the 35 hotspots of biodiversity worldwide, encompassing more than 17,000 vascular plants and an estimated number of fungi exceeding 100,000 species (Feng and Yang, 2018).

In sharp contrast, in most Europe and neighboring countries the biodiversity of vegetation and associated mycota is relatively low as a result of local extinctions caused by geological and climatic changes during past epochs, such as the harsh and destructive Pleistocene glaciations (Dennis, 1986; Molina and Trappe, 1994; Lodge *et al.*, 2004; Yang, 2005; Yu *et al.*, 2020) which caused the disappearance of several living organisms. Only a limited proportion of plants and fungal species survived after the regression of glaciers and it is therefore not a casuality that fungal biodiversity in Europe appears to be the poorest when compared to the greater wealth of other continents of the northern hemisphere, notwithstanding the much longer tradition of European mycological studies. Such a scenario is further corroborated by the current geographical distribution of the hypogeous genus *Alpova*, which is apparently common in Corsica, south-eastern Europe and throughout the eastern Mediterranean basin and even south into the relictual alder forests of North Africa, while it seems to be virtually absent in continental and western Europe, despite the abundant presence of its obligate symbiotic partner *Alnus* in the same area (Moreau *et al.*, 2013b). Pleistocene glaciations might explain this uneven distribution pattern with difficulties for these hypogeous fungi to follow their host trees (*A. glutinosa* (L.) Gaertn. and *A. incana* (L.) Moench) in the process of re-colonization of northern and western Europe from refuges in south-eastern Europe where they remained confined due to adverse climatic conditions (Moreau *et al.*, 2013b). Of course, further investigations in riparian areas of Carpathians, Caucasus and the Middle East might yield additional neglected *Alpova* species in the years to come (Moreau *et al.*, 2013b) and could help better understanding their biogeographic history.

In contrast to ECM species belonging in the Tricholomataceae and Cortinariaceae (especially *Cortinarius* (Pers.) Gray and *Inocybe* (Fr.) Fr.), both of which being extremely rare across the tropical belts (Buyck, 2001; Watling *in litt.*) and particularly in Africa (Bougher and Lebel, 2001), numerous Discomycetes and several genera in the orders Agaricales (especially *Amanita* and saprothrophic Tricholomataceae), Russulales and Boletales are best represented in this kind of environment, where they form ECM symbiosis mainly with members of the Fagaceae and Dipterocarpaceae in south-eastern Asia, and with Fabaceae subfamily Caesalpinoideae in subsaharan Africa and northern South America (Corner, 1972b; Singer *et al.*, 1983; Alexander and Högberg, 1986; Alexander, 1989, 2006; Newbery *et al.*, 1997; Henkel *et al.*, 2002; Lodge *et al.*, 2004; Watling and Lee, 2007). The occurrence of a multitude of boletoid genera constrained to the tropical belt may be explained with an inefficient dispersal ability or due to their failure in enduring low temperatures, resulting in a limitation or impossibility of several lineages to migrate towards temperate and subarctic ecosystems of both hemispheres (Tedersoo and Smith, 2013).

Being members of the Boletales mostly ECM and considering much unlikely, although possible, their colonization of remote geographical areas via overland long-distance dispersal, their migration might be explained with a reasonable host switch during both ancient geological times and more recent historical periods (Watling, 1981, 1988; Wolfe and Bougher, 1993; Kretzer *et al.*, 1996; Fischer *et al.*, 1997; Halling *et al.*, 2007b; Wilson *et al.*, 2012). Host shifts can no doubts be considered a successful evolutionary strategy that mediated migration routes of ECM species and their symbiotic partners between Asia and North America

across the Bering Strait (Beringian corridor). This migration is easily deductible from the disjunct geographic distribution in eastern Asia and north-eastern North America of certain species complexes, species pairs or species populations like those of *Suillus decipiens* (Peck) Kuntze/*S. spraguei* (Berk. & M.A. Curtis) Kuntze s.l., *S. americanus* (Peck) Snell/*S. sibiricus* (Singer) Singer, *S. clintonianus* (Peck) Kuntze, *Aureoboletus mirabilis* (Murrill) Halling, *Harrya chromapes* (Frost) Halling and *Sutorius eximius* (Peck) Halling (Wu *et al.*, 2000; Mueller *et al.*, 2001; Wu *et al.*, 2016a; Nguyen *et al.*, 2017; Zhang *et al.*, 2019) or otherwise from the presence in these two continents of phylogenetically closely related taxa in shared genera of the Boletaceae, such as *Bothia* Halling, T.J. Baroni & Manfr. Binder, *Retiboletus* Manfr. Binder & Bresinsky, *Pulveroboletus* and *Veloporphyrellus* L.D. Gómez & Singer (although in neither case disjunct populations belonging in the same species have been identified) (Li *et al.*, 2014a; Zeng *et al.*, 2015, 2016, 2017). Furthermore, a bi-directional exchange of plant-fungal communities between the American and Asian continents via Beringian connection would appear to be ascertained in the case of *Rhizopogon*. Dealing with the symbiosis *Pseudotsuga-Rhizopogon*, Mujic *et al.* (2019) placed the origin of this mutual relationship along coastal regions of the Pacific Northwest of the USA and Canada with a single event of co-migration to Asia and two independent migrations eastwards to the mountainous areas of western North America (Mujic *et al.*, 2019). This latter region constitutes at present a major center of diversity of *Rhizopogon* species, where the high speciation and diversification rate of *Rhizopogon* was probably driven by the specificity of host association with conifers (Molina and Trappe, 1994). Geographycally meaningful is also the disjunct geographic distribution in the paleotropics (eastern and south-eastern Asia) and neotropics (Central and northern South America) of the genera *Erythrophylloporus* and *Rugiboletus* G. Wu & Zhu L. Yang (Vadthanarat *et al.*, 2019b; Kuo and Ortiz-Santana, 2020).

In the western hemisphere Nearctic symbiotic macromycetes (including boletes) along with their ECM plant partners moved from north-eastern North America south to Central and subsequently northern South America (as demonstrated, for example, by the strong morpho-genetic affinities between the temperate species *Tylopilus balloui* (Peck) Singer and the similar neotropical *T. leucomycelinus* (Singer & M.H. Ivory) R. Flores & Simonini and *T. oradivensis* Osmundson & Halling) across the mandatory route of the Mesoamerican isthmus (Panamanian land bridge) which connected postglacial Laurasian remnants with Gondwanan remnants between the late Miocene and the Pliocene (Halling, 1989, 1997, 2001; Wolfe and Bougher, 1993; Halling and Mueller, 2002; Halling *et al.*, 2008; Flores Arzù, 2020). Similarly to ECM boletes, some saprothrophic genera, such as the lignicolous *Gyrodontium* Patouillard, may have followed a similar migration pattern (Robledo *et al.*, 2014).

Although L.D. Gómez observed that "Es muy interesante anotar la escasa correspondencia entre las boletáceas neotropicales y las del continente africano y subcontinente indio…" (Gómez, 1997), speculations have been proposed on possible mycofloristic relationships between Latin America and central Africa, as in the case of the genus *Fistulinella* (Guzmán, 1974), yet these theories are presently premature in their essential defining lines and still awaiting for molecular confirmation, at least as far as the Boletales are concerned.

Another example of ancient geographic migrations is correlated to the ancestral population of the "Chromapes group" (including genera in the subfamily Zangioideae such as *Australopilus* Halling & Fechner, *Harrya* Halling, Nuhn & Osmundson, *Royoungia* Castellano, Trappe & Malajczuk, etc.) which likely originated in Laurasia and subsequently migrated from eastern Asia south to Australia during the glaciations of Miocene-Pliocene (which provoked the lowering of sea level and the resulting formation of intercontinental stepping-stones fragments or land bridges), initially moving into the Indo-Malayan region then across the junction known as Wallace's Line, passing through Papua New Guinea and finally reaching the northern offshoots of Australia across the Torres Strait (Wolfe and Bougher, 1993; Halling, 2001; Halling and Mueller, 2002; Yang, 2005; Halling *et al.*, 2008; Feng and Yang, 2018). As pointed out by Wang *et al.* (2018) in relation to the ancestral area reconstruction of the lactarioid genus *Multifurca* Buyck & V. Hofst. (Russulaceae), the migration across Australasia might have also happened by island hopping. Although a bidirectional interchange between Asian and Australian continents via south-eastern Asia cannot be excluded (Halling *et al.*, 2008; Feng and Yang, 2018), as also stressed by Bougher and Lebel (2001) with respect to gasteroid and secotioid fungi associated with *Eucalyptus* and by Trappe *et al.* (2013) for the hypogeous false-truffle *Solioccasus* Trappe, Osmundson, Manfr. Binder, Castellano & Halling, a unidirectional migration from the northern hemisphere to the southern hemisphere can be virtually extended to all pileate-stipitate Australian boletes such as *Boletellus*, *Pulveroboletus* and *Strobilomyces* (Watling, 2001a-c) as well as to sequestrate hypogeous genera like *Rossbeevera* T. Lebel & Orihara owing to animal mycofagy (Orihara *et al.*, 2016b).

An alternative explanation for the current intercontinental distribution pattern might rely on a relictual presence based on a broad original occurrence of boletes in the Pangea and therefore prior to the continental drift which started between 180 mya (Varga *et al.*, 2019) and 100 mya (Heckman *et al.*, 2001; Halling *et al.*, 2008, 2015). This latter theory is strictly correlated with the ecological vicariance (Tedersoo and Smith, 2013) and had already been proposed by Trappe (1977) for hypogeous fungi. According to this scenario fungal species imprisoned into isolation on plates drifting were then likely affected by genetic drift, mutation, speciation and extinction processes that have later occurred at a regional scale in each continent independently due to locally different climatic and ecological phenomenons (Horak, 1983; Wang and Qiu, 2006; Halling *et al.*, 2008), determining the noteworthy biodiversity we can currently observe (Halling *et al.*, 2012a,b, 2015).

On the other hand, in the case of *Austropaxillus* the hypothesis of transoceanic long-distance dispersal (Li *et al.*, 2009) with later vicariance events is the only plausible theory to explain the current disjunct transantarctic distribution pattern of this genus in Australasia (Papua New Guinea, Australia, Tasmania, New Caledonia, New Zeland) and South America (Chile, Argentina) (McNabb, 1969; Bresinsky *et al.*, 1999) in association with *Nothofagus* (subgenera *Nothofagus* and *Lophozonia*), since the origin of *Austropaxillus* is dated to some 23 mya, therefore posterior to the Gondwana break-up (90–80 mya) (Vergara, 2014). Moreover, it seems likely that the absence of ECM association of *Austropaxillus* with *Nothofagus* subgenera *Trisyngyne* and *Fuscospora* could be elucidated with the extinction of fungal species due to changed climatic and edaphic conditions over geological times, which

Plate 1 **a** *Boletus aereus* Bull. (MG830); **b**: *Cyanoboletus pulverulentus* (Opat.) Gelardi, Vizzini & simonini (MG708); **c**: *Rubroboletus rhodoxanthus* (Kromb.) Kuan Zhao & Zhu L. Yang (MG496); **d**: *Tylopilus griseipurpureus* (Corner) E. Horak (MG521); **e**: *Rubinoboletus rubinus* (W.G. Smith) Pilát & Dermek (MG805); **f**: *Pseudoboletus parasiticus* (Bull.) Šutara on *Scleroderma citrinum* Pers. (MG806). Photographs by M. Gelardi.

eventually resulted inadequate for their survival and reproduction (Vergara, 2014). The biogeographic connection between South American and Australasian biotopes was already postulated by Horak (1983) with respect to the co-evolution of *Austropaxillus* species with their *Nothofagus* host trees and has recently acquired further support by the close phylogenetic relationships of several fungal lineages, both in phyla Basidiomycota and Ascomycota (Truong *et al.*, 2017).

However, a significant discrepancy of fungal biodiversity appears to be obvious based on present distribution and abundance of the Boletales in each continent, for instance when comparing European with eastern Asian mycobiota. Large-scale geotectonic upsets that occurred in Paleocene and Eocene as a result of the collision of the Indian tectonic plate with the Asian continent and the ensuing formation of the Himalayan range approximately 55 mya, led to the uplift of the Qinghai-Xizang (Tibet) Plateau during the late Pliocene and Pleistocene and to the current topography of Hengduan Mountains, determining a gradual drying up with an increasing aridity of the huge surface area of western and north-western China, which became a natural barrier that virtually interrupted migration routes of fungal species between eastern Asia and Europe (Yang, 2005). Such a transformed orogenesis accompanied by dramatic landscape and climate changes determined a progressive geographic isolation which may have promoted ideal conditions for an independent evolution of species that were previously evenly distributed throughout the Eurasian continent, leading to an allopatric speciation of different populations of the same species and shaping a remarkable fungal diversification in southern and south-western China (Yang, 2005; Feng and Yang, 2018), as investigated by a number of taxonomic studies focused on *Boletus* s.str., *Zangia* Yan C. Li & Zhu L. Yang, *Phylloporus*, *Retiboletus*, *Pulveroboletus*, *Heimioporus*, *Aureoboletus*, *Strobilomyces* and many other boletoid genera (Li *et al.*, 2011b; Feng *et al.*, 2012, 2016a; Zeng *et al.*, 2013, 2016, 2017, 2018; Wu *et al.*, 2014, 2016a; Cui *et al.*, 2015; Zhang *et al.*, 2019; Han *et al.*, 2020; etc.). However, moving northward fungal differentiation tends to reduce gradually due to the presence of a natural bridge connecting Europe and Asia, which is represented by the immense Russian-Siberian forests that facilitate exchanges among different populations and therefore contribute to a lower genetic divergence (Petersen and Hughes, 2007; Pers. Obs.). Despite the Qinghai-Tibet Plateau represents a physical barrier hampering the migration of species from eastern to

Plate 2 **g** *Paxillus involutus* f. *eburneus* Gelardi, Segneri & Vizzini (MG670); **h**: *Gyroporus castaneus* (Bull.) Quél. (MG494); **i**: *Pisolithus* sp. (MG799); **j**: *Astraeus hygrometricus* (Pers.) Morgan (MG804); **k**: *Suillus bellinii* (Inzenga) Kuntze (MG794); **l**: *Chroogomphus mediterraneus* (Finschow) Vila, P é rez-de-Greg. & G. Mir (MG786). Photographs by M. Gelardi.

western areas or vice versa, the Himalayan orographic system may act by converse as a corridor for dispersal of fungal biota occurring in subalpine forests and alpine meadows like *Boletus reticuloceps* (M. Zang, M.S. Yuan & M.Q. Gong) Q.B. Wang & Y.J. Yao and *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, which both have an omogeneous distribution throughout the mountain range (Thapa *et al.*, 2014; Feng and Yang, 2018).

Based on available data species in the Boletales appear to be mostly restricted to single continents or specific geographic regions, but with some notable exceptions. The combination of a broader geographic distribution and lower fungal biodiversity in cold-temperate and boreal ecosystems have been phylogenetically highlighted for the genera *Leccinum*, *Chroogomphus* and *Suillus* (den Bakker *et al.*, 2007; Li *et al.*, 2009; Shi *et al.*, 2013), for example with respect to the Eurasian *Chroogomphus rutilus* (Schaeff.) O. K. Miller, *C. purpurascens* (Lj.N. Vassiljeva) M.M. Nazarova, *Suillus asiaticus* (Singer) Kretzer & T.D. Bruns, *S. cavipes* (Klotzsch) A.H. Smith & Thiers (Li *et al.*, 2009; Nguyen *et al.*, 2017; Scambler *et al.*, 2018) and also to *Paxillus obscurisporus* C. Hahn and *Chamonixia caespitosa* Rolland in the Paxillaceae and Boletaceae, respectively (Vellinga, *in litt.*; Orihara *et al.*, 2016a), as well as for some species in the Sclerodermataceae, Suillaceae and Boletaceae with a molecularly confirmed intercontinental holarctic distribution such as *Suillus americanus* (Peck) Snell, *S. granultus* (L.) Roussel, *S. luteus* (L.) Roussel (this latter being the most easily encountered *Suillus* species in the world!) (Nguyen *et al.*, 2017), *Boletus edulis* Bull. (Dentinger *et al.*, 2010; Feng *et al.*, 2012; Cui *et al.*, 2015), *Imleria badia* (Fr.) Vizzini, *Leccinum scabrum* (Bull.) Gray, L. *rotundifoliae* (Singer) A.H. Smith, Thiers & Watling, *Porphyrellus porphyrosporus* Fr. & Hök) E.-J. Gilbert, *Strobilomyces strobilaceus* (Scop.) Berk., *Buchwaldoboletus lignicola* (Kallemb.) Pilát, *Tylopilus felleus* (Bull.) P. Karsten and *Scleroderma venenatum* Y.Z. Zhang, C.Y. Sun & Hai J. Li (den Bakker *et al.*, 2007; Zhu *et al.*, 2014; Wu *et al.*, 2016a; Han *et al.*, 2017, 2018, 2020; Orihara and Smith, 2017; Jo *et al.*, 2019; Farid *et al.*, 2020; Zhang *et al.*, 2020). Apparently more uncommon seem to be cases of disjunct distribution in Europe and North America, exemplified by the genera *Alessioporus* and *Pulchroboletus* Gelardi, Vizzini & Simonini and by the species *Leccinum holopus* (Rostk.) Watling, *L. rufum* (Schaeff.) Kreisel, *L. variicolor* Watling, *L. versipelle* (Fries & Hök) Snell, *L. vulpinum* Watling, *Astraeus pteridis* (Shear) Zeller and *Gomphidius glutinosus* (Schaeff.) Fr. (den Bakker and Noordeloos, 2005; den Bakker *et al.*, 2007; Phosri *et al.*, 2013; Gelardi *et al.*, 2014; Farid *et al.*, 2017;

Plate 3 **m** *Gomphidius glutinosus* (Schaeff.) Fr. (MG613); **n**: *Rhizopogon roseolus* (Corda) Th. Fr. (MG782); **o**: *Coniophora puteana* (Schumacher) P. Karst. (MG404); **p**: *Tapinella atrotomentosa* (Batsch) Šutara (MG722); **q**: *Serpula lacrimans* (Wulfen) J. Schrot. (MG450); **r**: *Leucogyrophana mollusca* (Fr.) Pouzar (MG394). Photographs by M. Gelardi.

Frank *et al.*, 2017; Crous *et al.*, 2019; Morgado and Geml, 2020; Rossi *et al.*, 2020). It is not to be excluded, however, that future studies could reveal a circumboreal distribution by unveiling the presence of these taxa in the Asian continent, too.

A south-eastern Asian origin and subsequent diversification of several taxa in the Boletales and notably of a number of genera in the Sclerodermatineae (*Calostoma* Desvaux, *Astraeus*, *Gyroporus*, *Pisolithus*, *Scleroderma*) and Boletaceae (*Boletus* s.str.) has been suggested in recently published molecularly-based studies (Dentinger *et al.*, 2010; Feng *et al.*, 2012; Wilson *et al.*, 2012; Phosri *et al.*, 2014). The main hotspot for porcini mushrooms (*Boletus* s. str.) diversity is unquestionably south-eastern Asia and such a richness is consistent with the paleotropic origin of this genus (Dentinger *et al.*, 2010), which might have likely arisen in Australasia or the Indo-Malayan area (although a Gondwanian origin cannot be excluded) with at least two subsequent migration events towards Europe and at least four events of radiation to North and Central America across the Bering land bridge followed by vicariance/speciation at regional level between the Middle Miocene and Early Pliocene (Feng *et al.*, 2012).

An additional hypothesis namely "boreotropical migration" has been proposed by J.A. Wolfe to explain the paleotropical disjunction of living organisms in Africa and Eastern Asia (Wolfe, 1975; Morley, 2000; Zachos *et al.*, 2001) and has recently been applied to the genus *Strobilomyces* (Han *et al.*, 2018). This theory is based on the assumption of a migration of biota through the megathermal forests which extensively covered the northern hemisphere during Paleocene and Eocene, reaching their maximum range in the Eocene before to gradually contract due to global cooling between Middle Eocene and Early Oligocene and finally reducing to tropical and equatorial African and south-eastern Asian regions, thus determining the fragmentation of boreotropics and the disjunct intercontinental distribution of plants and fungi (Morley, 2000, 2003). Relying on such a scenario the African ancestral origin of *Strobilomyces* has been inferred on account of molecularly-based studies comprising a worldwide species sampling and estimated to have occurred in Early Eocene, around 50 mya (range comprised between 65 and 35 mya) (Han *et al.*, 2018). An onset migration event from Africa to south-eastern Asia took place between 50 and 43 mya across the Strait of Gibraltar and then from Europe eastward along the northern margin of the Tethys Sea (the southernmost and most tropical region of Laurasia during the Eocene), leading to the origin and separation of the *Strobilomyces confusus* Singer clade (encompassing species

characterized by echinulate-tuberculate to cristate basidiospores) and the *S. strobilaceus* (Scop.) Berk. clade (comprising species with entirely reticulate basidiospores). Subsequently, two main dispersal events happened starting from south-eastern Asia; one headed south to Australasia between the Oligocene and Miocene (36–11 mya) through the passageway generated by the collision of Sundaland and Australia at the beginning of Miocene and the other northward to East Asia during the Miocene (23–6 mya) via the collision of northern Borneo with the continental margin of southern China, followed by an additional dispersal route into Japan (which was connected to mainland Asia until the Last Glacial Maximum before being separated by the East China Sea basin). Later on, at least four independent dispersal events from East Asia to North America across the Bering Strait (whose land bridge repeatedly appeared and disappeared in the course of different geological periods owing to drastic climatic fluctuations), then to Central America (Late Miocene, 8–3 mya), and an additional most recent migration from East Asia to Europe (Late Miocene-Pliocene, 7–1.5 mya) occurred (Han *et al.*, 2018; Feng and Yang, 2018). The increasing of *Strobilomyces* species diversification rate in East Asia in the Miocene has been likely the result of a massive expansion of host plants in the northern hemisphere after a long-term retention in south-eastern Asia caused by low temperature (Han *et al.*, 2018, 2020). Most of the phylogenetic species in *Strobilomyces* appear to have a restricted geographical distribution, showing a high degree of endemism (Han *et al.*, 2018). The hypothesis of boreotropical migration and Tethys region as a corridor for dispersal and species exchanges has also been invoked to address the discontinuous distribution of floristic components in Europe and south-western China (Sun, 2001) and to justify the distribution range of the genus *Hydnum* L. which, unlikely boletes, encompasses several species shared by Europe and China (Feng *et al.*, 2016b; Feng and Yang, 2018).

In a couple of recent monographic works devoted to morphological and phylogenetic revision of the genus *Gyroporus* on a global scale, Davoodian *et al.* (2018, 2020) highlighted the existence of numerous sister relationships between East Asian and North American clades as well as phylogenetic connections between African and Australian lineages. Furthermore, they inferred a Gondwanan/southern hemisphere origin of the "castaneus clade" (comprising species related to *G. castaneus* (Bull.) Quél. s.l.) with subsequent radiations in the northern hemisphere and a Laurasian/northern hemisphere origin of the sister lineages "cyanescens clade" (comprising species related to *G. cyanescens* (Bull.) Quél. s.l.) and *G. longicystidiatus* Nagasawa & Hongo s.l. clade with a derived clade in the southern hemisphere (Davoodian *et al.*, 2018, 2020).

Another challenging topic in the biogeography of boletes that is unfortunately far from being assessed due to the lack of targeted compelling studies is the predictive distribution mapping of European and East Asian bolete species with respect to their eastern and western limits, respectively. There are some questions arising in this context; first of all, what is the easternmost natural geographic limit of apparently strictly European boletes and, likewise, what is the westernmost limit of eastern Asian boletes that are currently thought to be confined to East Asia? Secondly, there exists an intermediate geographic region anywhere in central Asia where the distribution range of certain European and eastern Asian boletes overlap? Thirdly, do the ecological requirements and abundance of these species vary when they are found in remote ecosystems far away from their fundamental center of distribution? And finally, may latitude or altitudinal gradient as well as climatic factors such as precipitations, soil moisture levels and temperature influence their overall geographic distribution extent? Whatever the case, these queries like many others related to fungal distribution patterns presently remain unaddressed and a substantial amount of work is needed to solve uncertainty surrounding specific ecological preferences and to clarify unexplained evolutionary biogeographic issues. **Plates 1–3**.

Notes on the Edibility, Toxicity and Poisonings, Therapeutic Properties, Ethnomycological Traditions, Cultivation, Consumption and Marketing

Most representatives of the order Boletales are notoriously edible, highly nutritive and constitute a relevant ethnomycological non-timber forest resource, especially for many poor communities in developing countries. On the other hand, boletes collecting represents an amusing recreational activity for foragers in developed countries. Their consumption as food by human beings dates back to the Stone Age, more precisely to the Upper Paleolithic (Magdalenian period), based on the identification via radiocarbon techniques of the spores of boletoid and agaricoid species from a tooth plaque of a 18,700 years old woman found at El Miron Cave, Cantabria, northern Spain (Power *et al.*, 2015).

It is undeniable that, among edible boletes, members of *Boletus* s. str. (commonly called "king boletes", "ceps" or "porcini mushrooms") can nowadays probably be considered the most iconic wild edible mushrooms worldwide and their demand is steadily growing. Porcini mushrooms are sought after, popular and largely appreciated forest-occurring fungi because of their agreeable organoleptic qualities, prized flavor and delicious taste and are commonly used as an exquisite ingredient for a large variety of processed foods. Due to the high gastronomic interest, they are extensively harvested for self-consumption and for sale purposes and currently traded at high prices on both local and global scale, generating alternative relevant economic incomes for rural communities (Hall *et al.*, 1998, 2003; Yun and Hall, 2004; Kirk *et al.*, 2008; Sitta and Floriani, 2008; Feng *et al.*, 2012; Mello, 2012; Sitta and Davoli, 2012; Peintner *et al.*, 2013; Dentinger and Suz, 2014; Wu *et al.*, 2019; Gelardi, 2020; Li *et al.*, 2021). Several other species in the Boletales are innocuous after prolonged and complete cooking, including a number of red-pored boletes such as *Suillellus luridus* (Schaeffer) Murrill, *S. queletii* (Schulzer) Vizzini, Simonini & Gelardi, *Neoboletus luridiformis* (Rostkovius) Gelardi, Simonini & Vizzini (= *Boletus erythropus* Persoon s. auct. non s. orig.) (Sitta *et al.*, 2020), *N. magnificus* (W.F. Chiu) Gelardi, Simonini & Vizzini, *Rubroboletus sinicus* (W.F. Chiu) Kuan Zhao & Zhu L. Yang, *R. esculentus* Kuan Zhao, Hui M. Shao & Zhu L. Yang (Zhao and Shao, 2017), *Exsudoporus frostii* (J.L. Russell) Vizzini, Simonini & Gelardi (Bessette *et al.*, 2016) and *Xanthoconium affine* (Peck) Singer (Razanamparany *et al.*, 1986), just to name a few. likewise, in tropical Africa in order to avoid

intoxications derived from diverse cultural attitudes and cooking techniques used in different villages, the consumption of *Phlebopus* species is recommended after boiling (De Kesel *et al.*, 2017). However, caution should always be used when consuming edible red-pored boletes and, as a general rule and aside from porcini mushrooms which could also be consumed raw (even though they are somewhat difficult to digest), all other edible boletes and allied species would require pre-treatments prior to safe consumption and should by prudence be eaten only after prolonged cooking so as to make them harmless and to avoid unpleasant effects.

In the traditional Chinese folk medicine some species are considered curative or therapeutic and are used for healing various diseases, such as *Pulveroboletus ravenelii* (Berk. & M.A. Curtis) Murrill s.l. (treatment of lumbago and skelalgia, limb numbness, and improvement of the system of meridians and collaterals) (Liu, 1984; Wu *et al.*, 2013; Zeng *et al.*, 2017), whereas others appear promising in the biomedical and pharmacological fields, for example *S. placidus* (Bonorden) Singer s.l. (Liu *et al.*, 2009), *Paxillus involutus* (Batsch) Fr. s.l. (Wang *et al.*, 2012; Zmitrovich *et al.*, 2019a), *Scleroderma* spp. (Dai and Yang, 2008), *Psiloboletinus lariceti* (Singer) Singer (Denisova, 2010), *Boletus edulis*, *B. craspedius Massee*, *Fistulinella wolfeana* Singer & J. García, etc., with antioxidant properties (Tsai *et al.*, 2007; Ahmed *et al.*, 2015; Robles-García *et al.*, 2016; Dimitrijević *et al.*, 2017) and other species with potential antifungal activity against pathogenic filamentous fungi (Batool *et al.*, 2019). Certain taxa of the genera *Hortiboletus*, *Gyroporus*, *Rhizopogon* and *Tapinella* exhibit antibiotic and anticancer properties (Sasek and Musilek, 1967; Zheng *et al.*, 2006: Dai and Yang, 2008; Kim and Lee, 2009; Wu *et al.*, 2013), while antibacterial activities were observed from the acetone extracts of some species of *Buchwaldoboletus* (Madhosingh, 1966). Most recently, antioxidant, antimicrobial, anti-inflammatory and neuroprotective effects have been ascertained for the sequestrate mushroom *Octaviania asterosperma* Vittadini s.l. (Sevindik *et al.*, 2020).

A few species are inconstantly poisonous even if well-cooked (Ammirati *et al.*, 1985; Rumack and Spoerke, 1994; Benjamin, 1995; Alexopoulus *et al.*, 1996; Hall *et al.*, 2003; Mazza, 2008; Merelet *et al.*, 2012; Sarc *et al.*, 2013; Tolgor *et al.*, 2014; Chen *et al.*, 2016; Wu *et al.*, 2019; Sitta *et al.*, 2020), provoking acute gastrointestinal distress with symptoms such as nausea, violent vomiting and diarrhea and sometimes also sweating, headache, fever and hyperprocalcitonemia, such as *Rubroboletus satanas* (Lenz) Kuan Zhao & Zhu L. Yang and *Neoboletus venenatus* (Nagasawa) G. Wu & Zhu L. Yang, from which active principles responsible for intoxications (bolesatine and bolevenine, respectively) have been isolated (Kretz *et al.*, 1989, 1991; Ennamany *et al.*, 1998; Brunelli, 2007; Matsuura *et al.*, 2007), but also *Rubroboletus pulcherrimus* (Thiers & Halling) D. Arora, N. Siegel & J.L. Frank (Benjamin, 1995), *Rubroboletus pulchrotinctus* (Alessio) Kuan Zhao & Zhu L. Yang (Sitta *et al.*, 2020; Lezzi pers. comm.), *Heimioporus japonicus* (Hongo) E. Horak (Li *et al.*, 2011a; Tolgor *et al.*, 2014; Chen *et al.*, 2014, 2016; Zeng *et al.*, 2018), *Boletus huronensis* A.H. Smith & Thiers (Voitk, 2009; Bakaitis, 2012), as well as some species of *Pulveroboletus* (Sun *et al.*, 2012; Chen *et al.*, 2014; Wu *et al.*, 2014) and a certain number of species of *Scleroderma* (Stevenson and Benjamin, 1961; Arora, 1986; Bau *et al.*, 2014), although in the latter genus at least one entity endemic of Yunnan Province (south-western China), viz. *S. yunnanense* Y. Wang, is surprisingly known to be edible (best recommended when immature) and locally commercialized (Wang *et al.*, 2020; Yu *et al.*, 2020). Boletes with bitter taste (*Caloboletus*) as well as species traditionally assigned to *Boletus* sect. *Luridi* Fr. (currently belonging in several genera including *Neoboletus*, *Rubroboletus* Kuan Zhao & Zhu L. Yang, *Suillellus*, *Imperator* G. Koller et al., etc.) and species of *Leccinum* and *Leccinellum* (in these two genera the stipe must be discarded being fibrous and indigestible) cause constant gastrointestinal poisoning when eaten raw or in case of incomplete cooking (Bessette *et al.*, 2000, Sitta *et al.*, 2020). Applying the guidelines provided by Sitta *et al.* (2020), species generally declared toxic such as *Tylopilus felleus* (Brunelli, 2007), *Gyroporus ammophilus* (M.L. Castro & L. Freire) M.L. Castro & L. Freire (Castro and Freire, 1995) and *Hygrophoropsis aurantiaca* (Wulfen) Maire should better be treated as occasionally or doubtfully poisonous depending on different ways of preparation. The European species *Suillellus luridus* and *Imperator torosus* (Fr. & Hök) Assyov et al. have been suspected to provoke coprinic syndrome with simultaneous intake of alcoholic beverages (Zeitlmayr, 1955; Budmiger and Kocher, 1982; Kiwitt and Laatsch, 1994; Flammer, 2008), but at least for *S. luridus* in the few reported cases in literature the intoxication might have likely derived from inappropriate preparation (insufficient cooking) or/and overconsumption and in any case attributable to a gastrointestinal context (Gry and Andersson, 2014; Sitta *in litt.*). Sporadic poisonings with gastrointestinal upset or even with allergic reactions with an immunohemolytic syndrome (Bobrowski, 1966) have been pinpointed for some thermophilic European Mediterranenan species of *Suillus* associated with two-needled pine trees, namely *S. mediterraneensis* (Jacquetant & J. Blum) Redeuilh, *S. luteus* (L.) Roussell and *S. collinitus* (Fr.) Kuntze (Prager and Goos, 1984; Lavorato, 1996; Sitta, 1997; Flammer and Horak, 2003). Indeed, *Suillus* species with viscid pileus are variably laxative (the entire fruiting body, not only the pileus cuticle!) (Sitta *et al.*, 2020). A single case of intoxication with rabdomyolytic syndrome due to abundant consumption in consecutive meals af a *Leccinum* sp. mixed with *Boletus edulis* s.l. has been recently reported by Chwaluk (2013). The basidiospores of the dangerous dry rot resupinate *Serpula* spp. growing attached to indoor wooden structures of private properties were responsible of cases of asthma (Watling, 2008) and *Hydnomerulius pinastri* (Fr.) Jarosch & Besl was indicated as the probable cause of breathing problems (allergic alveolitis) (Stone *et al.*, 1989).

The collective species *Paxillus involutus* s.l. is potentially lethal, being responsible of the Paxillus syndrome (also known as hemolytic syndrome or allergic cytotoxic syndrome), an allergenic syndrome characterized by gastroenteritis followed by acute renal failure caused by an autoimmune hemolytic anemia reaction (formation of immunocomplexes or antibodies on red blood cells) that occurs with repeated eating of the swine fungus and may lead to the death of hypersensitive patients (Winkelmann *et al.*, 1982, 1986; Flammer, 1985; Pohle, 1995; Habtemariam, 1996; Anthowiak *et al.*, 2003; Brunelli, 2007; Assisi *et al.*, 2008). A heart attack casualty indirectly caused by the ingestion of the poisonous American bolete *Rubroboletus pulcherrimus* has been reported in literature (Benjamin, 1995). In addition, a case of fatal poisoning of a woman from an acute muscarinic syndrome occurred in Queensland (Australia) after eating mushrooms putatively belonging in the genus *Rubinoboletus* (Pauli and Foot, 2005).

Various species from the Waghi Valley in the Western Highlands of Papua New Guinea have long been considered hallucinogenic by local indigenous tribes (the so-called Nonda Mushrooms of the Kuma people), viz. *Boletus kumaeus* R. Heim, *B. flammeus* R. Heim, *B. manicus* R. Heim, *B. nigroviolaceus* R. Heim (dubitatively contaxic with *Sutorius australiensis* (Bougher & Thiers) Halling & Fechner, according to Halling *et al.*, 2012a), *B. reayi* R. Heim, *Heimioporus anguiformis* (R. Heim) E. Horak and *Retiboletus nigerrimus* (R. Heim) Manfr. Binder & Bresinsky (Ross, 1936; Reay, 1959, 1960, 1965, 1977; Heim, 1963, 1965, 1966, 1972, 1973, 1978; Heim and Wasson, 1964, 1965; Watling 2001c; Toro, 2004; Treu and Adamson, 2006). Although *B. manicus* has undergone chemical testing and revealed to contain three indolic substances (Heim, 1965, 1972), scientific evidence supporting the occurrence of magic properties in these boletes has never been effectively produced, hence this phenomenon inducing an ostensible temporary madness with a range of irrational behaviors should presumably be considered a simple ceremonial staging, a sort of cultural institution, a theatrical play or a social catharsis, rather than actual effects induced under the influence of certain alleged psychotropic fungal species (Clarke, 1965; Reay, 1977; Toro, 2004; Treu and Adamson, 2006; Horak pers. comm.). Regrettably, the ethnomycological tradition of the "mushroom madness" has gradually declined and faded away among villagers over the last decades and has nowadays almost completely gone lost (Treu and Adamson, 2006). Similar psychoactive effects have been reported from consumption of a few bluing boletes in the Far East, namely *Butyriboletus roseoflavus* (Hai B. Li & Hai L. Wei) D. Arora & J.L. Frank, *Lanmaoa asiatica* G. Wu & Zhu L. Yang (these two species being considered even more favorite, renowned and appreciated in Yunnan Province than porcini mushrooms, see Wang *et al.*, 2004!) and *Neoboletus magnificus* (W.F. Chiu) Gelardi, Simonini & Vizzini (Yu *et al.*, 2020), as well as hallucinogenic would appear the Asian *Heimioporus japonicus* according to Chen *et al.* (2016) and Zeng *et al.* (2018). However, even in the aforementioned cases such a weird phenomenon equally appears implausible and should be adequately investigated for confirmation. Curiously, a reversible neurotoxicity experience after consumption of *Scleroderma cepa* Pers. has recently been reported from Germany (Haberl *et al.*, 2016).

The longest culture of consumption, preservation and trade of porcini mushrooms and related edible boletes is from Europe (especially the mycophilic Italy but also eastern European countries, as well as Finland, France, Portugal and to a lesser extent Spain) and is dated back to the ancient Greeks and Romans (Buller, 1914), although only in recent times it has achieved a much larger, international scale (Sitta *et al.*, 2007a; Sitta and Davoli, 2012; Peintner *et al.*, 2013). The practice of collecting boletes for culinary purposes in the New World is historically and intimately connected to the Italian immigrants who settled in North America at the beginning of the 20th century (Arora, 2008), starting a mycophilic tradition that was previously practically unknown. In Mexico and Central America (especially central and western Guatemala) wild edible boletes constitute an integral part of the fungal diet of several rural ethnic groups, being ethnomycological ancestral traditions consolidated since the pre-Columbian times, especially the Mayan culture (Flores Arzù, 2020; Pérez-Moreno *et al.*, 2020). Conversely, in Costa Rica and Panama consumption of mushrooms is generally low and the latter country has even been defined as mycophobic (Pérez-Moreno *et al.*, 2020). Along with other members of the family Boletaceae, porcini mushrooms are traditionally harvested during the rainy season by rural communities and offered fresh for sale in local roadside food markets as well as in urban stores in eastern and south-eastern Asia, particularly in China (especially Yunnan Province), in order to earn additional income to supplement the household economy (Wang and Liu, 2002). Indeed, members of the Boletaceae constitutes the bulk of traded wild edible mushrooms in China, with 50,000 tons of boletes gathered and sold in local and international markets (Wang *et al.*, 2020) and a total of 74 bolete species belonging in 27 genera offered for sale in the sole Yunnan Province (23% of the total mushroom species) (Pérez-Moreno *et al.*, 2020; Yu *et al.*, 2020). In addition, at least four Chinese porcini species (*B. bainiugan* Dentinger, *B. meiweiniuganjun* Dentinger, *B. sinoedulis* B. Feng *et al.*, *B. shiyong* Dentinger) are commonly exported sliced and dried, brined, powdered, processed or otherwise preserved to Europe due to the increasing demand of this gourmet delicacy (Wu and Lu, 2006; Sitta *et al.*, 2007b; Sitta and Floriani, 2008; Feng *et al.*, 2012; Dentinger and Suz, 2014; Feng and Yang, 2018; Gelardi, 2020; Wang *et al.*, 2020). At present, around 1000 tons of dried porcini are annually exported to Europe and the USA, for a total value of USD 19 millions (Yu *et al.*, 2020), thus generating a significant international business. Similarly, allochthonous porcini species introduced to South Africa have long been exported to Europe (Sitta *et al.*, 2007b). Among epigeous gasteroid and hypogeus Boletales, members of *Astraeus* and *Rhizopogon* are commonly harvested and traded in local mushroom markets in Sichuan and Yunnan Provinces (Sitta *et al.*, 2007b; Wang *et al.*, 2020). Extensive cultivation trials on industrial scale of some species of *Phlebopus*, namely *P. portentosus* (Berk. and Broome) Boedijn and *P. spongiosus* Pham and Har. Takah. (the only two species of the Boletales that can be successfully cultivated under artificial conditions) with the production of basidiomes without host plants have recently started in south-western China, Thailand and Vietnam (Ji *et al.*, 2011; Kumla *et al.*, 2015; Le Thi *et al.*, 2017; Raghoonundon *et al.*, 2021), resulting in Yunnan Province in a daily yield of more than 400 kg of fresh mushrooms (Ji, 2016). *Phlebopus portentosus* is one of the most frequently consumed boletes in Asia and is considered an excellent source of protein and carbohydrates (Raghoonundon *et al.*, 2021). Although in sub-Saharan Africa there exists an ancient traditional ethnomycological knowledge related to the domestic consumption, drying and storage of non-timber forest products such as edible mushrooms with a special reference to the Sudanian woodlands in West Africa and the Zambezian miombo woodlands in Central, East and Southern Africa, boletes appear to be very rarely eaten by local indigenous people, mostly because of the soft texture and their general tendence to change color when injured (Eyi Ndong *et al.*, 2011; Härkönen *et al.*, 2015; De Kesel *et al.*, 2017; Pérez-Moreno *et al.*, 2020). As far as South America is concerned, information on traditional mushrooms consumption by endemic people is scanty and the overall knowledge of fungal diversity is still incomplete. However, *Suillus* and *Rhizopogon* species growing in exotic pine plantations in the Patagonian Andean forests of Chile and Argentina are seasonally harvested and traded locally and abroad (Sitta *et al.*, 2007a; Barroetaveña and Toledo, 2020; Pérez-Moreno *et al.*, 2020). On the other side of the Pacific Ocean, no ancient tradition of human consumption of boletes or even wild edible mushrooms as a whole appears to exist in New Zealand and the

lack of knowledge concerning the edibility of mushrooms is due to the relatively recent historical colonization of the islands. Currently, the only bolete species consumed in New Zealand are those imported accidentally from Europe alongside their exotic plant partners (Pérez-Moreno *et al.*, 2020).

Conclusions

The diversity of the Boletales in Europe and North America and to a lesser extent in East and south-eastern Asia is relatively well assessed (although new species continue to be described from Europe and North America annually, despite more than 200 years of taxonomic efforts!) with a discrete proportion of species having been sequenced in recent times, as opposed to the tropical regions of Central and northern South America as well as Australasia where a more balanced taxonomic and phylogenetic sampling is still awaiting. On the other hand, temperate South America, subsaharan Africa and northern Australia remain nowadays severely undersampled or practically ignored in this regard. Corner (1972b) and Singer (1986) claimed that bolete taxonomy can be properly understood only on the basis of a deep acquaintance of tropical species. Unfortunately, tropical habitats and the temperate belt of the southern hemisphere appear so far largely underexplored from the mycological viewpoint (Hawksworth and Rossman, 1997; Watling *et al.*, 2002; Lee, 2005; Dentinger *et al.*, 2010; Tedersoo *et al.*, 2010; Tedersoo and Smith, 2013), and a relevant fungal biodiversity might be uncovered from these remote geographic areas in the next future. It is therefore obvious that a better knowledge coupled with ongoing molecular phylogenetic investigation primarily applied to tropical mycota is presently determining and will continue to accomplish a rapid and dramatic taxonomic reassessment of historically established genera (Watling, 2008), leading to detection of unexpected mycological novelties, endorsing a better scientific investigation of under-studied or neglected fungal groups, incentivating studies on their distribution, adaptation capabilities and response to climate changes, promoting awareness and conservation actions, ensuring monitoring and sustainable management of fungal inheritance and thus providing new insights into the origins, biological interactions and the fascinating dynamics of boletes biogeographic patterns.

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Functional Traits of Stipitate Basidiomycetes

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The Rationale

Macrofungi (here: mushrooms) are highly variable in terms of basidiomes, spores, and mycelia (**Plate 1**) relating to their characteristics, i.e., to their appearance, their internal structures, or their microscopic attributes.

Centuries ago, fungal traits have led to developing a taxonomy and identification keys but not for gaining insight into fungal performance. In plant science, traits have also been used for decades to better understand plant ecology (Shipley *et al.*, 2016). Only recently, mycologists realized that appreciating trait-based functions deepens considerably the understanding of fungal behavior (e.g., Aguilar-Trigueros *et al.*, 2015; Halbwachs and Bässler, 2015; Halbwachs *et al.*, 2016).

What are functional traits? Traits are the tools for survival, reproduction, and dispersal (Violle *et al.*, 2007). In plants, e.g., a flower's color attracts insects, which may collect pollen and pollinate other blossoms. Another example is the acorn seed: its wings can carry the seed over long distances. Poisonous leaves deter herbivores (Taiz *et al.*, 2007). In fungi, analogous morphological and physiological characteristics are observed. Think of basidiome pigmentation, spore ornaments, and poisonous taxa. Fungi are generally highly variable due to environmental and/or genetic factors (pheno- and genotypic plasticity, Dawson and Jönsson, 2019), and show a plethora of traits. Their ecological functions are often not obvious. For example, why do most Boletales rely on poroid and not lamellate hymenophores, or why have some basidiomes stunted stipes? In any case, most traits come at costs. Especially pigments and other secondary metabolites need considerable energy input for synthesis, probably leading to trade-offs. Trade-offs are imminent because the energy budget of organisms is inevitably limited (Mayhew, 2006).

A word of warning: The general notion that everything in nature has a function is still under scientific debate. One should be aware that nature is the result of evolution, which is, in general, not a directed process but the result of try and error. As Jacob (1977) so aptly coined it: "evolution is a tinkerer".

Functional fungal traits refer to their morphology, physiology, and behavior of mycelia, basidiomes, and spores. The following sections provide insight into the connection of such traits with vital fungal functions. Note that many interpretations are based on plausibility and not on evidence.

The Mycelium

Morphological Traits

Although basidiomycete mycelia are abundant, e.g., in forest soils (**Fig. 1**), our understanding of their distribution, dynamics, and functioning is limited.

The contribution of individuals, i.e., physically and (or) physiologically delimited mycelia (genets), to nutrient translocation and cycling in forest soils is across species still largely unknown (Cairney, 2005). It is self-evident that the extent (coverage) of mycelia in the substrate echoes its ability to extract nutrients. However, there is more to mycelial morphology, as the following sections show.

Plate 1 Spores, mycelia, and various mushrooms. Spores Linas Kudzma CC BY-SA 3.0, courtesy Gernot Friebes, mycelia Bob Blaylock, Tobi Kellner CC BY-SA 3.0, mushrooms courtesy of Shawn Fischer.

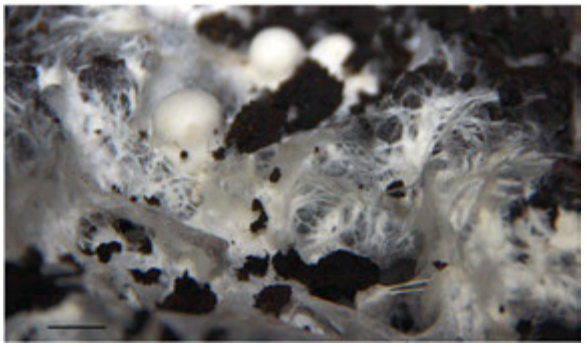

Fig. 1 Mycelium of *Agaricus bisporus* with basidiome embryos (primordia), bar 1 mm. Pradejoniensis CC BY-SA 3.0.

Mycelial coverage, mycelial biomass

The ability to form extensive mycelia, for instance in *Armillaria* (Rizzo *et al.*, 1995), is coupled with competitive (Brasier, 1999) and reproductive fitness (Dawson *et al.*, 2018). The size of species-specific mycelia may limit the size of basidiomes.

Vertical distribution

Generally, mycelia of saprotrophic fungi occur in the higher and younger organic layers, while ectomycorrhizal taxa prefer lower strata where humus accumulates and make nitrogen available for host plants (Lindahl *et al.*, 2007; Scattolin *et al.*, 2008). This spatial distribution reflects the decomposer qualities of the two guilds.

Hyphal cords and rhizomorphs

Cords and rhizomorphs are employed by some species when nutritious patches occur at a distance (Clémençon *et al.*, 2012), thus having the edge over less competitive species.

Ectomycorrhizal mantles

Ectomycorrhizal Agaricomycetes colonize host plant roots by forming hyphal mantles, which act as interfaces for exchanging nutrients and other beneficial compounds (Smith and Read, 2008). There exists a host of different mantle types in terms of morphology, texture, structure, and pigmentation (Agerer, 1987–2002). The ecological implications of the mantle types largely remain in the dark. Commonly, the mantles store nutrients for the development of sporocarps (Taylor and Alexander, 2005).

Extramatrical mycelia, exploration types

Ectomycorrhizal fungi show typical extramatrical mycelia. They can be classified into long-, medium- and short-distance, contact, and pick-a-back, which may represent trade-offs between mycelial coverage and decomposition abilities (Agerer, 2001) (see below Section "Guild-Specific Trait Syndromes").

Common mycorrhizal networks (CMN)

Ectomycorrhizal mushrooms form hyphal networks that connect them among themselves and host trees ("wood-wide-web") and distribute water, nutrients an and signaling compounds (Simard *et al.*, 2012), even if the latter are mixed stands (Simard *et al.*, 2015). In this way, CMN contribute to stabilizing forest ecosystem resilience to disturbance (Rhodes, 2017; Gilbert and Johnson, 2017).

Hyphal wall thickness

Thick-walled mycelial hyphae store nutrients (carbohydrates) (Clémençon *et al.*, 2012), which may be interpreted as a way to "sit out" unfavorable conditions (e.g., dry spells, extreme temperatures), thus improving stress tolerance. A number of species develop mycelia with relatively thin hyphal walls and low chitin content, e.g., waxcaps, which may increase the efficiency of nutrient uptake (Halbwachs *et al.*, 2013).

Clamp connections

Clamp connections in mycelial hyphae are the regular case in Agaricomycetes. They signify the development of a dikaryotic mycelium produced by meiosis leading to diploidy. It gives these fungi the advantage of recombination, i.e., high genetic adaptability (Moore *et al.*, 2011). It should be noted that in clamp-less, aseptate hyphae, i.e., coenocytic hyphae, the occurrence of multiple nuclei may be an alternative way of keeping up genetic adaptability (Moore *et al.*, 2011).

Mitospores

These basically asexual spores such as arthroconidia and chlamydospores serve as a back-up system when adverse conditions do not permit fructification. Mitospores occur, e.g, in *Asterophora*, *Pleurotus*, *Lentinellus*, *Rhodotus*, and *Volvariella* and can be dispersed by animals and perhaps wind (Clémençon *et al.*, 2012).

Fig. 2 *Polyporus tuberaster* (Jacq.) Fr. with its typical sclerotium. Alan Rockefeller CC BY-SA 3.0.

Mycelial cysts and cystidia

In, e.g., *Coprinellus*, *Pholiota*, *Stropharia*, and *Pleurotus* occur allocysts and thrombocysts. *Auriscalpium*, *Russula*, *Lentinellus*, and *Stropharia*, for example, may produce cystidia (Gleo- or acanthocytes) (Clémençon *et al.*, 2012). The function of these structures is unclear but could be excretory organs producing substances that alleviate biotic and abiotic stress, e.g., latex-producing gleocystis in *Russula* (see below Table x, Section "Basidiome Physiology").

Sclerotia and pseudosclerotia

Like thick hyphal walls, sclerotia and pseudosclerotia store carbohydrates or portions of the substrate to outlast adverse conditions (Clémençon *et al.*, 2012) (**Fig. 2**). Pseudosclerotia are mostly tough hyphal aggregates, which may become large as a football (Macfarlane *et al.*, 1978).

Physiological Traits

Mycelia do not only access and transport nutrients and water but are, at the same time, chemical factories. They produce an abundance of compounds, mainly enzymes and secondary metabolites, such as extrahyphal enzymes to cleave, e.g., cellulose, hormones, toxins, thermal protectors, and fungivore deterrents.

Many mushrooms have mycelia resistant to microbial and predator attacks (e.g., *Collembola*). Melanins (dark pigment) are well known for their protective qualities (Bell and Wheeler, 1986) (see Box 1). For instance, Bjorbaekmo *et al.* (2010) reported that many fungi associated with *Dryas*, an alpine/arctic shrub, show melanized mycelia. Moreover, some volatile organic compounds (VOC) and other secondary metabolites show repelling qualities (Moore *et al.*, 2014; Spiteller, 2015). Especially melanins are costly to synthesize, which probably leads to a trade-off with, e.g., mycelial growth (Koide *et al.*, 2014; Siletti *et al.*, 2017). Conversely, Crowther *et al.* (2014) found that strong competitors cannot tolerate desiccation well. On the other hand, some species may not only tolerate a specific stressor such as heat but a combination of stressors (Treseder and Lennon, 2015) by employing heat shock proteins and antifreeze compounds (mainly sugars and some lipids). Apart from house-keeping enzymes and hormones, compounds that protect against biotic and abiotic hazards are crucial for survival and fruiting competence.

If not growing in patches not yet colonized by other fungi, many mycelial hyphae show the ability to fend off hyphae of other fungi (competitive exclusion or growth deadlock: Cairney, 2005). Ectomycorrhizal fungi often outcompete saprotrophic taxa ("Gadgil effect", Cairney, 2005; Gadgil and Gadgil, 1975), though this effect varies with soil horizons and season (Peršoh *et al.*, 2018). The antagonistic mycelial interaction between different taxa is often controlled by excreting secondary metabolites that suppress the growth of foreign hyphae (Kües *et al.*, 2018) or even kill them (Silar, 2012). Not only chemical but also physical defense mechanisms can be observed, e.g., blocking foreign mycelia by hyphal proliferation at the point of contact (Widden, 1997). Ectomycorrhizal taxa and lichens are renowned for their ability to dissolve mineral matter, among others, to enable the uptake of mineral phosphorus, which is not readily soluble (Gadd, 2017).

Box 1 Functional properties of fungal melanins

(1) Structural enforcement (cell walls) against osmotic and turgor forces
(2) Desiccation protection
(3) Thermal stress protection
(4) Protection against ionising radiation (UV, γ-radiation etc.)
(5) Salt and pH stress protection
(6) Mopping up heavy toxic metals
(7) Antioxidant
(8) Protection against lytic enzymes
(9) Fending off microbial attacks
(10) Enhancing virulence of plant pathogens
(11) Converting radioactive and other radiation to energy for metabolic processes

Melanized hyphae

The degree of hydrophobicity of, e.g., ectomycorrhizal mycelia, seems connected to substrate water content (Smits *et al.*, 2003) and nutrient distribution (Unestam and Sun, 1995). Hydrophilic mycelia are more water stress-tolerant, hydrophobic mycelial strands transport nutrients from more distant patches. Hydrophobicity may dramatically increase with age (Smits *et al.*, 2003). The ecological implications of this phenomenon are unknown.

Behavior

Little is known about behavioral traits in mycelia. Their traits seem primarily relate to autecological functions:

Aerotropism

Hyphae grow away from the colony center towards higher oxygen concentrations (Ugalde, 2006) to exploit new resources.

Autotropism

Hyphae show positive autotropism by growing hyphal branches (Ugalde, 2006) to possibly form a network, which may optimize metabolite distribution in the mycelium. Negative autotropism prevents hyphae from growing back to older parts of the mycelium (Ugalde, 2006), where resources have become depleted.

Chemotropism

Fulton (1906) found that hyphae of, e.g., *Coprinus cinereus* grow towards higher nutrient concentrations, while Gooday (1975) found little evidence that resource gradients affect hyphal growth.

Phototropism

Reinert (1959) reported negative phototropism, which is perfectly plausible regarding substrate exploitation and desiccation prevention.

Thigmotropism

Contact stimuli direct hyphae away from obstacles towards crevices, cavities, and pores (Gow, 1994; Perera *et al.*, 1997).

Mycelial persistence

Combative taxa like *Armillaria*, possess mycelia that live several years up to several hundred years (Burnett, 2003). Although it can be expected that small and ephemeral Agaricomycetes, which colonize $\pm$ fresh litter, develop short-lived mycelia, little is known about mycelial longevity (Burnett, 2003).

Host specificity

Most lignicolous saprotrophic mushrooms are promiscuous, i.e., occur on deciduous and coniferous wood but may have preferences (Krah *et al.*, 2018). Some only colonize deciduous, others only coniferous substrates, palm trees, ferns, or grasses. Only a few species are associated with specific host genera, such as *Mucidula mucida* with *Fagus* (Knudsen and Vesterholt, 2012).

Ectomycorrhizal taxa are more often host-specific, and some are even Genus-specific, e.g., *Suillus grevillei* with *Larix*, *Lactarius torminosus* with *Betula* and *Cortinarius archeri* with *Eucalyptus* (Molina *et al.*, 1992).

It can be expected that such traits form a functional consortium (syndrome) to optimize the investment of resources/energy.

The Basidiome

Basidiomes of Agaricomycetes (mushrooms) carry the machinery for sexual reproduction and dispersal. Reproductive and dispersal fitness depends on morphological and physiological adaptations of basidiomes to abiotic and biotic environmental conditions and the resulting ecological niches (Schoener, 2009; Halbwachs *et al.*, 2016). These adaptations become manifest in functional traits or their combinations (Crowther *et al.*, 2014; Shipley *et al.*, 2016; Perez–Harguindeguy *et al.*, 2013).

Basidiome Size and Number

The size range of mushrooms is overwhelming: some species develop cap diameters smaller than 1 mm, others attain up to 1 m (**Plate 2**). Larger basidiomes survive longer and produce more spores than smaller ones (Moore *et al.*, 2008). Species with (many) small basidiomes tend to fructify and perish faster and are thus adapted to rapidly changing and potentially perilous conditions (Bässler *et al.*, 2016).

Mycelium of a given size may either produce few large fruit bodies or numerous small fruit bodies. Indications of such a trade-off have been found by Bässler *et al.* (2015). Small fruit bodies have the advantage that a fungus can quickly utilize ephemeral resources for dispersal and reproduction. Larger fruit bodies can sustain longer periods and maximize their spore production, thus having a competitive advantage.

Basidiome Architecture

Although the standard blueprint of mushrooms is based on stipe, cap, and hymenophore (spore-producing tissue), these fungi show differing shapes (**Fig. 3**) (Halbwachs *et al.*, 2016). The basic characteristics of fruit bodies serve multiple purposes, mainly relating to structural stability, protection against hazards, and dispersal optimization.

Apart from implications for dispersal and reproduction, basidiome shapes predominantly have adaptive qualities relating to abiotic and biotic threats (**Table 1**).

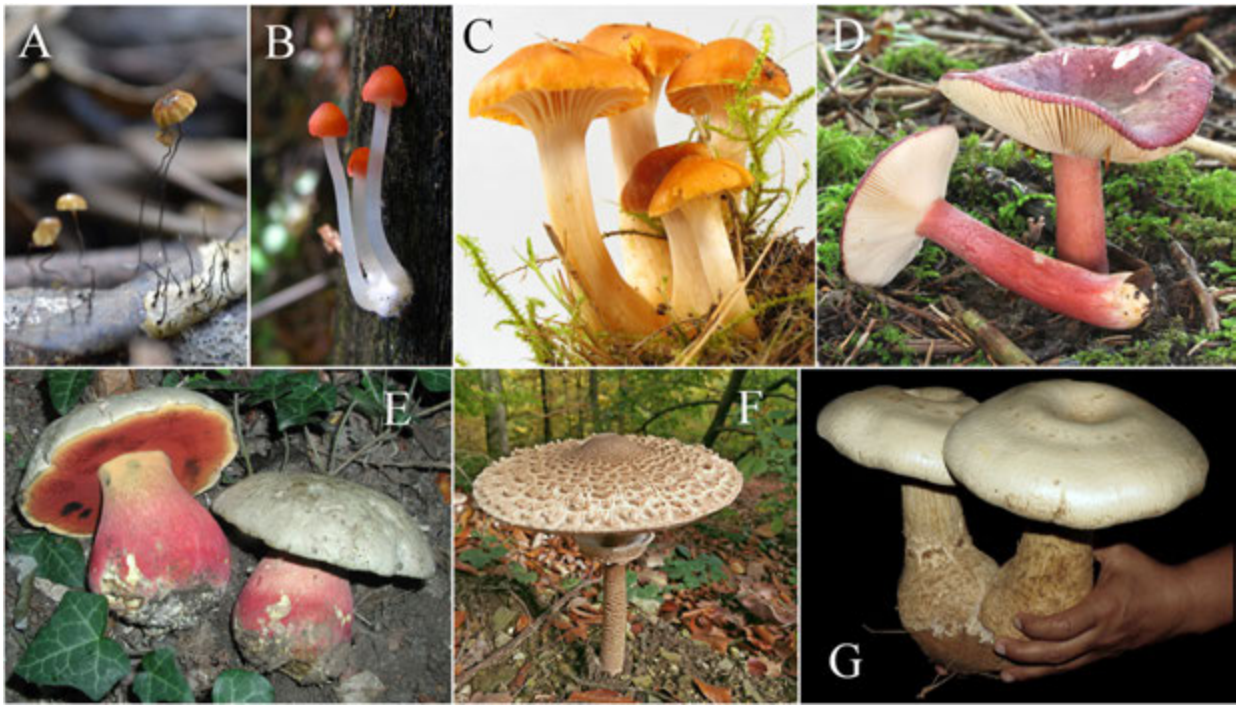

Plate 2 Examples of mushroom sizes. A: *Cryptomarasmius corbariensis*, ~5 mm; B: *Atheniella adonis*, ~12 mm; C: *Cuphophyllus pratensis*, ~70 mm; D: *Russula queletii*; E: *Rubroboletus satana*, ~30 cm; F: *Macrolepiota procera*, ~40 cm; G: *Macrocybe titans*, ~100 cm. A Stefan Mintoff CC BY-SA 3.0, B Dan Molter CC BY-SA 3.0, D H. Krisp CC BY-SA 3.0), E courtesy Boris Assyov, F Kreuzschnabel CC BY-SA 3.0, G J. Teodoro Chivata Bedoya CC BY-SA 3.0.

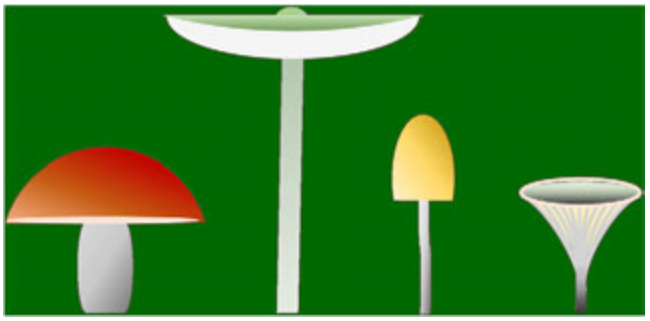

Fig. 3 Main basidiome types. From left to right: convex, plane with umbo, conical/bell-shaped, funnel-shaped.

Table 1 Main features of basidiome architecture and their putative ecological implications. Examples are depicted in **Plate 3**

Architectural features	*Examples*	*Interpretation*
Slim cap	*Coprinus comatus*	Keeps humidity in the sporophore, minimal investment in trama
Tall habit	*Amanita bisporigera, A. crocea*	The hymenophore is raised above the still air layer next to the ground so that air movement can carry away the spores
Stocky habit	*Lactarius turpis*	Traps humidity, spore dispersal predominantly by invertebrates, withstands harsh winds
Cap convex to flat	*Boletus aereus*	When fleshy, a good water and nutrient reservoir for spore generation and release, barrier to the sporophore, insulation against excessive temperatures, optimal space for the sporophore, turbulent air reaches sporophore without obstruction
Cap funnel-shaped (infundibuliform)	*Trogia* sp.	Unobstructed access to vectors, minimal investment in trama. Whether the cap depression serves as water-collecting structure, is unknown
Cap bell-shaped, conical	*Psilocybe mexicana*	Cap often loosely attached to stipe and thus keeps lamellae in a vertical position regardless of stipe angle ("gravitational suspension"), minimal investment in trama
Cespitose (clustered) growth	*Hypholoma fasciculare*	Overcomes spatial constraints: one large fruit body would be squeezed at the tree trunk, traps humidity
Pellis scaly*(squamulose), wooly (tomentose), bristly (strigose) etc.	*Sarcodon imbricatus, Lactarius pubescens*	Insulate against heat, bar UV radiation, reduce desiccation, may deter fungivores, may be water-repellent
Glutinous coating*	*Gliophorus psittacina*	Probably protects against desiccation and/or bars fungivores
Hydrophobic pellis	*Boletus aereus*	Hydrophobic cap coating prevents excessive water and probably pathogens penetrating deeper tissue strata (Lugones *et al.*, 1996)
Veils, cortinas, volvas, and rings*	*Amanita jacksonii*	These structures are remnants of the universal veil, which encases the emerging fruit body. They serve as protection against fungivores and desiccation, especially the young fruit body
Cap umbo	*Amanita crocea*	Probably for bracing the junction between stipe and cap and for keeping the cap horizontally
Tapered stipe	*Amanita bisporigeraGomphidia roseus*	Upwards: suggestive of a trade-off between biomass economy, fruit body stability and geotropic orientation of the hymenophore Downwards: stability ensured by surrounding substrate
Stipes with transversal density gradient	*Macrolepiota fuliginosa*	Hollow, cavernous or loosely stuffed stipes possess a higher tensile strength than full stipes, which improves the stability of tall mushrooms
Eccentric stipe	*Pleurotus ostreatus*	Prevents caps to become squeezed at tree trunks (entry point for microorganisms!)
Bulbous stipe	*Amanita bisporigera*	Anchor to substrate

*Defensive structures.

Note: Halbwachs, H., Simmel, J., Bässler, C., 2016. Tales and mysteries of fungal fruiting: How morphological and physiological traits affect a pileate lifestyle. Fungal Biology Reviews 30, 36–61.

Micro- and macrobial pests largely constrain the longevity of mushrooms. Consequently, fungi are equipped with defensive structures. Numerous morphological features physically deter fungivores, particularly when fungi are still young. At the same time, such structures may give protection against abiotic hazards. None of these traits can prevent damage; they merely extend the lifetime of fruit bodies and thus the sporulation period(s). It should be noted that basidiome size is correlated with longevity (Moore *et al.*, 2008). Some structural basidiome traits seem not to serve a function, for example, undulate or crenate cap margins, which deserves further research (see B and I in **Plate 3**).

Plate 3 A: *Amanita caesara*, B: *Lactarius pubescens*, C: *Coprinus comatus*, D: *Amanita bisporigera*, E: *Gliophorus psittacinus*, F: *Gomphidius roseus*, G: *Macrolepiota fuliginosa*, H: *Amanita crocea*, I: *Lactarius turpis*, J: *Trogia* sp., K: *Boletus aureus* L: *Chlorophyllum brunneum*, M: *Psilocybe mexicana*, N: *Pleurotus ostreatus*, O: *Sarcodon imbricatus*, P: *Hypholoma fasciculare*. A Yaqui CC BY-SA 3.0, B H. Krisp CC BY 3.0, C Tommy Kronkvist GNU FDL, D Dan Molter CC BY-SA 3.0, E Stu's Images CC BY-SA 4.0, F Björn S. CC BY-SA 2.0, G courtesy of Peter Karasch, H Ingrid Tiitre CC BY-SA 4.0, I Ron Pastorino CC BY-SA 3.0, J Liz Popich CC BY-SA 3.0, L Zdeněk Kubeš CC BY 3.0, M Alan Rockefeller CC BY-SA 4.0, N Andreas Kunze CC BY 2.0, O H. Krisp CC BY 3.0, P Jean-Pol Grandmont CC BY-SA 3.0.

Hymenophores

There are basically four hymenophore architectures (**Plate 4**):

Each architecture exhibits a typical hymenial surface area per area unit of the cap underside. Gills provide the most efficacious architecture, followed by tubes, teeth, and smooth hymenophores (Pöder, 1983) (**Fig. 4**).

Lineages with less efficacious hymenophores must either have a compensatory feature or have other advantages. Ways for compensating hymenial area loss could include larger fruit bodies or the production of multiple fruit bodies, thus increasing the size of the hymenophore-bearing cap. Another way could be a higher spore packing density by producing more elongate spores while maintaining their volume.

The role of cystidia in many taxa is multifaceted. They act as secretory organs that are possibly involved in hymenial water regulation, as stabilizers that maintain gill patterns during development (Moore *et al.*, 1998), as spacers to keep basidia apart (Largent *et al.*, 1978), and as fungivore deterrents (Nakamori and Suzuki, 2007).

Basidiome Physiology

Like mycelia, fruit bodies make use of a wide array of enzymes and secondary metabolites, especially for fending off fungivores or hazardous microorganisms (Halbwachs *et al.*, 2016). Guild-specific characteristics are treated below in Section "Guild-Specific Trait

Plate 4 The four hymenophore types: A lamellate (gills) (*Megacollybia platyphylla*), B poroid (tubes, pores) (*Leccinum manzanitae*), C hydnoid (teeth) (*Hydnellum aurantiacum*), D smooth (*Craterellus cornucopioides*). A Thomas Pruß CC BY-SA 3.0, B Richard Sullivan CCBY-SA 3.0, C Leah Bendlin CC BY-SA 3.0, D Wolfgang Wallner CC BY-SA 3.0.

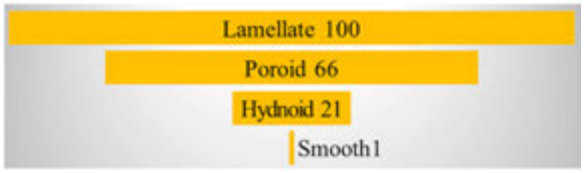

Fig. 4 Relative hymenophore area efficacies compared to lamellate hymenophores.

Plate 5 A: *Gyroporus cyanescens*, B: *Hygrocybe punicea*, C: *Cortinarius archeri* (), D: *Mycena aurantiomarginata*. A Aorg1961 CC BY-SA 3.0, C Ian Dodd CC BY-SA 3.0, D Alan Rockefeller, CC BY-SA 3.0.

Syndromes". In the following, important secondary metabolites found in mushrooms and their putative ecological function are accounted for.

Pigments

Most pigments can be attributed to functions such as antibiosis (e.g., blueing in many boletes when bruised) (Spiteller, 2015), bacterial defense (e.g., yellow mycenaaurin in *Mycena aurantiomarginata*) (Jaeger and Spiteller, 2010), antioxidant (e.g., betalains in some *Hygrocybe*) (Belhadj Slimen *et al.*, 2017; Bresinsky and Kronawitter, 1986), toxicity (e.g., blue-violet L-Dopa-based neurotransmitter in *Cortinarius*) (Gill, 2003; Misu and Goshima, 2006) and thermal regulation (e.g., melanins in *Boletus aureus*, see Krah *et al.*, 2019). For further details on pigments, refer to Gill and Steglich (1987) and Gill (2003). See examples in **Plate 5**.

Yellow seems to particularly attract flies and beetles (Menzel and Shmida, 1993; Prokopy and Owens, 1983). In horticulture, yellow sticky traps are widely applied to minimize insect infestations. It has been speculated that bright colors, especially hues from yellow to red, would serve as a warning cue to predators (aposematism) (Komárek, 2003). As yet, such an interaction could not be proved.

Volatile organic compounds (VOCs)

1-octen-3-ol deters slugs, e.g., in *Clitopilus prunulus* (Wood *et al.*, 2001) but also lures insect vectors (Combet *et al.*, 2006), an impressive case of trait multi-functionality and chemical economy in fungi. Also, other VOCs may be attractants for vectors (Boddy and Jones, 2008), e.g., the carrion smell of Phallales, which attracts flies. Some mushrooms virtually produce odor bouquets (Kües *et al.*, 2018). There are indications that toxic fungi develop warning odors (Sherratt *et al.*, 2005).

Taste compounds

Bitter or pungent compounds may deter some mammalian fungivores (Spiteller, 2015); other, e.g., sugary compounds may attract vectors (Dawson *et al.*, 2018).

Toxins

Many mushrooms are toxic for fungivores (Spiteller, 2008), albeit selectively. For instance, *Hypholoma fasciculare*, which is toxic to humans, is readily consumed by roe deer (authors' observation). Hallucigenous substances, like psilocybin, often lead to an aversion to a fungus (Camazine, 1983) or may cause an intoxicated vector to erratically move and thereby disperse spores more widely (Boyce *et al.*, 2019).

Latex

Some taxa (mainly *Mycena*, *Lactarius*, *Lactifluus*) produce, upon exposure to oxygen, milk that then turns to latex, which may act as wound closure to prevent microorganisms from entering the trama.

Texture

Another physiological aspect of basidiomes is texture. Basidiomes of most Agaricomycetes are monomitic (consisting exclusively of generative hyphae). They are fairly rigid when young due to hydraulic pressure (turgor) (Beecher *et al.*, 2001). Senescence sets in when trama loses water and, in consequence, becomes soft. Di- and trimitic fruit bodies (also possessing skeletal and/or binding hyphae) are tougher than monomitic ones, for example, *Megacollybia*, and *Trogia*, a trait that leads to extended longevity (Dawson *et al.*, 2018). The frequently observed considerable force exerted by mushrooms to break through compacted soil is not connected to a tough texture. This force is purely hydraulic and amounts to around 0.5 Newton/mm^2 (Halbwachs and Bässler, 2012), which means that e.g., an emerging basidiome of *Agaricus bisporus* may lift a weight of several kilograms, a quality that may be instrumental in skeletal and root-rich soils.

Luminescence

Bioluminescence in fruit bodies (**Fig. 5**) possibly attracts arthropod vectors (Sivinski, 1981; Oliveira *et al.*, 2015) since their green sensitivity matches the emission peak of luminescent fungi (Shimoda and Honda, 2013; Desjardin *et al.*, 2008) in the *Omphalotus*, *Armillaria* and mycenoi5 lineages (Desjardin *et al.*, 2008).

Basidiome Behavior

Basidiomes show some physical reactions to environmental cues:

Gravitropism (geotropism)

Mushrooms need to orient their hymenophore in a way that spores can be discharged without being obstructed. In most taxa, the stipe bends to maintain a horizontal position of the cap; some species can adjust their hymenophores (Moore *et al.*, 1996).

Phototropism

Phototropism may take over from gravitropism to enable the basidiome to grow out from, e.g. a crevice into the open. Then, geotropism takes over again to bring the cap into a horizontal position (Halbwachs *et al.*, 2016). For many taxa, blue light is required for basidiome initiation (Corrochano and Galland, 2006).

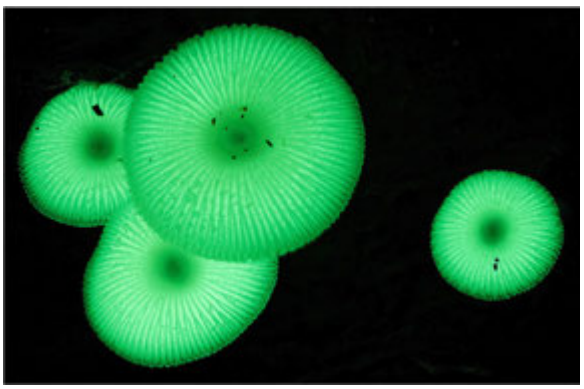

Fig. 5 *Mycena chlorophos*, a sub-tropical luminescent saprobic mushroom. Steve Axford CC BY-SA 3.0.

Thigmotropism

Negative thigmotropism, combined with gravitropism, leads to an avoidance of obstacles, such as dense turf, which may result in contorted stipes (**Fig. 6(A)**) (Halbwachs *et al.*, 2016).

Dwarfing

Dwarfing may occur in dry years (Halbwachs *et al.*, 2016) or due to low temperatures (Halbwachs and Simmel, 2018). See also Section "Trait Variability" in this chapter.

Fairy rings

Fairy rings, i.e., the simultaneous development of multiple fruit bodies along a circle or arc, predominantly occur in grassland under dry conditions (Griffith and Roderick, 2008) (**Fig. 6(B)**). They can attain diameters of several hundred meters and an age of several hundred years (Shantz and Piemeisel, 1917). Fairy rings may be visible signs of competitive competence without any specific function.

Phenology

Fructification timing, though largely determined by environmental factors such as precipitation and temperature (Halbwachs *et al.*, 2016), has a genetically controlled component (Selosse *et al.*, 2001). There are indications that many Agaricomycetes avoid fructification during the peak season of fungivorous insects (Halbwachs, 2019) (see also below Section "Guild-Specific Trait Syndromes").

The Spore

Fungal spores combine two capacities critical for fungi, namely reproduction and dispersal. The basidiomycete mushrooms reproduce sexually by merging germ tubes that emerge from different spores. The morphological diversity among all mushroom clades is legendary (**Fig. 7**). Be it size, shape, ornamentation, pigmentation, or other features, most of their functions are still subject to speculation, albeit with some plausibility.

Spores Size

In mushrooms, mean spore size (length) ranges between ca. 2.5 μm (e.g., *Cystolepiota luteohemisphaerica*) and 20.5 μm (*Hymenopellis megalospora*). Even more impressive are the volume differences of these species. The latter is ca 300 times more voluminous. For a given hymenial surface area and a given spore shape, the investment in biomass of large spores is higher than for small spores. This may seem counterintuitive. But as a matter of fact, spore volume follows a cubic function of the diameter (Halbwachs *et al.*, 2018).

Fig. 6 A: waxcaps with contorted stipes caused by thigmotropism, B: fairy ring of *Chlorophyllum* sp.

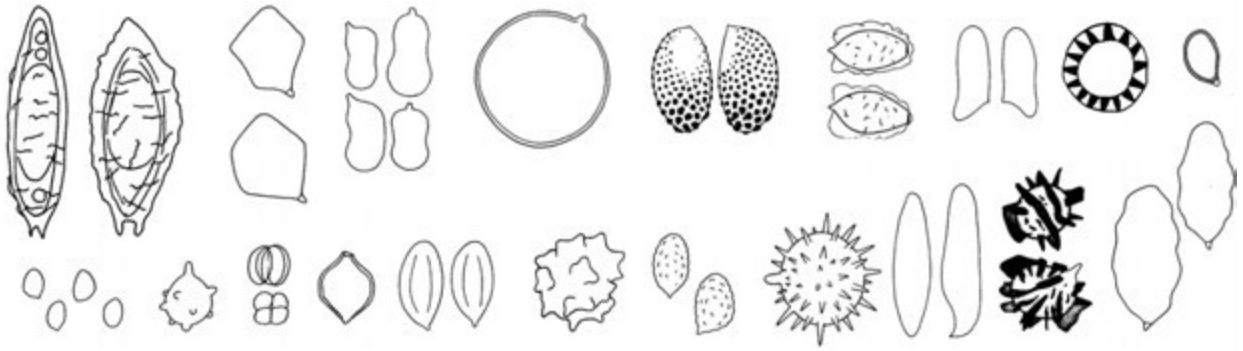

Fig. 7 Various basidiospores. Courtesy of Henning Knudsen.

The ecological implications of spore size are summarized as follows.

Discharge

The larger a spore, the further it is discharged because the spore length determines the size of Buller's drop (Stolze–Rybczynski, 2009). The larger Buller's drop, the more forceful it hits the spore's adaxial area and is severed from the basidium (Clémençon *et al.*, 2012). This may be the reason that in poroid mushrooms, spores become larger with tube diameter (Stolze–Rybczynski *et al.*, 2009).

Wind dispersal

Small spores are further dispersed by wind than large spores (Norros *et al.*, 2014). This due to the lower settling speed of small spores (Edmonds, 1979).

Impaction

Large spores have a higher impact on substrates facilitating establishment while small spores more easily circumvent obstacles during their flight (ruderal mushrooms) (Halbwachs and Bässler, 2015).

Desiccation resistance

There are indications that spore size can be related to fruiting phenology and humidity. Kauserud *et al.* (2010) found that basidiomycete assemblages have, on average larger spores under dry conditions than in humid regimes and hypothesized that it is a strategy to store water for germination.

Dormancy

Large spores contain carbon nutrients that prolong survival (Hawker and Madelin, 1976). See the next section for a size-shape syndrome in ectomycorrhizal mushrooms.

Germinability

Small spores readily germinate, which is advantageous, e.g., at higher latitudes where the window of opportunity is narrow (Cline and Zak, 2014).

Spore Shape

Most mushroom spores are fusiform to oval (oblong, ellipsoid) or globose, some ovoid, and some irregularly shaped (e.g., bean-shaped (phaseoliform), sausage-shaped (allantoid) and more), see Largent *et al.* (1978). Putative functions of such spore shapes are compiled in the sections below.

Discharge

Oblong spores discharge further because the spore length determines the size of Buller's drop (Stolze–Rybczynski, 2009). Perhaps, oblong spores produce elongate adaxial drops, thus increasing the discharge momentum by higher leverage.

Wind dispersal

Studies are showing that spore shape does not affect dispersal distance (Crandall, 2016; Hussein *et al.*, 2013). In contrast, e.g., Peay *et al.* (2012) and Tulloss (2005) reported that elliptical spores are carried further by wind than round ones. This is corroborated by studies that showed that elliptical spores occur more often in open forests (chaparral) (Botnen *et al.*, 2019) and nutrient-deprived settings (Halbwachs *et al.*, 2017). The contradictory findings need to be disentangled.

Impaction, substrate attachment

Allantoid spores may be well suited for clinging to woody surfaces; litter dwellers mostly have spherical spores (Calhim *et al.*, 2018).

Dormancy

Globose spores exhibit the smallest surface area compared to oblong ones with the same volume. In this way, a minimum surface area is exposed to microbial attacks and threatened by desiccation (Carlile *et al.*, 2001), which may be important for the ectomycorrhizal guild where spores need considerable time to get in contact with host rootlets. This correlates to spore size, which is phylogenetically rooted (Bässler *et al.*, 2015).

Production (numbers)

Evidently, more elongate spores can be produced per hymenial surface area unit than globose ones with the same volume. However, elongate spores are more costly to produce than globose ones having the same volume in the sense that internal spore structures are needed to prevent distortion (Kauserud *et al.*, 2008).

The ecological relevance of most spore shape variants has still to be elucidated. It could well be that, e.g., a bean-like shape bears no ecological meaning at all but is a mutational oddity that does not entail any disadvantage or advantage for a fungus.

The Spore Wall

Mushroom spores are encased in a multi-layered, protective membrane. Smooth and unpigmented (hyaline) ones have two layers; all other types that show ornamentation and/or pigmentation have five layers (Clémençon *et al.*, 2012). Spore wall traits are related to numerous functions that are connected to reproductive and dispersal fitness.

Ornamentation

Smooth spores seem to be dispersed predominantly by wind (Dawson *et al.*, 2018), and are typical for terrestrial saprotrophic mushrooms (Calhim *et al.*, 2018). Ornamented spore walls appear to be instrumental for dispersal by vectors, especially in the ectomycorrhizal guild (Halbwachs *et al.*, 2015; Vašutová *et al.*, 2019) (**Plate 6-A**), and for better clinging to woody substrates (Calhim *et al.*, 2018). Unexpectedly, ornamentation does not significantly contribute to air dispersal efficacy (Roper *et al.*, 2008), at least in ascomycetes.

Pigmentation

About 50% of all mushrooms produce melanized (dark) spores, at least in Scandinavian countries (Halbwachs *et al.*, 2015) (**Plate 6-B**) for protection (see Box 1). Fungal melanins are costly to produce (Rohlfs and Churchill, 2011) and require thick spore walls (Garnica *et al.*, 2007). To a lesser extent, pigments other than melanins also have protective qualities, e.g., carotenoids (Ruiz–Herrera, 2012; Gooday, 1981).

Appendages

Winged (calyptrate) spores as they occur in *Coprinopsis* (**Plate 6-C**) and *Galerina* have an extended outer wall layer (myxosporium). One may speculate that this trait slows down sedimentation, which would result in long-distance dispersal by wind, though Jones (2006) surmised that appendages (in ascomycetes) serve attachment to substrates rather than dispersal efficacy.

Hydrophobicity

Hydrophobic spores (mostly ornamented > Lotus effect) and are electrostatically attracted by water drops (Davies, 1961) and cuticles of arthropods (Ruddick and Williams, 1972). Therefore, hydrophobic spores can be transported by mist and insects. Rainfall washes out any spore larger than 2 μm (Henis *et al.*, 1987).

Germ pore

Germ pores have presumably evolved to allow thick-walled spores to quickly germinate, especially in coprophilous taxa (Garnica *et al.*, 2007) (**Plate 6-D**). Except for a few species (e.g., in *Inocybe*), spores of ectomycorrhizal taxa do not show germ pores (Halbwachs *et al.*, 2015), which may relate to prolonged dormancy.

Wall thickness

Thick spore walls are instrumental for taxa, which use aggressive substrates, e.g., dung dwellers (Garnica *et al.*, 2007) (**Plate 6-D**). Moreover, thick-walled spores may show prolonged dormancy (memnospores), while thin-walled xenospores are better suited for dispersal and quick germination (Dix and Webster, 1995). Note that there seems to be a correlation between spore wall thickness and spore size (Halbwachs and Bässler, 2020).

Plage (suprahilar depression)

The plage is a hydrophilic spot (**Fig. 8**) adjacent to the apiculum where Buller's drop hits the supra-apicular drop when the spore is discharged (Clémençon *et al.*, 2012). See also Sections "Spore Size" > "Discharge".

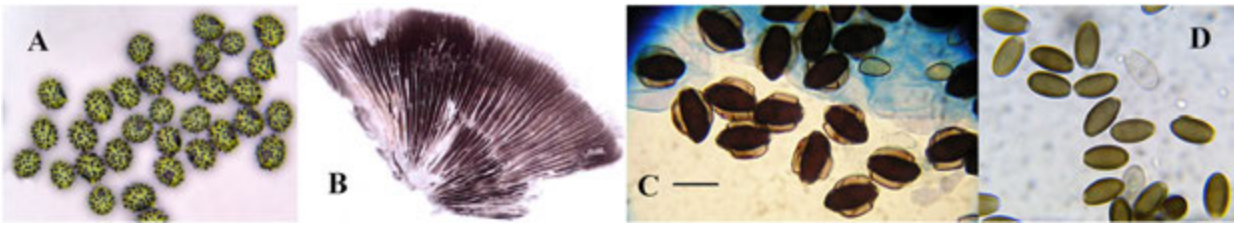

Plate 6 *Russula queletii* spores, bar 10 μm; B: *Psathyrella candolleana* spore print, bar 10 mm; C: Calyptrate spores of *Coprinopsis semitalis*, bar 10 μm; D: *Panaeolus olivaceus*, spores thick-walled with germ pore, bar 10 μm. A courtesy of Sven Kögel, B Adam Singer CC BY-SA 3.0, C courtesy of Andreas Gminder, D Byrain, CC BY-SA 3.0.

Guild-Specific Trait Syndromes

In the saprobic and ectomycorrhizal guilds, some specific morphological and behavioral traits are recognized.

Morphological Differences

Mycelial patterns differ between ectomycorrhizal and saprotrophic mushrooms. The former form hyphal mantles around rootles ("sheaths") that allow exchanging nutrients, water, and bioactive compounds (Smith and Read, 2008). Moreover, ectomycorrhizal mushrooms produce extramatrical mycelia and rhizomorphs of differing structures (Agerer, 2001) (**Plate 7**). The so-called exploration types are categorized according to their spatial outreach (**Table 2**).

Exploration types dynamically interact in the sense of competition and co-existence modulated by soil characteristics (Agerer, 2013). Some physiological features can be attributed to exploration types, e.g. the production of extracellular phenoloxidases by short- and medium-distance types (Agerer, 2001) or increased phosphorus uptake by long-distance foragers (Agerer, 2013). Nonetheless, the link between nutritional physiology and exploration type is not strict and often phylogenetically founded (Agerer, 2013).

Basidiomes of saprobic taxa are, on average, smaller than those of ectomycorrhizal mushrooms, probably because the latter obtain sufficient and reliable carbon from their hosts without directly investing in its uptake (Bässler *et al.*, 2015).

Spore traits also show some guild-specific characteristics. Contrary to saprobic mushrooms, the ectomycorrhizal guild shows more (hydrophobic) ornamentation and less thick-walled spores, while pigmentation is equally distributed between the guilds (Halbwachs *et al.*, 2015). However, the thick wall-melanized trait syndrome dominates in the saprobic guild, which could be an adaptation to wind dispersal and preferences for aggressive substrates. In the latter case, coprophilous taxa show a conspicuous

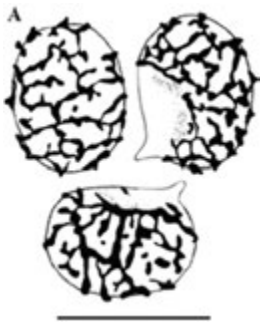

Fig. 8 Spores of Fig. *Lactarius laeticolor* with plage, bar 10 μm. Courtesy of Annemieke Verbeken.

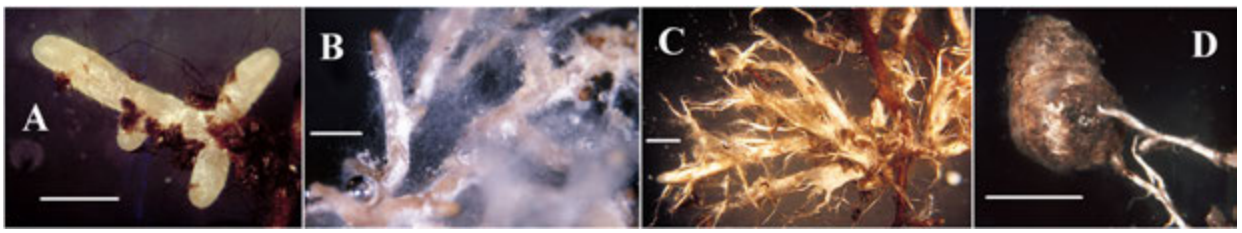

Plate 7 The predominant exploration types: A: contact (*Chroogomphus rutilus*), B: short-distance (*Hebeloma sacchariolens*), C: medium distance (*Cortinarius semisanguineus*), D – long-distance (*Suillus plorans*); bars A-C 1 mm, D 0,5 mm. A-D, source www.deemy.de, with permission.

Table 2 The main exploration types in ectomycorrhizal fungi and their foraging characteristics (subtypes omitted); contact type set at 0

	Relative space occupation	*Example (see Plate 7)*
Contact	0	*Chroogomphus rutilus*
Short-distance	1	*Hebeloma sacchariolens*
Medium-distance	2.5	*Cortinarius semisanguineus*
Long-distance	19	*Suillus plorans*

Note: Agerer, R., 2001. Exploration types of ectomycorrhizae. Mycorrhiza 11, 107–114. Weigt, R.B., Raidl, S., Verma, R., Agerer, R., 2012. Exploration type–specific standard values of extramatrical mycelium–a step towards quantifying ectomycorrhizal space occupation and biomass in natural soil. Mycological Progress 11, 287–297.

triple trait syndrome. Their spores are thick-walled, melanized, and larger than their non-coprophilous allies (Halbwachs and Bässler, 2020). The authors hypothesized that thick spore walls limit the spore volume left for nutrients needed for germination and that the general tendency within the agaricoid clade to produce thick-walled spores (Garnica *et al.*, 2007) could have facilitated the switch to a coprophilous lifestyle. The latter possess germ pores, which allows quick germination (Garnica *et al.*, 2007).

The more often occurring ornamentation in spores of the ectomycorrhizal guild has probably co-evolved with soil-dwelling arthropods. It increases the chances of the spores to hitch a ride and get in the vicinity of host rootlets to become established (Calhim *et al.*, 2018). Germ pores do practically not occur in the ectomycorrhizal guild (Halbwachs *et al.*, 2015), except for a few *Inocybe* and *Amanita* species, at least in Scandinavian countries (Knudsen and Vesterholt, 2012).

Physiological Differences

There are considerable differences in basidiome pigmentation between the saprobic (SAP) and the ectomycorrhizal (ECM) guilds. For one, ECM mushrooms in Europe are, on average, significantly darker than their mutualistic cousins, which is probably an important factor in thermal regulation (Krah *et al.*, 2019). Second, ECM basidiomes show 33% more often bright reddish hues (light purple to vermilion, saturation $>50\%$) compared to SAP mushrooms (α of χ^2 test <0.001) (based on the data set of Krah *et al.*, 2019), a trait that is predominantly associated with repelling and toxic as well as free radical scavenging qualities (Gill and Steglich, 1987).

It seems plausible that ectomycorrhizal mushrooms invest more often in toxic metabolites than the saprotrophic guild when since the latter need to invest in a more complex enzymatic toolset (N. Arnold, pers. communication).

Behavioral Differences

As stated above, ectomycorrhizal mushrooms have more pronounced host preferences than, e.g., wood decomposers. The ecological implications are twofold. Host specificity blocks competition, as the host does not accept other fungal taxa. On the other hand, dispersal and reproduction of host-specific fungi are more constrained than in promiscuous taxa, which have a broad choice of hosts (Molina *et al.*, 1992). This may not be overly limiting because host stands tend to be monotypic, especially at higher latitudes (Perry *et al.*, 2013). At arctic biomes, ectomycorrhizal mushrooms tend to be more promiscuous, probably because resource constraints select for optimizing establishment opportunities (Botnen *et al.*, 2014). One would expect that ectomycorrhizal mushrooms would preferably fruit during the late season when carbon allocation from the hosts is highest (Högberg *et al.*, 2010). Another study looking at ectomycorrhizal fruiting phenology of the ca. 2300 mushrooms described in the Funga Nordica (Knudsen and Vesterholt, 2012) revealed that this is not the case. Ectomycorrhizal and saprotrophic mushrooms behave quite similarly; both show a bimodal fruiting pattern (Halbwachs, 2019) (**Fig. 9**).

This surprising result points to two mechanisms. (1) Many ectomycorrhizal mushrooms accumulate nutrients in their mycelia, which can be used to fruit early in the next year, (2) the conspicuous fruiting drop during July may be an adaptation of avoiding fungivorous insects (Boddy and Jones, 2008) such as Mycetophilidae and Muscidae being most abundant during June to July (in Canada: Bolduc *et al.*, 2013). It is speculated that this pattern may be a strategy to also avoid competing soil microbiota by entering a mycelial diapause during which the formation of primordia is suppressed.

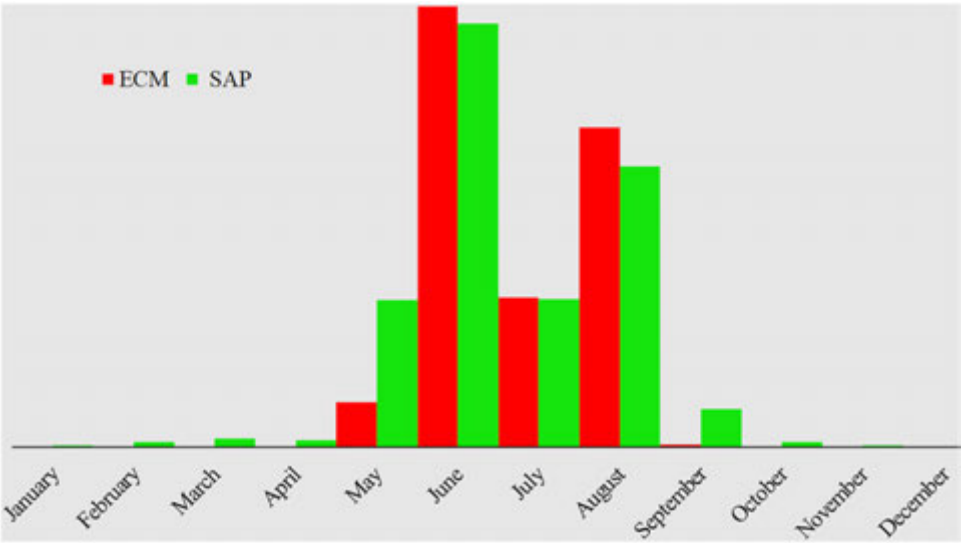

Fig. 9 Fruiting phenologies of Scandinavian ectomycorrhizal (ECM) and saprotrophic (SAP) mushrooms.

Future Directions

This compilation of macrofungal traits reveals numerous speculations and deficits. Therefore, efforts need to be increased to close such gaps, above all:

(1) Ancestral state analyses of traits relating to survival and reproduction, thus gaining a deeper understanding of trait functioning and syndromes.
(2) Applying comparative genomics to study the genetic foundation of the high morphological and physiological diversities.
(3) Investigating genets to elucidate trade-offs between mycelial and basidiome biomass of fungal guilds.
(4) Mining and analyzing data on mushroom traits to clarify the adaptive significance of phenotypes in view of environmental challenges, especially survival and reproductive fitness.
(5) Analysis of trait impacts on assemblages and ecosystem functioning.

The functional traits of Ascomycetes remain grossly understudied.

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Fossil Ascomycota and Basidiomycota, With Notes on Fossil Lichens and Nematophytes

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Introduction

Fungi are remarkably diverse, occur in virtually every type of environment, and affect the lives of other organisms in many different ways. Molecular studies suggest that there may be up to 3.8 million species of fungi today; however, only approximately 120,000 of them are formally described and named (Hawksworth and Lücking, 2017).

As carbon heterotrophs, fungi have evolved different nutritional modes and mastered various levels of cooperation with, and exploitation of, other organisms to acquire carbon (Dighton and White, 2017). Many degrade organic compounds such as lignin and cellulose and, through this recycling, return minerals to the soil and CO_2 to the atmosphere (Baldrian and Valášková, 2008; Hatakka and Hammel, 2010). Others partner with bacteria (Devau *et al.*, 2018), or team up with algae and cyanobacteria to form lichens (Büdel and Scheidegger, 2008), while still others enter into symbioses with plants (Brundrett and Tedersoo, 2018). Fungi have also evolved intimate relationships with animals (e.g., fungus-growing ants; see Chapela *et al.*, 1994); some even thrive within the animal, in anaerobic environments (Trinci *et al.*, 1994). On the other hand, parasitic fungi negatively affect the performance of other organisms and, as pathogens they are causative agents of many diseases (Harper and Krings, 2020).

Although the earliest reports of fossil fungi date back more than 150 years e.g., (Unger, 1847), the fungal component has been largely ignored until recently when interpreting fossil ecosystems. However, already the scattered historical evidence indicated that fungi were drivers of many crucial ecosystem functions also in the past. Paleontologists today take increased effort to document fossil fungi and assess the roles they played in the environments in which they lived. As a result, there is an increasing body of literature on fossil fungi, including studies that document complex levels of biological interaction.

Documentation of fossils is the only method to gain appreciation for the diversity of fungi through geologic time (Taylor *et al.*, 2015). Because this contribution is nested within the section "Macroscopic Fungi", which covers the Ascomycota and Basidiomycota with large fruiting bodies, it focuses on the fossil record of these two fungal lineages. The examples presented are not meant as an exhaustive review, but rather to demonstrate the types and quality of the evidence present. The fossil record of lichens is also briefly surveyed because these life forms today typically comprise members of the Ascomycota and Basidiomycota as mycobiont.

Fungal Evolution

The origin of the Fungi is estimated at between 660 Ma and up to 2.6 Ga ago based on molecular clock data and paleontological evidence [for the oldest (putative) fungal fossils, see Bengtson *et al.*, 2017; Loron *et al.*, 2019; Bonneville *et al.*, 2020; and the survey by Krings *et al.*, 2017b], and the divergence of the fungus-animal lineage from the plant lineage at between 780 Ma and up to 2.5 Ga ago (Lücking *et al.*, 2009; Berbee *et al.*, 2017).

The earliest fungi were aquatic. Just when these organisms first colonized the land surface remains an enigma, and how this arrival corresponds with the evolution of land plants an even further perplexing problem (Taylor *et al.*, 2015). There is evidence to suggest that fungi arrived on land approximately 1.0 Ga ago, when land plants had not yet evolved and the terrestrial ecosystems consisted of no more than biofilms and microbial mats made up of cyanobacteria in addition to various other microbes (Beraldi-Campesi, 2013). Nevertheless, these primeval ecosystems were no doubt critical for the early diversification of fungal nutritional modes and life histories. Although one can only speculate about the events during the terrestrialization of plants some 515–485 Ma ago (Morris *et al.*, 2018), a mutualistic symbiosis between some alga and a fungal partner likely was one necessary prerequisite to the establishment of plant life on land (Delaux *et al.*, 2015; Brundrett *et al.*, 2018). As terrestrial plants and ecosystems evolved, they progressively grew in stature, biomass, nutrient demand, and rooting depth to extensively colonize the continental land masses (Krings *et al.*, 2012). Molecular data and fossils indicate that the Fungi diversified, too, and readily became adapted to new types of environments and carbon sources. The largest morphological diversity in fungi today occurs in the Dikarya (i.e., Ascomycota and Basidiomycota), and is the expression of a long evolutionary success story (**Fig. 1**).

Modes of Fossil Preservation

The quality of information provided by fossil remains of Ascomycota and Basidiomycota depends on the way the fossils are preserved. For example, many types of propagules (e.g., conidia, ascospores) are durable structures that readily become preserved in a wide variety

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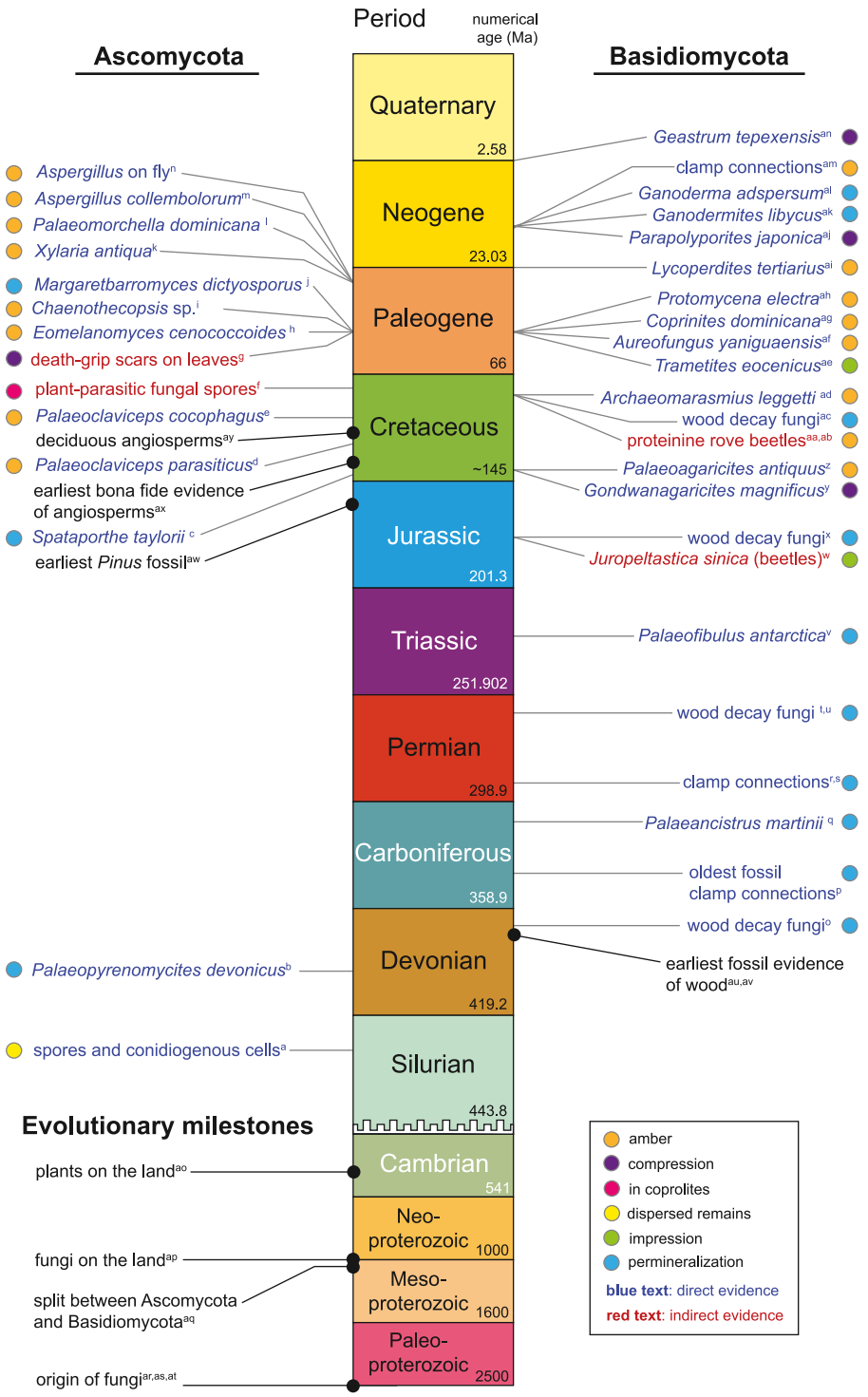

Fig. 1 Summary of selected examples of ascomycotan and basidiomycotan fossils used in this article plotted on the late Precambrian and Phanerozoic stratigraphic chart. Stratigraphic chart based on Cohen *et al.* (2013 updated). Major milestones in the evolution of life on land are illustrated to place the information into a broader context. References corresponding to taxa and milestones can be found in Appendix 1.

of settings and, consequently, are often recovered as dispersed microfossils by acid digestion of sediments and rocks (Kalgutkar and Jansonius, 2000). While these remains can be used in stratigraphic studies based on palynomorph assemblages (e.g., Graham, 1962; Pieńkowski *et al.*, 2011), and some appear to have relevance also as proxy indicators of paleoecological events (e.g., Lageard and Ryan, 2013; Rampino and Eshet, 2018), they do not normally represent a major source of information on the biology of ancient fungi. Conversely, the complex fruiting bodies (basidiomata) of free-living macrofungi, especially Agaricomycotina, are highly informative

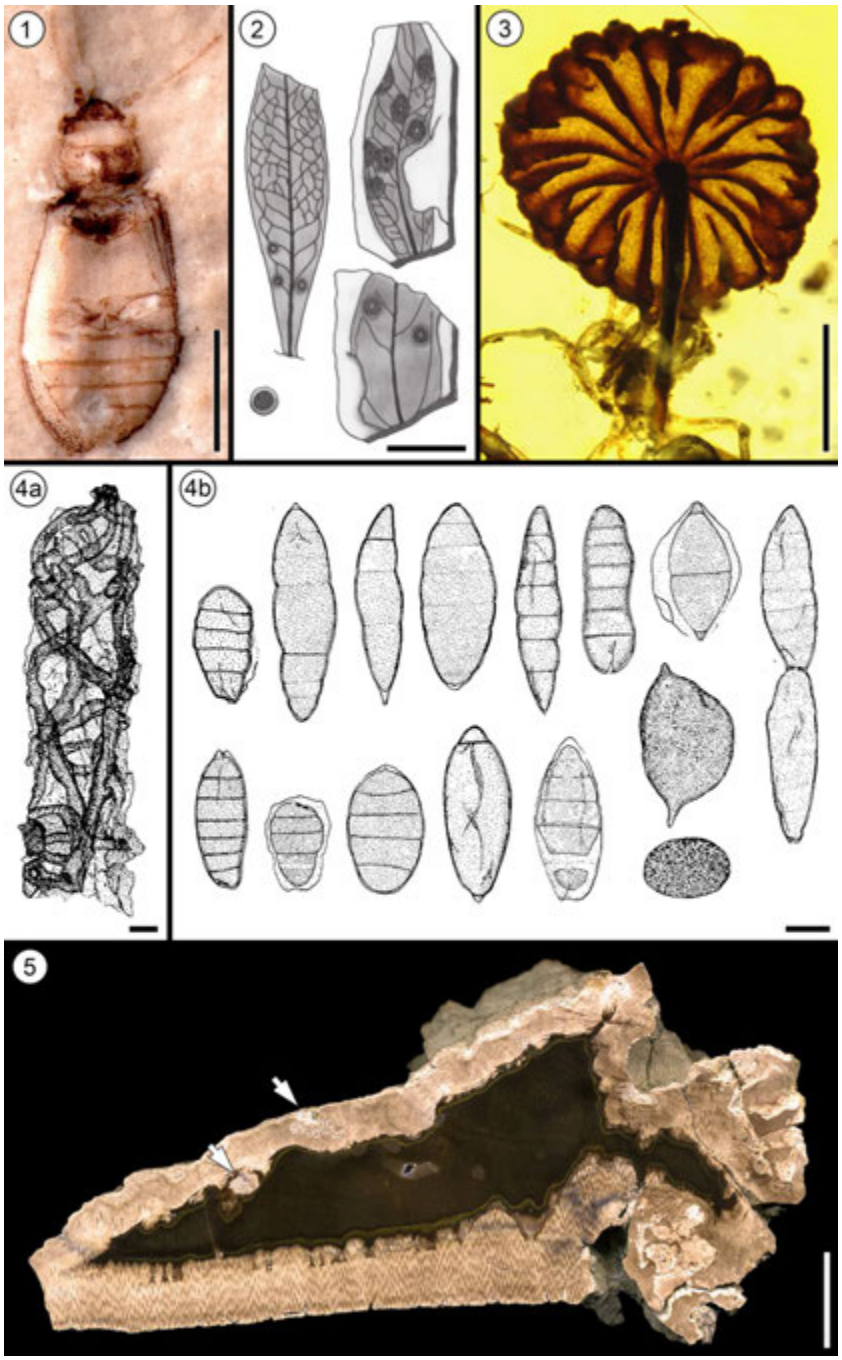

Plate 1 Examples of preservation types of ascomycotan and basidiomycotan fossils. Fig. 1 Impression of tooth-necked fungicolous fungus beetle; Middle Jurassic of China (from Cai *et al.*, 2014: Fig. 1B); bar 1 mm. Fig. 2 Parasitic *Sclerotium cinnamomum* Heer on cinnamon leaf from Rixhöft Lignite (Rozewie, Poland) (from Heer, 1869: Pl. 7, Figs. 21–22b); bar ca. 10 mm, anamorph(?) Ø left upper corner ca. 1 mm. Fig. 3 Amber fossil of mushroom similar to the extant genus *Marasmius*; Myanmar, Middle Cretaceous (ca. 100 Ma) (from Cai *et al.*, 2017: Fig. 1a); bar 1 mm. Fig. 4 Fungal fragments associated with coprolites (Upper Silurian $\sim$425 Ma); 4a: fragment with hyphae; 4b: various ascospores (from Sherwood-Pike and Gray, 1982: Fig. 2 G + 6); bars 10 μm. Fig. 5 Longitudinal section through well preserved bracket fungus (*Ganodermites libycus*); Libya, Lower Miocene (ca. 20 Ma); original image of specimen in SNSB-BSPG collection in Munich, Germany, accession number SNSB-BSPG 1997 I 80; note coprolite-filled feeding tunnels of fungivorous arthropods (arrows); bar 10 mm. Reproduced from Cai, C., Lawrence, J.F., Ślipiński, A., Huang, D., 2014. First fossil tooth-necked fungus beetle (Coleoptera: Derodontidae): Juropeltastica sinica gen. n. sp. n. from the Middle Jurassic of China. European Journal of Entomology 111, 299–302. Heer, O., 1869. Miocene baltische Flora, W. Koch, Königsberg. Cai, C., Leschen, R.A., Hibbett, D. S., Xia, F., Huang, D., 2017. Mycophagous rove beetles highlight diverse mushrooms in the cretaceous. Nature Communications 8, 14894.

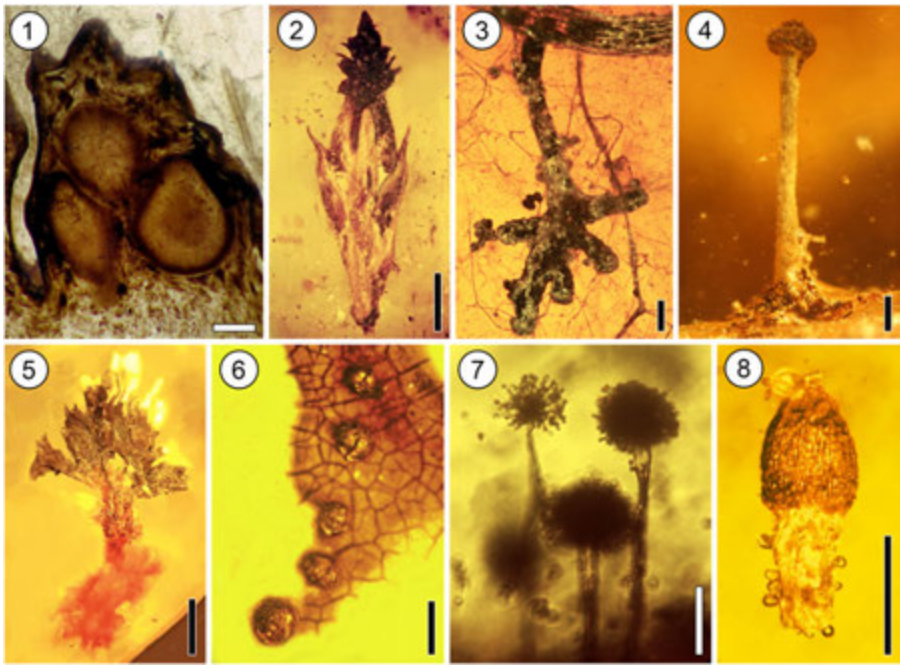

Plate 2 Fossil Ascomycota. Fig. 1 Longitudinal section through perithecia of *Paleopyrenomycetes devonicus* Taylor, Hass, Kerp, Krings et Hanlin, an ascomycete that colonized an early club moss during the Devonian; bar 100 μm. Fig. 2 *Palaeoclaviceps parasiticus* on a grass floret; Myanmar amber, Lower Cretaceous (ca 100 Ma); bar 1.6 mm. Fig. 3 *Dipterocarpus* rootlet wrapped in hyphal mantle typical of ectomycorrhizal; India, Lower Eocene (ca. 52 Ma). The fungus, an ascomycete, has been named *Eomelanomyces cenococcoides* Beimforde, Dörfelt et A.R. Schmidt; bar 100 μm. Fig. 4 *Chaenothecopsis* sp. from Baltic amber (Eocene, ca 40 Ma); bar 200 μm. Fig. 5 *Xylaria antiqua* in Dominican amber (ca 15–20 Ma); bar 2.4 mm. Fig. 6 Miocene pycnidia, *Palaeomycus epallelus*, in Dominican amber (ca 15–20 Ma); bar 0,82 mm. Fig. 7 *Aspergillus* conidia in Dominican amber (ca 15–20 Ma); bar 100 μm. Fig. 8 *Paleomorchella dominicana* in Dominican amber (ca 15–20 Ma); bar 1 mm.

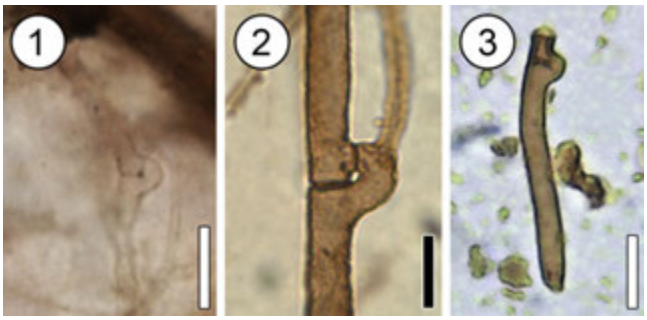

Plate 3 Clamp connections from different periods in geologic time.Fig. 1 Oldest fossil evidence to date of hyphae with clamp connection (Mississippian, ca 330 Ma); original image, copyright M. Krings, bar 10 μm. Fig. 2 Lower Permian (ca 290 Ma); original image of hypha in thin section from SNSB-BSPG collection in Munich, Germany, accession number SNSB-BSPG 1971 XVII 535; bar 5 μm. Fig. 3 Miocene (ca 14 Ma), bar 10 μm. Reproduced from Halbwachs, H., 2019a. Detecting fungal spores and other micro-fossils in amber and copal by solvent treatment. Palynology 44. Available at: https://doi.org/10.1080/01916122.2019.1633436.

biologically; however, most are short-lived and disintegrate within a few days (Nagy *et al.*, 2017), with next to no chance of becoming preserved in recognizable form. As a result, these structures are exceedingly rare as fossils. Common modes of preservation where fungal macrofossils might be found include amber, impression and compression, as well as petrifaction and permineralization (Taylor *et al.*, 2015). Moreover, there are several lines of indirect evidence that can be used to infer the presence of fungi in past ecosystems.

Amber

Amber, a solidified and fossilized tree resin produced by various conifers and angiosperms, is an excellent preservation medium that provides detailed insights into the diversity of soft-bodied organisms (Seyfullah *et al.*, 2018). Today amber is the most productive source of new information on fossil Ascomycota, Basidiomycota, and lichens (Kettunen *et al.*, 2018, 2019; Halbwachs,

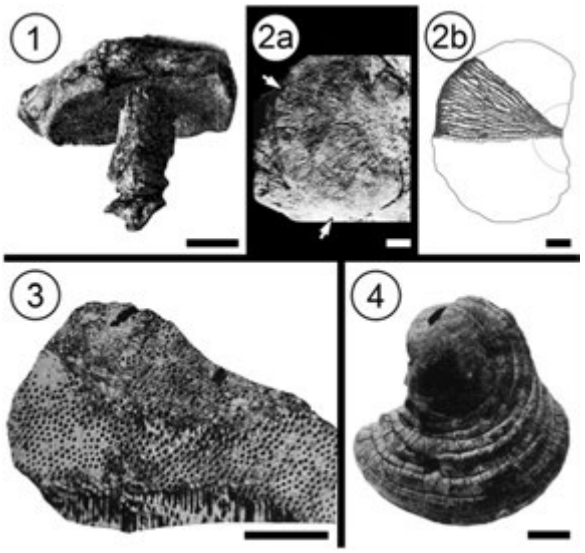

Plate 4 Fossil basidiocarps.Fig 1 "*Pseudopolyporus*", like many other supposed Paleozoic larger "fungi" this form was later ascribed to a different origin (Pirozynski, 1976), bar 10 mm; Fig. 2 *Trametites eocenicus* (arrows) (**Fig. 1**); Fig. 3 Outline of *T. eocenicus* redrawn from Knobloch and Kotlaba (**Fig. 1**) retrieved from https://advance.science.sfu.ca/fungi/fossils/Kalgutkar_and_Jansonius/index.php?-link=, Late Eocene (ca 38 Ma), bar 10 mm. Fig. 4 *Fomes idahoensis* (Brown, 1940), Early Pliocene (ca 4 Ma), bar 5 mm. Fig. 5 *Fomes applanatus* (Chaney *et al.*, 1936), Pleistocene (0.01–2.6 Ma), bar 20 mm. Reproduced from Hollick, A., 1910. A new fossil polypore, Pseudopolyporus carbonicus, Carboniferous of W.Va. Mycologia 2, 93–94. Knobloch, E., Kotlaba, F., 1994. Trametites eocenicus, a new fossil polypore from the Bohemian Eocene. Czech Mycology 47, 207–213.

2019b) (**Plates 1–3**). However, almost all amber comes from Cretaceous and Cenozoic strata, which is too geologically recent to record the origin or early evolution of most major lineages of fungi.

Impressions and Compressions

Impression fossils are negative reliefs that lack any organic remnants of the original organism. Some of the earliest reports of fossil macrofungi consisted of leaf impressions with various spots on them interpreted as fungi (e.g., Unger, 1847; Meschinelli, 1898) (**Plates 1-1**). Conversely, compressions are a type of preservation in which some (or all) of the original organic matter still remain, albeit usually altered in the form of coalification. Compressions of leaves may also show specks or black spots that have been interpreted as fungi (Pirozynski and Weresub, 1979) (**Plates 1–2**). Moreover, during the chemical preparation (maceration) of compressed leaves for cuticular analysis, fungi otherwise not seen become visible and, as a result, represent an expansive source of new information on associations of endophytic and epiphyllous fungi with ancient plants (e.g., Dilcher, 1965; Krings, 2001; Worobiec and Worobiec, 2017). In fact, our current knowledge of Mesozoic and Cenozoic epiphyllous Ascomycota is based primarily on chemically cleared leaf compressions.

Petrifactions and Permineralizations

Petrifactions and permineralizations are two types of preservation that provide exceptional resolution of (parts of) organisms that became immersed in water with high concentrations of dissolved minerals (e.g., silica, calcium carbonate) (**Plates 1–5**). Once the mineral is precipitated and hardened, the organism is entombed in rock and often faithfully preserved down to the finest anatomical details. The principal difference between petrifaction and permineralization is that the cell walls of the former are replaced by minerals, whereas they are still organic in the latter. Petrified or permineralized evidence of Ascomycota and Basidiomycota is known since the Devonian and Carboniferous, respectively, and usually occurs in the form of hyphae and propagules in cells and tissues of plant parts preserved by means of petrifaction or permineralization (Taylor *et al.*, 2015); however, there are also instances in which larger parts of these fungi (e.g., polyporalean basidiomes) are petrified or permineralized (e.g., Fleischmann *et al.*, 2007).

Indirect Evidence

One type of fossil that is not itself a fungus, but that may contain evidence of ancient fungi, is coprolites, fossilized animal feces or dung. Fungi are often well preserved in coprolites, and may provide information on environmental conditions, animal diet, and

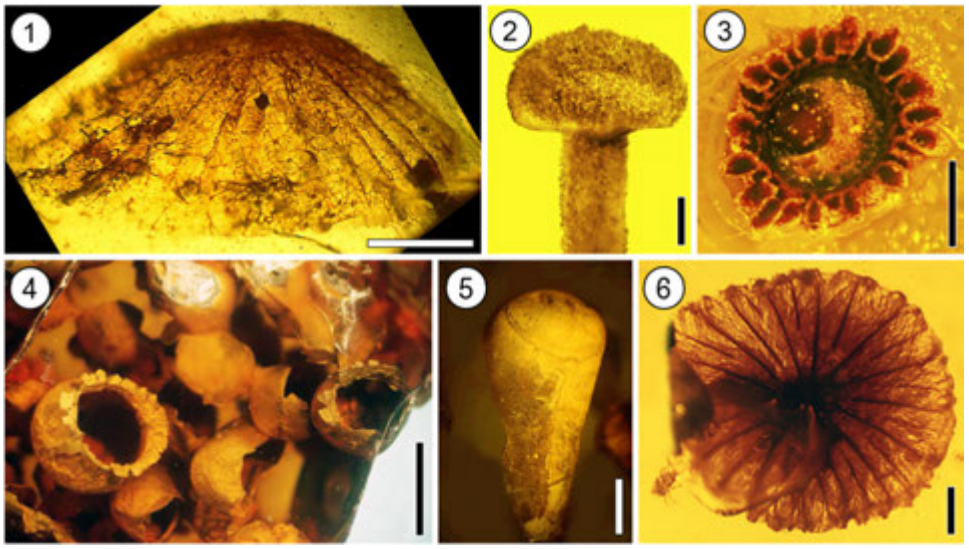

Plate 5 Fossil gilled mushrooms in amber. Fig 1 *Palaeoagaracites antiquus*, Myanmar amber (Cretaceous, 15–20 Ma), bar 0.5 mm. Fig. 2 *Gerontomyces lepidotus*, Myanmar amber (Cretaceous, 15–20 Ma), bar 0.2 mm. Fig. 3 *Nidula baltica*, Baltic amber (Eocene, 38–47 Ma), bar 0.4 mm. Fig. 4 *Palaeogaster micromorpha*, Myanmar amber (Cretaceous, 15–20 Ma), bar 3 mm. Fig. 5 *Lycoperdites tertiarius*, Mexican amber (Miocene, 13–19 Ma), bar 2 mm. Fig. 6 *Coprinites dominicana*, Dominican amber (Miocene, 15–20 Ma), bar 0.5 mm.

sometimes even on fungal interactions (**Plates 1–4**). For example, coprolites of herbivorous dinosaurs have been described that contain remains of plant-parasitic fungi, and thus document the presence of this interaction in the fossil record (Sharma *et al.*, 2005).

Other indirect evidence occurs in the form of fossilized fungivorous animals. One interesting study infers the existence of diverse Early Cretaceous Agaricomycotina, as well as a specialized trophic interaction and ecological community structure, from the presence of diverse rove beetles (Staphylinidae) in amber from Myanmar (Cai *et al.*, 2016b, 2017). Moreover, several studies describe polypore fungus beetles from amber that add support to the hypothesis that polyporous mushrooms diversified during the Mesozoic (e.g., Soriano *et al.*, 2014; Cai *et al.*, 2016a; Yun *et al.*, 2018). Indirect evidence of the presence of fungi in certain paleoenvironments also occurs in the form of impression and compression fossils of mycophagous (feeding on fungi) animals. An excellent example of this is a fossilized derodontid beetle from the Jurassic of China (Cai *et al.*, 2014) (**Plates 1–1**).

Information on fungal evolution also comes from geochemical biomarkers (Marynowski *et al.*, 2013), as well as from molecular data obtained from extant organisms. For example, wood-degrading Ascomycota and Basidiomycota constitute one of the major drivers of carbon cycling in forest ecosystems today (White, 2003; Lonsdale *et al.*, 2008). Our current conception of the evolutionary history of these fungi and their roles in ancient ecosystems is largely derived from molecular studies, while fossils (e.g., silicified wood showing characteristic patterns of degradation and decay symptoms) have only been used in a limited sense (see review by Harper *et al.*, 2017).

Fossils of Ascomycota and Basidiomycota

Although some molecular clock analyzes set the Ascomycota-Basidiomycota split at 1000–1490 Ma (see Lücking and Nelsen, 2018), which would place their origin in the Proterozoic, the geologic history of these two fungal lineages based on credible fossil evidence only extends back into the Paleozoic (Taylor *et al.*, 2015).

Ascomycota

The Ascomycota (sac fungi) is the largest group of extant Fungi, with ~65,000 described species that thrive as saprotrophs, parasites of plants, animals, and other fungi, and those forming symbiotic associations in lichens (see below); Ascomycota also form mycorrhizal relationships with plants (Tedersoo *et al.*, 2009). The principal character is the ascus, a sac-like structure (technically a meiosporangium) in which fusion of nuclei takes place that is followed by meiosis and the formation of non-motile ascospores (Pöggeler *et al.*, 2018). However, numerous ascomycetes are asexual (anamorphic), i.e., they lack a sexual phase (perfect stage); at least, a sexual stage has not been described or observed to date in these forms.

Chains of multiseptate spores and conidiogenous cells from the Silurian of Sweden are the oldest fossils to date thought to belong to the Ascomycota (Sherwood-Pike and Gray, 1985). The oldest structurally preserved fossil ascomycete is *Paleopyrenomycites devonicus* from the Lower Devonian Rhynie chert (Taylor *et al.*, 2005). This fossil occurs in leaf-like appendages of the

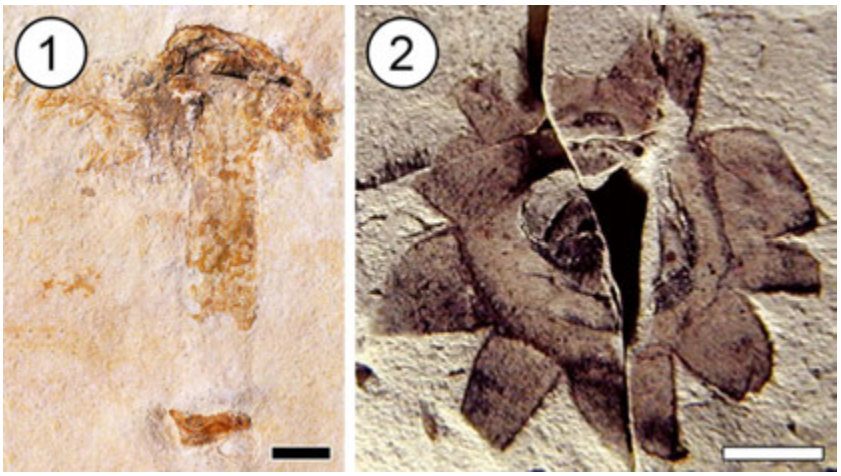

Plate 6 Compression fossils of Basidiomycota. Fig. 1 *Gondwanagaricites magnificus*, a gilled mushroom from the Cretaceous (113–120 Ma) of Brazil; gill remains well recognizable in top left portion, bar 5 mm. Fig. 2 Carbonized compression of an earthstar, Puebla/Mexico, Miocene to Pleistocene(?) (2–14 Ma?), possibly earlier (Lower Oligocene, ca. 30 Ma; Fig. 2), bar 5 mm. Reproduced from Magallon-Puebla, S., Cevallos-Ferriz, S.R., 1993. A fossil earthstar (Geasteraceae; Gasteromycetes) from the late Cenozoic of Puebla, Mexico. American Journal of Botany 80, 1162–1167.

early land plant *Asteroxylon mackiei* and consists of perithecia characterized by ostiolate necks protruding from the host epidermis through stomatal pores. Lining the interior of the perithecium are paraphysis interspersed with asci containing up to 16 ascospores.

Mesozoic and Cenozoic deposits have yielded abundant remains of Ascomycota (Taylor *et al.*, 2015); noticeable are several permineralized specimens (e.g., Mindell *et al.*, 2007; Bronson *et al.*, 2013), amber fossils of fruiting bodies (ascocarps or ascomata), including *Paleomorchella dominicana*, the first fossil of a morel (Poinar, 2016), and *Xylaria antiqua*, a fossil attributable to the family Xylariaceae (Poinar, 2014), both preserved in Miocene amber from the Dominican Republic, as well as records in Eocene Baltic amber of asexual reproductive structures of anamorphic fungi with probable affinities to the present-day genera *Periconia*, *Penicillium*, and *Scopulariopsis* (Tischer *et al.*, 2019).

Equally noticeable is the diversity of hyphae, fruiting bodies (e.g., thyriothecia, pycnidia), and hyphopodia (i.e., outgrowths from the mycelium that attach the fungus to the host) of microthyriaceous fungi on leaves. The vast majority of these remains have been obtained through maceration of compression fossils (e.g., Dilcher, 1965; Sun *et al.*, 2015; Bannister *et al.*, 2016), but there are also a few specimens in amber (Poinar, 2018). For the most part, the nutritional modes of fossil leaf-inhabiting fungi remain unknown; however, some authors note morphological similarities to modern plant pathogens such as *Asterina*, *Vizella*, and *Trichothyrina*, thus inferring the nutritional mode as parasitism (Taylor *et al.*, 2015).

There is also fossil evidence of other types of organismal interactions involving members of the Ascomycota, much of which is preserved in Cretaceous and Cenozoic amber. For example, from lower Eocene amber from India, Beimforde *et al.* (2011) report an ectomycorrhizal fungus, *Eomelanomyces cenococcoides*, which closely compares to an extant member of the Dothidiomycetes. An interesting case of parasitism occurs in the form of a spectacular specimen of a fungus described as *Paleoophiocordyceps coccophagus*, which was a parasite of Cretaceous scale insects (Sung *et al.*, 2008). This fossil provides the oldest compelling evidence of animal parasitism by fungi and is characterized by several synnemata emerging from the head of the host. Another case of animal parasitism comes from the Eocene and documents adaptive manipulation of ants by a parasitic ascomycete in the form of stereotypical death grip scars preserved in compressed angiosperm leaves (Hughes *et al.*, 2011). An interaction involving an ascomycete is also evidenced by a *Claviceps*-like sclerotium (*Palaeoclaviceps parasiticus*) that occurs on a grass floret entombed in Burmese amber (Poinar *et al.*, 2015). This fossil establishes the existence of intricate interactions between the ascomycete family Clavicipitaceae and flowering plant family Poaceae in the Early-mid Cretaceous. Moreover, Poinar *et al.* (2015) suggest that, if animals ingested the infected grass, they may have felt the effects of the psychotropic compounds produced by the fungus. Hallucinogenic substances produced by fungi often cause aversion for this fungus (Camazine, 1983), or they incite the intoxicated vector to erratically move around, thereby dispersing the spores of the fungus more effectively (Boyce *et al.*, 2019).

Resin exudates provide plants with protection against parasites and phytopathogenic microorganisms; however, some highly specialized fungi are also known to grow exclusively on these exudates (Beimforde and Schmidt, 2011; Beimforde *et al.*, 2017). The presence of resinicolous ascomycetes of the genus *Chaenothecopsis* in Eocene Baltic and Oligocene Bitterfeld ambers documents that these ascomycetes were already well adapted to their special ecological niche by the Eocene, and their morphology has since remained remarkably constant (Tuovila *et al.*, 2012). Baltic amber has also yielded an exquisite example a springtail overgrown by conidiophores of the fossil fungus *Aspergillus collembolorum* (Dörfelt and Schmidt, 2005). A second fossil *Aspergillus* comes from Dominican Republic amber and occurs in the form of tufts of catenulate chains of conidia covering the abdomen of a fly (Thomas and Poinar, 1988).

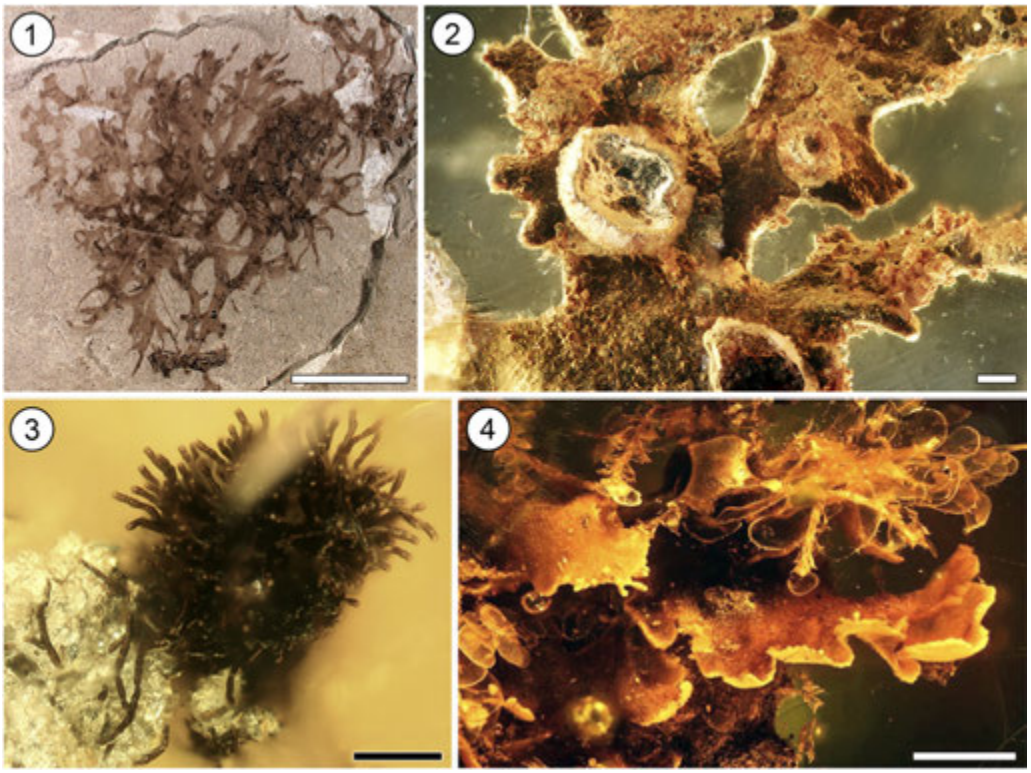

Plate 7 Fossil lichens. Fig. 1 Compression fossil from Middle Jurassic of Mongolia (ca 170 Ma) interpreted as a fruticose lichen; original image; bar 5 mm. Fig. 2 Foliose lichen from Baltic amber (Eocene, ca 38–47 Ma) (from Hartl *et al.*, 2015: Fig. 1 C); bar 5 mm. Fig. 3 *Taeniolella* aff., a crustose lichen preserved in Bitterfeld amber (Oligocene, ca 23.8–25.3 Ma) (from Kettunen *et al.*, 2017: Fig. 4A); bar 100 μm. Fig. 4 The squamulose *Phyllopsora magna* from Dominican amber (Miocene, ca 15–20 Ma) (from Kaasalainen *et al.*, 2018: Fig. 2A); bar 1 mm. Reproduced from Hartl, C., Schmidt, A., Heinrichs, J., Seyfullah, L., Schäfer, N., *et al.*, 2015. Lichen preservation in amber: Morphology, ultrastructure, chemofossils, and taphonomic alteration. Fossil Record 18, 127–135. Kaasalainen, U., Heinrichs, J., Renner, M.A., Hedenäs, L., Schäfer-Verwimp, A., *et al.*, 2018. A Caribbean epiphyte community preserved in Miocene Dominican amber. Earth and Environmental Science Transactions of the Royal Society of Edinburgh 107, 321–331.

Basidiomycota

The Basidiomycota constitutes a major phylum of the kingdom Fungi and, with today more than 31,000 described species, are second in species numbers to the Ascomycota (He *et al.*, 2019). The diagnostic trait is sexual reproduction via meiospores that develop on projections of a club-shape structure termed the basidium (Coelho *et al.*, 2017). Another feature generally attributed to the Basidiomycota is the clamp connection, a hyphal protrusion that develops at cell division to facilitate maintenance of the dikaryon condition by providing a bypass for one of the nuclei (**Plate 3**).

The oldest generally accepted basidiomycete fossils with this feature occur in a structurally preserved fern stem from the Mississippian (Lower Carboniferous) of France, and comprise abundant septate hyphae with clamp connections that pass from cell to cell (Krings *et al.*, 2011) (**Plate 3**–1). Similar hyphae in a Middle Pennsylvanian (Upper Carboniferous) fern rhizome from North America have been named *Palaeancistrus martinii* (Dennis, 1970). Development of hyphal branches from clamp-connections, which is a common feature among certain modern basidiomycetes (Routien, 1948), has been documented for both these Carboniferous forms. Slightly younger, Early Permian hyphae with clamp connections have been discovered in a tree fern root mantle from Germany (Krings *et al.*, 2017a) (**Plates 3**–2, **3**) and in a gymnosperm stem from China (Wan *et al.*, 2017). Mesozoic evidence of clamp connections comes from the Triassic of Antarctica (Osborn *et al.*, 1989), and Cenozoic clamp-bearing hyphae have been documented in Miocene amber from Sumatra (Halbwachs, 2019a) (**Plates 3**–**4**). Silicified gymnosperm wood degraded by wood-decaying fungi from the Permian and Triassic of Antarctica show irregular areas lacking cells, and septate hyphae with clamp connections (Harper *et al.*, 2017). The decay pattern in these fossils is comparable in appearance to present-day rots caused by basidiomycetes. Basidiomycota have also been identified as the causal agents for decay patterns in conifer wood specimens from the Jurassic of Argentina (Sagasti *et al.*, 2019) and the Cretaceous of China (Tian *et al.*, 2020) based on the presence of clamp-bearing hyphae in the decayed areas of the wood.

Molecular clock data have been used to suggest that the complement of enzymes necessary in lignin degradation evolved in fungi during the Early Permian, and that the decline of organic carbon at the end of the Carboniferous was directly correlated to the evolution of fungal lignin-degradation (Floudas *et al.*, 2012). Most, however, dismiss this hypothesis and regard the decline of

coal at the end of the Carboniferous as a result of a combination of climatic and tectonic factors (Nelsen *et al.*, 2016). Moreover, the oldest persuasive fossil wood showing disease symptoms suggestive of wood-degrading fungi comes from the Upper Devonian (Stubblefield *et al.*, 1985), and thus predates the age estimate given by Floudas *et al.* (2012) for the evolution of fungal lignin degradation by roughly 75 million years.

Fruiting bodies of Basidiomycota (basidiocarps or basidiomata) are exceedingly rare (or rarely recognized) as impression or compression fossils; however, there are quite a number of specimens preserved in amber and as permineralizations. Foremost among the Basidiomycota with a somewhat more extensive fossil record are the polypores (bracket fungi, conks), a morphologically heterogeneous assemblage of more than 1000 extant species in many families. Basidiomata of these fungi are often persistent (sometimes for several years) and possess a robust texture (Clémençon *et al.*, 2012), and thus are more likely to become fossilized than other types of basidiomata (**Plate 4**). Although there have been some reports of polyporous basidiomata from the Paleozoic and early Mesozoic (e.g., Herzer, 1895; Hollick, 1910; Fohrer and Simon, 2002, 2003), most of these specimens have subsequently been discounted or reinterpreted as other organisms (e.g., Kiecksee *et al.*, 2012). On the other hand, Cenozoic deposits have yielded numerous persuasive specimens of fossil polypores, including *Trametites eocenicus* from the Eocene of Bohemia (Knobloch and Kotlaba, 1994), *Parapolyporites japonica* (Tanai, 1987) from the Miocene of Japan, *Ganodermites libycus* from the Miocene of Libya (Fleischmann *et al.* 2007), and *Ganoderma adspersum* from the Miocene of the Netherlands (Fraaye and Fraaye, 1995).

The fossil record of gilled mushrooms is almost entirely comprised of specimens preserved in amber (Poinar, 2016) (**Plate 5**). However, the most remarkable recent discovery is *Gondwanagaricites magnificus*, a fossil mushroom from the Lower Cretaceous of Brazil that does not occur in amber, but rather is preserved as a mineralized replacement in laminated limestone (Heads *et al.*, 2017) (**Plate 6**). The oldest amber fossil of a gilled mushroom, *Palaeoagaricites antiquus* from the Lower Cretaceous of Myanmar, consists of a portion of a cap, 2.2 mm in diameter, with 16 radial furrows (Poinar and Buckley, 2007). The systematic position of *P. antiquus* remains unresolved; however, morphology and habitat (conifer trunks) suggest affinities with the genera *Mycena*, *Marasmius* or *Collybia*. Several 1 cm-sized gilled mushrooms are known from Eocene amber of the Dominican Republic. One of them is *Coprinites dominicana*, a fossil believed to have affinities with the modern genus *Coprinus* (Poinar and Singer, 1990), while another, *Protomycena electra*, is similar to present-day members of the genus *Mycena* (Hibbett *et al.*, 1997). *Aureofungus yaniguaensis* is the third gilled mushroom known from Dominican amber (Hibbett *et al.*, 2003). Other fossil gilled mushrooms are more difficult to accurately place within a phylogenetic framework. One of them is *Archaeomarasmius leggetti* (Hibbett *et al.*, 1997), known from Cretaceous amber from East Brunswick, New Jersey (USA), which appears similar to the extant genera *Marasmius* and *Marasmiellus*.

Fossils attributable to other lineages of the Basidiomycota have also been described, including *Lycoperdites tertiarius*, a member of the Gasteromycetes (puffballs) that is based on a set of 23 basidiomata at different stages of maturity, all preserved in a single piece of late Oligocene–early Miocene Mexican amber (Poinar, 2001). Another fossil gasteromycete, *Geastrum tepexensis*, is a compression fossil of an earth star from the Miocene–lower Pleistocene of Mexico (Magallon-Puebla and Cevallos-Ferriz, 1993) (**Plate 6**).

Lichens

Lichens are mutualistic associations of fungi and algae or cyanobacteria, or both, with body plans (thalli) and physiological properties that are different from either of the partners (Büdel and Scheidegger, 2008). The fossil record of lichens is exceedingly incomplete and includes only a few bona fide examples older than Cretaceous (Lücking and Nelsen, 2018). The crux of the matter is that the presence of both partners must be demonstrated to establish the existence of a fossil lichen. Furthermore, some degree of stable interdependence between the partners needs to be present. Most pre-Cretaceous fossils, interpreted as lichens or lichen-like associations, fail one or both of these criteria.

Early Devonian rocks from the Welsh Borderland have yielded *Cyanolichenomycites devonicus* and *Chlorolichenomycites salopensis*, two charcoalified bona fide lichen thalli that display thallus anatomies, similar to present-day heteromerous lichens (Honegger *et al.*, 2013). The thallus of *C. devonicus* is composed of a cortex several cell layers thick and a medulla of septate hyphae and presumed colonies of *Nostoc*-like cyanobacteria. On the other hand, fractured sections of *C. salopensis* show septate hyphae intermixed with globose cells thought to represent a green alga morphologically comparable to the common extant lichen photobiont *Trebouxia*. Perhaps the most widely known fossil interpreted as a lichen is *Winfrenatia reticulata* from the Lower Devonian Rhynie chert in Scotland (Taylor *et al.*, 1997). Specimens occur in the form of superimposed layers of parallel hyphae, the uppermost of which are folded vertically into loops that form a pattern of ridges and depressions. Extending from the walls of the depressions are hyphae that form a three-dimensional network of lacunae. Coccoid cyanobacteria surrounded by a prominent sheath regularly occur within the lacunae of the hyphal net. However, Lücking and Nelsen (2018) recently have questioned the lichen nature of *W. reticulate*, and suggested the fossil rather represents a cyanobacterial colony with fungal hyphae growing through it.

One of the few Mesozoic fossils believed to represent lichens is *Daohugouthallus ciliiferus*, an impression from the Jurassic of China of a small thallus composed of elongate primary axs that give rise to lateral and terminal branches (Wang *et al.*, 2010) (**Plate 7**–1). Filiform appendages morphologically resembling cilia arise from the thallus. On the other hand, *Honeggeriella complexa* from the Lower Cretaceous of British Columbia, Canada, is a permineralized bona fide lichen thallus that consists of a thick upper

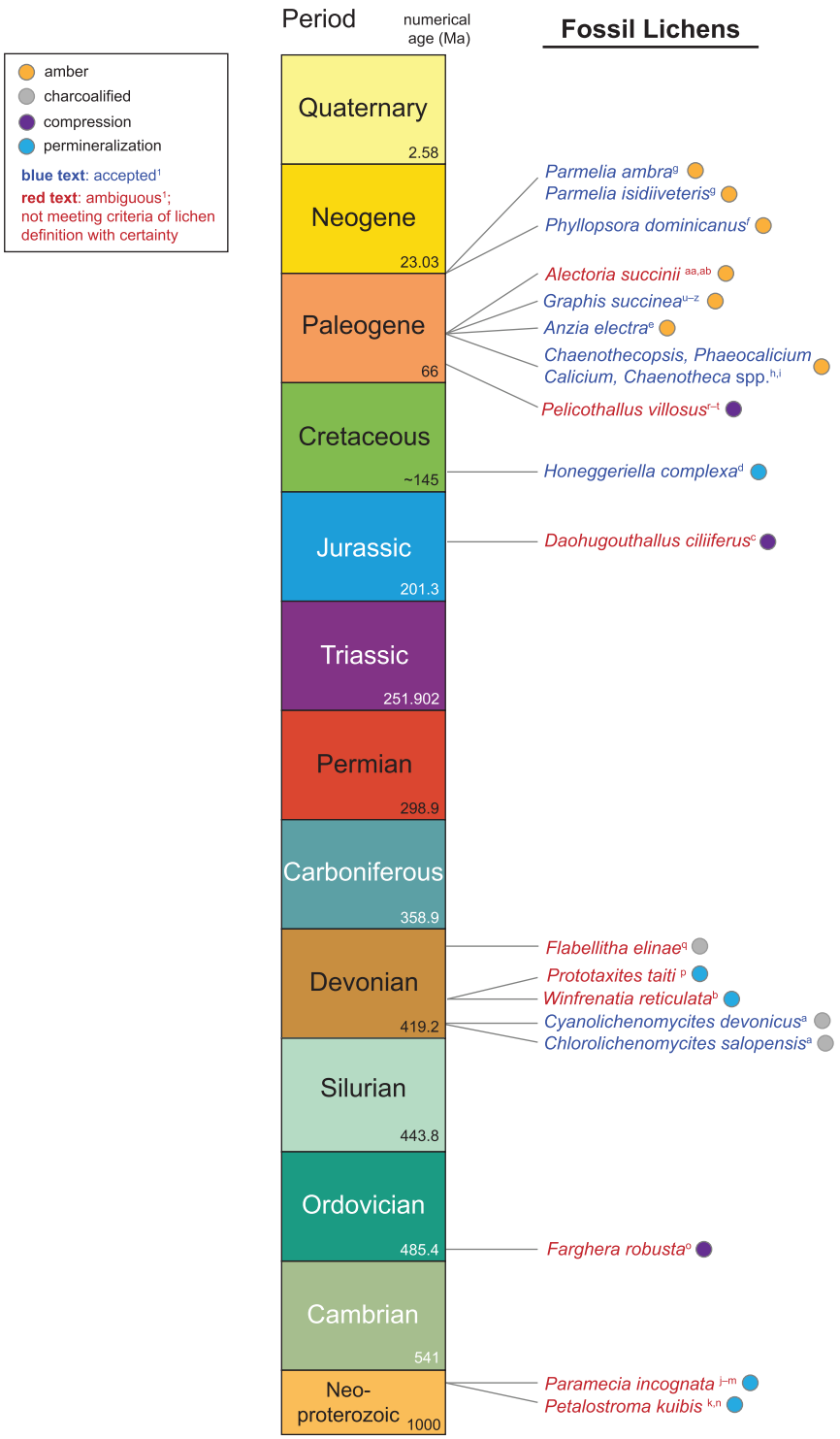

Fig. 2 Selected fossil lichens plotted on the late Precambrian and Phanerozoic stratigraphic chart. Stratigraphic chart based on Cohen *et al.* (2013 updated). [1]Accepted and ambiguous statuses of fossil lichens is based on Lücking and Nelsen (Tab. 23.1). References corresponding to taxa can be found in Appendix 2. Reproduced from Lücking, R., Nelsen, M.P., 2018. Ediacarans, protolichens, and lichen-derived Penicillium: A critical reassessment of the evolution of lichenization in fungi. In: Krings, M., Harper, C.J., Cúneo, N.R., Rothwell, G.W. (Eds.), Transformative Paleobotany: Papers to Commemorate the Life and Legacy of Thomas N. Taylor. London, San Diego, CA, Cambridge, MA, Oxford: Elsevier/Academic Press Inc. pp. 551–590.

Fig. 3 *Prototaxites* in a reconstructed Devonian landscape, visualized by Geoffrey Kibby; image courtesy Geoffrey Kibby, British Mycological Society.

cortex and a slightly thinner lower cortex, both constructed of thick-walled hyphae that are described as forming a plectenchymatous structure (Matsunaga *et al.*, 2013). The medulla contains globose cells interpreted as the photobiont. The interaction between the photobiont and mycobiont is evidenced by narrow hyphae and appressoria on the surface of the photobiont cells. While the mycobiont is most likely ascomycetous, the nature of the photobiont is difficult to interpret.

While there have been relatively few reports on Mesozoic lichen fossils, there are many more from the Cenozoic, most of which are based on specimens in amber (Hartl *et al.*, 2015) (**Plate 7**–2). For example, more than 150 lichen fossils recently have been identified from Eocene European amber (Kaasalainen *et al.*, 2017), including several forms that hosted lichenicolous (lichen-inhabiting) microfungi (Kaasalainen *et al.*, 2019). This discovery is interesting because lichenicolous fungi live exclusively on lichen thalli, and their nutritional modes range from parasitism to commensalism (Rambold and Triebel, 1992). Another study of lichen-inhabiting fungi in Paleogene amber indicates that the fossil associations are essentially identical to their modern analogs, thus suggesting that the origin of this relationship must extend back much further, likely to the Mesozoic (Kettunen *et al.*, 2016).

An excellent example of the exquisite preservation of lichens in amber is *Anzia electra*, a small thallus characterized by prominent spongiostratum (i.e., a layer of anastomosing hyphae on the lower surface of certain lichens, not effective in attachment) cushions reported from Eocene Baltic amber (Rikkinen and Poinar, 2002). The fossil belongs to *Anzia* sect. *Anzia*, the closest living relatives of which are found in east Asia and eastern North America, thus suggesting that *Anzia* sect. *Anzia* once was widespread in North America, Europe, and Asia, but later became extinct in Europe.

Two fossil thalli assignable to the lichen family Parmeliaceae have been described in Oligocene-Miocene amber from the Dominican Republic (Poinar *et al.*, 2000). One of them, *Parmelia ambra*, is a foliose thallus with dichotomously organized lobes, and rhizines arising from the lower surface. The second, *P. isidiiveteris*, is characterized by sinuate lobes with multiple, unevenly scattered isidia. Another lecanoralean lichen preserved in Dominican amber is *Phyllopsora dominicanus* (see also **Plate 7**–4), characterized by a squamiform to subfoliose thallus with the overlapping squamules each approximately 1.0 mm wide (Rikkinen and Poinar, 2008). Finally, Baltic and Bitterfeld amber has yielded fossils of lichens displaying alectorioid morphologies (Kaasalainen *et al.*, 2015), as well as calicioid fungi and lichens, which all can confidently be assigned to modern genera, including *Calicium*, *Chaenotheca*, *Chaenothecopsis*, and *Phaeocalicium* (Rikkinen, 2003; Rikkinen *et al.*, 2018).

Nematophytes

There are numerous fossils in the Silurian and Devonian for which the biological affinities remain unknown; some of these have variously been interpreted as fungi. This is especially true of the nematophytes, a group of organisms constructed entirely of interlaced tubes (Kondas, 2018). The largest nematophyte, *Prototaxites* (**Fig. 3**), occurs in the form of compressed or permineralized trunks, some of which are 1.25 m in diameter and > 8 m long, and are constructed of three size classes of septate and non-septate tubes or hyphae and, in certain sections of the trunks, there are what are interpreted as growth increments.

The most comprehensive study suggests that this fossil may have had affinities with the Basidiomycota (Hueber, 2001); its heterotrophic nutritional mode is also supported by isotopic analysis (Boyce *et al.*, 2007; Hobbie and Boyce, 2010). While Hueber's (2001) interpretation of *Prototaxites* as a huge basidiomycete fruiting body figures prominently in the literature, there are also several other hypotheses that have been advanced, including some type of marine alga, a rolled-up mat of a liverwort, a representative of an extinct lineage of organisms that lacks modern analogs, and some type of lichen surveyed by Taylor *et al.* (2010). Moreover, a petrified *Prototaxites* specimen from the Lower Devonian Rhynie chert in Scotland has recently been used to interpret this form as a member of the Ascomycota with inoperculate, polysporous asci lacking croziers (Honegger *et al.*, 2017).

Future directions

The preceding paragraphs indicate that there is a remarkable abundance of fungal remains in the fossil record. However, systematic studies of fungal lineages based on fossils are rare, due primarily to inherent problems connected with the incompleteness of the fossil record of the fungi. Moreover, when fungi were reported in the past they were rarely placed within a broader context. During the last 35 years, however, there has been an increasing awareness of fossil fungi and the pivotal roles they played in ancient ecosystems, which has been stimulated by a generally growing scientific interest in the microbial world and the interrelatedness of all organisms today. There are multiple approaches and avenues of investigation that require an understanding of fossil fungi. We believe that the solution to many important questions pertaining to the evolutionary history of fungi and characteristics of ancient ecosystems can be answered through collaborative and synergistic research efforts between mycologists, ecologists, chemists, and paleontologists, as well as perhaps a few other specialists, to fully integrate the fossil record of these organisms with modern approaches, such as geochemistry, experimental paleontology, fungal genomics, and phylogenetic analysis, and with the record of the organisms with which the fungi have interacted throughout geologic time. For example, most amber fossils represent organisms that once lived together in complex forest ecosystems ("amber forests"). Amber fossils, therefore, not only provide insights into the morphology of different types of organisms, but also yield valuable direct and indirect evidence of organismal associations and interactions, such as fungus gnats and mushrooms, that can best be assessed by collaboration of specialists from different areas of expertize.

Permissions

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The Cultivation of Macrofungi

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Introduction

Fungi interact with other organisms in ecosystems as decomposers and mutualistic or pathosistic symbionts, providing a number of ecosystem services (Millennium Ecosystem Assessment, 2005; Wallace, 2007). The main ecosystem services to which fungi contribute include nutrient recycling in the biogeochemical cycle (Gadd, 2007), soil health (Frac *et al.*, 2018), CO_2 storage (thanks to the fungi's contribution to the development of the tree component) (Leake, 2001), as well as the use that man has made of it in the food, medical and industrial fields (Brown, 2019; Comandini and Rinaldi, 2020; Gargano *et al.*, 2017; Hall *et al.*, 2007; Hall and Zambonelli, 2012a,b; Palanivel *et al.*, 2010; Polizeli *et al.*, 2005; Wasser, 2014). Especially in the food field the parts of fungus generally used are the sporomata or fruiting bodies in other words, the macrofungus that is visible to the naked eye.

Although macrofungi are a small part of the wide Kingdom of Fungi, they have a very high socioeconomic value for different human populations worldwide. Indeed, several macrofungal species were at first collected and consumed as wild products, and later they were cultivated and exploited for edible and/or medical purposes. The main species known worldwide are: button mushrooms (*Agaricus bisporus* (J. E. Lange) Imbach), porcini mushrooms (*Boletus* spp.), oyster mushrooms (*Pleurotus* spp.), morel mushrooms (*Morchella* spp.), truffles (*Tuber* spp.), Reishi (*Ganoderma* spp.), and Shitake (*Lentinula edodes* (Berk.) Pegler).

Unfortunately, not all macrofungal species are easily cultivable, and some species still need to be harvested as wild mushrooms. Probably the most famous example in the world is *Tuber magnatum* Picco (Italian white truffle), whose cultivation still remains very difficult, despite the extensive efforts of researchers around the world (Zambonelli *et al.*, 2015). In addition to the *T. magnatum*, there are many highly prized species consumed in many parts of the world, for which it would be interesting to develop and improve cultivation techniques, such as *Cordyceps* spp., *Tricholoma matsutake* (S. Ito & S. Imai) Singer (*ex T. nauseosum* (A. Blytt) Kytöv.), *Boletus* spp., *Lactarius* spp., *Suillus* spp., *and Amanita* spp. (Hall and Wang, 1998; Winkler, 2008). Today, the main cultivated species are saprobes (i.e., decomposers), which grow mainly on woody organic substrates. However, in the last century, thanks to the development of mycological, agronomical, and biomolecular knowledge, the cultivation of mycorrhizal mushrooms has also become a reality, in particular some truffles species, such as *Tuber melanosporum* Vittad., *Tuber aestivum* (Wulfen) Pers, and *Terfezia* spp., and some epigean species, such as *T. matsutake*, *Lactarius* spp. (Chevalier and Desmas, 1977; Mannozzi-Torini, 1981; Martin *et al.*, 2010; Morte *et al.*, 2012; Palenzona, 1969; Rubini *et al.*, 2014; Wang *et al.*, 2012; Yamanaka *et al.*, 2020). These acheivements made the cultivation of mycorrhyzal species a concrete economic reality in different rural areas in the world, such as Spain, France, Italy, New Zealand, and the United States (Di Piazza *et al.*, 2013; Guerin-Laguetie *et al.*, 2009; Hall and Zambonelli, 2012a,b; Lefevre, 2008; Pacioni and Lamolinara, 2011; Trappe *et al.*, 2009; Reyna and Garcia-Barreda, 2014; Serrano-Notivoli *et al.*, 2016; Zambonelli *et al.*, 2017).

In recent decades, the boost of biotechnologies has propelled the cultivation of macrofungi for enzyme productions (Fazenda *et al.*, 2008), bioremediation of polluted environments (Meharg and Cairney, 2000) and biomaterial productions for green building (Appels *et al.*, 2019; Girometta *et al.*, 2019), as well as for medical and nutraceutical purposes (Phan *et al.*, 2012; Wasser, 2014). More recently, due to the growing need to develop a circular economy, some authors have proposed to cultivate mushrooms in the agricultural supply chains to "closing loops" (Banasik *et al.*, 2017; Grimm and Wösten, 2018; Yang *et al.*, 2016), in order to create virtuous cycles with very low impacts.

Undoubtedly, owing to fungi heterogeneity, it is not easy to assess the state of the art of macrofungi cultivation, because many species are exploited for different purposes, some of them connected to popular traditions and some connected to restricted areas of the world.

Due to the different methods of cultivation and management, saprotrophic and ectomycorrhizal fungi are often treated in different ways, so that the economic data are often fragmented not only geographically but also according to the type of cultivation. For example, there is a limited number of saprotrophic species distributed and used all over the world that are common to different areas. Precisely for this small but widely used group of species, in 2013 the global trade of mushroom industry was estimated to be about $63 billion, where the major revenues (54%) are related to cultivated edible mushrooms (Royse *et al.*, 2017).

The purpose of this chapter is to give an overview about the huge world of macrofungi cultivation, addressing the main aspects related to the main cultivated species, cultivation techniques and information about biotechnological applications.

Cultivation of Saprotrophic Species

Cultivated mushrooms are often saprotrophic macrofungi species able to grow on decaying organic matter. Most of them preferably decompose plant residues as a carbon source. This characteristic makes these fungi essential for the carbon cycle in natural ecosystems and particularly suitable for human cultivation (Stamets, 1994, 2005). Among the "saprotrophic obligated or facultative" mushrooms there are species difficult to cultivate due to their needs, for example some species belonging to genera *Cordyceps* (Raethong *et al.*, 2020) and *Morchella* (Liu *et al.*, 2018). For these cases, more in-depth studies are needed in order to

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identify the trophic needs that allow for completion of the life cycle to optimize the process for sporomata production. Despite these particular cases, many other species can be successfully cultivated. Today 90% of the saprotrophic species cultivated worldwide belong to 5 genera: *Lentinula*, *Pleurotus*, *Auricularia*, *Agaricus* and *Flammulina* (Royse *et al.*, 2017). Regarding the technical aspects of the cultivation of macrofungi, the first step is to select a standardized substrate for cultivation of the target species. Once the substrate has been selected, the cultivation process is relatively simple and allows you to carry out one to more production cycles per year. Conversely, for the cultivation of symbiotic macrofungi the inoculation of the mycelium on a host plant is mandatory, making the management and development of symbiotic species much more difficult and slower.

The cultivation industry of saprotrophic macrofungal species is distributed worldwide. There are 3 main macro areas of production in the world: (1) Asia (in particular China, India and Japan); (2) Europe (Poland, Spain, France, Italy, and the Netherlands); (3) the United States of America. In these areas is concentrated 90% of the world's cultivation, processing and marketing of mushrooms (Royse *et al.*, 2017).

Among the species cultivated, due to its worldwide distribution and resilience *Pleurotus ostreatus* (Jacq.) P. Kumm. is the most studied species of mushroom, and it can be considered a sort of "model-species." *P. ostreatus* is probably the most studied because it is quite resistant and its laboratory and industrial management is rather simple. *P. ostreatus* is cultivated and studied for various purposes such as food, nutraceutical, biomaterial and bioremediation (Appels *et al.*, 2019; Girmay *et al.*, 2016; Sánchez, 2010; Vaverková *et al.*, 2018; Yang *et al.*, 2016; Yang *et al.*, 2019). These multiple uses testify to the great versatility of these organisms.

Main Steps

The cultivation of saprotrophic macrofungi depends on the specific trophic requirements of the selected species, the facilities available, the technologies applied in the process, and the purpose of cultivation (such as scientific, medical or food). Therefore, these aspects must be carefully considered before starting a cultivation. In general, any type of process of saprotrophic macrofungi cultivation involves five fundamental phases: (1) isolation; (2) substrate preparation; (3) inoculum; (4) incubation; (5) cultivation; (6) harvest and transformation. These phases, detailed in the following paragraphs, should be adapted to the specific situations.

Isolation

Isolation of the mycelium in axenic culture is essential for cultivating any macrofungal species. The mycelia of the most common species (e.g., *Agaricus*, *Pleurotus*, *Lentinula*, etc.) can be found quite easily because they are traded by several companies that produce mushrooms or by other companies in the agri-food sector. For less common species or for other scientific purposes such as carrying out molecular or biochemical study or making comparative studies between strains of different species, it is necessary to isolate mycelium in axenic culture directly from wild sporomata. For this purpose it is necessary to take portions of mycelium from fresh sporomata by operating in conditions of maximum sterility, using a laminar flow hood. The most common media used for the isolation of mushrooms are Potato Dextrose Agar (PDA), Malt Extract Agar (MEA) and Oatmeal Agar (OA); the recipes of the most common media are found in all the main manuals of mycology, such as Gams *et al.* (1987).

The main steps of isolation from fresh sporomata can be summarized as follows:

(1) Collect fresh and healthy sporomata (not attacked by insects or other parasites).
(2) Remove a portion of mycelium from the innermost part (in sterile conditions).
(3) Place the portion collected on an agarized nutrient medium (PDA, MEA, OA).
(4) Incubate in a thermostat (24°C for many species) and check the growth daily for possible contamination.
(5) In case of contamination by other fungi or bacteria it is necessary to transfer part of the developed mycelium into a new petri dish. Repeat this operation until the pure culture is obtained.
(6) Transfer the mycelium onto an agar slant tube and store at 4°C or with long-term preservation methods.

Long-term preservation of the isolated strains is an essential issue for any mycology laboratory and for any industry wishing to work with living fungal strains. Throughout scientific history preservation techniques have evolved to reach high levels of technological sophistication for the best long-term preservation of fungal strains. The long-term preservation of macrofungi is in some cases more difficult as they grow as mycelium and often they do not produce forms of resistance. However, in recent years, some authors have indicated different protocols for preserving at −80°C different mycelia from withe-rot fungi (Voyron *et al.*, 2009) and truffles (Piattoni *et al.*, 2017), and have tested ultra-low freezing (−120°C) on *Ganoderma lucidum* (Leonardi *et al.*, 2018). These indications about long-term preservation methods are very precious for building banks to collect strains of particular interest.

Substrate, inoculum and incubation

The preparation of the substrate is a basic step for a successful cultivation. Minge Duggar (1904) more than 100 years ago highlighted differences in the colonization rate, mycelial texture and sporomata morphology of fungal species grown on different material. For this reason, the composition of the substrate must be assessed according to the nutritional needs of each cultivated species. Recently, several authors (Haneef *et al.*, 2017; Raethong *et al.*, 2020) investigated how the characteristics of the different substrates affect the physicochemical properties of the mycelium during its development. These findings open a new scenario for exploitation of macrofungal cultivation to make biomaterials (Appels *et al.*, 2019; Girometta *et al.*, 2019). Suitable substrates need balanced nutrients, proper humidity and homogeneous, compact but aerated structure, preferably with high surface-to-volume

ratio. An appropriate level of water activity is necessary to activate properly hydrolytic and oxidative enzymes to trigger the metabolic activity and enable mycelial colonization.

Regardless of the type of application, the substrates must be placed in heat-resistant plastic bags to be autoclaved. A proper amount of spawns must be prepared on different substrates such as grains, bran, cereals, sawdust and shavings. Once the sterilization phase is over, when the substrates have reached room temperature again, it is possible to proceed with the spawn inoculum. The substrates inoculated must be incubated for a period ranging from 20 to 120 days, depending on the specific needs of the inoculated species.

Cultivation and harvest

In this phase the well colonized substrates need to be placed in the plant facility to trigger primordia generation. Fruiting is a very delicate phase; it is necessary to maintain the environmental parameters stable in particular, to keep temperature and relative humidity within the ranges that allow the mycelium to develop primordia and bring them to maturity. These parameters vary according to the cultivated species, but for many species temperatures range from 18°C to 26°C and the relative humidity must be maintained around values of 80%–90% (Ferri *et al.*, 2007). Sudden changes in temperature or relative humidity during this phase can cause production decline or even the loss of all products. For these reasons, several factors must be taken into account when arranging for cultivation in the plant, such as the specific requirement of the species, the type of cultivation to be undertaken, and the climate of the area where the cultivation takes place. We classify the plants into three different types: free/open field, semi-controlled and controlled management. Based on the different plants, we have three different levels of control of the environmental parameters that influence development and consequently the collection of sporomata, as shown below.

- Free/open field: same conditions as in nature; the substrates are planted in natural or semi-natural environments that mimic the natural conditions of growth and development of the species. Ripening and harvesting period is identical to natural conditions.
- Semi-controlled: cultivation takes place in a protected environment (e.g., greenhouses, shaders, cultivation tunnels) where only a few parameters are monitored and partially controllable. Ripening and harvesting period longer than natural conditions.
- Controlled management: cultivation takes place inside incubation cells where it is possible to fine-tune all the parameters that influence growth. Ripening and harvesting can take place cyclically regardless of seasonal cycles.

Pests and Adversities of Cultivated Saprotrophic Mushrooms

Macrofungi, as well as other cultivations, are threatened by pests that can lower the quality of the product and reduce the final mushroom yield. The micro-environmental conditions inside of the cultivation area during the growing phase (in particular temperature and high relative humidity) can lead to the onset of rapidly spreading infections that can compromise the cultivation. Thus, attention must be paid to preventing the spread of pathogens, especially in intensive installations in closed and semi-closed environments. Arthropods and other microfungal species are the main threats to macrofungal cultivation, but there are also less well-known cases of contamination caused by bacteria and viruses.

Among the most common pests affecting cultivated macrofungal species on a world scale, we highlight the adversities of *Agaricus bisporus* caused by sciarid flies, for example *Lycoriella auripila* Winn and *Lycoriella Ingenua* Dufour (Grewal, 2000; White, 1997), which lay their eggs inside freshly pasteurized or inoculated compost. These flies cause double damage, as the larvae feed on the substrate, and the adults can act as vectors for many other diseases. The *Diptera* belonging to the genus *Megaselia* are often also vectors of fungal pathogens in *A. bisporus* cultivation (Jess and Schweizer, 2009). Among fungal-induced pathologies *A. bisporus*, like other cultivated fungi, is very susceptible to attacks of *Verticillium* spp. and *Trichoderma* spp., in particular *Trichoderma harzianum* Rifai (Kosanović *et al.*, 2013).

Also, *Pleurotus ostreatus* is susceptible to different pathologies caused by organisms of different origin; the most common fungal infection is caused by species belonging to genera *Mycogone*, *Trichoderma* and *Fusarium* (Ferri *et al.*, 2007). Since *P. ostreatus* is a widely cultivated species, different studies have also evaluated possible solutions for preventing its diseases. For example, studies have focused on the sensitivity of different *Trichoderma* pathogenic species to prochloraz (Innocenti *et al.*, 2019), and other research demonstrates the possibility of using *Aureobasidium pullulans* (de Bary) G. Arnaud as a biocontrol agent against the species *Trichoderma pleuroti* S. H. Yu & M. S. Park and *Trichoderma pleuroticola* S. H. Yu & M. S. Park without affecting the *P. ostreatus* growth and final production (Roberti *et al.*, 2019).

Among the better known threats to *Lentinula edodes* (Berk.) Pegler cultivation are the arthropod pests caused by coleoptera *Dacne picta* Crotch (Ohya, 1993; Sato *et al.*, 1998), *Lepidoptera*, for example *Paragona multisignata*, *Diomea cremata* (Yoshimatsu and Nakata, 2003; Yoshimatsu and Kawashima, 2016) and *Diptera*, in particular *Camptomyia* sp. The mechanisms of infection are not yet fully understood, but some authors hypothesize that the harm of green mould (*Trichoderma* spp) is caused by secondary attacks of these insects (Kim *et al.*, 2016; Wang *et al.*, 2016). In 1993 the mite *Luciaphorus auricularia* was identified as a pathogen of *Auricularia polytricha* (current name *Auricularia nigricans* (Sw.) Birkebak) and *A. auricula-judae* (Bull.) Quél. In China (Zou *et al.*, 1993) this mite can lead to the loss of up to half of the production.

It is clear that pathogens of cultivated macrofungi are numerous, and most of them are still unknown. In recent years, thanks to an increased environmental awareness and to the need to increase food safety, some researchers have started to test biological control agents against the most common threatening organisms, such as the microfungi of the genus *Trichoderma* and the sciarids belonging to the genus *Lycoriella*. For this purpose, two biofungicides, based on *Bacillus subtilis* and tea tree oil respectively, were

tested against several *Trichoderma* species, highlighting the efficiency of *B. subtilis* against all *Trichoderma* tested (Kosanović *et al.*, 2013). Concerning pests, the predatory mite *Hypoaspis miles* Berlese has shown great potential in protecting *A. bisporus* cultivation from the *Sciaridae* belonging to genus *Lycoriella* (Jess and Schweizer, 2009). These studies have proposed environmentally friendly alternatives to the use of traditional chemical products, stimulating research in this field aimed at limiting the use of synthetic products which, in addition to being harmful to the environment, are detrimental to the health of consumers.

Cultivation of Ectomycorrhizal Species

Ectomycorrhizal fungi (hereafter ECM), living in obligate symbiosis with woody plants, play a key role in forest ecosystems. Thanks to this close interaction, ECM fungi contribute to the development and improvement of multiple ecosystem services for communities living within and/or close to forest areas (Donnini *et al.*, 2013; Zotti *et al.*, 2013). For example, the edible ECM species are the source of valuable food products (*Amanita caesarea* (Scop.) Pers., *Cantharellus cibarius* Fr., *Boletus* spp, *Lactarius* spp, *Morchella* spp, *Rhizopogon* spp, *Terfezia* spp, *Tricholoma matsutake* and *Tuber* spp.). Some of these species are not only a source of food but also pillars on which the economies of several villages and small towns in rural areas worldwide are based (Wang and Hall, 2004). Due to the nature of this obligate symbiosis, ECM fungi are mainly harvested as wild products, because the cultivation of ECM is difficult and it has always been considered a kind of "magic secret". For this reason, for many centuries ECM cultivation was limited to empirical evidence from natural history scholars who unfortunately were not yet aware of the details of mycorrhizal symbiosis (Dessolas *et al.*, 2007). Some of these empirical practices, providing good results, are still in use and are useful to improve and progress of cultivation techniques. Recently, for example, Murat *et al.* (2016) highlight how the empirical technique of "trapping truffles" seems to have positive effects on the production of *Tuber melanosporum* orchards. However, at the end of the seventeenth century, thanks to the first observation and descriptions of ectomycorrhizal structure (Frank, 1885), did rational research about the cultivation of macrofungal ECM begin. In fact, the discovery and description of ectomycorrhizal structure triggered a process that about 85 years later, at the end of the 1960s (1969), led to the cultivation of valuable ECM species, in particular truffles, in a rational way. The first data relative to the production of mycorrhizal plants with truffles were published in 1969 by Fontana and Palenzona. Today, thanks to decades of research in the mycological, biomolecular and agronomic fields, we can cultivate many ECM species for food purposes (Hall and Zambonelli, 2012a,b), forest restoration, decontamination from pollutants (Assad *et al.*, 2020; Futai *et al.*, 2008), and from their cultivation we can derive important positive effects on the ecosystem and the surrounding environment.

However, today the majority of ECM fungi cultivated are edible species. The best results have been achieved with the cultivation of different species of genus *Tuber*. Among the best-known examples we can mention *Tuber melanosporum*, grown mainly in Spain, France and Italy (Reyna and Garcia-Barreda, 2014; Samils *et al.*, 2008; Sourzat, 2017), though it has also been introduced in some areas in North America (Lefevre, 2012), Australia and New Zealand (Hall *et al.*, 2007), whereas *Tuber aestivum* is perhaps the most cultivated truffle species worldwide. Thanks to its ecological plasticity, *T. aestivum* is successfully cultivated in almost all of Europe – France, Italy, Spain, Portugal, Sweden, United Kingdom, Germany and Finland – with excellent results (Molinier *et al.*, 2016; Shamekh *et al.*, 2014; Stobbe *et al.*, 2013; Wedén *et al.*, 2009, 2013). *Tuber borchii* Vittad. is another less known species cultivated locally in Italy (Iotti *et al.*, 2016), while in North America among the native species of hypogean ECM *T. oregonense*, *T. gibbosum* are the most appreciated and sought-after species, and, although there is no ancient tradition, several initiatives have been underway in recent years aimed at enhancing and improving the visibility of these products that have a great potential (Lefevre, 2008, 2012). Unfortunately, as mentioned above, it is not yet possible to cultivate *Tuber magnatum* Picco rationally.

Also, the epigean species are very difficult to cultivate; for example, the first report about the production of *Cantharellus* fruit bodies dates back to 1997 (Dannel & Camacho, 1997). This work also hypothesized the possibility of cultivating *T. matsutake*, which is another economically interesting epigean species. Other epigean ECM species successfully cultivated around the 2000s are *Lactarius deliciosus* (L.) Gray, *Lyophyllum shimeji* (Kawam.) Hongo (Wang and Hall, 2004). Many efforts have since been made to study the cycle of other species such as *Boletus* or *Amanita*, to develop methodologies and protocols to increase production in naturally productive areas (Águeda *et al.*, 2008). For example, several experiments have been carried out in northern Europe to stimulate production in the forests of the region, while in Spain and France, particularly in the so-called "mycoforests" of the Rone-Alpes region, forestry practices are applied that take into consideration the effects on the production of macrofungi (Büntgen *et al.*, 2017; Pierangelo and Rolland, 2014). Today only sylvicultural practices are effective to stimulate the production of these species in areas that are already productive; this practice is called mycoselviculture (Savoie and Largeteau, 2011).

It is very difficult to estimate the economic income derived from ECM cultivation because the data available concerns only a few species, and often data are from both cultivated and wild products. Despite the paucity of the data, we can state with certainty that it is a large market capable of sustaining many people who live in different parts of the world, especially in rural areas. Below are some examples of what happens worldwide. For example, in Spain, the estimated annual production of the *B. edulis* species complex from the autonomous community of Castilla y León is 8500 tons, worth approximately € 38 million (Martínez-Peña *et al.* (2006–2008); Ortega-Martínez and Martínez-Peña, 2008). In Italy the satellite-activities of truffle cultivation are essential for sustaining several villages in the Apennine mountains (Donnini *et al.*, 2013; Iotti *et al.*, 2012). In France 80% of the production is still essentially in the southeast, mainly in the Drome, the Vaucluse and the Alpes de Hautes Provence, being the three principal departments (in 1999/2000 the national total was 30 tons, of which 27.4 came from the southeast and 3.6 from the southwest). Even if these data indicate a large market, the real value is likely to be much higher.

Ectomycorrhizal Plant Production

As already mentioned, to cultivate ECM mushrooms it is essential to plant trees inoculated with the desired species. Over the years, the research in the field of mycorrhized seedling production has gone through different methods of inoculating plant roots: in sterile, semi-sterile and non-sterile conditions (Repáč, 2011). Basically, mycorrhizal synthesis can be induced in three different ways: sporal inoculation, radical approximation, and mycelial inoculation. Over the years, these techniques have been studied in the laboratory to be perfected and put into practice directly by specialized nurseries. Using well mycorrhized seedlings, the grower is only tasked with planting plants already inoculated. An interesting option among the three fundamental techniques is the one proposed by French researchers who, referring to ancient truffle-growing manuals, foresee the inoculation in situ (Dessolas *et al.*, 2007). The advantage of this method is that it no longer takes place in the laboratory but is done in the field when the plants are planted, reducing costs. However, this practice has been rejected in recent years by some authors, as it appears to have a high rate of contamination by other ECM species during the colonization process.

All of these techniques obviously have advantages and disadvantages, so it is always necessary to choose the most suitable technique according to the production needs, the availability of sporomata to produce suitable quantities of inoculum and the macrofungal species you want to use. The inoculation methods used for the production of mycorrhizal plants are described in detail below.

Spore suspension inoculum

The protocol of sporal inoculation is one of the most widely used for the production of mycorrhized seedlings. Given the simplicity of the method it is probably the most ancient. In the past truffle hunters had empirically used this technique to prepare the inoculum to stimulate truffle production of potentially suitable plants. In the twentieth century, thanks to advances made by scientists, this method was standardized by researchers to make it more effective (Chevalier and Desmas, 1977; Chevalier and Grente, 1974; Chevalier and Poitou, 1990; Fassi and Fontana, 1967; Fontana and Palenzona, 1969).

The preparation of the seedlings (from seed or micropropagation) is made in semi-sterile conditions, in order to limit as much as possible the presence of antagonistic species. Mature and well-identified sporomata, previously sterilized, are grinded in order to obtain a mush of spores suspended in distilled sterile water; inoculation by immersion of the preparation obtained on the root systems of the young plants (Repáč, 2011). Through this method, the spores contained in the truffle, germinating near the root apparatus, will infect the roots of the plants. This process has an average duration of 6–7 months.

This method obviously presents several problems, since it is necessary that the sporomata of the selected species must be characterized by the production of a high quantity of fertile spores, and they must be available in abundant quantities without excessive cost. Even if mild sterilizations can be carried out, the microflora present in the sporomata and near the fertile parts could affect the success of the colonization. Therefore, it is difficult to obtain a homogeneous and repeatable result, because the success is affected by multiple factors arising from the quality of the material found. For example, unwanted species can be inoculated, especially when using production waste or cleaning residues from truffle cleaning. These products can often be mixed of different species, and therefore you could get different plants than you expect. Furthermore, several authors have pointed out that the sexuality of mycelium has a significant influence on the success of colonization and fructification; therefore, the choice of material and the preparation of the inoculum require careful preparation (Iotti *et al.*, 2016; Rubini *et al.*, 2014).

Spore-based inoculations have many advantages. The inoculum is relatively cost effective and easy to prepare, and it requires less time, no specialized equipment and less specialized training, compared with other methods. Spores are obtained by crushing whole sporomata (in the case of truffles) or only the mushroom hats (in the case of porcini or mushrooms with gills) using a mortar and pestle, an electric mill or a blender. To facilitate the grinding of fungal tissue, sterile water coupled with sand or other types of fine abrasive particles can be added to the crushing process.

Radical proximity

This method involves the use of a mycorrhized "mother plant"; it is very effective because it exploits the high infectivity of the hyphae produced by the active mycorrhizas (Iotti *et al.*, 2012). This method works well for those species that have low germinability of the spores and for which the mycelia have difficulty performing mycorrhizal symbiosis because they have low infectivity and suffer from competition with other mycelia (Gregori, 2002). For example, it is empirically exploited in some areas of Italy by truffle hunters who take seedlings and suckers from already productive areas to plant them in other areas. This method saves the purchase cost of the ascomata, which is very high especially for fine species, so that it can have a positive impact on the price of mycorrhization on the seedlings. However, to make this method effective over time, it is important to maintain a healthy of mycorrhization in the "mother plants" by having highly trained personnel monitor the state of mycorrhized roots and check for the presence of competing species.

In order to be able to carry this method out on a large scale there are certainly high initial costs, as a large number of mother plants need to be owned. However, if the mother plants are managed well over time, the gains should make it possible to justify the high initial planting costs.

Mycelium-based inoculum

This procedure is probably the most recent and technologically advanced one; it involves the isolation of mycelium in axenic culture and its multiplication in vitro in special nutritive soils. Once satisfactory quantities of mycelium have been obtained, it is used to inoculate young seedlings by adding it to the soil. Mycelium has been considered the most suitable source of inoculation

for infecting plants with ECM fungal strains (Marx and Kenney, 1982). However, the large-scale nursery application of this type of inoculum depends heavily on the saprobic potential of the fungal species of interest and its ability to produce large amounts of mycelium under axenic conditions.

Compared to previous methods, this method makes it possible to produce seedlings free of foreign fungi and with a high degree of mycorrhization. In addition, through this method it is possible to inoculate with specific fungal genotypes specially selected on the basis of specific needs, for example, infectivity and affinity to the host plant, high tolerance of, or ability to degrade, particular pollutants, and resistance to high environmental stress. Among the negative aspects, however, it should be noted that this method requires specific, rather expensive structures and instrumentation, and highly specialized personnel. Moreover, not all fungi have mycelia vitality after isolation and in vitro cultivation; in fact, many studies are carried out to find new culture media to make it easier to isolate and multiply the species that are more difficult to cultivate in the laboratory. One of the first protocols related to this technique is that of Molina and Palmer (1982), but unfortunately this is time-consuming and difficult to apply. Today, on the basis of past protocols, improvements have been made and many steps have been optimized. In fact, it is possible to perform mycorrhizal synthesis with mycelial cultures for many edible ectomycorrhizal ascomycetes and basidiomycetes, both in greenhouse and in vitro (Águeda *et al.*, 2008; Danell and Camacho, 1997; Endo *et al.*, 2014; Giomaro *et al.*, 2005; Zambonelli *et al.*, 2008). However, it is widely used only for some edible ECM species such as *Lactarius deliciosus* and *Tricholoma matsutake*. In 2016 the first data on the production of *Tuber borchii* ascomata were published 8 years after the planting of plants inoculated with pure crops, highlighting the possibility of using this technique to produce truffle plants instead of the more widely used sporal inoculum (Iotti *et al.*, 2016, 2012).

Adversities of Cultivation of ECM Mushrooms

On the basis of what has been described, it is clear that the cultivation of ECM fungi, due to their symbiotic mutualism, still presents particular difficulties that require the deepening of knowledge about several problems in order to find effective solutions. One of the main problems is certainly due to the high affinity of different species with the host plant. In fact, as it is known for each a plant species there are many fungal species that can do symbiosis, and, since it is not a pathological aspect, it is impossible to notice this competition without specific morphological and/or molecular investigations of the root system. In this case the competition between mycelia could lead to considerable economic damage in terms of lack of production.

Examples of competition between mycelium, for example, are reported within truffle cultivations where more adaptable and less valuable species can threaten the success of a truffle plantation. For example, the competitiveness of *Tuber brumale* Vittad. against *Tuber melanosporum* and *Tuber aestivum* was recently highlighted in a 14-year-old plantation that sowed seedlings inoculated with these three truffle species in adjacent plots. The results confirm the competitiveness of *T. brumale* against *T. aestivum* and *T. melanosporum* due to its great ability to colonize the soil around its ectomycorrhizae. However, its competitiveness is limited to the transept and has never been found within the *T. melanosporum* plot. These results show that, under optimal conditions for *T. melanosporum* and *T. aestivum*, the greatest risk of contamination with *T. brumale* is due to incorrect greenhouse activity (Ori *et al.*, 2018).

The Cultivation of Macrofungi for New Biotechnology

Recently, fungi were fruitfully exploited in a number of fields different from the food industry. Many researchers proposed to employ this kind of organism for bioremediation applications (mycoremediation) and biomaterial production (Appels *et al.*, 2019; Fazenda *et al.*, 2008; Girometta *et al.*, 2019; Meharg and Cairney, 2000).

Mushrooms can bioaccumulate and biodegrade a high range of organic and/or inorganic toxic compounds by means of the production of specific enzymes (e.g., lignin peroxidases and manganese peroxidases) and secondary metabolites (e.g., organic acids; Kapahi and Sachdeva, 2017; Vishwakarma, 2019). Not only saprotrophic but also white/brown rotting and ectomycorrhizal (ECM) fungi are well known in bioremediation technologies (Gadd, 2007; Vishwakarma, 2019). Elevated concentrations of toxic metals and radionuclides can often occur in sporomata of saprotrophic and ECM fungi in many polluted environments (Gadd, 2007; Liang and Gadd, 2017), favoring plants's colonization and playing a central role in the biogeochemical cycles of a site (e.g., *Thelephora terrestris Ehrh.*; Cecchi *et al.*, 2019). Conversely, white-rot fungi are well known to degrade petroleum hydrocarbons not only in terrestrial environments but also in seawater (e.g., *Phanerochaete chrysosporium* Burds., *Pleurotus ostreatus*, *Trametes versicolor*, *Bjerkandera adusta* (Willd.) P. Karst., *Lentinula edodes*, *Irpex lacteus* (Fr.) Fr.; Kumar *et al.*, 2018; Prasad, 2017). Furthermore, the surprising performance of mushrooms in biotechnology has been explored to produce new biomaterials for packaging (Haneef *et al.*, 2017), thermal and acoustic insulation (Girometta *et al.*, 2019) and a broad variety of design objects and furniture (e.g., https://grow.bio/), and to replace plastic in electronics (Vasquez and Vega, 2019) and produce biodegradable "bioblocks" from agricultural waste (Joshi *et al.*, 2020).

Conclusions

People have always attempted to cultivate different species of mushrooms, including the valuable hypogean species mainly for food interests and recently for the possible exploitation in the field of bioremediation. Today, the best results in the world involve the cultivation of different saprotrophic species, whereas ectomycorrhizal species, with a few exceptions, are difficult and expensive

to cultivate. The studies devoted to improving fungal production and to making possible cultivation of new species of fungi for different purposes are in progress all over the world. Currently, issues related to mycoselviculture and growth incentives are generating a great deal of debate, since these techniques aim to increase the production of those species whose development cycles we are not able to control.

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Macrofungi as Food

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Introduction

Macrofungi (also referred to collectively as "mushrooms") have been an important component of human food for thousands of years, with the earliest reports of macrofungi consumption coming from Spain (18,700 years ago), China (5000–6000 years ago) and Egypt (4600 years ago) (Chang, 2006; Power *et al.*, 2015; Straus *et al.*, 2015; Zhang *et al.*, 2015). Because of their pervasive use in dishes across disparate cultures, there is also a long history of developing mushroom cultivation techniques to ensure steady supply. The earliest attempts to cultivate macrofungi were found in Asia, followed by efforts in Europe and eventually in North America. The artificial cultivation of *Auricularia* sp was described 1400 years ago from China in *Tang Materia Medica* by Tang Sugong. In Europe, evidence suggests that France introduced the first cultivated mushroom in the 17th century (Zhang *et al.*, 2015).

Given the long history of human consumption of macrofungi, it is only in recent times that we have described and truly embraced macrofungi as a staple part of global human cuisine, as shown by an exponential increase in production and trade of mushrooms. This rising recognition, awareness and use has also led to the consumption of more species and spurred technological development for cultivated species as well as research on the ecology and sustainable management of wild species, the majority of which cannot be cultivated.

We present an overview of cultivated and collected edible macrofungi and their trade and commercial value. We discuss challenges faced by growers and traders as a result of increasing market demands as well as the importance of mushrooms to the food industry. Despite a long history of collecting wild mushrooms, uncertainty and confusion still exist on which species can be safely eaten. We also discuss a new classification system aimed at resolving different opinions and assessing published case reports (Li *et al.*, 2021).

Types of Edible Macrofungi

Edible macrofungi can be classified into three trophic groups: ectomycorrhizal, saprobic and parasitic. They originate from two main sources: species that are wild-harvested and those that are cultivated. Generally speaking, it is only the saprobic fungi that are cultivated, though new techniques are emerging for the partial cultivation of several ectomycorrhizal species. Approximately 60 edible species are commercially cultivated out of a potential 100 species identified by Chang and Miles (2004).

Wild edible mushrooms are mainly saprobic or ectomycorrhizal. Edible parasitic fungi are few in number; perhaps the most common species, *Armillaria mellea*, mainly colonizes the wood of tree species. Edible parasitic fungi also include several plant pathogens, including maize smut and sorghum smut (Boa, 2004). Ectomycorrhizal fungi form a symbiotic association with tree roots, exchanging nutrients assimilated from the soil for photosynthetically derived carbohydrates from the host tree (Deckmyn *et al.*, 2014).

The most widely cultivated macrofungi are *Agaricus bisporus*, *Lentinus edodes*, *Pleurotus* species, *Flammulina velutipes*, *Auricularia auricula* and *Volvariella volvacea* (Kabel *et al.*, 2017; Chang and Wasser, 2012; Bellettini *et al.*, 2019; Zhang *et al.*, 2013). Some progress has been made in the cultivation of ectomycorrhizal fungi, though this is only manageable for a few species and cannot be conducted independent of a host tree. Well-known examples include *Tuber melanosporum* (Hall *et al.*, 2007), and *T. indicum* (Hu *et al.*, 2010). Young trees must first be inoculated with the truffle fungus. Once the ectomycorrhiza form, the host trees are transplanted and managed in truffières, akin to fruit orchards. It can take up to 10 years for trees to become productive.

Wild Macrofungi

Wild edible macrofungi have diverse morphological features and shapes (**Fig. 1**). Some are typical mushrooms with a stalk and cap (sometimes referred to by scientists as "agarics"), while others are fan-shaped, ball-like, disc-like, saddle-shaped, cup-like and so on. Widely consumed types include typical mushrooms, boletes, chanterelles and truffles, though species preferences vary considerably throughout the world.

A recent study reported the existence of 2189 wild collected edible species (Li *et al.*, 2021) of which 2006 can be consumed safely, and a further 183 species that require pretreatment before safe consumption or were associated with allergic reactions. Harvesting

Fig. 1 Diverse types of wild edible mushrooms collected from forests. a: *Amanita hemibapha*, b: *Clitocybe gibba*, c: *Boletus valens*, d: *Clavaria flava*, e: *Boletus bicolor*, f: *Bovista pusilla*, g: *Laccria amethystina*, h: *Hygrocybe conica*, i: *Hydnum repandum*, j: *Cordyceps miltaris*, k: *Cystoderma amianthimum*, l: *Helvella crispa*, m: *Lactarius deliciosus*, n: *Lepiota magnispora*, o- *Phallus haitangensis*, p: *Lyophyllum fumosus*, q: *Lycoperdon perlatum*, r: *Russula area*, s: *Tricholoma myomyces*, t: *Laccaria laccata*, u: *Suillus pictus*, v- *Tricholoma bakamatsutake*, w- *Suillus luteus*, x: *Xerula radicat* (Photos: Huili Li).

Table 1 Most commonly harvested and traded mushroom species from around the world

Scientific name	*Region*	*References*
Astraeus hygrometricus	Asia	(Dell *et al.*, 2005)
Boletus edulis	Europe, Asia, North America	(Pilz and Molina, 2002)
Morchella conica	Asia	(Mortimer *et al.*, 2012)
Ophiocordyceps sinensis	Asia	(Mortimer *et al.*, 2012)
Phlebopus portentosus	Asia	(Mortimer *et al.*, 2012)
Termitomyces eurhizus	Asia	(Mortimer *et al.*, 2012)
Thelephora ganbajun	Asia	(He *et al.*, 2011)
Tricholoma matsuake	Aisa, North America, Erurope	(Wang *et al.*, 1997)
Tuber indicum	Aisa	(Mortimer *et al.*, 2012)
Agaricus campestris,	Erurope	Peintner *et al.*, 2013
Boletus badius	Erurope	(Peintner *et al.*, 2013)
Cantharellus cibarius	Erurope	(Peintner *et al.*, 2013)
Craterellus curnucopioides	Erurope	(Peintner *et al.*, 2013)
Lactarius delicosus	Erurope	(Peintner *et al.*, 2013)
Morchella esculenta	Erurope	(Peintner *et al.*, 2013)
Cantharellus formosus	North America	(Pilz and Molina, 2002)
Cantharellus subalbidus	North America	(Pilz and Molina, 2002)
Hydnum repandum, Hydnum umbilicatum	North America	(Pilz and Molina, 2002)
Tricholoma magnivelare	North America	(Pilz and Molina, 2002)
Morchella sp.	North America	(Pilz and Molina, 2002)
Leucangium carthusianum	North America	(Pilz and Molina, 2002)
Tuber gibbosum	North America	(Pilz and Molina, 2002)

commercial macrofungal species from the wild, such as matsutake (*Tricholoma* sp.), boletes (*Boletus* sp.), truffles (*Tuber* sp.), morels (*Morchella* sp.) and various *Lactarius* species (e.g. *L. deliciosus*) is commercially lucrative in many countries and provides essential income for collectors and their families (Boa, 2004; de-Román and Boa, 2006; Yeh, 2000; Cai *et al.*, 2011). The most harvested and traded wild edible macrofungi in Asia, Europe and North America are presented in **Table 1**.

Many studies have confirmed the economic importance of wild edible macrofungi as key non-timber forest products (NTPs) (Cunningham and Yang, 2011; Christensen *et al.*, 2008; Zambonelli *et al.*, 2012). Accelerating demand has led to concerns about excessive harvesting and associated environmental damage, both affecting the sustainability of wild collections (Mortimer *et al.*, 2012). Harmful practices, including raking the ground surrounding trees for emerging mushrooms, may already have affected production for some of the most-sought after species. Exports of *Tricholoma matsutake* and Bolete species both decreased from 2010 to 2015 due to diminishing yields (**Table 2**). Management schemes have attempted to establish sustainable guidelines for harvesting wild edible macrofungi (Yu and Liu, 2005; Luoma *et al.*, 2006; Pilz and Molina, 2002), but ensuring good practices and policing laws aimed at regulating harvests are not straightforward. In response to concerns about sustainability, many countries have adopted corresponding measures; in Europe, for example, 16 countries have enacted relevant legislation and others have implemented guidelines (Peintner *et al.*, 2013). Setting limits for how much can be collected could potentially contribute to increased productivity (de-Miguel *et al.*, 2014). An interdisciplinary approach to sustainable management developed for *Thelephora ganbajun* took into consideration the needs of farmers and policies aimed at protecting local habitats and ecology (He *et al.*, 2011). de-Miguel *et al.* (2014) found that in pine forests, macrofungal production has a direct relationship with forest management: intense schemes (higher rate of felling) increased total mushroom yield, while non-intense forest management schemes (lower rate of felling) decreased total mushroom yield.

Some studies have looked more carefully at harvesting intensity (Hopping *et al.*, 2018). Along a longitudinal transect, Ruiz-Almenara *et al.* (2019) collected wild mushrooms, for seven months in the year, with each collection lasting two days, and continuing for 5–9 years, and found that this did not affect the diversity and distribution of species. Egli *et al.* (2006) found no correlation between intensity of harvesting and yields or species richness. Trampling had a temporary reduction on mushroom numbers, though allowing sites to "rest" enabled population recovery. Pilz and Molina (2002) also indicated that harvesting wild will not impair resources in the short term. We agree that reasonable macrofungi harvesting does not necessarily negatively impact macrofungal yield and species richness, but overharvesting can impair both (Evans, 1997). One possible explanation is because harvesting macrofungi involves only

Table 2 Total foreign export volume and value of *Tricholoma matsutake* and Boletes from 2010 to 2015 in Yunnan Province

Year	*Total output of foreign export (/t)*	*Total foreign exchange (/10000 dollar)*	*Total output of foreign export (/t)*	*Total foreign exchange (/10,000 dollar)*
2010	1203.15	4724.73	10,499.15	7511
2011	764	5750	12,431	9178
2012	964	4859	9142	6675
2013	781	4058	7708	5921
2014	708.9	3837	7041.3	4852
2015	432.4	2455	8366	4965

Note: Adapted from Wang, T.-T., Zhao, C.-Y., Chen, X., Guo, X., Wu, S.-R., 2017. Analysis and recommendations on current status of Yunnan Province edible fungi industry during the"12th Five Year" period. Edible Fungi of China 36, 70–75.

collecting sporocarps, leaving underground mycelium and the surrounding habitat relatively undisturbed. Thus, habitat protection and management is critical to macrofungal production and distribution (Evans, 1997). The ultimate aim of managing wild edible macrofungi is to achieve sustainable production, yet there continues to be a severe lack of related knowledge in many countries. Due to marked differences in the types of habitats inhabited by wild edible macrofungi, working closely with local communities will be needed in future to develop site-specific management strategies through rigorous and participatory scientific inquiry (Brown *et al.*, 2018).

Cultivated Macrofungi

The 12 species (out of 60 overall) that are commonly cultivated at scale are: *Agaricus bisporus*, *Lentinus edodes*, *Pleurotus eryngii*, *Volvariella volvacea*, *Hericium erinaceus*, *Auricularia auricula-judae*, *Ganoderma lucidum*, *Grifola frondosa*, *Flammunlina velutipes*, *Tremella fuciformis*, *Pholiota microspora* and *Coprinus comatus*. The two main cultivation methods consist of growing the fungi on substrates or using submerged fermentation. Most commercial edible macrofungi are cultivated in bags or using compost beds. The choice of substrate and cultivation technique has a strong influence on the nutritional content of mushrooms. Techniques are constantly being updated and improved, particularly in recent years, such as on *Ganoderma lucidum*, *Lentinus edodes* and *Schizophyllum commune* (Elisashvili, 2012). Nonetheless, growing mushrooms on substrates remains time consuming and labor intensive. Submerged fermentation is more rapid and capable of generating large quantities of mycelia and extracellular bioactive products usable as food ingredients and flavoring agents (Lu *et al.*, 2020).

The general cultivation process for edible macrofungi includes two phases: laboratory-based work (spore/sporocarp tissue isolation, pure culture, stock culture, spawn preparation) and cultivation in growing houses (culture media production, inoculation culture, cultivation management, spawn running and harvesting) (Lee *et al.*, 2015) (**Fig. 2**). Maintaining sterility during pure mycelia isolation and spawn production is crucial. Growth conditions need to be controlled as well, appropriately adjusting factors like temperature, relative humidity, oxygen, pests and diseases (Thawthong *et al.*, 2014).

Health and Nutritional Benefits Provided by Macrofungi

In addition to their culinary value, macrofungi can contribute significantly to the nutritive value of a meal. Macrofungi are rich in proteins, amino acids, vitamins and dietary fiber, also containing a wide array of compounds that provide health benefits. Macrofungi consumption can thus deliver manifold benefits to human health.

The protein content of macrofungi can be extremely high for a non-meat based food source, with a number of species reaching protein levels exceeding 30% (**Table 3**) (Kumar *et al.*, 2013; Valverde *et al.*, 2015; Sanmee *et al.*, 2003; Maga and Joseph, 1981). Macrofungi are also a good source of carbohydrates, with many species having carbohydrate levels above 50% (**Table 3**). Conversely, fatty acids and cholesterol content are relatively low, further enhancing their health benefits (Ribeiro *et al.*, 2009). Macrofungi are a natural source of many vitamins (B1, B2, B12, C, D and E) (Heleno *et al.*, 2010; Mattila *et al.*, 2001). Vitamin D is essential to human health and generally available as Vitamin D3, which is largely found in animal-based products, such as meat or eggs; however, research has shown that mushrooms can also be valuable source of Vitamin D2, D3, and D4 (Cardwell *et al.*, 2018). Wild-harvested mushrooms have higher levels of Vitamin D than cultivated mushrooms, as UV light is required for Vitamin D production in mushrooms (Valverde *et al.*, 2015). Thus, if cultivated mushrooms are exposed to an artificial source UV light, they have the potential to also produce vitamin D, thereby enhancing the food value of cultivated mushroom products (Cardwell *et al.*, 2018). Furthermore, macrofungi are rich in minerals (selenium, potassium, iron, copper, zinc, manganese) essential for a balanced diet (Sánchez, 2004). Selenium, commonly found in edible macrofungi, is an essential trace mineral important to maintaining a healthy immune system, improves fertility in adults and has also been linked with a reduction in the risk of cancer, thyroid disease and asthma.

Dietary fibers, which are carbohydrate polymers that show resistance to digestion and absorption by the human digestive tract (Alimentarius, 2015), play an important role in human health. Fibers are useful in preventing and treating diabetes as well as in mitigating cardiovascular disease and colon cancer (Kaczmarczyk *et al.*, 2012; Kendall *et al.*, 2010). The cell wall components of

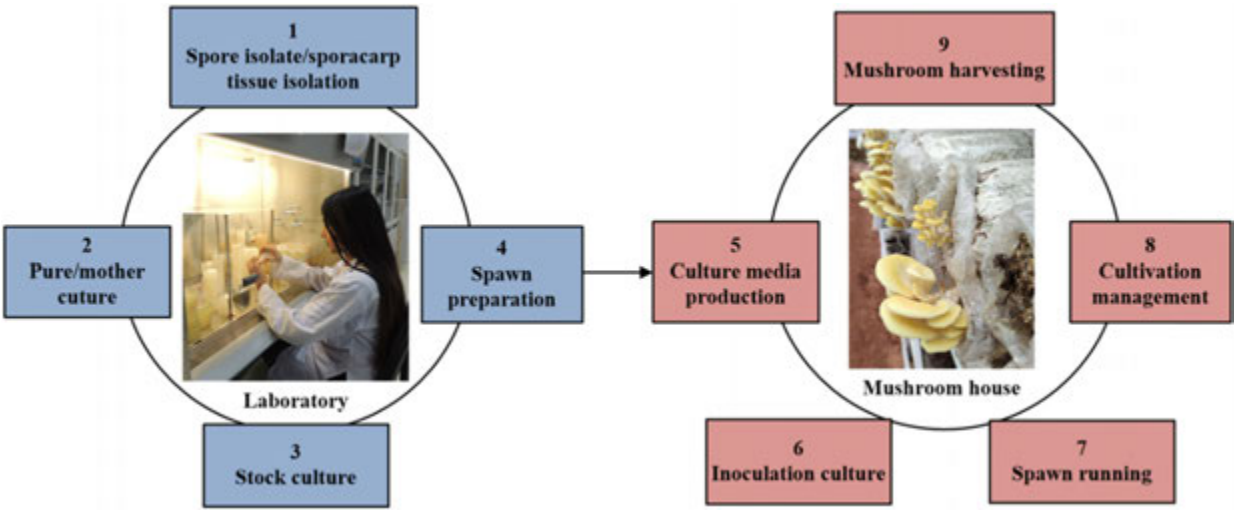

Fig. 2 Macrofungi cultivation process.

macrofungi (chitin and polysaccharides like mannans and *β-D*-glucans) are not easily digested by the human digestive tract and thus comprise a form of dietary fiber (Cheung, 2013). Although the content of dietary fiber varies between macrofungal species (**Table 3**), eating macrofungi on a daily basis can provide up to 25% of the required daily dose of fiber for humans (Cheung, 2013).

Macrofungi are rich in essential amino acids such as threonine, leucine, isoleucine, methionine, lysine, tryptophan, histidine, phenylalanine and valine (Yuwa-amornpitaK *et al.*, 2020; Valverde *et al.*, 2015). Additional amino acids found in macrofungi include glutamic acid, aspartic acid and glutamate. Essential amino acids are vital to human health, as the human body cannot synthesize these amino acids and needs to receive them via a balanced diet. These amino acids are important for the building of proteins, transport of nutrients within the body and maintaining an effective immune system.

Traditional medicine systems have a long history of using benefits associated with macrofungi in the treatment of illnesses. Indeed, the list is extensive and increasingly recognized by modern medicine. *In vivo* and in vitro testing has indicated that macrofungi can be used in antitumor, cancer-related, hypoglycemia, hypotension, diabetes and cardiovascular treatments; macrofungi are also characterized by immunostimulatory, neuroprotective, anti-neuroinflammatory, cytotoxic, anti-hyperlipidemic, antifungal, antibiotic, antiviral, antioxidant and hepaprotective properties (Valverde *et al.*, 2015; Chang and Wasser, 2012; Finimundy *et al.*, 2013; Yu *et al.*, 2009; Zhang *et al.*, 2011). A comprehensive list of macrofungal species and associated medical benefits is shown in **Table 4**. Some well-known examples of the medical applications of macrofungi include *Ganoderma lucidum* (Reishi) in antitumor, antioxidant and antimicrobial treatments (Ferreira *et al.*, 2015); *Hericium erinaceus* (Lion's mane fungus) in nerve damage treatments, as it possesses neuroregenerative properties (Wong *et al.*, 2012); and *Cordyceps* sp. in diabetes treatments and to improve human immune systems, as well as the uptake of oxygen by the blood (Shashidhar *et al.*, 2013; Mehra *et al.*, 2017). Furthermore, *Inonotus obliquus* (Chaga) is an effective treatment against tumors, diabetes and cardiovascular disease (Balandaykin and Zmitrovich, 2015).

Economic Value and Social Perspectives of Edible Macrofungi

Edible mushrooms are cultivated in more than 100 countries (Gupta *et al.*, 2018). The global consumption of edible macrofungi is around 12.74 million tons and global production is estimated to reach 20.84 million tons by 2026 (Research and Markets, 2018). The macrofungi industry was valued at approximately $63 billion in 2013, $34 billion of which stems from cultivated edible macrofungi, $24 billion from medicinal macrofungi and $5 billion from wild harvested macrofungi (Royse *et al.*, 2017). Over 30 billion kg of macrofungi were produced in China in 2013 (China Edible Fungus Association, 2014), accounting for 87% of total global production. The rest of Asia produced about 1.3 billion kg, while the EU, the Americas, and other countries combined produced about 3.1 billion kg. Five main genera constitute around 85% of the world's macrofungi supply. *Lentinula* is the major genus, contributing about 22% of the world's cultivated macrofungi. *Pleurotus* constitutes about 19% of the world's output while *Auricularia* contributes around 17%. The other two genera, *Agaricus* and *Flammulina*, account for 15% and 11% of the volume, respectively. Currently, only 10 countries in the world exceed 100,000 tons of production, namely the United States, Japan, the Netherlands, South Korea, Poland, Vietnam, Spain, France, Thailand and the United Kingdom.

In European countries, truffles are a multi-million-euro industry. In France, Italy and Spain, truffles are foraged in natural forests and also cultivated in truffle farms. *Tuber melanosporum* production is estimated to be worth approximately 23.5 million USD annually in France (Escafre and Roussel, 2006), 8.8 million USD in Spain and 4.8 million USD in Australia (Duell, 2012). In the past decade, the price that famers receive for one kilogram of *Tuber melanosporum* in Europe ranged from less than 176 USD to

Table 3 Nutrient composition of selected edible macrofungi (% dry weight)

Species	*Proteins*	*Carbohydrates*	*Fatty acids*	*Calorie (kcal/kg)*	*Ash*	*Crude fiber*	*Reference*
Agaricus arvensis	32.87	32.91	–	–	0.18	–	(Kumar *et al.*, 2013)
Agaricus bisporus	14.1	74	2.2	325	9.7	8.0–10.4	(Valverde *et al.*, 2015)
Agaricus langei	35.14	34.83	–	–	14.1	–	(Kumar *et al.*, 2013)
Aricularia auricula-judae	36.3	33.23	–	–	7.07	6.9	(Kumar *et al.*, 2013)
Boletus aestivalis	32.76	52.07	–	–	14.97	–	(Kumar *et al.*, 2013)
Boletus edulis	7.39	9.23	1.70	–	1.15	8.0	(Caglarirmak *et al.*, 2002)
Cantharellus cibarius	34.17	47	–	–	7.78	11.2	(Kumar *et al.*, 2013)
Craterellus odoratus	15.5	65.1	3.0	–	8.1	–	(Sanmee *et al.*, 2003)
Calocybe indica	3.22	6.38	–	500.3	2.3	–	(Zahid *et al.*, 2009)
Lactarius glaucescens	18.6	52.9	9.2	–	8.5	–	(Sanmee *et al.*, 2003)
Lactarius piperatus	2.67	6.5	0.18	–	1.15	–	(Caglarirmak *et al.*, 2002)
Heimeiella retispora	21.1	44.5	6.0	–	11.6	–	(Kumar *et al.*, 2013)
Lentinus edodes	4.5	87.1	1.73	772	6.7	7.3–8.0	(Valverde *et al.*, 2015, Cheung, 2013)
Lepiota lilacea	28.12	49.33	–	–	8.09	–	(Kumar *et al.*, 2013)
Lepiota magnispora	27.55	35	–	–	7.07	–	(Kumar *et al.*, 2013)
Phaeogyroporus potentosus	24.2	46.4	2.8	–	17.8	–	(Sanmee *et al.*, 2003)
Pleurotus ostreatus	7.0	85.9	1.4	416	5.7	7.5–8.7	(Cheung, 2013, Valverde *et al.*, 2015)
Pleurotus florida	3.29	4.37	–	355.1	1.27	–	(Zahid *et al.*, 2009)
Pleurotus eryngii	11.0	81.4	1.5	421	6.2	–	(Valverde *et al.*, 2015)
Pleurotus sajor-caju	37.4	55.3	1.0	–	6.3	–	(Valverde *et al.*, 2015)
Pleurotus giganteus	17.7	78.0	4.3	364	–	–	(Valverde *et al.*, 2015)
Pleurotus pulmonaritus	37.63	43.4	–	–	10.17	–	(Kumar *et al.*, 2013)
Lactarius hygrophoroides	44.93	42	–	–	2	–	(Kumar *et al.*, 2013)
Russula alboareolata	21.2	41.6	9.5	–	17.6	–	(Kumar *et al.*, 2013)
Russula lepida	18.3	60.1	5.6	–	7.6	–	(Kumar *et al.*, 2013)
Russula nigricans	22.6	56.3	4.8	–	6.7	–	(Kumar *et al.*, 2013)
Russla virences	20.0	54.7	4.3	–	11.3	–	(Kumar *et al.*, 2013)
Russla xerampelina	22.4	55.83	4.5	–	6.7	–	(Kumar *et al.*, 2013)
Schizophyllum commune	22.5	32.4	–	–	10.1	–	(Kumar *et al.*, 2013)
Tremmella fuciformis	4.6	94.8	–	0.2	0.4	1.4	(Cheung, 2013)
Trhicholoma giganteum	16.1	70.1	–	4.3	5.0	4.5	(Cheung, 2013)
Lepista irina	26.12	50.2	–		3.16	–	(Kumar *et al.*, 2013)
Tuber melanosporum	23.3	66.2	–	2.2	8.3	27.9	(Cheung, 2013)
Melanoleuca grammopodia	36.27	33.04	–		4.13	–	(Kumar *et al.*, 2013)
Volvariella volvacea	30.1	50.9	–	6.4	12.6	11.9	(Cheung, 2013)

Note: Adapted from Carneiro, A.A., Ferreira, I.C., Dueñas, M., *et al.*, 2013. Chemical composition and antioxidant activity of dried powder formulations of Agaricus blazei and Lentinus edodes. Food Chemistry 138, 2168–2173. Kalač, P., 2013. A review of chemical composition and nutritional value of wild-growing and cultivated mushrooms. Journal of the Science of Food and Agriculture 93, 209–218. Phan, C.-W., Wong, W.-L., David, P., Naidu, M., Sabaratnam, V., 2012. Pleurotus giganteus (Berk.) Karunarathna & KD Hyde: Nutritional value and in vitro neurite outgrowth activity in rat pheochromocytoma cells. BMC Complementary and Alternative Medicine 12, 1–11. Reis, F.S., Martins, A., Vasconcelos, M.H., Morales, P., Ferreira, I.C., 2017. Functional foods based on extracts or compounds derived from mushrooms. Trends in Food Science & Technology 66, 48–62. Valverde, M.E., Hernández-Pérez, T., Paredes-López, O., 2015. Edible mushrooms: Improving human health and promoting quality life. International Journal of Microbiology 2015. Kumar, R., Tapwal, A., Pandey, S., et al., 2013. Macro-fungal diversity and nutrient content of some edible mushrooms of Nagaland, India. Nusantara Bioscience 5, 1–7. Zahid, M.K., Barua, S., Haque, S.I., 2009. Proximate composition and mineral content of selected edible mushroom varieties of Bangladesh. Bangladesh Journal of Nutrition 61–68. Caglarirmak, N., Unal, K., Otles, S., 2002. Nutritional value of edible wild mushrooms collected from the Black Sea region of Turkey. Micologia Aplicada International 14, 1–5. Sanmee, R., Dell, B., Lumyong, P., Izumori, K., Lumyong, S., 2003. Nutritive value of popular wild edible mushrooms from northern Thailand. Food Chemistry 82, 527–532.

more than 939 USD. The price for retail customers can be even higher; in Paris and London, it is not uncommon for prices to reach 2347–4695 USD per kg. This encourages European truffle farmers to sell directly to markets. Moreover, additional economic gains result from the impact of truffle sales on agritourism, local mycological gastronomy, value-added truffle products, truffle fairs and retail markets. In total, the economic impact of *Tuber* was estimated at 117 million USD per year in Italy alone (Gregori, 2007; Doménech and Barreda, 2014).

Many rural people in developing countries rely upon commercial harvesting of wild macrofungi as an important source of household income. For example, there is a strong tradition of collecting and consuming wild edible macrofungi in eastern Finland, a region where the Karelian people settled from Russia. Around 25% of Karelian families collect wild mushrooms and sell them at markets. Boa (2004) showed that total amounts sold in local markets can be considerable. In Malawi, money earned by local mushroom collectors is not insignificant, and local markets for wild edible macrofungi are currently expanding. In Turkey, around 11 tons of fresh *Lactarius delicious* are annually sold in 13 villages. The total annual value of four key wild edible species is around 100,000 USD (Boa, 2004). Perhaps the most notable example of the role that wild harvesting of mushrooms has on local economies can be seen in Yunnan Province of China. Households and, at times entire communities, can generate their annual

Table 4 Bioactive compounds extracted from edible macrofungi

Edible macrofungal species	*Metabolites with antioxidant activity*	*Metabolites with Antimicrobial activity*	*Metabolites with Cytotoxic and/or Antiproliferative activity*	*Metabolites with other biological activities*
Canthharellus cibarius	Polysaccharide	–	Polysaccharide	Polysaccharide (immunomodulatory and neuroprotective activities)
Craterellus cornucopioides	Polysaccharide	–	Craterellin C (20)	Polysaccharide extract (antimutagenic effects, antihyperglycemic and anti-inflammatory activities)
Fistulina heptica	–	Cinnatriacetins A (37) and B (38)	–	–
Hydnum repandum	–	–	Repandiol (39)	–
Laccaria amethystea	–	Laccaridiones A (41) and B (42)	Laccaridiones B (42)	–
Lycoperdon pyriforme	–	Compounds 63 and 64	Compound 65	Compounds 63 and 64 (nematicidal activity)
Ramaria botrytis	Polysaccharide	–	A novel ubiquitin-like protein	Glucan (immunostimulating activity)
Sarcodon imbricatus	–	Polysaccharide	–	Polysaccharide (immunoenhancement and anti-myelosuppressive activities)
Termitomyces microcarpus	–	–	Dimethylincisterol; 5α,8α-epidioxy-(22*E*,24R)-ergosta-6,22-dien-3β-ol	–
Thelephora ganbajun	Ganbajunins A-B (105 – 106); Ganbajunin C (107); 3-O-methylatromentin	–	Polysaccharide	Ribonuclease (inhibitory activity toward HIV-1 reverse transcriptase); Polysaccharides (antidiabetic activity)
Volvariella bombycina		Isodeoxyhelicobasidin	Compound 113	Isodeoxyhelicobasidin (human neutrophil elastase (NHE) activity); Compound 113 (inhibitory effects on melanogenesis)

Note: Adapted from Thu, Z.M., Myo, K.K., Aung, H.T., *et al.*, 2020. Bioactive phytochemical constituents of wild edible mushrooms from southeast Asia. Molecules 25, 1972.

income during the wet season, from the sales of wild mushrooms, with thriving towns being established around the key purpose of trading in mushrooms.

Seasonal Availability and Preservation

Wild mushrooms occur in natural habitats inside small fruiting windows, often during rainy season, leading to predictable seasonal availability for timing harvests. Fruiting conditions vary considerably across hemispheres and climatic conditions (Montoya *et al.*, 2014). For example, the fruiting season of *Tricholoma matsutake* lasts from July to October, in the Northern Hemisphere, in which the soil temperature needs to average 19°C and relative humidity around 80%, (Yamanaka *et al.*, 2020). *Tuber indicum*, however, fruits between August to November in the Northern Hemisphere (García-Montero *et al.*, 2010). Because of their transient but predictable pattern of occurrence, wild edible macrofungi are considered delicacies and typically associated with a brief, seasonal flurry of economic activity around their collection and sale (Garibay-Orijel *et al.*, 2006). Developing commercial cultivation schemes under controlled conditions extends the fruiting window of these rare commodities. Given the heightened worldwide demand for edible macrofungal consumption, farmers in less developed regions of the world are now well positioned to increase production of cultivated mushrooms, bolstering rural incomes and mitigating environmental strain associated with wild overharvesting (Zhang *et al.*, 2018).

The fruiting bodies of fresh wild edible macrofungi typically have a short shelf life (1–3 days) at ambient temperatures (Diamantopoulou and Philippoussis, 2015; Ruan-Soto *et al.*, 2017). To meet this challenge, techniques have been developed to preserve fresh fruiting bodies beyond this short period of edibility, particularly in the preservation of *Agaricus* sp, *Lentinus edodes* and *Pleurotus* sp (Akbarirad *et al.*, 2021). Preservation methods can be divided into two types: short-term preservation (cooling, minimal processing, packaging) and long-term preservation (freezing, canning, pickling, drying) (Diamantopoulou and Philippoussis, 2015). Preservation methods can be further divided into thermal preservation (drying, cooling), physical preservation (packaging, irradiation, pulsed electric field) and chemical preservation (washing, coating, ozone, electrolyzed water) (**Fig. 3**) (Zhang *et al.*, 2018). Modern industrial technologies have significantly improved preservation methods. However, local communities around the world continue to predominantly rely upon four traditional methods of macrofungi preservation: drying, pickling, freezing and canning (Ruan-Soto *et al.*, 2017).

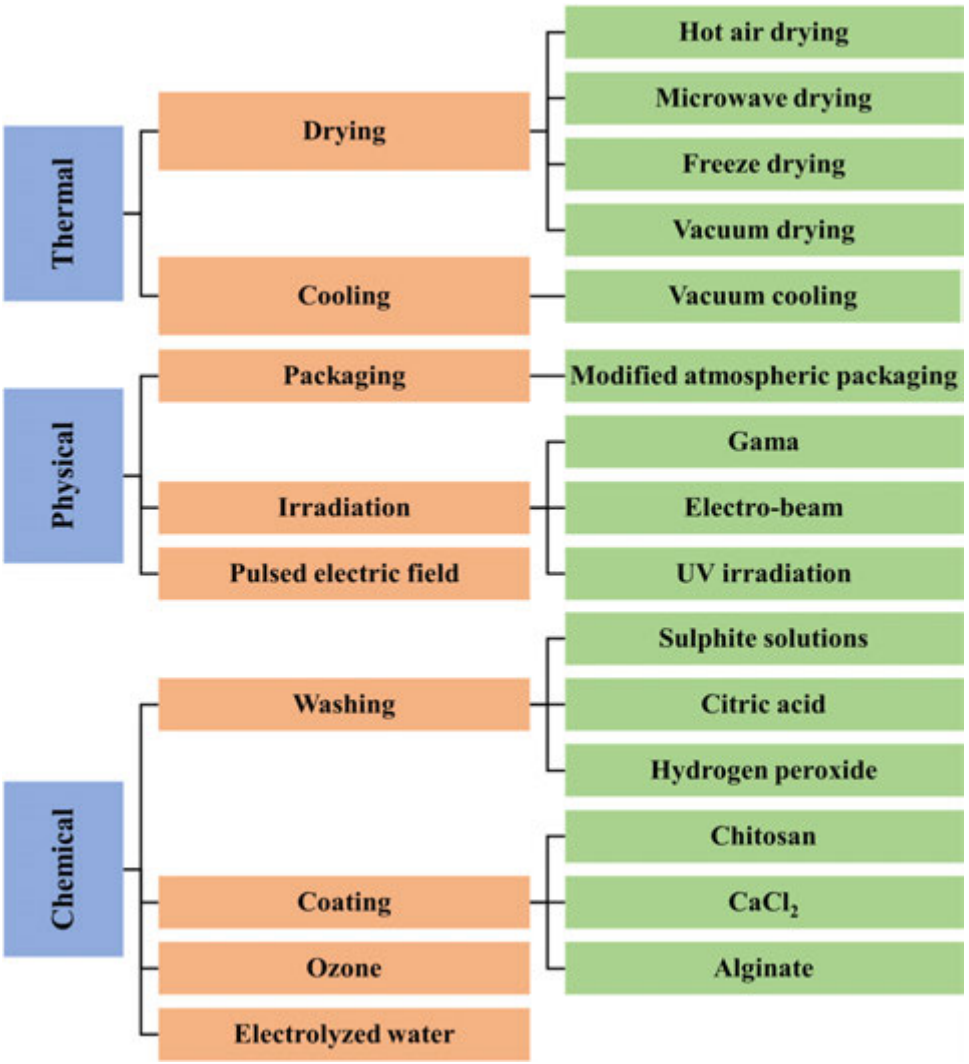

Fig. 3 Preservation methods of edible macrofungi Adapted from Zhang, K., Pu, Y.-Y., Sun, D.-W., 2018. Recent advances in quality preservation of postharvest mushrooms (Agaricus bisporus): A review. Trends in Food Science & Technology 78, 72–82.

Socio-Cultural Aspects of Macrofungi as Food

The concept of mycophilic and mycophobic peoples, countries and regions proposed by Wasson and Wasson (1957) has greatly influenced research and popular understanding of cultural attitudes and preferences for wild edible macrofungi. While it is a helpful concept for understanding cultural preferences, behaviors and depth of macrofungal knowledge, the concept is less useful in recognizing that behaviors and choices change over time. For example, local knowledge of safe-to-eat species can be easily lost as rural people migrate to urban centers, while new traditions can be introduced as people emigrate. Ukrainians who migrated to northeastern Argentina after World War 1 brought with them a vibrant tradition of collecting and eating wild macrofungi, infusing it into the weak existing local traditions.

Polish immigrants in the United Kingdom are carrying on their home tradition of visiting forests in late summer and autumn to seek out edible macrofungi in increasing numbers. The same applies to Italians and other "mycophilic" nationalities as they have been transplanted around the world. The acquisition of new eating habits involving wild macrofungi can also be reversed when reports of poisoning are widely published, especially through social media.

Simpler explanations for why some people do not eat wild macrofungi, despite local availability, also exist. Svanberg and Lindh (2019), describe how Swedes have greatly strengthened their predilection for wild macrofungi in the post-industrial era largely through informal educational and social events. Greater confidence in identifying species and the chance to gather "free food" led to behavioral changes. A loss of habitat and suitable tree species with edible ectomycorrhizal associations may also contribute to weak or absent traditions in a given area.

Much of our knowledge of local traditions around the collection and eating of wild macrofungi is anecdotal and poorly documented, though there has been a significant increase in ethnomycological studies (e.g., Nanagulyan *et al.* (2020) for Armenia

Table 5 Morbidity and mortality following consumption of wild mushrooms reported from different countries and their regions

Country/Region	*Annual average number of poisoned people*	*Annual average number of fatalities*	*Period*
Belarus	125 (2013–2015)	4	2006–2015
Bulgaria (Varna)	75	1	1991–2015
China	337	72	2004–2014
China (Guizhou)	70	9	2004–2013
China (Yunnan)	146	20	1985–2000
China (Yunnan)	662	25	2001–2006
Czechia (Prague Toxicological center)	146	7	1991
France	8000–10000[1]	–	1998
Iran	1247	19	2018
Iran (Razi Hospital of Rasht)	15	–	2006–2012
Italy (Province of Parma)	21	–	1996–2016
Japan	192	1	2001–2010
Japan	184	2	1988–1997
Japan	364	2	1959–1988
Japan	500–600	15	–
Japan (Nagano)	20	–	1970–1994
Mexico	–	10	2005–2006
Nepal	–	15–20	–
Poland	–	31	1931
Russia	1128	67	1995–2007
Russia	806	26	2005, 2010, 2013–2016
Spain (Barcelona)	15	1	2013–2016
Switzerland	248[1]	–	1966–2014
Switzerland	376[1]	–	1995–2009
Switzerland	8[2]	0	1995–2009
Taiwan	4	0	1986–1993
Thailand	–	8	2008–2014
Turkey (Middle Black Sea region)	53	–	2002–2007
Ukraine	361	31	2005–2014
Ukraine		112	2000
Ukraine (Lugansk oblast)	39	5	2001–2010
United States	7976 (1993)	20	1900–1994
United States	–	100–200	–
United States	8314	–	2001–2011
United States	9208	3	1989
United States	7000–9000	–	–
United States (27 from 37 regional poison centers)	3420	–	1987–1988
United States (Florida)	86	0	2003–2007
United States and Europe	–	100–200	–
Western Europe	–	50–100	–
Zambia	50	6	1980–1981

Note: Adapted from Govorushko, S., Rezaee, R., Duanov, J., Tsatsakis, A., 2019. Poisoning associated with the use of mushrooms: A review of the global pattern and main characteristics. Food and Chemical Toxicology 128, 267–279; 1-Number of calls to Poison Information Centers
2-Hospitalization of patients with macrofungi poisoning.

and Lucaj *et al.* (2021) for Lao PDR). The primary motivations for collecting wild edible macrofungi include earning money (Cai *et al.*, 2011) and self-consumption. Edible macrofungi comprise a valuable source of nutrition (Boa, 2004), particularly for poorer rural communities, or are simply a source of gastronomic intrigue and variety in middle- and higher-income countries. The harvesting of matsutake (*Tricholoma magnivelare*) in Bhutan (Namgyel, 2000) only attracted the attention of local people when the opportunity for commercial trade with Japan was identified.

The introduction of tree species to an area with novel edible macrofungi associates can boost local incomes as well as establish more positive attitudes toward mushrooms. *Pinus nigra* was used extensively in afforestation schemes in Castile Leon in Spain, introducing abundant and valuable harvests of *Lactarius deliciosus* (de-Romàn and Boa, 2004). The income potential of these chance occurrences is not fully understood, in part because the commercial harvesting of valuable species is unregulated in many countries and veiled in secrecy, driven in part by a desire to protect collection sources and avoid fees and taxes (Dyke and Newton, 1999). A stark contrast in attitudes to edible macrofungi in Finland (Härkönen, 1998) reflects the ethnic origins of collectors and

consumers. Those from Sweden have traditionally had a much weaker tradition of collecting and consuming wild mushrooms compared to those originally from Karelia, a region in neighboring Russia.

The direct nutritional value of wild macrofungi harvests to rural communities is also poorly understood. Yields are unpredictable, tightly linked with weather conditions, and the window of opportunity for harvesting fresh macrofungi is small. Some rural communities preserve excess harvests in oil or dry macrofungi, particularly *Boletus* and allied species, a practice common in China, Russia and countries in eastern and southern Africa with miombo woodland. In these circumstances, preserved macrofungi can offer diverse nutrients that are accessible in food-poor times of the year (Afonkin, 2013).

Food Safety

Food safety has been a hot topic for the assessment of human health all over the world. Although wild edible mushrooms have many benefits to human health, poisoning incidents caused by picking and consuming poisonous macrofungi continue to occur every year around the globe, posing a serious threat to human health. A number of accidents have been reported in Europe, north America and Asia (Chen, 2014). For example, in North America, according to the latest annual report on macrofungi poisoning issued by the North American Fungi Association, about 100 people are poisoned by eating poisonous macrofungi every year. In the Piemont region of Italy, there were 177 macrofungi poisoning incidents involving 318 people from 2002 to 2009 (Zicari *et al.*, 2011). In Asia, there were 569 poisoning incidents from eating wild macrofungi from 2001 to 2010, and of these, 1920 people were poisoned and 10 died (Yamaura, 2013). Likewise, from 2004 to 2014 in China, there were 3701 poisoning incidents and 786 deaths (He *et al.*, 2019). The morbidity and mortality of wild macrofungi are summarized in the **Table 5**. The available data suggest that China, Russia and the Ukraine have the highest mortality rates from macrofungi poisoning. Mortality rates from macrofungi poisoning are also high in Belarus, Poland, Turkey, Iran, Nepal and Mexico. However, this must be seen in the context of an estimated total of 200–250 deaths occurring worldwide in any one year (Govorushko *et al.*, 2019).

Some species can only be eaten with careful cooking methods or pretreatment. For example, before cooking, *Bulgaria inquinans*, the fresh fruiting body must first be washed in clean water before being rubbed with light alkali water until all the black spores on the surface have been washed away. Additionally, *Bulgaria inquinans* contains a bioactive photosensitive toxin (Diisobutyl phthalate), which can produce allergic symptoms such as the swelling of the mouth and burning pain of the skin under sunlight when eaten without being properly cleaned (Bao *et al.*, 2019). It is thus necessary to clarify contradictory or ambiguous information to avoid future poisoning incidents. Furthermore, it is imperative to develop a structured definition of macrofungal edibility to strengthen knowledge and confidence in consuming wild species while also reducing consumer anxiety around edible fungi (Li *et al.*, 2021).

Besides innately poisonous macrofungal species, edible macrofungi can also be contaminated from outside sources, such as from the environment, during processing, degradation and spoilage. The most common outside sources of contamination are heavy metals, pesticides, processing contaminants, radioactive isotopes, microbial contamination, inherited toxins and allergens. Previous studies have indicated that many edible species of macrofungi can potentially accumulate high concentrations of heavy metals, of which mercury, cadmium, lead, and arsenic have received the most research attention. For example, *Lycoperdon perlatum*, *Macrolepiota rhacodes* and *Lepista nuda* have been identified as potent accumulators of lead (Kalač and Svoboda, 2000; Sesli *et al.*, 2008). *Boletus edulis*, *Marasmius oreades* and *Tricholoma georgii* can accumulate a high-concentration level of Cd and Hg (Kalač and Svoboda, 2000). Related research also found that approximately 26%–72% of arsenic toxicity was significantly reduced by cooking (Chiocchetti *et al.*, 2020). In addition, macrofungi are a perishable food, easily contaminated by other microbes; therefore, eating spoiled macrofungi should be avoided.

We propose six recommendations to address these issues: (1) industry, importers and restaurants should only trade or serve macrofungi that are widely known to be edible and correctly identified; (2) obligatory training for business operators that produce foodstuff with macrofungi or who import macrofungi should be enforced to prevent poisoning events; (3) food inspectors should have a general knowledge about which macrofungi are edible; (4) consumers should consume edible macrofungi, both fresh and processed, only when they are confirmed to be safe and labeled accordingly; (5) new tools for regulating trade and industry as well as for public food inspection should be developed; and (6) a list of guidelines informed by intelligent risk assessments for edible macrofungi should be generated, which can be used in accordance with general food safety requirements (Niksic *et al.*, 2016).

Conclusion

Macrofungi have been shown to be an important source of food and nutrition for humans, contributing to our health and development. Although macrofungi have been eaten by humans for thousands of years, it is only in recent history that the true value of macrofungi as a food has been embraced, as reflected in the massive upsurge in the production and trade of macrofungi. This increase is likely the result of gains made in our own understanding of the nutritional composition of macrofungi as well as the recognition of macrofungi as a functional food and as a positive contributor to human health. However, despite the widespread use of macrofungi as a food, knowledge gaps still exist around which macrofungi are considered safe and suitable to eat. Complicating the picture is that an element of cross-cultural subjectivity may influence determining the edibility of macrofungal species. This subjectivity may cloud the reality of what can and cannot be eaten. Thus, a concerted effort should be made to not

only further research the food safety aspects of macrofungi but also to present and refine a standardized system for categorizing the edibility of macrofungi.

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Overview: Human Fungal Pathogens

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Human pathogenic fungi are a major cause of morbidity and mortality, and the incidence of fungal diseases continues to rise and new fungal pathogens, including the identification of cryptic species, are emerging. Recent estimates of the incidence of fungal diseases conclude that there over 300 million people globally who develop a serious invasive infection every year and close to a billion individuals have cutaneous fungal diseases (GAFFI, 2018). Annual incidence estimates for leading invasive mycoses include ~700,000 invasive *Candida* infections, ~500,000 *Pneumocystis* pneumonias, ~250,000 patients with invasive aspergillosis, ~220,000 episodes of cryptococcal meningitis, and ~100,000 cases of histoplasmosis. Moreover, there are an estimated 1.5 million deaths annually attributed to fungal diseases (Bongomin *et al.*, 2017).

General recognition of fungi as major human pathogens did not occur until the 1980s, and was associated with a marked increase in invasive mycoses linked to the HIV epidemic, the increased use of invasive procedures and intensive care units, and the administration of immunosuppressive drug therapies (Nucci and Marr, 2005). Moreover, increased global travel, climate change, and novel immunotherapies are contributing to the increased rates of fungal diseases. These chapter of the encyclopedia on human pathogenic fungi has over 40 chapters that broadly cover key concepts in the biology of human fungal pathogens, details major etiologic agents of human mycoses and disease syndromes, and reviews approaches to diagnosis and treatment of fungal diseases encountered in our healthcare systems.

Chapters are dedicated to the epidemiology of yeast and mold infections. Insights into epidemiology have led to life-saving interventions in specific patient populations, such as in various prophylactic antifungal regimens provided to transplant patients. However, the chapters also reveal areas of new risks for fungal diseases. For example, the advent of new immunomodulatory therapeutics being administered in cancer care as well as other diseases has led to specific alterations in immunological cascades that are placing those receiving these novel therapeutics at risk.

The chapters on specific pathogens as well as frequently encountered diseases, ranging from infections of the skin to the central nervous system, effectively present the biology of human pathogens and the disease processes that occur. Certain fungi, like *Candida albicans* and *Pneumocystis jirovecii*, are primary human commensals whereas the vast majority of fungal pathogens are acquired from the environment. The variety of human pathogenic fungi span three different phyla Zygomycota, Ascomycota, and Basidiomycota. Depending on the species, the pathogenic morphologies of these fungi can appear as molds, pseudohyphal forms, yeast or other unique structures, such as the spherules of *Coccidioides* species. These various morphologies are challenging to the host immune system, particularly in the setting of phase transition, where the immune cells are faced with structures that can be antigenically quite different from that originally acquired from the environment.

The chapters in this encyclopedia also highlights emerging and re-emerging fungi. The broad use of antifungals in humans, animal husbandry, and agriculture along with changes in climate, increased incursion of humans into new environments, and profound immunosuppression of certain patient populations have resulted in a surprising number of clinical cases of human mycoses by new or re-emerging pathogens. Examples in these chapters include *Candida auris*, a global threat due to its rapid emergence and resistance to antifungal drugs, and *Emergomycosis* species, which have also occurred globally. Molecular biology has also influenced this process as previously described single species have now been shown to actually consist of several cryptic and/or divergent species. For example, *Paracoccidioides brasiliensis* and *P. lutzii* diverged at least 30 million years ago, whereas the cryptic species with the *P. brasiliensis* complex evolved within the past few to 12 million years ago (Turissini *et al.*, 2017). Furthermore, certain fungi have been renamed based on deeper analyzes of genomes, such as the major fungal pathogen in Southeast Asia *Penicillium marneffei* becoming *Talaromyces marneffei* (**Fig. 1**).

Effectively diagnosing and managing of human mycoses is critical to the outcome of these diseases; yet, despite our current approaches for identifying and treating these diseases, mortality rates of invasive mycoses remain at ~40%. The chapters review the classic approaches to identifying fungi in clinical situations and also detail newer advances that are improving our ability to efficiently diagnose patients. Point of care tests, such as the lateral flow assay for cryptococcosis, are especially important in low resource countries. Molecular tests and applications of matrix-assisted laser desorption/ionization (*MALDI*) time-of-flight (TOF) mass spectrometer approaches have also accelerated our capacity to diagnose these complex diseases. Antifungal medications used for systemic therapy currently target either cell membrane (ergosterol) synthesis or stability, synthesis of cell wall beta-glucan, or RNA synthesis. Exceptions include the use of trimethoprim-sulfamethoxazole for treatment of pneumocystosis or paracoccidioidomycosis. Chapters in the encyclopedia review the mechanisms of action, the spectrum of activities of and resistance mechanisms to these drugs as well as their unfortunate toxicities. Additionally, a chapter presents information about new drug development, which is essential to improve our armamentarium for application to the care of patients with invasive mycoses. Harnessing the immune system to combat fungal diseases is an exciting area of study, and a chapter is presented that focuses on immunotherapy for fungal infections.

Fungal diseases remain a major scourge on humanity. Although investigators and healthcare providers have dramatically improved our knowledge about fungi and our capacity to care for patients with fungal diseases, an increased focus is necessary to

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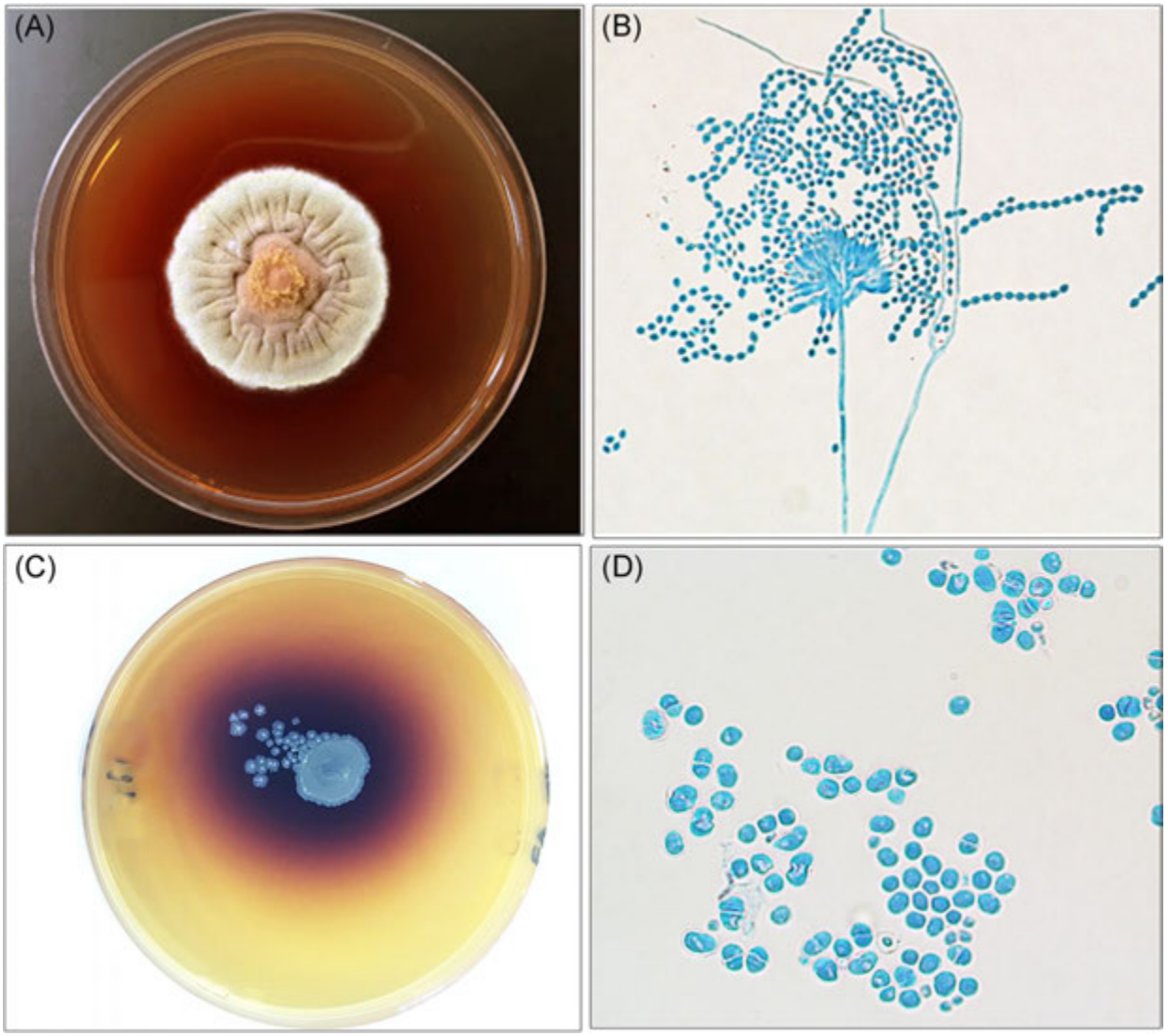

Fig. 1 The human pathogenic fungal pathogen *Talaromyces marneffei*. Fig. 1. Colony appearance *T. marneffei* on potato dextrose agar at 25°C (A) and brain heart infusion agar at 37°C (C). Microscopic morphology of mycelium (B) and binary fission yeast or yeast-like arthroconidia (D) (brightfield microscope; magnification, ×100).

truly combat fungal infections, which too often are neglected in terms of public policy and research agendas (Rodrigues and Nosanchuk, 2020). The chapters on human fungal pathogens in this encyclopedia provide the reader with a broad and solid background on fungal biology and the diseases currently encountered.

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Polyenes and Amphotericin B

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Introduction to Fungal Pathogens and Antifungal Drugs

The discovery of penicillin by Fleming in 1928 was a breakthrough moment in the history of Medicine, showing that microorganisms can produce active compounds of medical use (Fleming, 1929). Since then, the development of effective antimicrobials has been a priority for the treatment of infectious diseases. The high impact that pathogenic microorganisms have in our society has resulted in the use of multiple drugs that have helped to control their incidence. However, there is still a great need to find new antimicrobial therapies to treat these diseases. Some examples are opportunistic fungal diseases, which have increased their incidence in our society due to the emergence of immunosuppressed individuals, such as patients who suffer severe abdominal surgery or who are under immunosuppressive therapy (transplants, cancer, etc), or the use of parenteral nutrition or central venous catheters (Leroy *et al.*, 2009; Puig-Asensio *et al.*, 2014).

The main fungal pathogens belong to *Candida*, *Cryptococcus* and *Aspergillus* genera, but there are many others able to cause disease with a lower prevalence. One of the main problems of these infections is the high mortality associated, which ranges between 20%–90%, depending on multiple factors, from both the pathogen and the immunological state of the patient.

The main antifungal families used to treat invasive fungal diseases are azoles, echinocandins, fluoropirimidines and polyenes. Each family has advantages, but they also show limited activity against some fungal species (Gomez-Lopez *et al.*, 2008).

Azoles (mainly fluconazole, itraconazole, voriconazole, posaconazole and isavuconazole) inhibit ergosterol biosynthesis. It is important to highlight that fungal membranes do not contain cholesterol, but ergosterol, which is the main sterol that maintains the proper fluidity of the membrane in this kingdom. The most recent antifungal family used in clinic belongs to echinocandins (caspofungin, micafungin and anidulafungin). They inhibit the synthesis of an important component of the fungal cell wall, β-1,3-glucan, weakening the integrity of this structure. Fluoropirimidines (5-fluorocytosine, also known as flucytosine) inhibit DNA and RNA synthesis. But the antifungals that present the strongest fungicidal activity belong to the polyenes family, which will be the main topic of this chapter. Chemically, these compounds are polyunsaturated molecules with multiple double and triple bonds. There are several polyenes that have medical use. In particular, some of these polyenes have antifungal activity, mainly, filipin, natamycin, nystatin and amphotericin B, being this last one the compound with the strongest fungicidal activity among antifungal drugs.

Amphotericin B. An Old Drug, But Still With Many Secrets Behind

In this review, we will give a brief overview of the most relevant aspects of Amphotericin B (AmB). This molecule was found by the Squibb Institute for Medical Research from cultures of a filamentous bacteria (*Streptomyces nodosus*) isolated from the soil collected in the Orinoco River region of Venezuela (Dutcher, 1968; Cereghetti and Carreira, 2006). The compound had amphoteric properties (ability to react either as acid or base), and for this reason it was named amphotericin. Originally, two types of amphotericins, A and B, were identified for their antifungal activity. Amphotericin B showed the strongest *in vitro* efficacy, so it was the main drug to treat fungal diseases since the early 60s, until new antifungal families were introduced in clinical practice decades later.

The molecular structure of AmB was determined in the 60s. AmB is a large polyene macrolactone composed of a ring of 38 carbon atoms (**Fig. 1**). The structure of AmB confers both hydrophilic and hydrophobic properties. The hydrophilic region is comprised by one of the sides of the ring (carbons 3–15) that contains several hydroxyl groups. The other side of the ring (carbons 20–33) contains multiple double bonds, conferring hydrophobic properties. The macrolactone also contains a ring formed by a bond between carbons 13 and 17. Finally, Amphotericin B is glycosylated, and it contains the sugar mycosamine at carbon 19. This antifungal is water-insoluble, and its preparation requires the use of organic solvents, such as DMSO. The structure and chemical properties described above will explain the biological properties of this molecule.

Mechanisms of Action

Since its discovery, AmB has been an "enigmatic" molecule because it has taken many years to understand and characterize its mechanisms of action. Furthermore, it is still not fully known how this molecule exactly exerts all the effects that have been reported in literature. But if we want to send a clear message to the reader, that would be that AmB is a "multitask" molecule because it has several mechanisms of action. Classically, it has been postulated that the main target of this antifungal is the plasma membrane, but many other studies also support that amphotericin B can also cause intracellular damage. So far, three main mechanisms of action have been attributed to AmB, and will be described in the next sections.

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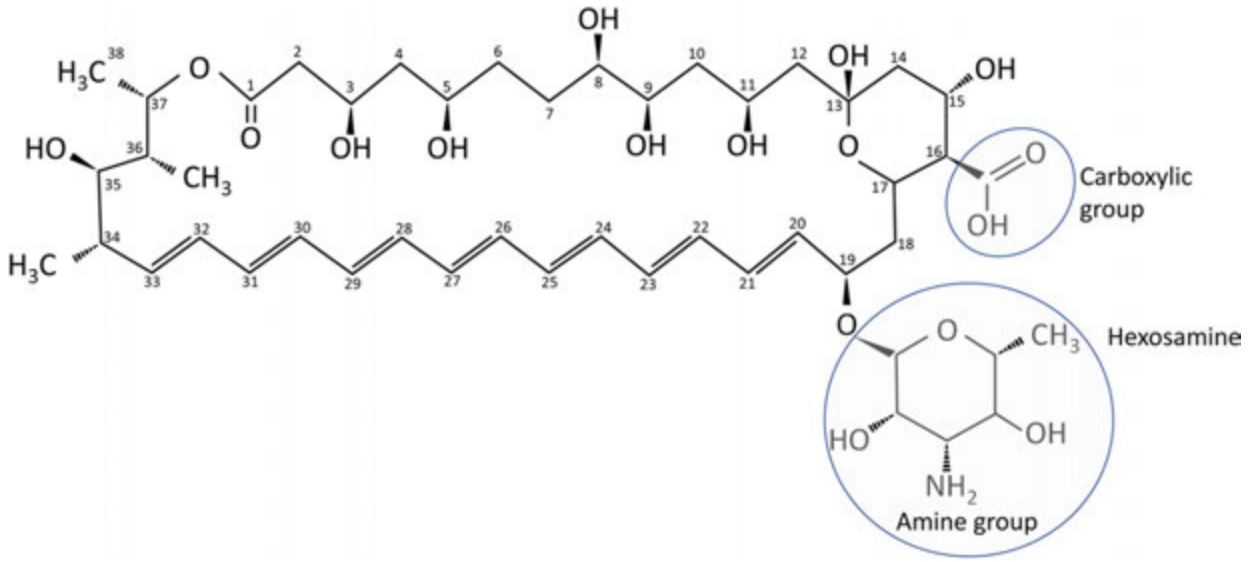

Fig. 1 Chemical structure of AmB.

Pore Formation at the Membrane

Polyene compounds are known to affect the cell membrane, and the specific effect of AmB for fungi was determined when it was found that it had a greater affinity for ergosterol than for cholesterol (Kotler-Brajtburg *et al.*, 1974). The first studies that proposed the "classical mechanism of action" were performed in the 70 s and suggested that AmB formed a pore at the membrane, increasing the permeability (Marty and Finkelstein, 1975; de Kruijff *et al.*, 1974; de Kruijff and Demel, 1974). The ability to form pores is due to the chemical structure. The main model of action suggests that several molecules of AmB (4 – 12) form an orientated structure. The inner part (lumen) of the channel would be composed by the polyol region, staying the polyunsaturated side at the external part, interacting with sterols (1:1 ratio). The diameter of the pore depends on the concentration of AmB at the membrane, and ranges from 0.16 to 16 nm (Yang *et al.*, 2013; Kaminski, 2014). The exact mechanism by which the antifungal interacts with ergosterol remains to be fully elucidated, but the mycosamine ring plays a key role in binding to the sterol (Wilcock *et al.*, 2013).

The antifungal conformation in the membrane can be very variable. For example, it has been described that at low concentrations, AmB does not fully penetrate the membrane, but stays perpendicularly to the phospholipids, in a flat position on the surface (Mouri *et al.*, 2008). At higher concentrations it can penetrate between the phospholipids and forms aggregates as macrocomplexes forming pores. Furthermore, when AmB molecules assemble to form pores, they can arrange into different conformations (Cohen, 1998, 2010). One of them is called the non-aqueous conformation, in which the AmB molecules adopt a V-shape, and only allow the movement of monovalent cations ($K+$ or $Na+$). On the other hand, AmB can also associate and form a channel in an open conformation (aqueous conformation), in which the antifungal molecules are in a parallel position with the phospholipids. The main factor that determines the formation of aqueous or non-aqueous pores is the ergosterol concentration, being the non-aqueous conformation formed in the absence of ergosterol. The aqueous type channels allow the diffusion of solutes such as monosaccharides. In addition, the aqueous channels can be formed in two different ways: as double barrel or single barrel. In the double barrel state, two AmB molecules interact longitudinally, acquiring a parallel orientation with the phospholipids of the membrane. When the AmB molecules associate to form pores, two barrels are formed. The COOH group of each AmB molecule is at the edge of the membrane and the OH group at carbon 35 is at the inner part, interacting with the other AmB molecule. In the single barrel model, AmB molecules do not interact longitudinally with any other, producing a single pore which causes a thinning of the membrane (**Fig. 2**).

In conclusion, the general idea that AmB forms pores is very simplistic as this molecules can adopt different conformations with different properties, which highlights the complexity of the effects of AmB at the membrane.

Ergosterol Sequestration

During decades, it has been believed that the mechanism of action of AmB was based on its ability to form pores and increase membrane permeability. However, later studies have demonstrated that this process is not the only one involved in the fungicidal effect of AmB. One of the alternative mechanisms proposes that AmB produces killing through binding to ergosterol without pore formation. Evidence of this alternative model was provided by the characterization of the effects of the polyene natamycin on yeasts, where it was found that this compound strongly bound to ergosterol and inhibited growth of *S. cerevisiae* without altering

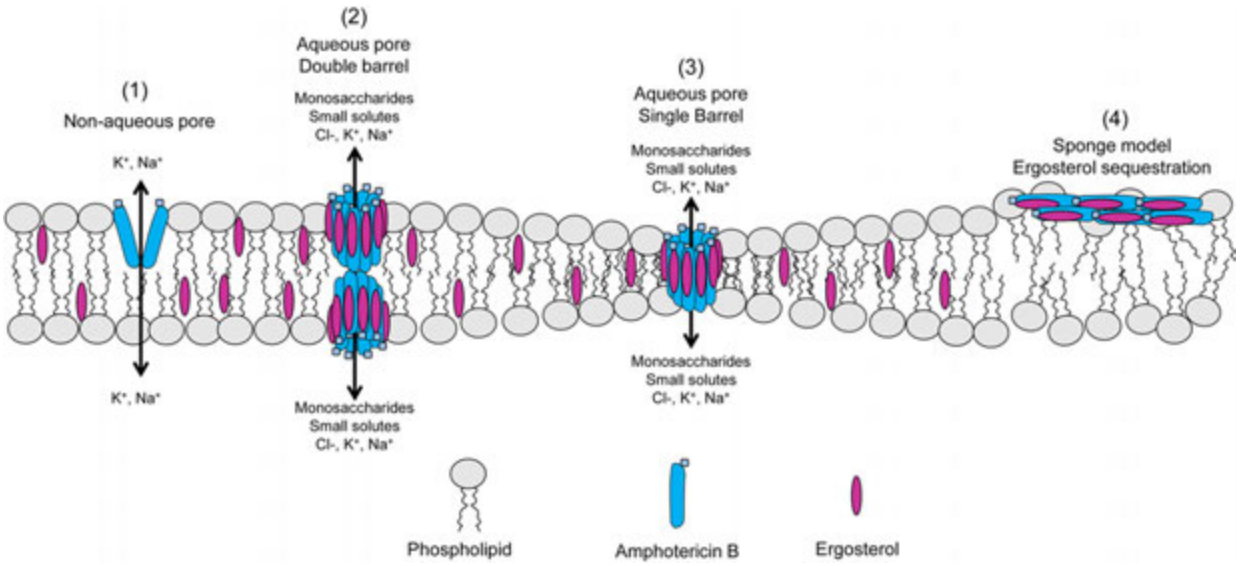

Fig. 2 Different conformations of AmB in the plasma membrane. (1) Non-aqueous conformation, in V-shape, that allows the pass of small cations and is ergosterol-independent. (2) Aqueous confirmation in the double barrel state, which allows the pass of small solutes, cations and anions. (3) Aqueous conformation in the single barrel state. (4) Sponge model, in which AmB does not arrange in pores, but forms aggregates at the surface that sequester ergosterol from the phospholipids, causing a disorganization of the membrane.

the plasma membrane permeability (te Welscher *et al.*, 2008). Further evidence is derived from the characterization of the role of the different regions of the molecule in pore formation and binding to ergosterol. Channel formation largely depends on the carboxylic group (C16) and on the hydroxyl at C35, and modifications of these groups still result in active amphotericin molecules (Carmody *et al.*, 2005; Szpilman *et al.*, 2008a,b; Palacios *et al.*, 2007). In this sense, it has been shown that binding of AmB to ergosterol is enough to kill the fungal cells (Gray *et al.*, 2012), and this antifungal can accumulate, forming large aggregates acting like "sponges" that extract ergosterol from the membrane (Anderson *et al.*, 2014). In this way, AmB would interfere with many of the functions that ergosterol has in the cell (cell fluidity, endocytosis, and vacuole fusion, among others) and lead to cell death. These models are also supported by the fact that alterations of the mycosamine group, required for ergosterol binding, result in a lack of fungicidal activity (Croatt and Carreira, 2011; Wilcock *et al.*, 2013).

In summary, the effects of AmB on the fungal membrane have been largely demonstrated due to its affinity to ergosterol. The mechanisms described so far (pore formation and the sponge model) are not exclusive, and there is strong evidence to believe that both take place simultaneously. Although several authors conclude that the role of pores might not be the relevant one, the fact that AmB has several effects on the membrane explains the strong fungicidal action of this antifungal.

Beyond the Membrane: Oxidative Burst and Intracellular Damage

In the previous sections, we have briefly described the effects that AmB has on the fungal membrane due to its affinity to ergosterol, but AmB has additional mechanisms of action beyond the plasma membrane. Several studies have dissociated the fungicidal effect from the increased permeability of the membrane induced by AmB (Chen *et al.*, 1978). In addition to the "sponge" model described above, many studies demonstrate that AmB can induce accumulation of reactive oxygen species (ROS) in the cell (Sokol-Anderson *et al.*, 1986; Belenky *et al.*, 2013; Mesa-Arango *et al.*, 2014; Sangalli-Leite *et al.*, 2011; Phillips *et al.*, 2003). These molecules are important regulators of multiple cellular processes, but when their concentration increase, they can alter the chemical structure of proteins, lipids and nucleic acids, causing important damage to the cells. In this way, AmB causes damage not only at the membrane, but also at multiple cellular levels.

The role of free radicals seems to be more important than the effects at the plasma membrane. For example, AmB has reduced antifungal activity under hypoxic conditions (Gale *et al.*, 1977), a fact that cannot be related to channel formation at the membrane. On the other hand, many studies indicate that induction of oxidative damage is one of the main killing mechanisms induced by AmB. It has been demonstrated that AmB has the same effects on some molecules (i.e., lipid peroxidation and protein carbonylation) as those induced by ROS (Blum *et al.*, 2008; Mesa-Arango *et al.*, 2014). Moreover, antioxidants protect the cells from killing produced by this antifungal (Sokol-Anderson *et al.*, 1988).

The mechanism by which AmB induces oxidative stress is not known. ROS are secondary subproducts of mitochondrial respiration, and it has been shown that this organelle, along with the respiration process, play a role in AmB-induced oxidative stress (Mesa-Arango *et al.*, 2014).

Another strong evidence of the role of ROS in AmB mechanism of action has been shown from the study of the filamentous fungus *Aspergillus terreus*. This mold has reduced susceptibility to this antifungal compared to other *Aspergillus* species such as *A. fumigatus*, despite both fungal species have similar ergosterol content at their membranes. In the case of this fungus, several chapters have associated its increased resistance to an increase in the response to oxidative stress, based in antioxidant enzymes such as superoxide dismutase (SOD) and catalase (Jukic *et al.*, 2017; Blum *et al.*, 2008).

Spectrum of Action and Resistance

As stated above, AmB is the antifungal that has more deleterious effects on the fungal cells including membrane, proteins, lipids and DNA, and for this reason, it is the most potent licensed antifungal available. Fungal resistance to AmB is very rare. There are very few yeast species with reduced susceptibility (i.e., some *Trichosporon* spp). However, resistance to AmB is more frequent among filamentous fungi, such as *A. terreus*, *Lomentospora prolificans*, and some *Fusarium spp*.

Secondary resistance to AmB is extremely rare, as it is required that the cells acquire simultaneously several mechanisms to resist all the challenges induced by AmB. The main known mechanism of resistance is a reduction in the ergosterol level, which has been reported in some *Aspergillus*, *Candida* and *Cryptococcus* spp (Kim and Kwon-Chung, 1974; Woods *et al.*, 1974; Safe *et al.*, 1977; Drutz and Lehrer, 1978; Vandeputte *et al.*, 2007; Currie *et al.*, 1995; Kelly *et al.*, 1994; Hitchcock *et al.*, 1987; Sanglard *et al.*, 2003). This is normally achieved by mutations in the *ERG* genes, required for ergosterol biosynthesis. However, in other cases, AmB resistance does not correlate with a decrease in ergosterol at the membrane, as is the case with *A. terreus* described above (Dannaoui *et al.*, 2000), although this has also been described in other species (Joseph-Horne *et al.*, 1996). In this sense, AmB resistance can occur when there is an inhibition of the oxidative burst, which can be due to a decreased production of ROS or an increase of detoxifying mechanisms. Since ROS are mainly produced in the cell at the mitochondria, several processes that affect its function influence the susceptibility to this antifungal. For example, strains with diminished respiration rate are less susceptible to AmB (Geraghty and Kavanagh, 2003; Mesa-Arango *et al.*, 2014). In addition, inhibition of fungal respiration with rotenone, an inhibitor of respiratory complex I (NADH dehydrogenase), produces resistance to AmB (Mesa-Arango *et al.*, 2014). In agreement, similar results have been found when there is an increase in enzymes that detoxify ROS such as superoxide dismutase or catalase (Gonzalez-Parraga *et al.*, 2011; Sokol-Anderson *et al.*, 1988; Mesa-Arango *et al.*, 2014; Guirao-Abad *et al.*, 2020; Linares *et al.*, 2013). This data is supported by works that demonstrate that yeast cells respond in the presence of AmB with the activation of signaling pathways involved in adaptation to stress, such as the MAPK Hog1 route (Mesa-Arango *et al.*, 2014; Guirao-Abad *et al.*, 2020).

Characterization of some *Candida* AmB resistant isolates has shown that, in fact, there is accumulation of several of the mechanisms described above in the same strain: mutations in *ERG* genes, decreased respiration rates and increase in ROS detoxifying enzymes (Vincent *et al.*, 2013; Mesa-Arango *et al.*, 2014). If resistance to AmB requires the accumulation of several mutations, this might explain the low incidence of secondary resistance to this antifungal. But there is another aspect that may contribute to the low resistance rate to AmB. In addition to the low probability that several mutations that confer resistance occur in the same strain, it is important to consider that virulence depends on the ability of the fungi to grow and replicate *in vivo*. In this sense, some of the mutations that confer resistance to AmB, such as those that reduce the ergosterol concentration or the respiratory rate, affect also to the replication of the yeasts. This produces a reduction of the fitness of the cells, and since the ability of these mutants to replicate *in vivo* is compromised, it is also more unlikely that they cause disease compared to WT susceptible strains (Vincent *et al.*, 2013).

Amphotericin B Toxicity and Lipidic Formulations

Although AmB is the most effective antifungal, its clinical use is limited by its toxicity and secondary effects (see detailed reviews in (Laniado-Laborin and Cabrales-Vargas, 2009; Loo *et al.*, 2013)). As AmB shows low affinity to other sterols, such as cholesterol, it can cause deleterious effects also in mammalian cells. The main toxic effect induced by AmB occurs in the kidneys. Nephrotoxicity might occur during the first two weeks of treatment, and this effect correlates with an increased mortality of the patients. There are several mechanisms by which AmB induces nephrotoxicity, including a direct effect on the function of renal tubules and renal vasoconstriction. In addition, AmB can bind to some immune receptors (i.e., TLR) and in this way, it also behaves as an inmunomodulator, and alters the release of pro- and anti-inflammatory cytokines (Ben-Ami *et al.*, 2008; Mesa-Arango *et al.*, 2012). As a consequence, there are multiple side effects, such as chills, fever, hypotension, headaches, tachycardia, nausea and vomiting. Finally, it has been described that in a number of patients, AmB can inhibit erythropoiesis and reduce the number of platelets, inducing anemia and thrombocytopenia (Yeo *et al.*, 2006; Mahmud *et al.*, 2009; Kulpa *et al.*, 1981; Chan *et al.*, 1982).

The first licensed AmB in 1959 was a presentation with sodium deoxycholate (Fungizone), which is the most toxic formulation. Years later, lipidic formulations have been developed to reduce the toxic effects of AmB (Adler-Moore and Proffitt, 2008; Dupont, 2002; Torrado *et al.*, 2008; Hamill, 2013). So far, there are three different types of formulations: AmB lipidic complex (Abelcet), AmB colloidal dispersion (Amphotec) and liposomal Amphotericin B (AmBisome). Compared with the deoxycholate formulation, these formulations have significantly reduced toxicity and increased efficacy, being the liposomal AmB form the one showing the lowest nephrotoxicity. However, its high price limits its wide use in developing areas, where the incidence of some invasive fungal diseases, such as cryptococcosis, is of a great concern.

Clinical Uses of AmB

AmB has been the main choice for the treatment of invasive fungal diseases, and it shows activity against most of the pathogenic fungal species, such as *Candida*, *Cryptococcus*, *Histoplasma*, *Paracoccidioides*, *Coccidioides*, most *Aspergillus* spp (except *A. terreus*), *Mucorales* and *Fusarium*. Despite this, the new antifungal families (triazoles and echinocandins) have displaced the use of polyenes due to their toxicity or the price of the lipidic and liposomal formulations. For example, echinocandins have become the first choice to treat invasive candidiasis, and voriconazole is used for invasive aspergillosis. Still, AmB remains the first choice to treat some fungal diseases such as cryptococcosis. It is also used as secondary therapy in cases in which there is failure in the treatment with some of the first choice antifungals. This is the case for azole-resistant *A. fumigatus* or echinocandin-resistant *C. glabrata*, which is a species with intrinsic diminished susceptibility to azoles. Another example is the emerging pathogen *C. auris*, which is intrinsically resistant to fluconazole and has a particular ability to adapt to echinocandins, being in this case AmB one of the main therapeutic alternatives.

One of the main limitations for the use of AmB is its administration route. Although many attempts to develop oral formulations have been done (Cuddihy *et al.*, 2019), these have been so far unsuccessful because it is poorly absorbed from the gastrointestinal tract. For this reason, AmB formulations are injected through parenteral administration directly into the blood, and their concentration and distribution depends on the formulation administered (Loo *et al.*, 2013). It strongly binds to plasma proteins, and it has a poor diffusion to some body regions, such as the cerebrospinal fluid, aqueous humor, bile and amniotic and pericardial fluids. Finally, AmB is slowly eliminated by the body having an elimination half time above 7 days.

Conclusions

Amphotericin B was the first antifungal isolated and used in clinical practice, and paradoxically, after 60 years of use, we still do not fully understand how it kills the fungal cells. Many studies have demonstrated that AmB is a multifaceted molecule that has several mechanisms of action: pore formation, ergosterol sequestration and induction of oxidative burst. For decades, the only role attributed to this molecule was an increase in plasma membrane permeability due to channel formation, but in the last years, there are strong evidences supporting that ergosterol sequestration and induction of reactive oxygen species are the main mechanisms of action. More research is still needed to fully understand all the effects of AmB on the cells. The main limitation for its wide use is the associated toxicity. Since this antifungal exerts the strongest fungicidal activity compared to the other families, it is also important to design future strategies that will help to reduce its toxicity and, in this way, improve the efficacy and outcome of the patients.

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Azole Antifungal Drugs: Mode of Action and Resistance

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Glossary

Antifungal susceptibility testing These methods allowed to study how susceptible/resistant is a fungal species particular isolate to the tested antifungal agents.
Aspergillosis The set of diseases caused by *Aspergillus* spp. which comprises a variety of clinical manifestations.
***Aspergillus* spp.** The most frequent genus of filamentous moulds isolated in clinical samples, causing a wide range of infections.
Azole drugs Heterocyclic compounds that contains a ring with nitrogen substitutions. In the case of triazoles, the ring contains three nitrogen atoms. In the case of moulds, triazoles are currently the antifungal drug class more commonly used to treat these infections.
Azole resistance The set of intrinsic features or subsequent alterations that enables fungi to survive in presence of azole drugs.
Broth microdilution plates A method used to test the susceptibility of a microorganism to some compounds where the drug is diluted in broth in a microtiter plate.
***Candida* spp.** A major human yeast pathogen that causes both mucosal and deep tissue infections.
Candidiasis The set of diseases caused by the different *Candida* species, including a variety of clinical manifestations.
Cyp51A/Erg11p Cytochrome P450 14-α demethylase enzyme, the target of azole drugs.
Demethylation inhibitors A drug family that inhibits a key enzyme involved in ergosterol biosynthesis, the 14-α sterol demethylase, impairing the fungal growth. Imidazoles and triazoles are the most used compounds of this family.
Efflux pumps Proteins that selectively admit or excludes chemicals so that they can regulate intracellular concentration of different compounds.
Ergosterol Main sterol in membranes of fungal cells.
Fungicides Chemical compounds used mainly in agriculture to kill moulds and their spores in crops.
Invasive fungal diseases An infection caused by fungal species characterizes by the invasion of a tissue, usually due to the lack of an effective immune response.
Minimum inhibitory concentration The lowest concentration of a compound which does not enable growth of a specific microorganism.
Multi-azole resistance A set of characteristics that enables fungi to survive in presence of several azole drugs.
Point mutation Is a type of changes in the DNA sequence in which one single nucleotide base is added, deleted or substituted for another nucleotide.
Transcription factors Transcription factors include a wide number of proteins that initiate and regulate the transcription of genes.

Introduction

Invasive fungal diseases (IFD) are life-threatening infections and the main cause of mortality and morbidity in immunocompromised patients or in high risk patients who suffer from immunodeficiencies, hematological malignancies, solid organ transplant recipients, or those with chronic obstructive pulmonary disease or receiving high-dose and continued corticosteroid therapy (Low and Rotstein, 2011; Akan *et al.*, 2013; Rüping *et al.*, 2008; Walsh and Gamaletsou, 2013).

The most common fungal pathogens involved in invasive diseases are *Candida albicans* and *Aspergillus fumigatus* (Schwartz and Patterson, 2018; Pagano *et al.*, 2011). However, other *Candida* non-*albicans* species as well as other filamentous fungi, have been increasingly reported and quite often causing disseminated infections in immunocompromised hosts (Alcazar-Fuoli and Mellado, 2014; Picazo *et al.*, 2008; Friedman and Schwartz, 2019).

In spite of the improvements in diagnosis, and antifungal therapy for treating and preventing fungal infections, morbidity and mortality rates associated with these infections remains very high (Neofytos *et al.*, 2009). The most important factors are associated with the status of the host; however, other aspects can also have a major impact. Among them, fungal diagnosis is not straightforward and therefore antifungal therapy is often initiated too late. The key issue for a successful recovery is the prompt treatment with the appropriate drug, avoiding, when possible, drug toxicity (Badiee and Hashemizadeh, 2014; Pianalto and Alspaugh, 2016; Wiederhold, 2017).

This situation becomes more complicated because emerging resistance to existing antifungals is a current problem (Garcia-Rubio *et al.*, 2017; Pristov and Ghannoum, 2019) and a poor response to antifungals has been described in clinical isolates (Pfaller, 2012; Schwartz and Patterson, 2018).

However, some new molecular tools can provide a valuable amount of novel information that eventually might be applied to improve patient management. The most remarkable one is whole genome sequencing (WGS) which has emerged as a useful tool

for the analysis of genetic differences between fungal isolates, their genetic background, and even to know how antifungal resistance was developed (Garcia-Rubio *et al.*, 2018).

Antifungals in Clinical Use

The current antifungal drugs target a limited number of cellular processes. Most of them interfere with the ergosterol biosynthesis, which is the main sterol in fungal membranes. The need for new antifungal agents is undeniable since current therapeutic choices to treat IFD are limited to three major classes: polyenes, echinocandins and azole drugs (Patterson, 2005; Campoy and Adrio, 2017).

Amphotericin B (AMB) is included in the polyene group of drugs, and it has been the standard treatment for invasive fungal infections during decades. Ergosterol is the target of this compound, but due to the high toxicity to the host, probably owing to the interaction with cholesterol host-cell membranes, its used has been compromised and is no longer administrated as first choice treatment (Ostrosky-Zeichner *et al.*, 2003).

The use of echinocandins-caspofungin (CSF), micafungin (MCF) and anidulafungin (ANF)- has significantly improved the outcome of patients with these infections (Patil and Majumdar, 2017) as they have favorable safety profiles and a broad spectrum of activity. Their effectiveness is based on the blockage of cell-wall synthesis by inhibiting β-(1,3)-D-glucan synthase (Turner *et al.*, 2006). They are the primary treatment for invasive infections caused by most of *Candida* species (Bennett, 2006; Wiederhold, 2016) and are only effective in combination with azoles for treating *Aspergillus* spp. infections (Perlin, 2007; Panackal, 2016).

Azole drugs include fluconazole (FCZ), itraconazole (ITZ), voriconazole (VRZ), posaconazole (PSZ), and isavuconazole (ISZ). The last of them has been recently introduced in clinical use as a new extended-spectrum triazole, and its activity against *Aspergillus* has been proved (Miceli and Kauffman, 2015). All of them are well tolerated and are active against diverse fungi (Andes and Dismukes, 2011). Besides, triazoles are orally available, which makes them essential for long-term therapy. FCZ is well established as a leading drug in the setting of prevention and treatment of mucosal and invasive candidiasis (Charlier *et al.*, 2006). Although VRZ is recommended as first-line therapy for invasive aspergillosis (IA), ITZ is still commonly used for chronic and allergic non-invasive forms of aspergillosis, while PSZ was shown to reduce the number of invasive fungal infections in neutropenic patients (Patterson, 2005).

The emergence of antifungal resistance is a current problem that could be influenced by the widespread use of these compounds, especially azoles, as antifungal agents both in the clinical and the agricultural setting (van der Linden *et al.*, 2011; Pfaller, 2012; Manavathu *et al.*, 1998).

Drug resistance among fungi species represents the primary cause of treatment failure in patients with invasive fungal infections and an important and alarming health problem, since once the resistance is acquired to a compound, it could lead to cross-resistance to other drugs of the same family (Sanglard, 2016).

This manuscript reviews the current understanding of azole drug resistance with special emphasis on the molecular resistance mechanisms that have been described mainly in *Candida* and *Aspergillus* spp.

Definition of Resistance

Resistance to antifungal drugs could be defined as the set of intrinsic features or alterations that enables fungi to survive in presence of these substances.

Generally, resistance can be classified as either microbiological or clinical. Microbiological resistance depends directly on the microorganism and can be classified as primary or intrinsic, when an organism is resistant to a drug prior to exposure, and secondary or acquired resistance, which is developed in response to the exposure against an antifungal agent. Clinical resistance has an impact on treatment failure despite of its susceptibility *in vitro*. This may happen when the drug used is not able to reach the infected site in enough concentration or/and the host immune response is not able to eradicate the fungi (Kanafani and Perfect, 2008). Also pharmacokinetic and pharmacodynamics parameters of the drug have an important role (Nucci and Perfect, 2008).

How Resistance is Determined? Antifungal Susceptibility Testing

Antifungal susceptibility testing (AST) refers to the ability of a specific organism to grow *in vitro* in the presence of a particular drug. The standardization of a methodology, which allows reproducible and reliable results, has been thoroughly explored. Accordingly, Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antibiotic Susceptibility Testing (EUCAST) have published guidelines for testing *Candida* and *Aspergillus* isolates using standardized broth microdilution methods (Arendrup *et al.*, 2012; Clinical Laboratory Standards Institute, 2008a,b; Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing, 2008; Pfaller *et al.*, 2009; Verweij *et al.*, 2009). These methodologies allow the *in vitro* establishment of antifungal cut-off as well as the identification of resistant isolates that is crucial to choose the most suitable treatment.

The AST reference methods are based in broth microdilution plates that can easily be prepared and frozen in the clinical laboratory until susceptibility testing is performed. In general, the incubation period is usually 24–48 h, and after that the minimum inhibitory concentration (MICs) are read. However, the difference between MICs reading at 24 and 48 h is minimal for some isolates, and does not alter the interpretative category (Alastruey-Izquierdo *et al.*, 2015). For visual interpretations the end point for azoles is the point presenting substantial reduction in growth and could also be referred to as 80% reduction in growth when compared with growth control. However, when the microdilution method is used and read by spectrophotometer absorbance, 50% of growth inhibition is the value that best approximates the visual endpoint (Rex *et al.*, 2001).

Both procedures, CLSI and EUCAST, have been extensively employed and it has been demonstrated that are able to characterize the mould susceptibility through MICs to antifungal drugs (Alastruey-Izquierdo *et al.*, 2015). However, breakpoints to allow discrimination between susceptible and resistant strains *in vivo* have not been established for all species (Johnson, 2008). Therefore, *in vivo* correlation studies are urgently needed to develop interpretative MIC clinical breakpoints (CBPs) for the different antifungals used for treating these infections.

Azoles: Mode of Action

The use of mould active azoles caused a revolution in medical mycology due to their broad spectrum and their reduced toxicity compared to amphotericin B and has clearly improved patient survival (Sheehan *et al.*, 1999; Neofytos *et al.*, 2009).

The chemical families that inhibit C-14 demethylation are imidazoles, which are mostly used topically, and triazole drugs. They are classified chemically based on the number of nitrogen atoms in the azole ring (two for imidazoles and three for triazoles) (Andes and Dismukes, 2011). Collectively, these compounds are called sterol demethylation inhibitors (DMIs) and are widely used in both the clinical (as treatment or profilaxis choice for fungal infections) and in the environmental setting (as fungicides in agriculture) (Kelly *et al.*, 1995; Hollomon, 2017).

In the mammalian cells, cholesterol is one of the most important sterols present in the cell membrane. However, the major sterol in the fungal cell is a different one, the ergosterol, which makes it a suitable antifungal target. The triazoles target fungal cell growth by inhibiting a key enzyme involved in ergosterol biosynthesis, the 14-α sterol demethylase of the cytochrome P450s Cyp51 family, that are encoded by *ERG11* in yeast and *cyp*51 gene in moulds. This enzyme removes the methyl group at position C-14 of precursor sterols. The inhibition of the ergosterol synthesis at that level produces the accumulation of toxic sterols, such as lanosterol or eburicol, which substitute the methylated sterols and deplete ergosterol from fungal membrane, leading to cell membrane instability, growth impairment, and subsequent cell death (Kelly *et al.*, 1995; van den Bossche *et al.*, 1995; Waterman and Lepesheva, 2005; Parker *et al.*, 2014) (**Fig. 1**).

Differential antifungal potency between azoles is due to changes in their affinity for the enzyme and their different toxicity and drug interaction profiles depend on the cross-inhibition of various human cytochrome P450 enzymes (Lat and Thompson, 2011).

Since the first azole compounds were synthesized (imidazoles, such as miconazole, clotrimazole, and ketoconazole) some chemical modifications have been made in order to increase their activity and reduce cell toxicity, giving rise to the triazoles (Campestre *et al.*, 2017). Five triazole compounds (fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole) have been clinically approved and are currently widely used for the prevention and treatment of several life-threatening fungal infections (EMA, 2012a,b). Fluconazole (FCZ) is fungicidal in most species of *Candida* and was considered first-line agent in the past, however clinical trials have demonstrated that echinocandins can improve rate of survival (Tagliaferri and Menichetti, 2015). With regard to filamentous moulds, FCZ have no clinical relevant activity. *Aspergillus* spp. are intrinsically resistant to FCZ (Leonardelli *et al.*, 2016), unlike ITZ, VCZ, and PSZ which have been shown to have good *in vitro* and *in vivo* activity against these fungi. However, ITZ and VRZ are only fungistatic against *Candida* species (Greer, 2003). At present, VCZ is the most common antifungal compound used for invasive aspergillosis (IA). Isavuconazole (ISZ), approved for IA treatment in 2015 (Seyedmousavi

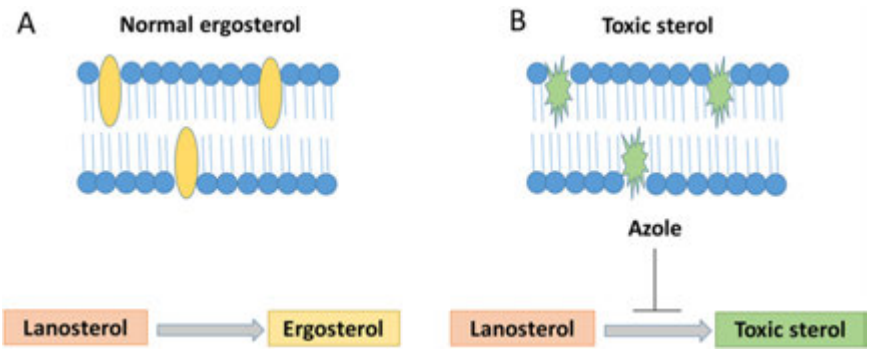

Fig. 1 (A) Normal ergosterol biosynthesis pathway resulting in cell growth (B) Ergosterol biosynthesis via the inhibition of Erg11/Cyp51 produces the accumulation of toxic sterols and subsequent cell death.

et al., 2015), has a broad spectrum of *in vitro* activity and *in vivo* efficacy against a wide range of yeasts and moulds including *Aspergillus* spp., and *Candida* spp. (Perkhofer *et al.*, 2009; Guinea *et al.*, 2008; Espinel-Ingroff *et al.*, 2013; Howard *et al.*, 2013).

Other newer triazole compounds are still under investigation (Turel, 2011). For instance, albaconazole (ABZ) has shown *in vitro* activities against pathogenic yeasts, and filamentous fungi and has been proven effective in some animal models (Miller *et al.*, 2004; Bartroli and Uriach, 2005).

Azole Drug Resistance Mechanisms

The resistance mechanisms to antifungal agents can differ between groups of drugs mainly due to the mode of action of each class of antifungal compound. As mentioned before for antifungal drugs in general, also two types of resistance have been described within azoles, the primary or intrinsic resistance and the secondary resistance (Anderson, 2005; Andes and Dismukes, 2011; Cowen *et al.*, 2015). The first results from the natural interaction between the fungal species and the azole compounds and occurs regardless of previous azole exposure (White *et al.*, 1998). In addition, fungal azole resistance can be explained by acquired secondary resistance mechanisms, which have been studied at molecular level and will be extensively explained here. The development of this type of resistance can evolve by the selection of the most resistance species in the host or by enabling the emergence of a resistant clone within a fungal species (Verweij *et al.*, 2016). Clinically, the main problem of secondary resistance is the selection of these resistant organisms during the azole treatment (Andes and Dismukes, 2011), and consequently, the restriction of treatment options (Rodriguez-Tudela *et al.*, 2008a). Therefore, a better understanding of mechanisms that underlie azole resistance is critical in order to avoid and prevent resistance development (Snelders *et al.*, 2008).

Azole resistance can develop through multiple mechanisms including: (1) target alteration or (2) its overexpression, (3) up-regulation of multidrug transporters, (4) transcription factors that play a role in cellular changes conferring tolerance to drug stress or toxicity or, (5) through biofilm sequestration.

Azole Resistance Mechanisms in *Candida* Spp.

The extensively use of FCZ and other triazoles due to their effectiveness against this genus, as prophylaxis, empirical therapy or diagnostic-driven therapy, has caused a selective pressure which has led to the emergence of less susceptible species and secondary resistance among primarily sensitive isolates (Cowen *et al.*, 2015).

Azole resistance in *Candida* spp. has been widely reported and well-studied. There are several mechanisms that can lead to acquired azole resistance in species of *Candida* genus (**Fig. 2**). The most commonly reported resistance mechanism is the decrease in the intracellular azole concentration by the activity of some efflux pumps (**Fig. 2.1**), encoded by the ATP-binding cassette (ABC) transporters or by the major facilitator superfamily genes (MFS) (Kanafani and Perfect, 2008; Sanglard, 2016). Induction of *Candida* drug resistance by increased expression of *CDR* genes, which encode efflux pumps, tends to reduce the accumulation of all azole drugs and it is often sufficient for antifungal resistance in certain species; i.e., *CDR1* and *CDR2* genes in *Candida albicans* and CgCDR1, CgCDR2 and CgSNQ2 genes in *Candida glabrata*. Conversely, efflux pumps encoded by multidrug resistance (*MDR)* genes are usually selective for fluconazole (Pfaller, 2012) and are restricted to MDR1 from *C. albicans* (Sanglard *et al.*, 1995; Calabrese *et al.*, 2000) and *Candida dubliniensis* (CdMDR1 and CdCDR2) (Moran *et al.*, 1998).

The upregulation of ABC and MFS transporters is controlled by different transcription factors (TFs) (**Fig. 2.2**). In *C. albicans*, *CDR1* and *CDR2* are regulated by TAC1 (Coste *et al.*, 2004), and also *CDR1* by another regulator called MRR1 (Morschhäuser *et al.*, 2007). In *C. glabrata*, CgPdr1 is a transcriptional factor involved in azole resistance via upregulation of ABC genes including, at least, CgCDR1, CgCDR2 and CgSNQ2 (Ferrari *et al.*, 2009; Vermitsky *et al.*, 2006).

Another resistance mechanism consists in increasing the number of drug targets (**Fig. 2.3**), so a higher azole concentration is needed to saturate all target molecules, leading to azole resistance. In *C. albicans*, the overexpression of the target gene (*ERG11*) mediated by the transcription factor UPC2 (**Fig. 2.3**) has been associated with azole resistance (Dunkel *et al.*, 2008; Lohberger *et al.*, 2014).

Finally, acquisition of point mutations in the azole target *ERG11* (**Fig. 2.4**), which encodes the enzyme 14α-lanosterol demethylase in *Candida* spp., results in amino acid substitutions that decrease the affinity of the target (Erg11p) for azoles (Lamb *et al.*, 2000). Several mutations Erg11p have been described but only a few of them have been directly related to azole resistance (Morio *et al.*, 2010). For example, Y132F/H and G464S/D have been shown to confer azole resistance in *C. albicans*, *C. dubliniensis* and *Candida tropicalis* (Perea *et al.*, 2001; Chau *et al.*, 2004; Kelly *et al.*, 1999; Forastiero *et al.*, 2013; Vandeputte *et al.*, 2005). Recently, combined mutations in both, *ERG11* and another enzyme of the ergosterol biosynthesis pathway encoding a C-5 sterol desaturase (*ERG3*) seem to be an emerging azole resistance mechanism in *C. albicans* and *C. tropicalis* (Morio *et al.*, 2012; Forastiero *et al.*, 2013; Eddouzi *et al.*, 2013; Jiang *et al.*, 2013; Martel *et al.*, 2010). However, the innate fluconazole resistance phenotype of *Candida krusei* seems to be due to the low sensitivity of the azole target (Erg11p) to azole drugs in combination with the constitutive expression of the multidrug efflux pump Abc1p (Lamping *et al.*, 2009). Only one study has addressed the molecular mechanisms underlying azole resistance in *C. parapsilosis*, concluding that similarly to *C. albicans*, its tolerance to azoles involves the activation of efflux pumps and/or their increased expression (**Figs. 2.1** and **2.3**) (Silva *et al.*, 2011).

Much less is known in the development of extra or intracellular compartments to sequester azoles. *Candida* spp. is able to form biofilm in some specific growth conditions (Ramage *et al.*, 2005; Desai and Mitchell, 2015). Biofilm formation has also been recognised

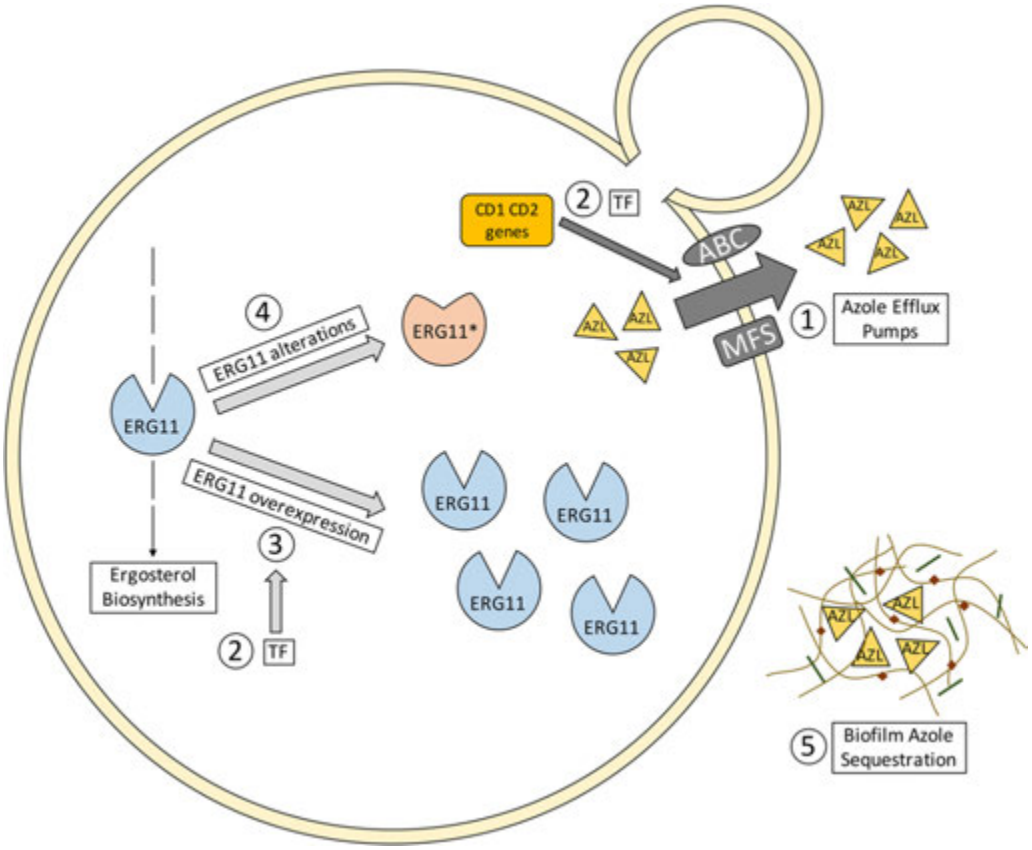

Fig. 2 Schematic representation of the main azole resistance mechanism in *Candida* spp.

as a potential factor for fungal drug resistance due to the production of an exopolymeric material that inhibits or restricts penetration of antifungal drugs (Taff *et al.*, 2013). This process has been clearly documented in *C. albicans* azole resistance (Mitchell *et al.*, 2015; Bonhomme and d'Enfert, 2013) and also has been described in *Candida auris* (Kean *et al.*, 2018). In relation to *C. auris,* one of the most troubling changes in the epidemiology of invasive candidiasis (Rhodes and Fisher, 2019), it is worth mentioning its rapidly worldwide emergence and its potential for efficient nosocomial transmission (Friedman and Schwartz, 2019; Kordalewska and Perlin, 2019). It is generally accepted that most isolates are multi-drug resistant (Arendrup *et al.*, 2017). Thus, some Erg11p specific mutations strongly associated with azole resistance have already been described (Chow *et al.*, 2018; Lockhart *et al.*, 2017; Rhodes *et al.*, 2018).

Azole Resistance Mechanisms in *Aspergillus* Spp.

The genus *Aspergillus* comprises 344 species (Samson *et al.*, 2014) and many of them have been described as pathogenic in humans and animals (Sugui *et al.*, 2014). In *A. fumigatus* in particular, azole resistance is well studied due to its clinical importance since it is the predominant etiological agent isolated from immune-compromised patients (Segal, 2009; Vermeulen *et al.*, 2015). Azoles are the main antifungal drugs that are used both in agriculture as well as in the clinical setting. However, the existing selective pressure in the environment and in the patients generates different azole resistance mechanisms (Bueid *et al.*, 2010; Snelders *et al.*, 2008; Camps *et al.*, 2012a) (**Fig. 3**). As a consequence, there may be found different azole susceptibility patterns based on their resistance mechanism, with increasing MICs to one, two, or all triazoles (Rivero-Menendez *et al.*, 2016; Garcia-Rubio *et al.*, 2017).

Therefore, different azole resistance profiles can be mainly attributed to specific amino acid changes in Cyp51A protein, which are caused by single-point mutations in the azole target gene, *cyp51A* encoding a 14-α sterol demethylase (**Figs. 3.1** and **4**).

The main triazole resistance mechanisms most commonly described when the acquired resistance is generated during extended periods of azole treatment involves the following amino acid substitutions:

A mutation in position glycine 54 (G54) can result in amino acids changes such as G54E, G54V, G54R and G54W. Clinical strains with these mutations show resistance with high MICs to ITZ and also to PSZ but not to VRC (Diaz-Guerra *et al.*, 2003; Nascimento *et al.*, 2003; Mann *et al.*, 2003).

Another important mutation is located at methionine 220 (M220), which can change to M220V, M220K, M220T and M220I. This mutation leads to ITZ resistance and reduced susceptibility to PSZ, and VRZ (Mellado *et al.*, 2004; Chen *et al.*, 2005).

Apart from these there are other single point mutations that should be mentioned. The point mutation G448S (Bellete *et al.*, 2010; Pelaez *et al.*, 2012; Krishnan Natesan *et al.*, 2012) confers resistance to VRZ and moderate ITZ and PSZ resistance while the

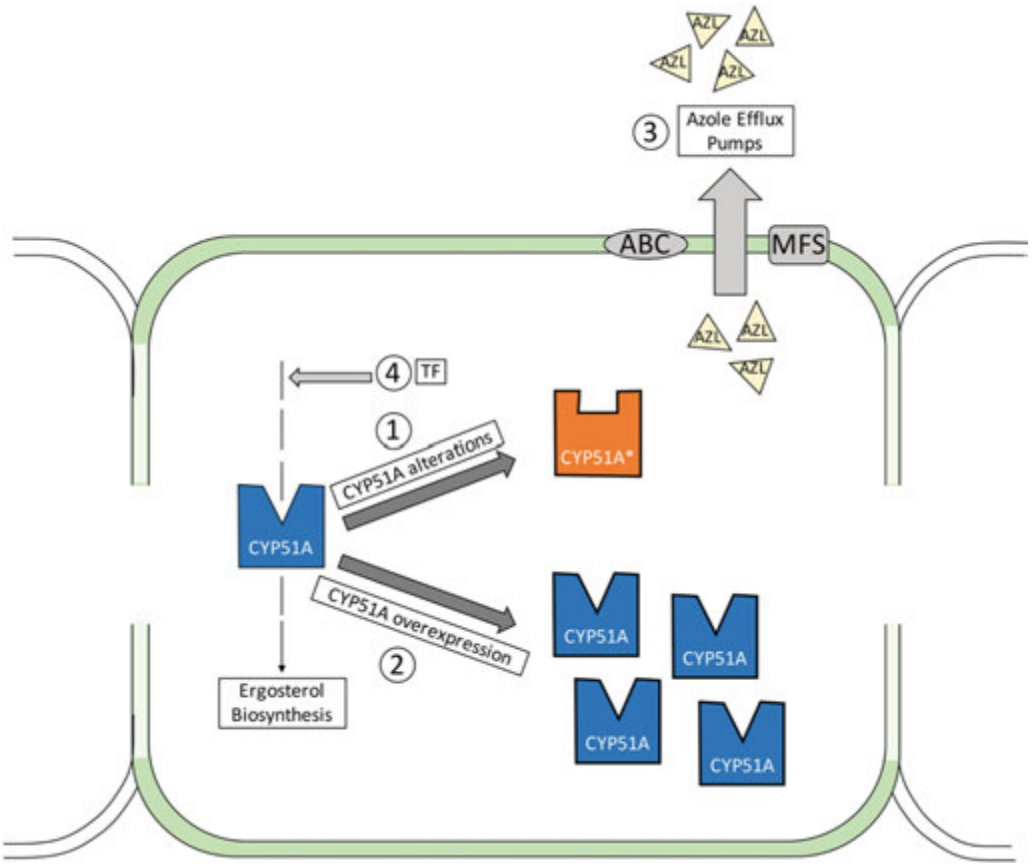

Fig. 3 Schematic representation of the main azole resistance mechanism in *Aspergillus* spp.

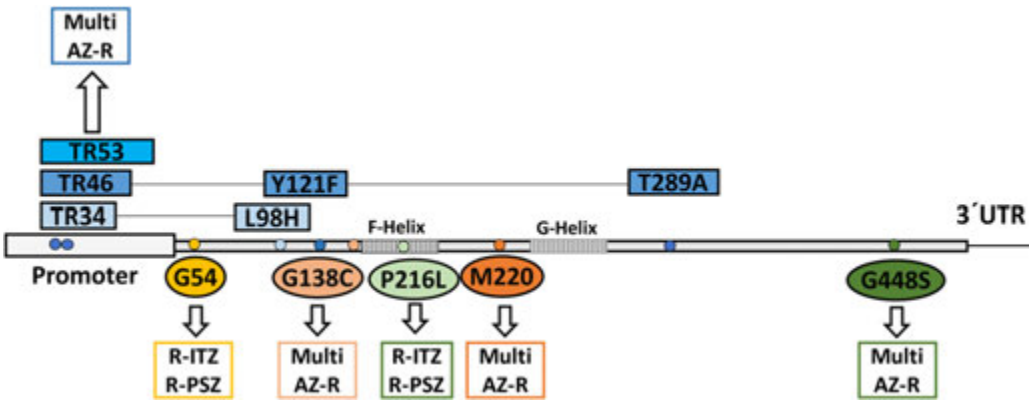

Fig. 4 Schematic representation of the *A. fumigatus* main azole resistance mechanisms involving promoter and *cyp51A* gene modifications and their corresponding azole susceptibility profiles.

G138C substitution has been described in a multiazole resistant strain reported from patients under azole treatment (Howard *et al.*, 2006; Albarrag *et al.*, 2011). The last single point mutation described directly related to azole resistance was P216L (Camps *et al.*, 2012b; Hagiwara *et al.*, 2014; Dabas *et al.*, 2018).

Other single-point mutations have been associated with a reduced azole susceptibility profile or azole resistance (G138R, N22D, F165L, F219C, F219I, D262Y, A284T, Y431C, G432, G434C, T440A, N479D and Y491H) (Howard *et al.*, 2009; Mortensen *et al.*, 2011; Bueid *et al.*, 2010; Manavathu *et al.*, 2003; Howard *et al.*, 2006; da Silva Ferreira *et al.*, 2004; Alanio *et al.*, 2011; Escribano *et al.*, 2011; Bader *et al.*, 2013) but their role in azole resistance remains to be fully clarified. Also two combinations of Cyp51A amino acid substitutions (F46Y, M172V and D255E or F46Y, M172V, N248T, D255E and E427K) (Alanio *et al.*, 2012; van der Linden *et al.*, 2015; Rodriguez-Tudela *et al.*, 2008b; Howard *et al.*, 2009; Snelders *et al.*, 2009; Zhao *et al.*, 2013; Chowdhary *et al.*, 2015; Kidd *et al.*, 2015; Prigitano *et al.*, 2014; Özmerdiven *et al.*, 2015; Hurst *et al.*, 2017; Shalhoub *et al.*, 2015; Wang *et al.*, 2014) have been described among

clinical and environmental strains showing a susceptibility profile with higher azole MICs than wild-type *A. fumigatus* strains. However, some authors have claimed that these substitutions are not associated with azole resistance (Escribano *et al.*, 2011) because their azole MICs normally remained below the accepted threshold defined for resistant isolates (Verweij *et al.*, 2009), while other authors concluded that they could be associated with a poorer azole treatment response (Alanio *et al.*, 2012).

Triazole resistance in *A. fumigatus* can evolve during therapy, but resistant isolates are also being detected in azole-naive patients, with evidence to suggest acquisition of resistant isolates from the environment. In this context, a new group of resistance mechanisms in which *cyp51A* overexpression is involved together with Cyp51A mutations has been described (**Figs. 3.1** and **3.2**). It has been speculated that this resistance mechanism derived from environmental origin due to extended use of demethylation inhibitors (DMIs) as agricultural fungicides.

Normally, it is caused by a particular genetic alteration that consists in tandem repeats (TR) integration of various sizes (34, 46 or 53 bp) in the *cyp51A* gene promoter and in most cases in combination with one or more point mutations in Cyp51A. All of them confer a multi-azole resistance profile. The most common tandem repeat insertion is the TR34/L98H (Mellado *et al.*, 2007) which confers pan-azole resistance. This particular mechanism conferring multi-azole resistance has been observed most frequently in clinical isolates in the Netherlands where it has also been found in the environment, suggesting a possible environmental origin of the resistance (Snelders *et al.*, 2008, 2009). The next most common mechanism is the TR46 integration in combination with two point mutations, Y121F and T289A, which mainly leads to VRZ resistance (van der Linden *et al.*, 2013).

In addition, there are some *cyp51A*-independent *A. fumigatus* azole resistance mechanisms, much less common that the ones mentioned previously. For example, the decrease in the intracellular azole concentration by active efflux systems, such as ATP-binding cassete (ABC) transporters, or the major facilitator superfamily (MFS) (**Fig. 3.3**) (Sanglard, 2016). Also, some transcription factors have been reported as contributing to azole resistance in *A. fumigatus* (**Fig. 3.4**), such as SrbA (Willger *et al.*, 2008), a transcriptional regulator belonging to the sterol regulatory element binding protein (SREBP), the transcription factor HapE (Camps *et al.*, 2012c; Gsaller *et al.*, 2016) and AfYap1 which is regulated by oxidative stress (Qiao *et al.*, 2010). Finally, mutations in the mitochondrial complex I probably have a role in azole drug resistance through membrane alterations (Bromley *et al.*, 2016).

Although *A. fumigatus* azole resistance is the main concern in this field, other human pathogenic species also show azole resistance (Richardson and Lass-Flörl, 2008). Azole resistance caused by amino acid changes in Cyp51A target have been linked to *Aspergillus lentulus* (Alcazar-Fuoli *et al.*, 2011; Mellado *et al.*, 2011), another species from *Aspergillus* section *Fumigati* that shows high VCZ and ITZ MICs (Alcazar-Fuoli *et al.*, 2008; Alastruey-Izquierdo *et al.*, 2014). The same azole profile is shown by *Aspergillus viridinutans* (Lamoth, 2016). Other species within the *Fumigati* section, such as *Aspergillus fumigatiaffinis* and *Neosartorya pseudofischeri* (Alcazar-Fuoli *et al.*, 2008; Balajee *et al.*, 2005) have high triazoles MICs while *Neosartorya udagawae* only has high VCZ MICs (Vinh *et al.*, 2009).

Conclusions

In conclusion, the development of antifungal drug resistance is the inevitable and logical consequence of their broad use in different settings, although the frequency and clinical relevance is, at present, unknown. However, it is acknowledged that azole resistance or even fungal decreased azole susceptibility have a negatively impact on treatment response and the outcome of IFDs. Further understanding of antifungal resistance should be promoted at different levels:

(1) The study of molecular mechanisms of antifungal drug resistance is the most valuable strategy to resistance development control and also in helping to develop safer and more active molecules able to avoid them. In the meanwhile, it is important the correct use of the available tools: epidemiological surveillance of resistance emergence and to use all the efforts towards prompt diagnosis in order to accomplish an adequate and effective treatment.
(2) Reference microdilution methods have established the guidelines for AST. However, the standardization of additional methods, such as the E-test, colorimetric MIC determinations, and newer antifungal susceptibility methods is lacking. This could make susceptibility testing less time consuming and, eventually more accessible to hospital labs. As new antifungal agents are introduced, the AST will have to be further modified to accommodate special concerns with these drugs and an expanded spectrum of fungal pathogens.
(3) Research on new antifungal agents as well as new combinations of antifungal drugs would be more than welcomed to avoid or to minimize resistance. Particularly, the challenges of emerging fungal pathogens underscore the need for the development of new antifungal agents and strategies.

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Echinocandins

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Background and Structure

The naturally occurring precursors, pneumocandins, to modern-day echinocandins were originally discovered in the 1970s (Patil and Majumdar, 2017). After numerous attempts to chemically modify these precursors as safe and effective drugs, caspofungin acetate was the first developed and approved for clinical use in the United States in 2001. Currently, there are three echinocandins available for clinical use and include caspofungin, micafungin, and anidulafungin. They represent a medication class that is critically important for the prevention and treatment of invasive fungal infections, in particular invasive candidiasis, and rank second to triazoles in overall antifungal use (Toda *et al.*, 2019; Vallabhaneni *et al.*, 2018). Additional related agents are in development, including the long acting echinocandin rezafungin (formerly CD101 and SP3025), and a non-echinocandin glucan synthase inhibitor ibrexafungerp (formerly SCY-078 and MK-3118).

Each of the 4 echinocandins are large, semisynthetic structures that contain a hexacyclic lipopeptide core composed conserved amino acid residues (e.g., 3,4-dihydroxyhomotyrosine and 3-hydroxy-proline) (Balkovec *et al.*, 2014; Zambias *et al.*, 1992) (**Fig. 1**). Unique structural components to each echinocandin are due to side chain modifications that affect pharmacokinetic properties such as water solubility, half-life (in the case of rezafungin), and decreased toxicity compared to parent compounds. Ibrexafungerp, which targets to the same cellular process as echinocandins, is chemically and structurally different from echinocandins. Enfumafungin, an acidic terpenoid, was discovered through natural product screening and first described in 2000 (Onishi *et al.*, 2000). Ibrexafungerp is a derivative of enfumafungin developed with enhanced antifungal potency and pharmacokinetic properties (Davis *et al.*, 2019).

Mechanism of Action, Spectrum of Activity, and Susceptibility Testing

Echinocandins competitively inhibit the fungal cell wall enzyme $(1\rightarrow3)$-β-D-glucan synthase, which is responsible for synthesis of an essential polysaccharide (β-1,3 glucan) necessary for cell wall integrity (Douglas *et al.*, 1997). Against yeast that contain this

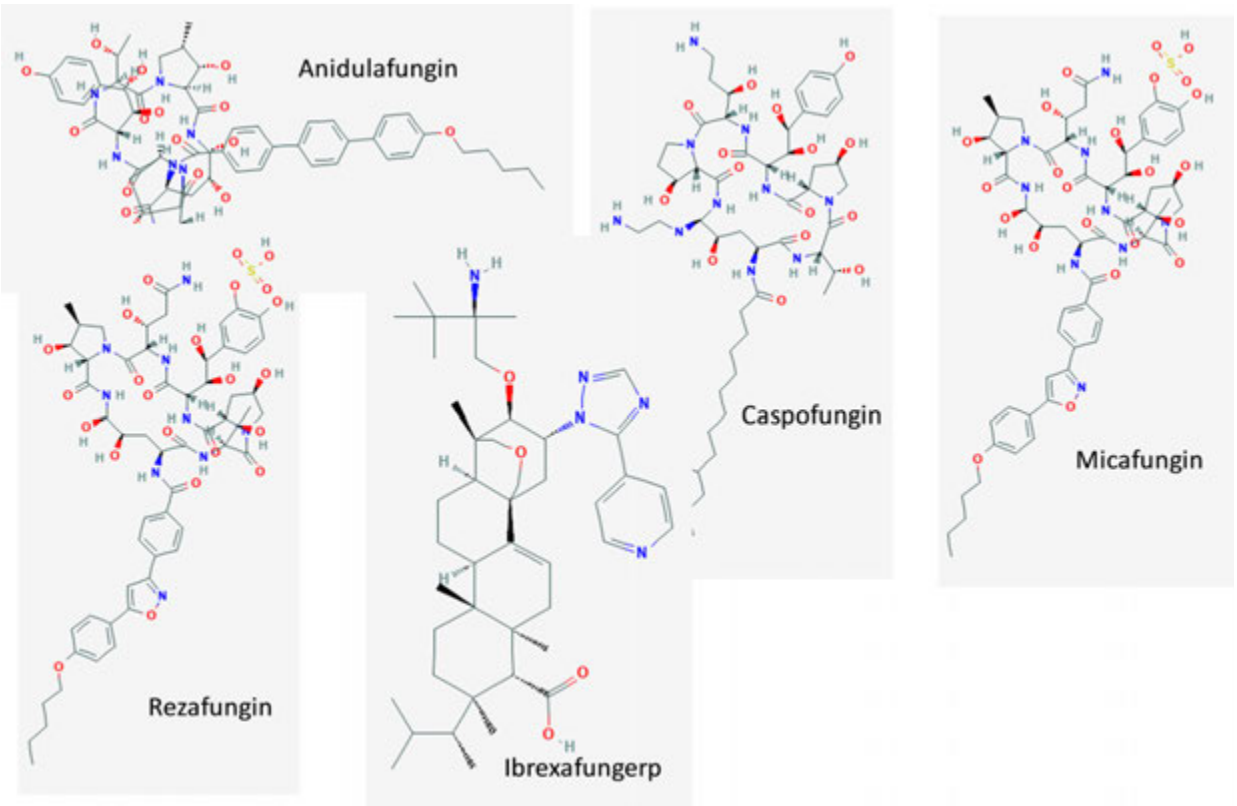

Fig. 1 Echinocandin chemical structures (pubchem.gov).

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critical cell wall enzyme and structure, echinocandins are cidal as they result in cell wall instability and resultant lysis. Therefore, susceptibility testing for echinocandins against yeast involves determining the minimum inhibitory concentration (MIC), defined as the drug concentration that yields no visible growth by eye. Each of the three approved echinocandins (caspofungin, micafungin and anidulafungin) demonstrate potent in vitro activity with very low MICs against *Candida* spp. including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*, and *C. krusei* (Chandrasekar and Sobel, 2006; Pfaller *et al.*, 2003; Vazquez and Sobel, 2006). Higher MICs have been demonstrated for *C. parapsilosis*, *C. lusitaniae*, and *C. guillermondii*; however, pre-clinical and clinical studies have demonstrated these agents to be useful for the treatment of infections due to these pathogens despite higher MIC values (Barchiesi *et al.*, 2006; Fernandez-Ruiz *et al.*, 2014; Pfaller *et al.*, 2006, 2008).

Against *Aspergillus* spp, echinocandin effect is fungistatic as β-1,3 glucan synthesis is limited to the hyphal tips (Bowman *et al.*, 2002). Susceptibility testing, therefore, is different for echinocandins when tested against *Aspergillus* spp., with the primary endpoint of drug effect read as the minimum effective concentration, or MEC. This is determined visually as the lowest concentration of drug that results in aberrant hyphal growth in contrast to the long, infrequently branched hyphal elements noted in controls (Kurtz *et al.*, 1994). Finally, in addition to *Candida* and *Aspergillus* spp, echinocandins also have potent in vitro effect against the cyst form of *Pneumocystis jirovecii* (Schmatz *et al.*, 1990).

The spectrum of activity does not include some notable fungal pathogens, including filamentous fungi other than *Aspergillus* spp (e.g., Mucorales, *Fusarium* spp, *Scedosporium/Lomentospora* spp), *Cryptococcus* spp, *Trichosporon* spp, or the endemic dimorphic fungi (Almyroudis *et al.*, 2007; Cuenca-Estrella *et al.*, 1999; Diekema *et al.*, 2003). In general, the lack of activity against these other pathogenic fungi is felt due to diminished presence or lack of β-1,3 glucan (Aguilar-Zapata *et al.*, 2015). It should be noted, the two novel agents in development that are discussed in this review, rezafungin and ibrexafungerp, have similar effect and spectrum given they also target the fungal cell wall enzyme $(1\rightarrow3)$-β-D-glucan synthase (Pfaller *et al.*, 2013, 2017a,b, 2016; Schell *et al.*, 2017).

Resistance to Echinocandins

Intrinsic resistance, as noted above, is due to diminished presence or lack of the target enzyme $(1\rightarrow3)$-β-D-glucan synthase. For example, *Cryptococcus* spp and *Trichosporon* contain β-1,6 glucan as the predominant cell wall polysaccharide (Aguilar-Zapata *et al.*, 2015; Kauffman and Carver, 2008). In general, acquired antimicrobial resistance can be due to drug efflux, altered drug target, altered drug penetration, or protection via enzymatic degradation or protection proteins. Multidrug-resistant (MDR) transporters, while important for resistance for other agents including triazoles, are not a significant mechanism of drug resistance for echinocandins (Niimi *et al.*, 2006). Thus, when echinocandins were newly developed there was lack of cross-resistance in *Candida* spp that contained triazole resistance (e.g., fluconazole resistance) secondary to drug efflux (Bachmann *et al.*, 2002; Richards *et al.*, 2008). Nevertheless, resistance has emerged to echinocandins for *Candida* spp. leading the CDC to list drug-resistant *Candida* as a major medical threat (CDC, 2019). Echinocandin resistance is most prevalent in *C. glabrata*, with rates that vary geographically from 0% to 10% (Alexander *et al.*, 2013; Astvad *et al.*, 2018; Fraser *et al.*, 2019; McCarty *et al.*, 2018; Pfaller *et al.*, 2019; Toda *et al.*, 2019) and *C. auris*. Rates of resistance for *C. auris* is more difficult to accurately define as we continue to learn more about the incidence of this emerging pathogen, what clinical breakpoints should be used to adequately define resistance, and the epidemiological distribution and frequency of resistance phenotypes and genotypes (Lockhart, 2019; Lockhart *et al.*, 2017).

Echinocandin resistance is primarily mediated through alterations in the catalytic subunits of the glucan synthase enzyme (Fksp) (Douglas, 2001, 1997; Perlin *et al.*, 2015). Specifically, "hot-spot" areas located within the genes *FKS1* and *FKS2* have been identified along with specific mutational changes that confer echinocandin resistance (Garcia-Effron *et al.*, 2010, 2009; Katiyar *et al.*, 2006; Pham *et al.*, 2014; Shields *et al.*, 2015; Zimbeck *et al.*, 2010). To date, there are numerous reported unique mutations that yield heterogeneous changes in echinocandin MIC. In resistance development models, it appears that *FKS1/2* mutational change is a final step in response to stress from drug exposure, but other adaptational changes, primarily driven by cell stress response pathways, to echinocandin exposure occur prior to *FKS* mutational change (Healey and Perlin, 2018). These pathways may explain the clinical phenomena of echinocandin tolerance, which is when an infection due to *Candida* spp are not responding to echinocandin therapy despite not having a genetic resistance determinant (e.g., *FKS* mutation) and previously exhibiting phenotypic susceptibility (Healey and Perlin, 2018; Perlin *et al.*, 2017; Wiederhold *et al.*, 2005). Clinical reports of *FKS* mutations leading to increased echinocandin MIC and subsequent poor patient outcome have increased in the past decade, most alarmingly multi-drug (triazole and echinocandin) resistant *C. glabrata* (Alexander *et al.*, 2013; Beyda *et al.*, 2014; Farmakiotis *et al.*, 2014; Pfaller *et al.*, 2012; Vallabhaneni *et al.*, 2015). Additionally, the emergence of multi-drug resistant *C. auris* has occurred, with multiple reports indicating resistance mechanisms are similar to other *Candida* spp. and secondary to stress response pathways leading to *FKS* mutational changes in the drug target (Biagi *et al.*, 2019; Chowdhary *et al.*, 2018; Kordalewska *et al.*, 2018). Reduced susceptibility to echinocandins noted in some species groups, for example *C. parapsilosis* and *C. guillermondii*, are mediated through naturally present Fks amino acid substitutions in the wild-type population that render reduced susceptibility to echinocandins (Perlin *et al.*, 2017). While the MIC may be higher in these isolates, clinically they respond well to echinocandin therapy and pre-clinical models suggest further alterations that lead to overt resistance may adversely affect fitness and stress tolerance (Papp *et al.*, 2018).

With the discovery that *FKS* mutational changes were the primary driver for overt resistance in *Candida*, laboratory generated *FKS* mutant *A. fumigatus* strains were produced that demonstrated similar in vitro resistance to echinocandins (Gardiner *et al.*, 2005; Rocha *et al.*, 2007). However, clinical observation of acquired echinocandin resistance due to *FKS* in *Aspergillus* has been reported in only a single patient with chronic pulmonary aspergillosis (Jimenez-Ortigosa *et al.*, 2017). While echinocandins are

used less frequently in invasive aspergillosis, primarily limited to salvage or combination use, it is striking how infrequent this has been observed for this pathogen and warrants further investigation.

Resistance to both rezafungin and ibrexafungerp has been demonstrated in vitro and amongst select clinical isolates in surveillance programs. Not surprisingly, rezafungin resistance appears to be quite similar to the other echinocandins, but may have a higher barrier to resistance given its pharmacological advantages including high Cmax and prolonged half-life (Locke *et al.*, 2016). In contrast, ibrexafungerp is quite interesting in that it targets glucan synthase slightly differently than the echinocandins, and therefore it has been shown that specific mutational changes both within and outside of the traditional hot-spot locations of *FKS1/2* (Jimenez-Ortigosa *et al.*, 2017), are necessary to produce resistance. Therefore, ibrexafungerp resistance does not necessarily overlap with echinocandin resistance, which helps explain surveillance susceptibility data in which ibrexafungerp has been found to have relatively low MICs for a number of echinocandin resistant isolates (Jimenez-Ortigosa *et al.*, 2014; Pfaller *et al.*, 2013) and has been effective in animal models with echinocandin resistant isolates (Wiederhold *et al.*, 2018).

Biofilm formation is an important aspect, especially within *Candida* spp., allowing for colonization of mucosal surfaces, attachment to medical devices, and persistent human disease within a sequestered environment (Nobile and Johnson, 2015). *Candida* biofilms are a dense, complex extracellular matrix structure composed of organism and host derived proteins and macromolecules (Desai *et al.*, 2014; Gulati and Nobile, 2016; Mitchell *et al.*, 2016). Functionally, it provides a sanctuary from the human immune system and provides a physical barrier for drug penetration that can mediate drug tolerance and/or drug resistance (Desai *et al.*, 2014; Kernien *et al.*, 2017). Pre-clinical in vivo studies examining administration of echinocandins via antifungal lock therapy, whereby the echinocandin is instilled into a biofilm infected central venous catheter and allowed to dwell for significant periods of time, are promising compared to other therapeutic strategies and drug classes (Basas *et al.*, 2019; Salinas *et al.*, 2019). An advantage to echinocandin therapy appears to be the combined direct antifungal effect as well as improving the immune system's ability to target and kill pathogenic yeast within the biofilm (Hoyer *et al.*, 2018; Katragkou *et al.*, 2015; Larkin *et al.*, 2018). Therefore, echinocandins may be the preferred drug class for biofilm-related *Candida* infections pending further clinical studies.

Pharmacokinetics and Pharmacodynamics

Pharmacologic Properties

The pharmacology of the three clinically available echinocandins are relatively similar (Eschenauer *et al.*, 2007; Kauffman and Carver, 2008). They are available in intravenous (IV) form only as they are poorly absorbed through the gastrointestinal system (<10% bioavailability). Each has a relatively long half-life, with caspofungin exhibiting the shortest at 9–11 h, micafungin 11–17 h, and anidulafungin the longest at 24–26 h. Due to this and pharmacodynamic properties (see below), once daily IV administration is clinically utilized for each of the available echinocandins. After IV administration, they distribute widely and effectively into numerous tissue sites including the liver, spleen, lung, and kidney. However, owing to their large, complex structure and high protein binding, they do not penetrate well into the CSF or eye. Indeed, a recent examination of serum and CSF caspofungin levels in 13 patients demonstrated 11 of 13 had below the limit of detection of caspofungin in CSF whilst having therapeutic concentrations in the serum (Strenger *et al.*, 2017). Additionally, urinary excretion of unmetabolized drug is very low for all three leading to limited effectiveness at this site. This latter pharmacokinetic property also means that dose adjustments for renal impairment are unnecessary for the group. The echinocandins are metabolized to inactive metabolites in the tissues, which are primarily excreted by the biliary system. Finally, in general the echinocandins are very well tolerated without significant toxicities or adverse effects. In fact, due to their safety profile, they are often the agents of choice for prophylaxis or treatment in those with significant organ dysfunction and most notably, in those with significant liver disease or serious drug interactions where triazoles are contraindicated (Andes *et al.*, 2016; Shibata *et al.*, 2017; Verma *et al.*, 2017). Therefore, the main limiting factor in echinocandin use is the need for once daily IV administration, which necessitates maintenance of intravenous access over prolonged periods in already at risk populations of patients, such as immunosuppressed hematology-oncology patients. The two novel therapies in development, rezafungin and ibrexafungerp, both aim to mitigate this limitation through different pharmacological properties.

Rezafungin, a novel echinocandin in pre-clinical development, exhibits similar bioavailability, distribution, metabolism, and safety as the three approved echinocandins. One significant difference though is worth noting. Rezafungin exhibits a prolonged T1/2 of approximately 80 h following the first dose and 150 h following subsequent doses (Sandison *et al.*, 2017). This property makes once weekly intravenous dosing for the treatment or prevention of fungal infection a possibility for rezafungin, for which it is currently in clinical trials.

Ibrexafungerp, a non-echinocandin glucan synthase inhibitor, is currently in pre-clinical trials for the treatment of *Candida* spp or *Aspergillus* spp infections. Oral bioavailability is significantly higher at 35%–51% (Davis *et al.*, 2019) compared to the echinocandins, allowing for both IV and oral formulations. While still in development, it appears that it follows similar other pharmacological properties as the echinocandins in terms of distribution, tissue penetration, half-life, metabolism and elimination.

There is significant limitation, though, to highly homogenous phase I/II preclinical studies to characterize drug pharmacokinetics. Many patients in whom these therapies are ultimately used are quite heterogenous, with highly variable physiology as well as numerous confounding comorbid diseases and clinical conditions. This can lead to discrepancies between the predicted

pharmacokinetic exposures (e.g., AUC) based on preclinical studies and the observed pharmacokinetic exposures, for example, in a population of critically ill patients. Therefore, in recent years, pharmacokinetic characterization has been undertaken in patients, most commonly those critically ill in the intensive care unit (ICU), as this population is most at risk for exposure variability due to altered distribution, metabolism, and elimination of drugs (Parker *et al.*, 2015). Data is consistent between the three approved echinocandins in that lower than expected drug exposures are noted in ICU patients, with significant covariates that predict suboptimal exposures including increased body weight (i.e., obesity), increased severity score (e.g., SOFA or APACHE), and hypoalbuminemia (Boonstra *et al.*, 2017; Bruggemann *et al.*, 2017; Ferriols-Lisart *et al.*, 2017; Garcia-de-Lorenzo *et al.*, 2016; Jullien *et al.*, 2017; Lempers *et al.*, 2015; Maseda *et al.*, 2018; Wasmann *et al.*, 2019). When evaluated from a pharmacodynamic perspective, many of these studies demonstrated that dose escalation of 1.5–3x the usual dosage may be necessary, especially for obese patients or those with very high severity scores, to optimize AUC exposures and achieve the pharmacodynamic target exposures in this patient population. Another area of concern in terms of suboptimal exposures in clinical medicine occurs for those patients in the ICU on continuous renal replacement (i.e., hemodialysis) therapy. In general, echinocandin exposures are not affected by continuous venovenous hemodiafiltration (Aguilar *et al.*, 2014; Perez-Pitarch *et al.*, 2018; Roger *et al.*, 2017; Vossen *et al.*, 2017). However, these studies did demonstrate that echinocandin exposures were lower than expected for many of these critically ill patients on hemodialysis, confirming the aforementioned studies that critically ill patients may have suboptimal exposures. It should be noted, however, specific differences in drug adsorption to newer dialysis membranes, and in particular AN69 surface-treated and polymethylmethacrylate filters, has been observed suggesting adsorption and need for dose adjustments in CRRT may need to be evaluated with further developments in dialysis filtration technology (Honore *et al.*, 2014). Additionally, extracorporeal membrane oxygenation (ECMO), has become an important respiratory and cardiac life support system utilized in a number of clinical scenarios. However, the ECMO circuit and system can lead to drug extraction and sequestration, thus lowering physiological exposure to the patient. Indeed, over a 4 h period micafungin loss was almost 50% in an experimental ECMO circuit, and by 24 h loss was approximately 75% (Watt *et al.*, 2017). Given the diversity of ECMO and hemofiltration technology, including but not limited to filter chemistry, types of plastics, and coatings/polymers within the systems, ongoing evaluation of these technologies and how they impact drug pharmacokinetics is needed. This also suggests that development and utilization of a therapeutic drug monitoring assay to perform dose adjustments based on individual drug concentrations may be an important aspect of future clinical care for certain patient populations.

Pharmacodynamics

The study of pharmacodynamics integrates drug pharmacokinetics, a measure of in vitro drug potency (MIC), and therapeutic effect (Craig, 1998; Drusano, 2007). The overarching goal is to identify exposure-response relationships that predict therapeutic efficacy in order to design optimal dosing regimens for clinical use as well as set clinical susceptibility breakpoints. Practically, this involves [1] determining which of three PK/PD indices – 24 h Area under the drug concentration curve (AUC)/MIC, Maximum concentration (Cmax)/MIC, or the percentage of the dosing interval in which drug concentrations exceed the MIC (Time > MIC) – correlates with efficacy, and [2] what is the magnitude of that PK/PD index associated with a specific therapeutic outcome such as net stasis or 1-log reduction in burden. In general, pharmacodynamic studies may be performed using in vitro models (e.g., hollow-fiber) or in vivo models (e.g., immune compromised mouse). As a group, the echinocandins have been studied extensively utilizing both methods. Results from studies specifically examining exposure-response relationships for micafungin, caspofungin, and anidulafungin against *Candida* spp have demonstrated concentration-dependent killing effects and prolonged post-antifungal effects (Andes *et al.*, 2008a,b; Ernst *et al.*, 1999, 2002; Gumbo *et al.*, 2006; Louie *et al.*, 2005). Both Cmax/MIC and 24 h AUC/MIC have been shown to be the pharmacodynamic indices associated with therapeutic effect. Similar results in experimental studies against *Aspergillus* spp have been observed (Groll *et al.*, 2001; Petraitiene *et al.*, 2002; Petraitis *et al.*, 1998, 2002; Wiederhold *et al.*, 2004), as well as more contemporary studies with rezafungin and ibrexafungerp (Lakota *et al.*, 2017; Lepak *et al.*, 2015, 2018a,b, 2019). These results indicate the most efficacious method to administer these drugs to patients is via large, infrequent doses to maximize Cmax and AUC exposures. Additionally, given the prolonged half-life's and favorable safety profile, investigations into escalating doses and administering even less frequently (as infrequently as once weekly) has been successfully demonstrated in animal models (Gumbo *et al.*, 2007; Lepak *et al.*, 2016) and patient pharmacokinetic studies (Muilwijk *et al.*, 2018). As discussed above, rezafungin, with its significantly prolonged half-life, has been shown in animal model studies to be effective therapy in extended interval dosing design, and clinical studies are underway utilizing a once-weekly dosing schedule to take advantage of the pharmacokinetic and pharmacodynamic profile of this agent.

Pharmacodynamic target exposures have been explored for a variety of Candida spp in studies that have varied from 24 to 96 h depending on infecting pathogen (Andes *et al.*, 2010, 2008a,b; Lepak *et al.*, 2012, 2015, 2018a, 2019, 2017, 2018b). It is generally accepted that free drug concentrations are microbiologically active while protein bound drug is inactive, thus having accurate protein binding estimates and correcting for protein binding by reporting free drug PD target exposures, is critically important especially when protein binding is quite high as is the case for the echinocandins. Outcomes examined in these studies include the PD exposure (24 h AUC/MIC) necessary to produce a net static effect (fungal burden at the start of therapy is the same as fungal burden at the end of therapy) and for 1-log decrease in fungal burden (i.e., 1-log kill). Free drug 24 h AUC/MIC targets for net stasis against *C. albicans* have varied between 12 and 28 for micafungin, caspofungin, and anidulafungin. Target exposures for *C. glabrata* and *C. parapsilosis* have been consistently lower with ranges of 2.9–14 and 5–17, respectively. Interestingly, similar studies

often utilizing the same isolates in studies with rezafungin and ibrexafungerp have demonstrated numerically lower PD target exposures. In the case of rezafungin, this may be due to the extremely long half-life with very high loading concentrations upfront in order to maintain 24 h AUCs over the 7 day treatment period (Lakota *et al.*, 2017). The only drugs to be studied, from a pharmacodynamic perspective, against other *Candida* species include micafungin and rezafungin. Both have shown promising effects and exposure response relationships against *C. auris* (Lepak *et al.*, 2018a, 2017), and rezafungin additionally against *C. tropicalis* and *C. dubliniensis* (Lepak *et al.*, 2019). PD target studies against *Aspergillus* spp are limited, primarily due to the fact that this drug class slows hyphal growth at the tips but does not kill the fungus.

Clinical Use of Echinocandins

Multiple clinical trials have been performed with micafungin (de Wet *et al.*, 2004, 2005; Huang *et al.*, 2012; Kohno *et al.*, 2004; Kuse *et al.*, 2007; Ostrosky-Zeichner *et al.*, 2005; Pappas *et al.*, 2007; van Burik *et al.*, 2004), caspofungin (Arathoon *et al.*, 2002; Betts *et al.*, 2009; Kartsonis *et al.*, 2002; Mora-Duarte *et al.*, 2002; Ostrosky-Zeichner *et al.*, 2014; Villanueva *et al.*, 2001, 2002), and anidulafungin (Krause *et al.*, 2004a,b; Reboli *et al.*, 2007; Ruhnke *et al.*, 2012; Winston *et al.*, 2014). In these trials, echinocandins have been demonstrated to be safe and effective for prevention of fungal infection, empiric therapy for those with suspected fungal infection, and for the treatment of candidiasis. Currently, the major role for echinocandin use is the latter, and specifically as a first-line (i.e., preferred agent) for patients with invasive candidiasis. Initially, clinical trials were able to demonstrate a clear safety advantage and potential efficacy advantages of echinocandins over amphotericin B (Arathoon *et al.*, 2002; Kuse *et al.*, 2007; Mora-Duarte *et al.*, 2002; Villanueva *et al.*, 2001). Against fluconazole, individual trials often showed relatively comparative safety and efficacy of fluconazole versus echinocandins for the treatment of candidiasis (de Wet *et al.*, 2004, 2005; Krause *et al.*, 2004b; Villanueva *et al.*, 2002). A meta-analysis of randomized controlled trials in patients with Candidemia or invasive candidiasis demonstrated two factors critical to improved patient survival: echinocandin use for initial therapy and removal of infected central venous catheters (Andes *et al.*, 2012). A more recent randomized controlled trial confirmed these results where isavuconazole was shown to be not non-inferior to caspofungin for the treatment of invasive candidiasis in adults (Kullberg *et al.*, 2019). Response rates were significantly improved in caspofungin treated patients, although it should be noted that mortality (a secondary endpoint) was not significantly different between the two. Nonetheless, based on the evidence, echinocandins have become the preferred initial therapy for patients (adults and children) with candidemia or invasive candidiasis based on both their safety and efficacy (Andes *et al.*, 2012; Pappas *et al.*, 2016; Tsekoura *et al.*, 2019).

A number of studies have also examined the use of echinocandins as prophylaxis for at risk populations (e.g., neutropenic patients) to prevent infection and in general have performed better than fluconazole, presumably due to additional effects against *Aspergillus* spp (van Burik *et al.*, 2004; Winston *et al.*, 2014). The use of echinocandins, though, for prophylaxis is limited with the development of newer triazoles, such as posaconazole, that are extremely effective in prophylaxis settings to prevent mold infections. Thus, echinocandin use for prophylaxis is clinically limited to situations in which triazoles are contraindicated or for certain populations in which *Candida* infections are particularly more likely than filamentous fungal infections, for example liver transplant recipients. Additionally, while the echinocandins are largely interchangeable, only caspofungin has FDA label approval for febrile neutropenia and micafungin has FDA labeled approval for fungal prophylaxis in neutropenia (Drugs@FDA, n.d.).

The use of echinocandins as prophylaxis or empiric therapy for those with risk factors for or clinically suspected invasive candidiasis is a more nuanced situation. For patients in the ICU, who are at risk for central line infections and intra-abdominal infections where *Candida* may play a prominent role as infecting pathogen, a number of trials have failed to show benefit to prophylaxis or pre-emptive approach to antifungal therapy, including echinocandins (Knitsch *et al.*, 2015; Ostrosky-Zeichner *et al.*, 2014; Pelz *et al.*, 2001; Schuster *et al.*, 2008; Timsit *et al.*, 2016). That being said, there may be clinical factors that lead to a high suspicion of invasive candidiasis, and whether empirical use of an echinocandin versus fluconazole may be preferred for these patients often hinges on the local incidence (Lepak and Andes, 2011). Thus, as a matter of routine in the ICU, echinocandin prophylaxis, pre-emptive, or empirical therapy cannot be recommended at this time.

Echinocandin studies against invasive aspergillosis are limited and only caspofungin carries labeled approval for salvage therapy in patients refractory to or intolerant of first-line options (Drugs@FDA, n.d.) such as voriconazole and isavuconazole. Clinical studies have examined caspofungin use as first-line or second-line therapies, but are limited by their non-randomized or retrospective design, heterogenous patient populations and wide response rates (Cornely *et al.*, 2011; Egerer *et al.*, 2012; Herbrecht *et al.*, 2010; Kartsonis *et al.*, 2005; Maertens *et al.*, 2006, 2004; Viscoli *et al.*, 2009). Data exists for micafungin demonstrating similar response rates as caspofungin (Denning *et al.*, 2006; Ji *et al.*, 2017; Kontoyiannis *et al.*, 2009). Anidulafungin monotherapy data for invasive aspergillosis consists of case-reports only. Anidulafungin was however, a component of the largest randomized, double-blinded, placebo-controlled trial examining the effects of combination therapy (Marr *et al.*, 2015). Prior to this, numerous pre-clinical in vitro and in vivo models, as well as uncontrolled retrospective studies, had suggested combination therapy consisting of an echinocandin with a triazole (such as voriconazole, posaconazole, or isavuconazole) may be more beneficial than monotherapy with a triazole alone. The primary outcome was mortality at 6-weeks, which was not statistically different between voriconazole monotherapy and voriconazole with anidulafungin. There was a trend in favor of combination therapy and in certain subgroups. The newer therapies, rezafungin and ibrexafungerp, are currently in clinical trials and their role in therapy remains to be determined at this point.

Echinocandins in Special Populations

Pediatrics

Echinocandin use in pediatric patients has dramatically risen as studies and formal approval for pediatric use has matriculated over the last decade in Europe and the United States. In fact, echinocandin use in hospitalized children increased over 300% from 2005 to 2015 in the United States (Downes *et al.*, 2018). Pediatric use of echinocandins is mainly limited to micafungin and caspofungin, both of which have sufficient studies to warrant approval for pediatric specific indications and dosing.

Micafungin has been studied and approved for use in neonatal and pediatric populations for the treatment of invasive candidiasis, esophageal candidiasis, and for prophylaxis of candida infections in stem cell recipients (Drugs@FDA, n.d.; Manzoni *et al.*, 2014). Original approval was for pediatric patients $\geq$4 months in age with dosages based on weight and indication (varying between 1–3 mg/kg IV daily with a daily maximum of 50–150 mg); however, more recent approval has been added for pediatric patients under 4 months with a recommended dose of 4 mg/kg IV daily (Drugs@FDA, n.d.). It is important to note, though, a number of investigations have demonstrated altered pharmacokinetics in newborns based on both age and weight (Heresi *et al.*, 2006; Seibel *et al.*, 2005), suggesting higher doses may be necessary for pre-term and term neonates (Ascher *et al.*, 2011; Caudle *et al.*, 2012; Hope *et al.*, 2010). Additionally, preclinical rabbit models of *Candida* meningoencephalitis, a rare but recognized complication of invasive candidiasis in the neonatal period, demonstrated that micafungin penetrated brain parenchyma infected by hematogenous seeding during invasive candidiasis and that micafungin could be used successfully for therapy (Hope *et al.*, 2008). The pharmacodynamic targets for successful therapy demonstrated in the in vivo study are, however, optimally met by a dosage range of 10–25 mg/kg IV daily in neonates (Benjamin *et al.*, 2010; Hope *et al.*, 2008; Hope *et al.*, 2010). Additionally, as *Candida* meningoencephalitis can be difficult to diagnose and CSF cultures are not reliably positive in this disease, some suggest that if an echinocandin is to be used in a neonate with candidemia it should likely be used at a dosage of at least 10 mg/kg IV daily. Indeed, a recent phase III study of micafungin at 10 mg/kg in infants with invasive candidiasis was shown to be well tolerated and efficacious for invasive candidiasis (Benjamin *et al.*, 2018). It is important to note, though, that clinical efficacy data for any echinocandin, including micafungin, for use in meningoencephalitis is lacking.

Caspofungin has approval for pediatric patients for the treatment of invasive or esophageal candidiasis, treatment of suspected fungal infection in febrile neutropenia, and additionally has approval for salvage therapy for those with invasive aspergillosis refractory to or intolerant of other therapies for ages 3 months and older (Drugs@FDA, n.d.; Fisher *et al.*, 2019; Tsekoura *et al.*, 2019). Dosing is done in body surface area, with a loading dose of 70 mg/m^2 IV and maintenance dose of 50 mg/m^2 IV daily thereafter.

At this time, anidulafungin does not have specific approval in the United States or Europe related to pediatric use. A number of studies and anecdotal reports have been completed with encouraging results (Benjamin *et al.*, 2006; Roilides *et al.*, 2019; Rosanova *et al.*, 2017; Wilke, 2013), but dosing and indication of anidulafungin use in pediatric populations is still in the process of study and refinement.

Geriatric

No dose adjustments are necessary for any of the three approved echinocandins based on age. For each drug, when response and safety data was analyzed based on age, no significant difference was noted between geriatric and younger populations (Drugs@FDA, n.d.). Some pharmacokinetic studies report elevated echinocandin drug exposure in the geriatric population, which could be secondary to changes in lean body mass and volume of distribution, as well as metabolism and drug excretion. For example, caspofungin AUC was approximately 28% higher in the geriatric population (Drugs@FDA, n.d.); however, there was no apparent clinical significance, both in terms of efficacy and safety, from this increased drug exposure.

Pregnancy/Lactation

The echinocandins as a group have been classified as pregnancy class C drugs (Drugs@FDA, n.d.). For each, this designation is largely based on animal studies demonstrating that fetal harm may occur; and for each, there is a lack of sufficient data in pregnant women to adequately establish and/or inform the risk of adverse effects to human embryo and fetal development. Each of the echinocandins is secreted in the milk of lactating rats, but data does not exist to confirm this in humans. Even if present, echinocandins are not absorbable through the gastrointestinal tract, and thus would not be expected to produce a systemic exposure in a breast-feeding infant. That being said, local effects on the infant's gastrointestinal microbiome are plausible. Therefore, a risk: benefit analysis based on the benefits of breastfeeding, the mother's clinical need for echinocandin therapy, potential alternative therapies, and risk of echinocandin gastrointestinal exposure by the infant should be considered.

Drug–Drug Interactions

Echinocandins are poor substrates for hepatic metabolism enzymes (Niwa *et al.*, 2014), and therefore drug-drug interactions are uncommon for the group (Drugs@FDA, n.d.; Patil and Majumdar, 2017). The most clinically important drug interactions are summarized below, and are separated into interactions in which an interacting drug changes (i.e., increases or decreases) the

echinocandin exposure and interactions in which the echinocandin changes the interacting drug's exposure. Two drugs can interact with caspofungin to change the drug exposure. Rifampin reduces caspofungin exposure by approximately 30% and cyclosporine increases caspofungin exposure by approximately 35%. Cyclosporine also interacts with anidulafungin, leading to an anidulafungin AUC increase of approximately 22%. There are no known drug interactions that increase micafungin exposure. Caspofungin interacts with tacrolimus to decrease the tacrolimus exposure by approximately 20%. Micafungin interacts with three drugs, it decreases cyclosporine clearance by approximately 16% and increases sirolimus and nifedipine AUC exposure by about 21% and 18%, respectively. Anidulafungin does not appear to have any interactions that affect the pharmacokinetics of other drugs.

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Allylamines, Morpholine Derivatives, Fluoropyrimidines, and Griseofulvin

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Introduction

In this chapter, we address non-polyene, non-echinocandin and non-azole antifungal agents, such as allylamines, morpholine derivatives, fluoropyrimidines, and griseofulvin. These antifungals are commonly used to treat superficial and cutaneous mycoses, frequently caused by dermatophytes, via oral or topical administration. We focus on the brief history, pharmacodynamics and pharmacokinetics, toxicity effects and some strategies to improve the pharmacological characteristics of these antifungals, such as the production of synthetic analogues and the development of new formulations based in drug carrier systems.

Allylamines

The functionalized allylamines are synthetic compounds, with the two main pharmaceutically important representatives being naftifine and terbinafine (**Fig. 1**). Both are active on dermatophytes and are indicated for treatment of skin and nail mycoses (Petranyl *et al.*, 1984). Allylamines specifically inhibit ergosterol biosynthesis at the squalene epoxidation step by inhibiting squalene epoxidase enzyme, causing a fungistatic or fungicidal effect by the accumulation of a toxic concentration of squalene and ergosterol depletion (Ryder, 1992). This excess of squalene is deposited into lipid vesicles, and these function as lipid sponges that remove lipids from the cell membrane, causing instability and shrinkage, leading to fungal cell death (Ryder, 1992).

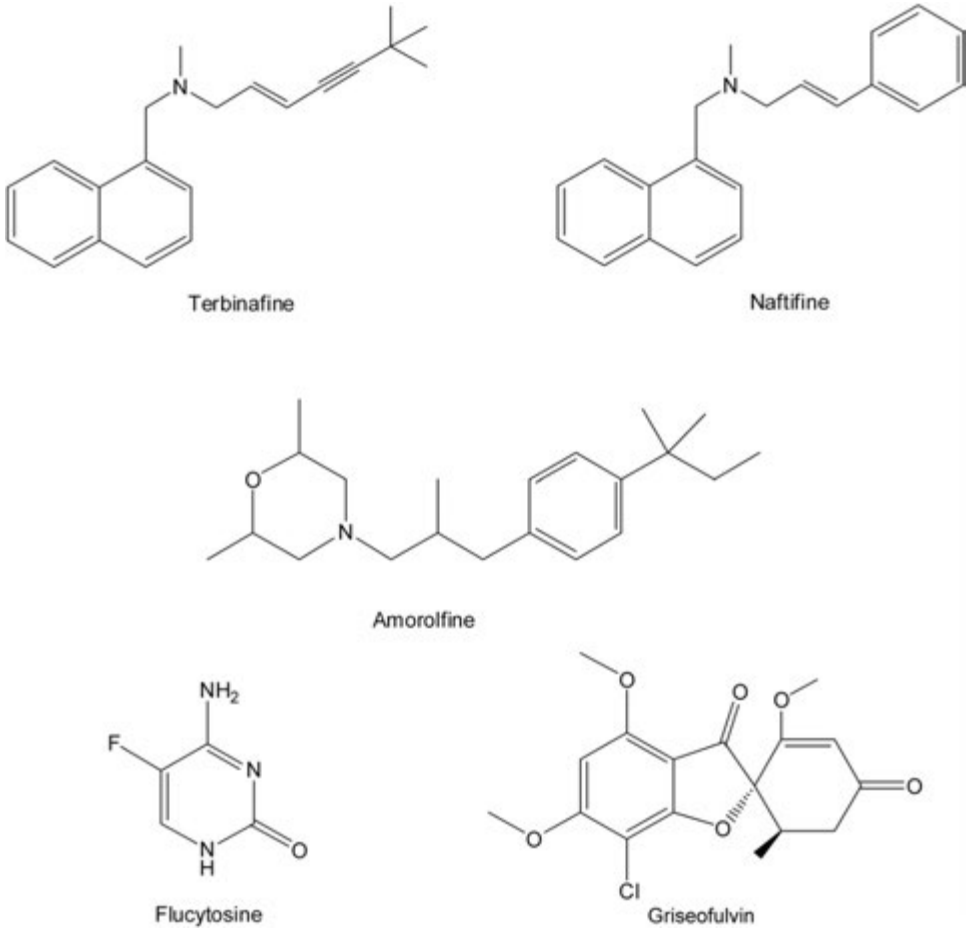

Fig. 1 Chemical structures of antifungals allylamines (terbinafine and naftifine), morpholine derivatives (amorolfine), fluoropyrimidines (flucytosine), and griseofulvin.

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Naftifine is obtained by acid hydrolysis of heterocyclic spironaphthalenones (Petranyl *et al.*, 1984) and it is considered a fungicide *in vitro* against dermatophytes *Trichophyton* spp., *Microsporum* spp., and *Epidermophyton* spp., but is not active against *Candida* spp. (Georgopoulos *et al.*, 1981). Terbinafine is a synthetic analogue of naftifine (Ryder, 1992); it has a fungicidal effect against dermatophytes and also demonstrates antifungal action against *Candida* spp. and *Cryptococcus* spp. due to a high chemical affinity for squalene epoxidase, but shows variable activity *in vitro* (Barchiesi *et al.*, 1998; Lee and Fothergill, 2003). Both drugs are used topically (cream or gel formulations), but only terbinafine is given orally for systemic absorption due to the increased selectivity (Newland and Abdel-Rahman, 2009).

Terbinafine is unaffected by the presence of food when administered orally and has a bioavailability of 70%–80%, with maximal plasma concentration achieved within 2 h following administration of 250 mg (0.8–1.5 μg/ml) (Jensen, 1990). The terbinafine half-life was calculated to be 0.8–1.2 h and its elimination is approximately 16 h. It is completely metabolized in the liver, generating 15 metabolites that are excreted in the urine; in addition, no parent drug is detected in the urine (Jensen, 1990). Terbinafine is a highly lipophilic and keratinophilic compound and, thus, accumulates in the stratum corneum of the skin and nails (Ryder and Frank, 1992). For this reason, allylamine drugs are used effectively to treat dermatophytosis of the skin and nails (Sahni *et al.*, 2018).

Terbinafine is generally well tolerated, but adverse effects include mild and transient forms associated with its oral administration, such as gastrointestinal symptoms, and its preference for the skin may result in cutaneous rash and urticaria (Friedlander *et al.*, 2002; Elewski *et al.*, 2008).

Although terbinafine is effective in the treatment of skin and nail mycoses mainly caused by dermatophytes, this drug also has *in vitro* antifungal activity against other fungi, such as *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Penicillium marneffei*, *Sporothrix* spp. and chromoblastomycosis agents at low concentrations (Ryder *et al.*, 1998; Barchiesi *et al.*, 1998; Jessup *et al.*, 2000; Kirkpatrick *et al.*, 2005; Mendes *et al.*, 2010; Guerra *et al.*, 2012; Borba-Santos *et al.*, 2015). However, it was less effective against *Blastoschizomyces capitatus* and *Trichosporon beigelii* (Kirkpatrick *et al.*, 2005).

Synergistic *in vitro* antifungal activity has been demonstrated with terbinafine in combination with azoles or amphotericin B for many important fungal pathogens, including *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., zygomycetes, *Fusarium* spp., *Paecilomyces* spp., dematiaceous molds, and the highly resistant *Scedosporium prolificans* (Vazquez, 2008; Gómez-López *et al.*, 2003; Ortoneda *et al.*, 2004a,b; Barchiesi *et al.*, 1997; Biancalana *et al.*, 2011; Barchiesi *et al.*, 1998; Meletiadis *et al.*, 2000; Kirkpatrick *et al.*, 2005; Ryder and Leitner, 2001; Guerra *et al.*, 2012). Azole agents and terbinafine block different steps of the ergosterol biosynthesis pathway, supporting the mechanism of synergistic action (Meletiadis *et al.*, 2000).

In addition to the utilization of terbinafine in the treatment of dermatomycosis, interest has expanded to include its use alone or in combination with azoles for a range of cutaneous and subcutaneous mycoses, such as sporotrichosis, eumycetoma, and chromoblastomycosis (Esterre *et al.*, 1998; Chapman *et al.*, 2004; N'diaye *et al.*, 2006; Dolton *et al.*, 2014), and to treat resistant or refractory fungal infections (candidiasis, cryptococcosis and aspergillosis), as is described in numerous reported cases (Revankar *et al.*, 2008; Villars and Jones, 1992; Schiraldi and Colombo, 1997; Ghannoum and Elewski, 1999; Steinbach *et al.*, 2003; Gómez-López *et al.*, 2003; Krishnan-Natesan *et al.*, 2008).

There are few studies on resistance to allylamines; one of the limitations for this is the lack of standardized susceptibility testing to antifungals for dermatophyte fungi. Recently, 15 *T. interdigitale* and 5 *T. rubrum* isolates exhibiting high MIC values for terbinafine were evaluated for mutations in the squalene epoxidase gene, and the higher tolerance to terbinafine was related to a change of phenylalanine to leucine in the 397th position of the target enzyme, which may lead to a failure of terbinafine to block ergosterol biosynthesis (Rudramurthy *et al.*, 2018).

New formulations are being developed to reduce allylamine drug toxicity, to increase skin penetration, and to improve biodistribution and antifungal efficacy. Formulations based in colloidal nanocarriers containing naftifine were assayed in models *in vitro* in pig skin and *in vivo* in human skin; these nanocarriers increased transport of naftifine into deeper layers of the skin, improving topical drug delivery (Erdal *et al.*, 2016). Terbinafine contained in lipid- and polymeric-based carriers were also developed and tested on animal models of fungal infections (Chen *et al.*, 2012; Vaghasiya *et al.*, 2013; Wavikar and Vavia, 2013; Tayel *et al.*, 2013). The terbinafine in lipid nanoparticles caused a significant reduction in *C. albicans* CFU counts compared to the conventional formulation (Wavikar and Vavia, 2013; Vaghasiya *et al.*, 2013). The formulation of terbinafine-loaded solid lipid nanoparticles was also shown to significantly increase penetration of the skin layers, increasing its bioavailability (Chen *et al.*, 2012). Terbinafine in polymeric nanoparticles (composed of chitosan, soybean lectin or Pluronic® F68) was tested in a *Fusarium* fungal corneal keratitis model and was able to increase ocular bioavailability of terbinafine by twofold compared to the conventional formulation (Tayel *et al.*, 2013).

Another important strategy for improving the pharmacokinetic profile and antifungal activity of allylamines is the synthesis of structural analogues. Some naftifine analogues were shown to have activity on *T. rubrum* and *T. mentagrophytes in vitro*, and had even greater effects on yeasts, such as *C. neoformans* and *C. albicans* (Abonia *et al.*, 2018); terbinafine analogues were screened for antifungal activity against *C. albicans*, and similar results to terbinafine were found (Kharkar *et al.*, 2009).

In addition, butenafine is a benzylamine derivative that also shows potent fungicidal activity, particularly against etiological agents of superficial mycoses, dermatophytes, *Aspergillus*, and dimorphic and dematiaceous fungi (Singal, 2008). Its mechanism of action is similar to allylamines, and a cream formulation is recommended for the treatment of interdigital tinea pedis, tinea cruris, and tinea corporis (Singal, 2008; El-Gohary *et al.*, 2014).

Morpholine Derivatives

Morpholine derivatives are synthetic heterocyclic compounds containing amine and ether functional groups. They have demonstrated inhibitory activity on plant, animal and human pathogenic fungi (Polak, 1983). Among the morpholine derivatives, amorolfine (**Fig. 1**) is the most studied molecule and is used in the treatment of human fungal disease; nevertheless, in agriculture, aldimorph, fenpropimorph, fenpropidin, tridemorph, and dodemorph are used as fungicides and are highly toxic for human use (Jachak *et al.*, 2015).

Morpholine derivatives are ergosterol biosynthesis inhibitors, which may have a fungicidal effect against the most fungal species (Haria and Bryson, 1995). The antifungal activity occurs by the inhibition of two enzymes, Δ^8–Δ^7 isomerase and sterol Δ^{14}-reductase, in the post-lanosterol/cycloartenol step of the ergosterol biosynthetic pathway, which leads to ergosterol depletion and the accumulation of intermediates, ignosterol and lichesterol (Müller *et al.*, 2013). Treatment with amorolfine may lead to morphological changes in fungal cells, such as shortening of germ tubes, enlargement in the diameter of hyphae, excessive branching in *Botrytis* spp. and in the dermatophytes (Polak, 1988). In addition, the cell wall thickness is significantly increased and chitin deposits are included inside and outside (Polak, 1992). This fungicidal effect is dependent on both the drug concentration and the exposure time (Polak-Wyss, 1995).

Currently, the morpholine derivative amorolfine is recommended to treat dermatophytosis in human skin and nails (Kaul *et al.*, 2017). In *in vitro* assays, dermatophytes ($\sim$15 species) are susceptible to amorolfine at concentrations lower than 2 μg/ml (Adimi *et al.*, 2013; Favre *et al.*, 2003; Li *et al.*, 2004). Polak (1992) also demonstrated the antifungal efficacy of amorolfine on dermatophytes, and against other fungi; however, it did not appear to be effective when given orally. Mice infected with *T. mentagrophytes* responded to the combined therapy of amorolfine with terbinafine, itraconazole or griseofulvin in a dose-dependent manner, showing a significant reduction in the appearance of mycotic foci compared to untreated animals (Polak-Wyss, 1995).

Topical amorolfine nail lacquer 5% is commonly used for the treatment of human onychomycosis as a monotherapy or in combination with other antifungals, such as griseofulvin, ketoconazole, itraconazole or terbinafine (Haria and Bryson, 1995; Polak *et al.*, 2004); importantly, amorolfine in combination with systemic antifungal agents was better than the monotherapy using itraconazole or terbinafine (Feng *et al.*, 2017).

Administration of amorolfine nail lacquer 5% once or twice weekly is 60%–71% effective in the treatment of toenail onychomycosis, and complete cure is observed after six months of the antifungal therapy in 38%–46% of patients (Hay, 2010). Following topical application of amorolfine, the rate of absorption through the human thumbnail reaches an initial peak after 5 h and decreases slightly, then rises again to peak higher still at 25 h and decreases slightly (Franz, 1992).

Research to improve the antifungal efficacy of morpholine derivatives is ongoing. Jachak *et al.* (2015) synthesized modified morpholines with incorporated silicon, which exhibited potent antifungal activity against *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. neoformans* (MICs from 0.25 to 4 μg/ml), similar to the results observed with amorolfine (MICs from 0.125 to 1 μg/ml).

Fluoropyrimidines

The anticancer and antimicrobial activities of fluoropyrimidines were first described in 1957 by Heidelberger and collaborators. The fluoropyrimidines, such as 5-fluorouracil (5-FU), capecitabine and tegafur, are antimetabolite drugs widely used in the treatment of colorectal and breast cancers (Wilson *et al.*, 2014). The mechanism of action of fluoropyrimidines consists of the erroneous incorporation of 5-FU into RNA and DNA and inhibition of thymidylate synthase (TYMS), which results in the inhibition of protein and DNA biosynthesis (Wilson *et al.*, 2014).

5-fluorocytosine (flucytosine or 5-FC, **Fig. 1**) is also an antimetabolite drug, being a fluorinated analogue of cytosine. It was synthetized in 1957 at Roche Laboratories as potential anticancer agent, but did not demonstrate to be effective in tumor treatment (Heidelberg *et al.*, 1957; Heidelberger *et al.*, 1958). Years later, 5-FC showed antifungal efficacy using murine models of candidiasis and cryptococcosis, and was recommended for treatment of both invasive fungal infections thereafter. Although 5-FC has high antifungal activity on *Candida* and *Cryptococcus*, it was also shown to have lesser inhibitory effects on *Aspergillus* spp., dematiaceous fungi and etiological agents of chromomycosis, such as *Phialophora* spp., *Cladosporium* spp. and *Exophiala* spp. (Benson and Nahata, 1988).

5-FC itself has no antifungal effect. It must be taken up by the fungal cells via a transporter, cytosine permease, and converted first into 5-fluorouracil (5-FU) by cytosine deaminase, and then into the metabolite 5-fluorodeoxyuridine, which inhibits RNA and DNA synthesis (Polak and Scholer, 1980; Polak, 1977). The conversion from 5-FC to 5-FU inside the fungal cell is a crucial step for the antifungal effect, as fungi lacking cytosine deaminase are not sensitive to 5-FC (Polak and Scholer, 1980; Polak, 1977).

Unfortunately, 5-FC as a single antifungal agent in clinical use is problematic owing to its relatively weak antifungal efficacy and the fast development of fungal resistance by gene mutations in cytosine permease or cytosine deaminase (Polak, 1977). A 5-FC primary resistance rate of 1%–7% is observed for *Candida* and *Cryptococcus* isolates (Cuenca-Estrella *et al.*, 2001; Govender *et al.*, 2011; Loyse *et al.*, 2013). Thus, 5-FC in monotherapy is not recommended; only 5-FC in combination with amphotericin B has been proven to be synergistic for the treatment of cryptococcosis using *in vitro* assays, animal models and clinical trials (Montgomerie *et al.*, 1975; Schwarz *et al.*, 2006; Schwarz *et al.*, 2007; Dromer *et al.*, 2008). Currently, 5-FC combined with amphotericin B for 2 weeks followed by maintenance with fluconazole has been considered the gold standard treatment for cryptococcal meningitis (Perfect *et al.*, 2010; World Health Organization, 2018). Other synergistic combinations, such as 5-FC with caspofungin, were also evaluated for the treatment of candidiasis (Charlier *et al.*, 2015).

5-FC is available in tablet form (250 and 500 mg) and as an intravenous formulation (10 mg/mL in saline) (World Health Organization, 2007). It is well absorbed by the gastrointestinal tract (76%–89%), with peak plasma concentration occurring approximately 2 h after oral administration (Cutler *et al.*, 1978; Vermes *et al.*, 2000). Because 5-FC is soluble in water, it gets into the urine, peritoneal fluid, cardiac tissue and the eye in sufficient concentrations. 5-FC also has great blood-brain barrier penetration, an important characteristic for the treatment of cryptococcal meningitis (Daneshmend and Warnock, 1983; Kethireddy and Andes, 2007).

5-FC is excreted almost entirely by glomerular filtration (90%) and dose reduction is necessary for patients with renal dysfunction (Cutler *et al.*, 1978). 5-FC can cause renal toxicity, and for this reason, it is important to monitor renal function during 5-FC clinical use (Vermes *et al.*, 2000).

Oral 5-FC is generally well tolerated by patients because mammalian cells do not have cytosine deaminase (Vermes *et al.*, 2000); however, it occasionally causes gastrointestinal symptoms, such as nausea and diarrhea, skin rashes and suppression of bone marrow function (Vermes *et al.*, 2000). At high 5-FC serum levels, a transient hepatomegaly and altered transaminases may be observed. 5-FC also induces teratogenic effects at concentrations lower than those used for treatment of cryptococcal meningitis (Vermes *et al.*, 2000).

5-FC derivatives had been synthetized with the aim of increasing antifungal activity and reducing the rate of resistance development. Pyrimidin-one and -thione (often known as DHPMs), structural analogues of 5-FC, were tested in combination with amphotericin B or fluconazole for activity against *Candida albicans* strains and showed synergistic antifungal effects *in vitro*, which could lower the required dosages of antifungal drugs for the treatment of invasive fungal diseases (Wani *et al.*, 2017).

In the design of new drugs, the hybridization approach is a worldwide practice strategy for developing molecules with higher activity and less drug resistance due to the possible alternative action mechanisms of hybrids (Fang *et al.*, 2017). Hybrids of 5-FC and fluconazole were designed and synthesized and presented potential antifungal effects (Fang *et al.*, 2017).

Griseofulvin

Griseofulvin was isolated from *Penicillium griseofulvin* in its pure form for the first time by Brian and collaborators in 1939 (Oxford *et al.*, 1939), and its chemical structure was elucidated between 1952 and 1959 (Petersen *et al.*, 2014) (**Fig. 1**). Griseofulvin is a secondary metabolite, which can also be produced by other fungi genus as *Aspergillus*, but the majority of these are found in *Penicillium*, where at least 16 species are known to be griseofulvin producers (Larsen *et al.*, 2005).

The natural product griseofulvin is an antifungal used for treatment of superficial mycoses and dermatophytosis in humans and animals (Gull and Trinci, 1973). In the last decade, there has been a renewed interest in griseofulvin due to its antimitotic and antiproliferative activities against various types of cancer cells and antiviral effects (Petersen *et al.*, 2014). The mode of action of griseofulvin is still poorly understood, but disruption of microtubule dynamics in both fungal and mammalian cells has been proposed (Osment, 1969; Gull and Trinci, 1973; Petersen *et al.*, 2014).

Griseofulvin has a fungistatic action by inhibition of microtubule assembly; it interacts with microtubules to affect the formation of the mitotic spindle, causing abnormal chromosomal rearrangements and, as a final consequence, inhibits fungal replication (Gull and Trinci, 1973). It has *in vitro* activity mainly on several species of *Microsporum*, *Epidermophyton* and *Trichophyton*, which are responsible for hair and skin diseases (dermatophytosis) in both humans and animals (Gentles, 1958; Moriello and DeBoer, 1995; Chadeganipour *et al.*, 2004; Gupta *et al.*, 2009).

Patients presenting long-standing onychomycoses had been treated with griseofulvin previously, demonstrating limited therapeutic success (Scholz and Meinhof, 1991); and higher MIC values of griseofulvin may be associated with therapeutic failure (Gupta *et al.*, 2009). Griseofulvin is currently not recommended for treatment in monotherapy, as there are other alternatives, such as allylamines, amorolfine and azoles (Rajagopalan *et al.*, 2018). Although a large variety of oral and topical antifungal protocols are available for the treatment of dermatophytosis, the efficacy of these drugs is variable, with treatment failure occurring in up to 40% of patients, possibly due to resistance phenomena, as with the treatment of *M. canis* infection, for example (Aneke *et al.*, 2018).

Griseofulvin can be administered topically or orally as a crystal suspension. It is poorly absorbed by the gastrointestinal tract due to its low water solubility, and varies considerably; absorption is increased by decreasing of the crystal size associated with a greasy diet (Steinbach and Dvorak, 2012). Griseofulvin is deposited in keratin precursor cells and becomes bound to newly formed keratin (Steinbach and Dvorak, 2012). It is metabolized by liver microsomal enzymes and excreted in the urine and feces (Gupta *et al.*, 1994).

Griseofulvin has a long-standing history of safety; however, some side effects have been described, such as cutaneous rash, urticaria, nausea, vomiting, diarrhea, headache, fatigue, proteinuria and leukopenia (Steinbach and Dvorak, 2012). Because it is metabolized in the liver, increases of hepatic enzymes in the serum can occur (Steinbach and Dvorak, 2012).

Analogues of griseofulvin have been isolated for their antifungal and anticancer activity. The first griseofulvin analogue bearing an N-isopentane moiety, and the first example of a naturally occurring N-containing griseofulvin derivative, was isolated from *Penicillium griseofulvum* CPCC 400528 (Zhang *et al.*, 2017). Other griseofulvin analogues were also isolated from the mushroom, *Xylaria cubensis* (Paguigan *et al.*, 2017). In addition, fluorinated analogues were synthesized from previously isolated griseofulvin, and these compounds were strongly active against a panel of cancer cell lines and demonstrated antifungal activity against *Microsporum gypseum* (Paguigan *et al.*, 2017). Thus, aside from being an antifungal drug, griseofulvin and its derivatives may also be a clinically-viable candidate for cancer chemotherapy.

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New Targets for the Development of Antifungal Agents

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Introduction

Disease and death caused by fungal infections have transitioned from a rare curiosity to a major global health problem (Robbins *et al.*, 2016). An estimated 1.5–2 million people die of fungal infections each year (Denning and Jugessur, 2016). The therapeutic arsenal has been limited, and the available antifungal drugs actually suffer from limitations in the route of administration, high toxicity, a narrow spectrum of activity, detrimental drug interactions, the development of drug resistance, and poor bioavailability in target tissues (Brown *et al.*, 2012).

In this context, new safe molecules with efficient antifungal activities are needed. In this chapter, we discuss some of the alternatives to conventional antifungals for treating fungal diseases. New targets in fungal cells for the development of new antifungals, including inhibitors of chitin synthesis, protein and lipid synthesis, efflux and proton pumps and folate synthesis, as well as cell stress regulators and molecules from drug repurposing, are described here (details are shown in **Table 1**). Some of these molecules are in the clinical trial phase (I and II), while others are still in preclinical phase studies.

Cell Wall

GPI Anchor

APX001, the N-phosphonooxymethyl prodrug of APX001A, is a first-in-class broad-spectrum antifungal compound that inhibits Gwt1, an enzyme which is required for cell wall localization of glycosylphosphatidylinositol (GPI)-anchored mannoproteins in fungi (Zhao *et al.*, 2018; Viriyakosol *et al.*, 2019). The active compound, APX001A, demonstrated potent *in vitro* activity against clinically important fungal species, including echinocandin- and fluconazole-resistant isolates (Pfaller *et al.*, 2019). The antifungal efficacy of APX001 was demonstrated in *in vivo* murine models of systemic candidiasis with *Candida albicans* and *Candida auris* (Zhao *et al.*, 2018; Wiederhold *et al.*, 2019) and invasive pulmonary aspergillosis (Gebremariam *et al.*, 2018); APX001 alone or in combination with fluconazole was effective in the treatment of cryptococcal meningitis (Shaw *et al.*, 2018). In addition, APX001 and its derivatives were also highly effective *in vitro* against *Coccidioides* spp. isolates, and *in vivo* against *Coccidioides imitis* in an experimental pneumonia mouse model, reducing fungal burden in the lungs and preventing dissemination, similarly to fluconazole (Viriyakosol *et al.*, 2019). The preclinical efficacy and safety data lend support to the clinical trials (phase II), now in the patient recruitment phase, that will evaluate the efficacy and safety of APX001 in patients with candidemia (NCT03604705).

Chitin Synthase

Nikkomycins X and Z, isolated from *Streptomyces tendae* (Tu 901 strain) (Dähn *et al.*, 1976), are competitive inhibitors of chitin synthase, which is responsible for chitin polymer production, an important component of the fungal cell wall (Hector *et al.*, 1990; Gaughran *et al.*, 1994). Nikkomycins X and Z showed promising *in vitro* activity against *C. albicans* and *C. immitis* (McCarthy *et al.*, 1985; Hector *et al.*, 1990), and the synergistic effect of nikkomycin Z with echinocandins or azoles was shown against *C. albicans*, *Candida glabrata*, and *Aspergillus fumigatus in vitro* (Li and Rinaldi, 1999; Ganesan *et al.*, 2004; Sandovsky-Losica *et al.*, 2008; Verwer *et al.*, 2012; Kovács *et al.*, 2019). Nikkomycin Z was found to be highly effective for the treatment of coccidioidomycosis in animal models (Hector *et al.*, 1990; Shubitz *et al.*, 2013). Notably, nikkomycins had pronounced antifungal activity against *C. immitis* due to the higher chitin content in the cell wall (Li and Rinaldi, 1999). Importantly, clinical trials for nikkomycin Z had been conducted, in which its safety and pharmacokinetics were evaluated in both healthy individuals and those diagnosed with *Coccidioides* pneumonia (NCT00614666 and NCT00834184) (Nix *et al.*, 2009). Unfortunately, the spectrum of activity of nikkomycin Z on other systemic mycoses is limited; as a result, studies were conducted to improve its efficacy. Novel nikkomycin analogs, Px and Pz, developed by mutasynthesis, showed strong inhibitory activity on *C. albicans* (Niu and Tan, 2013; Feng *et al.*, 2014). Structural analogs were synthesized with enhanced chitin synthase inhibitory activity in *C. albicans* (Obi *et al.*, 2000).

Lipids

Acetyl-CoA Synthetase

AR-12 is a celecoxib derivative that inhibits the activity of acetyl-CoA synthetase (ACs), an enzyme involved with acetate metabolism, in fungal cells. In most yeast species, acetate represents an important carbon source for the generation of acetyl-CoA; ACs

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Table 1 New targets and antifungal compounds

Compounds	*Molecular target*	*Target species*	*MIC values*	*In vivo*
APX001	GPI synthesis	*Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Scedosporium* spp.	0.008–0.5 μg/ml	Candidiasis, cryptococcosis, aspergilosis and coccidiodomycosis (murine)
Nikkomycins Z and X	Chitin synthase enzyme	*Candida* spp., *C. immitis*	0.77–0.25 μg/ml synergism with azoles and echinocandins	Coccidiodomycosis (murine)
AR-12	Acetyl-CoA synthetase	*Candida* spp., *C. neoformans, Fusarium* spp., *B. dermatiditis, H. capsulatum, C. immitis*	1–4 μg/ml synergism with fluconazole	Cryptococcosis (murine)
Quinoclidines	SQS	*Candida* spp.	1 to >16 μg/ml	Not
Celastrol	SQS	*Aspergillus* spp.	830 nM	Not
Azasterols	24-SMT	*C. albicans, C. neoformans, P. brasiliensis, Pneumocystis carinii, P. jirovecii*	≤0.06 to >16 μg/ml 200 nM to 10 μM	Not
Arylguanidines	24-SMT	*Candida* spp., *Aspergillus* spp., Dermatophytes	≤0.06 to 16 μg/ml	Not
Aureobasidin A	Ipc1	*C. albicans, S. cerevisiae, C. neoformans*	0.25 to >4 μg/ml	Not
Defensins	GlcCer	*C. albicans*	5–10 μM	Candidiasis (murine)
BHBM and DO	GlcCer	*C. neoformans, Candida* spp., *A. fumigatus, H. capsulatum, Pneumocystis* spp.	0.125 to >32 μg/ml	Candidiasis, cryptococcosis and pneumocystosis (murine)
Milbemycin	ABC transporter	*Candida* spp.	3.2–6.4 μg/ml synergism with azoles	Systemic candidiasis (murine)
Unnarmicin A and C	ABC transporter	*S. cerevisiae, C. albicans*	80–320 μM synergism with azoles	Not
Tellurides	ABC transporter	*S. cerevisiae, C. albicans*	100 μM reverse resistance; MIC (2–5 μM) and synergism with azoles	Not
Omeprazole and derivatives	Proton pump	*Cryptococcus* spp., *Candida* spp., *S. cerevisiae, A. fumigatus*	0.06–1000 μg/ml synergism with azoles	Not
Sordarins	Fungal elongation factor 2 (EF2)	*Candida* spp., *C. neoformans, P. carinii* (currently named *P. jirovecii),* filamentous fungi	0.004 to >64 μg/ml	Candidiasis and histoplasmosis (murine)
Tavaborole	tRNA synthetase	Dermatophytes, *Candida* spp., *C. neoformans, Malassezia* spp., *A. fumigatus, F. solani*	≤0.5–8 μg/ml	Approved as topical treatment for onychomycosis
Antifolates	Dihydropteroate synthase (DHPS)	*A. fumigatus, Cryptococcus* spp.	4 to ≥256 μg/ml synergism with trimetropin	Not
Antifolates	Dihydrofolate reductase (DHFR)	*C. albicans, C. glabrata*	0.2–1024 μg/ml	Not
Cyclosporine A and tacrolimus	Calcineurin	*C. albicans, Cryptococcus* spp., *Aspergillus* spp., *Sporothrix* spp., *Trichosporon* spp., *Malassezia* spp., Mucorales	1 to >64 μg/ml synergism with azoles, amphotericin B and caspofungin	Not

(*Continued*)

Table 1 Continued

Compounds	*Molecular target*	*Target species*	*MIC values*	*In vivo*
Geldanamycin	Hsp90	*Candida* spp., *Aspergillus* spp., *Mucor* spp., *Fusarium* spp., *Paecilomyces* spp.	2 to >16 μg/ml synergism with azoles and echinocandins	Candidiasis and aspergillosis (*Galleria mellonella* and murine)
Efungumab or Mycograb	Hsp90	*Candida* spp.	128–256 μg/ml synergism with fluconazole, caspofungin, and amphotericin B	Candidiasis (murine)
Miltefosine	Not fully elucidated	*Candida* spp., *Cryptococcus* spp., *Paracoccidioides* spp., *H. capsulatum, C. posadasii, Sporothrix* spp., *Aspergillus* spp., *F. solani, Scedosporium* spp., *Pythium* spp., and Dermatophytes	0.12 to >64 μg/ml	Candidiasis and cryptococcosis (*Galleria mellonella* and murine)
OIPC	Not fully elucidated	*C. albicans, Aspergillus* spp.	1–4 μg/ml	Candidiasis (murine)
Sertraline	Not fully elucidated	*Cryptococcus* spp., *Candida* spp., *S. schenckii, T. asahii*	2–8 μg/ml synergism with azoles and amphotericn B	Cryptococcosis (murine)
Tamoxifen	Not fully elucidated	*S. cerevisiae, C. albicans, C. neoformans*	4–64 μg/ml	Candidiasis and cryptococcosis (murine)
Verapamil	Calcium channel	*C. albicans, A. fumigatus, F. solani, Scedosporium* spp., *Trichoderma* spp., *Rhizopus* spp.	853–2731 μg/ml synergism with azoles and polyenes	Aspergillosis and gastrointestinal-colonization by *C. albicans* (murine)
Benzimidazoles	β-tubulin	*C. neoformans*	0.095–0.125 μg/ml	Cryptococcosis (murine)

Note: GPI: glycosylphosphatidylinositol; SQS: squalene synthase; 24-SMT: Sterol C24-methyltransferase; Ipc1: inositol phosphorylceramide 1; GlcCer: glucosylceramide; Hsp90: Heat shock protein 90; BHBM: N′-(3-bromo-2-hydroxybenzylidene) – 2-methylbenzohydrazide; DO: 3-bromo-N′-(3-bromo-4-hydroxybenzylidene) benzohydrazide; OIPC: Oleylphosphocholine.

activity seems to be essential in *C. albicans* (Carman *et al.*, 2008) and is required for virulence in *C. neoformans* (Hu *et al.*, 2008). The inhibition of fungal ACs results in autophagy, decreased histone acetylation, and loss of cellular integrity, while it also down-regulates host chaperone proteins that reduce the immune response (Koselny *et al.*, 2016b; Kushwaha *et al.*, 2017; Mourad and Perfect, 2018). AR-12 has a broad spectrum of activity on planktonic cells of *Candida* spp., *Cryptococcus neoformans*, *Fusarium* spp., Mucor, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *C. immitis* (Baxter *et al.*, 2011; Koselny *et al.*, 2016a,b). AR-12 also demonstrated antibiofilm effects on *C. albicans* biofilms (Baxter *et al.*, 2011) and, in combination with fluconazole, showed synergistic and fungicidal effects on *C. neoformans in vitro* (Chabrier-Roselló *et al.*, 2013). In a murine model of disseminated cryptococcosis, the combination of AR-12 and fluconazole reduced the fungal burden in the brain, which was not observed when the drugs were used alone (Koselny *et al.*, 2016a). In addition, safety and tolerability of AR-12 were evaluated in a phase I clinical trial for its use as an anticancer agent (NCT00978523).

Ergosterol Biosynthesis

Both ergosterol and cholesterol are synthesized from acetyl-CoA via a series of enzymatic reactions that are identical between fungi and animals up to the synthesis of lanosterol, the key branching point. The azoles primarily block ergosterol synthesis by inhibiting lanosterol-14α-demethylase (Erg11), the first primary target in the fungal membrane that is not present on the host cell membrane (Perfect, 2017). Squalene synthase (SQS) and sterol C24-methyltransferase (24-SMT) inhibitors block upstream and downstream steps of lanosterol synthesis, respectively. Despite the extensive and exciting reports highlighting the potential of SQS and 24-SMT inhibitors as promising lead compounds or antifungal drugs *in vitro*, little has been translated to *in vivo* and clinical studies.

Squalene synthase (SQS)

Known inhibitors of human SQS, a target for hypercholesterolemia and atherosclerosis therapies, include zaragozic acids, quinuclidines, benzoxazepines and substituted morpholines (Kourounakis *et al.*, 2011). Quinuclidine-based SQS inhibitors, such as E5700 and ER-119884, were originally developed by Eisai (Tokyo, Japan) as cholesterol- and triglyceride-lowering agents for human diseases, but later demonstrated important antiprotozoal activities *in vitro* and against acute Chagas disease in a murine model (Urbina *et al.*, 2004). Both E5700 and ER-119884 inhibited growth and altered the lipid profile and ultrastructure of the multidrug-resistant *C. tropicalis* strain, and, thus, could act as leads for the development of new compounds against the multidrug-resistant *Candida* species (Ishida *et al.*, 2011b). Similarly, an arylquinuclidine derivative (WSP1267) effectively inhibited the *in vitro* growth of a collection of *Candida* clinical isolates, including several non-*albicans* and azole-resistant strains (Ishida *et al.*, 2011a). Using a different approach, Song and colleagues screened a library of 744 compounds against *Aspergillus flavus* SQS, and the best inhibitor, celastrol, was found to be a noncompetitive inhibitor of *Aspergillus* SQS with inhibitory effects on *A. fumigatus*, the main causative agent of invasive aspergillosis in humans (Song *et al.*, 2019).

Sterol methyltransferase (SMT)

Azasterols 20-piperidin-2-yl-5α-pregnan-3β-20(R)-diol (AZA) and 24(R,S),25-epiminolanosterol (EIL) are steroid compounds and are known inhibitors of 24-SMT in fungi (Song and Nes, 2007). EIL antifungal activity was described for *C. neoformans* and *C. albicans*, with a potency comparable to itraconazole (Ishida *et al.*, 2009; Nes *et al.*, 2009). AZA also showed potent activity against *C. albicans* (Ishida *et al.*, 2009) and *Pneumocystis carinii* (Urbina *et al.*, 1997); interestingly enough, AZA showed the highest potency against the dimorphic fungus *Paracoccidioides brasiliensis* (Visbal *et al.*, 2003). Sulfur-containing sterols, such as 25-thialanosterol and 25-thialanosterol iodide, also demonstrated *in vitro* disruption of ergosterol biosynthesis in *C. albicans* via inhibition of 24-SMT (Kanagasabai *et al.*, 2004; Leaver, 2018). Additionally, α-Bisabolol, a natural phenylpropanoid found in *Matricaria recutita* and *Plinia cerrocampanensis* essential oils, impairs the growth of *A. fumigatus* via inhibition of microsomal 24-SMT (Jahanshiri *et al.*, 2017). Abafungin is the first member of a novel class of synthetic antifungal compounds, the arylguanidines, and presents potent *in vitro* antifungal activity against several medically important fungi, including dermatophytes, *Candida* sp. and *Aspergillus* sp. (Borelli *et al.*, 2008). Preliminary studies suggested that abafungin inhibits transmethylation at the C-24 position of the sterol side chain, which is catalyzed by 24-SMT, while a secondary mechanism involves a direct effect on the fungal cell membrane (Borelli *et al.*, 2008). Finally, the 24-SMT inhibitor 22-hydrazone-imidazolin-2-yl-chol-5-ene-3b-ol (H3) impaired fungal growth and sterol composition of the dimorphic fungi *Sporothrix schenckii* and *Sporothrix brasiliensis*, confirming 24-SMT as a promising target for novel antifungal therapies, including emergent species, like *Sporothrix* spp. (Borba-Santos *et al.*, 2016).

Sphingolipid Biosynthesis

Sphingolipids are important in cellular metabolism and can have a relevant role in the regulation of fungal virulence. Therefore, blocking the synthesis and/or function of specific fungal sphingolipids is an attractive area for the development of new antifungals. Although the most frequent sphingolipid in mammalian cells is sphingomyelin, there are two main groups described in fungal cells: inositol phosphorylceramide (IPC) and hexosylceramides, such as glucosylceramide (GlcCer); therefore, inhibitors of IPC and GlcCer synthesis could potentially be developed into new antifungal drugs (Rollin-Pinheiro *et al.*, 2016).

Inositol phosphorylceramide synthase (IPCs)

Aureobasidin A (AbA) is a natural compound that inhibits inositol–phosphoryl ceramide synthase 1 (IPCs1), particularly in yeasts such as *C. albicans*, *S. cerevisiae* and *C. neoformans* (Aeed *et al.*, 2009). In addition to decreased IPC production, AbA treatment results in the accumulation of ceramide to a toxic level (Cerantola *et al.*, 2009) and, in *C. albicans*, AbA impairs germ tube elongation and affects *in vitro* biofilm formation (Tan and Tay, 2013). Despite having good activity against yeasts, AbA showed reduced activity against molds like *Aspergillus* sp., but structural modifications aiming to broaden its spectrum of activity are very promising. A recent agreement between AureoGen, a small pharmaceutical company involved in the research and development of AbA, and Merck may advance the development and potential commercialization of the first fungal IPCs1 inhibitor (Rollin-Pinheiro *et al.*, 2016).

GlcCer synthase

Natural products have been shown to target GlcCer directly to affect fungal growth. Defensins from plants and insects, such as RsAFP2 (*Raphanus sativus* antifungal protein 2), are active against *C. albicans* strains; moreover, *C. glabrata* (known to lack GlcCer) are resistant to RsAFP2, suggesting that this could be the target of defensins (Tavares *et al.*, 2008). RsAFP2 directly impairs *C. albicans* yeast–hyphae transition, a crucial step for pathogenesis, and induces the accumulation of ceramides, which leads to apoptosis (Thevissen *et al.*, 2012). Two synthetic compounds that might potentially target GlcCer, N′-(3-bromo-2-hydroxybenzylidene)-2-methylbenzohydrazide (BHBM) and 3-bromo-N′-(3-bromo-4-hydroxybenzylidene)benzohydrazide (DO), showed *in vitro* and *in vivo* antifungal activity against most of the medically important fungi, including *C. neoformans*, *Candida* spp., *Pneumocystis* sp., *A. fumigatus* and *H. capsulatum*. Besides having broad antifungal activity, BHBM and DO were also well tolerated by animals and were additive to current antifungals (Mor *et al.*, 2015).

Efflux Pump

Many fungal species acquire azole resistance via overexpression of efflux pumps; it is the most common antifungal resistance mechanism (Cannon *et al.*, 2009; Zavrel and White, 2015). Two classes of efflux pumps have been found in fungi: ATP-binding cassette (ABC) proteins and major facilitator superfamily (MFS) pumps. ABC proteins constitute a large class of drug efflux pumps that use ATP hydrolysis to translocate compounds across cell membranes (Del Sorbo *et al.*, 2000). In this context, one possible option to tackle resistance could be the use of transporter inhibitors, especially in combination with antifungals.

Milbemycins (A3 and A4) and their derivatives, known as ABC transporter inhibitors, are products of fermentation made by *Streptomyces* species (Kumar and Jha, 2017). Milbemycins showed intrinsic antifungal activity and, importantly, they caused a reduction in the MIC values of fluconazole and voriconazole for *C. albicans* and *C. glabrata* (Silva *et al.*, 2013; Walker *et al.*, 2014). In an invasive candidiasis murine model, treatment with milbemycin A3/A4 decreased the fungal burden in the infected tissues and acted synergistically with fluconazole (Silva *et al.*, 2013).

Unnarmicins (A and C) were isolated from the marine bacterium *Photobacterium* (Tanabe *et al.*, 2007; Oku *et al.*, 2008), and both increased the susceptibility of the ABC transporter-overexpressing *S. cerevisiae* strain to fluconazole; most importantly, these compounds were also able to strongly sensitize azole-resistant *C. albicans* clinical isolates (Tanabe *et al.*, 2007).

In addition, organic compounds containing tellurium reduced *S. cerevisiae* growth by disrupting its ATPase function (Chan *et al.*, 2007; Reis de Sá *et al.*, 2014). Moreover, these compounds reversed the resistance phenotype to fluconazole in ABC transporter-overexpressing *S. cerevisae* and *C. albicans* strains; the authors then attributed the ABC transporters as targets for these compounds (Reis de Sá *et al.*, 2014).

Proton Pump

Proton pump inhibitors, such as omeprazole, rabeprazole, lansoprazole, pantoprazole, and esomeprazole, act as agents with antifungal activity or reverse-acquired resistance to azoles (Afeltra and Verweij, 2003). These compounds inhibited *Cryptococcus* spp., *Candida* spp. and *S. cerevisiae* (Küçükaslan *et al.*, 2013; Brilhante *et al.*, 2019). Omeprazole also has an antifungal effect and is known to inhibit the fungal plasma membrane proton ATPase Pma1 of *C. albicans* and *S. cerevisae* (Monk *et al.*, 1995; Manavathu *et al.*, 1999). Importantly, when proton pump inhibitors are combined with azole agents, a significant reduction in MIC values of both drugs is observed, suggesting a synergistic action, as seen in *A. fumigatus* (Afeltra *et al.*, 2004).

Protein Synthesis

Elongation Factor 2 (EF2)

Sordarins, such as zofimarin, BE31405, xylarin, hipoxysordarin, neosordarin, and GR135402 and derivatives, were isolated from various fungal species and are potent and selective inhibitors of translation in fungal cells, interacting with the fungal EF2 to stabilize the EF2-ribosome complex (Liang, 2008; Vicente *et al.*, 2009). *In vitro* studies demonstrated the activity of sordarins against *Candida* spp., *C. neoformans*, *P. carinii* (currently called *P. jirovecii*), and some filamentous fungi (Herreros *et al.*, 1998; Odds, 2001). *In vivo* activity was observed in murine models of candidiasis and histoplasmosis, where treatments increased animal survival and decreased the fungal

burden (Graybill *et al.*, 1999; Aviles *et al.*, 2000; Kamai *et al.*, 2005). Recently, sordarins and synthetic derivatives showed potent fungicidal and pan-fungal activities (Chakraborty *et al.*, 2016; Wu and Dockendorff, 2019; Zhang *et al.*, 2019).

Transfer RNA Synthetase

Tavaborole (AN2690), a benzoxaborole compound, exerts its antifungal activity by blocking protein synthesis and inhibiting cytoplasmic leucyl-aminoacyl transfer RNA (tRNA) synthetase (Markham, 2014). *In vitro* studies showed that tavaborole is effective for dermatophytes (Markham, 2014) and several other fungi, such as *Candida* spp., *C. neoformans*, *Malassezia* spp., *A. fumigatus* and *F. solani*, presenting a broad action spectrum (Gupta and Daigle, 2014). Clinical trials showed that tavaborole has characteristics that allow for favorable penetration into and permeation throughout the human nail plate and can be safe and effective when used topically for the treatment of toenail onychomycosis (Del Rosso and Plattner, 2014; Jinna and Finch, 2015). Tavaborole 5% solution was approved by the US FDA in July 2014 for use as a topical treatment for onychomycosis as an alternative to the conventional antifungals (Gupta and Daigle, 2014).

Folate Biosynthesis

Antifolates are chemical compounds that interfere with folate biosynthesis, an important pathway for the biosynthesis of purines, pyrimidines and several amino acids (Visentin *et al.*, 2012). Two important steps in folate biosynthesis involve dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR).

Dihydropteroate Synthase (DHPS)

DHPS catalyzes the conversion of PABA (para-aminobenzoate) to dihydropteroate (Minato *et al.*, 2015), and the main DHPS inhibitors are sulfonamides, a class of synthetic molecules that act as competitive inhibitors (Griffith *et al.*, 2018). The sulfonamides, such as sulfamethoxazole, sulfadiazine, sulfadoxine and others, are usually recommended to treat bacterial infections and are frequently used in combination with DHFR inhibitors due to increased resistance in monotherapy (Griffith *et al.*, 2018). Besides having antibacterial activity, DHPS inhibitors have demonstrated antifungal activity against *Trichophyton longifusus*, *Microsporum canis*, *C. albicans*, *C. glabrata*, *A. flavus*, and *F. solani* (Chohan *et al.*, 2006).

Dihydrofolate Reductase (DHFR)

DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, an essential cofactor for the synthesis of purines, thymidine, glycine, and methionine, which affects DNA replication, repair and methylation, and its blocking mechanism presents an interesting strategy for drug discovery (Daly *et al.*, 1994; Paulsen *et al.*, 2013; Al-Rashood *et al.*, 2014; Alotaibi *et al.*, 2017). DHFR is currently targeted by methotrexate (MTX) and trimetrexate (both anticancer), trimethoprim (TMP, antibacterial), pyrimethamine (PYR, anti-protozoan) (Alotaibi *et al.*, 2017), proguanil (antimalarial) and others (Gregson and Plowe, 2005). Some of these molecules also show *in vitro* antifungal activities; PYR inhibited growth of *C. albicans*, whereas TMP and MTX were not effective alone in inhibiting *C. albicans*, but MTX in combination with azole agents led to a synergistic effect (Navarro-Martínez *et al.*, 2006; Yang *et al.*, 2019). In the last decade, researchers have developed new compounds with higher selectivity and greater affinities for DHFR in *C. albicans* and other species (Vuppala *et al.*, 2019), such as propargyl-linked antifolate derivatives (G-Dayanandan *et al.*, 2014), quinazolines (Al-Rashood *et al.*, 2014), some triazoles (Hassan *et al.*, 2013), Furo compounds, and pyrrolo pyrimidines (Cody and Schwalbe, 2006).

Although DHPS and DHFR inhibitors had reduced antifungal activity, the combination of sulfamethoxazole with trimethoprim exhibits a synergistic effect and has been used for years to treat paracoccidioidomycosis in Latin America (Shikanai-Yasuda *et al.*, 2017).

Cell Stress Regulators

Calcineurin

Calcineurin is a conserved Ca^{2+}-calmodulin-activated protein phosphatase 2B belonging to the phospho-protein phosphatase family of enzymes, and its signaling plays diverse roles in fungi in regulating stress responses, morphogenesis and pathogenesis (Juvvadi, Lamoth and Steinbach, 2014; Juvvadi *et al.*, 2017). Calcineurin is the target of immunosuppressive drugs such as cyclosporine A and tacrolimus (FK506) (Steinbach *et al.*, 2007). Many studies show that calcineurin inhibitors are effective against clinically important fungi, such as *C. albicans*, *Cryptococcus* spp., *Aspergillus* spp., *Sporothrix* spp., *Trichosporon* spp., *Malassezia* spp., and Mucorales (Stie and Fox, 2008; Shirazi and Kontoyiannis, 2013; Kubiça *et al.*, 2016; Borba-Santos *et al.*, 2017; Ianiri *et al.*, 2017). Additionally, calcineurin inhibitors have a synergistic effect with antifungal drugs, such as azoles, amphotericin B and caspofungin (Shirazi and Kontoyiannis, 2013; Kubiça *et al.*, 2016).

Heat Shock Protein 90 (Hsp90)

This chaperone regulates crucial cell responses to both cell membrane and cell wall stresses and has been extensively validated as a regulator of virulence and antifungal drug resistance (Huang *et al.*, 2019). Geldanamycin and Efungumab (Mycograb) act on Hsp90. Studies show that geldanamycin has activity on yeast and filamentous fungi in combination with azoles and echinocandins in *in vitro* and *in vivo* models (Cowen *et al.*, 2009; Zhang *et al.*, 2013; Lamoth *et al.*, 2014). Mycograb (a monoclonal antibody), when combined with fluconazole, caspofungin, and amphotericin B, has synergy against the *Candida* species *in vitro*, and in a murine model of invasive candidiasis *in vivo* (Bugli *et al.*, 2013). Moreover, the efficacy and safety of Mycograb for cryptococcal meningitis were evaluated in phase II clinical trials (NCT 00324025 and NCT00847678).

Drug Repositioning

The repositioning of drugs refers to the establishment of a new pharmacological indication for a drug that was previously approved for another purpose (Truong *et al.*, 2018), offering attractive benefits, as toxicological and pharmacokinetic profiles have been established previously, accelerating development time and avoiding substantial costs associated with costly clinical trials (Krysan, 2015; Truong *et al.*, 2018).

Miltefosine (MFS)

Initially developed as an anticancer agent and currently used in the treatment of certain breast cancer types, MFS is an alkyl phosphosphocholine compound that has been repurposed for the treatment of leishmaniasis (Sundar and Chakravarty, 2015). MFS has shown impressive broad-spectrum antifungal activity *in vitro*, covering most medically important fungi, including yeasts (*Candida* spp. and *Cryptococcus* spp.), dimorphic fungi (*Paracoccidioides* spp., *H. capsulatum*, *C. posadasii*, *Sporothrix* spp.), dermatophytes and several other filamentous fungi (Widmer *et al.*, 2006; Tong *et al.*, 2007; Brilhante *et al.*, 2014, 2015; Imbert *et al.*, 2014; Borba-Santos *et al.*, 2015; Compain *et al.*, 2015; Rossi *et al.*, 2017; Loreto *et al.*, 2018). MFS was effective against *Candida* spp. biofilms *in vitro* (Vila *et al.*, 2013, 2016), including the emerging multidrug-resistant species *C. auris* (Wall *et al.*, 2019), and reduced the biomass of *F. oxysporum* biofilms (Vila *et al.*, 2015b). Most importantly, MFS was effective as a topical treatment for oropharyngeal candidiasis in a murine model (Vila *et al.*, 2015a), confirming its promising application for superficial and mucosal fungal infections. However, conflicting studies demonstrated variable translation of MFS *in vivo* activity against disseminated cryptococcosis (Widmer *et al.*, 2006; Wiederhold *et al.*, 2013). Moreover, MFS reduced the fungal infection burden from *C. albicans* and *C. gattii* in the larval model of *Galleria mellonella* (Spadari *et al.*, 2019).

The mechanism of action of MFS in fungi is still unknown, but signaling to an apoptotic-like cell death via activation of metacaspase Mca1 by ROS accumulation in the mitochondria was observed in *S. cerevisiae* (Biswas *et al.*, 2014), and similar results were obtained for *C. neoformans* (Spadari *et al.*, 2018). Moreover, MFS induces other physiological effects, including increased plasma membrane permeability and melanin accumulation (Borba-Santos *et al.*, 2015; Rossi *et al.*, 2017; Spadari *et al.*, 2018).

Strategies including the synthesis of structural analogs and the development of drug delivery systems were applied to MFS to reduce its cytotoxicity. Encapsulation of MFS in alginate nanoparticles decreased toxic effects and infection in the *G. mellonella* model (Spadari *et al.*, 2019), while a few structural analogs of MFS showed enhanced *in vitro* inhibition of *C. albicans* (Vila *et al.*, 2013) and *Sporothrix* spp. (Borba-Santos *et al.*, 2016). Oleylphosphocholine (OlPC) is the most extensively explored MFS analog to date. OIPC is effective *in vitro* and *in vivo* against *C. albicans* biofilms (Holtappels *et al.*, 2017). OIPC showed excellent *in vitro* activity against *Aspergillus* spp.; however, its therapeutic efficacy in an acute mouse model for invasive aspergillosis was less convincing, and other clinically relevant animal models should be further evaluated (Paulussen *et al.*, 2015). Compared to MFS, OlPC exhibited fewer side effects when tested *in vivo* and has good oral bioavailability. Further pharmacokinetic and pharmacodynamic data will confirm OIPC as a potential candidate for clinical studies.

Sertraline

Antidepressant drugs such as sertraline showed antifungal effects alone and in combination with azoles or amphotericin B *in vitro* against *Cryptococcus* spp., *Candida* spp., *S. schenckii* and *T. asahii* (Zhai *et al.*, 2012; Cong *et al.*, 2016; Costa Silva *et al.*, 2017; Oliveira *et al.*, 2018; Villanueva-Lozano *et al.*, 2019). Additionally, sertraline efficiently reduced the fungal burden in the brain and spleen of mice infected with *C. neoformans* (Zhai *et al.*, 2012; Trevinõ-Rangel *et al.*, 2016). Its mechanism of action on fungi is not fully elucidated; however, a fungicidal effect which occurs by damaging the plasma and mitochondrial membranes to activate apoptotic signaling pathways was described for *Candida* spp. (Costa Silva *et al.*, 2017); inhibition of protein synthesis by sertraline was observed for *Cryptococcus* spp. (Zhai *et al.*, 2012). Currently, sertraline use is in clinical trials (phase III) for oral treatment of cryptococcal meningitis alone or in combination with AMB and FLC (NCT 01802385 and NCT 03002012).

Tamoxifen

Tamoxifen is a selective estrogen receptor modulator used in breast cancer treatments (Ngan *et al.*, 2019). Its *in vitro* antifungal activity was observed against *S. cerevisiae* (Wiseman *et al.*, 1989), *C. albicans* (Beggs, 1993; Dolan *et al.*, 2009) and *C. neoformans* (Hai *et al.*, 2019). Tamoxifen reduced the fungal burden in a murine model of disseminated candidiasis and cryptococcosis, and researchers have proposed tamoxifen as an alternative for neurocryptococcosis due its ability to cross the blood-brain barrier and accumulate in the brain at levels well above the MIC values (Morello *et al.*, 2003; Dolan *et al.*, 2009; Butts *et al.*, 2014). The antifungal activity of tamoxifen is the result of its effects on multiple physiological processes, such as membrane damage and interference with calcium homeostasis, and studies show that its activity correlates with calmodulin inhibition (Dolan *et al.*, 2009; Butts *et al.*, 2015). Clinical trial studies (phase II) are beginning to evaluate the efficacy, feasibility, and safety of tamoxifen in combination with standard therapies (amphotericin B and fluconazole) in the treatment of cryptococcal meningitis (NCT03112031).

Verapamil

A typical calcium channel blocker of the phenylalkylamine class, verapamil is widely used in the treatment of angina pectoris and hypertension (Strigun *et al.*, 2011). The *in vitro* antifungal activity of verapamil led to a significant decrease in the *C. albicans* hyphal development and the ability to adhere to a polystyrene surface (Yu *et al.*, 2014). Moreover, treatment with verapamil led to a remarkable decrease in gastrointestinal-colonizing fungal cells (Yu *et al.*, 2014). Verapamil alone, however, had poor antifungal activity (Homa *et al.*, 2017). Studies suggest that the combination of verapamil with azoles or polyenes shows synergistic effects and is a promising therapy for *C. albicans*, *A. fumigatus*, *F. solani*, *Scedosporium* spp., *Trichoderma* spp., and *Rhizopus* spp. (Krajewska-Kułak and Niczyporuk, 1993; Afeltra *et al.*, 2004; Homa *et al.*, 2017; Nazik *et al.*, 2017). Recently, the combination of verapamil with itraconazole controlled fungal infection with *A. fumigatus* in an invasive aspergillosis murine model by improving the survival rate of animals and reducing the fungal burden in the lungs (Zeng *et al.*, 2019).

Benzimidazoles

These molecules were developed primarily as anthelmintic agents for human and veterinary medicine (Cruz *et al.*, 1994). Benzimidazole drugs, such as albendazole, mebendazole, and flubendazole, have potent *in vitro* activity against *C. neoformans*, and flubendazole is effective in murine models of cryptococcal meningitis (Joffe *et al.*, 2017; Nixon *et al.*, 2018). The authors suggested that benzimidazoles inhibit fungal growth by blocking β-tubulin polymerization for microtubule formation, disrupting mitosis (Cruz *et al.*, 1994), and interfering with fungal morphology, biofilm formation, cell proliferation and intracellular parasitism (Joffe *et al.*, 2017).

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Immunotherapy of Fungal Infections

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Glossary

Adaptive immunity Microbe-specific, antigen-educated, mature T- and B-cells, which create long-term immunological memory with recall function, initiate cross-talk between immune components via biochemical messenger proteins known as "cytokines" and "chemokine's," and recruit other immune effectors to the site of host–microbe interaction.

Adjuvant Pharmacological or immunological agents which, when coadministered with the vaccines, modulates the quantum and/or kinetics of the immune response due to the vaccine.

Barrier immunity Physical impediments to the entry of a microbe into the human body. For example, the skin, external surface epithelium, and internal linings of the upper and lower respiratory, gastrointestinal, and genitourinary tracts.

Colonization A state following infection in which a microbe inhabits a niche in the host and host–microbe interaction, which is not clinically apparent, results in elimination of the microbe or transition to other states, such as disease or latency.

Commensalism A state following infection in which the outcome of host–microbe interaction results in neutral, cooperative, symbiotic, or beneficial outcomes for the host and there is no host damage.

Damage-response framework (DRF) A unifying hypothesis to account for microbial pathogenesis and infectious diseases as a function of host–microbe interaction. The DRF asserts that the relevant outcome of host–microbe interaction is damage in the host, which can stem from host factors, microbial factors, or both. All outcomes of infection can be reconciled as the amount/degree of damage stemming from host–microbe interaction. This dispenses with the need for terms to characterize microbes that assume they or impaired hosts are solely responsible the pathogenesis of infectious diseases, such as pathogen, commensal, and opportunist. The DRF considers the balance between the host response and host damage, defines disease as one possible outcome of host–microbe interaction, and recognizes that the host response may result in damage due to either an insufficient or excessive immune response. These concepts provide the rationale for immune-based therapies for microbial diseases.

Damage Impairment of normal host physiology, biology, immunity, and/or other functions that may stem from host factors, microbial factors, or both.

Effector leukocytes White blood cells that perform specific immune defense functions. Some examples are: T-lymphocytes or "T-cells," which may be helper (T_H) or regulatory (T_{reg}) cells bearing CD4 antigen on surface, or cytotoxic (T_c) bearing CD8 antigen; B-lymphocytes or "B-cells," which secrete antibodies; dendritic cells (DC); natural killer (NK) cells; monocytes, which become macrophages on entering the tissues; polymorphonuclear neutrophils (PMNs), amongst others.

Exposure Point of actual contact between a host and a microbe.

Host immunity Aggregate of immune mechanisms that enable a host to resist development of damage stemming from host–microbe interaction.

Immunomodulation Aggregate of processes in which immune-reactive components of either host defense or microbe switch on, turn off, regulate, enhance or decrease the quantum of immune response and inflammation in the host.

Immunotherapy Therapies in which components of the immune system and/or microbial components are administered to reduce the amount/degree of host damage stemming from host–microbe interaction. Immunotherapies can be active, for example, vaccines, or passive, for example, delivery of components of the immune system.

Infection Acquisition of a microbe by a host.

Infectious disease A clinical state that reflects an outcome of host–microbe interaction that arises in a susceptible host when the amount or degree of host damage reaches a clinically apparent or assessable state.

Inflammation Aggregate of biochemical messenger-mediated processes by which effector leukocytes are brought to the site of immune unrest, such as host–microbe or host–allergen interaction. Because of the natural functions of these effectors, excessive inflammation results in damage to the host tissues at the site of these interactions.

Innate immunity Broad spectrum, nonspecific, rapidly active immune defense provided by effector leukocytes (cell-mediated immunity) and circulating antibodies produced by subsets of naïve B-lymphocytes (antibody-mediated immunity). These components are capable of killing microbes via various mechanisms.

Latency/persistence A state following infection in which a microbe remains in the host in a manner that does not result in its elimination; there is no clinically apparent damage, but transition to a state of disease can occur in the setting of immune impairment and/or other processes.

Monoclonal antibody Antibodies secreted by a single B-cell. These antibodies recognize a single determinant on the eliciting antigen. Monoclonal antibodies can be isolated from polyclonal responses and/or produced by molecular engineering from single B-cells by a variety of techniques.

Opsonization A process in which microbial cells or microbe-derived materials are coated with antibodies or small protein fragments generated by the complement system. This marks the cells/materials for further action by professional phagocytes (DCs, monocyte/macrophages, PMNs) and/or cytotoxic cells.
Pathogenesis Aggregate of processes that shifts the physiological state from normal host physiology, biology, immunity, and functions to damage.
Phagocytosis and antigen presentation The process of engulfment of microbial cells/products by certain immune effectors, referred to as phagocytes. The engulfment creates a fluid-filled space ("vacuole") in which the microbes/products are trapped. Next, reactive chemical substances and/or enzymes are released in the vacuole, which kill the microbes and/or digest the products into much smaller fragments. These fragments are associated with certain membrane-bound protein structures called major histocompatibility complexes (MHCs) and presented to T- and B-cells for their education, maturation and generation of immunological memory. This process is referred to as "antigen presentation."
Polyclonal antibody response Population of antibodies secreted by different B-cells in response to an antigen. The resultant immunoglobulin's generally recognize multiple determinants of eliciting antigen.
Trained immunity A relatively new concept in which NK cells and monocyte/macrophages may undergo extra-chromosomal or epigenetic reprogramming to generate short-term immune memory.
Vaccine A single or a combination of antigen(s) which, when administered to a host induce/s an immune response to the administered component/s that results in microbe-specific immunity as well as immunological memory. Administration of a vaccine, known as "vaccination" or "immunization," elicits immune responses in the host that prevent the development of disease. Active vaccination is used to induce immunity prior to exposure or infection with a microbe. Passive vaccination is used to provide exogenous preformed immunity to treat or prevent disease. Passive and active vaccination each augment the ability of the host to limit damage stemming from the host–microbe interaction.
Virulence factors Microbial factors and/or components that confer or enhance a microbe's capacity to cause damage in a host. Many such factors allow the microbe to evade host defenses, thrive in inhospitable intracellular environments, and be invulnerable to cytotoxic host immune mechanisms.

Introductory Concepts

Host–Microbe Interaction, Infection, Disease, Damage

The term "infectious disease" refers to a clinical state that occurs as a result of infection with a microbe when the amount/degree of damage to host tissues resulting from a host–microbe interaction becomes clinically apparent and/or assessable. However, an infectious disease does not represent necessarily a single outcome, but is rather one possible outcome of a host–microbe interaction (Pirofski and Casadevall, 2002). Just as a host exhibits (an) immune response to a microbe, microbes exist in hosts in different states. The first step in the pathogenesis of an infectious disease is exposure, which is the (first) point of actual contact (or the possible risk of contact) between a host and a microbe. Infection signals the acquisition of a microbe by a host. There are multiple portals by which a microbe can infect a host, including respiratory, gastrointestinal, cutaneous, and hematogenous sites. However, not all states of microbial existence within a host lead to a clinically symptomatic or detectable/assessable state; thus neither exposure nor infection alone is sufficient to predict the occurrence of the state of disease. The states that are asymptomatic include commensalism, colonization, and latency, which differ from one another only by the amount or degree of host damage that results from the host–microbe interaction. However, these states can transition to disease in the setting of host immune impairment and defects, such as a breakdown in barrier immunity, insufficient and excessive immune responses, and physiological immune impairments that occur throughout the lifespan of the host. "Successful" host responses can lead to the elimination of a microbe, a process that antimicrobial therapy also seeks to accomplish. This theory of infectious disease/microbial pathogenesis was put forth as the damage-response framework (DRF) by Casadevall and Pirofski (1999, 2003b) and Pirofski and Casadevall (2002, 2008).

Although quantification of disease as a function of host damage is a central tenet of the DRF, measureable indices of damage remain relatively limited to radiographic findings, perturbations in leukocyte counts and other hematologic indices, and alterations in temperature and physiological state as measured by blood pressure and hemodynamic parameters. At present, measures of inflammation apart from fever and blood counts are not available clinically. Nonetheless, the recognition that infectious diseases reflect an outcome of host–microbe interactions has led to efforts to characterize the host response to a microbe as a prelude to designing newer therapeutic modalities, including immune-based therapies. Such therapies, known as "immunotherapy," employ active immunization with microbial components, and/or passive administration of host immune system components to reduce host damage stemming from host–microbe interaction. This chapter will focus on the application of this modality to management of medical mycoses. Notably, immunotherapy has been a mainstay of treatment for cancer for some time (Raval *et al.*, 2014). While we have done our best to comprehensively review this topic, we apologize in advance for any and all omissions readers might identify.

Host–Fungal Interaction and Virulence Factors

The outcome of fungal interaction with the host determines the state of the fungus in the host (Pirofski and Casadevall, 2015). Therefore, consideration of immunotherapy for fungal diseases must incorporate the unique challenges that fungi pose to host immunity (Casadevall and Pirofski, 2012). The ability of some fungal organisms to cause disease can be a function of certain structural and secreted components, often called "virulence factors." Many of these factors enable the fungus to evade host defenses, thus facilitating its ability to live and disseminate in a host. The main structural component that impacts the latter is the fungal cell wall. Composed of complex polysaccharides (e.g., chitin, α- and β-glucans, etc.), lipids (such as ergosterol) and glycoproteins, the cell wall provides a structural and functional barrier that protects fungal cells from host immune-mediated damage and osmotic lysis. While the cell wall is a crucial component of all fungi, cell walls of the yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* are uniquely further protected by a complex polysaccharide capsule, a significant virulence factor with immunomodulatory properties (O'Meara and Alspaugh, 2012). Microscopic asexual spores ("conidia") of filamentous fungal ("mold") organisms are ubiquitous and covered with a tough cell wall that protects them against both environmental and host immune challenges. For instance, the hyphae (aerial structures) and conidia of many molds contain highly hydrophobic surface proteins called hydrophobins (Bayry *et al.*, 2012) that protect conidia from immune-mediated damage, such as for *Aspergillus fumigatus* (Rambach *et al.*, 2015). In turn, conidial interaction with the host immune system can result in allergic and other inflammatory states. A number of medically important fungi, for example, species of *Candida*, *Histoplasma*, *Coccidioides*, *Paracoccidiodes*, *Blastomyces*, and *Sporothrix*, exhibit dimorphism, whereby they exist in either a hyphal or a yeast/yeast-like state, with each state producing unique virulence properties. In addition, many fungi produce toxic secondary metabolites ("mycotoxins" such as gliotoxin, aflatoxin, fumonisin, etc.) that are capable of causing disease via carcinogenicity, immunosuppression, allergy, and/or metabolic derangement (Bennett and Klich, 2003).

Management of Fungal Diseases

Current antifungal chemotherapy

The current foundation of antifungal therapy consists of different classes of systemic and topical agents, including polyene macrolides, triazoles, echinocandins, a pyrimidine nucleoside analog, and other inhibitors of fungal DNA/RNA or protein synthesis. Antifungals in clinical use are safe and reasonably well tolerated. Although they can lead to fungal elimination in the setting of intact immunity, many patients with fungal diseases have varying degrees of immune impairment, a context in which fungal elimination is notoriously difficult to achieve. In addition, the use of antifungal agents is subject to drug–drug interactions, narrow activity spectrum, variable pharmacokinetics/pharmacodynamics, inherent or acquired resistance to the agent, and inadequate clearance of the fungal burden from tissues or organs (Olson *et al.*, 2012; Autmizguine *et al.*, 2014). Limitations in the use and/or efficacy of antifungal chemotherapy underscore the importance of efforts to develop immunotherapeutic approaches to prevent and treat fungal diseases.

Rationale for immunotherapy

Immunotherapy can offer adjunctive, preventative, and/or therapeutic options. This concept is bolstered by the fact that even when antifungal therapies are effective, successful management often involves engagement of host immunity. Following direct fungicidal and/or fungistatic action of an antifungal agent, the host immune system takes over further fungal elimination and scavenging of dead fungi and toxic byproducts. This process employs inflammatory mediators (cytokines and chemokines), released by innate immune cells either in response to the fungal pathogen per se, or via the immunomodulatory effect of antifungal agents (Olson *et al.*, 2012). Therefore, control of host damage, ranging from fungus-mediated damage to damage stemming from the host response, is necessary for successful management of fungal diseases.

Host immunity and antifungal defense

Host immunity represents the sum total of a multitude of immune mechanisms that enable the host to resist the development of fungal and other microbial diseases, including (1) barrier immunity, (2) specialized immune cells (cell-mediated immunity, "CMI") in the blood and other organs, and (3) antibodies and other proteins (humoral/antibody-mediated immunity, "AMI"), produced by these cells. In the following sections, these mechanisms are discussed in the specific context of antifungal defenses.

The first line of defense is intact "barrier immunity." These barriers, which include the skin, external and internal epithelial surfaces (including those lining the interiors of the upper and lower respiratory, gastrointestinal, and genitourinary tracts), limit/control/impair fungal invasion of the blood, brain, and other tissues (Borghi *et al.*, 2014; Dühring *et al.*, 2015). Upon breaching this first line, fungi (yeast or mold) encounter cellular components of "innate immunity," nonspecific effector leukocytes, such as polymorphonuclear neutrophils (PMNs) and mononuclear (monocyte/macrophage, and dendritic cells/DC) phagocytes as well as circulating natural killer lymphocytes ("NK cells") and certain subsets of naïve B-lymphocytes ("B-cells") (Drummond *et al.*, 2014). The phagocytes engulf ("phagocytose") and kill fungi via enzymatic lysis (Sorrell and Chen, 2009; DeLeon-Rodriguez and Casadevall, 2016). NK cells can kill fungi directly or via destruction of immune cells with internalized fungi (Djeu *et al.*, 1988; Levitz and Dupont, 1993; Murphy *et al.*, 1993; Park *et al.*, 2009; Bouzani *et al.*, 2011; Schmidt *et al.*, 2011). Additionally, NK cells and monocyte/macrophages have recently been implicated in short-term innate immune memory (referred to as "trained

immunity") via epigenetic reprogramming independent of other effectors (Netea, 2013), which helps mount a resistance to disease/damage caused by lethal systemic fungal infections.

Fungal fragments resulting from intra- or extracellular lysis, as well as secreted fungal materials, serve as determinants for antigen-presenting cells (APCs), such as primarily macrophages, DCs, and to a lesser extent, two subsets of B-cells (B1 and Marginal Zone/MZ B-cells) (Zhong *et al.*, 2007; Hohl *et al.*, 2009; Ersland *et al.*, 2010; Drummond *et al.*, 2014; Huston *et al.*, 2016). APCs educate two important cellular components, the follicular subset of B-cells ("B2 B-cells") and T-lymphocytes ("T-cells"), thereby bridging nonspecific, rapidly active innate immunity and fungus-specific "adaptive immunity." Adaptive immunity consists of antigen-educated, fungus-specific, mature T- and B-cells, which create immunological memory as well as various CMI effectors that are recruited to areas of host–fungus interaction (in aggregate, "inflammation"). Adaptive responses are particularly essential to deal with fungi, many of which have the capacity for facultative intracellular survival and live dormantly within phagocytes and tissues (Sebghati *et al.*, 2000; Coelho *et al.*, 2014). Effector T-cells involved in antifungal immunity are primarily of three types, namely, (1) surface CD4 glycoprotein-bearing ($CD4^+$) T-helper (T_h) cells, which play a crucial role in maturation of antigen-educated B-cells into antibody-secreting plasma cells and memory B-cells, and activation of cytotoxic T-cells (T_c) and macrophages; (2) surface CD8 glycoprotein-bearing ($CD8^+$) T_c or "Killer T" cells, with the ability to directly kill fungal cells and fungus-infested host cells as well as generate long-term immune memory (van de Veerdonk and Netea, 2010; Nanjappa *et al.*, 2012; Verma *et al.*, 2014; Gibson and Johnston, 2015); and (3) a subset of $CD4^+$ T-cells with immune-suppressive regulatory functions ("T_{reg}" cells). Of note, in the context of the DRF, T_h1 and T_h17 cells mediate the induction of inflammation (and, consequently, collateral tissue damage) in response to fungi, whereas T_{reg} cells are responsible for the modulation of excessive inflammation and maintenance of peripheral tolerance; interestingly, B1 and B2 B-cells work reciprocally in promoting the differentiation of T-cells into T_h or T_{reg} lineages (Zhong *et al.*, 2007).

Central to host defense against fungi are secreted molecules released by CMI effector cells (Roy and Klein, 2012; Mueller-Loebnitz *et al.*, 2013; Rohatgi and Pirofski, 2015). These include: (1) chemical messengers (proteins "cytokines" and "chemokines") and other signaling molecules with wide-ranging mediatory and modulatory actions, for example, the immunomodulatory pro- (such as: interferon/IFN-γ, tumor necrosis factor/TNF-α, and interleukin/IL-17/22) and anti-inflammatory (such as: IL-4, IL-10; transforming growth factor/TGF-β) cytokines important in antifungal defense (Datta *et al.*, 2008; Wüthrich *et al.*, 2012; Mueller-Loebnitz *et al.*, 2013; Lauvau *et al.*, 2015; Rohatgi and Pirofski, 2015; Trautwein-Weidner *et al.*, 2015), (2) proteins with cytotoxic functions (such as: perforins, granzyme, granulysins, α- and β-defensins, etc.) (Schmidt *et al.*, 2011; Zhang and Gallo, 2016), and (3) members of the complement system, an innate defense system whose activation leads to cascading biochemical reactions generating small proteins which help kill fungi by direct cytotoxic actions or indirectly by coating the fungal cells (or fungal materials), thereby marking them (a process called "opsonization") for action by phagocytes or NK cells. In addition, antibodies, the principal component of AMI, are immunoglobulin proteins secreted by both naïve and antigen-educated B-cells, with or without help from $CD4^+$ T-cells. AMI is significant and important in host defense and resolution of fungal infections. High-affinity antibodies elicited by mature B-cells bind specifically to antigens on the surface of, or secreted by, fungal cells, thereby triggering a number of cellular and humoral mechanisms that lead to fungal elimination (Rodrigues *et al.*, 2007; Casadevall and Pirofski, 2012; Wüthrich *et al.*, 2012; Verma *et al.*, 2014; Rohatgi and Pirofski, 2015). Functions of AMI include: (1) promotion of phagocytosis ("antibody-dependent cellular phagocytosis") and killing by other effectors ("antibody-dependent cell-mediated cytotoxicity"/ADCC), (2) direct killing of fungal cells via complement-mediated membrane damage, (3) clearance of fungal products, (4) immunomodulation, and (5) modification of fungal metabolism/gene expression (Casadevall and Pirofski, 2005). In addition, low-affinity, naturally occurring antibodies secreted by naïve and innate-like B1 and MZ B-cells without prior antigenic stimulation that bind evolutionarily conserved microbial determinants, for example, β-glucans, likely enhance innate antifungal immunity (Panda and Ding, 2015; Rohatgi and Pirofski, 2015).

Most fungal infections do not result in disease in normal hosts with normal immunity. The state of host immunity, which ranges from immunocompetent (when immune defenses are active and appropriate) at one end of the spectrum to immunocompromised (when immune defenses are insufficient or inappropriate) on the other largely determines host susceptibility to fungal diseases. It is important to note that a variety of conditions and/or therapies, including HIV infection/AIDS, cancer, immunosuppressive chemotherapy for treatment of cancer and inflammatory diseases, biological response modifiers for inflammatory diseases, hematological or solid organ transplantation, disruption of normal skin and/or mucosal barrier immunity due to surgery, burns, and in-dwelling catheters impair immunity and render patients vulnerable to a wide array of microbes that do not generally cause disease in people with intact immunity (Pirofski and Casadevall, 2015). These microbes include fungi, such as *C. neoformans*, *Candida albicans*, *Histoplasma capsulatum*, *Aspergillus*, *Pneumocystis* and *Trichosporon* species, *Zygomycetes*, among others (Badiee and Hashemizadeh, 2014). However, it must also be noted that fungal diseases also occur in immunocompetent people (Casadevall and Pirofski, 1999, 2007a). Colonization of the nasal cavity by filamentous fungi (*Aspergillus*, dematiaceous fungi, hyaline molds, *Mucor*, etc.) may lead to sinonasal tract diseases in healthy individuals (Casadevall and Pirofski, 1999; Callejas and Douglas, 2013). *H. capsulatum*, which causes disseminated histoplasmosis in immunocompromised patients, may lead to chronic granuloma formation and fibrosing mediastinitis in apparently normal individuals with a strong immune response (Casadevall and Pirofski, 1999). *C. gattii* can cause pulmonary and central nervous system disease in the setting of normal or compromised immunity (Franco-Paredes *et al.*, 2015). Thus fungal diseases cannot be defined independently of the immune status of the host, reinforcing a central tenet of the DRF, namely, that infectious diseases can only occur in a susceptible host, providing a rationale for the use of immunotherapy for fungal diseases (Pirofski and Casadevall, 2008).

Antifungal Immunotherapy

The idea behind antifungal immunotherapy is to augment the host immune response to fungi. While in some instances it might recapitulate the "normal"/natural immune response, in others it might leverage the ability of mediators that are not part of the normal/natural response to limit/eliminate the fungus and/or damage stemming from host–fungus interaction. Many of the foregoing immune mechanisms hold promise for immunotherapeutic applications. On the basis of their impact on the host response, immunotherapeutic modalities can be divided into two main categories: active and passive.

Active Immunotherapeutic Modalities

"Active" immunotherapy is an exogenously delivered modality designed to stimulate a host immune response to the antigen/s that are administered. Vaccines are the main type of active immunotherapy. Active vaccination induces a specific response that provides the host with a preexisting immune armamentarium to the relevant microbe as well as immunological memory that makes it possible for the host to respond again in the future. Currently licensed vaccines that elicit microbe-specific immunity are composed of inactivated viruses (e.g., influenza), live-attenuated viruses (e.g., measles, mumps, rubella, varicella), or a component of a virus or bacteria (e.g., hepatitis B surface antigen, toxoids, capsular polysaccharides). In theory, vaccines can also elicit nonspecific immunity via immunization with a cross-reactive antigen present on multiple microbes (Casadevall and Pirofski, 2003a). Functionally, antimicrobial vaccines are primarily prophylactic, with the goal of helping the host develop immunity to microbial infections if/when exposure occurs. Although some antiviral vaccines, for example, rabies, can be used therapeutically very soon after exposure, current vaccines for other infectious diseases are not used in this mode. Of note, the concept of therapeutic vaccines has increasingly been leveraged for treatment of cancer (Melero *et al.*, 2014). Given that many fungal diseases occur long after infection and stem from an immune breakdown in a latent state ("reactivation"), therapeutic vaccines could hold promise for prevention and/or management of reactivated fungal infections.

The benefit of active vaccination is that it induces enduring immunity. On the other hand, its reliance on the host's ability to develop an immune response limits its utility in the setting of impaired immunity. One additional concern is that a beneficial immune response can take time to develop, since multiple rounds of vaccination might be needed for sufficient immunity to develop. Nonetheless, despite being less immunogenic, most current vaccines are effective in patients with impaired immunity, such as those with HIV, hematologic malignancies, and on corticosteroid therapy (Nanjappa *et al.*, 2012); also, see review in Spellberg (2011). In this regard, adjuvants (such as CpG oligodeoxynucleotides/ODN) and immunomodulators, capable of enhancing the immunogenicity of the vaccines, hold further promise for immunocompromised patients (Spellberg, 2011).

Fungal vaccines

At present, there are no licensed vaccines for fungal diseases. Nonetheless, there are ample data to suggest that vaccines eliciting AMI and/or CMI could provide a rational, effective approach to preventing fungal disease. As noted above, the multitude of independent, overlapping and/or redundant mechanisms that constitute antifungal immunity offer the promise of protection via vaccines even in the setting of certain types of immune impairment (Spellberg, 2011; Iannitti *et al.*, 2012; Nanjappa *et al.*, 2012; Verma *et al.*, 2014). In this discussion, vaccines that elicit AMI will be considered first. AMI is the main mediator of protection for all currently licensed vaccines and extensive preclinical data show that AMI can enhance host resistance to a number of fungi, including *Candida* spp. and *C. neoformans*. In fact, the rationale for these vaccines is partly derived from the observation that specific antibodies develop in course of the normal response to these yeasts (see discussions in Torosantucci *et al.*, 2009; Sandini *et al.*, 2011; Datta and Subramaniam, 2014; Rohatgi and Pirofski, 2015). In addition, many preclinical studies provide strong evidence that certain conserved determinants can generate cross-reactive antibodies with a broad spectrum of activity against numerous medically relevant fungi, and vaccines which elicit antibody responses to specific and cross-reactive fungal antigens mediate protection in animal models. Several examples are discussed below.

One well-characterized, antigenic determinant that elicits AMI is the capsular polysaccharide (glucuronoxylomannan/GXM) of *C. neoformans* (Maitta *et al.*, 2004a; Datta *et al.*, 2008). A conjugate vaccine consisting of tetanus toxoid and GXM was protective against *C. neoformans* challenge in mice (Devi, 1996). Given that this GXM conjugate elicited antibodies with both protective and non-protective functions (Mukherjee *et al.*, 1995) and that capsular polysaccharides are often poorly immunogenic even when conjugated to protein carriers, peptide mimotopes of GXM that focus the antibody response on determinant(s) that elicit protective antibodies were investigated as a vaccine target for *C. neoformans* (see review in Pirofski, 2001). Indeed, a peptide mimotope-conjugate vaccine (tetanus/diphtheria toxoid conjugated to a peptide epitope mimicking a GXM determinant recognized by a protective human GXM monoclonal antibody) elicited GXM-reactive antibodies that were protective against cryptococcal challenge in mice (Maitta *et al.*, 2004b; Datta *et al.*, 2008). However, not all GXM mimotopes elicit a protective antibody response (Valadon *et al.*, 1996). Like GXM, *Candida* cell wall components β-(1,2)-mannobiose [$\beta(\text{Man})_2$] and -triose [β-$(\text{Man})_3$] are poorly immunogenic by themselves; however, when conjugated to certain synthetic T-cell epitope peptides expressed during human candidiasis, β-mannotriose elicited complement-fixing antibodies that protect against disseminated and vulvovaginal candidiasis in mouse models (Xin *et al.*, 2008). Interestingly, different antigens from the same fungus can elicit different types of immunity. For example, for the dimorphic fungus *Sporothrix schenckii*, the yeast form and its exoantigens induce a T_h1/T_h17 response that is protective in mice, whereas cell wall derived-*S. schenckii* glycoprotein antigens elicit antibodies that protect against

S. schenckii and *Sporothrix brasiliensis* strains by enhancing effector cell fungal opsonization, induction of IL-12 and IFNγ, and inhibition of yeast adhesion to fibroblasts (Portuondo *et al.*, 2016).

In the context of AMI-eliciting antigens, an important example is a vaccine candidate based on a recombinant secretory aspartyl proteinase (Sap2) of *C. albicans*. This vaccine, designed primarily for therapeutic use in recurrent vulvovaginal candidiasis, exhibited a strong and encouraging outcome in a Phase 1 clinical trial (De Bernardis *et al.*, 2015). The Sap2 enzyme, despite its adhesin-like functions and ability to digest host immunity-associated proteins, has minimal immunogenicity in *Candida*-colonized subjects, and the vaccine (from Pevion Biotech, Switzerland), delivered via virosomes (viral envelopes carrying the vaccine antigen) appears to confer protection by driving the generation of adequate levels of Sap-neutralizing antibodies in both mouse models and humans (De Bernardis *et al.*, 1997, 1999, 2015; Sandini *et al.*, 2011).

With respect to vaccine antigens that elicit cross-reactive antibodies, *C. neoformans* melanin elicits antibodies that prevent growth of phylogenetically distant melanin-bearing fungi, including the yeast, *C. neoformans* and the mold *Fonsecaea pedrosoi* (Rosas *et al.*, 2001; Alviano *et al.*, 2004), and may also protect against *Paracoccidioides brasiliensis* and other melanogenic fungi (Casadevall and Pirofski, 2007b). Other determinants common between *C. neoformans* and *F. pedrosoi* are glucosylceramides, which are glycosylated sphingolipids that are part of the fungal plasma membrane and cell wall. These determinants elicit antifungal antibodies that inhibit the growth of fungi in vitro and protect mice against lethal challenge models (Nimrichter *et al.*, 2004; Rodrigues *et al.*, 2007). Surface mannans of *C. albicans* or the capsular galactoglucoxylomannan of *Cryptococcus laurentii* elicit cross-reacting antibodies capable of inhibiting growth of a variety of *Candida* and cryptococcal species (Machova *et al.*, 2015). The exopolysaccharide of the fungus *Curvularia brachyspora* (chemically, mostly galactomannan) elicits antibodies that cross react with glucogalactomannan and β(1,3)-glucan exopolysaccharides from, respectively, *Aspergillus terreus* and *Paecilomyces variotii* (Menolli *et al.*, 2014). A well-known example of a cross-reactive fungal determinant is the cell wall component laminarin/β(1,3)-glucan, which is common to the phylogenetically disparate fungi *Candida*, *Aspergillus*, and *C. neoformans*. A glycoconjugate (laminarin conjugated to diphtheria toxoid) vaccine elicits protective antibodies in murine models against lethal challenge by all three fungi (Torosantucci *et al.*, 2005, 2009; Rachini *et al.*, 2007).

The two fungal vaccines currently most advanced in the clinical pipeline are both for candidiasis. In addition to the aforementioned Sap2-based vaccine, another candidate vaccine is based on one of two *C. albicans* adhesin proteins from the same gene family (*ALS*), namely ALS1p and ALS3p, which are involved in recurrent vulvovaginal candidiasis, biofilm formation, and fluconazole resistance (Roudbarmohammadi *et al.*, 2016). Recombinant antigens prepared from the N-termini of both ALS1p (rALS1p-N) and ALS3p (rALS3p-N) protected immunocompetent and immune-suppressed mice from disseminated and oropharyngeal candidiasis with *C. albicans* and non-*albicans* species (Ibrahim *et al.*, 2006; Spellberg *et al.*, 2006) rALS3p-N, which induces similar CMI, but broader AMI than its ALS1 counterpart, is the moiety included in a vaccine (from NovaDigm Therapeutics, United States) that is currently in Phase 1 clinical trials with encouraging results (Spellberg *et al.*, 2008a; Baquir *et al.*, 2010; Schmidt *et al.*, 2012). Interestingly, although the rALS3p-N-based vaccine-mediated protection via T_h1/T_h17 CMI in mice, serum antibody titers predicted vaccine efficacy against *Candida* (Spellberg *et al.*, 2008a; Lin *et al.*, 2009). Notably, this vaccine also protected mice against systemic infection with various, including methicillin-resistant strains of *Staphylococcus aureus* entirely via CMI without B-cell help (Spellberg *et al.*, 2008b; Lin *et al.*, 2009). In an interesting contrast, another recombinant vaccine for *Candida* being developed by NovaDigm is completely dependent on AMI; the vaccine antigen is the N-terminal region of another GPI-anchored cell surface protein, Hyr1 that is expressed on *C. albicans* hyphae and responsible for the yeast's resistance to phagocytic killing. Antibodies elicited against Hyr1 directly neutralize this function rendering the yeast susceptible to killing by neutrophils and tissue phagocytes and the rHyr1-N vaccine as well as passively administered anti-rHyr1 antibody protect immunocompetent and immune-suppressed mice against disseminated candidiasis with *C. albicans* and non-*albicans* species (Luo *et al.*, 2011).

The concept that AMI and CMI are distinct arms of immunity that pertain to certain microbes, for example, AMI mediates protection against extracellular microbes and CMI mediates protection against intracellular microbes, has been challenged by the discovery of antibody functions that augment and modulate cellular immunity (Casadevall and Pirofski, 2011). Nonetheless, certain vaccine antigens are currently understood to work primarily through adaptive CMI, especially $CD4^+$ and $CD8^+$ T-cells that provide direct protection and immunological memory. For example, immunization with a peptide (P10) derived from gp43, a major antigen of the dimorphic fungus *P. brasiliensis*, reduced organ fungal burden in a murine *P. brasiliensis* respiratory infection model alone and synergistically with various antifungal drugs. In this model, protection correlated with higher levels of the pro-inflammatory T_h1 cytokines IL-12 and IFNγ, and lower levels of the anti-inflammatory T_h2 cytokines IL-4 and IL-10 (Marques *et al.*, 2006). Similar T_h1-mediated protection was afforded by *P. brasiliensis* heat shock protein (Hsp)-60 (de Bastos Ascenço Soares *et al.*, 2008). Cryptococcal mannoproteins (terminally mannosylated glycoproteins) are recognized by mannose-binding molecular pattern receptors on DCs, which in turn induce in vitro antigen-specific proliferation of $CD4^+$ and $CD8^+$ T-cells obtained from both HIV+ and HIV– individuals (see Levitz *et al.*, 2015). T_h1-immunity, mediated by IFNγ, was also responsible for protection against lethal *Coccidioides posadasii* respiratory challenge in mice immunized with recombinant coccidioidal proteins, the cell wall component Ag2/PRA, and secreted protein CSA (either alone, or together as a bivalent unit) (Shubitz *et al.*, 2006), or a synthetic T-cell reactive peptide derived from *C. posadasii* cell wall that is homologous to fungal aspartyl proteases (Tarcha *et al.*, 2006). A variety of studies with *A. fumigatus* proteins have shown the importance of T_h1-immunity in aspergillosis. Immunization with the recombinant hyphal protein Aspf3 protected immune-suppressed mice from pulmonary aspergillosis in a $CD4^+$ T-cell dependent manner (Diaz-Arevalo *et al.*, 2011). Additionally, Aspf3 (also known as Pmp20), Crf1 (a cell wall glucanase), and Gel1 (a 1,3-β-glucanosyl transferase) induced a strong T_h1 response and proliferation in both healthy individuals and patients with

invasive aspergillosis (IA), and T_h1 cells recognizing all three *A. fumigatus* proteins were cross-reactive with other *Aspergillus* species as well as with *C. albicans* and other filamentous molds, zygomycetes and sordariomycetes, with release of IFNγ and IL-17 (Stuehler *et al.*, 2015). Crf1-induced $CD4^+$ memory T_h1 cells that are protective against *A. fumigatus* also protected mice against lethal *C. albicans* challenge (Stuehler *et al.*, 2011).

Studies with antigens and allergens from *A. fumigatus* show that fungal antigenic determinants can induce pro-inflammatory T_h17 and anti-inflammatory T_{reg} cells along with or independent of T_h1/T_h2 responses. Immunization with membrane-associated GPI-anchored proteins, such as Crf1, Gel1, and another aspartyl protease, Pep1, induced specific antibody production and activated T_h1/T_{reg} cells along with T_h17, while inhibiting T_h2 cells, and this correlated with protection of immune-suppressed mice from an *A. fumigatus* challenge (Bozza *et al.*, 2009). The role of T_h17 cells, and the T_h17-secreted cytokines, IL-17 and IL-22, in antifungal immunity has been controversial: T_h17/IL-17-mediated inflammatory mechanisms are protective in oropharyngeal candidiasis, murine PCP, pulmonary histoplasmosis and aspergillosis; whereas, on the other hand, mucosal *C. albicans* and *A. fumigatus* infection recruits T_h17 cells that cause exuberant inflammation and tissue damage (see Wuthrich *et al.*, 2011a). It is likely that T_h17/IL-17 function in the course of fungal disease is modulated by several factors, such as the disease state, site of infection, inoculum, and the degree of proliferation of T_h17 cells and timing/locus of IL-17 elicitation. Vaccines might be able to focus the T_h17/IL-17-mediated inflammation to mediate protection. For example, subcutaneous immunization with live (*H. capsulatum*) or live-attenuated strains (*Blastomyces dermatitidis*, *C. posadasii*) promotes differentiation of naïve T-cells into T_h17 cells that produce IL-17, IL-22, and IFNγ and recruit PMNs and phagocytes into lungs, which protects mice from lethal pulmonary challenge with these fungi independently of T_h1 cells (Wuthrich *et al.*, 2011a). IL-17A was an obligate requirement for successful vaccine immunity in these models (Wuthrich *et al.*, 2011a). A subcutaneous canine vaccine containing the same live-attenuated *B. dermatitidis* strain as above was found to elicit IFNγ, TNFα, and granulocyte macrophage-colony stimulating factor (GM-CSF) from peripheral lymphocytes in response to stimulation with *Blastomyces* cell wall antigens, and this response was found to be independent of any pre-immunization exposure to *B. dermatitidis* (Wuthrich *et al.*, 2011). Similarly, Pep1-vaccine-induced protection against pulmonary *A. fumigatus* was mediated by IFNγ, IL-17A, and IL-10, in addition to $CD4^+$ and $CD8^+$ T-cells (*albeit* via different activation mechanisms). However, Pep1 was still protective in the absence of IL-17A in the setting of chronic granulomatous disease (De Luca *et al.*, 2012). Similar protection was also observed with Crf1 and Gel1. These studies highlight the idea that an understanding of the cellular and molecular correlates of the immune (and inflammatory) response to fungi makes rational vaccine design eminently possible.

In the setting of compromised immunity (especially neutropenia) natural and antigen-induced $Foxp3^+$ T_{reg} cells produce high levels of IL-10 and TGFβ and induce a protective tolerance to *A. fumigatus* that is protective and counters the chronic immunopathology observed in experimental models, patients with IA and allergic bronchopulmonary aspergillosis (ABPA) (see Bedke *et al.*, 2014), and *Pneumocystis carinii* in experimental *Pneumocystis* pneumonia (PCP) (Hori *et al.*, 2002). Notably, T_{reg}-mediated immunosuppression via IL-10 and TGFβ has been implicated in fungal persistence, but may contribute to immunological memory (see Romani and Puccetti, 2006). Tr1 cells are presumed to protect from T_h1/T_h17-mediated inflammatory processes, including autoimmunity (Bedke *et al.*, 2014). Along these lines, immunization with a peptide (p41), an immunodominant epitope within Crf1, and zymosan used as an adjuvant, induced Tr1, or $CD4^+Foxp3^-$ T_{reg} cells, that produce high IL-10 relative to IFNγ but no IL-4. In murine IA models, Crf1/p41-induced Tr1 cells reduced inflammatory infiltrates and neutrophils in the lungs with no concomitant effect on lung fungal burden and suppressed overall expression of T_h1/T_h17-related transcription factors in thoracic lymph node T-cells. However, CpG ODNs, when used as adjuvants in parallel experiments showed the opposite effects, namely, induction of $Foxp3^+$ T_{reg} cells, an increase in IFNγ levels, and expression of T_h1/T_h17-related transcripts (Bedke *et al.*, 2014). These observations indicate that the choice of adjuvant and vaccine antigen influence the balance between fungal elimination, inflammation, and damage control in the setting of high or low degrees of inflammation.

Specific challenges to fungal vaccine development

Fungal organisms can cause a broad spectrum of diseases ranging from self-limited cutaneous lesions to acute and chronic pulmonary disease to disseminated and deadly systemic disease, but most fungi do not cause disease in individuals with intact immunity. Therefore, it was not until the medical advances, introduction of cytotoxic therapies and regular use of intravascular catheters, and broad-spectrum antibiotic development that began in the 1950s and were followed by the HIV/AIDS pandemic in the late 1970s, that fungal diseases assumed medical relevance. However, even with the subsequent unprecedented expansion of populations of people with various degrees of immune suppression that render them vulnerable to fungal diseases with high morbidity and mortality, invasive mycoses do not garner the degree of attention by clinicians, scientists and policymakers as do bacterial and viral diseases. This under-appreciation is a likely reason why the development of vaccines for fungal diseases has lagged behind those for other microbes (Spellberg, 2011) and why to date only two vaccines for one fungal disease have advanced to clinical trials. This is worrisome, especially because fungal infection is very common and the risk of disease is high if the host immunity falters. For instance, the Mississippi and Ohio River valley in the United States is endemic for the dimorphic fungus, *H. capsulatum* (Nett *et al.*, 2015) with high mycelial concentration in the regional soil. Another area of environmental risk is the Pacific Northwest of Canada and the United States, where a low level outbreak of cryptococcosis due to *C. gattii* has been emergent since 1999 (Datta *et al.*, 2009a,b). Therefore, fungal vaccines are a still unmet, but crucial clinical need that is likely to increase with the confluence of possible shifts/increases in fungal habitats related to global warming, growth of solid organ transplantation, and continued development of therapies for malignancy and inflammatory diseases that impair host immunity.

Since vaccine-mediated immunity (AMI and CMI) depends upon the host's immunological status, a common concern for all vaccines is their efficacy in the setting of impaired immunity. Although this concern has been allayed for certain antifungal vaccines (Spellberg, 2011; Nanjappa *et al.*, 2012), and rational vaccine design can lead to the configuration of vaccines and developments of adjuvants that skew the response in the most advantageous way for a given patient. In this regard, the emerging field of precision medicine provides hope that vaccine/adjuvant combinations that will optimize the type of immune response that will benefit (an individual) patient can be identified. Other challenges to vaccine design, such as poor immunogenicity or immunosuppressive properties of certain fungal antigens, such as GXM (O'Meara and Alspaugh, 2012) can likely be overcome by the use of adjuvants that counteract the undesired properties of an antigen, and/or discovery of novel antigens with more beneficial effects. Another important challenge to fungal vaccine development is that many fungal diseases arise from reactivation of a latent focus. In this regard, fungal vaccines that are designed for high risk patients could need to exert a direct therapeutic effect and/or be targeted to processes that mediate dissemination. Finally, there are important regulatory and fiscal barriers to fungal vaccine development (see Spellberg, 2011; Hamad, 2012; Moriyama *et al.*, 2014).

Passive Immunotherapeutic Modalities

Passive immunotherapy is a modality, whereby preformed products of the immune response are exogenously administered to the host. This is in contrast to the aforementioned active immunotherapeutic modalities (such as vaccines) where the administered product is given to induce a host immune response. Passive immunotherapy is usually focused on the microbe and can be effective largely independently of the host immune status. Therefore, this modality can be rapidly deployed to provide immediate immunity, and is generally suitable for use in immunocompromised patients. Passive immunotherapy leverages a number of pathogen-specific cellular or humoral effector mechanisms, including poly- or monoclonal antibodies (mAbs) with fungistatic or fungicidal functions, administration of chemical messengers (cytokines/chemokines) associated with innate and adaptive responses, adoptive transfer of antigen/pathogen-primed immune cells from a donor to a recipient, and antimicrobial peptides (AMPs) directly acting on the microbe.

Passive antibody therapy for fungal diseases

Passive administration of preformed antibody is a powerful anti-infective strategy with roots in late 19th century that was validated as the first form of antimicrobial therapy in the first quarter of the 20th century. Antibodies, with their signature ability to recognize a specific cognate antigen, mediate antimicrobial activity through a variety of mechanisms, including: promotion of phagocytosis via opsonization, activation of complement, neutralization of viruses and toxins, sequestration of soluble antigens, modulation of inflammatory processes, direct ADCC, and impact on microbial metabolism (reviewed in Casadevall and Pirofski, 2011). With the advent of antibiotics in early 1940s, steep logistical and biological challenges impacted the feasibility of passive antibody therapy ("serum therapy"), which led to the virtual abandonment of this modality, except for niche conditions such as treatment of toxins produced by tetanus, *Clostridium botulinum*, snake/scorpion venom, and certain viral diseases. However, introduction of hybridoma technology and mAbs in the 1970s, as well as currently available tools based on them, have led to the ability to overcome the difficulties of serum therapy. Although, to date, interest in passive antibody therapy for infectious diseases has not risen to pre-antibiotic era levels, mAbs are widely used in oncology and rheumatology, and a number of agents are in advanced trials for infectious diseases.

Immunotherapy with mAbs

Protective mAbs against fungal antigens

Fungal disease pathogenesis elicits antibody responses to fungal surface and intracellular antigens, such as polysaccharides, proteins, pigments, and glycoproteins/glycolipids (Casadevall and Pirofski, 2007b), but the protective role of such antibodies has been debated. Experimental and clinical observations prior to the 1990s suffered from certain methodological limitations that likely led to inconsistent results concerning the ability of antibodies to protect against disease with *C. neoformans* and *C. albicans*, the yeasts most extensively studied (see Casadevall, 1995). However, subsequent experimental studies, many of which were performed with mAbs produced via hybridoma technology, provided strong evidence for that AMI can mediate protection against fungi. Such studies corroborated the clinical observation that certain invasive fungal infections occur in patients with primary antibody deficiencies, such as common variable immunodeficiency/CVID, X-linked agammaglobulinemia/XLA, and Good syndrome (Macura *et al.*, 2003; Lucas *et al.*, 2010; Marr *et al.*, 2012; Malphettes *et al.*, 2015). Although, historically, direct associations between antibody deficiency and invasive fungal disease were largely lacking, more recent studies have linked antibody repertoire defects to the pathogenesis of certain fungal diseases (Hori *et al.*, 2002; Rohatgi and Pirofski, 2015). In this regard, the generation of mAbs made it possible to interrogate the response to a fungus and identify specific antibodies with the ability to alter the host response to fungi to benefit the host. Generation and description of protective mAbs against *C. neoformans* (Dromer *et al.*, 1987) and murine *P. carinii* (Gigliotti and Hughes, 1988) in the late 1980s, and subsequently against a number of medically important fungi (including *A. fumigatus*, *C. albicans*, *H. capsulatum*, and *P. brasiliensis*) (see Casadevall and Pirofski, 2012) provided strong evidence to support the feasibility of using mAbs as agents of immunotherapy.

A discussion of antifungal mAbs is incomplete without consideration of antibody functional efficacy and structure–function relationships. mAb-based studies revealed that the same B-cell may produce antibodies to the same antigen, but with disparate

Table 1 Protective antibodies to fungal determinants

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
Aspergillus species	β-1,3 Glucan (cell wall)	Vaccine antigen with diphtheria toxoid	Protected mice against lethal challenge with *Aspergillus fumigatus* conidia	In vivo; also, immune serum inhibited conidial growth in vitro	Torosantucci *et al.* (2005)
	Pep1p protease; GPI-anchored Gel1p and Crf1p; α- and β-1,3-glucans (cell wall)	Vaccine antigen	Increased survival, reduced fungal burden in lung and brain; reduced inflammatory cell recruitment in lung	In vivo; vaccine antigens delivered with CpG or via DC pulsing conferred T_H1-dependent protection, with T_{reg} and/or T_H17 activation	Bozza *et al.* (2009) and Stuehler *et al.* (2011)
	Asp *f3* allergen (peroxisome)	Vaccine antigen	Elicits protective IgG2a response against invasive aspergillosis; induces CMI which clears fungal elements from lungs	In vivo; corticosteroid-immunosuppressed mouse model of pulmonary aspergillosis	Ito *et al.* (2006)
	Asp *f16* allergen	Vaccine antigen with CpG ODN adjuvant	Increased survival; reduced lung fungal burden; better lung pathology index; strong DTH response	In vivo; murine pulmonary aspergillosis model; protection mediated by T_H1 responses with increased IFN-γ and reduced IL-4 and IL-10; vaccine induces DC maturation and activation, and stimulates innate CMI	Bozza *et al.* (2002)
	Heat-killed *Saccharomyces cerevisiae*	Vaccine antigen with alum adjuvant	Antigen promotes T-cell proliferation and production of IFNγ, IL-6 and IL-17A, and induces antibodies to fungal glucan and mannan	Heterologous protection against various yeast and molds	Un-published data summary in Stevens *et al.* (2011)
	100 KDa cell wall glycoproteins	mAb (IgG1, "A9")	High affinity binding to *A. fumigatus* hyphal cell wall peptides; hyphal growth inhibition	In vitro; also, protected mice against lethal systemic challenge and reduced fungal burden in vivo	Chaturvedi *et al.* (2005)
Blastomyces dermatidis	Attenuated (BAD-1 adhesin knockout) mutant strain; cell wall/membrane antigen derived from vaccine strain	Vaccine antigen	Prolonged survival in murine pulmonary and systemic infection models, lowered fungal burden (even with non-isogenic strains), and enhanced DTH and type 1 cytokine responses	In vivo; protection mediated by strong vaccine-induced $CD4^+$ T-cell responses, with IFN-γ; protection adoptively transferable via lymphoid cells; requires presence of adapter protein Card9, which leads to T_H17 cell development	Wuthrich *et al.* (2000) and Wang *et al.* (2014)
			Safe, tolerable, and immunogenic in dogs	In vivo; elicits T-cell response marked by IFNγ and GM-CSF	Wuthrich *et al.* (2011)
Candida albicans	β-1,3 Glucan (cell wall)	Vaccine antigen with diphtheria toxoid or MF59 adjuvant	Protected mice against both systemic and mucosal (vaginal) candidiasis	In vivo; protection also via passively transferred immune serum, immune vaginal fluid, polyclonal antibody to β-glucan	Torosantucci *et al.* (2009) and Pietrella *et al.* (2010)
	Candida mannans (cell wall)	Vaccine antigen complexed with liposomes	Prolonged survival, reduced organ fungal burden in systemic model, and accelerates clearance of fungus from vaginal tissue	In vivo; murine systemic infection model, using both normal and SCID mice, and intravaginal infection model; protection mediated by AMI	Han and Cutler (1995) and Han *et al.* (1998)
	Secretory aspartyl proteinase (Sap2) native or recombinant (rSap2t)	Vaccine antigen in "CT" adjuvant	Protected against experimental vaginal candidiasis, accelerated fungal clearance from the vagina	In vivo; rat intravaginal model; vaccination elicited Sap2-specific antibodies; protection also depends on T-cell response	De Bernardis *et al.* (1997) and Sandini *et al.* (2011)
			Improved survival in mice		Spellberg *et al.* (2008a)

Table 1 Continued

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
	Agglutinin like sequence adhesins (Als1p/Als3p/rAls3p-N)	Vaccine antigens in alum adjuvant		In vivo; murine systemic model; adjuvant necessary for protection; protection mediated by strong vaccine-induced T-cell responses	
			high IgG and IgA titers	Phase 1 dose escalation study volunteers	Moriyama *et al.* (2014)
	Recombinant Hyr1p (cell wall)	Vaccine antigen	Improved survival in mice, reduced lung fungal burden. Also, active against non-*albicans Candida* species	In vivo; murine systemic immune competent or –compromised model; protection mediated by neutralization of virulence function of Hyr1p (which hinders phagocytosis) and enhancement of PMN function	Luo *et al.* (2011)
	Fructose bisphosphate aldolase (cell wall)	Vaccine antigen	Prolonged survival, reduced organ fungal burden	In vivo; murine systemic infection model; DC-based immunization; peptide not restricted by MHC-II; protection via AMI; recognized by human salivary IgA	Xin and Cutler (2011) and Calcedo *et al.* (2012)
	Live-attenuated *C. albicans* tet-NRG1 strain	Vaccine antigen	Improved survival in healthy and B-cell deficient, but not T-cell deficient, mice	In vivo; murine systemic infection model	Saville *et al.* (2009)
	C. albicans whole sonicate (cell lysate)	Vaccine antigen to prepare IgY antibody in chicken egg yolk	Reduced fungal adherence to human pharynx carcinoma cell line FaDu	In vitro; also, in vivo, decreased fungal burden, and limited dissemination of oral candidiasis in mice	Ibrahim el *et al.* (2008)
	Sap2 native or recombinant (rSap2t)	mAb (IgG1, "GF1"; IgM)	Passive transfer of anti-rSap2t mAbs reduced vaginal fungal burden	In vivo; rat intravaginal model	De Bernardis *et al.* (1997) and Sandini *et al.* (2011)
		Genetically engineered V_H and V_L domain antibodies	Inhibited *C. albicans* adherence to rat vaginal epithelial cells and accelerated fungal clearance	In vitro and in vivo; effect seen with both fluconazole-susceptible and fluconazole-resistant *C. albicans*	De Bernardis *et al.* (2007)
	β-1,3 Glucan (cell wall)	mAb (IgG2b, "2G8")	mAb Binding showed direct antifungal, growth-inhibiting action against *C. albicans*; protected mice against vaginal candidiasis	In vitro and in vivo	Torosantucci *et al.* (2009) and Pietrella *et al.* (2010)
	Cell wall mannoproteins	mAb (IgM, "C7"); mAb against MP58	Protected mice against invasive candidiasis	In vivo; C7 binds *C. albicans* cell wall mannoprotein, and als3	De Bernardis *et al.* (1997), Sevilla *et al.* (2006), Viudes *et al.* (2004), and Brena *et al.* (2007)
	β-1,2-Linked mannotriose (cell wall)	mAb (IgM; B6.1)	Prolonged survival, reduced organ fungal burden in systemic model, and accelerates clearance of fungus from vaginal tissue	In vivo; murine systemic infection model, using both normal and SCID mice, and intravaginal infection model	Han and Cutler (1995) and Han *et al.* (1998)
	HSP90 (cell surface)	mAbs	Prolonged survival after challenge with fluconazole-sensitive or –resistant *C. albicans*, improved renal clearance	In vivo; murine acute and chronic models of invasive candidiasis; protection associated with neutralization of protein	Matthews *et al.* (1995)

(*Continued*)

Table 1 Continued

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
				binding properties of HSP90	
		Human recombinant mAb ("mycograb")	Active against a wide range of *Candida* species; synergy with Amphotericin B; reduced organ fungal burden in murine candidiasis	Preclinical assessment	Matthews *et al.* (2003)
	Fructose Bisphosphate aldolase	mAb (IgM, "E2–9")	Prolonged survival, reduced organ fungal burden	In vivo; murine systemic infection model	Xin and Cutler (2011)
Candida dubliniensis	Mannan	Vaccine antigen conjugated to human serum albumin	Engaged both AMI and CMI; Increased anti-mannan IgM, IgG and IgA levels, induced $CD4^+$ T-cells and T_H1 cytokines, activated $CD25^+$ B-cells	In vivo, in immunized rabbit	Paulovicova *et al.* (2007)
Cryptococcus neoformans	GXM (cell surface)	Vaccine antigen conjugated to tetanus- or diphtheria-toxoid	Promoted survival, reduced organ cryptococcal burden	In vivo; murine systemic infection model; protection mediated via AMI	Devi (1996), also see Zaragoza *et al.* (2009)
	P13 Peptide mimotope of GXM	Vaccine antigen conjugated to tetanus- or diphtheria-toxoid	Prolonged survival, reduced serum GXM levels	In vivo; murine acute and chronic systemic infection model; protection mediated via AMI, T_H1 cell responses, and vaccine-induced immunomodulation	Datta *et al.* (2008)
	CneF (culture filtrate antigen complex); cell surface mannoprotein purified from CneF	Vaccine antigens	Promoted survival, decreased organ fungal burden, reduced organ inflammatory damage	In vivo; murine systemic infection model; T-cell dependent protection mediated via TNF-α, IFN-γ, and IL-2; mannosylation of peptide antigen is critical for optimal T-cell responses	Specht *et al.* (2007) and Mansour *et al.* (2004)
	β-1,3 Glucan (cell wall)	mAb (IgG2b, "2G8")	Reduced brain and liver fungal burden in both immune competent and neutropice mice	In vivo; murine systemic cryptococcal infection model; also, in vitro, binds to cryptococcal cell wall, inhibits growth, reduces capsular thickness	Rachini *et al.* (2007)
	GXM	mAb	Prolonged survival and decreased fungal burden in *C. neoformans*-infected mice	In vitro and in vivo; well-documented beneficial effects in murine systemic and pulmonary infection models	Maitta *et al.* (2004a), Dromer *et al.* (1987), Sanford *et al.* (1990), Mukherjee *et al.* (1992), Fleuridor *et al.* (1998), and Beenhouwer *et al.* (2007)
	Glucosyl-ceramide (cell wall glycolipid)	Polyclonal Ab (IgG1) purified from patient sera	Inhibited cell budding and cell growth in a complement-independent manner	In vitro; effect demonstrable in both acapsular and encapsulated cryptococcal strains	Rodrigues *et al.* (2000)
	Melanin (cytoplasm)	mAb ("11B11," "6D2")	Promoted survival, reduced organ cryptococcal burden	In vivo; murine systemic infection model; also, in vitro, mAb reduces the growth rate of melanized *C. neoformans* cells	Rosas *et al.* (2001)
Cryptococcus gattii	Cell wall/cytoplasm associated proteins	Vaccine antigen	Promoted survival, reduced lung cryptococcal burden,	In vivo; murine pulmonary infection model; protection via increased pro-	Chaturvedi *et al.* (2014)

Table 1 Continued

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
			increased *C. gattii*-specific antibody production	inflammatory T_H1 cytokine recall responses	
Coccidioides posadasii	Pmp1 (peroxisome), recombinant	Vaccine antigen with lipid-based adjuvant	Reduced organ fungal burden, prolonged survival	In vivo; murine systemic and pulmonary infection models; also, antigen reacted with sera of patients of coccidiodomycosis	Orsborn *et al.* (2006)
	Pep1 aspartyl protease (cell wall and secreted)	Vaccine antigen with CpG ODN adjuvant	Promoted survival and reduced fungal burden	In vivo; T-cell reactive antigen; murine pulmonary coccidioidomycosis model	Tarcha *et al.* (2006)
	Ag2/Pra + recombinant PrP2 (both Proline-rich cell wall proteins with distinct T-cell epitopes)	Vaccine antigens in adjuvant	Improved survival, reduced lung fungal burden	In vivo; murine pulmonary infection model; combination of antigens provided better protection than single antigens	Herr *et al.* (2007)
	Heat-killed or live *S. cerevisiae*	Vaccine antigen delivered subcutaneously (HKSc) or orally (Live Sc)	Promoted survival and reduced organ fungal burden in murine *C. posadasii* systemic infection model	In vivo; antigen promotes T-cell proliferation and production of IFNγ, IL-6 and IL-17A, and induces antibodies to fungal glucan and mannan, which may account for heterologous protection	Liu *et al.* (2011) and Capilla *et al.* (2009)
Coccidioides immitis	Attenuated live mutant strain	Vaccine antigen in EP67 adjuvant	Increased survival, reduced inflammatory pathology, lowered fungal burden in lung	In vivo; murine pulmonary infection model; protection mediated by initial PMN migration; EP67 adjuvant (C5a analog) helps down-regulate later PMN presence in lungs and promotes protection; requires T_H17	Hung *et al.* (2012) and Wuthrich *et al.* (2011)
	Polypeptides containing MHC-binding T cell epitopes	Vaccine antigen in CpG or Glucan particle adjuvant	Reduced in fungal burden; antigen in glucan particle was more efficient in promoting survival	In vivo; murine pulmonary infection model; protection mediated by $CD4^+$ and $CD8^+$ T-cells	Hurtgen *et al.* (2012)
Fonsecaea pedrosoi	Melanin (cytoplasm)	Vaccine antigen eliciting AMI	Purified antibodies against melanin inhibited fungal growth, increased phagocytosis and destruction of conidia	In vitro	Alviano *et al.* (2004)
	Glucosyl-ceramide (cell wall)	mAbs	Reduced fungal growth, enhanced phagocytosis of conidia by murine macrophages	In vitro; protection mediated via mAbs specific to *F. pedrosoi* glucosyl-ceramide antigens	Nimrichter *et al.* (2004)
Histoplasma capsulatum	Live yeast	Vaccine antigen	Reduced lung fungal burden and prolonged survival in immunocompromised mice	In vivo; CD4-depleted mouse pulmonary infection model; protection dependent upon MHC-I-restricted memory $CD8^+$ T-cells secreting TNF-α, IFN-γ, and GM-CSF (in absence of CD4)	Wuthrich *et al.* (2003)
	Sec31 protein, recombinant	Vaccine antigen	Promoted survival in both sublethal and lethal infections, and reduced lung and spleen fungal burdens	In vivo; murine pulmonary infection model; protection dependent upon presence of $V\beta4^+$ T-cells in the lungs	Scheckelhoff and Deepe (2006)
		Vaccine antigen			

(*Continued*)

Table 1 Continued

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
	HSP60 (cell surface), recombinant		Prolonged survival, limited fungal replication	In vivo; induces protective immune responses dependent on Vβ 8.1/8.2$^+$ CD4$^+$ T-cells; requires T_H17; in absence of CD4$^+$ T-cells, DCs stimulate CD8$^+$ T-cells for protection	Wuthrich *et al.* (2011), Gomez *et al.* (1995), and Lin *et al.* (2005)
		mAb (IgG1, IgG2a)	Prolonged survival, reduced fungal burden in *H. capsulatum*-infected mice	In vivo; increased IL-12 and TNF-α, decreased IL-4 and IL-10. Also, in vitro, reduced intracellular fungal survival and increased phagolysosomal fusion of macrophages	Guimaraes *et al.* (2009)
	Histone H2B-like protein (cell surface)	mAb	Protected mice against lethal pulmonary histoplasmosis	In vivo; protection mediated via enhancements in IL-4, IL-6, and IFN-γ in the lungs. Also, mAb alters intracellular fate of *H. capsulatum*	Nosanchuk *et al.* (2003)
Paracoccidioides brasiliensis	Recombinant yeast protein PB27 (cell surface and cytosolic)	Vaccine antigen	Prolonged survival in mouse systemic infection model, reduced organ fungal burden and lung granuloma	In vivo; protection mediated by high IgG2b and IgG1, as well as IFN-γ and TGF-β; enhancement of fluconazole efficacy	Fernandes *et al.* (2011)
	HSP60	Vaccine antigen	Reduced fungal burden and lung damage, protected mice against moderate and lethal pulmonary challenge	In vivo; T_H1-dependent protection, centered around IFN-γ	de Bastos Ascenço Soares *et al.* (2008)
	Heat-killed *S. cerevisiae* expressing recombinant gp43	Vaccine antigen	Reduced fungal growth and lung damage; enhanced IFNγ and IL-12 in lung and spleen	In vivo; T_H1-dependent protection; murine systemic infection model	Assis-Marques *et al.* (2015)
	P10 (peptide derived from gp43)	Vaccine antigen, as fusion peptide with FLiC or in complete/ incomplete Freund's adjuvant	Reduced fungal growth and lung damage; strong specific immune response even in immune compromised hosts; action synergistic with antimicrobial agents	T_H1-dependent protection (P10 has a CD4$^+$ T-cell epitope); murine pulmonary infection model (healthy or immune-suppressed via steroid)	Braga *et al.* (2009) and Munoz *et al.* (2014)
	Mycobacterium leprae HSP65	DNA vaccine	Reduced organ fungal burden and organ damage in murine systemic infection; functions as an immunomodulator	In vivo; protection mediated via priming strong T_H1 response and downregulating T_H2 cytokines	Ribeiro *et al.* (2010)
	gp43 (Secreted)	mAb 3E	Reduced fungal burden and lung inflammation; enhanced IFNγ levels in lungs	In vivo; murine pulmonary and systemic infection models; also, in vitro 3E enhanced phagocytosis of yeast cells	Buissa-Filho *et al.* (2008)
	gp70 (intracellular/ secreted)	mAb specific for gp70	Diminished lung granulomas in mice with *P. brasiliensis* infection	In vivo; mAb counteracts adverse, disease-enhancing effects of gp70	de Mattos Grosso *et al.* (2003)
Pneumocystis carinii (murine); *Pneumocystis jirovecii* (human)	Major cell surface glycoprotein (MSG)	Vaccine antigen	Promoted survival, and reduced lung fungal burden and injury in infected rats	In vivo; immunocompromised rat model of *Pneumocystis* pneumonia; vaccine elicited AMI	Theus *et al.* (1998)
	p55			In vivo; protection mediated by both CMI and AMI in	Feng *et al.* (2011)

Table 1 Continued

Fungus	*Determinant*	*Agent*	*Effect*	*Notes*	*Reference*
		DNA vaccine targeting protein p55	Reduced organism burdens; improved histological scores	immunosuppressed rat model	
	Cell surface Glycoprotein A (gpA)	mAb 2B5	In murine *P. carinii* pneumonia model, mAb selectively destroyed gpA variant-bearing *P. carinii* subpopulation by attacking trophozoites	In vivo; mAb specific for one variant of gpA of murine *P. carinii*	Gigliotti *et al.* (1996)
	Kex 1 (kexin-like protease); kex 1 homolog, recombinant antigen A12	mAb (IgM, "4F11"; IgG switch variant, "4F11G1")	Protected SCID mice against *Pneumocystis* pneumonia, reduced fungal burden	In vivo; mAbs bind to intracellular, membrane-bound antigens; 4F11G1 also recognized multiple epitopes on human *P. jirovecii* isolates	Gigliotti *et al.* (2002) and Wells *et al.* (2004, 2006)

Abbreviations: AMI, antibody-mediated immunity; CMI, Cell-mediated immunity; CSF, colony stimulating factor; DC, Dendritic cells; DTH, delayed type hypersensitivity; GM, granulocyte macrophage; GXM, Glucuronoxylomannan; HSP, heat shock protein; IFN, interferon; IL, interleukin; mAbs, monoclonal antibodies; MHCs, major histocompatibility complexes; ODN, oligodeoxynucleotides; PMNs, polymorphonuclear neutrophils; SCID, severe combined immunodeficiency.

epitope specificity and drastically different protective efficacy (Mukherjee *et al.*, 1995). Again, GXM-reactive murine mAbs of isotypes IgG1 and IgG3 derived from the same B-cell were, respectively, protective and non-protective/disease-enhancing following cryptococcal challenge in mouse models (Yuan *et al.*, 1995, 1997). Similar observations were made with human IgM mAbs prepared against GXM in transgenic mice, with different binding specificity, germline heavy-chain variable region gene usage, and protective efficacy (Maitta *et al.*, 2004a). Additionally, there is evidence that for fungal infections, the extent of antibody-mediated protection may vary depending on the isotype/subtype and titer of the fungus-specific antibody (Taborda *et al.*, 2003) and the genetic (major histocompatibility complex/MHC) background of the host (Rivera and Casadevall, 2005) among other factors. In aggregate, this body of evidence supports the concept that the presence of non-protective fungus-specific antibodies in polyclonal immune serum may reduce the degree of protection afforded by the protective antibodies therein. This might have been a driver of the initial inconsistencies observed in serum therapy. Indeed, monoclonal forms of such non-protective antibodies have now been described for *C. albicans*, *H. capsulatum*, and *C. neoformans* (Casadevall and Pirofski, 2007b). Any design and utilization of mAbs as immunotherapeutic agents for specific fungal infections must, therefore, consider that fungi can elicit antibodies that do and do not benefit the host in the setting of fungal infection. On the other hand, in the vast majority of normal individuals who rarely develop disease with most fungi, the aforementioned polyclonal antibody response might balance pro- and anti-inflammatory responses with the result being damage control. Therefore, a response that might limit damage in normal individuals might be insufficient in those with impaired immunity who are likely to have/develop a higher fungal burden and/or a breakdown in latency that leads to dissemination.

Studies to develop protective mAbs against fungi have often progressed in tandem with the search for vaccine antigens, including fungal cell wall components (e.g., glucan/mannan polysaccharides, glycoproteins, glycolipids), non-cell wall surface molecules (e.g., cryptococcal GXM, *Histoplasma* Hsp60), cytoplasmic/nuclear proteins (including histone-like proteins and those involved in signaling pathways) and other processes. For instance, the aforementioned vaccine studies on β-glucan yielded the cross-reactive mAb 2G8, which was protective against *C. neoformans*, *C. albicans*, and *A. fumigatus* (Torosantucci *et al.*, 2005, 2009; Rachini *et al.*, 2007). Further examples of protective mAbs against fungal antigens of interest to immunotherapy are summarized in **Table 1**.

Of note are two mAb-based therapeutic agents that advanced to clinical development. Efungumab (Mycograb) is a recombinant single-chain (scFv) anti-HSP90 antibody of human origin, which protected mice from *Candida tropicalis* (Matthews *et al.*, 2003; Nooney *et al.*, 2005; Hodgetts *et al.*, 2008) and potentiated the action of antifungals of various classes upon coadministration. Combined with Amphotericin B, caspofungin (an echinocandin), or fluconazole, Mycograb was more effective against eight clinical isolates of *C. neoformans* than the individual drug treatments alone, especially in the case of Amphotericin B, which additionally resolved *C. albicans*, *Candida krusei*, and *Candida glabrata* infections (Nooney *et al.*, 2005). Mycograb with caspofungin increased the caspofungin-susceptibility of *Candida* species (Matthews *et al.*, 2003; Nooney *et al.*, 2005; Hodgetts *et al.*, 2008), and was effective and well tolerated in fungal sepsis when combined with liposomal Amphotericin B or caspofungin (Sutherland and Ellis, 2008). In a clinical trial with patients with candidemia, Mycograb reduced *Candida*-attributable mortality and showed improved clearance of fungal cultures (Pachl *et al.*, 2006). Unfortunately, production difficulties and other issues forced the abandonment of this potentially promising therapy. The other agent is mAb 18B7, a mouse-derived IgG1 against *C. neoformans*, which was studied in a clinical trial and found to be safe and to reduce serum GXM when used at high doses (Larsen *et al.*, 2005). However, further clinical development of this promising mAb has been hampered by funding issues. These examples illustrate how nonscientific factors can negatively influence the development of therapeutic options.

Radioimmunotherapy

Developed initially for cancer treatment, radioimmunotherapy (RIT) for infectious diseases is an immunotherapeutic modality in which antibodies labeled with radionuclides deliver lethal irradiation to a target cell. The GXM-reactive IgG1 mAb 18B7 (mentioned above) and *Histoplasma* cell wall protein-reactive IgM mAb 9C7 labeled with β-emitter rhenium-188 or α-emitter bismuth-213 were protective against, respectively, experimental *C. neoformans* and *H. capsulatum* infections in a radiation dose-dependent manner (Dadachova and Casadevall, 2006, 2009). In experimental models of high-burden cryptococcal infections, the therapeutic effect of RIT was mediated via fungus-targeting mechanisms (e.g., direct killing or induction of fungal apoptosis), and reduction of fungal metabolic activity (Dadachova *et al.*, 2006; Bryan *et al.*, 2009) as well as host-focused alterations of the cytokine milieu (IL-2, IL-4, IL-10, TNFα, and IFNγ) (Dadachova *et al.*, 2006). Interestingly, in vitro exposure of macrophage-like J774.16 cells or epithelial-like CHO cells to radiolabeled (via antibody binding) *C. neoformans* resulted in minimal bystander loss of membrane integrity, viability and metabolic activity (Bryan *et al.*, 2013), thereby indicating the relative safety of this method. Successful application of *Aspergillus*-specific mAbs (JF5) for RIT of invasive pulmonary aspergillosis was recently reported (Thornton, 2014).

Immunomodulation via mAb

Although mAb-based mechanisms discussed so far are fungus-targeting via antibodies that neutralize and/or kill fungi (reviewed in Casadevall and Pirofski, 2012) certain antibodies, whether host-derived or exogenous, are able to variably modulate the host immune response to benefit the host. For example, mAb3B4, specific for a *C. albicans* germ tube surface antigen, modulated phagocytosis and bound keratin, enhancing B1 B cell compartment repopulation (Li *et al.*, 2007). Anti-CD40 mAbs, explored for their protective potential against systemic *C. neoformans* infection, correlated with an increase in IFNγ and TNFα production and upregulation of MHC-II expression on brain microglia (Zhou *et al.*, 2006). In a mouse model of fungal sepsis, mAb-mediated inhibition of negative costimulatory molecules, such as programmed cell death 1 (PD-1), its ligand (PD-L1), or T-lymphocyte antigen-4 (CTLA-4), enhanced survival of mice (Chang *et al.*, 2013b) mAbs capable of inducing anti-inflammatory mechanisms have shown promise in inflammatory disorders. In a murine PCP model, an anti-CD3 mAb reduced T-cell induced inflammatory damage to lung tissue and improved outcome via depletion of $CD4^+$ and $CD8^+$ T-cells (Bhagwat *et al.*, 2010), and the anti-IgE mAb (Omalizumab) successfully treated a cystic fibrosis patient with antibiotic- or prednisone-refractory ABPA (Kanu and Patel, 2008).

A most interesting example of mAb-mediated immunomodulation is afforded by *P. brasiliensis* (paracoccidioidomycosis) in which asymptomatic infection or mild disease is associated with T_h1-immunity, whereas severe disease involves suppression of delayed type hypersensitivity (DTH) responses and T_h2-immunity. In murine models of paracoccidioidomycosis, A/J are genetically resistant, able to induce mixed T_h1/T_h2 responses with IFNγ from protective $CD8^+$ T-cells, whereas B10.A mice are susceptible, due to $CD4^+CD25^+Foxp3^+$ T_{reg}-mediated induction of inhibitory $CD4^+$ T-cell anergy via IL-10, TGFβ, and indoleamine 2,3-dioxygenase/IDO. However, even in resistant A/J mice, T_{reg} cells inhibited localization of inflammatory T-cells to the site of infection (Felonato *et al.*, 2012). Therefore, an anti-CD25 IgG mAb, which depleted T_{reg} cells and impaired IDO expression, negated the T_{reg}-immunosuppressive functions, reduced organ fungal burden, and increased survival of infected B10.A mice (Felonato *et al.*, 2012). This example underscores the importance of molecular mechanism-based design of immunotherapeutic modalities that take the balance of inflammation and fungal clearance into account.

Specific challenges to development of mAb-based immunotherapies

The appeal of therapeutic mAbs for infectious diseases is substantial; they augment host immunity, do not induce antifungal resistance or directly affect the microbiome, and provide immediate therapeutic protection regardless of host immune status, thereby overcoming a major limitation of vaccines. Nevertheless, mAb-based therapy poses some unique challenges. In comparison to broadly active antimicrobial drugs, the enhanced specificity of mAbs requires that the identity of the causative microbe be known, and for efficacy, the antibody preparation must be specific to antigenic determinants available on the microbe. Therapeutic antibody is also best administered early in the course of disease. The possibilities of adverse reactions remain; for instance, anti-mAb host immune responses, leading to allergy, infusion-related reactions, compromised immunity, and gastrointestinal- and cardiovascular-related complications, potential toxicity, unexpected pharmacodynamics following administration (Bhatia *et al.*, 2009; Chen and Cleck, 2009; Hansel *et al.*, 2010), and occasional adverse reactions, as observed during Phase-I evaluation of mAb 18B7 (Larsen *et al.*, 2005). Increasingly, however, these concerns are allayed, as rapid diagnosis is now available via various technologies, and it is possible to leverage the mAb technology to produce high affinity antibodies to almost any determinant, while limiting toxicity. Safety profiles of mAb-based immunotherapies are excellent, and potential benefits, significant; therefore, the use of mAbs in treatment and prevention of infectious, including fungal, diseases is a worthwhile pursuit (see discussion in Casadevall and Pirofski, 2015).

AMPs

AMPs are evolutionarily conserved, short endogenous polypeptides that are naturally produced during the innate immune response. They are classified as possible immunotherapeutic agents, because their properties allow them to kill microbes at micromolar concentrations via cytotoxic abilities, including permeabilization and destabilization of the microbial plasma membrane, disruption of metabolic processes, activation of internal processes with lethal consequences, and damage to critical intracellular targets (Zasloff, 2002). The mammalian body secretes AMPs (e.g., defensins, cathelicidins, histone-derived peptide buforin II, etc.) from epithelial cells constitutively and in response to microbes to protect external and internal/luminal surfaces. In

addition, neutrophil-derived AMPs are powerful chemoattractants for CMI components and thereby act as sentinels for adaptive immunity (Zasloff, 2002). However, despite their potential for therapeutic applications (broad spectrum, rapid onset activity, and relatively low possibility of resistance emergence in microbes), development of AMPs as adjunct therapies has faltered mainly because of pharmacokinetics-associated delivery issues (natural AMPs are subject to proteolytic and pH-mediated damage) and significantly high cost of production (Seo *et al.*, 2012). Nonetheless, there are several synthetic and semisynthetic derivatives of natural AMPs under consideration for immunotherapy for fungi, especially as adjuncts to conventional antifungal agents.

AMPs that work through membrane-destabilizing mechanisms include salivary histatins, a family of small cationic histidine-rich AMPs secreted by human salivary glands endowed with plasma membrane-destabilizing/damaging properties by virtue of which they are active against various *Candida* species (*C. albicans, C. glabrata*, and *C. krusei*), *Saccharomyces cerevisiae*, *C. neoformans*, and *A. fumigatus* (Tsai and Bobek, 1998; Edgerton and Koshlukova, 2000). One of the most potent Histatins, salivary Histatin-5, induces loss of mitochondrial transmembrane potential in *C. albicans*, triggering free radical generation leading to cell death (Helmerhorst *et al.*, 2001) P-113, a Histatin-5 derivative, has potent broad-spectrum candidacidal activities (*C. albicans, C. glabrata, Candida parapsilosis*, and *C. tropicalis*) including against azole-resistant strains (Rothstein *et al.*, 2001). Human lactoferrin (hLF) 1-11 is a synthetic AMP, derived from lactoferrin (an innate immunity-associated, iron-binding glycoprotein), which targets *C. albicans* mitochondria and triggers extracellular synthesis and secretion of ATP, which in turn leads to membrane pore formation via surface ATP-binding sites (Lupetti *et al.*, 2000). hLF is active against fluconazole-resistant *C. albicans* and was safe and well tolerated in healthy and bone marrow transplant recipient volunteers (Lupetti *et al.*, 2003; Brouwer *et al.*, 2011). Other lactoferrin-derived synthetic peptides include Lfpep and kaliocin-1, which exhibit fungicidal activity against fluconazole- and amphotericin B-resistant *C. albicans* strains. Kaliocin-1, unlike Lfpep, works via a nonpore forming mechanism that involves an interaction with the microbial mitochondrial membrane (much like Histatin-5) (Viejo-Diaz *et al.*, 2005; Yount *et al.*, 2007). Palmitoyl-lys-ala-DAla-lys is a short synthetic lipopeptide AMP with detergent-like effects and membranolytic activities against *A. fumigatus* in vitro that prolonged survival in an immunocompromised mouse model of aspergillosis (Vallon-Eberhard *et al.*, 2008). Other AMPs include Pleurocidin (an α-helical cationic AMP secreted by the flatfish White Flounder) and its semisynthetic analogs, which has strong candidacidal antifungal activity via disruption of microbial plasma membrane (Jung *et al.*, 2007), and Trappin-2, a neutrophil-derived cationic serine protease inhibitor with strong antifungal activity against *A. fumigatus* and *C. albicans* via disruption of target cell membranes (Baranger *et al.*, 2008). Finally, synthetic peptides that are structurally based on the characterized bactericidal domain of CAP37 (a neutrophil-derived cationic AMP) exhibit significant fungicidal activity against fluconazole-sensitive and -resistant isolates of *C. albicans* blastoconidia, *Candida guilliermondii*, *C. tropicalis*, *Candida pseudotropicalis*, *C. parapsilosis*, and *Candida dubliniensis*. This activity is likely exerted via binding of these AMPs to the fungal membrane through a yet-unidentified receptor leading to membrane disruption (Pereira *et al.*, 2010). In the context of AMPs, special mention must be made of Echinocandins (caspofungin, micafungin, anidulafungin), which are semisynthetic lipopeptides already approved by Food and Drug Administration (FDA) for clinical use as antifungal agents with activity against *C. albicans* and *C. glabrata*, *P. carinii*, *Saccharomyces* and *Aspergillus* species, exerted via fungal cell wall destabilization and lysis due to inhibition of the synthesis of a crucial wall component, β(1,3)-D-glucan (Denning, 2003).

The membrane destabilization functions of AMPs depend on their amphipathic nature to facilitate their transmembrane translocation. Thus hydrophobicity is an important physicochemical characteristic that influences their function, and must be taken into consideration during the synthesis of these peptides. For instance, increasing the hydrophobicity of analogs of D-V13K (an α-helical AMP) conferred enhanced antifungal activity against ascomycetes (*Aspergillus nidulans*, *Scedosporium prolificans*, and *C. albicans*), but reduced the activity against zygomycetes (*Absidia corymbifera*, *Rhizopus*, and *Rhizomucor* species). Notably, this increased their general hemolytic effects (Jiang *et al.*, 2008), which is undesirable for clinical applications. On the other hand, for Pleurocidin analogs, reduction in hydrophobicity via polar amino acid substitutions decreased their hemolytic activity, while retaining the antifungal properties (Sung and Lee, 2008).

Some AMPs that work via other distinct mechanisms include the neuropeptide α-melanocyte stimulating hormone (α-MSH) and its synthetic analogs. These peptides are candidacidal via activation of excessive cyclic adenosine monophosphate (cAMP), thereby disrupting the cAMP-mediated signaling pathways essential for *Candida* gene expression. A synthetic analog $(CKPV)_2$ has excellent activity against the azole-resistant species *C. krusei* and *C. glabrata*, along with significant anti-inflammatory activity via inhibition of TNFα production from host neutrophils (Cutuli *et al.*, 2000; Gatti *et al.*, 2006; Capsoni *et al.*, 2007). Chicken albumen AMP Cystatin is a cysteine-protease inhibitor with fungal growth inhibition effects on azole-sensitive *C. albicans*, *C. parapsilosis*, and *C. tropicalis* comparable to fluconazole. This antifungal activity, distinct from its anti-protease function, is not antagonistic toward azole or polyene antifungals and not vulnerable to efflux-mediated azole-resistance mechanisms in *C. albicans* (Kolaczkowska *et al.*, 2010). Synthetic peptide PLD-118 (derivative of naturally occurring β-amino acid, cispentacin) inhibits fungal isoleucyl-tRNA synthetase, and thereby, protein synthesis, which led to cessation of growth and significant dose-dependent fungal clearance in an immunocompromised rabbit model of experimental oropharyngeal and esophageal candidiasis with fluconazole-resistant *C. albicans* clinical isolates (Petraitis *et al.*, 2004).

In addition to direct antifungal actions, some AMPs can also work by modulating host immunity. The hematopoiesis-regulating synthetic peptide SK&F 107647 expanded $CD11b^+$ monocytes and neutrophils in healthy animals, and significantly reduced organ fungal burden when used in combination with low doses of amphotericin B in persistently neutropenic rabbits with disseminated candidiasis (Lyman *et al.*, 1999). In a mouse model, administration of peptides recognized by T-cells, such as *A. fumigatus* ribotoxin (Aspf1), induced immunological tolerance to *Aspergillus* allergens responsible for ABPA via modulation of cytokine production and reduction in pro-inflammatory responses to *A. fumigatus* (Svirshchevskaya *et al.*, 2000). The spider

hemocyte-derived cationic AMP, gomesin enhanced pro-inflammatory cytokines TNFα, IFNγ, and IL-6 and reduced organ fungal burden in a murine model of disseminated and vaginal candidiasis (Rossi *et al.*, 2012). There are also a host of other small cationic peptides, either induced by IL-6/TNF-α or constitutively expressed, that can modulate cytokine production, alter host gene expression, and/or minimize inflammation (Zhang *et al.*, 2000; Bowdish *et al.*, 2005).

Killer toxins and killer peptides: A functionally different mAb and AMP-based approach

Certain yeasts, described as "killer strains," secrete protein toxins known as "killer toxins" (KT) with broad-spectrum antimicrobial activity against susceptible ("KT-sensitive") fungi, bacteria and viruses across wide phylogenetic distances. The secreted toxin interacts with primary and secondary KT receptors (KTRs) on the surface of KT-sensitive microbes, is taken up by endocytosis, and eventually reaches the nucleus where it causes cell-cycle arrest by interfering with DNA synthesis. Alternatively, some toxins disrupt membrane integrity by forming pores (Schmitt and Breinig, 2006). Two such killer yeasts, *Wickerhamomyces anomalus* (formerly known as *Pichia anomala* and *Hansenula anomala*) and *Williopsis saturnus* var. *mrakii* (formerly known as *Hansenula mrakii*), secrete, respectively, *Pa*KT and HM-1 KT. In vivo, two types of antibodies may be generated in response to KT-sensitive fungal species, against the fungal KT and/or against surface KTR, both of which may neutralize KT action by blocking the interaction between KT and KTR. However, anti-idiotypic mAbs (raised against such anti-KT/anti-KTR antibodies) can in turn neutralize these blocking antibodies, thereby permitting KTs to regain their lethal function (reviewed in Magliani *et al.*, 2012) a strategy that has been effective in *C. albicans* (Polonelli *et al.*, 1997). Interestingly, these anti-idiotype mAbs (which prevent the anti-KT/anti-KTR antibodies from binding to KT/KTRs) mimic the internal structural image of the KTs themselves as reflected in the retention of the KT-like lethal activity against KT-sensitive microbes (Polonelli *et al.*, 2003). As an example, K20, an anti-idiotypic mAb generated against KT-neutralizing mAb KT4, was fungicidal in vitro against KT-sensitive *C. albicans* (Polonelli *et al.*, 2014).

The discovery of naturally occurring anti-idiotypic polyclonal antibodies with KT-like functions ("KT-mimicking antibodies," KAbs) in the sera of human candidiasis patients that were able to strongly inhibit *P. carinii* attachment to lung cells in murine models (Seguy *et al.*, 1997) corroborated the rationale for the aforementioned "anti-idiotypic" immunotherapy. Since then, monoclonal anti-idiotypic KAbs have demonstrated in vitro and in vivo activity against *A. fumigatus* (Cenci *et al.*, 2002) fluconazole-susceptible or -resistant *C. albicans* (Polonelli *et al.*, 2003), and various *Candida* species (Manfredi *et al.*, 2005). The discovery that cell surface KTR structure may contain β-glucans (β-1,3- and 1,6-glucan) (Polonelli *et al.*, 2003) expanded the scope of KAb activity to phylogenetically distant fungal pathogens, all containing cell wall β-glucans. mAb and recombinant single-chain variable-fragment (scFv) antibodies generated against HM-1 KT also showed significant action against *Aspergillus* (Selvakumar *et al.*, 2006c), *C. albicans* and non-*albicans* species (Selvakumar *et al.*, 2006b) and *Cryptococcus* species (Selvakumar *et al.*, 2006a) via inhibition of β(1,3)-glucan synthase and in *Aspergillus* hyphae, inhibition of metabolic activity (Selvakumar *et al.*, 2006b).

The research on KTs and anti-idiotypic mAbs also led to the discovery of synthetic killer peptides (KPs), a different class of effector AMPs. Derived from the sequence of anti-idiotypic mAb or corresponding scFvs, these KPs retain the functional internal image of the KT and serve as broad-spectrum antifungal molecules. A decapeptide KP fragment P6, derived from the sequence of scFv H6 antibody, retained the ability to bind β-glucan and, therefore, the original candidacidal activity both in vitro and in experimental models of rat vaginal and systemic candidiasis (Polonelli *et al.*, 2003). KP was able to kill a large number of *Candida* strains regardless of their antifungal drug-resistance (Polonelli *et al.*, 2003; Manfredi *et al.*, 2005), *Candida* in biofilms (Manfredi *et al.*, 2007), *P. brasiliensis* (Travassos *et al.*, 2004), and capsular and acapsular *C. neoformans* (Cenci *et al.*, 2004) in vitro and in mice with marked reduction of organ fungal burden and enhanced survival. Interestingly, in addition to direct antifungal activity, KP bound MHC-II and CD16/32 on DCs to modulate expression of MHC-II and pro-inflammatory costimulatory molecules (Cenci *et al.*, 2004, 2006). KP was also active against the fungal phytopathogen *Fusarium oxysporum* (Donini *et al.*, 2005), which can cause disease in neutropenic patients.

Immunotherapy via nonspecific immunomodulation

Cytokine therapy

The principle of nonspecific, adjunctive immune modulation with use of pro- or anti-inflammatory, and/or regulatory cytokines to treat fungal disease is based on the role these mediators play in natural human antifungal immunity. However, as put forth in the DRF, such therapy must establish a balance between pro-inflammatory and anti-inflammatory effects of these mediators to prevent detrimental unresponsiveness or tissue damage from excessive inflammation. This is especially important for a disease like cryptococcal meningitis that can occur in the setting of either a weak/insufficient immune response as in HIV/AIDS or due to the immunosuppressive therapy used for solid organ transplantation as well as in the setting of resurgent immunity due to immune-reconstitution in HIV-infected persons receiving antiretroviral therapy or apparently normal immunity (Datta *et al.*, 2008). In addition, extrinsically introduced cytokines often show synergistic effects with coadministered antifungal agents. Immunomodulatory properties have been demonstrated with various common antifungals, such as polyenes (amphotericin B, nystatin) (Sau *et al.*, 2003; Razonable *et al.*, 2005a,b) azoles (voriconazole) (Simitsopoulou *et al.*, 2008; Salvenmoser *et al.*, 2010), and (as above) echinocandins (caspofungin, micafungin) (Moretti *et al.*, 2012, 2014), in cell lines in vitro and murine models of *A. fumigatus* and *C. albicans* infections. Notably, the toll-like receptors (TLRs) these agents engage under different conditions influence whether pro- or anti-inflammatory cytokines are induced (Simitsopoulou *et al.*, 2008; Salvenmoser *et al.*, 2010; Moretti *et al.*, 2012, 2014). This knowledge, in concert with the tenets of the DRF, makes treatment approaches that combine antifungals and cytokines feasible for certain fungal infections.

Data supporting the use of cytokine-based immunotherapy emanate from research and clinical observations, some of which have been discussed above in the section on CMI-stimulating vaccines. IFNγ, the prototypical pro-inflammatory cytokine released from CD4$^+$ and CD8$^+$ T-cells and NK cells, is an important candidate due to its ability to induce T_h1-mediated antifungal immunity and activate effector phagocytes to exert fungicidal effects. Therapeutic use of IFNγ is supported by the observations that candidiasis and aspergillosis, fungal diseases with different pathogenesis, are each susceptible to T_h1-type, especially IFNγ-mediated responses (Netea *et al.*, 2003; Chai *et al.*, 2010), and that T_h1-immunity to *A. fumigatus* is cross-protective with *C. albicans* (Stuehler *et al.*, 2011). In addition, there is some clinical evidence for a benefit of IFNγ in invasive fungal infections (Hubel *et al.*, 2002) especially in immune-suppressed populations. For example, in a clinical trial in patients with invasive candidiasis and/or aspergillosis, adjunctive immunotherapy with recombinant IFNγ (in conjunction with an echinocandin) improved leukocyte immune responses via upregulation of HLA-DR expression and enhancement of pro-inflammatory cytokines associated with both innate and adaptive immunity (Delsing *et al.*, 2014). In HIV-associated cryptococcal meningitis, short-term combination therapy with IFNγ with amphotericin B markedly enhanced clearance of *C. neoformans* from the brain with minimal adverse effects (Jarvis *et al.*, 2012). In addition, IFNγ levels in cerebrospinal fluid correlated positively with treatment outcome (Siddiqui *et al.*, 2005; Jarvis *et al.*, 2015; Mora *et al.*, 2015) and negatively with detrimental immune-reconstitution inflammatory syndrome (IRIS) (Chang *et al.*, 2013a). Also, adjunctive IFNγ in association with antifungals showed encouraging results, including in salvage therapy of refractory disease (Pappas *et al.*, 2004; Gamaletsou *et al.*, 2012).

In addition to IFNγ, granulocyte (G) and GM-CSF have received attention as adjunctive therapy to modulate antifungal immunity, with the caveat that data on their clinical use are largely restricted to small studies or case reports. G-CSF and GM-CSF are of particular interest, since their ability to reduce/reverse neutropenia and enhance functions of neutrophils and macrophages (Georgopapadakou and Walsh, 1996) is likely to be beneficial in fungal infections where phagocytosis and inflammatory mechanisms are required for defense. Encouraging results in vitro and in animal models have provided the rationale for human use (Chiou *et al.*, 2000; Hubel *et al.*, 2002). In HIV-positive children, antifungal function of neutrophils against *A. fumigatus* hyphae (which is diminished in HIV infection, even in absence of neutropenia) was restored via ex vivo stimulation with G-CSF (Roilides *et al.*, 1993). Used in conjunction with azole antifungals, recombinant G-CSF and GM-CSF both enhanced candidacidal activity of neutrophils and monocytes in vitro (Vora *et al.*, 1998). In a clinical trial with HIV-positive patients, recombinant human GM-CSF as adjunct therapy showed encouraging results in fluconazole-refractory oropharyngeal candidiasis (Vazquez *et al.*, 2000). GM-CSF was shown to help maintain pro-inflammatory IL-1α, TNFα, and macrophage inflammatory protein (MIP)-1α responses of mouse bronchoalveolar macrophages challenged with *Aspergillus* conidia in vitro (Brummer *et al.*, 2003), and, in a murine model of pulmonary aspergillosis, intranasally administered GM-CSF increased macrophage activation and reduced lung fungal burden (Quezada *et al.*, 2008). In an interesting case, long-term, continuous treatment with intravenous GM-CSF followed by short-term subcutaneous G-CSF in a diabetic patient with isolated chronic mucocutaneous candidiasis resulted in complete remission, improvement in monocyte and neutrophil functions, along with stimulation of T_h17 cells (including CD4$^-$CD8$^-$ TCR$\gamma\delta$ cells) and suppression of inappropriate IFNγ secretion (Wildbaum *et al.*, 2013). Recently, a case of relapsing *C. albicans* meningoencephalitis in the setting of primary immunodeficiency associated with a genetic defect in T_h17 immunity and defective IL-17 and IFNγ synthesis achieved complete remission and improvement in IL-17 secretion with G-CSF treatment (Celmeli *et al.*, 2016).

Promising results have also been seen with pro-inflammatory cytokines (such as IL-12 and IL-17) in both in vitro and mouse models of various fungal infections. Protective effects of IL-12 treatment (mediated by IFNγ) were demonstrated in mice infected with *H. capsulatum* (Zhou *et al.*, 1995) and *C. neoformans* (Decken *et al.*, 1998). Another Th1 cytokine, IL-18, was shown to work in close conjunction with IL-12 and IFNγ to protect mice against disseminated cryptococcosis (Antachopoulos and Walsh, 2012). Treatment of *Coccidioides immitis*-challenged mice with recombinant IL-12-secreting J774 macrophages protected the mice by reducing organ *C. immitis* burdens and stimulating secretion of IFNγ (Jiang *et al.*, 1999). Immunocompetent mice responded to *P. carinii* challenge by releasing IL-12, and exogenously administered IL-12 enhanced *P. carinii* clearance via inflammatory cell recruitment into lung tissue with release of IFNγ, TNFα, and IL-12 in immunocompetent and CD4$^+$ T-cell depleted mice (Ruan *et al.*, 2008).

CD4$^+$ T_h17 cells elicit IL-17, IL-21, and IL-22 function that bridges innate and adaptive immunity to mucosal infections (Khader *et al.*, 2009). IL-17 mediates protection against various bacteria and fungi via induction of CMI mediators (such as G-CSF, IL-6, and IL-8/CXCL8), neutrophil differentiation and migration, and AMPs, such as β-defensins and S100As see above, and reviewed in Matsuzaki and Umemura (2007) and Khader *et al.* (2009). The recent discovery of a genetic predisposition to chronic mucocutaneous candidiasis (Huppler *et al.*, 2012) has led to the recognition of the role of IL-17 in resistance to mucosal fungal infections. Human diseases linked to IL-17 signaling defects that lead to reduced IL-17 and T_h17 impairment have been associated with autoantibodies, mutations in the IL-17 gene or its receptor, and other mutations (e.g., in STAT-3, responsible for autosomal dominant hyper-IgE syndrome) (reviewed in Khader *et al.*, 2009; Huppler *et al.*, 2012). In murine respiratory *P. carinii* and *A. fumigatus* infection models, early induction of IL-17 (along with IL-23, which drives lineage commitment of Th17 cells) plays a pivotal role in fungal clearance (Rudner *et al.*, 2007; Werner *et al.*, 2009). Notably, and important in the context of the DRF, IL-17 modulates the expression and/or pro-inflammatory properties of IL-22 to protect against acute tissue damage at mucosal sites and in the lungs following various infectious and other pathological conditions (Sonnenberg *et al.*, 2010). The critical importance of this modulation is highlighted by the observation that heightened and persistent T_h17 responses driven by dendritic cell-derived IL-23 are responsible for inflammatory host tissue damage via neutrophils in murine models of candidiasis and aspergillosis, with no concomitant restriction of fungal growth (Zelante *et al.*, 2007). The balance between protective and inflammatory effects of

T_h17 cells is under intense research, and data indicate that T_h17 mucosal immunity may need further downstream regulators to achieve this outcome (Khader *et al.*, 2009).

Preclinical in vitro and in vivo murine studies have demonstrated the promise of a few other cytokines against fungal infections. In a murine model of *C. albicans* sepsis, IL-7 (a pluripotent cytokine that induces proliferation and activation of naïve and memory T-cells) increased lymphocyte function (including expression of adhesion molecules and IFNγ production), DTH responses, and host survival (Unsinger *et al.*, 2012). TNFα (closely associated with IL-12/IL-18) was beneficial in a murine model of disseminated cryptococcosis (Kawakami *et al.*, 1999). Notably, the potential importance of TNFα is further highlighted by the observed increase in risk for fungal disease during treatment with TNFα antagonists (such as the mAbs infliximab or adalimumab), or the anti-inflammatory TNF receptor Fc fusion protein etanercept (Weinblatt *et al.*, 1999). Patients who received a TNF inhibitor (TNFi) had an increased incidence of *Pneumocystis jirovecii* (human variant of *P. carinii*) pneumonia and other fungal infections (Chirch *et al.*, 2014; Grubbs and Baddley, 2014). In an ex vivo cell culture system, the type 1 cytokine IL-2-activated fungistatic and fungicidal activities of T-cells ($CD4^+$ and $CD8^+$), T-lineage NK cells, and $CD16^+/56^+$ non-T-lineage NK and LAK cells against encapsulated *C. neoformans* (Levitz and Dupont, 1993), and a combination of an anti-CD40 antibody (CD40 is a member of TNFα receptor family) and IL-2 (mediated via IFNγ) was protective in murine cryptococcal infection (Zhou *et al.*, 2006, 2007).

Immunotherapy of rare mold infections via immunomodulation

Certain molds, including the septate, filamentous sordariomycetes *Fusarium* and *Scedosporium*, non-septate zygomycetes (such as *Rhizopus* and *Mucor*), and certain yeast different from *Candida* and *Cryptococcus* (such as *Trichosporon*), have emerged as important causes of high morbidity and mortality in patients undergoing myeloablative therapy and/or stem cell/bone marrow and/or solid organ transplantation (Katragkou and Roilides, 2012). Conventional antifungal therapies are often of extremely limited efficacy in diseases caused by molds, in large part because these agents are unable to eradicate the causative fungus in the setting of severely impaired immunity. Therefore, approaches that augment host immune responses via immunotherapy make sense, with the caveat (usual for this modality) that not all such methods have been tested in randomized clinical trials (reviewed in Katragkou and Roilides, 2012). Nevertheless, these examples provide hope that understanding of the pathophysiology of disease pathogenesis can be leveraged to direct immunotherapy. For instance, given the clinical importance of host neutropenia in development of zygomycosis, transfusion of granulocytes stimulated by recombinant G-/GM-CSF or IFNγ has shown promise in research (in vivo) models and limited clinical cases, alone or in conjunction with conventional antifungals. Notably, the lipid-lowering drug group, statins, also appear to have broad-spectrum and pleiotropic abilities to modulate immune and inflammatory functions and are fungicidal against zygomycetes, *albeit* with variable modes of action and fungal susceptibilities (see Katragkou and Roilides, 2012). Similarly, stimulation of neutrophils with IFNγ, GM-CSF, and/or IL-15, as well as granulocyte transfusion, has shown beneficial effects (in research models and isolated clinical cases) against *Scedosporium* and *Fusarium*, both fungi with relatively high innate resistance to administration of antifungals alone (Katragkou and Roilides, 2012).

The importance of negative immunomodulation as therapy for fungal infections

The DRF underscores an important principle of microbial pathogenesis, namely, that host–microbe interaction can lead to host tissue damage (the hallmark of "disease") stemming from an exuberant and unmodulated host inflammatory response. Excessive inflammation has been observed in the central nervous system of otherwise normal patients with *C. gattii* (Panackal and Williamson, 2015), and dysregulated inflammation has been implicated in the pathogenesis of ART-induced *Cryptococcus*-associated IRIS (Chang *et al.*, 2013a; Eschke *et al.*, 2015; Jarvis *et al.*, 2015; Panackal and Williamson 2015; Kao and Goldman 2016). Along the same lines, excessive inflammation has been implicated in various manifestations of candidiasis (see Jabra-Rizk *et al.*, 2016). In such situations, adjunct therapeutic interventions that dampen the inflammation in a generalized or targeted manner may be beneficial.

Adoptive cell transfer

For the past two decades, numerous in vitro and ex vivo studies have evaluated various strategies to prime, enhance, or modulate the antifungal immunity afforded by components of innate and adaptive CMI, such as granulocytes, DCs, NK cells, lymphokine-activate killer (LAK) cells, and $CD4^+$ or $CD8^+$ T-cells. For instance, adoptive transfer of Th1-committed $CD4^+$ T-cells (secreting IFNγ and IL-2) from mice immunized with crude antigen from *A. fumigatus* conferred protection to neutropenic naïve mice against aspergillosis (Cenci *et al.*, 2000). Similarly, upon adoptive transfer, cytotoxic $CD8^+$ T-cells generated against *Aspergillus* peptide antigen f16 (Aspf16) increased the survival time of *A. fumigatus*-infected immunocompromised mice (Sun *et al.*, 2012). In allogeneic bone marrow transplant recipients with IA, adoptive transfer of donor-derived anti-*Aspergillus* T_h1 cells, generated via ex vivo exposure of donor T-cells to *Aspergillus* antigens in a CMI context, conferred immunity to recipients and the T_h1 cells retained their proliferative abilities. However, it was also observed that commonly used immunosuppressants, such as cyclosporin A, mycophenolic acid, glucocorticoids, and rapamycin reduced the overall numbers of anti-*Aspergillus* T_h1 cells, raising concerns about the utility of this modality for organ transplant recipients (Tramsen *et al.*, 2014). Adoptive T-cell transfer has also shown promise in experimental murine infections with dimorphic fungi. Administration of *Histoplasma*-reactive $CD4^+$ T-cells followed by pulmonary challenge with *H. capsulatum* reduced the fungal tissue burden of nude C57BL/10 mice or irradiated heterozygous nude (nu/+) C57BL/6 mice (but not normal or neutropenic mice) (Allendoerfer *et al.*, 1993), and adoptive transfer of IFNγ-producing $CD4^+$ T-cells expressing $V\alpha2^+J\alpha49^+/V\beta^+J\beta1^+$ TCR generated against cell wall/membrane components of *B. dermatitidis* protected against lethal experimental murine blastomycosis (Wuthrich *et al.*, 2007).

APCs, such as multifunctional DCs, play a key role in bridging innate and adaptive immunity against fungi. Pulsing human or murine DCs with live fungal components (*Aspergillus* conidia or hyphae) or RNA derived from these components led to their functional maturation. Such mature DCs-activated antigen-specific, IFNγ-secreting T-cells in situ and upon adoptive transfer to naïve mice, contributed to rapid recovery of immunocompetence in mice receiving allogeneic bone marrow transplants with experimental aspergillosis (Bozza *et al.*, 2003). Similarly, priming DCs and other APCs with pentadecapeptides (PPCs) spanning the Aspf16 coding sequence expanded the APC pool and generated potently cytotoxic T-cells (Ramadan *et al.*, 2005; Zhu *et al.*, 2008), with the ability to kill *Aspergillus* conidia and DCs pulsed with Asp *f16*-PPC or *Aspergillus* culture-extract (Ramadan *et al.*, 2005). Autologous DCs, pulsed with conidial lysates from an *A. fumigatus* environmental strain and cocultured with neutrophils in presence of IL-2, IL-7, and IL-15, resulted in a >30-fold increase in effector and memory $CD4^+$ T cell numbers (Gaundar *et al.*, 2012). Because these adoptive transfer strategies exhibit secondary effects upon recipient effector cells that induce activation, such transfers are often equated with vaccinations. However, by virtue of their inherent functional plasticity, certain subsets of DCs are also able to modulate and dampen exuberant inflammatory responses via T_{reg} induction (Perruccio *et al.*, 2004).

Granulocytes, including neutrophils, are crucial effector cells in antifungal immune defense. Invasive fungal infections often occur in the setting of neutrophil dysfunction or frank neutropenia (a common presentation of many hematological disorders and a known complication of cancer chemotherapy) and are associated with high mortality under such conditions despite appropriate antifungal therapy (Hubel *et al.*, 2001). Transfusion of granulocytes ("GTX") from healthy donors, stimulated by G-CSF or steroids, in conjunction with antimicrobial therapy was shown to improve clinical outcome in neutropenic patients with various infectious diseases, including IA and *Candida* sepsis, when administered therapeutically (Hubel *et al.*, 2001; Illerhaus *et al.*, 2002) or as prophylaxis for neutropenic patients at risk for invasive fungal infections (Illerhaus *et al.*, 2002; Kerr *et al.*, 2003); also see this Cochran meta-analysis (Estcourt *et al.*, 2015). The feasibility, tolerance and efficacy of combination of antifungal-GTX was demonstrated in several clinical studies in neutropenic pediatric and young adult patients with cancer and history of proven mold or yeast infections (Grigull *et al.*, 2006a,b; Sachs *et al.*, 2006; Seidel *et al.*, 2009). G-CSF-activated GTX reconstituted absolute neutrophil counts and effectively cleared *Zygomycete* infections (Pagano *et al.*, 2009). In patients with severe aplastic anemia, GTX administered in conjunction with G-CSF improved survival rates from fungal infections (aspergillosis, cryptococcosis, and candidiasis) and hematopoietic recovery (Wang *et al.*, 2014a,b). GTX has been shown to result in significant clinical improvement in invasive fusariosis, a disease relatively refractory to available antifungals; however, the efficacy was much reduced if the neutrophil recovery was hampered due to underlying conditions (Kadri *et al.*, 2015). In this chapter, the decrement in neutrophil response to GTX was attributed to HLA alloimmunization (Kadri *et al.*, 2015), which remains an important concern in the otherwise well-tolerated GTX modality (for a discussion of concerns, see Hubel *et al.*, 2001). Commonly occurring mild and manageable reactions to GTX aside, this modality can lead to more severe side effects, including hypotension and acute lung injury, whose mechanism is poorly understood. A more critical, potential complication is alloimmunization or graft-versus-host disease (GVHD) secondary to GTX; however, irradiation of GTX products prevents GVHD, and alloimmunization appears to be a problem only in case of underlying neutrophil dysfunctions. Prophylactic GTX prior to bone marrow transplants may cause delayed engraftment if the granulocytes are sourced from an incompatible donor; however, a lymphotoxicity assay prior to GTX may establish donor compatibility (Hubel *et al.*, 2001). Therefore, even though the therapeutic potential of GTX has not yet been proved in controlled clinical trials, this modality appears to hold promise as a safe, low-toxicity and efficacious option to protect neutropenic patients from invasive fungal diseases.

In addition to granulocytes, two other cell types tested in studies were: (1) HL-60, a human myeloid, phagocytic cell line derived from a cancer patient, and (2) mesenchymal stem cells/MSCs, which are multipotent, non-hematopoietic progenitor cells that can differentiate into multiple lineages, and have excellent proliferative potential, low immunogenicity, and immunomodulatory properties. HL-60 cells can be activated to differentiate toward a mature neutrophil phenotype, and administration of activated, irradiated HL-60 cells improved the survival of neutropenic mice with candidemia (Spellberg *et al.*, 2007). In murine models, MSCs have shown promise for treatment of liver fibrosis and hepatocarcinoma as well as bacterial infections and allergic airway inflammations, and may show promise for fungal diseases also. A single colony-derived IL-17-producing subset of bone marrow MSCs inhibited *C. albicans* growth in vitro and promoted survival of mice with candidiasis (Yang *et al.*, 2013). In an in vitro study with a murine macrophage cell line J774A.1, murine bone marrow MSCs exerted anti-inflammatory effects by decreasing TNFα and increasing IL-10 elicited by macrophages in response to *A. fumigatus* conidia, and also reduced fungal growth (Cho *et al.*, 2016).

Other immune effector cell lineages, including NK cells, monocytes and B-cells, have been evaluated for their protective potential via adoptive transfer. Human NK cells inflict direct damage to *A. fumigatus* cells via release of IFNγ, a mechanism that is separate from NK cytotoxicity stemming from toxic proteins (Bouzani *et al.*, 2011). Bacillus Calmette-Guerin (BCG)-vaccination in healthy volunteers helps generate trained immunity in NK cells, which may react with non-related non-mycobacterial microbes. BCG-induced trained NK cells enhanced the survival of SCID mice with disseminated candidiasis (Kleinnijenhuis *et al.*, 2014). C-C chemokine receptor 2 (CCR2)- and Ly6C-expressing inflammatory monocytes are effectors involved in the response in systemic candidiasis during active infection; in mice depleted of CCR2, adoptive transfer of such monocytes early in the infection promotes fungal clearance from kidney and brain (Ngo *et al.*, 2014). In mice lacking lymphocytes due to type-I IFN receptor deficiency ($IFrag^{-/-}$), adoptive transfer of B-cells helped maintain early hematopoietic progenitor activity during immune response to *Pneumocystis* infection and replenished the bone marrow in an IL-10- and IL-27-dependent manner (Hoyt *et al.*, 2015).

Table 2 Features of antifungal immunotherapy approaches

Modality	*Type*	*Specificity*	*Mechanism(s) of action*	*Advantages*	*Concerns*
Active	vaccine	Host	1. Induces fungus-specific adaptive immune response 2. Generates immunological memory 3. Acts via antibody-mediated and cellular effector mechanisms 4. Has the potential to act therapeutically, either to limit damage from reactivation of latent fungal infection or to augment fungal clearance in an ongoing or reactivated infection	1. Ample research, clinical data, and success with vaccines for other microbes support the approach 2. Independent, overlapping and/or redundant mechanisms are harnessed to produce comprehensive immunity 3. Has the potential to induce broad spectrum (multi-strain/microbe) activity 4. May work even in certain settings of immune suppression	1. Poor immunogenicity and/or immunosuppressive properties of certain fungal antigens (rational vaccine design and right adjuvants may help) 2. May require immune competence to be effective (rational vaccine design and right adjuvants may help) 3. May induce pathogen strain replacement (unlikely for conserved fungal determinants) 4. Cost, disbelief, and nonscientific impediments to development
Passive	mAbs	Fungus	Direct and indirect antifungal activities, including: 1. Neutralization and/or blocking of fungal antigens 2. Opsonization of fungal cells 3. Complement- and antibody-dependent cytotoxicity 4. Direct membrane damage leading to cellular lysis 5. Alterations in gene expression	1. Supported by robust preclinical data that has demonstrated antibody-mediated immunity be beneficial in host defense against fungi 2. Direct and indirect action, dependent on or independent of host immune status 3. Immediate action 4. Enables next-generation modalities, for example, radioimmunotherapy 5. Narrow spectrum of activity 6. Excellent safety profile	1. Narrow spectrum of activity, requiring fungal etiology to be identified before therapy (advances in molecular biology assist in rapid production of specific mAbs) 2. Early administration required for maximum efficacy (better diagnostic options may help) 3. Possibility of adverse reactions 4. Nonscientific issues hampering development
		Host	Immunomodulation, polarization of effector cells, repopulation of specific cell subsets		Balance of pro-inflammatory action and fungal clearance is an important consideration
Passive	AMP	Fungus	1. Direct cytotoxic activity via membrane destabilization, disruption of cellular metabolism, and signaling cascades 2. Chemoattractants for adaptive immune effectors	1. Endogenous products of innate immune response 2. Effective at small concentrations 3. Broad spectrum 4. Rapid onset activity 5. Relatively low possibility of resistance emergence in fungi 6. May act synergistically with antifungals	1. Pharmacokinetics-associated delivery issues 2. High cost of production
		Host	Immunomodulation		
Passive	KT, KP	Fungus	Similar to mAbs (KT) and AMP (KP)	Effective in in vitro, and in vivo preclinical models against yeasts and molds	Narrow spectrum of activity, difficult to produce, paucity of data
Passive	Cytokine therapy	Host	1. Nonspecific, adjunctive immune modulation 2. Pro-, anti-inflammatory and regulatory functions 3. Mobilization of effector immune response against fungi	1. Leverages the natural immune functions of these molecules 2. May act synergistically with antifungals 3. Benefits seen in isolated clinical cases	1. May cause excessive inflammation, tissue injury, or induce tolerance (can be carefully calibrated to achieve balance) 2. Paucity of data
	Adoptive transfer cell	Host		Repopulation of immune subsets following ex vivo activation (effective especially in case of cellular immune defects in recipients)	1. Leverages the natural immune functions of these effector cells 2. Modulates immune responses to fungal antigens 3. Benefits seen in isolated clinical cases and in clinical trials – safe, low-toxicity, efficacious

Table 2 Continued

Modality	Type	Specificity	Mechanism(s) of action	Advantages	Concerns
			Possibility of severe side effects, alloimmunization and graft vs. host disease (can be clinically managed)		

Abbreviations: AMP, antimicrobial peptide; KT, killer toxin; KP, killer peptide; mAbs, monoclonal antibodies.

Fungal allergy and immunotherapy

Mold allergens are spores and/or submicron-size mycelial fragments from filamentous fungi, such as *Alternaria*, *Cladosporium*, *Penicillium*, and *Aspergillus*, up to 28 fungal genera, that elicit 107 variants of allergens. They are ubiquitous, and significant etiological agents of respiratory allergy, rhinitis and asthma. These allergens, mostly proteins (enzymes, Hsps, and peroxisomal proteins), are capable of inducing strong IgE- or T-cell mediated inflammatory reactions and, therefore, are potential candidates for use in immunotherapy to suppress inflammatory pathways or induce antigen-specific responses that modify disease progression (Twaroch *et al.*, 2015). However, severe adverse reactions from mold extracts in early studies hindered the development of efficient immunotherapies. A promising targeted approach used allergen-DNA transfected DCs to generate allergen-specific IgG4 but not IgE, and stimulated autologous $CD4^+$ and $CD8^+$ T-cells to secrete T_h1 cytokines (Konig *et al.*, 2007). Newer approaches, such as recombinant hypoallergenic allergen derivatives (with IgE epitopes deleted or host IgG generated to neutralize allergen IgE epitope) and peptide-based allergen vaccines (with targeted mutations to lower IgE binding), aim to induce tolerance to the allergen by reducing IgE production, downregulating T_h2-mediated responses, and generating more T_{reg} cells (Twaroch *et al.*, 2015). Chronic inflammation associated with allergic fungal sinusitis does not respond well to standard treatment options, but has been amenable to adjunct sublingual immunotherapy (drops of allergens in metered, sublingual doses), which lowers serum IgE levels with no adverse effect (reviewed in Melzer *et al.*, 2015).

Concluding Remarks

Notwithstanding the availability of antifungal agents, the burden of invasive mycoses, especially in immunity-impaired populations, demands the exploration of new approaches to prevention and treatment. Human antifungal vaccines and other immunotherapies would represent a significant step in that direction. The promise of immunotherapy for fungal diseases lies in the ability of this modality to leverage the host immune response to achieve a beneficial effect that induces fungal clearance and/or modulates the inflammatory response to limit host damage (summary in **Table 2**). Although various circumstances have hindered the progress of developmental efforts, the advent of new technologies, platforms to dissect the immune response, and rapid diagnostics make the prospect of active and/or passive immunotherapy for fungal diseases eminently logical and feasible. Vaccines for a wide variety of microbial infections have a long track record of safety, success globally, and economic benefit. Therefore, management of mycotic diseases via vaccines and other immunotherapeutic interventions deserves vigorous consideration and support. Advances in our understanding of fungal pathogenesis have revealed previously unappreciated opportunities to influence the immune response to the benefit of the host with fungal disease. Continued research into host–fungus interactions in the pathogenesis of fungal disease holds the key to the development of specific and nonspecific immunotherapeutic modalities to treat fungal disease in the future.

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Diagnosis of Fungal Infections

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Glossary

Antigen A molecule capable of inducing an immune response in the host organism.
Commensal fungi Fungal species that live in or within human body without harming or affecting it.
Complement fixation A diagnostic test which is based on the process of binding serum complement to the product formed by the union of an antibody and the antigen for which it is specific.
Cross-reactivity (of a antigen detection method) When an antibody directed against one specific antigen is successful in binding with a different antigen.
Differential diagnosis The differentiation between two or more conditions which share similar symptomatology.
Endemic mycoses Mycoses caused by fungal species that have specific geographic distributions such as *Histoplasma capsulatum*, *Paracoccidioides* spp., *Coccidioides* spp., and *Blastomyces dermatitidis*.
Environmental fungi Fungal species that are commonly present in the environment.
Enzyme Immune Assay (EIA) A test that measures the presence or concentration of a molecule in a solution through the use of an antibody or an antigen, which is linked to an enzyme. These enzymes allow for detection because they produce an observable color change or light in the presence of certain reagents.
EORTC/MSG criteria A standard set of definitions of invasive fungal disease which aim is to improve the quality of clinical studies. These criteria have been established by the Mycoses Study Group of the European Organization for Research and Treatment of Cancer and the National Institute of Allergy and Infectious Diseases.
False negative (of a diagnostic test) A negative result given by the test that actually is positive.
False positive (of a diagnostic test) A positive result given by the test that actually is negative.
Immunodiffusion A diagnostic test which involves diffusion of the antigen and the antibody through a substance such as agar. The antigen and the antibody bind with each other and form an insoluble immuno-precipitate.
ITS region Internal transcribed sequence region of the ribosomal DNA that has been classically used for the identification of fungal species.
Latex-agglutination A diagnostic test for the detection of antigens or antibodies in a sample. When a sample containing the specific antigen (or antibody) is mixed with an antibody (or antigen) which is coated on the surface of latex particles, a visible agglutination occurs.
MALDI-ToF MS technology Matrix-assisted laser desorption ionization time of flight mass spectrometry is an identification method based on the acquisition of a mass spectrum from an unknown microorganism and its comparison with a database of reference spectra to determine the organism identity.
Opportunistic fungal infections Infection by a fungus that normally does not cause disease but becomes pathogenic when patient's immune system is impaired and unable to articulate a response to the infection.
Point of care (POC) diagnostic method Medical testing at or near the site of patient care. These methods are characterized for being simple, fast, and cheap.
Polymerase chain reaction (PCR) A laboratory molecular technique used to make multiple copies of a segment of DNA.
Sensitivity (of a diagnostic test) The proportion of true positives that are correctly identified by the test.
Specificity (of a diagnostic test) The proportion of true negatives that are correctly identified by the test.

Introduction

Human fungal infections encompass a broad spectrum of diseases caused by more than 600 species of fungi present in the environment (see "Relevant Websites section"). They can affect any part of the body producing superficial infections (skin and nails), mucosal infections, chronic and allergic infections, and finally, they also can invade tissues causing an invasive fungal infection (IFI). IFI is the most serious clinical entity with a high mortality since mainly affect immunosuppressed patients with poor general condition.

Diagnosis of these diseases is difficult and frequently delayed due to the lack of suspicion and/or the lack of suitable diagnostic tests. In many parts of the globe, particularly in low income countries, some of these infections are neglected diseases than could be easily solved with skilled personnel and adequate diagnosis (Oladele *et al.*, 2019). In developed countries, the main problem is that these invasive infections occur in highly immunosuppressed patients with a poor outcome in absence of an early diagnosis. The increasing use of immunosuppressive drugs for treating serious medical conditions have favored the rise of opportunistic fungal infections in these regions.

The overall mortality due to fungal infections is around 1,600,000 people per year (Bongomin *et al.*, 2017). The most common species involved in invasive disease are *Aspergillus* spp. *Candida* spp. *Cryptococcus* spp., *Pneumocystis jirovecii* and fungi responsible of

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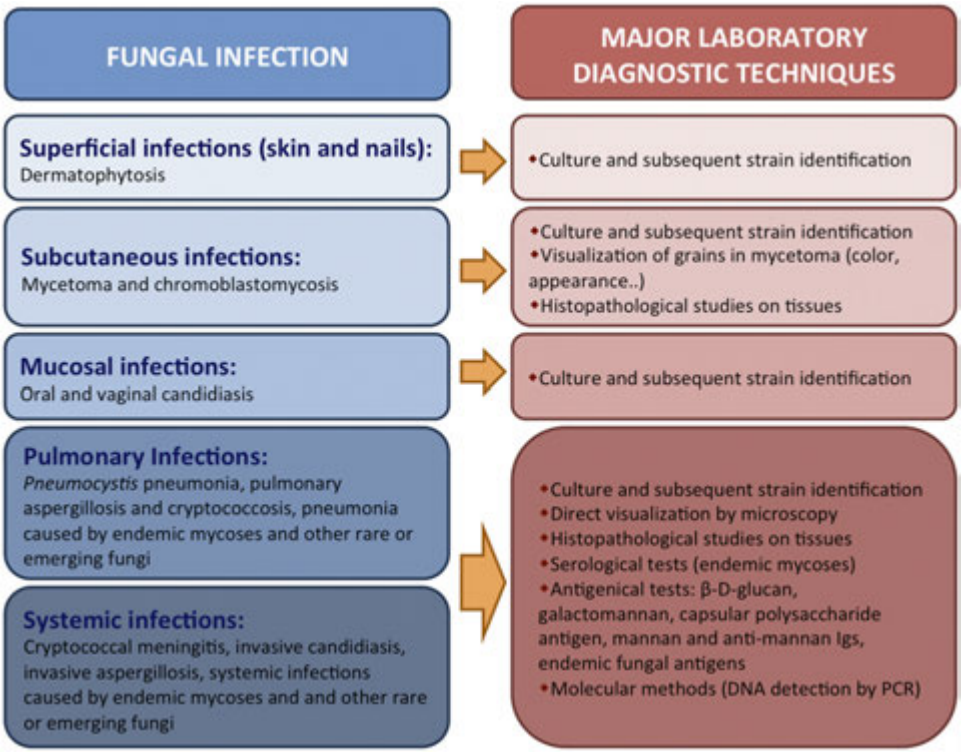

Fig. 1 Main human fungal infections and the corresponding laboratory techniques commonly used for their diagnosis.

endemic mycoses (*Histoplasma capsulatum*, *Paracoccidioides* spp., *Coccidioides* spp., and *Blastomyces dermatitidis*). Nevertheless, when a patient has several predisposing factors for a prolonged period of time, almost any fungal species can cause an infection.

The clinical profile of mycosis varies greatly depending on the causal agent, the location of the infection, and the predisposing factors of the patient. Regardless of the patient's clinic, the diagnosis is always difficult. Classical diagnosis methods have limitations as they lack of a suitable sensitivity and specificity. Recently, new diagnostic tests have been developed but many of them are still used in combination with classical methods because of the lack of validation and consensus among laboratories. Moreover, some of these new techniques are too expensive for resource challenging countries. This review describes the methods routinely employed in the Mycology Laboratories and their usefulness in the diagnosis of the different clinical entities in the field of the Medical Mycology. In addition, it delves into the methods most recently developed and describes their use alone or in combination with classical methods.

Two figures have been included with the purpose of summarizing all these concepts. In **Fig. 1** it is shown how, depending on the kind of infection, the number of techniques used is greater and more specialized due to both the difficulty of the diagnosis and the severity of the infection. In **Fig. 2**, a brief summary of all diagnostic techniques that can be performed has been presented.

Conventional Diagnostic Methods

Conventional diagnostic methods are those used classically in the Laboratory of Microbiology. Although they are useful, they lack of sensitivity and specificity and should be used in combination with the new tools recently developed.

Culture

Isolating the fungus from a clinical sample using an adequate culture medium is considered the gold standard method for the diagnosis of a fungal infection. Specific media for fungal culture commonly used are Sabouraud dextrose agar (SDA), potato dextrose agar (PDA), malt extract agar (MEA), and, less commonly, brain heart infusion (BHI). Optimally, cultures should be incubated at 30°C ($\pm$1°C) during 7 days, but some fungal species require longer incubation periods, up to several weeks (Sutton, 2015). Although this technique is simple and cheap, it is time consuming and its diagnostic performance depends on sample origin, fungal species, underlying disease of the patient, clinical status, etc. Here we cite some examples: (1) Blood cultures, which has been used mainly in *Candida* spp. invasive infections, are frequently negative in other infections even if they are disseminated (Alexander and Pfaller, 2006), (2) Mucorales species are fast growing but the yield in cultures from clinical samples is low, probably due to the fragility of their hyphae (Ribes *et al.*, 2000), (3) endemic fungi are fastidious and slow-growth organisms that requires 3–4 weeks to

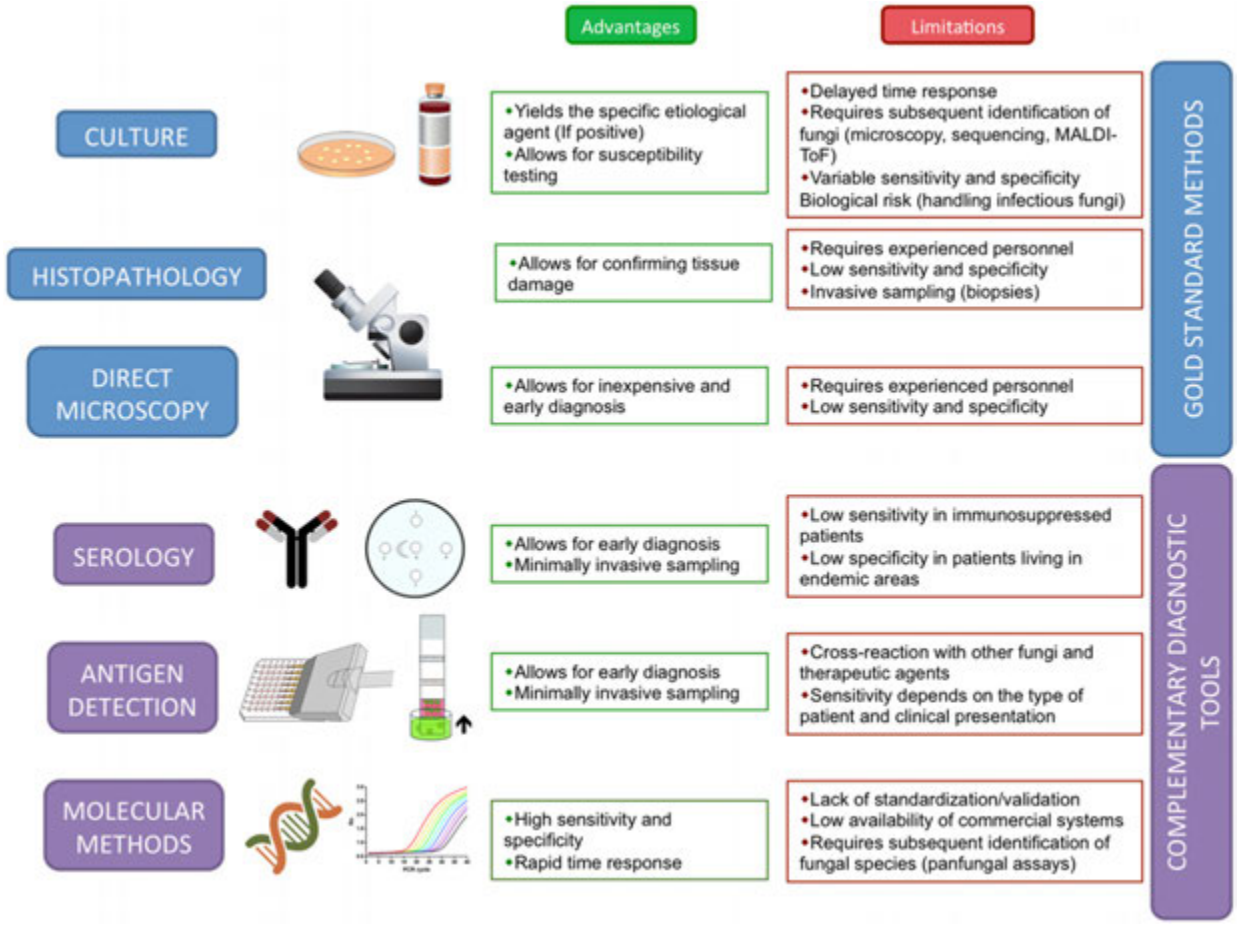

Fig. 2 Advantages and limitations of both classical and new fungal diagnostic methods.

growth (Buitrago *et al.*, 2011), etc. Finally, false positive results are common since fungi are usual contaminants of the laboratory and part of the saprophytic human flora (Cuenca-Estrella *et al.*, 2008).

Once the culture is obtained, the identification of the grown fungus is essential, especially in invasive disease, in order to establish an appropriate antifungal therapy. Classical fungal identification requires the visualization and identification of the different fungal structures by microscopy. In the case of yeasts, also biochemical tests of carbon assimilation and/or fermentation are carried out to complement microscopical observations (Willinger *et al.*, 2015). Microscopical identification requires very skilled and specialized personnel that can be common in a reference center but rare in a clinical setting. Recently, however, a fast identification is possible by using alternative techniques such as those based on mass spectrometry (MS) or molecular methods. In the last decade, MALDI-TOF technology has revolutionized microbial identification since it is easy to use and allows reducing costs as well as time response (Posteraro *et al.*, 2013). The sequencing of specific DNA targets, mainly the ITS region of the ribosomal has been very useful for identification of fungi and taxonomical studies being considered the gold standard method for fungal molecular identification (Perlin and Wiederhold, 2017) but this specialized approach is time consuming and requires an appropriate database for sequence comparison (Irinyi *et al.*, 2015).

Direct Visualization and Histopathology

Direct examination of clinical samples by microscopy to detect fungal structures is also a simple, cheap, and rapid method largely used in Microbiology laboratories. Samples are visualized after being treated with KOH 10% or stained with specific dyes. It is a very useful technique for some species as *Pneumocystis jirovecii* (immunofluorescent staining) (Tasaka and Tokuda, 2013) or *Cryptococcus neoformans* (Indian ink staining) (Baddley and Dismukes, 2011), but in general, it lacks sensitivity and negative results never rule out an infection by fungi.

Histopathology uses tissue samples to identify fungal structures by microscopy. If fungi are suspected, the specimens are first colored with stains that highlight the fungal wall as Gomori methenamine silver (GMS) or periodic acid-Schiff (PAS). This method, although is an important tool for the microbiologist, has also several limitations. Moreover, obtaining biopsies

of the affected tissue is a very invasive procedure and may be contraindicated in certain patients (Guarner and Brandt, 2011; Perfect, 2013). In other cases, the amount of material obtained is very limited making histopathological studies impossible.

Both methods enable the identification of fungal structures and they can provide a presumptive diagnosis while waiting for additional test for identifying the species involved, as the presence of common fungal elements such as hyphae or yeasts in samples or tissues does not provide sufficient information to identify the species (Lease and Alexander, 2011). The sensitivity for microscopic morphological techniques have been reported that varies from 20% to 80% (Guarner and Brandt, 2011). Finally, very skilled personnel is required for both methods as it is difficult to identify fungal structures in tissues and misidentifications are frequent. Diagnosis by these methods should be descriptive and correlated with clinical and epidemiological data.

New Diagnostic Methods

To overcome weaknesses of conventional diagnostic methods, alternative approaches based on the detection of antibodies, antigens, DNA, and other biomarkers have been developed and they are now routinely used as complementary diagnostic tools in clinical settings.

Antibody Detection

Complement fixation (CF), immunodiffusion (ID), and enzyme-immunoassay (EIA) are the most common techniques used to detect antibodies in the serum of patients with suspicion of fungal infection. Nowadays, these tests are mainly used for the diagnosis of endemic mycoses and chronic or allergic forms of aspergillosis. The sensitivity of these methods varies depending on the test (i.e., 59%–88% in chronic pulmonary aspergillosis; Page *et al.*, 2016) and/or the type of disease (i.e., 64%–97% for histoplasmosis; Falci *et al.*, 2017).

The main advantage of serological tests is the requirement of minimally invasive samples. Furthermore, results may be obtained when culture is negative and, if positive, the need of handling potentially infectious fungi is reduced (Kozel and Wickes, 2014). However, sensitivity is very limited in immunosuppressed patients due to the low antibody response and interpretation of serological results could be challenging since seropositivity remains long time after disease (Ramanan *et al.*, 2017; Richardson and Page, 2018).

Antigen Detection

The detection of fungal antigens in human body fluids has revolutionized the early diagnosis of invasive fungal infections. Several tests based on the detection of these antigens have been commercialized and included in the revised EORTC/MSG criteria for diagnosis of IFIs (Donnelly *et al.*, 2019). **Table 1** summarizes main characteristics, sensitivity, and limitations of each technique.

Detection of 1,3-β-D-glucan (BDG) for diagnosis of fungal infection

BDG is a component of fungal wall, except in *Cryptococcus* spp. and Mucorales, which is released during infection. There are several commercial assays developed but only Fungitell (Associates of Cape Code, Inc., East Falmouth, MA, USA), a chromogenic quantitative enzyme immunoassay (EIA), has been approved by FDA (Theel and Doern, 2013). The detection of this antigen in serum has a great sensitivity in some important fungal infections as invasive candidiasis (IC), invasive aspergillosis (IA), and *Pneumocystis* Pneumonia (PCP) (Ambasta *et al.*, 2015; Cuenca-Estrella *et al.*, 2012; Karageorgopoulos *et al.*, 2011). However, there are many factors affecting the performance of the technique: (1) It cannot discriminate at species level, (2) causes a high rate of false positive results, (3) is presented in a closed commercial system, which commonly implies the shipment of sample to a reference laboratory, and (4) the efficiency of this test is reduced in solid organ transplant (SOT) patients (Cuenca-Estrella *et al.*, 2011; Lu *et al.*, 2011; Perfect, 2013).

Detection of galactomannan (GM) and glycoproteins for diagnosis of IA

The detection of GM, a polysaccharide present in the cell wall of most *Aspergillus* spp. which is secreted during infection, is commonly performed by using the commercial assay Platelia *Aspergillus* Ag EIA (Bio-Rad, Marnes-la-Coquette, FR). This technique has a great efficiency in serum of patients with hematological malignancies or hematopoietic stem cell transplant (HSCT) recipients, but sensitivity is drastically reduced in SOT patients, especially lung transplant recipients. In addition to serum, GM can also be efficiently detected in other samples such as bronchoalveolar lavage fluid (BALF), advancing IA diagnosis (Ambasta *et al.*, 2015; Steinbach, 2015). Despite these strengths, several limitations, such as cross-reactivity with other fungi and false positive (i.e., antibiotic treatment) or false negative results (i.e., antifungal treatment), deeply affect sensitivity and specificity of the technique (Lease and Alexander, 2011).

Last years, a new diagnostic technology has been developed based on immuno-chromatographic assays performed in lateral-flow devices (LFD). These "pregnancy tests-like" assays are cheap, sensitive, easy to use, and have low turnaround time (Prattes *et al.*, 2016). The *Aspergillus*-specific LFD test is based on the detection of an extracellular glycoprotein (different from GM) secreted by *Aspergillus* spp. constitutively during active growth (Thornton, 2008). This test can be performed in serum and BALF samples with comparable sensitivity to GM detection and higher specificity, although it shows cross-reaction with *Penicillium* spp. (Heldt and Hoenigl, 2017).

Table 1 Characteristics of the most used antigen detection methods for the diagnosis of fungal infections

Antigen	*Organism*	*Sensitivity*	*Limitations*
BDG	Panfungal (except *Cryptococcus* spp. and Mucorales)	IC > 65% (S) IA 50–80% (S) PCP 95%–96% (S)	No discrimination at species level False positives BDG absent in *Cryptococcus* spp. and Mucorales Closed commercial system Low efficiency in SOT patients (66%)
GM	*Aspergillus* spp.	71% (S) > 70% (BALF)	False positives (cross-reactivity with other fungi, antibiotics, etc.) False negatives (antifungal therapy) Low efficiency in SOT patients (22%)
LFDA *Aspergillus*	*Aspergillus* spp.	68% (S) 86% (BALF)	Cross-reactivity with *Penicillium* spp. Pre-treatment (S) Semi-quantitative test
CPA	*Cryptococcus* spp.	70% (S) 90% (CSF)	Cross-reactivity with other fungi Low efficiency in pulmonary forms (25%–56%)
CPA (LFDA)	*Cryptococcus* spp	≈100% (S/CSF)	Semi-quantitative test
MNN/Anti-MNN Ig	*Candida* spp.	83% (S)	Low efficiency in non-*C.albicans* infections Not included in EORTC/MSG criteria
EFA	*H. capsulatum*	79% (S) 82% (U) 94% (BALF,MVista)	Cross-reactivity with other fungi Low efficiency in pulmonary forms in serum and urine samples Samples are required to be shipped to the company's facilities (MVista tests) Only tested in urine samples (IMMY test for *Histoplama* antigen detection)
	C. immitis	84% (S/U) 93% (CSF)	
	B. dermatitidis	57% (S) 90% (U)	

Source: BALF: Bronchoalveolar lavage fluid; BDG: 1,3-β-D-glucan; CPA: Capsular polysaccharide antigen; CSF: Cerebrospinal fluid; EFA: Endemic fungal antigens; GM: Galactomannan; IA: Invasive aspergillosis; IC: Invasive candidiasis; Ig: Immunoglobulin; MNN: Mannan; PCP: *Pneumocystis*pneumonia; S: Serum; SOT: Solid organ transplant; U: Urine.
Note: Reproduced from Original Work by Clara Valero.

Detection of capsular polysaccharide antigen (CPA) for the diagnosis of cryptococcosis

Latex-agglutination (LA) is the most used method for the detection of the CPA of *Cryptococcus* spp, particularly a component called glucuronoxylomannan (GXM), which is shed into blood and cerebrospinal fluid (CSF) during infection (Yauch *et al.*, 2005). This method can be performed in both serum and CSF being very useful for the diagnosis of cryptococcosis, especially in AIDS patients with cryptococcal meningitis, however the performance is reduced in patients with only pulmonary involvement (Baddley and Dismukes, 2011). An LFD is also available for the detection of CPA of *Cryptococcus* spp. with sensitivity even superior to the LA assay (Nalintya *et al.*, 2016).

Detection of mannan (MNN) for diagnosis of invasive candidiasis

The combined detection of MNN, a highly immunogenic component of *Candida* spp. cell wall that circulates in blood during infection, and anti-MNN Igs can be used for specific detection of *Candida* spp. in serum samples (Sendid *et al.*, 1999). EIAs quantifying MNN and anti-MNN Igs are commercialized as Platelia Candida Ag Plus and Platelia Candida Ab Plus (Bio-Rad, Marnes-la-Coquette, FR) and the combined detection shows acceptable sensitivity for the diagnosis of IC, although it is reduced in non-*albicans Candida* infections (Mikulska *et al.*, 2010).

Detection of endemic fungal antigens (EFA) for the diagnosis of endemic fungal infections

The detection of antigens produced by fungal species causing endemic mycoses such as *H. capsulatum*, *P. brasiliensis*, *C. immitis*, and *B. dermatitidis* represents a breakthrough in the diagnosis of endemic fungal infections.

The *H. capsulatum* polysaccharide antigen can be detected in both serum and urine samples with similar diagnostic value (Fandino-Devia *et al.*, 2016) and it can be performed by using two commercial assays: (1) *Histoplasma* Quantitative EIA Test (MiraVista Diagnostics, Indianapolis, IN, USA), and (2) the *Histoplasma* Antigen EIA kit (IMMY, Norman, OK, USA). MiraVista's test presents great efficiency in serum and urine samples from patients with disseminated infection, but it is reduced in patients with pulmonary forms of the disease (Hage *et al.*, 2015), in which BALF samples are more suitable for diagnosis (Hage *et al.*, 2011).

This test is only performed in MVista's facilities then is not accessible out of USA limiting their use. On the other hand, IMMY's test demonstrates a good agreement with MiraVista's but it has been only tested in urine samples (Falci *et al.*, 2017). Recently, a promising monoclonal *Histoplasma* galactomannan enzyme-linked immunosorbent assay has been developed by IMMY showing great performance and reproducibility (Cáceres *et al.*, 2018). MVista Diagnostics has also developed EIA tests for the diagnosis of coccidioidomycosis and blastomycosis. In the case of the detection of *Coccidioides* spp. antigen, it can be performed, in addition to serum and urine, in CSF samples. This means a great advantage in the diagnosis of coccidioidal meningitis, although sensitivity is lower in single respiratory infections (Kassis *et al.*, 2015). Finally, sensitivity for the detection of *B. dermatitidis* antigen varies deeply depending on the specimen employed, being more efficient in urine samples, however sample pre-treatment can enhance diagnostic performance (Smith and Gauthier, 2015). The main limitation of many of these assays is the high degree of cross-reaction with other fungi (Malcolm and Chin-Hong, 2013).

DNA-Based Methods/Molecular Methods

Methods based on the detection of DNA have been developed recently for the diagnosis of fungal infections. Their powerful advantages over conventional methods (simplicity, high specificity, and short turnaround time) have made them potential candidates to replace those traditional diagnostic assays (Arvanitis *et al.*, 2014).

PCR-based methods

The most relevant DNA detection methods are those that implement polymerase chain reaction (PCR). This technique is playing an increasingly role in the routine diagnostic of several laboratories of clinical microbiology as they offer a rapid, specific and sensitive detection of pathogens (Buchan and Ledeboer, 2014). Furthermore, quantitative or real-time PCR (qPCR) assays allow for determining the fungal DNA burden in patients by using non-specific DNA-binding dyes or fluorescently labeled probes (Kozel and Wickes, 2014). Despite of the advantages, most reported assays have been developed in house by different laboratories and just a reduced number of tests have been commercialized. Moreover, these techniques have several limitations: (1) The contamination risk of samples or the technique itself by environmental fungi, (2) the moderate amount of DNA in low invasive samples such as blood and serum, (3) the lack of standardization, and (4) the low availability of widely validated commercial systems (Alanio and Bretagne, 2017; Khot and Fredricks, 2009). Due to all these disadvantages, PCR has classically been excluded of the EORTC/MSG criteria for IFI diagnosis, however all efforts made last years have achieved its inclusion in the last update (Donnelly *et al.*, 2019).

Nowadays, there are two approaches for the diagnosis of fungal infections by using PCR:

(1) Specific qPCR assays: The greatest efforts have been made in the detection of species causing important fungal infections such as IC, AI, and PCP (**Fig. 3**). However, there are considerably fewer qPCR studies for the detection of fungi causing neglected infections as histoplasmosis or emerging fungal infections as those caused by *Scedosporium* spp. or *Fusarium* spp. among others. Most developed methods target specific multicopy regions of the ribosomal DNA and are performed by using conventional or real-time PCR. One interesting approach based on specific qPCR assays is multiplex qPCR (MRT-qPCR), since it allows for detecting several pathogens in the same reaction tube. These assays are very useful for the differential diagnosis of fungal species with non-specific symptomatology (Gago *et al.*, 2014) or with different antifungal susceptibility profiles (Alonso *et al.*, 2012; Bernal-Martinez *et al.*, 2013; Foongladda *et al.*, 2014), and also allows for the detection of mixed infections. Recently, many efforts have been made in Europe, to try to achieve a consensus in the development of PCR diagnostic methods with the purpose of being included in the international guidelines (FPCRI; see "Relevant Websites section").

(2) Panfungal or broad-range PCR assays (**Fig. 3**): These assays are very useful when there is not a clear suspicion of the fungus involved in the infection. Universal primers able to amplify any fungal species are used to detect fungal DNA in the clinical sample. Sensitivity values of these assays in paraffin-embedded biopsies range between 86%–94% in culture-proven cases and 64%–89% in cases confirmed by histopathology (Powers-Fletcher and Hanson, 2016). One of the main limitations of these techniques is the delay in response time due to the requirement of sequencing the amplified panfungal PCR product. Furthermore, specimen contamination and commensal fungi colonization of body sites of sampling could deeply affect the specificity of the technique (Khot and Fredricks, 2009). Some recent studies have developed other panfungal methods directed to reduce response time. One of these methods complements the use of panfungal primers with a melting curve analysis and probes able to detect genera or the group of fungi (Valero *et al.*, 2016). Others involve different identification techniques such as DNA microarray (Sakai *et al.*, 2014), electrospray-ionization MS analysis (Massire *et al.*, 2013), or T2 magnetic resonance (Pfaller *et al.*, 2016).

Non-PCR based methods

Alternative DNA-based methods for the diagnosis and identification on fungal infections are those based on in situ hybridization of labeled probes. The PNA-FISH (peptide nucleic acid-fluorescence in situ hybridization) method employs fluorescently labeled probes that bind complementary to fungal sequences and has been often used to identify *Candida* spp. from positive blood culture bottles. The probe color detected gives a preliminary indication of fluconazole susceptibility associated to the species (Klingspor *et al.*, 2018).

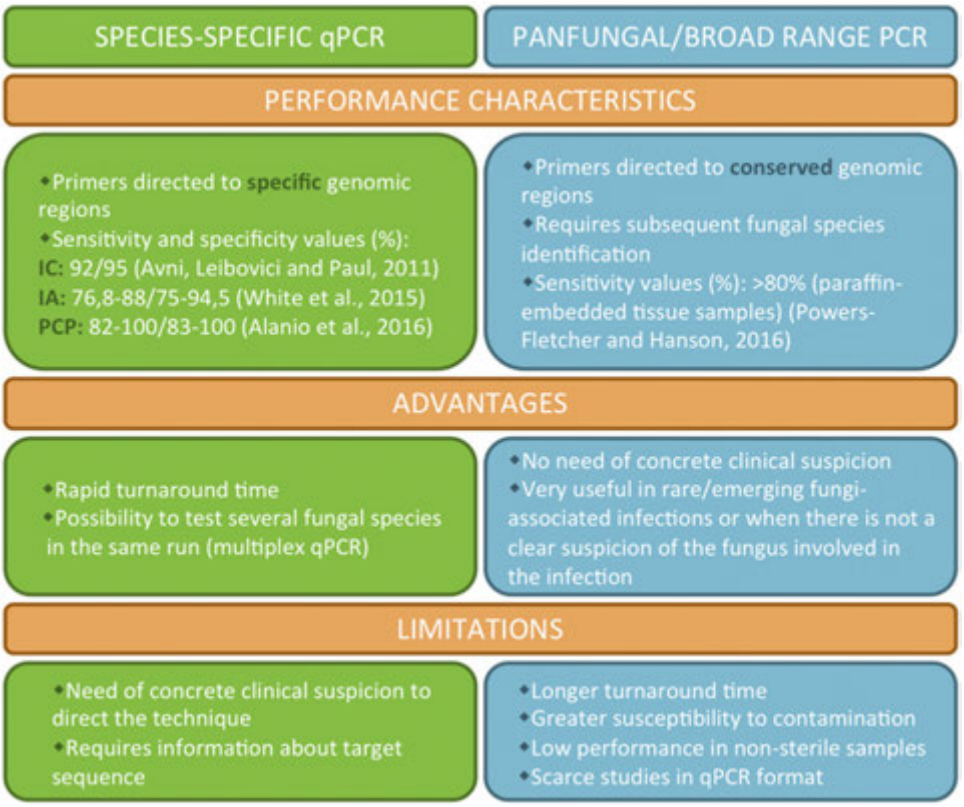

Fig. 3 Comparative scheme describing main characteristics, advantages, and limitations of both specific and panfungal PCR diagnostic techniques.

Similarly, chemiluminescent DNA probes are commercially available for the identification of endemic fungal species from positive cultures (Gomez, 2014) or tissues (Guarner and Brandt, 2011).

Future Directions and Conclusion

The aim of this chapter is to summarize those diagnostic methods commonly used for the diagnosis of fungal infections. As stated before, diagnosis of these infections is difficult and the combination of conventional and new techniques is essential for an early diagnosis. In addition, it is important to know the local epidemiology and prevalence of fungal infections as it can help choose the most appropriate diagnostic method that should be used (Lass-Florl, 2017).

Nevertheless, fungal diagnostics is an area under continuous evolution since novel tests and new applications of already existing techniques are in constant development. In that context, the search for new biomarkers (both from the fungal pathogen and the human host) is a main objective for researchers. The detection of new fungal cell wall polysaccharides and second metabolites such as mycotoxins (Escriva *et al.*, 2017) and volatile organic compounds (VOCs) (Acharige *et al.*, 2018) have been reported in last years. Regarding already established techniques, the application of MALDI-ToF MS technology directly in clinical samples (Rizzato *et al.*, 2015) have also gained great interest. In the case of the study of host factors, the increasing use of proteomic and bioinformatic approaches has promoted the research in this area with the aim of being able to predict susceptibility and risk of acquiring fungal infections as well as offering a personalized diagnosis (Lydon *et al.*, 2018). Although many work should be done in this sense in coming years, several efforts have been already made in concern of some fungal infections such as IA (Oliveira-Coelho *et al.*, 2015).

Alternatively, the development of "point of care" (POC) techniques have increased last years as an attempt to obtain robust and cheap methods to be used in less favored regions of the world. LFDs for the detection of *Cryptococcus* and *Aspergillus* are commercially available as previously mentioned, and one more for the diagnosis of histoplasmosis has just been released by MiraVista Diagnostics (Cáceres *et al.*, 2019).

In summary, fungal infections are currently recognized as a cause of significant morbidity and mortality, especially among immunocompromised patients. Consequently, a proper and early diagnosis is essential to establish a suitable antifungal therapy that could allow for improving patient's outcome. Although the ideal diagnostic method or strategy is still missing, several efforts have been made in last years, which have led to a new generation of diagnostic tools. Undoubtedly, so much remains to be done in this context, from widely standardizing and validating already developed tests through large multicenter studies to exploring

combination testing or adapting antigen and DNA detection methods to POC platforms. All this efforts will end in the construction of a proper panel of fungal diagnostic methods that could be available wherever it is required.

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Relevant Websites

https://www.isham.org/working-groups/european-aspergillus-pcr-initiative-eapcri
Fungal PCRInitiative (FPCRI).
https://www.gaffi.org
Gaffi - Global Action Fund for Fungal Infections.

Commensal to Pathogen Transition of *Candida albicans*

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Glossary

Attenuated (virulence) Reduced capacity to induce damage/disease.
Biofilm A structured community of microorganisms that adhere to each other and are commonly embedded within a self-produced extracellular matrix composed of polysaccharides, proteins, and DNA.
Candidiasis Infections caused by *Candida* species.
Colonization Growth (or prolonged survival) of microorganisms in a distinct (host) niche.
Commensal Microorganism that growths within a host without (negatively) affecting the host species.
Dysbiosis Disturbance of the microbiota leading to microbial imbalance and commonly reduced species diversity; often associated with increased abundance of a few taxa or species.
Filamentation The production of filamentous growth forms (hypha, pseudohypha).
Hypha Long, filamentous fungal cells, characterized by directed growth at the tip, branching, parallel cell walls, and the absence of constriction at the septa separating individual cells.
Hypha-associated Genes or proteins that are usually only expressed by hypha and not by yeast cells.
Inoculation Introduction of a microorganism into a host or growth medium.
Macronutrients Nutrients that provide energy, for example, carbohydrates and amino acids.
Microbiota Ecological community of commensal, symbiotic, and pathogenic microorganisms found at a distinct site/within a distinct host niche.
Micronutrients Nutrients required by organisms throughout life in small quantities as necessary cofactors for metabolism, for example, trace minerals and vitamins.
Morphogenesis The process by which an organism develops its shape. In the context of *Candida* it usually refers to the formation of hypha from yeast cells, whereas the term "reverse morphogenesis" is sometimes used to describe the formation of yeast cells from filamentous growth forms.
Morphotype Morphological growth form; *C. albicans* has different morphotypes, e.g., yeast, pseudohypha, and hypha.
Mycobiota Ecological community of fungi found at a distinct site/within a distinct host niche.
Nosocomial infection An infection contracted by a patient while under medical care.
Opportunistic infection An infection that usually does not occur in healthy hosts but only if predisposing factors (weakened immune system, breached epithelial barriers) are present that give the pathogen the opportunity to establish an infection.
Pathobiont A microorganism that under normal circumstances lives as a commensal but has the potential to cause disease.
Pathogen A microorganism that causes disease.
Pathogenesis The mechanisms leading to disease.
Pseudohypha Elongated fungal cells, characterized by constrictions between individual cells.
Pyroptosis A form of programmed cell death of immune cells that occurs most frequently upon infection with intracellular pathogens and leads to the release pro-inflammatory cytokines, thereby contributing to inflammation.
Virulence The ability of microorganisms to cause damage to a host.
Virulence factor Gene or attribute that contribute to the virulence of a pathogen.

Introduction: *Candida albicans* as Commensal and Pathogen

Candida albicans is an archetypical opportunistic pathogen: While it is the most common cause of systemic fungal infections, it is also carried by two out of three humans on mucosal membranes without causing any damage (Richardson and Rautemaa, 2009; Thewes and Hube, 2008; Kett *et al.*, 2011; Odds, 1987; Pfaller and Diekema, 2007). As a commensal, the fungus is commonly found within the oral cavity, gastrointestinal tract (GI tract), and on the urogenital mucosa. On all sites, it has to adapt to the local environment that differs significantly between various anatomical locations. Environmental conditions are on the one hand defined by properties of the local mucosa. On the other hand, *C. albicans* has to establish its niche within the complex microbiota that also varies depending on the anatomical site (Morgan *et al.*, 2013). Adaptation to these different niches requires versatile nutrient acquisition, metabolic flexibility, and stress resistance.

C. albicans' adaptability contributes to its success as an opportunistic pathogen. If the balance between host defense systems, microbiota, and *C. albicans* is disturbed, for example, due to immunosuppression, impairment of mucosal barrier integrity, or disturbance of the bacterial microbiota by antibiotics, *C. albicans* can cause both local and disseminated infections (Goncalves *et al.*, 2016; Kullberg and Arendrup, 2015; Suleyman and Alangaden, 2016). Mucosal infections can occur at all sites that are colonized with *C. albicans* and are often associated with specific risk factors that do not predispose to infections at other sites: For example, oropharyngeal candidiasis is very common in AIDS patients but these patients do not have an increased risk for other

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forms of candidiasis (Fidel, 2011). Chronic mucocutaneous candidiasis is often associated with mutations that impair Th17 immunity; although continuously suffering from invasive local candidiasis, whether these patients also have an increased risk for disseminated infection depends on the exact kind of mutation in the IL17 signaling pathway (Okada *et al.*, 2016). These examples illustrate the complexity and niche-specificity that applies to the host-pathogen interactions that control the commensal-to-pathogen shift in *C. albicans*.

In the following parts, we will summarize the current knowledge on which features make *C. albicans* a successful commensal focusing on the GI tract, and discussing some of the main attributes that contribute to its success as an opportunistic pathogen.

Candida albicans as a Commensal: Living with the Host and Microbial Neighbors

As mentioned above, both the environmental conditions and the present microbiota vary between different mucosal sites. Consequently, different aspects of *C. albicans* physiology contribute to varying extend to fungal survival in the different mucosal niches. In the following part, we focus on the GI tract; for further information on *C. albicans* as a commensal and pathogen in the oral and vaginal niche we refer the reader to recent comprehensive reviews (Cassone, 2015; Donders and Sobel, 2017; Goncalves *et al.*, 2016; Hebecker *et al.*, 2014; Lalla *et al.*, 2013; Peters *et al.*, 2014; Singh *et al.*, 2014; Bradford and Ravel, 2017; Yano *et al.*, 2018; Swidergall and Filler, 2017).

Fungal Factors That Contribute to *C. albicans* Survival in the Human Gut

In the human body, the GI tract is not only the major reservoir of *C. albicans*, but also the dominant source for candidiasis, a life-threatening systemic infection caused by this yeast (Mittal and Coopersmith, 2014). Research in fact provides evidence that the majority of systemic *Candida* infections are endogenous, so patients are infected with their very own *C. albicans* strains (Miranda *et al.*, 2009). As fungi were long underrepresented in microbiome studies due to technical reasons, the exact densities of fungi in the GI tract and other body niches are still poorly understood. However, there is increasing interest toward the entity of fungi living on and in the human body, termed mycobiota, and its interaction with the human host (Underhill and Iliev, 2014). In approximation, recent studies estimate at least 0.1% of all microorganisms (so 10^{11} out of 10^{14}) in the human GI tract to be fungi (Qin *et al.*, 2010; Ianiro *et al.*, 2016). In this context, it is interesting that *C. albicans* is among the few microbial species enriched in the patients upon intensive care, while the overall microbial diversity under these circumstances is decreased (Zaborin *et al.*, 2014). *Candida* colonization is also more common among patient with GI tract pathologies, like inflammatory bowel disease (IBD) or Crohn's disease (Kumamoto, 2011; Iliev *et al.*, 2012), and might contribute to pathogenesis by promoting inflammation following fungal overgrowth (Kumamoto, 2011). However, recent studies demonstrated also beneficial effects of fungal colonization, including but not limited to *C. albicans*, on the immune system and immunological disorders such as asthma (Jiang *et al.*, 2017; Wheeler *et al.*, 2016) and reviewed in Kumamoto (2016). Thus, even though the role of fungi as colonizers in the gut is not yet fully understood, it appears likely that fungi are important players in the intestinal microbiota, with the consequences of fungal colonization depending on underlying diseases or dysbiosis.

Antibiotic treatment reducing the number of bacteria is associated with increased fungal colonization (Underhill and Iliev, 2014). This indicates that *C. albicans* is well adapted to the GI tract and can grow to high densities if bacterial competition is reduced. This is supported by a recent study in germ free mice, in which a single inoculation results in stable, high level intestinal colonization (Böhm *et al.*, 2017). While colonization alone does not lead to infection (Koh *et al.*, 2008; Böhm *et al.*, 2017), increased colonization with *C. albicans* constitutes a risk factor for candidiasis (Muskett *et al.*, 2011). Thus, recent research increasingly focuses on the properties that allow the fungus to colonize mucosal surfaces and especially the gut (reviewed in Prieto *et al.*, 2016; Perez, 2019).

Within the length of the GI tract, and across the diameter of the intestine, several environmental parameters, including pH, oxygen and nutrient availability, and concentrations of hydrolytic enzymes and antimicrobial peptides, vary significantly (Zheng *et al.*, 2015a; Khutoryanskiy, 2015). Some of these factors are interconnected: The pH for instance ranges from highly acidic in the stomach to alkaline in the small intestine. It influences availability of micronutrients, such as iron, the proton gradient, that is important for nutrient uptake, and affects the efficacy of fungal enzymes (Davis, 2009). *C. albicans* can grow within a broad pH range, mediated by the sensing of environmental pH via the plasma membrane receptors Rim21 and Dfg16. The associated signaling cascade leads to activation of the transcription factor Rim101 at neutral to alkaline pH. Other factors involved in the response to changing pH are the cell wall β-glycosidases Phr1 and Phr2, Mds3, and calcineurin. Additionally, *C. albicans* is able to actively increase the local pH by metabolizing amino acids or polyamins, leading to the production and release of ammonia (Vylkova *et al.*, 2011). Interestingly, the pH affects the exposure of β-glucan, an important Pathogen-associated Molecular Pattern, on the cells surface of *C. albicans* and thereby influences fungal recognition by immune cells (Sherrington *et al.*, 2017). Similarly, β-glucan exposure is reduced at hypoxia (Pradhan *et al.*, 2018; Lopes *et al.*, 2018). Reduced exposure of β-glucan supports competitive fitness of *C. albicans* during intestinal colonization (Sem *et al.*, 2016), suggesting that environmental cues affecting cell wall architecture regulate commensal behavior of the fungus. In addition, it was recently shown that production of inflammatory mediators by the fungus contributes to intestinal fitness in the presences of phagocytes (Tan *et al.*, 2019). Together, this suggests an active role of the host immune system in shaping the mycobiota, even in the absence of inflammation. Furthermore, *C. albicans*

colonization affects not only the mucosal but also systemic immune responses, especially the response of innate immune cells to invading pathogens (Tso *et al.*, 2018; Shao *et al.*, 2019).

Although the gastrointestinal tract might appear rich in nutrients, specific types of carbon and nitrogen sources can be scarce in distinct microniches (recently reviewed in Fischbach and Sonnenburg, 2011; Flint *et al.*, 2012). Furthermore, *C. albicans* has to compete with other members of the microbiota for available nutrients. The metabolism of *C. albicans* is very versatile and the fungus can use a wide range of both carbon and nitrogen sources (Brown *et al.*, 2014; Ene *et al.*, 2014). Peptides, amino acids, sugars, and organic acids can be taken up by specific transporters and used to produce energy or as building blocks. As they commonly need to be released or extracted from macromolecules, *C. albicans* possesses a number of proteases and lipases to support nutrient liberation (Polke *et al.*, 2015). Typically, those enzymes include secreted aspartic proteases (Saps), lipases (Lips), and Plb phospholipases as well as several enzymes for the degradation of polysaccharides (Klis and Brul, 2015).

Overall, the gene expression of *C. albicans* colonizing the mouse gut appears to be distinct from other profiles. By comparing *C. albicans* cells invading host tissue during disease with *C. albicans* colonizing the mouse cecum, interesting similarities were revealed, but also niche- and mode-specific differences were detected (Thewes *et al.*, 2007; Walker *et al.*, 2009; Zakikhany *et al.*, 2007; Rosenbach *et al.*, 2010). The commensal gene expression profiles shared similarities with both, logarithmically growing cells and cells in stationary phase under standard in vitro conditions (Rosenbach *et al.*, 2010). The glucose metabolism was induced even though the gut is considered to be a low-glucose niche (Rosenbach *et al.*, 2010). Remarkably, although *C. albicans* grows predominantly as yeast in the mouse cecum (see also below), several genes that encode certain adhesins, were also expressed despite that they were previously thought to be hypha-associated (Herwald and Kumamoto, 2014; Rosenbach *et al.*, 2010).

So far, studies aiming at experimentally testing the contribution of factors to *C. albicans* fitness as a commensal of the gastrointestinal tract have focused mainly on transcription factors (TFs) due to their central role for controlling expression of physiological traits. One well studied TF is Efg1 that is involved in filament formation (Stoldt *et al.*, 1997). *EFG1*-deficient *C. albicans* strains are unable to form filaments under many in vitro conditions (Stoldt *et al.*, 1997). In colonization experiments using mice, Efg1 influences *C. albicans* survival in the GI tract as *EFG1*-deficient strains were found to be more efficient colonizers of the mouse gut (Pierce *et al.*, 2013). The stress response in the cecum was also induced in an Efg1-dependent manner (Pierce *et al.*, 2013). Further TFs required for *C. albicans* filamentation under certain laboratory conditions and important for GI tract colonization are Cph2 and Tec1 (Rosenbach *et al.*, 2010). Another transcription factor involved in the intestinal commensal state is Efh1, a paralog of *EFG1* whose deletion does not affect filamentation under in vitro conditions (Doedt *et al.*, 2004). Efh1 appears to actively promote *C. albicans* commensalism but is dispensable for systemic infections (White *et al.*, 2007). Using a signature-tagged mutagenesis approach, Pérez *et al.* tested a library of 77 TFs that did not affect growth in vitro to assess their ability to colonize the mouse gut. Thereby they identified six factors that contributed to colonization in antibiotic-treated mice: *TYE7*, *RTG1*, *RTG3*, *LYS144*, *HMS1*, and *orf19.3625* (Perez *et al.*, 2013). Rtg1, Rtg3, Tye7, and Lys144 affect expression of genes involved in nutrient acquisition and metabolism (Perez and Johnson, 2013; Rosenbach *et al.*, 2010). *ZCF8*, *ZFU2* and *TRY4* were recently identified as additional factors in a similar screening conducted in germ free mice (Böhm *et al.*, 2017). An alternative approach, using overexpression strains, identified *CRZ2*, a TF affecting both expression of cell wall-modifying enzymes and promoting resistance to acidic pH and bile salts (Znaidi *et al.*, 2018). Furthermore, *C. albicans* mutants lacking the gene *PHO4*, encoding a transcription factor involved in phosphate metabolism, were outcompeted by the wild type during colonization (Urrialde *et al.*, 2016). These results thus underline the role of metabolic adaptation and nutrient acquisition for fitness in the gut. Finally, the kinase Hog1, a central factor in the general stress response of *C. albicans*, was shown to contribute to intestinal fitness (Prieto *et al.*, 2014), indicating that *C. albicans* is exposed to stressful conditions in this environment.

The current knowledge implies that morphogenesis, metabolism, and stress response affect *C. albicans* survival in the gastrointestinal tract (Perez, 2019). This picture is certainly still incomplete and ongoing research will likely provide further information on distinct factors influencing the life of *C. albicans* as a gut commensal in the near future.

Morphology of *C. albicans* in the Gut

One of the most remarkable features of *C. albicans* is its morphological flexibility (Noble *et al.*, 2016). This includes growth as typical round, budding yeasts and filamentous growth either as long true hyphae or pseudohyphae. Both morphotypes are essential for virulence: Yeasts are highly proliferative and thought to be important for dissemination, while hyphae are important for invasion and tissue destruction (Gow *et al.*, 2002; Whiteway and Oberholzer, 2004; Jacobsen *et al.*, 2012). While yeasts, pseudohyphae, and hyphae are isolated from sites of infection, filamentous forms of *C. albicans* are found only rarely during gut colonization (Noble *et al.*, 2016; Kayser, 2010; White *et al.*, 2007). As the intestinal environment provides strong triggers for filamentation (e.g. 37°C, high CO_2, and peptidoglycan shed by bacteria) (Sudbery, 2011; Noble *et al.*, 2016; Wang and Xu, 2008), the absence of prominent filamentation is unlikely due to a lack of hypha induction but more likely mediated by other environmental factors. Furthermore, increasing evidence indicates that growth in the yeast form is favoring intestinal colonization (Böhm *et al.*, 2017).

In addition to normal yeasts, also known as white a/α cells, a gut-specific yeast-like morphotype termed GUT (gastrointestinally-induced transition) was recently identified in this particular niche (Pande *et al.*, 2013). GUT cells are phenotypically and metabolically distinct from other morphotypes and can be produced by passaging *C. albicans* through the murine gastrointestinal tract. GUT cells are characterized by overexpressing *WOR1*, encoding a transcription factor that is not expressed in white

cells and controls the white-opaque switching important for mating (Huang *et al.*, 2006) However, GUT cells are distinct from mating-competent opaque cells and remain heterozygous at the mating type locus. Compared to other yeast forms, GUT cells have an elongated shape with smooth surface leading to altered colony morphology. GUT cells outcompete white cells in murine colonization, possibly due to an adapted metabolism that facilitates highly efficient utilization of the nutrients available in the gut (Pande *et al.*, 2013).

Animal Models Used to Investigate *C. albicans* Commensalism in the Gut

Currently, our knowledge on specific factors required for commensalism in the gut is still limited and nearly exclusively deduced from studies in mice, and a few studies in rats (Koh, 2013). Still, there are significant differences between humans and mice that need to be kept in mind when transferring knowledge from one organism to the other. Differences that might influence pathogenesis of infections are, for example, the composition of blood leukocytes, the functionality of neutrophils, or the inflammatory response (Mestas and Hughes, 2004; Seok *et al.*, 2013; Ermert *et al.*, 2013; Warren, 2009). At the commensal state of *Candida*, there are also some factors to be considered when mimicking men in mice. Laboratory mice are usually not colonized with *C. albicans*, even though other *Candida* species such as *C. krusei* can be present (Dollive *et al.*, 2013). This might point to a distinct role of the murine gut microbiota mediating colonization resistance toward *C. albicans*. However, differences in exposure might affect these findings. Laboratory mice kept under hygienic conditions are probably more likely to harbor environmentally-derived yeasts (such as *C. krusei*) than *C. albicans* that is only found associated with warm-blooded hosts (Odds, 1987). Furthermore, timing of exposure might be relevant. Humans are believed to be colonized during birth or the first days of life when the gut microbiota is in its earliest stages. Indeed, also infant mice are more susceptible both to colonization and spontaneous dissemination of *C. albicans* (Guentzel and Herrera, 1982; Pope *et al.*, 1979). Furthermore, food composition affects colonization resistance (Kadosh *et al.*, 2016; Gunsalus *et al.*, 2016).

In general, however, many studies have shown that the gut microbiota of adult mice and other rodents mediates colonization resistance toward *C. albicans* (Kennedy and Volz, 1985a,b; Koh, 2013; Fan *et al.*, 2015). Therefore, depletion of bacteria is necessary to facilitate stable colonization of adult mice with *C. albicans* (see **Fig. 1**). Experimental procedures published in different studies vary in the choice of antibiotics and *C. albicans* inoculation regiment (selected recent examples in **Table 1**), but mutually a combination of antibiotics is used. The infectious dose appears to have less influence on long-term colonization as proliferation to high, stable colonization levels occurs within a few days, even after inoculation of a minimal dose of 100 *Candida* cells (Prieto and Pla, 2015). Importantly, colonization experiments with single *C. albicans* mutants might not always reveal the influence of genes on colonization efficiency and competition experiments might be required to detect fitness defects (Prieto *et al.*, 2014).

According to our current understanding, *C. albicans* generally does not spontaneously disseminate in significant numbers from the gut to the blood stream of adult mice (Ekenna and Sherertz, 1987; Koh, 2013). In order to achieve translocation and disseminated infection, impairment of the epithelial barrier and suppression of the innate immune system is required (Koh *et al.*, 2008). This can be achieved, e.g., by induction of colitis using the detergent dextran sulfate sodium and selective depletion of neutrophils. Alternatively, treatment with cyclophosphamide, a chemotherapeutic drug leading to the death of fast-proliferating cells, results in a combination of both, reduced barrier function of the enteric epithelium and immunosuppression via leukopenia (see **Fig. 1**). Consequently, mice treated with cyclophosphamide are more likely to develop disseminating candidiasis originating from the gut (Ekenna and Sherertz, 1987). Murine sepsis models based on mechanical disruption of the gut, such as cecal ligation and puncture, are not

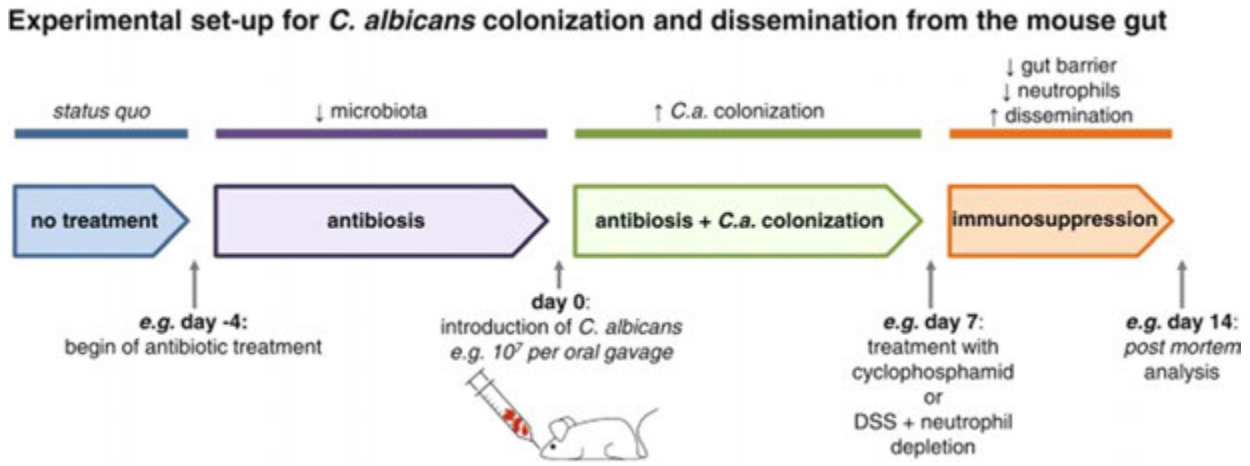

Fig. 1 Set-up for *C. albicans* colonization and dissemination from the mouse gut. Widely-used experimental strategies to reduce the pre-existing microbiota (blue), to colonize with *C. albicans* (C.a., green) and to induce dissemination (orange) from the mouse gut. Illustration by I.D. Jacobsen.

Table 1 Overview of colonization strategies with antibiosis prior to *Candida albicans* inoculation of mice

Antibiosis	*Inoculum*	*C. a. strain*	*CFUs/g feces*	*Time point p.i.*	*References*
Bacitracin Streptomycin Gentamycin	Oral gavage, 10^7 cells	CAF2	10^6–10^7	3 d	(Wiesner *et al.*, 2001)
Tetracycline Streptomycin Gentamycin	Oral gavage, 5 x 10^7 cells	DAY185	10^5	18 d	(White *et al.*, 2007)
Streptomycin Penicillin G	Drinking water, 10^7 CFUs/mL	SC5314, CAF2–1	10^7	21 d	(Koh *et al.*, 2008)
Tetracycline Streptomycin Gentamycin	Oral gavage, 5 $\times$ 10^7 cells	CKY101	10^7	3 d	(Pierce *et al.*, 2013)
Streptomycin Bacitracin Gentamycin	Oral gavage, 10^7 cells	CAF2	10^6–10^7	40 d	(Prieto *et al.*, 2014)
Streptomycin Bacitracin Gentamycin	Oral gavage, 10^6 cells	CAF2	10^7	3 d	(Prieto and Pla, 2015)
Streptomycin Penicillin	Drinking water, 10^7 CFUs/mL	SC5314	10^6–10^7	10 d	(Vautier *et al.*, 2015)

Note: Colonization strategies presented in different publication varied in choice of antibiotics, application procedure, dosage of *C. albicans* (*C.a.*) inoculum, and stability of colonization (by fungal load in feces) as finally the time point of analysis.
Source: Contributed by A. Elisabeth Greßler.

commonly used as a model for gut-derived candidiasis (Toscano *et al.*, 2011; Warren, 2009), but might be an alternative approach. A typical experimental set-up for a colonization-dissemination experiment as currently used in the field is depicted in **Fig. 1**.

Candida albicans as Part of the Microbiota

In addition to immunosuppression and breach of mucosal barriers, the disturbance of the microbiota constitutes a major risk factor for disseminated candidiasis. Long-term antibiotic treatment and malnutrition during intensive medical care are prominent causes of dysbiosis (Zaborin *et al.*, 2014). This indicates that the *C. albicans* population in the gut is usually controlled by commensal bacteria and possibly other fungi colonizing the respective niche in the body. This is mirrored in mouse models of *Candida* gut colonization: As mentioned above, extensive antibiosis prior to introduction of the fungus is necessary to achieve stable colonization with *C. albicans* (Koh, 2013; Fan *et al.*, 2015). Like the bacterial microbiota, the fungal "mycobiota" is more and more appreciated for its immune-modulatory properties, and not only as a source of infection (Gouba and Drancourt, 2015; Suhr and Hallen-Adams, 2015; Underhill and Iliev, 2014). To date, little is known about the exact mechanisms of microbial competition and microbe-microbe interaction and microbe-host cross-talk and how this affects host health and disease development. Yet, recent research provides evidence for an intricate interplay of many actors that exceeds simple competition for resources.

Notably, interactions between microbes are numerous and occur naturally on all surfaces of the human body, i.e., the skin, the lung, the oral cavity, the urogenital tract and the gastro-intestinal tract (Peters *et al.*, 2012; Peleg *et al.*, 2010). The specific nature of these interactions varies depending on the species involved, but it can be expected that some general concepts are conserved. To date only few studies have specifically addressed the interactions of *C. albicans* with intestinal bacteria. Here, we will briefly summarize the current knowledge on *Candida*-bacteria interplay in general (for more detailed information we refer to Polke *et al.* (2015), Allison *et al.* (2016) and Förster *et al.* (2016)).

Candida and bacteria can interact by (1) direct physical contact, (2) communication via released molecules, and (3) indirectly by modification of the environment and the host response. Given the high density of microorganisms on mucosal surfaces, direct contact is highly anticipated. Interestingly, different bacteria have been described form aggregates with *C. albicans* and are often found to bind fungal hyphae better than yeast cells (Peters *et al.*, 2010; Harriott and Noverr, 2010; Schlecht *et al.*, 2015; Brand *et al.*, 2008; Bamford *et al.*, 2009; Adam *et al.*, 2002; Kong *et al.*, 2015; Bagg and Silverwood, 1986; Harriott and Noverr, 2011; Holmes *et al.*, 1996; Jenkinson *et al.*, 1990; Metwalli *et al.*, 2013; Shirtliff *et al.*, 2009). Binding can involve both *C. albicans* and bacterial adhesins as well as mannosylation of fungal surface proteins (Schlecht *et al.*, 2015; Kong *et al.*, 2015; Tati *et al.*, 2016; Bamford *et al.*, 2009; Brady *et al.*, 2010; El-Sabaeny *et al.*, 2000; Holmes *et al.*, 1996; Klotz *et al.*, 2007; Silverman *et al.*, 2010; Wright *et al.*, 2013; Dutton *et al.*, 2014). In the oral cavity, these interactions can promote *C. albicans* colonization and persistence (Cannon and Chaffin, 2001; Shirtliff *et al.*, 2009). Furthermore, adhesion to invading *C. albicans* hyphae can aid bacteria that otherwise would be unable or little efficient to penetrate host cells, as it has been shown for *Staphylococcus aureus* (Kong *et al.*, 2015; Schlecht *et al.*, 2015; Alves *et al.*, 2014). Synergistic interactions of *C. albicans* and

bacteria are also found in mixed species biofilms, an important source of nosocomial infections (Shirtliff *et al.*, 2009; Hirota *et al.*, 2017; Taff *et al.*, 2013). Bacteria, including species present in the gut such as *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, or *Salmonella typhimurium*, have been found to also affect filamentation of *C. albicans*, an effect often mediated by small, secreted signaling molecules (Hogan *et al.*, 2004; Brand *et al.*, 2008; Peleg *et al.*, 2008; Kim and Mylonakis, 2011; Cruz *et al.*, 2013; van Leeuwen *et al.*, 2016; Graham *et al.*, 2017). Most of these interactions negatively affect hypha formation and it is thus tempting to speculate that it is the microbiota that keeps *C. albicans* in the less invasive yeast form in the gut (Förster *et al.*, 2016). Reducing bacterial microbiota by antibiotics might relief this restriction and thereby promote filamentation and invasion; however, yeasts are the dominant morphotypes of *C. albicans* also in the gut of mono-colonized germ free mice, indicated that interactions with bacteria are not necessary to promote this morphology in the intestine (Böhm *et al.*, 2017).

Recent studies indicate that the human gut microbiota is very diverse with more than 1000 bacterial species and up to 75 fungal genera described (Qin *et al.*, 2010; Mar Rodriguez *et al.*, 2015). It furthermore becomes increasingly clear that a balanced composition of bacteria and fungi is correlated to intestinal health, while dysbiosis is accompanied with a range of diseases (Wheeler *et al.*, 2016; Sokol *et al.*, 2016; Mittal and Coopersmith, 2014; Lagunes and Rello, 2016). The composition of the microbiota affects *C. albicans* survival in the gut and non-pathogenic gut-residing bacteria, i.e., Firmicutes and Bacteroidetes, are involved in maintaining *C. albicans* colonization resistance in mice (Fan *et al.*, 2015). One specific member of this group, *Bacteroides thetaiotaomicron*, was demonstrated to mediate this effect by inducing the production of host antimicrobial effectors (Fan *et al.*, 2015). Another group of bacteria with antagonistic relations to *C. albicans* are lactobacilli. Lactobacilli negatively affect *C. albicans* colonization both by direct mechanisms, modification of the environment, and indirectly via cross-talk with host cells (Wynne *et al.*, 2004; Romani *et al.*, 2015; Kelly *et al.*, 2015; Noverr and Huffnagle, 2004; Graf *et al.*, 2019). While not being the only source, lactobacilli are known for their capacity to produce short-chain fatty acids (SCFAs) in the gut. SCFAs were demonstrated to increase barrier function of the gut epithelium and inhibit *C. albicans* filamentation (Kelly *et al.*, 2015; Noverr and Huffnagle, 2004) and this mechanism might also contribute to colonization resistance and yeast growth.

Notably, while *C. albicans* is clearly affected by bacteria in the gut, the fungus likewise affects composition of the bacterial community and bacterial virulence. In mice, the introduction of *C. albicans* affects the composition and development of the intestinal microbiota after antibiotic depletion, leading to an enrichment of *Bacteroidetes* and *E. faecalis*, but slower regrowth of lactobacilli (Erb Downward *et al.*, 2013). The fungus is also associated with expansion of enterococci in the oral cavity (Bertolini *et al.*, 2019). *C. albicans* furthermore dampens the virulence of *P. aeruginosa* in a colonization-dissemination model by reducing the expression of bacterial virulence factors (Lopez-Medina *et al.*, 2015). In contrast, by modification of environmental pH, *C. albicans* leads to increased expression of *Staphylococcus aureus* virulence factors (Todd *et al.*, 2019a,b). In addition, *C. albicans* was also shown to promote growth of obligate anaerobic *Clostridium* spp. under aerobic conditions, potentially boosting their ability to colonize the human gut (van Leeuwen *et al.*, 2016; Fox *et al.*, 2014). However, the effects of *C. albicans* colonization on *Clostridium difficile* infection of the gut are contradictory (Markey *et al.*, 2018; Panpetch *et al.*, 2019). While we are just at the beginning of unraveling the triangle of *C. albicans*, gut-residing bacteria and the human host, these findings indicate that bacterial-fungal interactions affect not only the composition of the microbiota but might also significantly influence the development of either bacterial or fungal infections.

Features That Contribute to the Success of *C. albicans* as Pathogen

Virulence is a multi-faceted feature of microorganisms that includes the ability to thrive within host tissue, withstand rapid elimination by the immune system and the capability to cause tissue and/or organ damage. Keeping this in mind, it is not surprising that genes involved in various processes, from basic cell biology to specific interactions with host cells, have been demonstrated to affect the virulence potential of *C. albicans* (see da Silva Dantas *et al.* (2016) for an overview). Here, we will focus on factors that influence the interaction of the fungus with host cells, nutrient acquisition and will briefly discuss the role of the cell wall and morphogenesis for candidiasis.

Interaction of *Candida albicans* with Epithelial Cells

As a commensal *C. albicans* lives in close contact with – or depending on the presence of a mucus layer, in proximity to – epithelial cells without causing overt damage. This changes during infection characterized by increased fungal load, invasion into epithelial barriers (and underlying tissue), induction of an immune response and tissue damage. The factors that trigger the transition from commensalism to infection are only incompletely understood, but the disturbance of the microbiota clearly plays an important role (Fan *et al.*, 2015; Lopez-Medina *et al.*, 2015) and recently reviewed in Förster *et al.* (2016) and Höfs *et al.* (2016).

The first step toward infection is attachment of the yeasts to host cells, in the case of mucosal membranes epithelial cells (**Fig. 2**). This complex process is likely initiated by passive forces, e.g., hydrophobic cell–cell interactions, followed by specific interactions between fungal and host cell surface molecules (a comprehensive overview has been published by Moyes *et al.* (2015)). While some adhesins also occur on yeast cells, hyphae express adhesins, like Als3 and Hwp1, in larger number and more efficiently (Wächtler *et al.*, 2011; Phan *et al.*, 2007). Targeted host structures include cadherins, integrins, and components of the

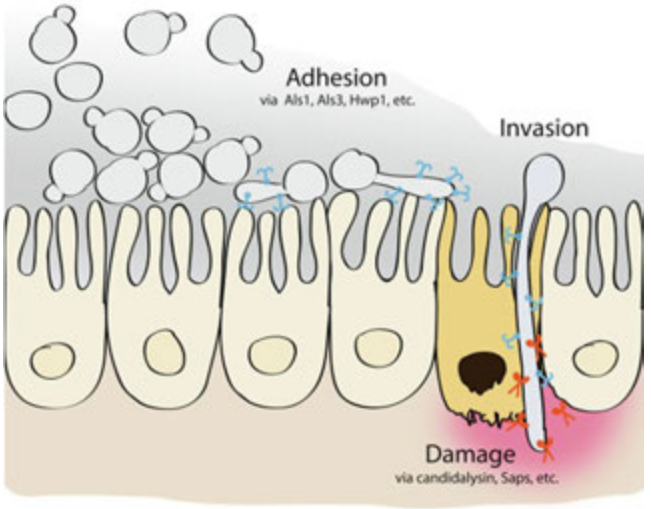

Fig. 2 Adhesion, invasion, and damage by *C. albicans* in the human gut. Major steps of the *C. albicans* life-cycle during the switch from commensal to pathogen. Hyphae-associated adhesins (blue anchors) mediate the attachment to enterocytes. Growing hyphae and subsequent release of candidalysin and proteases (orange scissors) and lipases penetrates enterocyte barrier and destroys underlying cell layers. Illustration by M.J. Niemiec and M. Polke.

extracellular matrix (Phan *et al.*, 2007; Zhu *et al.*, 2012; Chaffin, 2008). Of note, adhesion to host cells is a strong trigger of filamentation in vitro and hypha formation is important for the second step during infection: invasion (**Fig. 2**). Invasion into host cells occurs by two mechanisms, induced endocytosis and active penetration. Active penetration is a fungal-driven process that requires directed filamentous growth (Wächtler *et al.*, 2012). The current knowledge suggests that in addition to physical forces provided by the turgor pressure, hydrolytic enzymes, especially secreted aspartic proteases (Saps) support this process. Inhibition of aspartic proteases by pepstatin A significantly reduces invasion (Dalle *et al.*, 2010); the contribution of individual Saps to this process is however still controversially discussed (Naglik *et al.*, 2003, 2008). In contrast to active penetration, induced endocytosis requires the activity of host cells and occurs even with inactivated fungal cells. It is initiated by the recognition of distinct fungal surface proteins ("invasins") by host cell receptors, which triggers rearrangement of the cytoskeleton leading to uptake of the fungal cell. The first *C. albicans* invasin identified was Als3 that binds to cadherins on host cells (Phan *et al.*, 2007). Another known *C. albicans* invasin is Ssa1 and further host receptors include the EGF receptor and the human epidermal growth factor receptor 2 (Zhu *et al.*, 2012; Sun *et al.*, 2010). Importantly, as Als3 is a hypha-specific protein, both active penetration and induced endocytosis depend largely on hypha formation. Therefore, hyphae are considered to be the invasive morphology in *C. albicans*.

Invasion is a prerequisite for damage but not necessarily linked to damage of host cells: As the host membrane remains intact following initial invasion, forming a glove-like structure surrounding growing hyphae (Wächtler *et al.*, 2012; Zakikhany *et al.*, 2007), the host cell is not immediately lysed. Indeed, specific *C. albicans* mutants have been identified that show unaltered adhesion and invasion but significantly reduced damage in the interaction with epithelial cells. One of these mutants is *C. albicans eed1*Δ/Δ, lacking *EED1*, a factor that is essential for maintenance of hyphal growth and thus hyphal elongation. Following invasion, this mutant reverts back to yeast growth intracellularly, thereby reducing the strain on the host cell membrane afflicted by growing hyphae of wild type *C. albicans* (Zakikhany *et al.*, 2007; Martin *et al.*, 2011). Other examples are mutants lacking either *DUR31* or *HSP21*, that likewise show reduced hyphae formation following invasion (Mayer *et al.*, 2012a,b). Most important, however, was the discovery that an *ECE1* deletion mutant is highly attenuated in epithelial damage, despite normal filamentation. *ECE1* was already identified in the 1990s as one of the genes most highly expressed on hyphae compared to yeast cells (Birse *et al.*, 1993). Yet again, its function was only elucidated in 2016 (Moyes *et al.*, 2016): *ECE1* codes a protein that is cleaved into eight short peptides by Kex2. Peptide 3 generated by this cleavage adopts an α-helical structure and integrates into host cell membranes, leading to permeabilization and lysis. Due to this effect, the peptide was named candidalysin (Moyes *et al.*, 2016). Thus, candidalysin links hyphal morphology and damage beyond physical rupture by continuous filamentous growth and provides further explanation for the significant role of filamentation in pathogenesis (Wilson *et al.*, 2016).

Interaction of *Candida albicans* with the Immune System

Immune cells are rapidly recruited during candidiasis and especially innate phagocytes are efficient and essential effector cells during fungal infections (**Fig. 3**) (Lionakis *et al.*, 2011; Duggan *et al.*, 2015; Lionakis, 2014). In fact, impairment of the immune system represents a risk factor for candidiasis, but not all patients suffering from disseminated *C. albicans* infections are obviously immunocompromised (Nolla-Salas *et al.*, 1997; Yapar, 2014; Guery *et al.*, 2009). To be a successful pathogen, *C. albicans* needs to be able to either prevent recognition or survive antimicrobial mechanisms employed by immune cells to a certain extend. As the

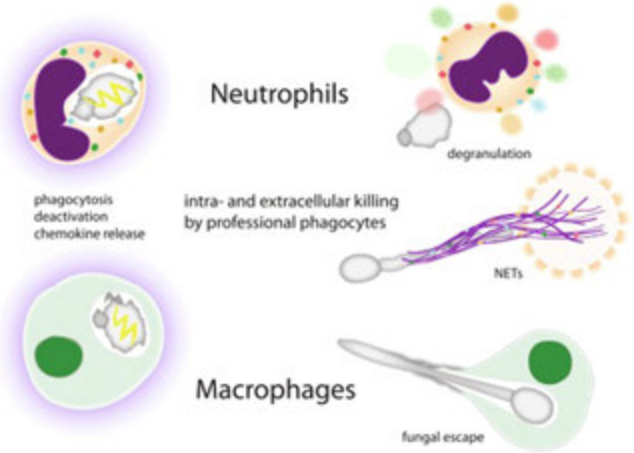

Fig. 3 Killing of *C. albicans* by professional human phagocytes. Killing of *C. albicans* by neutrophils and macrophages occurs intra- and extracellularly (IC/EC). IC: Internalization of *Candida* via phagocytosis leads to inactivation of fungus by ROS (yellow lightning) and host effector proteins. EC: Chemokines are released (purple glow) by activated phagocytes to recruit more phagocytes. Antifungal effector proteins are released into cell environment during degranulation. Neutrophils release extracellular traps (NETs) to entangle and kill *Candida*. Notably, *C. albicans* can outgrow macrophages, not neutrophils. Illustration by M.J. Niemiec.

immune response to *C. albicans* is addressed in detail in another module of this series, we will focus here on some of the mechanisms by which the fungus withstands elimination of the immune system.

The immune system can be roughly divided into cellular and humoral effector mechanisms. Humoral factors include antibodies, the complement system and antimicrobial peptides (AMPs). Antibodies are readily produced in humans and mice following exposure to *C. albicans* (Pitarch *et al.*, 2006, 2009, 2011; Mochon *et al.*, 2010) but naturally induced antibodies are considered to have only a limited protective effect (recently reviewed in Richardson and Moyes (2015)). The complement system and AMPs are evolutionary old and comprise two of the first lines of defense against invading microbes. Furthermore, AMPs are produced by mucosal epithelial cells even in the absence of a pathogenic threat and thereby control the commensal microbiota. Several AMPs, β-defensins, the cathelicidin LL-37 and histatin 5, have been shown to kill *C. albicans* in vitro (Chang *et al.*, 2012; Vylkova *et al.*, 2007a,b). It has furthermore been proposed that the reduced levels of histatin 5 observed in the saliva of HIV+ patients contribute to the increased risk of these patients for oropharyngeal candidiasis (Khan *et al.*, 2013). Although this and the hypothesis that AMPs control fungal growth on mucosal surfaces in vitro appear plausible, only few studies have been conducted to address this experimentally (Liao *et al.*, 2017; Fan *et al.*, 2015). Nonetheless, *C. albicans* also seems to have evolved AMP resistance mechanisms, like inactivation through proteolytic cleavage of AMPs by Sap9 and Sap10 and inactivation by a secreted glycodomain of Msb2 (Meiller *et al.*, 2009; Puri *et al.*, 2012; Swidergall *et al.*, 2013; Szafranski-Schneider *et al.*, 2012). Tolerance to AMPs can furthermore be induced by induction of compensatory mechanisms, for example, Pbs2 via the Hog1 stress response pathway, or stabilization of mitochondrial integrity via Ssd1 and Bcr1 (Jung *et al.*, 2013; Argimon *et al.*, 2011). Similarly, *C. albicans* interferes with complement activation by various means (recently reviewed in Cheng *et al.* (2012) and Luo *et al.* (2013)), including proteolytic cleavage of active complement components by Saps and binding of host factors that regulate complement activation. To which extend these immune evasion mechanisms contribute to pathogenesis is not fully clear; however, complement defects render mice more susceptible to systemic candidiasis (Ashman *et al.*, 2003; Tsoni *et al.*, 2009). Furthermore, opsonization by complement enhances phagocytosis and fungal killing (reviewed in Rambach and Speth (2009)), thereby linking complement evasion to other survival strategies of *C. albicans*.

Professional phagocytes, especially neutrophils and monocytes/macrophages, constitute cellular components of the first line of defense against *C. albicans* (Basu *et al.*, 2008; Kullberg *et al.*, 1999; van Enckevort *et al.*, 1999; Romani *et al.*, 1996; van 't Wout *et al.*, 1988). Following recognition of the fungus via interaction of pattern recognition receptors (PRRs) with fungal surface components acting as pathogen-associated molecular patterns (PAMPs) (recently reviewed in Erwig and Gow (2016), Miramon *et al.* (2013), and Plato *et al.* (2015)), immune cells become activated and either phagocytose the pathogen or, in the case of neutrophils, can undergo degranulation or form neutrophil extracellular traps (NETs) via programmed cell death to release antifungal effector molecules (Gazendam *et al.*, 2016; Stephenson *et al.*, 2016) (**Fig. 3**). The cell wall composition and, more importantly, exposure of distinct components on the surface significantly influences the efficacy of recognition and the subsequent response induced; thus, cell wall modifications are one way by which *C. albicans* can influence recognition by phagocytes and, additionally, also phagosome maturation (Lewis *et al.*, 2012; Bain *et al.*, 2014). These mechanisms are discussed in detail below.

Phagocytosis requires physical uptake of the pathogen and is therefore influenced by size and shape. *C. albicans* hyphae can grow to a length that prevents complete phagocytosis (reviewed in Erwig and Gow (2016)). Therefore, the ability of *C. albicans* to form filaments affects the interaction with phagocytes. However, yeast cells can readily be phagocytosed and phagolysosome maturation creates a hostile environment characterized by reduced pH, a high concentration of AMPs, reactive oxygen and nitrogen species (ROS and RNS, respectively), hydrolytic enzymes and nutrient starvation (Erwig and Gow, 2016; Miramon *et al.*,

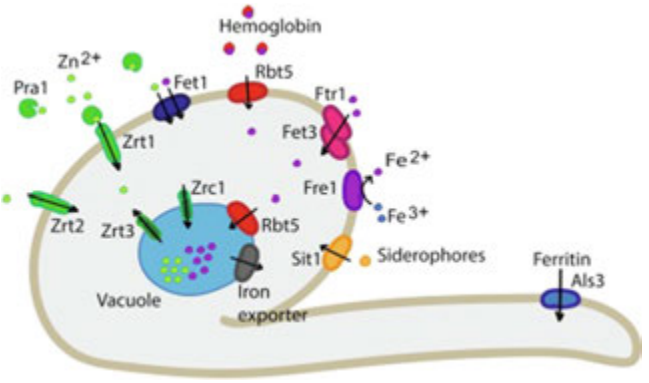

Fig. 4 Micronutrient acquisition by *C. albicans*. *C. albicans* imports, stores, and exports trace elements during its life-cycle. In the mammalian host, the availability of micronutrients is limited. Depicted are the transport systems for zinc and iron. Illustration by M. Kapitan.

2013). *C. albicans* is able to escape from macrophages by either triggering pyroptosis, a programmed cell-death pathway, or by affecting phagolysosome maturation, development of long hypha, and physical piercing of macrophages. Induction of pyroptosis by *C. albicans* is associated with hypha induction but does not require morphogenesis, but rather remodeling of the cell wall associated with increased 1,3 β-glucan exposure, and ergosterol (Uwamahoro *et al.*, 2014; Wellington *et al.*, 2014; O'Meara *et al.*, 2015, 2018; Koselny *et al.*, 2018). Furthermore, the fungal peptide toxin Candidalysin can trigger apoptosis (Kasper *et al.*, 2018).

Phagocytes typically produce significant amounts of ROS and RNS upon encountering *C. albicans* and oxidative mechanisms are important for killing of *C. albicans* (Aratani *et al.*, 2002; Sasada *et al.*, 1987; Vazquez-Torres *et al.*, 1996; Brothers *et al.*, 2013). The fungus responds by activating a general stress response that involves the protein kinase Hog1 and the more specific oxidative and nitrosative stress response pathway involving the transcription factors Cap1 and Cta4, respectively (Wang *et al.*, 2006; Alonso-Monge *et al.*, 2003; Arana *et al.*, 2007; Brown *et al.*, 2009; Smith *et al.*, 2004; Chiranand *et al.*, 2008; Patterson *et al.*, 2013; Day and Quinn, 2019). The activated stress response results in increased expression of detoxification systems (reviewed in detail in Erwig and Gow (2016), Dantas Ada *et al.* (2015) and Miramon *et al.* (2013)) that include superoxide dismutases (Sods) (Hwang *et al.*, 2002; Frohner *et al.*, 2009; Miramon *et al.*, 2012), catalase 1 (Nakagawa *et al.*, 2003; Wysong *et al.*, 1998), a glutathione-based system including glutathione peroxidases and reductases (Enjalbert *et al.*, 2007; Fradin *et al.*, 2005; Rubin-Bejerano *et al.*, 2003; Chaves *et al.*, 2007; Yadav *et al.*, 2011; Miramon *et al.*, 2014), and flavodoxin-like proteins (Li *et al.*, 2015), that are protective against oxidative stress. Resistance against nitrosative stress depends on the flavohemoglobin NO scavenger Yhb1 (Ullmann *et al.*, 2004). Upon phagocytosis, *C. albicans* furthermore upregulates certain metabolic pathways, including gluconeogenesis, enzymes for the utilization of alternative carbon sources, β-oxidation of fatty acids and the glyoxylate cycle, whereas glycolysis and protein synthesis are downregulated (Lorenz *et al.*, 2004; Fradin *et al.*, 2005). As the phagosome is generally considered to be poor in nutrients, this metabolic adaptation contributes to survival of the fungus in this specific environment (Fernandez-Arenas *et al.*, 2007; Miramon *et al.*, 2012). Finally, the upregulation of uptake systems for trace metals (discussed in detail below, see **Fig. 4**) by phagocytosed *C. albicans* cells likely contributes to overcome restriction of these micronutrients within host cells (Lorenz *et al.*, 2004).

The Cell Wall as a Dynamic Factor in *Candida*-Host Interactions

As the outer layer of the cell, the cell wall and associated proteins present the structures that mediate contact of *C. albicans* with the environment and host cells. While on the one hand providing the major outer physical structure for the cell and mediating resistance to adverse environmental conditions, the cell wall is also presenting pathogen-associated molecular patterns (PAMPs) that are recognized by PRRs on professional and associate immune cells (recently reviewed in Erwig and Gow (2016), Miramon *et al.* (2013), Plato *et al.* (2015), Barreto-Bergter and Figueiredo (2014), and Snarr *et al.* (2017)). Thus, the cell wall is critical for host-pathogen interactions of *C. albicans*. The fungal cell wall consists of different β-glucans, chitin and chitosan, which are located in an inner layer, and mannans attached to proteins in an outer layer (for details see Free (2013)). Most of the cell wall molecules are carbohydrates, which all contribute to immune recognition (reviewed in detail by Netea *et al.* (2008), Gow and Hube (2012), Zheng *et al.* (2015b), Plato *et al.* (2015), Becker *et al.* (2015), Erwig and Gow (2016), and Snarr *et al.* (2017)). The receptors involved in recognition and the response of immune cells differs depending on the cell wall moiety that is recognized: β-1,3 glucan interacts with the C-type lectin Dectin-1, which triggers a strong inflammatory response (Brown, 2011; Cheng *et al.*, 2011). Mannans can be recognized by Toll like receptors (TLRs), the Mannose Receptor DC-SIGN, Mincle and Galectin-3 (Netea *et al.*, 2008, 2006; Barreto-Bergter and Figueiredo, 2014), likewise inducing a proinflammatory response which is, in comparison to β-1,3 glucan, less pronounced (Gow and Hube, 2012). Chitin is sensed by NOD2, TLR9 and the mannose receptor, but in contrast to the other components can reduce inflammatory responses (Mora-Montes *et al.*, 2011; Wagener *et al.*, 2014). Thus, the relative exposure of the different carbohydrates determines the quality and quantity of subsequent immune responses. This is exemplified in studies of *C. albicans* mutants with defects in mannosylation which lead to increased exposure of inner cell wall components, resulting in altered interaction with immune cells (reviewed in Hall and Gow (2013) and Diaz-Jimenez (2017)).

Importantly, the cell wall composition and relative exposure of the different carbohydrate components is highly dynamic and influenced by the morphological state, environmental conditions and the interaction with immune cells (recently reviewed in Hall (2015)). Yeast and hypha differ in the types of glucan (Lowman *et al.*, 2003, 2014) and the amount of chitin, with chitin being more abundant in hypha (Munro *et al.*, 1998). Furthermore, the distribution of chitin differs; while it is found along the cell wall of hypha, it is concentrated at the bud scar in yeast (reviewed in Gow *et al.* (2012)). Furthermore, some mannoproteins are differentially expressed depending on the morphology, e.g., hypha-specific proteins like Hwp1 and Als3 (reviewed in Sundstrom (1999) and Trevijano-Contador *et al.* (2016)) and the mannan composition in yeast and hypha is different (Shibata *et al.*, 2007). As a consequence of these differences, immune cells respond differently to yeast and hypha (reviewed in more detail in Gow *et al.* (2011), Jacobsen *et al.* (2012), Erwig and Gow (2016), and Trevijano-Contador *et al.* (2016)).

The environment influences the cell wall composition and structure. Mannan structures (Kruppa *et al.*, 2011; Lowman *et al.*, 2011; Ene *et al.*, 2012) and the levels of glucan and chitin (Ene *et al.*, 2012), for example, vary depending on the culture conditions. As mentioned above, both pH and hypoxia affect the exposure of β-glucan (Sherrington *et al.*, 2017; Pradhan *et al.*, 2018; Lopes *et al.*, 2018). Lactate induces active β-glucan masking while cell wall stress induced by the antifungal echinocandins leads to increased chitin and enhanced β-glucan exposure both in vitro and during in vivo treatment (Walker *et al.*, 2008; Wheeler *et al.*, 2008; Lee *et al.*, 2012; Ballou *et al.*, 2016). Importantly, in the absence of antifungals, unmasking of β-glucan also occurs during systemic candidiasis in mice as a result of active cell wall remodeling in response to damage by neutrophil extracellular traps (Hopke *et al.*, 2016). Chitin can also be upregulated during infection, affecting recognition by Dectin-1, a phenomenon that varies between different *C. albicans* strains (Marakalala *et al.*, 2013). Increased chitin in *C. albicans* is furthermore associated with reduced virulence in a systemic mouse model (Lee *et al.*, 2012), highlighting the importance of the cell wall and its dynamic modifications for the interactions *C. albicans* with its host.

The Influence of Macronutrients on *Candida*-Host Interaction

As both a commensal and a pathogen, *C. albicans* can thrive at different anatomical sites. These differ not only in pH, oxygen levels and host defense systems but also regarding the quality and quantity of available carbon and nitrogen sources. Its success at the different niches suggests that *C. albicans* is metabolically versatile and has evolved means to assimilate a range of nutrient sources. Furthermore, the dissemination into various tissues during systemic infection requires fast adaptation to local nutrient availability. Indeed, *C. albicans* can utilize a vast variety of different sugars and amino acids (Brunke and Hube, 2013; Miramon and Lorenz, 2017) and a range of secreted hydrolytic enzymes contributes to the liberation of nutrients from complex molecules (Brunke and Hube, 2013; Naglik *et al.*, 2004; Schaller *et al.*, 2005). Complex regulatory networks mediate the response of *C. albicans* to nutrient starvation or alterations in the available carbon or nitrogen sources, leading to altered expression of hydrolases, transporters and metabolic pathways like gluconeogenesis, glycolysis or fatty acid β-oxidation (Barelle *et al.*, 2006; Askew *et al.*, 2009; Morschhauser, 2011; Ramachandra *et al.*, 2014; Sabina and Brown, 2009; Lee *et al.*, 2013). In comparison to the related baker's yeast *Saccharomyces cerevisiae*, substantial transcriptional and post-translational rewiring has occurred in *C. albicans*, contributing to the metabolic flexibility of *C. albicans* (Whiteway *et al.*, 2015; Sandai *et al.*, 2012; Ramirez and Lorenz, 2007).

In addition to providing the necessary energy for fungal survival, growth, and production of effector molecules, metabolic adaptation is directly linked via transcriptional networks to the expression of different fitness and virulence attributes such as morphogenesis, stress resistance, adhesions, and hydrolytic enzymes (Han *et al.*, 2011). Cell wall composition is also influenced by nutrients, especially carbon metabolism, and thereby metabolism indirectly influences the interaction with immune cells (recently reviewed in Hall (2015)). One well studied example is the growth of *C. albicans* on lactate in comparison to glucose and the changes caused by this. While most growth media used in the laboratory are rich in glucose, glucose availability is limited within the host. Thus, *C albicans* has to use alternative carbon sources such as lactate. Growth on lactate in vitro led to alterations in cell wall architecture and secretome, with consequences for adhesion, stress resistance, and interaction with immune cells: Lactate grown cells were phagocytosed less by macrophages while fungal escape was enhanced; at the same time lactate-grown *C. albicans* cells induced more IL-10 but less IL-17, thereby leading to a reduced proinflammatory response (Ene *et al.*, 2012, 2013). As a consequence, fitness of lactate-grown *C. albicans* cells in vivo was enhanced (Ene *et al.*, 2012).

Due to the link between metabolism and fitness/virulence traits, the adaptation of *C. albicans* to different environments and nutrient availability within the host simultaneously affects expression of virulence factors (recently reviewed in Brown *et al.* (2014)). This linkage of metabolic responses to environmental clues and virulence can be interpreted as "adaptive prediction" (Mitchell *et al.*, 2009), by which the availability of distinct nutrients serves as a clue for further subsequent environmental changes, e.g., attack by immune cells following translocation into the blood stream, leading to oxidative stress due to ROS production. From an evolutionary perspective, this linkage is likely to be highly advantageous to the fungus and likely contributes to its success in diverse anatomical niches both as a commensal and a pathogen. For more information and a summary of the open questions, we refer the readers to the review by Brown *et al.* (2014).

Acquisition of Micronutrients by *C. albicans*

Micronutrients are as essential for microbial growth as carbon- and nitrogen sources. Trace metals such as iron, zinc, copper, and manganese are essential co-factors in enzymes and stabilize three-dimensional protein structures (Waldron *et al.*, 2009; Palm-Espling *et al.*, 2012). However, their free availability in the host is limited by sequestration and binding to host molecules. Host-mediated nutrient depletion from invading pathogens is also termed 'nutritional immunity,' a mechanism that has gained increased attention in recent years, and is therefore described in some detail below.

Every aspect of nutritional immunity, from the host or pathogen perspective, reflects the co-evolutionary nature of these interactions. Best studied is the competition for iron (see also a recent review by Fourie *et al.* (2018)), but most concepts apply to all transition metals in eukaryotes (Sutak *et al.*, 2008; Weinberg, 1975). In the host, iron is mainly kept intracellular – free iron is practically unavailable in blood or other extracellular liquids, with exception of the gut. Iron is bound to transferrin in plasma and lactoferrin in external secretions. However, in order to grow within the host, microbial pathogens cope with these limitations. In case of direct physical contact between the host iron source and the pathogen, host iron-retaining proteins might be manipulated to release their iron load into the extracellular environment for uptake. Secondly, many bacteria and fungi secrete siderophores: Small molecules with exceptionally high iron affinity that free iron from host proteins followed by resorption into the pathogen cell. In response, mammals produce lipocalin-2, to intercept siderophores. This can in turn be counteracted by many pathogens which disguise their siderophores by glycosylation (Sutak *et al.*, 2008). Iron is a common co-factor in enzymes and one of the most important direct functions of iron during host-pathogen interactions is likely the detoxification of H_2O_2 by the iron-dependent heme-enzyme catalase (Hamilton and Holdom, 1999). Iron uptake in *C. albicans* occurs by a reductive and the non-reductive mechanism (Waldron *et al.*, 2009; Almeida *et al.*, 2009) (**Fig. 4**). In a nutshell, the high affinity reductive uptake system functions via the interplay of three enzymes. A reductase (Fre) converts ferric Fe^{3+} to ferrous Fe^{2+}. The actual uptake is mediated by a permease (Ftr) and a multicopper ferroxidase (Fet). *C. albicans* relies on this pathway to acquire iron from ferritin and transferrin (Knight *et al.*, 2005; Almeida *et al.*, 2008). Probably due to functional redundancy due to gene expansion of the reductases and multicopper oxidases, only the permease Ftr1 has been shown to be crucial in blood stream infections by *C. albicans*. Non-reductive Iron uptake is based on the import of iron-containing proteins, namely, hemoglobin/heme (recently reviewed by Roy and Kornitzer (2019)) and siderophores. *C. albicans* has receptors for heme (e.g., Rbt5p), but also for ferritin (reductive uptake). The ferritin receptor and adhesin Als3 is expressed only by hyphae (Almeida *et al.*, 2008). Siderophores are not produced by *C. albicans*, but they can be utilized as an iron source even if produced by other microbes (Almeida *et al.*, 2009).

In contrast to other sites in the human body, the gut is probably the only site where microbes encounter high iron levels. Accordingly, as a colonizer of this niche, *C. albicans* is able to adapt not only to iron limitation but also to relatively high iron concentrations. To do so, an intricate system of three transcriptional regulators has evolved that controls iron uptake in *C. albicans* (Polke *et al.*, 2015; Blankenship and Mitchell, 2011). The transcriptional repressor Sfu1 and the virulence regulator Hap43 dampen iron uptake and Sfu1 is required for persistence of *C. albicans* in the gut. In contrast, during blood stream infections, when iron is limited, iron uptake is upregulated by Sef1, a process essential for virulence in the systemic mouse model (Chen *et al.*, 2011). Sfu1 represses Sef1, thereby interconnecting the response to low and high iron and adaptation to distinct niches in the host.

Uptake of iron and copper are very tightly connected. The ferroxidase (Fet) is actually a copper-dependent enzyme, and the iron reductases (Fre) are indeed ferric/cupric reductases that reduce Cu^{2+} to Cu^{1+} through the oxidation of Fe^{2+} to Fe^{3+}, and vice-a-versa (Niemiec, 2015). Reduced Cu^{1+} is taken up by Cu transporters (Ctr). Ctr1 and Ctr3 are plasma membrane-localized, Ctr2 is vacuolar – and all three transport towards the cytosol. Interestingly, the number of *CTR* copies varies between fungal species and might affect virulence due to an effect on, e.g., hyphal growth (Ding *et al.*, 2014). During infection, pathogens face copper starvation as well as copper intoxication. For example, excess copper has been described as a mode of action against intraphagosomal *Mycobacteria* in macrophages (Rowland and Niederweis, 2012). Therefore, copper export and copper detoxifying metallothioneins are important to maintain copper homeostasis. Metallothioneins are conserved in prokaryotes and eukaryotes. A potential role in virulence was demonstrated in *Cryptococcus neoformans*, a facultative intracellular fungal pathogen, but not yet in *C. albicans*. However, *C. albicans* is known to export excess copper, indicating that fungi are primed to resist potential copper poisoning in vivo (Ding *et al.*, 2014). At the same time, copper is essential for pathogenic survival. Most fungal SODs, crucial to withstand superoxide stress, have copper in their active site. In *C. albicans*, Sod1, Sod4, and Sod6 are copper/zinc-dependent, Sod5 was recently described to be a Cu-only SOD (Gleason *et al.*, 2014). *C. albicans* extracellular Sod4 and Sod5 were demonstrated to detoxify superoxides produced during interactions with macrophages (Frohner *et al.*, 2009).

Of all metal-containing enzymes, 9% use zinc – so zinc is the second most abundant metal cofactor after manganese (Waldron *et al.*, 2009). Further, zinc stabilizes zinc-finger domains, a function that is especially important in eukaryotic organisms (Decaria *et al.*, 2010). Several studies have demonstrated the importance of zinc in pathogenic and non-pathogenic fungi. *C. albicans* is very sensitive to zinc starvation. Zinc can actually be the limiting factor if all other nutrients are available (Soll *et al.*, 1981; Bedell and Soll, 1979). Zinc acquisition in fungal pathogens is mediated by a small number of proteins and is conserved in *C. albicans* (**Fig. 4**): Zinc enters the cytoplasm via the Zrt plasma membrane transporters (CaZrt1, CaZrt2). From there, zinc can be transported further into the vacuole through ZnT transporters (in *C. albicans*: Zrc1). In return, zinc can be mobilized from the vacuolar pool by Zrt exporters (CaZrt3). Interestingly, no cellular zinc exporter has been described in fungi, yet. The homeostasis of zinc is regulated on two levels: the expression of Zrt importers, controlled by Zap1, and zinc import and export from subcellular organelles (Wilson *et al.*, 2012), resulting in biphasic zinc compartmentalization which is essential for virulence (Crawford *et al.*, 2018). Vacuolar storage might be especially important during infection, since it allows proliferation even under zinc-restricting conditions

(Simm *et al.*, 2007). In addition, *C. albicans* uses a zincophore system – a secreted protein that sequesters zinc from host cells and re-associates with CaZrt1 to deliver zinc to the fungal cell: Pra1 (Citiulo *et al.*, 2012). The expression of Pra1 is repressed under acidic conditions, but induced under zinc starvation or at neutral/alkaline pH (Citiulo *et al.*, 2012). Remarkably, Pra1 is not only beneficial for the fungus, it is actually immunogenic and immune-modulatory (Wilson, 2015). Amongst many functions, Pra1 can serve as a ligand to neutrophil integrin receptor $\alpha M\beta 2$ and promote neutrophil killing (Soloviev *et al.*, 2007). Probably due to this "side effect," Pra1 is in not conserved in all pathogenic fungi (Wilson, 2015). Zinc is furthermore important for biofilm formation, and involved in tolerance to intrinsic oxidative stress by the cytoplasmic Sod1 (Wu *et al.*, 2009). Sod1 is a Cu/zinc superoxide dismutase (SOD). Notably, the cell surface superoxide dismutases Sod4p and Sod6p that are crucial to survive host-derived ROS, have Cu/zinc as their active site (Frohner *et al.*, 2009).

In non-plant eukaryotes, manganese function is restricted to mitochondrial enzymes, including Sod2 and Sod3. The human host restricts access to manganese by releasing calprotectin that is capable of binding manganese and zinc (Kehl-Fie *et al.*, 2011; Sohnle *et al.*, 2000). Furthermore, manganese (and iron) are actively depleted from the macrophage phagosome (Kehl-Fie and Skaar, 2010). Manganese restriction is important during bacterial infections, but its role during fungal pathogenesis requires more investigation (Corbin *et al.*, 2008; Kehl-Fie *et al.*, 2011; Posey and Gherardini, 2000).

C. albicans Morphology – Does it Determine the Difference Between Commensalism and Infection?

As mentioned throughout the previous parts, the formation of filamentous growth forms during morphogenesis is generally thought to be essential for *C. albicans* virulence. This concept is based on the observation that *C. albicans* mutant strains locked in either the yeast or hyphal form were attenuated in their virulence in murine infection models (Lo *et al.*, 1997; Braun *et al.*, 2000, 2001; Murad *et al.*, 2001). Given that hypha have the ability to physically penetrate host cell barriers and hypha formation is associated with production of candidalysin (Brand, 2012; Moyes *et al.*, 2016), it appears obvious that hypha formation significantly affects the ability of *C. albicans* to penetrate tissue, damage host cells and cause disease. However, hypha formation is co-regulated with several virulence-associated factors, leading to rearrangement of the cell wall and thereby affecting the response of immune cells, and is further associated with stress response and metabolism (Inglis and Sherlock, 2013; Dantas Ada *et al.*, 2015; Monge *et al.*, 2006; O'Connor *et al.*, 2010). It is thus difficult to determine to which extend the formation of hyphae per se contributes to virulence (Kumamoto and Vinces, 2005). Most likely, both, yeast and hyphae, play distinct roles during the different steps of *C. albicans* infections (Gow *et al.*, 2002; Kumamoto and Vinces, 2005; Saville *et al.*, 2003).

While invasive growth and host damage are intrinsically linked during infection, commensalism is characterized by a balance between host and fungus that does not cause host damage (Gow and Hube, 2012). Interestingly, genetic analyzes revealed that colonization of the gut is associated with significant expression levels of hypha-associated gene expression, such as *EFH1*, *ECE1*, *RBT4*, and *RBT1* in yeast cells (d'Enfert, 2009; Doedt *et al.*, 2004; Rosenbach *et al.*, 2010; White *et al.*, 2007). Thus, transcriptional programs that were previously considered to be morphology-associated based on in vitro observations might be uncoupled from the filamentation program under distinct environmental conditions. Furthermore, *ECE1*, the gene encoding for candidalysin and required for host cell damage, is upregulated in the gut. This raises the question whether growth as yeast in the gut truly reflects a benign, commensal transcriptional program and whether separate commensal and pathogenic transcriptional programs exist. The fact that *C. albicans* intestinal colonization requires factors contributing to survival in phagocytes (Tan *et al.*, 2019), and that colonization stimulates innate immune mechanisms pathogens (Tso *et al.*, 2018; Shao *et al.*, 2019) suggests active interaction with the immune system; in combination with the advantage of yeast over filamentous morphologies in the gut, the yeast morphology in the gut is likely to be the consequence of complex interactions between host, intestinal environment, and *C. albicans*. Factors that have so far been only associated with *C. albicans* infections may be required for fungal survival in the gut. Indeed, recent gene network analyzes of transcription factors required for gut colonization and/or fitness during systemic infection revealed several connections between the regulons of transcription factors important for either commensalism or virulence (Perez and Johnson, 2013).

Further research directed at a better understanding the entire *C. albicans* biology, not only as a pathogen but also as a commensal on different mucosa, is required to decipher what it is exactly that makes this fungus a successful member of the human microbiota. Hopefully, this will contribute to eventually elucidate the specific factors that predispose patients to disseminated candidiasis.

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Candida psilosis Complex

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Glossary

Allele A variant of a locus.
Auxotrophy Inability of an organism to grow in the absence of a given organic compound.
Chemokines Type of cytokines that attracts specific celltypes to the site where they are secreted.
Clade Evolutionary term for a group of organisms including their common ancestor and all its descendants.
C-type lectin A type of lectin that requires Ca^{2+} for proper function.
Cytokine Small proteins that are secreted by various type of cells and play role in communication and interaction between cells.
Dectin-1 A C-type lectin playing important role in fungal recognition.
Desaturase An enzyme responsible for converting saturated chemical bonds to unsaturated ones.
Diploid A term used for organisms possessing two copies of each chromosome.
Endothelium Thin layer of endothelial cells covering the inner wall of blood – and lymphatic vessels.
Epithelium Thin layer of epithelial cells covering the inner and outer surfaces of the organs.
Ergosterol A type of sterol found in the fungal cell membrane to increase rigidity.
Glucan A D-glucose polymer in the fungal cell wall.
Heterozygosity A state of being heterozygous.
Heterozygote/ Heterozygous The organism possesses two different alleles in a given locus.
Homology Similarity of biological properties between species derived from the same ancestor.
Homozygote/ Homozygous The organism possesses the same alleles in the given locus.
Hybrid The resulting descendant of mating between two different species.
In silico Any approach (search, calculation, prediction, etc.) that utilizes a computer.
In vitro Any experiment performed outside of a living organism.
In vivo Any experiment performed on a living organism.
Lectin A protein that recognizes and binds carbohydrate molecules.
Lipase An enzyme that degrades fatty acids via hydrolysis.
Locus A given region of a genome.
Lysosome A membrane-bound organelle filled with hydrolytic enzymes in animal cells.
Macrophage Antigen presenting phagocyte in the tissues.
Mannan (O-, N-linked) A glucose polymer consisting of a linear backbone (α-1,6-bonds) with possible (α-1,2- or α-1,3-bonds) branches. It can be attached to –OH groups of Serine or Threonine of proteins that is called O-linked mannan, or to $-NH_2$ group of Asparagine that is known as N-linked mannan.
Mating (yeast) A well regulated process by which two individual cells (belonging to the opposite mating type) fuse together.
Mb Megabase, unit of the size of DNA. 1 megabase is 1.000.000 bases.
Meiosis A type of cell division, where the initial chromosome number (characteristic to the parent cell) is halved (in the daughter cell).
MIC Minimum inhibitory concentration is the lowest concentration of a specific drug that inhibits the growth of the microbe of interest.
Mitochondrion An organelle in the eukaryotic cell responsible for providing energy.
Mitosis A type of cell division, where the initial chromosome number (characteristic to the parent cell) does not change (in the daughter cell).
Neutropenia A condition where the concentration of neutrophil granulocytes is less than $1500 * \mu l^{-1}$.
Neutrophil granulocyte Short-lived white blood cells being present in the largest number in the blood of most mammals.
Null-mutant A strain of an organism lacking both alleles of a given locus.
Onycomycoses Infection of the nails caused by a fungus.
Orthology (genome) A type of homology where the homolog region is defined across species.
Paralogy (genome) A type of homology where the homolog region is defined in one species. It is typically represented by gene duplications.
PBMC Peripheral blood mononuclear cell, any blood cell possessing a round shaped nucleus.
PCR Polymerase chain reaction, a method used for multiply a specific region of the genome or DNA sequence.
Phagocyte A type of cells in the body that is capable of engulfing various organic or inorganic particles (fungus, bacteria, cell debris etc.).
Phagosome The organelle formed as a result of phagocytosis.
Phospholipases Enzymes responsible for hydrolyzing ester and ether bonds of phospholipids.
Phylogenetic reconstruction An *in silico* process by which the most probable relationship between species can be defined.
Plasmid A circular extrachromosomal element.
Primer A single oligonucleotide that act as starting point for DNA synthesis during PCR.

Promoter A DNA region found before a gene. This is where transcription factors can bind and therefore determine whether a gene is turned on or off.
Prototroph An organism that is capable of growing in the absence of organic nutrients.
QRT-PCR Quantitative real-time PCR, a PCR based method to determine the amount of a desired nucleotide sequence.
Receptor A protein that can receive and transfer chemical – or physical signals from the environment to promote a regulated cell response.
Recombinase An enzyme catalysing the recombination procedure.
Recombination A regulated process where different regions of the chromosomes are combined.
SNP Single nucleotide polymorphism or point mutation. A type of alteration in the nucleotide sequence where only one nucleotide is changed compared to a reference sequence.
Tetraploid A term used for organisms possessing four copies of each chromosome.
T_h cell Special type of immune cell being responsible for regulating the immune response by secreting specific cytokines.
Transcription factor Proteins that regulate the transcription.
Transcription A process by which the information encoded by a gene is converted into RNA.
Wild-type Any isolate without a genetic modification.

Candida parapsilosis Sensu Lato – General Introduction

Candida parapsilosis sensu lato species are diploid yeasts belonging to the so-called CUG clade of the Class *Saccharomycetes* (Division of *Ascomycota*). The term "*C. parapsilosis sensu lato*" refers to three very closely related but distinct species: *C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*, formerly known as *C. parapsilosis* Group I, II and III, respectively. Their genome sizes range between ~12.6–13.3 Mb and encode ~5700–6300 genes. Although they conserve remnants of mating related genes in their genomes, no complete sexual cycle has yet been observed in this group. Instead, recent results suggest that genetic diversity is increased through hybrid formation. These species are generally considered as human commensals, often associated with healthy human skin and mucosa. However, in the setting of immune compromise, these fungi cause illnesses ranging from mild superficial or mucosal infections to life-threatening systemic diseases. Strikingly, *C. parapsilosis sensu stricto* has a strong predilection for disease in low and very low birth weight neonates and is responsible for numerous outbreaks in neonatal intensive care units. Additional predisposing risk factors for disease from these pathogens include the use of intravenous catheters, antibiotics, neutropenia, HIV infection, cancer therapy and prolonged hospitalization. After identification, azole derivatives are generally administered, alternatively amphotericin B may be used or, if susceptible, echinocandins. Although significant advances in the understanding of the pathobiology of these yeasts have been made, there remain many questions regarding the specific tools that they harness to enable human infection. The virulence factors that have been identified to date, include adhesins and secreted hydrolytic enzymes (lipases and proteinases). In turn, host receptors involved with recognition and interaction with these yeasts include Toll-like receptor 4 (TLR4), Dectin-1, and Galectin-3. Additional insights have suggested that *C. parapsilosis sensu stricto* can modulate the host immune response by inhibiting the Th1/Th17 host response and inducing antigen production to hide from the host immune system. There are many unexplained questions regarding the biology of *Candida parapsilosis sensu lato* species, but their increased medical importance over the past two decades has resulted in numerous active investigations intended to elucidate a deeper understanding the pathogenesis of these species in order to develop improved preventative and therapeutic strategies.

Candida parapsilosis Sensu Lato Complex – History

The evolution of the nomenclature of the *Candida parapsilosis sensu lato* species is somewhat convoluted and emerged out of a lack of scientific tools as well as the absence of standardization of scientific naming. Moreover, communication between doctors, biologists and other scientists was very limited and ponderous in the late 1800s and early 1900s. At that time macroscopic/microscopic morphology and the capability of an isolate to grow or not to grow on/in different mediums were the basis for species identification (Castellani and Chalmers, 1919; Stovall and Bubolz, 1932; Ciferri and Redaelli, 1929). The limitation of these methods and the disarray of species naming are well represented by the publication of Ciferri and coworkers who found 121 synonym names for *Candida albicans* in the literature in 1938 (Cifferi *et al.*, 1938). In fact, the majority of *Candida* species were mostly described as *Monilia* in the literature 19th and the first half of the 20th century until the 1940s when a group of scientists proposed a fundamental change in the nomenclature based on the hosts these microbes infect. Since then, *Monilia* species were considered as plant pathogens and the name *Candida* was dedicated to yeasts infecting animals (Mackinnon and Artagaveytia-Allende, 1945; Skinner, 1947). There was also an attempt to standardize the classification process. Besides morphology, various cultivation methods were applied and fermentation properties were examined that helped avoid misidentification of the *Candida* species (Martin and Jones, 1940).

The first appearance of the name *Candida parapsilosis* in the literature was in 1932 by Langeron and Talice. However, this organism had actually been reported 6 years before by Pollacci and Nann in their scientific paper on a microbe called *Monilia*

onychophila, that later turned out to be *C. parapsilosis*. Unfortunately, many of these early reports are not available directly, but are known through references in subsequent publications (Talice, 1932; Martin and Jones, 1940; Talice, 1932). The earliest accessible publication is from 1928 written by a doctor named Ashford who described a patient suffering from diarrhea whose stool grew an interesting yeast. This microbe differed in its fermentation capabilities from the well known yeast at that time called *Monilia psilosis*, which had previously been isolated from stool and other patient samples. Ashford named this microbe *Monilia parapsilosis* and this is today known as *Candida parapsilosis* while *Monilia psilosis* is called *Candida albicans* (Ashford, 1928; Robin, 1923; Rogers, 1922).

C. parapsilosis gained importance in the 1970s when the number of medical case reports related to this yeast started to rise. Further reports underscored that the predilection for disease varied in *C. parapsilosis* compared to *C. albicans* as, for example, *C. parapsilosis* is often associated with neonatal candidiasis (Anonymous, 1977; Faix, 1983; Baley *et al.*, 1986). It was during this time period that significant advances in molecular biology techniques occurred that facilitated the use of genetic markers and related methods for species characterization and identification. By applying techniques such as restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNA (RAPD), the genetic background of *C. parapsilosis* was revealed to have diversity as demonstrated by sequence variances in the internal transcribed spacers (ITS) as well as in mitochondrial DNAs and rRNA domains. These data led to *C. parapsilosis* being separated into three different groups, called Group I, II and III (Scherer and Stevens, 1987; Lehmann *et al.*, 1992; Lin *et al.*, 1995; Nosek *et al.*, 2002b; Kurtzman and Robnett, 1998; Kurtzman and Robnett, 1997; Rycovska *et al.*, 2004). In 2005, Tavanti and coworkers used a multilocus sequence typing (MLST) technique to more deeply molecularly characterize these three groups and their work suggested that the *C. parapsilosis sensu lato* group should be divided into three individual species: Group I became *C. parapsilosis sensu stricto*, Group II was renamed to *C. orthopsilosis* and Group III was called *C. metapsilosis* (Tavanti *et al.*, 2005). The three species are impossible to distinguish based on macroscopic or microscopic characteristics. In general they are all incapable of fermenting maltose and present as round or elongated cells, which may form pseudohyphae (Ashford, 1928). Colonies are usually shiny, white or yellowish in color, but this feature depends on the media used for cultivation. The morphology of *C. parapsilosis sensu lato* colonies is very diverse (smooth, rough, snowball, crepe, crater and concentric) and can be influenced by compounds or chemicals used in the media (Enger *et al.*, 2001; Laffey and Butler, 2005). The species grow well at 30°C and 37°C, but none grow at 42°C. The holotypes have similar minimal inhibitory concentration (MIC) values against antifungal agents, and they also do not differ in either their biofilm forming capabilities or sugar assimilation profiles. Since morphological characterization cannot distinguish between these species, molecular methods are applied for species identification. Standard identification approaches are based on the localization of single nucleotide polymorphisms (SNPs) in putative alanyl-tRNA synthetase and secondary alcohol dehydrogenase genes compared to the historically most ancient species, *C. parapsilosis sensu stricto*. Primer pairs have also been developed that do not amplify any fragment from *C. metapsilosis* and *C. orthopsilosis* genome (Tavanti *et al.*, 2005).

Candida parapsilosis Sensu Lato – Epidemiology

The *Candida* strains that scientists studied in the 1940–50s were mostly isolated from oral, vaginal and stool samples and the outcome of these illnesses were rarely fatal (Seelig, 1966). Although *C. albicans* was the most abundant among them, some other, so-called non-*albicans* species were also represented. The emergence of *Candida* species as a significant human pathogen began in the decade of "Penicillin era", when healthcare workers began to encounter super-infections. This term was used when a patient was admitted to a hospital with a bacterial infection and was treated with an antibiotic that eliminated the pathogen, but a secondary infection occurred (a.k.a. a super-infection, an infection caused a microbe other than the original one). One of the early well-known pathogens responsible for super-infections was *Staphylococcus aureus* but later fungi also started to emerge (Colgan, 1956; Scales *et al.*, 1956; Woods *et al.*, 1951). The possible connection between the use of broad-spectrum antibiotics and super-infection was first discussed in 1949 by Harris, who proposed that antibiotic drugs eradicated the microbes causing the presenting disease, but also eliminated or at least attenuated normal gut colonizing bacteria facilitating the development of super-infections by certain organisms, such as *Candida* species (Harris, 1949). Other reports subsequently provided additional support for this concept of antibiotics disrupting homeostasis of normal flora (Bartels and Buchbinder, 1945; Pappenfort and Schnall, 1951; Janke, 1952; Huppert *et al.*, 1953; Sharp, 1954). Moreover, experimental evidence was provided by Foley and Winter in 1949, who demonstrated that the administration of penicillin *in vivo* to chicken embryos enhanced the lethality of *C. albicans* clinical strains (Foley and Winter, 1949).

In addition to modification by antimicrobials, the general health of the host's immune system was identified as a critical factor in resistance to *Candida*. Alterations in host immunity that facilitated *Candida* infection ranged from the increased access to the bloodstream via intravenous catheters to devastation of host effector cells through primary or secondary immune destruction. Along this line, by the late 1970s and early 1980s, two independent effects leading to immunodeficiency were recognized as being closely connected to candidiasis: (1) organ transplantation associated with artificial attenuation of the immune system to avoid organ rejection and (2) patients with acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV) (Sanger *et al.*, 1962; Klein *et al.*, 1984). The increasing rates of *Candida* species among immunocompromised patients and their association with nosocomial infections resulted in a fundamental change in research priorities. Early publications in the *Candida* field had focused primarily on species identification, characterization and case reports, but the rapid increase in clinical cases expanded investigations into *Candida* virulence, pathogenesis and molecular characteristics. Infection models were established, the essential compounds required for growth were identified and the chemical properties of the cell wall were determined

(Salvin *et al.*, 1952; Eisman *et al.*, 1953; Gebhardt and Hill, 1956; Roth *et al.*, 1957; Johnson, 1954; Sikl *et al.*, 1964). Since *C. albicans* has long been the dominant *Candida* species causing systemic infections, most of these publications report results primarily on *C. albicans*; however, scientific papers from the 1950s began to explore other *Candida* species, including *C. tropicalis*, *C. krusei*, *C. glabrata* and *C. parapsilosis* (De Eshougues *et al.*, 1951; Monod *et al.*, 1956; Rotter and Staib, 1958; Rook and Brand, 1950; Wickerham, 1957; Manchester and Georg, 1959).

C. parapsilosis sensu lato species are associated with humans (Weems, 1992). Interestingly, although *C. parapsilosis sensu stricto* has also been isolated from non-human sources like plants, seawater and soil, there have been no environmental *C. orhopsilosis* or *C. metapsilosis* strains isolated to date, but this does not necessarily mean that these species do not have a niche unrelated to humans (Fell and Meyer, 1967). There are differences in tissue colonization by these species with *C. parapsilosis sensu stricto* and *C. orthopsilosis* colonizing normal skin, *C. parapsilosis sensu stricto* and *C. metapsilosis* oral mucosa and *C. parapsilosis sensu stricto* vaginal mucosa (Underhill and Iliev, 2014; Findley *et al.*, 2013; Ghannoum *et al.*, 2010; Nyirjesy *et al.*, 2005). However, they are opportunistic pathogens as local and systemic disease can arise from colonized tissues. In addition to the above described risks of immunodeficiency and use of antibiotics as predisposing factors for disease secondary to these species, prolonged hospitalization, intravenous catheters, indwelling medical devices, surgical interventions, malignancy, neutropenia and burn injuries are all associated with invasive infections (Law *et al.*, 1984; Pan *et al.*, 2012 and reviewed in Trofa *et al.*, 2008). Additionally, parenteral nutrition, intravenous lipid emulsion and intubation are also important risk factors for infections due to *C. parapsilosis sensu stricto*, particularly in neonatal disease (Saiman *et al.*, 2000). *C. parapsilosis sensu stricto* is the predominant species identified in clinical disease followed distantly by *C. orthopsilosis* and *C. metapsilosis* (Gomez-Lopez *et al.*, 2008; Lockhart *et al.*, 2008; Tay *et al.*, 2009; Mirhendi *et al.*, 2010; Orsi *et al.*, 2010; de Toro *et al.*, 2011). Nevertheless, *C. orthopsilosis* and *C. metapsilosis* species have been isolated from ascites fluid, cerebrospinal fluid, joint fluid, abscesses, lung, blood, skin, nail, urine, central venous catheter and from superficial candidiasis (Lockhart *et al.*, 2008; Chen *et al.*, 2010; de Toro *et al.*, 2011; Asadzadeh *et al.*, 2016; Feng *et al.*, 2012; Silva *et al.*, 2009a; Schroder *et al.*, 2016).

C. parapsilosis sensu lato species cause candidemia (fungemia caused by *Candida* species), which is a dangerous condition when the fungus gets into the blood and therefore can disseminate throughout the body. Interestingly there is a remarkable difference between *C. albicans* and *C. parapsilosis sensu stricto* in this process. *C. albicans* is a normal colonizer of the gut and the female genitals and is transmitted from mother to child during and shortly after birth (called vertical transmission) (Nucci and Anaissie, 2001; Tiraboschi *et al.*, 2010; Farr *et al.*, 2015). Thus, people who develop *C. albicans* candidemia are usually infected by the strain they are colonized with. In contrast, *C. parapsilosis sensu stricto* does not need to undergo colonization after adhesion to cause such a disease. These infections are often associated with hospital environments (nosocomial infections), where medical devices and healthcare workers contribute to the horizontal transmission of this yeast (discussed in Trofa *et al.*, 2008). Nosocomial acquisition of *C. parapsilosis* has been deemed responsible for 9%–39% of adult infections (reviewed in detail in Trofa *et al.*, 2008). There are relatively few reports of candidemia due to *C. metapsilosis* and *C. orthopsilosis* (Gomez-Lopez *et al.*, 2008; Chen *et al.*, 2010; Blanco-Blanco *et al.*, 2014; Constante *et al.*, 2014; Ziccardi *et al.*, 2015); however, this could be due to inaccurate speciation within the *C. parapsilosis sensu lato* group.

The clinical relevance and manifestations of *C. parapsilosis sensu stricto* range from superficial to disseminated infections. Fungal meningitis, arthritis, ocular and urinary tract infection rarely occur due to *C. parapsilosis sensu stricto*. Fungal peritonitis is also a rare, but serious condition with high mortality rate, mostly caused by *Candida* species, among which *C. parapsilosis sensu stricto* has been found to be responsible for 26%–34% of cases (Chen *et al.*, 2004; Wang *et al.*, 2000; Warady *et al.*, 2000; Manzano-Gayosso *et al.*, 2003). *Candida* species are the second or the third most common cause of fungal nail infection (onycomycoses) (Mugge *et al.*, 2006; Jayatilake *et al.*, 2009). Although globally *C. albicans* is the dominant species causing onycomycoses, *C. parapsilosis sensu stricto* is the most prevalent species of the genus in some regions (Mugge *et al.*, 2006; Mujica *et al.*, 2004; Segal *et al.*, 2000; Fich *et al.*, 2014). Vulvovaginal candidiasis is a condition that 50% of the women over 25 years of age will experience at least once. It is mostly caused by *C. albicans* (85%–95%) while *C. parapsilosis sensu stricto* varies between 1.2%–8.9% (Geiger *et al.*, 1995; Sobel, 2007; Nyirjesy *et al.*, 2005 and reviewed in Trofa *et al.*, 2008). *C. parapsilosis sensu stricto* is also the second most common cause of fungal endocarditis, with a mortality rate of approximately 50% (Martin *et al.*, 1979; Ellis *et al.*, 2001; Garzoni *et al.*, 2007). The most striking observation regarding *C. parapsilosis sensu stricto* epidemiology, however, is its remarkable tendency to cause disease in low birth weight (less than 2500 grams) premature infants (Cheng and Yu, 1970; Rubinstein *et al.*, 1975; Brunner, 1975; Gugnani *et al.*, 1978; Yarchoan *et al.*, 1979; English, 1972; Falagas *et al.*2010). As noted above, parenteral nutrition, intravenous lipid emulsion and intubation are major risk factors for neonatal *C. parapsilosis sensu stricto* disease (Saiman *et al.*, 2000). In this specific group of patients, the prevalence of *C. parapsilosis sensu stricto* varies between 15.5%–65.1% (reviewed in Trofa *et al.*, 2008). In these neonates, the infection can affect almost all the body sites including the lungs, urine, retina and central nervous system (Carter *et al.*, 2008). In one study of a 15 year period (from 1981 to 1995) the prevalence of candidemia increased by 10-fold in a neonatal intensive care unit in the USA, with *C. albicans* as the dominant species during the first 10 years, but *C. parapsilosis sensu stricto* was responsible for up to 60% of the cases in the last 5 years (Kossoff *et al.*, 1998). Although this is an extreme example, the dominance of *C. parapsilosis sensu stricto* is obvious as it was demonstrated by a large scale study published in 2013 focusing specifically on neonatal candidemia. The authors summarized the results of 37 publications and found the average proportion of *C. parapsilosis sensu stricto* of all neonatal *Candida* infection was 33.4%. The infection did not show a homogeneous geographic distribution, it varied between 19.1 and 35.7% among continents (Europe 19.1%, Asia 24.7%, South America 29.1%, North America 33.8% and Australia 35.8%). The average mortality rate among neonates was approximately 10% (reviewed in Trofa *et al.*, 2008; Pammi *et al.*, 2013). However, the data presented a reciprocal correlation between birth weight and mortality rate, i.e., lower

the birth weight the higher the mortality rate. The mortality rates among infants weighing less than 1500 grams varies between 20% and 45%, and those who recover frequently suffer from long-term neurodevelopmental issues (Benjamin *et al.*, 2006; Fridkin *et al.*, 2006; Benjamin *et al.*, 2010; Miranda *et al.*, 2012; Friedman *et al.*, 2000). *C. metapsilosis* and *C. orthopsilosis* are infrequently reported as causes of neonatal candidemia (Oliveira *et al.*, 2014). Despite the clear association of *C. parapsilosis sensu stricto* with neonatal invasive disease, the ability of this fungus to preferentially affect this specific patient population remains unresolved.

Candida parapsilosis Sensu Lato – Diagnostics and Treatment

Until the early 1950s superficial and systemic *Candida* infections, independent of the species, were difficult to cure due to the lack of effective antimycotics (Duhig and Mead, 1951). The first antifungal drug available was nystatin that was followed by amphotericin B soon after (Hazen and Brown, 1950; Oura *et al.*, 1955; Sloane, 1955). These drugs are polyenes that function by destabilizing cell membrane ergosterol in fungi, causing an imbalance in ion homeostasis (reviewed in Ellis, 2002; Hammond *et al.*, 1974). These polyenes are challenged by their lack of absorption orally and their intravenous use is complicated by side effects, such as renal injury (Bell *et al.*, 1962; Giddings, 1962; Haber and Joseph, 1962). Nevertheless, amphotericin B was to the first antifungal that was able to cure systemic candidiasis, and newer formulations with less toxicity have been developed, particularly by admixing with lipids or intercalating the drug into liposomes (summarized in Dismukes, 2000). *C. parapsilosis sensu lato* isolates are susceptible to amphotericin B with very few exceptions, although *C. parapsilosis sensu stricto* is somewhat less susceptible than the other two members of the complex (Lockhart *et al.*, 2008; Silva *et al.*, 2009a; Ziccardi *et al.*, 2015; Lotfali *et al.*, 2016). Historically polyenes were followed by flucytosine (5-Fluorocytosine or 5-FC) in the late 1960s to treat candidiasis (Tassel and Madoff, 1968). This chemical is a fluorinated analogue of the base cytosine and can be uptaken by fungal cells. 5-FC itself does not possess antifungal activity however in the fungal cell it can be converted into derivatives inhibiting fungal RNA and DNA synthesis (Polak and Scholer, 1975; Waldorf and Polak, 1983). 5-FC has excellent oral bioavailability and can be effective in the setting of disseminated candidiasis (Tassel and Madoff, 1968). However, rapid resistance can occur with monotherapy, which limits the use of 5-FC (Hoeprich *et al.*, 1974; Ostrosky-Zeichner *et al.*, 2003). In the 1980s, azoles (that interfere with the ergosterol biosynthesis of fungi) became available and their ease of administration by the oral route has made them highly utilized (Sobel *et al.*, 1989). The azoles include ketoconazole, fluconazole, posaconazole, itraconazole, isavuconazole, and voriconazole. Of these, fluconazole is the most commonly used drug to treat *C. parapsilosis sensu lato* infections, since most of the isolates tested so far are susceptible (Pappas *et al.*, 2004). Although a publication from 2008 found that *C. parapsilosis sensu stricto* minimal inhibitory concentrations (MICs) were slightly lower than the ones of *C. orthopsilosis* and *C. metapsilosis*, a 2015 study established an opposite correlation (Ziccardi *et al.*, 2015; Lockhart *et al.*, 2008). These differences might be due to the limited sample numbers examined (especially in the case of *C. metapsilosis* and *C. orhopsilosis*) or it can be a geographical characteristic, but it is also possible that susceptibility is changing over time. At present, the echinocandins (anidulafungin, caspofungin, micafungin) are also frequently used prior to speciation of *Candida* identified in invasive disease (see Pappas *et al.*, 2016b). Echinocandins interfere with the β-1,3-glucan synthase 1, an enzyme responsible for cell wall biosynthesis of specific fungi, like *Candida*. *C. parapsilosis sensu stricto* displays a higher MIC value compared to *C. metapsilosis* and *C. orthopsilosis* as well as *C. albicans* (Ostrosky-Zeichner *et al.*, 2003; Pfaller *et al.*, 2006; Lockhart *et al.*, 2008). A possible explanation for this phenomena could be the diverse sequences of *FKS1* (the gene that encodes the synthase enzyme) orthologs in the three species (Garcia-Effron *et al.*, 2011). Moreover some authors reported strains of *C. parapsilosis sensu stricto* resistant to caspofungin (Ataides *et al.*, 2015; Ziccardi *et al.*, 2015). Due to the different mechanisms of these drugs, combination therapy utilizing antifungals with different mechanisms of action can be considered (Rex *et al.*, 2000; Vasquez *et al.*, 2002 and summarized in detail in Pappas *et al.*, 2004, 2016a).

Because of the different susceptible profiles of pathogens, accurate and rapid identification of the species is crucial for efficient antimicrobial therapy. To identify *Candida* species from clinical samples one can utilize molecular and non-molecular methods. The latter involves differential medias, carbohydrate assimilation/fermentation tests and microscopic examinations. Although these methods are relatively cost-efficient they are time consuming. There were also detection kits commercially available making these processes faster and more accurate but still the identification may take longer than one day. Molecular methods involving peptide nucleic acid – fluorescent *in situ* hybridization (PNA - FISH) designed to 26S RNA, polymerase chain reaction (PCR) applied alone or in combination with DNA digestion, quantitative real-time – PCR (qRT-PCR) with targeting dedicated genomic region of a specific pathogen were introduced, but these methods were much time intensive and costly (Rigby *et al.*, 2002; Flahaut *et al.*, 1998; Morace *et al.*, 1997; Maaroufi *et al.*, 2003). Due to the lack of special equipment and standardized protocols in many hospitals still non-molecular methods are in use (Hospenthal *et al.*, 2006 and reviewed in Silva *et al.*, 2012). More recently, techniques such as Luminex xTAG Fungal Analyte system, matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI - TOF MS) and loop-mediated isothermal amplification (LAMP) methods are being utilized in some institutions due to their high specificity and sensitivity (Babady *et al.*, 2011; Buchan and Ledeboer, 2013; Haas *et al.*, 2016; Trabasso *et al.*, 2015).

Candida parapsilosis Sensu Lato - Phylogeny

As noted, *C. parapsilosis sensu lato* species complex belongs to the Class *Saccharomycetes* (Division of *Ascomycota*/Kingdom *Fungi*) and they are members of the CUG clade, a group of yeasts that differs from most other organisms in how its members translate the CUG codon during protein synthesis. The universal codon usage CUG nucleotide triplet is generally translated to the hydrophobic amino acid leucine, but these fungi incorporate the hydrophilic amino acid serine instead (Santos and Tuite, 1995). The CUG clade also includes *Lodderomyces elongisporus* and *Debaryomyces hansenii* and certain *Pichia* species as well as other *Candida* species, like *C. albicans*, *C. dubliniensis*, *C. tropicalis* (Pryszcz *et al.*, 2015). Computational analyses have revealed that *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* are a closely related species complex in the CUG clade irrespective of the genomic regions chosen or the methods applied for calculations. By changing these parameters, however, the topology of the *C. parapsilosis sensu lato* species might be different in one publication compared to another. For example using only internal transcribed spacer 1 (ITS1) (a short non-coding genomic region located between 18S and 5.8S rDNA coding sequences) in combination with neighbor joining for phylogenetic reconstruction, *C. orthopsilosis* and *C. metapsilosis* clustered together separately from *C. parapsilosis sensu stricto* (Tavanti *et al.*, 2005). This topology was also found when the ITS1 - 5.8S rDNA - ITS2 region was the subject of analysis with the same approach (Robl *et al.*, 2014) or when the partial sequences of more than 1000 genes were analyzed using a maximum likelihood method (Riccombeni *et al.*, 2012). However, when this latter algorithm was applied for genomic sequences of mitochondrial proteins, the basal position of *C. metapsilosis* was established, while *C. orthopsilosis* and *C. parapsilosis sensu stricto* seem to be more closely related (Wu *et al.*, 2009). Similar results were obtained by a recent analysis applying the sophisticated PhylomeDB pipeline for all the ortholog genes of the three species (Pryszcz *et al.*, 2015).

Candida parapsilosis Sensu Lato – Genomics

Current methods allow for the whole genome sequencing of an organism within a few weeks. Understanding a species genome not only facilitates genetic manipulation, but the data can also be used for comparative analysis with other genomes to reveal genomic differences between species and even strains, and the data helps retrace evolutionary connections and trends. The genomes of *C. parapsilosis sensu lato* species complex conserves remarkable genetic evidences of how a species evolved on the way to becoming a human pathogen. Remarkably, all three members of the *C. parapsilosis sensu lato* group went through different evolutionary paths to become the pathogenic species that we know today. The first whole genome sequence available of the species complex was of *C. parapsilosis sensu stricto*. The genome of the clinical strain CDC 317 (isolated from a hand of a health care worker in the USA) was published in 2009 (Kuhn *et al.*, 2004; Butler *et al.*, 2009). This was followed by *C. orthopsilosis* and *C. metapsilosis* in 2012 and 2015, respectively (Riccombeni *et al.*, 2012; Pryszcz *et al.*, 2015). These three species are considered to be diploid organisms and they have not been observed to undergo either mating or meiosis. Mating requires two mating competent cells belonging to opposite mating type, as is a well described process in *Saccharomyces cerevisiae*. These types are determined by two loci in *S. cerevisiae* called mating type (MAT). The MAT locus can be either an "a" or an "α" idiomorph in this species (Haber, 2012). Homologous sequences of MATa and MATα idiomorphs can be found in other yeasts like *C. albicans* and they are also present (sometimes only partially) in the *C. parapsilosis* species complex where they are called mating-type-like (MTLa and MTLα) locus (Sai *et al.*, 2011).

Candida parapsilosis Sensu Stricto – The Single

Although more than 200 isolates of *C. parapsilosis* have been genetically analyzed, no MTLα idiomorphs have been found; therefore, there is genetically no chance for mating. Not having been found of course does not mean it does not exist, maybe it is very rare, or it has a specific geographic preference. However, the complete lack of a mating competent strain encoding MTLα idiomorph in its genome is just one reason explaining why mating might not occur in this species. Besides, a1 gene encoded within the MTLa idiomorph turned out to be a pseudogene (possibly due to a recent event), therefore it is not functional (Sai *et al.*, 2011; Logue *et al.*, 2005). Many authors have reported on the poor genetic variability among different *C. parapsilosis sensu stricto* clinical isolates, which points to the fact that this species might be highly clonal, meaning that the originally environmental species adapted to the human host only one time with the isolates causing human disease representing descendants of the same ancient strain (Lasker *et al.*, 2006; Butler *et al.*, 2009; Tavanti *et al.*, 2010). The first comprehensive whole genomic analysis of *C. parapsilosis sensu stricto* revealed molecular evidences suggesting that this transition might have actually happened more than once. The comparative genomic approach involved the reference strain CDC 317 and three newly sequenced strains: two environmental isolates (CBS 1954 from Italy and CBS 6318 from the USA), and one clinical strain GA1 (isolated from bloodstream infection in Hamburg, Germany) (Pryszcz *et al.*, 2013; Butler *et al.*, 2009; van Rij and Verona, 1949; Valach *et al.*, 2012; Gacser *et al.*2005). Pryszcz and coworkers established expansions of the arsenite transporter 3 (*ARR3*) gene (that encodes a protein required in baker's yeast to survive under high arsenic conditions) in clinical isolates (Ghosh *et al.*, 1999). One can assume that an organism living on the human skin does not have to face the selection pressure of arsenite, as it is highly toxic to the host. Therefore, the increased number of this gene presumably provides evolutionary advantage in the environment but not in/on the host. According to the molecular analysis these genetic expansions in the two clinical isolates occurred independently, meaning that the two strains might have been created in nature. If this is true, the "environment to human" transition of *C. parapsilosis sensu stricto* might not have been

a unique event and might have happened at least twice (Pryszcz *et al.*, 2013). Further analysis established few (5147) SNPs and 40 copy number variations (including deletions and duplications) between the four strains. Predicted gene numbers and genome sizes varied (between 5665 and 6293 genes and 13.030 and 13.138 Mb) mostly due to copy number variations. Heterogeneous distribution of SNPs and the footprints of flanking regions of deletions suggest that recombination events likely happened in *C. parapsilosis sensu stricto* (Pryszcz *et al.*, 2013). This is in line with the finding of Fundyga and colleagues, who had proposed that the minor sequence variations in the sequenced alleles could have been introduced by recombination events (Fundyga *et al.*, 2004). All these results taken together describe a largely homogenous genome and (in evolutionary steps) a very young species (estimated age of the *C. parapsilosis sensu stricto* species is about 10^6 years) (Pryszcz *et al.*, 2013; Fundyga *et al.*, 2004).

Candida orthopsilosis – Keep on Hybridizing

C. orthopsilosis species seems to consist of (at least) two different subspecies called Type 1 and Type 2. The latter is very likely formed due to a hybridization event. The signs of divergence and recombination within the species were described in 2005 and 2007 by Tavanti and coworkers by using RAPD and amplified fragment length polymorphism (AFLP) analysis (Tavanti *et al.*, 2005, 2007). Also in 2005, Iida and colleagues analyzed ITS sequences of *C. parapsilosis sensu lato* species, and identified two previously unknown groups of the complex. One was called Group IV, which showed the highest similarity to Group II (*C. orthopsilosis*) and one more group (named Group V) that seemed to be closely related to Group II and Group IV (Iida *et al.*, 2005). The genome of *C. orthopsilosis* (strain 90–125, a Type 2 isolate) was published in 2012. The 12.6 Mb genome contains 5700 protein-coding genes distributed on eight chromosomes (Riccombeni *et al.*, 2012). Based on the strains and sequences available, the genome of *C. orthopsilosis* is the only one in the complex so far that conserves evidence of mating in terms of MTL loci. This was established by Sai and colleagues, who investigated 16 *C. orthopsilosis* isolates and found both MATa/MATa (9 out of 16), MATα/MATα (5 out of 16) homozygous and MATa/MATα heterozygous (2 out of 16) isolates in both the subspecies, but no mating was observed under various laboratory conditions. One possible explanation was the incorrect maturation of the transcripts of MTLa1 and MTLa2 regulatory proteins (Sai *et al.*, 2011). Further evidence of mating was found a few years later with a comparative analysis of two *C. orthopsilosis* isolates (Riccombeni *et al.*, 2012; Pryszcz *et al.*, 2014). Surprisingly the newly sequenced MCO456 (ATCC96141) Type 1 (isolated in Texas, USA) strain showed a high degree of heterozygosity, as heterozygous sites affected 17% of the genome compared to only 0.1% in the reference 90–125 Type 2 strain (Tavanti *et al.*, 2005; Pryszcz *et al.*, 2014). *In silico* data revealed a mixed genome as rDNA cluster came from a close relative of Type 2 strain, the ITS region and MTL locus possibly derived from a Type 1 ancestor, suggesting that it is most likely that MCO456 Type 1 strain is a consequence of mating between two parental lineages (Pryszcz *et al.*, 2014). The minor (0.3%) divergence found between mitochondrial genomes of the subspecies also supports the mating hypothesis, as mitochondrion inheritance was found to be uniparental in *Candida* species (Ni *et al.*, 2011). Besides MCO456, another Type 1 isolate, AY2 (isolated in Singapore) was also investigated (Chan *et al.*, 2011). Interestingly there was over 99.9% identity found between the two Type 1 hybrids. This phenomenon that strains isolated from very distant geographic regions (Texas, USA and Singapore) share almost the same genome, point to the fact that the hybrid might have formed once, obtained evolutionary advantage in virulence and might have spread worldwide. However, this hypothesis had two weak points: (1) the sample number was limited, and (2) no homozygous Type 1 strain was involved in the analysis (Pryszcz *et al.*, 2014). This gap was filled in 2016, when a large scale comprehensive analysis of *C. orthopsilosis* isolates was performed involving 27 newly sequenced strains deriving from all over the world and associated with human infection. Among them only one isolate was found to be homozygous (like strain 90–125), whereas all the others were heterozygous. Phylogenetic reconstruction based on the SNP distribution in heterozygous regions clustered the isolates into four distinct groups. This indicates that hybridization in the *C. orthopsilosis* species is not a unique event, but occurred at least four times. A similar approach revealed that all the investigated isolates derived from hybridization events between the same parental lineages. To unify the nomenclature of the parental strains it was suggested to use "Parent A" and "Parent B" instead of "Type 1" and "Type 2". "Parent A" homozygous strain is equal to Type 2 isolate represented by 90–125. "Parent B" is a hypothetical strain that has not yet been identified. "Parent A" and "Parent B" are 5% different in genome sequence, and they possibly went through hybridization and formed "Type 1" hybrids. Upon hybridization the tetraploid state can be resolved by random chromosome loss resulting in a (nearly) diploid genome, which is a known process in *C. albicans* (Bennett and Johnson, 2003). It is likely that similar events could have happened to *C. orthopsilosis* hybrids, but the involvement of sexual processes can not be completely excluded (Schroder *et al.*, 2016).

Candida metapsilosis – The Virulent Hybrid

C. metapsilosis is the third member of the species complex and is least frequently associated with clinical disease (Gomez-Lopez *et al.*, 2008; Silva *et al.*, 2009a; Canton *et al.*, 2011; Feng *et al.*, 2012). Perhaps this was the reason why its genome was a missing link right until 2015, when the comparative analysis of the genome of eleven clinical isolate was published (Pryszcz *et al.*, 2015). Nearly 6000 protein-coding genes were identified in the 13.3 Mb genome. The results, similarly to *C. orthopsilosis*, revealed hybridization between two parental strains, being approximately 4.5% divergent in sequence. Molecular evidence suggests that all 11 strains involved in the analysis are derivatives of the same hybrid. However, as with *C. orthopsilosis*, it is not clear whether sexual processes were involved in the hybridization event. Although no MTLa genes had previously been found in 18 *C. metapsilosis* isolates by PCR, whole genome sequencing revealed strains carrying at least portions of both mating type loci; therefore, mating

can be considered as a possible mechanism for hybrid formation (Sai *et al.*, 2011; Pryszcz *et al.*, 2015). The fraction of the heterozygous regions varied between 54.5%–61.3% and 63.4%–68.5% across strains depending on the parameters used for *in silico* analysis, meaning that either the hybridization event is very recent or this heterozygous state is stable and loss of heterozygosity occurs slowly. Moreover, 84 deletions and 87 duplications were identified among the samples in question. This is in agreement with the results of Hensgens and colleagues who found *C. metapsilosis* species to be highly heterogeneous using AFLP genotyping (Hensgens *et al.*, 2009). Since all eleven *C. metapsilosis* isolates derived from clinical sources and they most likely represent descendents of the same hybrid, one might consider that by this process a new species emerged that was capable of adapting to human host and eventually acting as a pathogen under given circumstances (Pryszcz *et al.*, 2015). This hypothesis would have been supported further if the parental isolates were available, but unfortunately no such strains have yet been found. It is also not known if the parental strains are pathogenic or even still exist in the first place.

Candida parapsilosis Sensu Lato – Genetic Manipulation

The first genetically altered mutant of the complex was created in 2002. It was a galactokinase auxotroph strain of *C. parapsilosis* complemented with the functional allele of the gene cloned into a circular plasmid (Nosek *et al.*, 2002a). Since then, many homozygous deletion mutants were generated in *C. parapsilosis sensu stricto* according to the methods developed and improved during the last 15 years (Gacser *et al.*, 2005; Ding and Butler, 2007; Gacser *et al.*, 2007; Holland *et al.*, 2014). Efficient genetic manipulation requires three parameters optimized: (1) a selectable marker, (2) a target locus, (3) a method for transformation.

Selectable markers represent a class of reporter genes, as they "report" if they (and most likely the construct they are the part of) are in the cell or not. The ones used in *C. parapsilosis sensu lato* can be divided into two groups: dominant selectable markers and auxotrophy markers. The huge advantage of dominant selectable markers is that they can be used in prototroph strains (i.e., in practically any isolates). The first attempt of using such a system in *C. parapsilosis sensu stricto* was in 2005. Gacser and colleagues adopted a technique used in *C. albicans* that consisted of a drug called mycophenolic acid that can be neutralized by the enzyme inosine monophosphate dehydrogenase 3. The gene is encoded in *C. albicans*, and must be artificially overexpressed to take effect against the chemical (Kohler *et al.*, 1997; Gacser *et al.*, 2005). The major problem with dominant markers is that since *C. parapsilosis sensu lato* species are diploid, to delete both alleles of a given gene, two rounds of transformation have to be performed. But once the heterozygote is resistant to a given drug, how can one use the same drug again to create the homozygote? To resolve the problem, a system was developed that used a so-called recyclable selection marker. In *C. albicans*, the auxotrophy marker *URA3* is subsequently replaced with a dominant marker (nourseothricin acetyl transferase). The construct carrying the marker gene is flanked by two recognition sites of a site specific recombinase called flippase that upon activation removes all the sequences between the recognition sites, including the flippase, its promoter and most importantly the marker gene; therefore, the same cassette can be used again to remove the second allele (Kilby *et al.*, 1993; Morschhauser *et al.*, 1999; Staib *et al.*, 1999; Shen *et al.*, 2005). This technique was adopted by two different groups and used to perform the first targeted gene deletions in *C. parapsilosis sensu stricto* (Ding and Butler, 2007; Gacser *et al.*, 2007). Besides dominant selectable markers, auxotrophy markers are also possible to use. The drawback of this approach is the need of an auxotroph strain. Obviously if one decides to delete the same region in different prototroph isolates, this system is not usable. Pioneering work was done by Nosek and coworkers, who created the first auxotrophy selection system in *C. parapsilosis sensu stricto* utilizing a galactose auxotroph recipient strain and the galactokinase gene of *C. parapsilosis sensu stricto* isolated and cloned into a plasmid (Nosek *et al.*, 2002a). However to create knock out mutants (without the use of recyclable markers), the recipient strain must be a double auxotroph, which requires more work to create. But once the double auxotroph strain is available, the mutant generation is much more efficient then the recyclable technique in terms of time and effort. This method was elaborated by Noble and Johnson for *C. albicans* and used later by Holland and coworkers in *C. parapsilosis sensu stricto* to create large scale gene deletion libraries in these species (Noble and Johnson, 2005; Holland *et al.*, 2014).

Circular plasmids encoding an autonomously replicative sequence (ARS) can exist in *C. parapsilosis sensu stricto* (without integration into the genome), although they are not mitotically stable, meaning that without selection, they are eliminated rapidly (Nosek *et al.*, 2002a). Interestingly some circular constructs are stable without the ARS, while others are not. It is possible that the stable ones possess a region that can act as an "ARS-like" element (Nosek *et al.*, 2002a; Gacser *et al.*, 2005). In contrast to circular replicative elements, linear constructs (linearized plasmids or PCR products, etc.) tend to integrate by recombination into the genome and therefore be mitotically stable, which can be a targeted process or occur randomly. Randomly integrating constructs (or at least some of them) might have a special preference to dedicated regions of the genome, called hotspots (Gacser *et al.*, 2005). Targeted gene deletion can be achieved by adding homologous sequences of the flanking regions of the targeted locus in question to the ends of the linear constructs used for transformation. This can be performed by classical molecular techniques (amplification of homolog flankings by PCR, digesting and ligating them around the marker construct) or by PCR-mediated fusion as reported recently (Gacser *et al.*, 2007; Holland *et al.*, 2014). In general approximately 150 base pairs were found to be efficient, but later 400–500 or even 700 base pair long homologous sequences were used (Ding and Butler, 2007; Gacser *et al.*, 2007; Horvath *et al.*, 2012; Holland *et al.*, 2014; Grozer *et al.*, 2015). Even though it is called targeted, ectopic integration (when the construct does not integrate into the target locus, but some other region) can occur.

Once the construct is ready for transformation a reliable method is needed to allocate the DNA into the cell. To do so three methods were adopted and optimized for transforming *C. parapsilosis sensu lato*: (1) Biolistics, (2) chemical transformation, and (3)

electroporation. Biolistics (often referred as "gene gun") is a physical method that utilizes heavy metal (wolfram or gold) particles coated with the DNA construct that are accelerated by a rapid helium gas burst. The metal particles (and the DNA) reach a speed so great that they can hit through not only the membrane but also the cell wall of the yeast cells (Klein *et al.*, 1992). Although there is a publication of using biolistics to transform *C. parapsilosis sensu stricto* with replicative elements, it was not efficient and the procedure required special equipment (Zemanova *et al.*, 2004). Instead, chemical transformation and electroporation have become the standard everyday protocols to follow. Chemical transformation by the Lithium-acetate/single-strand DNA/polyethylene glycol method was the first approach successfully used to genetically modify *C. parapsilosis sensu stricto*. The advantage of the method is that it does not require a dedicated machine or equipment. This method yielded a transformation efficiency of 10^3 transformants μg^{-1} DNA using a non-integrative construct (Nosek *et al.*, 2002a). Although it was approximately ten times more efficient than biolistics it was still less than what had been achieved in other *Candida* species using electroporation (Zemanova *et al.*, 2004; Voronovsky *et al.*, 2002). The principles of electroporation were discussed by Shigekawa and Dower where the electroporator device applies a short direct current pulse that temporary opens pores in the plasma membrane through of which DNA molecules can pass (Shigekawa and Dower, 1988). As the cell wall represents an additional barrier, it must be weakened by (Tris-(hydroxymethyl)-aminomethane Ethylene-diamine-tetraacetate) Tris-EDTA, Lithium-acetate and Dithiothreitol treatment. Combining these methods resulted in an increase of two orders of magnitude μg^{-1} DNA in the transformation efficiency compared to chemical transformation, leading to the general use of this approach (Zemanova *et al.*, 2004; Nguyen *et al.*, 2011; Horvath *et al.*, 2012; Grozer *et al.*, 2015). However, it was subsequently determined that the efficiency of the electroporation and chemical method is highly strain dependent. For example, *C. parapsilosis sensu stricto* SR23 and GA1 isolates are more effectively transformed by using electroporation, whereas CLIB 214 and its derivatives are more amenable to chemical transformation (Zemanova *et al.*, 2004; Horvath *et al.*, 2012; Holland *et al.*, 2014; Perez-Garcia *et al.*2016).

Additionally, there is a study describing the first deletion mutant in *C. orthopsilosis*, which was generated using a recyclable nourseothricin selection marker that was introduced to the cells by electroporation to create *his1* and *leu2* homozygous deletion strains to screen possible mating events (Sai *et al.*, 2011). In contrast, no *C. metapsilosis* transformant has been reported to date.

Candida parapsilosis Sensu Lato – Virulence

Virulence is neither a property of a pathogen nor the host, rather it is a term that characterizes the outcome of the interaction between them. Virulence depends on the susceptibility of the host and properties related to the pathogen. These latter ones are called virulence factors, which, when altered, cause attenuated damage in a given system upon interaction (Casadevall and Pirofski, 1999). Cellular organisms developed protective mechanisms during evolution against obligate or opportunistic pathogens to maintain their own integrity. In higher eukaryotes the immune system is responsible for performing such tasks. It has humoral – (water soluble molecules, for example antibodies, complement proteins, defensins, etc.) and cellular components (phagocytes, B-cells, T-cells, etc.). It also can be divided to an ancient innate (monocytes, neutrophils, etc.) component and an evolutionary more recent adaptive branch (represented by B-cells and T-cells) (Batista and Harwood, 2009; Sarma and Ward, 2011; Ganz, 2003; Dale *et al.*, 2008; Crotty, 2015).

Candida cells are often associated with the skin and medical devices. In terms of pathogenesis, yeast cells have to overcome immune system defenses, most usually epithelial cells and phagocytes, to cause disease; therefore studies examining *C. parapsilosis sensu lato* virulence primarily focus on properties related to such interactions. Although overall tendency in virulence ranges from *C. parapsilosis sensu stricto* to *C. orthopsilosis* and *C. metapsilosis* in a decreasing order, the virulence attributes within species is a strain dependent characteristic (Sabino *et al.*, 2011; Nemeth *et al.*, 2013). Historically, the first attempt to gain insight into *C. parapsilosis sensu stricto* - host interaction *in vivo* was in 1962 by Andriole and Hasenclever, who investigated the survival of alloxan treated mice inoculated with different *Candida* species. They established that *C. parapsilosis sensu stricto* caused 40% mortality after one day of intravenous infection irrespectively of the alloxan treatment, and suggested the high dosage (10^8 yeast cells) as the most possible factor (Andriole and Hasenclever, 1962). This was proven a few years later by Goldstein and colleagues, who intravenously infected mice with lower inocula and monitored the animals for 21 days. They found that *C. parapsilosis sensu stricto* was cleared from all the organs examined (lung-heart, liver-spleen, kidneys, brain). However, clearance was significantly delayed when mice were treated with steroid (cortisone) (Goldstein *et al.*, 1965).

Morphology

C. parapsilosis sensu lato species typically grows as yeast-like cells, but *C. parapsilosis sensu stricto* and *C. orthopsilosis* strains are also able to form pseudohyphae (Nemeth *et al.*, 2013). The prefix "pseudo" is used because these filament-like structures are elongated, similar to true hyphae of *C. albicans*, but the two formations are fundamentally different. Pseudohyphae consist of mostly ellipsoid shaped cells that are attached to each other at the site of septation, while true hyphae are tube like structures with parallel sides without obvious constrictions between the cells and the first septum is located in the tube-like structure distant from the mother cell (reviewed in Berman and Sudbery, 2002). Filamentation can be induced by growing *C. parapsilosis sensu lato* species in the presence of 5% (V/V) CO_2 or in fetal bovine serum (FBS) containing media (Sabino *et al.*, 2011; Nemeth *et al.*, 2013). Different cell morphologies can affect colony morphology and biofilm forming abilities as well (discussed subsequently) (Laffey and Butler,

2005). Although the pseudohypha forming capabilities of the *C. parapsilosis sensu lato* species is poorly studied, it appears that in contrast to *C. parapsilosis sensu stricto* and *C. orthopsilosis*, *C. metapsilosis* is unable to form pseudohyphae. However, among *C. parapsilosis sensu stricto* and *C. orthopsilosis* isolates both pseudohypha producers and non-producers have been identified (Sabino *et al.*, 2011; Nemeth *et al.*, 2013; Gago *et al.*, 2014). Interestingly significantly larger proportion of *C. parapsilosis sensu stricto* strains cultivated from blood cultures formed filaments than environmental isolates (Sabino *et al.*, 2011). Pseudohypha formation might be important in host - pathogen interactions since pseudohypha-positive *C. orthopsilosis* isolates are less efficiently killed by J774.2 murine macrophages compared to pseudohypha negative cells. Additionally, this observation is also the case when the producer and non-producer strains of the species group are compared (Nemeth *et al.*, 2013).

Adherence and Biofilm Formation

C. parapsilosis sensu stricto has a strong predilection to adhere to medical devices, including implants, and the yeast has been frequently isolated from hands of healthcare workers, particularly in outbreak investigations (Ramage *et al.*, 2006; Bonassoli *et al.*, 2005). *C. parapsilosis sensu stricto* tends to form lower amounts and less compact biofilms than *C. albicans*. *C. albicans* biofilm on silicon elastomer is shallow and contains yeast-like, pseudohyphal – and hyphal elements embedded in a rich extracellular matrix (Andes *et al.*, 2004; Ramage *et al.*, 2004). In contrast, *C. parapsilosis sensu stricto* biofilms are thin and consists of yeast-like cells with a basal yeast layer but without significant extracellular matrix (Kuhn *et al.*, 2002). Interestingly biofilm forming capability of *C. parapsilosis sensu stricto* cells depends on their morphology. Different morphotypes correlate with distinct adhesion properties and varied colony morphologies (smooth, rough, snowball, crepe, crater and concentric) between which fungal cells can undergo a morphological "switch", a process known as phenotypic switching. Morphology is not conserved among strains, but it can be changed and/or facilitated by adding specific chemicals (Lott *et al.*, 1993; Enger *et al.*, 2001; Laffey and Butler, 2005). In addition to morphology, adherence and biofilm formation among *Candida* species strongly depends on the medium, pH and oxygen tension (Hawser and Douglas, 1994; Ramage *et al.*, 2006; Silva *et al.*, 2009b). Although biofilms are formed by the different *C. parapsilosis sensu lato* species, there is significant variability in their biofilm forming capabilities. *C. orthopsilosis* and *C. parapsilosis sensu stricto* biofilms on silicon elastomer material are similar in terms of dry weight in mature biofilms (48 h), but in contrast to *C. orthopsilosis*, *C parapsilosis sensu stricto* tends to have a solid lag phase, while *C. orthopsilosis* biofilms form rapidly. In contrast, *C. metapsilosis* forms less biofilm (Lattif *et al.*, 2010). Similarly, *C. parapsilosis sensu stricto* and *C. orthopsilosis* are more adherent and form more biofilm *in vitro* (with human buccal epithelial) and *in vivo* (in experimental mouse vaginal candidiasis model) compared to *C. metapsilosis* (Bertini *et al.*, 2013). However, the overall adherent properties of *C. parapsilosis sensu stricto* are weak or moderate compared that to those of *C. albicans* in vascular endothelium and human epithelial models (Klotz *et al.*, 1983; Bendel *et al.*, 1995). Notably, the factors involved in *C. parapsilosis sensu stricto* adherence are poorly understood. Agglutinin-like sequences (Als) are glycosylphosphatidylinositol (GPI) anchored cell surface proteins responsible for adhesion, and they compose a large and well characterized family in *C. albicans*, and Als3 is one of the best characterized adhesin (Hoyer, 2001; Phan *et al.*, 2007; Hoyer *et al.*, 2008; Hoyer and Cota, 2016). A recent study reported the involvement of the *C. albicans ALS3* ortholog gene CPAR2_404800 in *C. parapsilosis sensu stricto* adhesion. The homozygous mutant lacking CPAR2_404800 was attenuated in its ability to adhere to buccal epithelial cells *in vitro* and to bladder epithelium in a murine urinary tract infection model compared to wild-type (Bertini *et al.*, 2016). Additionally, the regulation of biofilm formation in *C. parapsilosis sensu stricto* is still unclear. However, the ortholog of *C. albicans BCR1* gene (a regulator of biofilm formation) in *C. parapsilosis sensu stricto* (*CpBCR1*) encodes a transcription factor that is required for adhesion and biofilm formation on silicone surface. In *C. albicans* Bcr1 is a positive regulator of *HWP1* that encodes a GPI-anchored protein responsible for adhesion to buccal epithelial cells (Staab *et al.*, 1999; Nobile and Mitchell, 2005; Nobile *et al.*, 2006b; Mayer *et al.*, 2013). Although *C. parapsilosis sensu stricto* lacks the ortholog of *HWP1*, it encodes one of its close relatives, *CpRBT1*, and this gene has been found to be under the regulation of CpBcr1 (Braun *et al.*, 2000; Ding and Butler, 2007). Interestingly, while in *C. albicans* Bcr1 promotes the expression of *ALS1* and *ALS3* in addition to *HWP1*, CpBcr1 appears to promote *CpRBT1*, but not the *CpALS* genes (Nobile *et al.*, 2006a; Ding and Butler, 2007). This suggests that the induction of *ALS3* genes in the two pathogens occurs through different regulatory pathways.

Secreted Enzymes

Secreted enzymes play vital roles in nutrition acquisition in nature and during interaction with the host. Indeed, the host environment is limited in accessible carbon and nitrogen sources. To digest such macromolecules, *Candida* species secrete proteinases, lipases and phospholipases. The production of phospholipase in *C. parapsilosis sensu stricto* is strain dependent and their involvement in virulence remains in question (Mohan das and Ballal, 2008; Pakshir *et al.*, 2013; Ramos Lde *et al.*, 2015; Shirkhani *et al.*, 2016). However, proteinases and lipases are well characterized and linked to virulence. Genes of these proteins expanded and compose gene families in certain pathogenic species that have resulted in the divergence of the enzymes in terms of substrate specificity, inducibility and expression pattern (Monod *et al.*, 1994; Hube *et al.*, 2000; Naglik *et al.*, 2004; Schaller *et al.*, 2005).

Genomic analysis of the *C. parapsilosis sensu lato* species complex revealed a remarkable expansion of secreted aspartyl proteinase (in *parapsilosis*) (*SAPP*) genes. While in *C. orthopsilosis* 11 such ORF were identified, the genome of *C. metapsilosis* and *C. parapsilosis sensu stricto* encode 14 potential *SAPP*s (Pryszcz *et al.*, 2015). The ability of *C. parapsilosis sensu stricto* to secrete proteinases and their association with virulence was already established in the 1980s (Macdonald, 1984; Ruchel, 1984; Ruchel

et al., 1986). By the 1990s, secreted proteinases were shown to degrade secretory Immunoglobulin A, and isolates from patients with *C. parapsilosis sensu stricto* vulvovaginitis were found to secrete significantly higher amount of proteinases than isolates from asymptomatic carriers (Douglas, 1988; Agatensi *et al.*, 1991). Although proteinase secretion is a general property of *C. parapsilosis sensu stricto*, examinations of *C. metapsilosis* and *C. orthopsilosis* have revealed both producers and non-producers (Sabino *et al.*, 2011; Nemeth *et al.*, 2013; Gago *et al.*, 2014). In *C. parapsilosis sensu stricto SAPP1* and *SAPP2* have been investigated in detail. The proteins encoded by these genes differ in their substrate specificity, enzymatic activity and expression patterns (Merkerova *et al.*, 2006; Hruskova-Heidingsfeldova *et al.*, 2009). *SAPP1* is duplicated, the paralogs are distinguished as *SAPP1a* and *SAPP1b*, and they are similar in nucleotide sequence, except only one nucleotide that however, does not alter the protein sequence (Horvath *et al.*, 2012). The mature protein localizes to the cell wall, and it is expressed only when exogenous protein is present as the sole nitrogen source in the media (Vinterova *et al.*, 2011; Hruskova-Heidingsfeldova *et al.*, 2009). The disruption of the alleles of *SAPP1a* and *SAPP1b* (Δ/Δ*sapp1*) in *C. parapsilosis sensu stricto* increased the resulting mutant's susceptibility to human serum and reduced its resistant to primary human phagocytes (peripheral blood mononuclear cells (PBMCs) and PBMC-derived macrophages (PBMC-DM)) in terms of phagocytosis and killing compared to wild-type yeasts (Horvath *et al.*, 2012). The expression of *SAPP2* appears to be constitutive, as it has been detected in every media examined, but the expression is significantly increased in the Δ/Δ*sapp1* mutant compared to wild-type (Hruskova-Heidingsfeldova *et al.*, 2009; Horvath *et al.*, 2012).

Based on computational predictions, lipases represent another major secreted family of pathogenic *Candida* species. In *C. albicans* there are 10 lipase genes identified to date, and some of them have been associated with virulence (Hube *et al.*, 2000; Stehr *et al.*, 2004; Schofield *et al.*, 2005). *C. parapsilosis sensu stricto* have been biochemically studied since the 1990s, and more recent studies have examined their potential utilization for biotechnological purposes (Briand *et al.*, 1995; Vaysse *et al.*, 1997; Neugnot *et al.*, 2002; Rodrigues *et al.*, 2016; Kannoju *et al.*, 2017). In the *C. parapsilosis sensu lato* group, the predicted numbers of lipase genes are 4 (*C. parapsilosis sensu stricto* and *C. orthopsilosis*) and 5 (in *C. metapsilosis*) (Pryszcz *et al.*, 2015). The higher number of lipase genes in *C. metapsilosis* is in contrast to laboratory observations, as no lipase producer *C. metapsilosis* strain has yet been identified, suggesting that the predicted genes are not functional (Nemeth *et al.*, 2013). However, it is possible that the induction media were not specifically optimized for *C. metapsilosis*. *C. parapsilosis sensu stricto* and *C. orthopsilosis* have both lipase positive and negative isolates. Notably, *C. parapsilosis sensu stricto* and *C. orthopsilosis* lipase producer strains are able to cause significantly more damage to J774.2 macrophages *in vitro* than non-producer ones (Nemeth *et al.*, 2013). Targeted removal of *Candida parapsilosis sensu stricto* lipase genes (*CpLIP*) *CpLIP1* and *CpLIP2* demonstrated their role in nutrient acquisition and virulence. Although both genes were removed, reintroduction of only one allele of wild-type *CpLIP2* totally complemented both the examined phenotypes, suggesting that *CpLIP1* (and other predicted lipase genes) might not be actively transcribed or proteolytically active under specific circumstances (Neugnot *et al.*, 2002). In the Δ/Δ*cplip1-cplip2* mutant the overall lipase production was abolished (suggesting that other predicted lipases might not be expressed) and an apparent growth deficiency in mediums containing fatty acids as a sole carbon source was observed. Moreover CpLip2 is also involved in biofilm formation on polyethylene, silicon and polystyrene surfaces and, more importantly, in virulence both *in vitro* and *in vivo*. The null-mutant was less resistant to phagocytosis and killing by murine macrophages, mature and immature human PBMC derived dendritic cells and PBMC-DM *in vitro*, and in a peritoneal mouse and a rat neonate model (Gacser *et al.*, 2007; Nagy *et al.*, 2011; Toth *et al.*, 2014a; Trofa *et al.*, 2011). Moreover, the phagocytes responded with higher chemokine and inflammatory cytokine expression on RNA and protein levels in *in vitro* interactions (Nagy *et al.*, 2011; Toth *et al.*, 2014a). The possible reason of the more efficient uptake was investigated by Toth and colleagues, who found an increase in macrophage (J774.2) migration towards lipase null-mutant fungal cells compared that to the wild-type yeasts (Toth *et al.*, 2015). In another *in vitro* system utilizing PBMC-DMs, the Δ/Δ*cplip1-cplip2* strain avoided phagosome - lysosome fusion (and consequently macrophage killing) less effectively than wild-type cells, and induced a significantly higher expression and secretion of TNFα, IL-1β, and IL-6 inflammatory cytokines (Toth *et al.*, 2014a). These findings indicate that secreted lipases might be the tools *C. parapsilosis sensu stricto* utilizes to attenuate host inflammatory responses.

Host Interaction

Recognition of pathogens upon interaction occurs between so-called pathogen associated molecular patterns (PAMP) and the receptors of the host cells called pattern recognition receptors (PRR). PAMPs are evolutionary conserved molecules that are indispensable components of a given pathogen (i.e., double stranded RNA of certain viruses, lipopolysaccharide of bacteria, β-glucan components of the fungal cell wall, etc.). PRRs are germ-line encoded receptors that can bind one or a few types of PAMPs, which triggers a cell response leading to phagocytosis and/or secretion of cytokines and other soluble factors. Such soluble factors are, for example, small molecular weight defensins, which are secreted primarily by neutrophils (α-defensins) and epithelial cells (β-defensins). Defensins are positively charged, cysteine rich proteins, often acting as pore-forming agents (reviewed in Ganz, 2003; Lehrer and Lu, 2012). *C. parapsilosis sensu stricto* induces significant release of α-defensins from primary human neutrophils and interactions with a human intestinal cell line, Caco-2, leads to an increased expression and secretion of human β-defensin 2 (HBD-2), although the difference compared to the non-infected Caco-2 controls was not significant (Gacser *et al.*, 2014).

In addition to the secretion of biologically active molecules, phagocytosis of pathogens and intracellular killing are effective mechanisms for eradicating invading microbes. After engulfment, a pathogen can be eliminated through various mechanisms including reactive chemical compounds, enzymatic digestion, ion chelators (Babior *et al.*, 2002; Aratani *et al.*, 1999; Vazquez-Torres *et al.*, 1996; Samaranayake *et al.*, 2001; Nikawa *et al.*, 1993). The first step of host-pathogen interaction is the recognition of

the microbe by the host effector cell. Successful pathogens have evolved numerous, specific paths to avoid the interaction between their PAMPs and the PRRs of the host. Although the involvement of *C. albicans* cellular components on host interactions has been intensively studied, our knowledge on such components of *C. parapsilosis sensu lato* remains limited (Ruiz-Herrera *et al.*, 2006; Chaffin, 2008; McKenzie *et al.*, 2010). *C. parapsilosis sensu stricto* cells are ingested and efficiently killed by J774.2 murine macrophages *in vitro*, and, during the interaction, the phagocytes respond with the transcription of inflammatory cytokines (IL-1β and TNFα) (Nemeth *et al.*, 2014). In terms of the components, the cell walls of the *C. parapsilosis sensu lato* species appear to be molecularly similar to the ones present in *C. albicans*, but their proportions and, consequently, the overall structures are diverse. Interestingly the cell wall porosity levels of the species complex is approximately twice as much as that of *C. albicans*, which is due to the shorter mannan chains attached to cell wall proteins in the *C. parpasilosis sensu lato* species (Shibata *et al.*, 1995; Estrada-Mata *et al.*, 2015). In line with this, the *C. parpasilosis sensu lato* species possess more glucan underneath a mannan layer, possibly as a result of a known compensatory mechanism to maintain cell wall strength (Estrada-Mata *et al.*, 2015; Walker *et al.*, 2013). The PRRs involved in the recognition and subsequent cytokine secretion playing role in *C. parapsilosis sensu lato* - phagocytes interaction are the C-type lectins Dectin-1 and mannose receptor (MR), which recognize β-1,3 glucan and N-linked mannans respectively, and toll-like receptor 4 (TLR4), binds O-linked mannans (Brown and Gordon, 2001; Medzhitov *et al.*, 1997; Tada *et al.*, 2002; Garner *et al.*, 1994; Yamamoto *et al.*, 1997; Netea *et al.*, 2006). All of these receptors are involved in TNFα secretion, while IL-1β production was found to be MR and Dectin-1 dependent. However, the involvement of TLR4 in IL-1β secretion might be a strain specific property since some reports describe TLR4 and Dectin-1 dependent IL-1β production (Toth *et al.*, 2013; Estrada-Mata *et al.*, 2015; Perez-Garcia *et al.*, 2016). Indeed the involvement of receptors in cytokine secretion induced by different isolates of the same species is a known phenomenon in *C. albicans* - host interactions (Marakalala *et al.*, 2013; Netea *et al.*, 2010). The lack of N-linked mannans in the outer layer leads to increased secretion of IL-1β, TNFα, IL-10 (anti inflammatory cytokine) and IL-6 from human PBMCs, and this compound is not necessary to efficient phagocytosis. Notably, *C. orthopsilosis* differed from the other two members of the group in terms of decreased TLR4 dependent IL-6 and MR dependent IL-10 secretion, which is consistent with the observation of the highly exposed glucan and chitin content of *C. orthopsilosis* compared to *C. parapsilosis sensu stricto* and *C. metapsilosis* (Estrada-Mata *et al.*, 2015).

C. parapsilosis sensu stricto and *C. albicans* induce the differentiation of distinct T-helper subpopulations. In contrast to PBMCs secreting increased amounts of Th1 and Th17 cytokines (IFNγ, IL-1β and IL-17, IL-22 respectively) upon challenge with *C. albicans*, interactions with *C. parapsilosis sensu stricto* induces the release of IL-10, a typical Th2 cytokine. Although the investigation of the T-helper response to *C. parapsilosis sensu stricto* is a very new field, recent work demonstrates that *C. parapsilosis sensu stricto* is able to switch the Th1/Th17 - Th2 bias towards the latter with a yet unknown mechanism to avoid the antifungal activity induced by Th1/Th17 cytokines (Toth *et al.*, 2013). Moreover, Galectin-3, another lectin type receptor that can be both anchored onto the cell surface and secreted, is involved with *C. parapsilosis sensu stricto* recognition in a systemic mouse infection model, as the fungal burden and the damage in the kidney of Galectin-3 knock-out mutants were higher compared to the ones of the wild-type mice. Interestingly, this study also found that the level of Galectin-3 in the sera of human neonates was significantly lower than in the samples of adults, which could be a possible explanation for why newborns are more susceptible to *C. parapsilosis sensu stricto* infection than adults (Linden *et al.*, 2013).

Successful pathogens utilize mechanisms by which they can avoid elimination by host mechanisms. It has already been established that *C. parapsilosis sensu stricto* is capable of replicating, forming pseudohyphae, aborting host cell mitosis and inducing exocytosis, therefore escaping from phagocytes and endothelial cells (Toth *et al.*, 2014b; Glass *et al.*, 2015). Additionally, endothelial cells might act as fungal reservoirs during infection. *C. parapsilosis sensu stricto* can be uptaken by these cells, and once internalized they can be hidden away from patrolling neutrophil granulocytes (Glass *et al.*, 2015). As shown above, *C. parapsilosis sensu stricto* secreted lipases are able to attenuate PBMC derived dendritic cell and PBMC-DM cytokine secretion, phagosome - lysosome fusion and affect phagocyte migration (Nagy *et al.*, 2011; Toth *et al.*, 2014a). Moreover, lipases might further modulate the immune response, such as through altering the prostaglandin profile of the host (Toth *et al.*,). Phospholipases can cleave membrane bound lipids resulting in the release of arachidonic acid, which can be processed further by the host to synthesize prostaglandins that can have either pro- or anti-inflammatory effects (Funk, 2001; Harris *et al.*, 2002). *C. parapsilosis sensu stricto* can produce prostaglandin E2 (PGE2), which is known to mediate T-cell response by promoting a Th2-type differentiation that, as mentioned earlier, is a poorly effective response to *Candida* (Grozer *et al.*, 2015; Harris *et al.*, 2002). In fact, *C. parapsilosis sensu stricto* induces human PBMC-DMs to produce more IL-10 and less IL-1β, IFNγ, IL-17 and IL-22 compared to PBMC-DMs challenged with *C. albicans* (Toth *et al.*, 2013). In contrast to *C. albicans*, removal of the gene *OLE2* (putative Δ9 fatty acid desaturase) from the genome of *C. parapsilosis sensu stricto* did not decrease PGE2 production. However, the *de novo* fatty acid biosynthesis changed, as demonstrated by an increase in palmitoleic acid and oleic acid, and the mutant was less resistant to human PBMC-DMs. Moreover it induced higher IL-10 (Th2 cytokine) secretion than the wild-type (Grozer *et al.*, 2015). Changes in the fatty acid synthesis can also lead to an impaired capacity to form biofilms on polystyrene and silicone surfaces as well as a decreased resistance against macrophage killing (Nguyen *et al.*, 2009). The involvement of fungal fatty acid synthesis in virulence is also elucidated by the fact that *OLE1* (a fatty acid desaturase) is responsible for maintaining cell membrane integrity and invasive growth of *C. parapsilosis sensu stricto*, as an *OLE1* mutant was less resistant to human serum and phagocytosis *in vitro* and showed attenuated virulence in intravenous mouse infection model (Nguyen *et al.*, 2011). The interactions of *C. metapsilosis* and *C. orthopsilosis* with host cells are poorly studied. However, *C. metapsilosis* is significantly less resistant to host effector cells compared to *C. parapsilosis sensu stricto* and *C. orthopsilosis* (Orsi *et al.*, 2010; Sabino *et al.*, 2011; Nemeth *et al.*, 2013), which is in line with the limited clinical cases of disease due to *C. metapsilosis*. Interestingly, BV-2 mouse microglial cells challenged with *C. metapsilosis*

effectively formed phagosomes containing the yeast cells, whereas *C. parapsilosis sensu stricto* partially inhibited this process (Orsi *et al.,* 2010). Studies in a Greater wax moth (*Galleria mellonella*) larvae survival model further demonstrated that *C. metapsilosis* is significantly less virulent compared to the other members of the *sensu lato* species and *C. metapsilosis* was also more effectively phagocytosed by *Galleria mellonella* haemocytes compared to the other two members of the complex (Nemeth *et al.,* 2013; Gago *et al.,* 2014).

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Candida auris: A New, Threatening Yeast

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Introduction

Candida auris is a novel and multidrug-resistant *Candida* species first reported in Japan in 2009 (Satoh *et al.*, 2009) and since then has been reported in 35 countries in five continents. This emerging yeast that can cause invasive infections and spread easily in healthcare settings is associated with high mortality. *C. auris* has some properties that make it very different from other species of *Candida*: (1) It is hard to identify using conventional phenotypic techniques and is commonly misidentified in clinical laboratories (Jeffery-Smith *et al.*, 2018); (2) Pathogenic isolates of *C. auris* have emerged independently in three continents simultaneously (Lockhart *et al.*, 2017b); (3) *C. auris* is very resistant to antifungals displaying distinct mechanisms of antifungal resistance and some strains have demonstrated high minimum inhibitory concentrations (MICs) to all three major classes of antifungal agents, extreme multidrug-resistance (XMDR), a feature not seen before in other *Candida* species (Forsberg *et al.*, 2019); and (4) *C. auris* persists for weeks in healthcare environments, can colonize patients perhaps indefinitely and is easily transmitted among patients causing healthcare-associated outbreaks, mainly in critically ill patients (Ruiz-Gaitán *et al.*, 2018a).

All these attributes make *C. auris* a "superbug" concerning microbiologists, clinicians, hospital epidemiologists and health authorities. In June 2016 the Centers for Diseases Control and Prevention (CDC) released clinical alert to healthcare facilities and provided guidelines to clinical management, laboratory diagnosis and infection control. Likewise, European Center for Disease Prevention and Control, World Health Organization, Pan American Health Organization, Public Health England, Spanish Ministry of Health, and South African National Institute of Communicable Diseases also issued similar epidemiological alerts in 2016.

The sudden and simultaneous appearance of *C. auris* throughout the world, as a human-infecting pathogen, remains a mystery to scientists. A recently published study suggests that the global transformation of *C. auris*, possibly a former plant saprophyte, into a deadly pathogen may be due to global warming, which could have forced *C. auris* around the world to adapt to higher temperatures, making it easier to infect humans with the helpful avian flora acting as intermediary hosts (Casadevall *et al.*, 2019).

Therefore, proper prevention and control of *C. auris* in healthcare facilities requires better knowledge of this new yeast, rapid detection and accurate methods of surveillance and identification, appropriate treatment and outcome monitoring of infected patients, infection control strategies adapted to this new species, and a coordinated public health response.

Microbiological Characteristics

Biology and Morphology

Phylogenetic

C. auris is closely related to *Candida haemulonii*, *Candida pseudohaemulonii*, *Candida duobushaemulonii*, and *Candida heveicola* in the Metschnikowaceae clade of *Candida* genus (Satoh *et al.*, 2009; Daniel *et al.*, 2014). Phenotypically, based on traditional biochemical methods only, these species are very similar and could be hard to correctly distinguish one species from the others of this complex (Kathuria *et al.*, 2015).

Colony morphology

C. auris can grow in different culture media. On chromogenic media (CHROMAagar *Candida* or CAN2) it develops pale purple to pink colonies; on Sabouraud dextrose agar, *C. auris* forms dull white to cream, smooth colonies, and on malt extract agar, it grows as butyrous, white-gray, smooth colonies (Lone and Ahmad, 2019). *C. auris* has high tolerance for heat and salinity (can grow at temperatures up to 42°C and in media contained 10% NaCl) but does not grow in presence of 0.01% cycloheximide (Jeffery-Smith *et al.*, 2018). These characteristics can be used in the presumptive identification of this pathogen from other species.

Cellular morphology

Microscopically, *C. auris* cells are oval or elongated and appears in single, pairs or in aggregates. The size (2–3 × 2.5–5 μm) and growth rate is comparable to *Candida glabrata* than to *Candida albicans* and unlike other *Candida* species, *C. auris* does not form hyphae, pseudohyphae, germ tube or chlamydospore on cornmeal agar (Satoh *et al.*, 2009). However, occasional rudimentary pseudohyphae formation has been found in *C. auris*, either in vitro or in vivo *Galleria mellonella* model, suggesting it could be a strain dependent condition (Borman *et al.*, 2016). The presence of at least two cellular morphologies of *C. auris* (aggregating and nonaggregating cells) has been reported (Borman *et al.*, 2016; Ben-Ami *et al.*, 2017; Sherry *et al.*, 2017). Aggregating *C. auris* isolates are produced by the inability of daughter cells to separate after budding and cannot be disrupted by mechanical action and/or detergents. Interestingly, in *G. mellonella* infection model studies, the nonaggregating isolates demonstrated greater pathogenicity

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than aggregating isolates (Borman *et al.*, 2016; Sherry *et al.*, 2017). Recently, a novel phenotypic switching system in *C. auris* has been reported which transits cells in three different cell types: typical yeast, filamentation-competent yeast and filamentous cells (Zhang and Hu, 2018). The heritable switch between the typical yeast and the filamentation-competent/filamentous phenotype is triggered by passage through a mammalian body, whereas the switch between the filamentation-competent and filamentous phenotype is nonheritable and is favored by low temperatures.

Biochemical features

The utilization of nitrogen and carbon sources by *C. auris* are distinct from other species of *Candida*. *C. auris* ferments glucose, trehalose (weak), sucrose (weak), but does not ferment maltose, lactose, galactose or raffinose. It assimilates glucose, maltose, D-trehalose, sucrose, D-melezitose, D-raffinase, soluble starch, galactitol, D-mannitol, citrate, sorbitol and Nacetyl-D-glucosamine (some strains) for carbon source. *C. auris* uses ammonium sulfate, cadaverine and L-lysine as nitrogen sources, but does not utilize sodium nitrate, potassium nitrate and ethylamine (Satoh *et al.*, 2009; Anil Kumar *et al.*, 2014; Prakash *et al.*, 2016).

Virulence

In *G. mellonela* and murine models, *C. auris* presents similar or slightly less virulence as *C. albicans, C. glabrata* and *Candida tropicalis* but greater virulence than *C. haemulonii* (Borman *et al.*, 2016; Ben-Ami *et al.*, 2017; Fakhim *et al.*, 2018). Most *C. auris* strains form biofilms to different degrees while some do not form biofilms at all (Chatterjee *et al.*, 2015; Oh *et al.*, 2011). *Biofilm formation* has been demonstrated with non-aggregate forming strains and, to a lesser degree, aggregate-forming strains of *C. auris*; these biofilms are thin and composed mainly of yeast cells with very limited extracellular matrix (Larkin *et al.*, 2017; Sherry *et al.*, 2017). Notably, *C. auris* biofilms had less biomass when compared with those of *C. albicans* but greater biomass than those of *C. glabrata*. The capacity of *C. auris* to produce/secrete other *virulence factors* as phospholipase, proteinase, hemolysins, aspartic proteases and the presence of oligopeptide transporters and mannosyl transferases is strain-dependent, at different degrees, and lower than *C. albicans* (Kumar *et al.*, 2015; Chatterjee *et al.*, 2015; Larkin *et al.*, 2017; Sherry *et al.*, 2017).

Identification

C. auris can be misidentified by the commercially available *biochemical*-based yeast identification systems (API-20C AUX, VITEK-2 YST, BD-Phoenix, MicroScan and Auxacolor) used in clinical laboratories worldwide. Due to the lack of *C. auris* in their databases, these systems do not identify or, worse, misidentify *C. auris* with other species such as *Rhodotorula glutinis, Candida sake* or *Saccharomyces cerevisiae* (API-20C AUX), as *C. haemulonii* or *C. famata, C. lusitaniae* or *C. famata* (VITEK-2YST VITEK), as *C. haemulonii* or *Candida catenulata* (BD-Phoenix), as *C. famata, C. lusitaniae, C. guilliermondii, C. catenulata, C. albicans, C. tropicalis* or *C. parapsilosis* (MicroScan), and as *S. cerevisiae* (Auxacolor) (Lone and Ahmad, 2019; Jeffery-Smith *et al.*, 2018). To avoid the identification problems of commercial techniques, the use of CHROMagar *Candida* medium supplemented with Pal's agar has been suggested as a simple low-cost method to distinguish between *C. auris* and *C. haemulonii* isolates (Kumar *et al.*, 2017). In our own experience the CHROMagar *Candida* medium supplemented with fluconazole (32 mg/L), used as a primary culture medium, is very useful for presumptive identification of *C. auris* in colonization surveillance cultures (unpublished data).

Currently, *molecular methods* or matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) are recommended for definitive *C. auris* identification. Sequencing of genetic loci, including D1/D2, RPB1, RPB2, and internal transcribed spacer (ITS) domains of the rRNA, has proven useful in the identification of *C. auris* (Chowdhary *et al.*, 2013; Sharma *et al.*, 2015), but it is unlikely to be available outside reference laboratories. Bruker Biotyper and bioMérieux VITEK MS *MALDI-TOF MS* identification platforms, using their latest updated libraries, properly identify *C. auris* and are the most reliable methods to identify this species in the majority of clinical laboratories (Lockhart *et al.*, 2017a). For the *typing* of *C. auris* isolates, a range of molecular techniques, including AFLP analysis, pulsed-field gel electrophoresis, M13 DNA fingerprinting or sequencing of genetic loci in addition to MALDI-TOF MS have been successfully implemented (Schelenz *et al.*, 2015; Girard *et al.*, 2016; Sharma *et al.*, 2015). Besides, *whole genome sequencing* (WGS) is increasingly being used to identification and typing of *C. auris* cases (Chatterjee *et al.*, 2015; Lockhart *et al.*, 2017b; Tsay *et al.*, 2017; Pchelin *et al.*, 2019). This method provides a better epidemiological analysis than all other. However, it is more expensive and requires higher skill and data processing capacity.

Other rapid culture-independent diagnostic techniques are under development, including polymerase chain reaction (PCR) and real-time PCR tests. These techniques will enhance the capacity to screen patients and identify new cases colonized by *C. auris* allowing a rapid implementation of infection control measures (Kordalewska *et al.*, 2017; Martínez-Murcia *et al.*, 2018; Ruiz-Gaitán *et al.*, 2018b; Leach *et al.*, 2018).

Antifungal Resistance

At present, because there are no established clinical breakpoints for this new specie, the MICs obtained for *C. auris* isolates have been compared to the breakpoints determined for other *Candida* species by the CLSI and EUCAST reference methodologies (CLSI, 2018; EUCAST, 2018). For epidemiological purposes, the CDC also maintains the conservative breakpoints already in use for other *Candida* spp.: $\geq$32 mg/L for fluconazole, $\geq$2 mg/L for amphotericin B, $\geq$4 mg/L for anidulafungin and micafungin, and $\geq$2 mg/L for caspofungin; for voriconazole and other triazoles, fluconazole resistance may be considered as

Table 1 In vitro antifungal resistance of 1044 *C. auris* isolates (data from 14 different studies from 10 countries in four continents)

Drug (no. of isolates)	*Average percentage of resistance or non-WT isolates (%)*
Fluconazole (939)	73.92
Isavuconazole (851)	4.13
Itraconazole (334)	39.14
Posaconazole (811)	28.75
Voriconazole (974)	35.92
Anidulafungin (903)	23.20
Micafungin (956)	18.36
Amphotericin B (894)	19.00

surrogate marker (Tsay *et al.*, 2017). Applying these breakpoints resistance to fluconazole exceeds 90%, while resistance to voriconazole may approach 60% of all *C. auris* isolates. Resistance to amphotericin B has been reported in up to 35% of isolates, whereas resistance to echinocandins has been between 2%–8%. Overall, more than 40% isolates were resistant to two antifungal classes (multi drug-resistant -MDR-), and about 4% of isolates have been resistant to all the three major classes of anti-fungal drugs, which makes them extreme drug-resistant (XDR) isolates, this status has never been observed before in *Candida* species (Lockhart *et al.*, 2017b; Chowdhary *et al.*, 2018; Ruiz Gaitán *et al.*, 2019a). Low MICs for the newer triazoles, such as posaconazole and isavuconazole, suggest that these antifungals maybe effective against *C. auris*. Arendrup *et al.* have proposed tentative CLSI and EUCAST epidemiological cut-off values (ECVs) for *C. auris* (Arendrup *et al.*, 2018). In a recent study, we have applied these ECVs to 73 bloodstream *C. auris* strains isolated during the Spanish outbreak (Ruiz-Gaitán *et al.*, 2018a) and to published MIC data of 14 studies from around the world that included more than 10 isolates each (Ruiz Gaitán *et al.*, 2019a). A total of 1044 isolates of *C. auris* were analyzed and the results are summarized in **Table 1**.

Although drug resistance mechanisms in *C. auris* are not established yet, these could be similar to those observed in other resistant *Candida* species: upregulation of efflux pumps (mainly ABC-type efflux pump) and biofilm formation for azole resistance (Ben-Ami *et al.*, 2017; Chatterjee *et al.*, 2015; Healey *et al.*, 2018), and gene mutations and biofilm formation for both, echinocandins or amphotericin B resistance (Sherry *et al.*, 2017; Kordalewska *et al.*, 2018; Hou *et al.*, 2019; Escandón *et al.*, 2018).

Persistence on Hospital Environment

One of the alarming characteristics of *C. auris* is its ability to persist on both dry and moist surfaces, bedding materials, floors, sinks, chairs, tables and other healthcare surfaces around infected or colonized patients, including medical equipment such as electrocardiogram leads, blood pressure monitoring cuffs, infusion pumps, and ventilators (Biswal *et al.*, 2017; Piedrahita *et al.*, 2017; Schelenz *et al.*, 2016; Ruiz-Gaitán *et al.*, 2018a). It has been confirmed that *C. auris* can persist on dry plastic surfaces for at least 2 weeks on culture and 1 month when their esterase activity (viability) is measured with a solid-phase cytometer (Welsh *et al.*, 2017). Therefore *C. auris* may spread through contact with contaminated environmental surfaces or fomites, and people, including healthcare workers, infected or colonized with *C. auris* shedding the organism. This could be one of the reasons of the outbreaks' spread and the difficulty in controlling them. In the *C. auris* outbreak reported from the United Kingdom in a neuroscience intensive care unit, with a total of 70 infected or colonized patients, the transmission of *C. auris* was found to be linked to reusable axillary temperature probes (Eyre *et al.*, 2018), confirming that this emerging pathogen can persist in the environment and be transmitted in health care settings.

Resistance to Disinfectant and Antiseptic Compounds

As stated above, contaminated surfaces may be a source of transmission of *C. auris*. Unlike other *Candida* species, *C. auris* is resistant to some disinfectant compounds commonly used in hospitals and healthcare facilities around the world, and this could be another reason that has made it difficult to control the outbreaks effectively. In consequence, the appropriate use of disinfectant compounds plays a major role in controlling the management and spread of this pathogen.

Although there are no established guidelines for decontaminating surfaces contaminated by *C. auris*, healthcare organizations have issued different recommendations. The CDC suggests the use of disinfectants effective against *Clostridium difficile* spores, while Public Health England suggests the use of sodium hypochlorite, alone or in conjunction with other products (Ku *et al.*, 2018). These recommendations have been based on an increasing number of studies, which demonstrated that sodium hypochlorite, peracetic acid, hydrogen peroxide and vaporized hydrogen peroxide resulted in the greatest reduction in *C. auris* CFU (Biswal *et al.*, 2017; Abdolrasouli *et al.*, 2017; Moore *et al.*, 2017; Cadnum *et al.*, 2017). On the other hand, acetic acid, ethyl alcohol, and quaternary ammonium compounds showed less effectiveness (Cadnum *et al.*, 2017). In addition, the use of mobile ultraviolet-C light room decontamination devices has been shown to be useful for killing *C. auris* from surfaces when used with a longer cycle time (30 minutes) and at a proper distance (2 meters) after patient discharge (de Groot *et al.*, 2019). The effectiveness of different

Table 2 Effectiveness of surface disinfectants against *C. auris*

Agent	*Concentration*	*Contact time*	*Efficacy*	*Reference*
Sodium hypochlorite (Clorox, Chlor-clean, HazTab)	≥1000 ppm 0.39%–0.65%, 10%	1 min	+ + +	Cadnum *et al.* (2017), Abdolrasouli *et al.* (2017), Moore *et al.* (2017)
Quaternary ammonium	2%	10 min	–	Cadnum *et al.* (2017), Biswal *et al.* (2017)
Peracetic acid - hydrogen peroxide (OxyCide)	1200 ppm	3 min	+ + +	Cadnum *et al.* (2017)
Ethyl alcohol (Purell desinfectant)	29.4 %	30 sec	+	Cadnum *et al.* (2017)
Acetic Acid	>5%	3 min	+	Cadnum *et al.* (2017)
Hydrogen Peroxide (Oxivir Tb, Clorox)	0.5%, 1.4%	10 min, 1 min	+ + +	Cadnum *et al.* (2017)
Vaporized hydrogen peroxide (BioQuell)	8 g/m^3	60 min	+ + +	Abdolrasouli *et al.* (2017)
UV light (UV- 360 Room Sanitizer)	2 meters	30 min	+ + +	de Groot *et al.* (2019)

disinfectants against *C. auris* are summarized in **Table 2**. After contact with infected or colonized patients, hand hygiene using soap and water may require the subsequent use of alcohol-based hand sanitizer for maximal disinfection.

Epidemiology

C. auris was first reported in 2009 following isolation from the external ear canal of a 70-year-old woman in a Tokyo geriatric hospital. Molecular analyzes and chemotaxonomic studies indicated that this strain represented a new species (phylogenetically related to *C. haemulonii* in the Metschnikowiaceae clade), and the authors proposed *Candida auris* (Latin for "ear") (Satoh *et al.*, 2009). Not long afterwards, the first three cases of bloodstream infection by *C. auris* were described in South Korea during a retrospective microbiology review (Lee *et al.*, 2011), including one case occurred in 1996 (the earliest known *C. auris* episode) and two cases in 2009. Isolates were initially misidentified as *C. haemulonii* and as *R. glutinis*, and were accurately identified as *C. auris* by molecular methods. Since then, *candidemia episodes* by *C. auris* have been reported from India (Chowdhary *et al.*, 2013), South Africa (Magobo *et al.*, 2014), Kuwait (Emara *et al.*, 2015), Venezuela (Calvo *et al.*, 2016), the United Kingdom (Schelenz *et al.*, 2016), the United States of America (Vallabhaneni *et al.*, 2016), Spain (Ruiz Gaitán *et al.*, 2017), Oman (Mohsin *et al.*, 2017), Pakistan (Lockhart *et al.*, 2017b), Israel (Ben-Ami *et al.*, 2017), Colombia (Parra-Giraldo *et al.*, 2018), Panama (Araúz *et al.*, 2018), United Arab Emirates (Alatoom *et al.*, 2018), Malaysia (Mohd Tap *et al.*, 2018), Germany (Kohlenberg *et al.*, 2018) and Greece (Stathi *et al.*, 2019). Infections by *C. auris* have also been described in Canada (Schwartz and Hammond, 2017), Japan (Iguchi *et al.*, 2018), Switzerland (Riat *et al.*, 2018), China (Wang *et al.*, 2018), Norway, Austria, Belgium and France (Kohlenberg *et al.*, 2018). Other countries such as Kenya, Thailand and the Netherlands have reported cases but detailed reports have not been published. Therefore, until August 31, 2019 *C. auris* has been reported from 35 countries in five continents; however, as this new species is misidentified by commonly available diagnostic methods, the burden of *C. auris* infection or colonization episodes is probably underestimated. **Fig. 1** shows the countries affected by *C. auris* infections since 1996, including published and unpublished single case reports. Since its first description, *C. auris* has become one of the most frequent causes of candidemia in some hospitals in India (Rudramurthy *et al.*, 2017), South Africa (Magobo *et al.*, 2014), Venezuela (Calvo *et al.*, 2016) and Spain (Ruiz-Gaitán *et al.*, 2018a).In South Africa it was the third most common cause of candidemia and was responsible for 14% of all cases during 2016–2017 (van Schalkwyk *et al.*, 2019). Outbreaks of *C. auris* infections have been reported in healthcare institutions in India (Anil Kumar *et al.*, 2014; Biswal *et al.*, 2017), the United Kingdom (Schelenz *et al.*, 2016), the United States (Vallabhaneni *et al.*, 2016), Venezuela (Calvo *et al.*, 2016), Pakistan (Lockhart *et al.*, 2017b), Colombia (Parra-Giraldo *et al.*, 2018; Morales-López *et al.*, 2017), Panama (Araúz *et al.*, 2018), Spain (Ruiz-Gaitán *et al.*, 2018a), and South Africa (Govender *et al.*, 2018) (**Fig. 2**). The largest outbreak reported in a single institution was observed in a Spanish hospital affecting more than 500 patients, including 90 episodes of candidemia and more than 400 colonized patients (Ruiz-Gaitán *et al.*, 2018a).

Phylogenetic analysis of isolates recognizes *four distinct C. auris clades* that cluster geographically and differ by tens of thousands of single-nucleotide polymorphisms (SNPs): The South Asian, South African, South American, and East Asian clades (Lockhart *et al.*, 2017b; Chatterjee *et al.*, 2015; Sarma and Upadhyay, 2017). Recently, an isolate representative of a potential fifth clade has been reported in a patient in Iran who had never traveled outside the country (Chow *et al.*, 2019). However, within the clades, high degree of relatedness was observed. With different clades from different geographical areas, it is suggested that the emergence of *C. auris* in each geographical region is independent and does not spread from a single source (Lockhart *et al.*, 2017b). The rise of *C. auris* as a human pathogen, however, is emerging simultaneously in five continents remains an enigma.

The ecological niche of *C. auris* is not known and although it has not been detected in the natural environment, related species have been detected in plants, insects, and sea/pool water (Jackson *et al.*, 2019). A recently published study suggests that the global transformation of *C. auris*, possibly a former plant saprophyte, into a deadly human pathogen may be due to global warming. This could have forced *C. auris* around the world to adapt to higher temperatures, making it easier to infect humans with the help of migratory birds acting as intermediary hosts and spreading agents (Casadevall *et al.*, 2019).

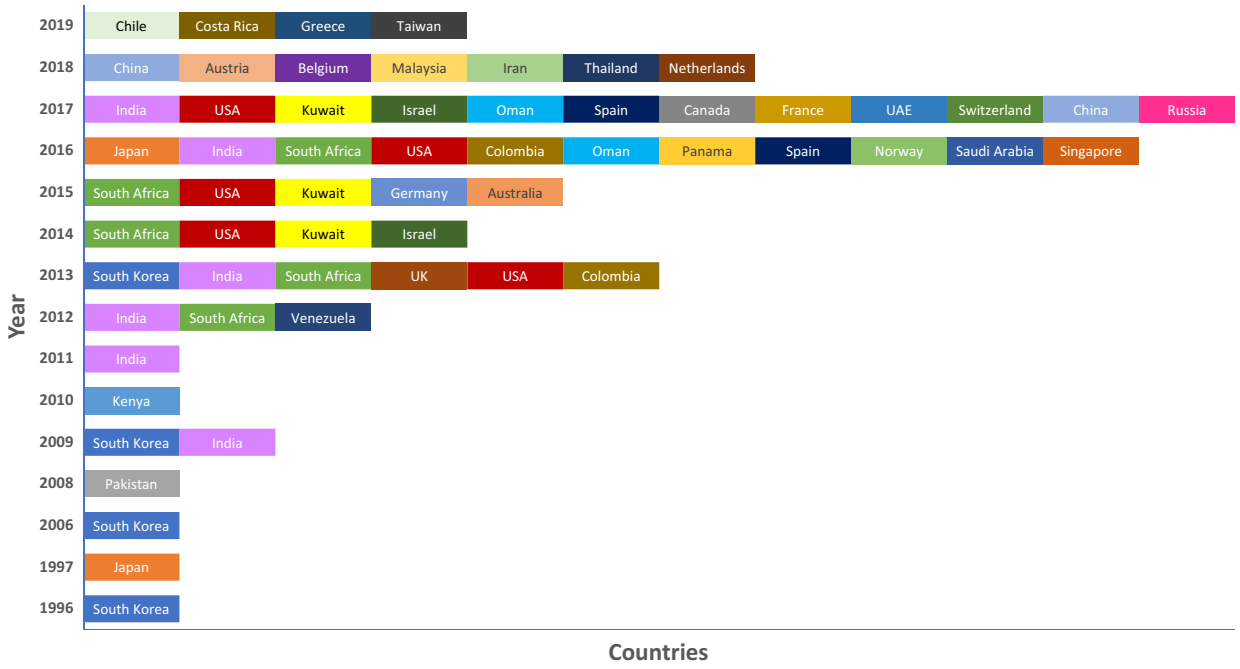

Fig. 1 Countries affected by *C. auris* infections since 1996–2019.

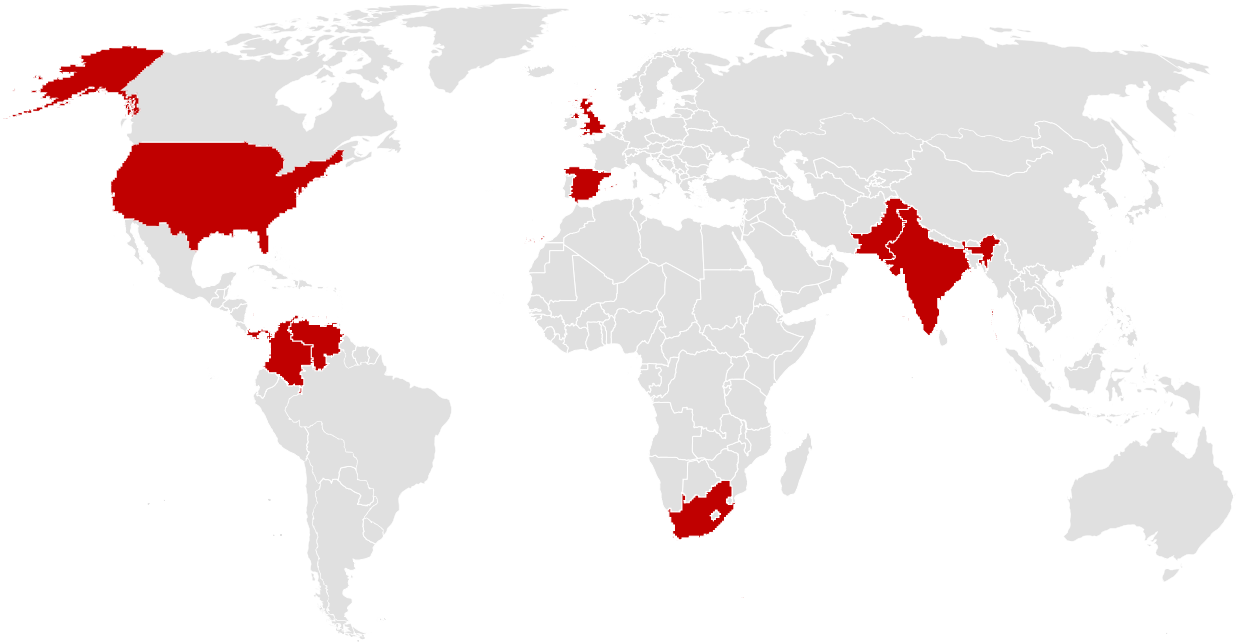

Fig. 2 Countries from which *C. auris* outbreaks have been reported as August of 2019.

Clinical Features

Colonization

C. auris has the ability to colonize multiple body sites including nose, mouth, ear canal, groin, axilla, rectum, urine, and wounds (**Fig. 3**). Persistent colonization is another characteristic of *C. auris* and has been isolated in patients for 3 months or more after initial detection despite the use of agents such as chlorhexidine, nystatin or echinocandins (Schelenz *et al.*, 2016; Vallabhaneni *et al.*, 2016).

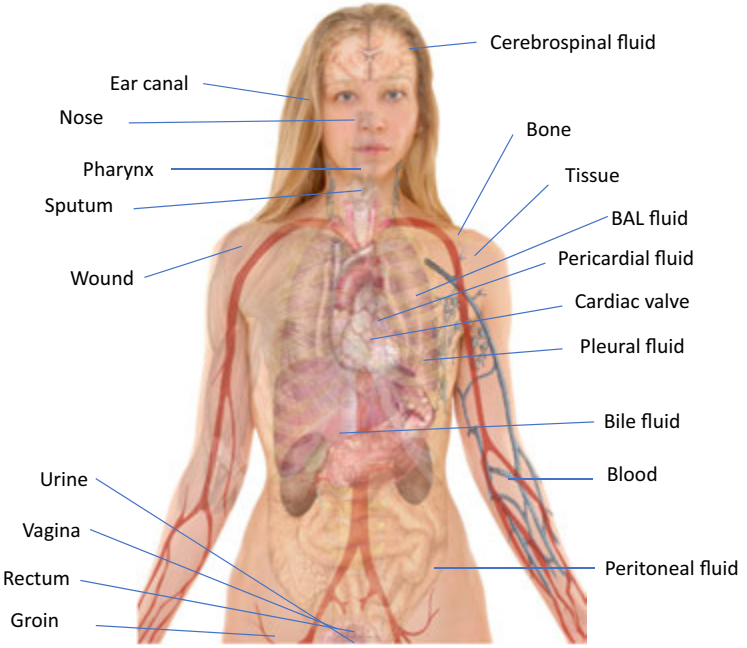

Fig. 3 Anatomical sites and clinical samples in which *C. auris* has been isolated in different studies.

These facts suggest the need to isolate colonized patients and to carry out weekly surveillance cultures until discharge and upon readmission to health care facilities. Patient isolation may be discontinued after three weeks of negative surveillance cultures. In large hospital outbreaks, such as in the United Kingdom and New York City, the screening sites in patients with the highest diagnostic performance for *C. auris* were the axilla and groin. In contrast, in the Spanish outbreak, rectum and urine were the most frequently colonized sites by *C. auris* (see "Relevant Websites section"; Kenters *et al.*, 2019).

Risk factors for *C. auris* colonization including prolonged stay in intensive care units, mainly in endemic countries, and contact with previously colonized patients or environment. It has been reported that the contact time for the acquisition of *C. auris* can be as short as 4 hours, and invasive infections can be acquired within 48 hours after admission to the critical care unit (Sarma and Upadhyay, 2017; Schelenz *et al.*, 2016; Jeffery-Smith *et al.*, 2018; Kenters *et al.*, 2019). The use of empirical antifungal therapy in colonized patients is not indicated and should only be considered if the patient presents a rapid clinical worsening (Kenters *et al.*, 2019).

Clinical Presentation

The clinical presentation of invasive infections by *C. auris* is similar to that of other species of *Candida*, including a wide spectrum of manifestations: candidemia, meningitis, osteomyelitis, otomastoiditis, chronic otitis media, intra-abdominal infections, pericarditis, pleural effusion, vulvovaginitis and bone infection (Satoh *et al.*, 2009; Chowdhary *et al.*, 2017; Khan and Ahmad, 2017; Sears and Schwartz, 2017; Morales-López *et al.*, 2017; Jeffery-Smith *et al.*, 2018; Spivak and Hanson, 2018; Khatamzas *et al.*, 2019). However, candidemia is the most frequent clinical manifestation of *C. auris* infection, as reported in a meta-analysis which included 16 countries and 742 isolates (Sekyere, 2018).

In the majority of reported cases, invasive infection by *C. auris* occurs in patients with altered immunity (systemic lupus erythematosus, diabetes mellitus, psoriasis, ulcerative colitis, etc.) who are in critical condition or have undergone invasive procedures (central venous catheter, urethral catheter, venovenous extracorporeal membrane oxygenation or major surgery) (Lee *et al.*, 2011; Chakrabarti *et al.*, 2015; Calvo *et al.*, 2016; Vallabhaneni *et al.*, 2016; Abdalhamid *et al.*, 2018; Alatoom *et al.*, 2018; Das *et al.*, 2018). Likewise, in most cases there is a history of administration of broad-spectrum antimicrobial agents or antifungal agents prior to the invasive candidiasis onset (Lockhart *et al.*, 2017b; Rudramurthy *et al.*, 2017; Ruiz-Gaitán *et al.*, 2019b). Some candidemia episodes have been observed in patients with severe comorbidities, including hematologic malignancies and solid organ transplantation. Afterward, invasive infection should be suspected in septic transplant recipients previously colonized by *C. auris* or hospitalized in units with positive environmental cultures, all in order to establish an early and adequate antifungal treatment (Azar *et al.*, 2017; López-Vilella *et al.*, 2019).

Outcome

C. auris attributable mortality infection is arduous to establish as most patients have a wide variety of comorbidities and co-infections that make this estimate difficult (Sekyere, 2018). In *C. auris* candidemia episodes, the estimated overall mortality rates range from 28%

Table 3 Reported cases of infection by *C. auris*

Type of infection or location of C. auris isolation	*No. of cases*	*References*
Candidemia	432	Kim *et al.* (2009), Lee *et al.* (2011), Chowdhary *et al.* (2013), Anil Kumar *et al.* (2014), Magobo *et al.* (2014), Chakrabarti *et al.* (2015), Kathuria *et al.* (2015), Sharma *et al.* (2015), Chatterjee *et al.* (2015), Emara *et al.* (2015), Lockhart *et al.* (2017b), Schelenz *et al.* (2016), Calvo *et al.* (2016), Al-Siyabi *et al.* (2017), Mohsin *et al.* (2017), Rudramurthy *et al.* (2017), Ben-Ami *et al.* (2017), (see "Relevant Websites section"), Morales-López *et al.* (2017), Vallabhaneni *et al.* (2016), Ruiz-Gaitán *et al.* (2019b), Tan and Tan (2018), Pchelin *et al.* (2019)
Central nervous system	12	Borman *et al.* (2016, 2017), Morales-López *et al.* (2017), Ruiz-Gaitán *et al.* (2018a), Khatamzas *et al.* (2019), Sayeed *et al.* (2019)
Endocarditis and other heart infections	3	Khillan *et al.* (2014), Ruiz-Gaitán *et al.* (2018a)
Respiratory tract (only includes BAL and pleural involvement)	18	Anil Kumar *et al.* (2014), Khillan *et al.* (2014), Kathuria *et al.* (2015), Borman *et al.* (2016), Prakash *et al.* (2016), Azar *et al.* (2017)
Otitis externa	23	Satoh *et al.* (2009), Vallabhaneni *et al.* (2015), Abastabar *et al.* (2019), Iguchi *et al.* (2018), Jung *et al.* (2019)
Abdominal	18	Kathuria *et al.* (2015), Prakash *et al.* (2016), Ruiz Gaitán *et al.* (2017), Morales-López *et al.* (2017), Tsay *et al.* (2017), Sayeed *et al.* (2019)
Skin and soft tissue (including surgical wounds)	13	Anil Kumar *et al.* (2014), Kathuria *et al.* (2015), Borman *et al.* (2016), Lockhart *et al.* (2017b), Tan and Tan (2018)
Bone	6	Morales-López *et al.* (2017), Tsay *et al.* (2017), Ruiz-Gaitán *et al.* (2018a), Tan and Tan (2018), Heath *et al.* (2019)
Persistent candidemia and breakthrough fungemia	13	Ruiz-Gaitán *et al.* (2018a), Alatoom *et al.* (2018)

to 60% (Lee *et al.*, 2011; Chowdhary *et al.*, 2013; Calvo *et al.*, 2016; Lockhart *et al.*, 2017b; Ruiz-Gaitán *et al.*, 2019b; Barantsevich *et al.*, 2019). Likewise, it has been observed that independently of the type of infection or the host's immune status, delay in the starting of an adequate antifungal therapy, either by erroneous identification or late diagnosis, increase the mortality rate up to 35% (Morales-López *et al.*, 2017). In the limited number of candidemia episodes reported in pediatric patients, mostly preterm new-born from Asia and South America, the mortality rate raised 30% (Lee *et al.*, 2011; Chowdhary *et al.*, 2013; Calvo *et al.*, 2016; Warris, 2018).

Regarding complications related to candidemia, in the outbreak reported in Spain complications after or during candidemia were observed in up to 12% of the episodes including spondylodiscitis, ventriculitis and endocarditis. Interestingly, all cases appeared despite appropriate antifungal treatment (Ruiz-Gaitán *et al.*, 2018a).

Although the available data are scarce, in addition to candidemia, other deep-seated infections caused by *C. auris* have been reported: one episode of pericarditis, one case of *C. auris* infection from a donor after lung transplant, and four cases of peritoneal infection, meningitis or bone infection (Khillan *et al.*, 2014; Mohd Tap *et al.*, 2018; Heath *et al.*, 2019) (**Table 3**).

Treatment

C. auris poses a real treatment challenge due to its high antifungal resistance rates. However, antifungal treatment is not recommended for *C. auris* isolates when the evidence suggest colonization rather than infection. Unlike other *Candida* species, there are no therapeutic guidelines for *C. auris* infections and therefore therapeutic options should be considered on each patient individually. Furthermore, the CDC recommend consulting with an infectious disease specialist before treating a patient infected by this species. While *C. auris* exhibit multidrug resistance, most isolates respond to echinocandins. Therefore, *echinocandins*, at standard dosing, are recommended as first-line treatment of invasive *C. auris* infections in adult patients. Prior susceptibility testing, however, is always required (Chowdhary *et al.*, 2016). For neonates, CDC recommend 1 mg/kg/day of *amphotericin B* deoxycholate or 5 mg/kg/day of liposomal amphotericin B in unresponsive cases (Tsay *et al.*, 2017). If after 5 days of treatment, clinical or microbiological improvement is not achieved, switching to a liposomal amphotericin B (5 mg/kg daily) or a *combination therapy* (echinocandin plus liposomal amphotericin B) must be considered. In addition, azoles plus echinocandins *in vitro* combinations have also been effective against *C. auris* (Fakhim *et al.*, 2017).

To date the appropriate duration of antifungal treatment for invasive *C. auris* infection is not known. In patients with echinocandin therapy new metastatic septic foci (meningitis, endocarditis, arthritis) have been observed even weeks after blood cultures became negative (Ruiz-Gaitán *et al.*, 2018a). Therefore, close *outcome monitoring* of patients with *C. auris* candidemia and extension of treatment beyond that contemplated in the current clinical guidelines (Cornely *et al.*, 2012; Pappas *et al.*, 2016) are recommended.

Due to the common resistance to fluconazole and voriconazole, and the emerging resistance to amphotericin B and echinocandins in some countries, the need for *new antifungal drugs* with activity against *C. auris* is evident. Fortunately, a number of new antifungal agents that are undergoing phase 2 or 3 clinical trials that have also been shown to be effective against *C. auris*. Ibrexafungerp, formerly SCY-078, is the first orally bioavailable 1,3-β-D-glucan synthesis inhibitor with a potent antifungal and antibiofilm activity against *C. auris* isolates and *C. auris* biofilms (Larkin *et al.*, 2017; Berkow *et al.*, 2017). VT-1598, a tetrazole-based drug that inhibits the ergosterol biosynthetic pathway, has also demonstrated *in vitro* and *in vivo* activity against *C. auris* (Wiederhold *et al.*, 2019a). Manogepix (APX001) is novel antifungal agent that exhibits potent, *in vitro* and *in vivo*, antifungal activity against *C. auris* and other drug resistant fungal species by inhibiting the fungal wall protein Gwt1 (Zhao *et al.*, 2018; Hager *et al.*, 2018a; Berkow and Lockhart, 2018b; Arendrup *et al.*, 2018; Wiederhold *et al.*, 2019b). Finally, rezufungin (formerly CD101) is a new echinocandin with a long half-life allowing intravenous administration of once a week, furthermore CD101 possesses potent antifungal activity against *C. auris* (Berkow and Lockhart, 2018a; Hager *et al.*, 2018b; Lepak *et al.*, 2018).

Infection Prevention and Control

The rapid acquisition of *C. auris* in health-care facilities in association with its multi-drug resistance and the high mortality rates related to invasive infection by this emerging pathogen highlight the need of prompt implementation of *infection prevention and control* (IPC) measures. Recommendations for infection control are the same for a patient infected or colonized with *C. auris* since both pose a risk for transmission. Until now, these recommendations have been adapted from strategies implemented for other pathogens like *Clostridium difficile* and multi-resistant Gram-negative bacilli. Recently, two European groups have just published recommendations specifically elaborated for *C. auris* in the light of experience acquired in recent years (Kenters *et al.*, 2019; Alastruey-Izquierdo *et al.*, 2019).

The implementation of early and strict IPC measures is essential to prevent the spread of *C. auris*. Furthermore, immediate notification of *C. auris* isolation to clinical and infection control teams, as well as to health authorities, is essential to implement IPC measures at all levels in a timely manner. The initial IPC measure is to isolate patients infected or colonized in single-patient rooms; if single rooms are not available, patients with *C. auris* must be cohorted in the same room, unit or box. Furthermore, healthcare personnel should strictly follow *contact precautions* when caring infected or colonized patients, including strict adherence to hand hygiene practices and proper use of personal protective equipment (disposable long-sleeved gown and gloves). Hand hygiene should be performed at the point of care using alcohol-based handrub conform EN 1500 (Ku *et al.*, 2018). Besides, the number of staff caring for these patients must be minimized and the allocation of dedicated staff should be considered wherever possible (Ong *et al.*, 2019).

After identification of a *C. auris* case, all patients sharing the same room, unit or box must be screened for *asymptomatic colonization* in the axilla and groin, including any other relevant sites (e.g., nose, urine, rectum, throat, wounds and catheter exit sites). In colonized patients, at least two negative assessments should be performed one week apart to discontinue IPC measures (Kenters *et al.*, 2019; Alastruey-Izquierdo *et al.*, 2019). When transferring patients colonized or infected by *C. auris* to other healthcare facilities, the staff at the receiving facility should be notified to implement the appropriate IPC and screening measures.

Although there are fewer data on the effectiveness of antiseptics against *C. auris* for skin *decolonization*, in high-risk patients and/or before mayor surgery procedures, selective oropharyngeal and/or digestive decontamination may be considered with chlorhexidine, oral nystatin or other antifungal drugs, taking into account the susceptibility profile of each isolate (López-Vilella *et al.*, 2019).

Any reusable *medical equipment* used in patients infected or colonized by *C. auris* should be cleaned and disinfected after every single use and, where possible, dedicated equipment should be used. The cleaning and disinfection of affected *patient rooms* should be implemented trice a day with a product effective against *C. auris* (as mentioned above). Additionally, after the patient's discharge the terminal cleaning and disinfection effectiveness should be monitored by culturing the surfaces around the patient (Kenters *et al.*, 2019; Alastruey-Izquierdo *et al.*, 2019).

Conclusion

Since its first description in 2009, *C. auris* has emerged as a serious nosocomial health risk, with widespread outbreaks in numerous hospitals worldwide. *C. auris* has subsequently been reported from a multitude of clinical manifestations, including colonization, mucosal infections, deep-seated infections, candidemia and even death. Infections caused by *C. auris* have been documented in healthcare institutions in 35 countries and is listed as the second leading cause of blood infection outbreaks in some affected institutions. The lack of appropriate identification, protection and containment procedures at some institutions has allowed *C. auris* to spread across the globe. This emerging pathogen can survive for months on exposed surfaces and medical equipment and is able to develop resistance to different disinfectant compounds. Furthermore, *C. auris* is very resistant to antifungals and some strains have demonstrated high MICs to the three major classes of antifungal agents, a feature not seen before in other *Candida* species.

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Further Reading

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Identification of Candida auris.

Immune Response to *Candida albicans* Infection

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Glossary

Antimicrobial peptides Small soluble defense molecules, which constitute key elements of the innate immunity as they can directly kill bacterial, viral and fungal pathogens.

DAMPs (damage-associated molecular patterns) Danger signals, usually host biomolecules, generated in injured tissues that can initiate and perpetuate a non-infectious inflammatory response; many DAMPs are nuclear or cytosolic proteins/DNA released outside the cell or exposed on the surface of the cell following tissue injury.

Emergency myelopoiesis Infection-activated proliferation and differentiation of bone marrow hematopoietic and progenitor stem cells toward the myeloid lineage, with the development of other lineages (lymphoid and erythroid) inhibited, in order to replenish mature myeloid cells consumed during infection.

Fungal colonization Growth of *C. albicans* on host tissues (gastrointestinal and urogenital tract) leading to a commensal relationship with the immunocompetent host; high level of colonization (due to antibacterial chemotherapy and/or immunosuppression) precedes de invasive infectious process, which often involves translocation of the pathogen across the gut mucosa to the bloodstream.

IFN-gamma (interferon-γ or type II interferon) Pleiotropic cytokine, predominantly secreted by activated T cells (CD4+ Th1 and CD8+ cells), as well by innate lymphoid cells (ILCs) and another immune cells ($\gamma\delta$ T), which plays an essential role in both innate and adaptive immune responses, by activating a number of phagocytic cell functions that favor pathogen clearance during infection.

ILCs (innate lymphoid cells) A family of developmentally related innate immune cells that belong to the lymphoid lineage, and are characterized by their lymphoid morphology, a lack of myeloid and dendritic cell phenotypic markers, and absence of antigen T- and B-cell receptors; although these cells do not respond in an antigen-specific manner, are involved in tissue development and remodeling, and are emerging as important effectors of innate immunity by secreting a variety of cytokines, including IFN-γ, which depends on particular ILC subsets.

Inflammasomes Cytosolic multiprotein complexes, formed by a PRR (e.g., NLR), an adapter protein (e.g., ASC), and caspase 1; their assembly and activation are triggered in response to microbial (PAMPs) and non-microbial danger (DAMPs) signals leading to caspase-mediated proteolytic activation of pro-IL-1β and pro-IL-18 into fully mature inflammatory cytokines.

Innate immune tolerance Down regulation of innate immune responses, such as proinflammatory cytokine production, probably through epigenetic changes, upon exposure of innate immune cells to microbial ligands; the most studied example is the endotoxin tolerance induced by successive injections of bacterial lipopolysaccharide (LPS).

Lectins A class of proteins that function as receptors with special carbohydrate-recognition domains, which are able to identify and bind to carbohydrates and their conjugates, forming aggregates; they are widely found in plants and animals, including mammals, and depending on their structure and function are classified into four types: C-, P-, I- and S-type lectins.

Morphogenetic transition (yeast-to-hypha transition) Process by which dimorphic fungal species, as *C. albicans*, switch between two growth patterns depending on environmental conditions. The transition from budding yeast to filamentous growth (hypha) initiates with the emission by the yeast of a germ-tube (nascent hypha), and it is associated with changes in cell wall structure and composition and in the expression of virulence factors.

Opsonins Molecules that enhance phagocytosis by binding to microbial cells during infection, and to dead/dying self-cells; major opsonins are the complement system, antibodies and other molecules (as the mannose binding lectin) which aid the immune system by a variety of mechanisms: favouring clearance of pathogens and dead self-cells by macrophages and neutrophils, activating the complement system, and targeting cells for destruction by natural killer cells.

Phagocytic synapse Massive reorganization of membrane proteins coordinated by the actin cytoskeleton of phagocytic cells that occurs upon recognition of β-glucan particles (zymosan or yeasts) by dectin-1, which clusters the receptor in a synapse-like structure, required for activation of dectin-1 signaling; this allows phagocytic cells to discriminate direct microbial contact from soluble ligands.

PAMPs/MAMPs (pathogen/microbial associated molecular patterns) Conserved molecules, broadly expressed in pathogen, as well as in non-pathogen microorganisms (bacteria, fungi, virus), and absent in mammals, that constitute molecular signatures that trigger immune responses upon recognition by innate immune cells through PRRs.

PRRs (pattern recognition receptors) Receptors that are predominantly expressed in innate immune cells that sense microbial presence through recognition of PAMPs/MAMPs, allowing discrimination between self and non-self by using a limited number of receptors, and initiating innate defense mechanisms.

Trained immunity Prolonged enhancement of function of the innate immunity after adequate priming (recognition of microbial ligands by host receptors) leading to a functional reprogramming associated to epigenetic changes. Macrophages and monocytes are among the main cells of the innate

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immune system that can be trained, leading to phenotypes better equipped to deal with the pathogen.

Type I interferons (IFN-α, IFN-β) Pleiotropic cytokine family, expressed by immune cells, that have diverse effects on innate and adaptive responses during infection; type I IFNs are essential for defense against viral infections, but may be detrimental to a diverse range of microbial infections by impairing host resistance; their role during candidiasis has not been unequivocally established yet.

Introduction: *Candida albicans*, an Opportunistic Fungal Pathogen

The genus *Candida* includes more than 300 disparately related species. *Candida spp*. are found in a diverse range of environmental niches, but only about twenty *Candida* species can cause human disease. Of these, more than 90% of invasive infections are caused by the five most common pathogens, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, being *C. albicans* the most common fungal pathogen for the immunocompromised host. The incidence of other species, such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis* has increased in the past few years, and also, other *Candida* species have been described as emerging pathogens able to cause disease in humans (i.e., *C. dubliniensis*, *C. guilliermondii*, *C. kefyr*, *C. famata*, *C. lusitaniae*, *C. auris*, *among* others) (Pelroth *et al.*, 2007; Daniel, *et al.*, 2014; Guinea, 2014; McCarty and Pappas, 2016). This review is focused on *C. albicans*, as the most prominent species causing candidiasis, based on frequency and virulence.

As a commensal harmless organism, *C. albicans* colonizes the mucosal surfaces of the body of approximately 50% of healthy individuals at any given moment. When the host defenses are impaired, the balance between the host and this commensal fungus may turn into a parasitic relationship. The nature and extent of the impairment of host immune responses determine the manifestation and severity of candidiasis, a term which includes superficial mucocutaneous (skin, oral, vaginal and gastrointestinal) infections as well as serious disseminated or invasive infections (blood-stream infection or candidemia and deep-seated candidiasis) with mortality rates that can reach 40% (Pelroth *et al.*, 2007; Pfaller and Diekema, 2007; Yapar, 2014).

C. albicans does not act as a passive element during the infectious process but actively participates in the establishment and progress of the infection by expressing a set of putative virulence factors, whose expression varies among strains and is often environmentally regulated. These fungal attributes include the yeast-to-hypha transition (morphogenetic conversion from budding yeast to the filamentous growth form or hypha), the secretion of hydrolytic enzymes (aspartyl-proteases and phospholipases, among others) and the cytolytic peptide toxin candidalysin, phenotypic switching (ability to switch between different cell phenotypes), antigenic variability, adhesion to inert (plastic) materials and host tissues and ligands, ability to develop biofilms, and immunomodulation of host responses (Naglik *et al.*, 2004; Mayer *et al.*, 2013; Poulain, 2015; Höfs *et al.*, 2016; Moyes *et al.*, 2016).

The fungal cell wall, as the outermost cellular structure, plays a major role in the interactions between the microorganism and the environment, including the host, and therefore in the pathogenicity of the fungus. The *C. albicans* cell wall is a complex and dynamic structure composed by a network of β-1,3 and β-1,6 glucans, chitin, and mannoproteins. Microfibrillar polymers (chitin and glucans) form a skeleton that accounts for its rigidity and morphology. Cell wall mannoproteins are attached to microfibrillar polymers and play a major role in fungal physiology, including interactions with the host. Mannan, a complex structure composed by polymers of mannose, is found in covalent association through N- or O-linkages with these cell wall proteins (mannoproteins), that expand the entire cell wall structure, from the periplasm to the external surface where they are dominant. The cell wall mannan of *C. albicans* contains α-1,2-, α-1,3-, α-1,6-, and β-1,2-linked mannopyranose units with few phosphate groups. Lipids are also present in the phospholipomannan complex (PLM), a type of extensively glycosylated glycosphingolipid with hydrophilic properties that plays a relevant role in host interactions. Significant differences in cell wall organization and composition have been described between budding yeasts and hyphae, such as chitin and β-glucan content, structure and exposure to cell surface, and mannan structure and mannoprotein expression (Gozalbo *et al.*, 2004; Poulain and Jouault, 2004; Ruiz-Herrera *et al.*, 2006; Arana *et al.*, 2009; Höfs *et al.*, 2016).

Host resistance to candidiasis involves the coordinated action of both innate and adaptive host immune responses, which are triggered following fungal recognition by immune and non-immune cells. Defense mechanisms are initially triggered by an inflammatory response mediated by the innate immune system that also induces and modulates the adaptive immune responses, which in turn regulates signals from the innate system. The most relevant aspects of these defense responses to *C. albicans* are described next.

Initial Recognition of *C. albicans*, Innate Immune Responses

C. albicans first entrance in the body occurs at the mucosal and epithelium surfaces. When the pathogen overcome this first barrier, *C. albicans* is recognized by resident immune cells, which trigger antifungal responses to attract and activate other immune cells in order to clear the fungus from the tissues.

PRRs Involved in *C. albicans* Detection

Candida sensing is based on a complex set of interactions involving a variety of host receptors, mainly PRRs (pattern recognition receptors), and fungal ligands considered as PAMPs (pathogen-associated molecular patterns). Major *C. albicans* PAMPs are cell wall components, such as the skeletal polysaccharides chitin and glucan (β-1,3-glucan and β-1,6-glucan) and mannan (mannoproteins); also, intracellular fungal components, such as nucleic acids (DNA, RNA), that are released following phagocytosis and killing of fungal cells, are ligands for some intracellular PRRs. Expression and cell surface exposition of some fungal PAMPs may differ between the yeast and hyphal forms of *C. albicans*, a phenomenon that determines significant differences in the immune responses triggered by both fungal morphotypes. Besides, expression of some fungal virulence factors is also co-regulated with the yeast-to-hypha transition, enabling the hyphae to be better equipped to develop the infectious process and to overcome the host immune responses (Poulain and Jouault, 2004; Poulain, 2015; Arana *et al.*, 2009).

The most relevant families of PRRs involved in *C. albicans* sensing are Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (**Fig. 1**).

TLRs constitute a family of PRRs expressed by most immune cell types (innate cells such as neutrophils, monocytes, macrophages, dendritic cells, as well as by B- and T-lymphocytes and natural killer cells), and non-immune cells, such as epithelial and endothelial

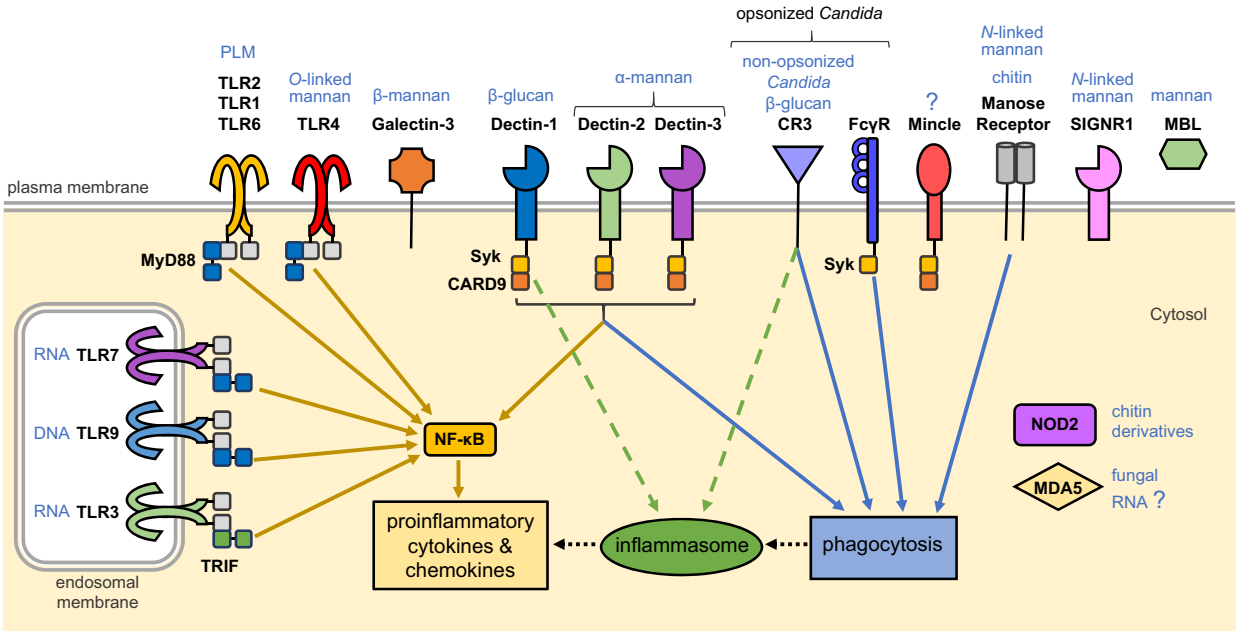

Fig. 1 *C. albicans sensing by innate immune cells. C. albicans* yeasts and/or hyphae are sensed by a variety of PRRs that recognize fungal ligands (PAMPs). Extracellular fungal ligands (basically cell wall components, such as glucans, N-linked and O-linked mannan moieties, chitin) are recognized by plasma membrane receptors, such as TLRs (mainly TLR2 and TLR4), CLRs (dectin-1, dectin-2 and dectin-3, MR and others: mincle, SIGNR1), galectin-3 (an S-type lectin), and the integrin CR3. Intracellular fungal ligands (DNA, RNA), released upon phagocytosis and killing, involves recognition by TLRs located at the endosomal membrane. Receptors for complement fragments and particularly FcγR also contribute to the recognition of *C. albicans* opsonized cells. Soluble (serum) MBL also contributes to *C. albicans* detection by binding mannan moieties and promoting opsonization. The simultaneous recognition of various PAMPs triggers complex signal transduction pathways (not shown in detail) leading to activation of immune responses. Signaling though TLR/MyD88 and Dectin-1/Syk/Card9 converge in the transcription factor NF-κB that activates expression of proinflammatory cytokines, whereas signaling through dectins, CR3, MR and FcγR induces phagocytosis of fungal cells. Other receptors, such galectin-3, SIGNR1, mincle, contribute to these responses. Collaboration among receptors in recognition of yeasts or hyphae and cross-talk among signal transduction pathways determines the tailored immune response to *C. albicans*. The inflammatory response is also generated by inflammasomes, that induce caspase-1-mediated activation of pro-IL-1β, a key cytokine in immunity against *C. albicans*; inflammasome complexes can be generated and activated following fungal recognition by some PRRs (Dectin-1, CR3, TLRs), and by endogenous molecules released following phagocytosis and phagosomal disruption. Other cytosolic receptors (NOD2, MDA5) also contribute to detection of fungal-derived ligands. CARD9: caspase activation and recruitment domain-containing protein 9; MyD88: myeloid differentiation factor 88; NF-κB: nuclear factor-κB; Syk: spleen tyrosine kinase; TRIF: TIR-domain-containing adapter-inducing interferon-β.

cells. Among TLRs, the plasma membrane-bound TLR2 (which forms homodimers and heterodimers with TLR1 and TLR6) and TLR4 recognizes mannan moieties associated to the fungal cell wall; β-1,2 mannosides present in phospholipomannan and mannoproteins constitute the major ligand for TLR2, whereas O-linked mannosyl residues, which are accessible on the yeast surface, are the ligand for TLR4 (**Fig. 1**). Endosomal TLR9, TLR7 and TLR3 sense microbial nucleic acids following endocytosis and pathogen degradation that causes release of DNA and RNA (**Fig. 1**). TLR9 recognizes DNA containing unmethylated CpG motifs (the high rate of methylation and low frequency of CpG motifs in mammalian DNA avoids its recognition by TLR9). TLR7, and probably TLR3, recognizes fungal RNA. Restriction of some TLRs to endosomal membrane is also critical for discriminating between self and non-self nucleic acids (Sasai and Yamamoto, 2013; Kawasaki and Kawai, 2014; Fradin *et al.*, 2015; Gil *et al.*, 2016).

C-type lectin receptors (CLRs) play critical roles in *C. albicans* sensing by innate immune cells, as they recognize special carbohydrate domains of the fungal cell surface (**Fig. 1**). Major ligands for CLRs are β-glucans and mannans. Dectin-1 is the receptor for fungal β-glucans, which is expressed by myeloid phagocytes, as macrophages, monocytes, dendritic cells and neutrophils; differential surface exposure and structural differences of β-glucans between yeasts and hyphae (or even between fungal strains) modulate their immunological properties. β-glucans are also recognized by human neutrophils through the integrin complement receptor 3 (CR3). Dectin-2, mainly expressed by macrophages, neutrophils and dendritic cells, is the functional receptor for N-linked α-mannan residues on the yeast and hyphal cell walls, and forms heterodimers with dectin-3, a CLR that recognizes α-mannans on the surface of *C. albicans* hyphae. Consequently, the simultaneous and differential recognition of mannans from yeasts and hyphae by dectin-2 and dectin-3, and the different accessibility of glucan to dectin-1 may account for differences in the immune responses triggered by yeast and hyphal forms of *C. albicans*. Highly branched N-linked mannosyl chains are also recognized by the mannose receptor (MR) expressed on macrophages; MR also participates in recognition of fungal chitin. DC-SIGN (dendritic cell-specific-ICAM-grabbing non-integrin) is a CLR present on myeloid cells, including dendritic cells, that recognizes also N-linked mannan. Mincle (macrophage inducible $Ca2+-$dependent lectin) is another member of the dectin-2 CLR family, expressed on monocytes/macrophages and neutrophils, which is involved in *C. albicans* recognition, although its ligand has been not well characterized yet. Galectin-3 is an S-type lectin receptor on the macrophages that recognizes β-mannan domains present both in phospholipomannan and mannoproteins. Mannan-binding lectin (MBL) is a soluble serum CLR that binds mannan-moieties on fungal surfaces (Brown and Gordon, 2001; Brown *et al.*, 2002; Poulain and Jouault, 2004; Taylor *et al.*, 2007; Hardison and Brown, 2012; Miramón *et al.*, 2013; Netea *et al.*, 2015; Richardson and Moyes, 2015).

In addition to detect PAMPs on the cell surface or in the lumen of endosomes or lysosomes, there is a cytosolic detection system to sense infection, which includes NOD-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). NOD2 is involved in the immune responses to chitin-derived components, and NLRs function as components of the inflammasomes, cytosolic multi-protein complexes that play an important functional role in the innate immune response to candidiasis (**Fig. 1**), and also have an effect on adaptive immune responses (Joly and Sutterwala, 2010; Joly *et al.*, 2012; Tomalka *et al.*, 2011). Recently MDA5, a member of the RIG-I-like receptors (a family of PRRs that sense cytoplasmic nucleic acids important for viral recognition) has been also described to play a role in immune responses to *C. albicans*, although the fungal ligand responsible for MDA5 activation has been not yet characterized (Jaeger *et al.*, 2015).

C. albicans cells usually express various cell-surface PAMPs whose expression may change among strains and morphotypes. Therefore, recognition of *C. albicans* by immune cells is a complex process that may involve the simultaneous or sequential activation of different PRRs. Consequently, collaboration among receptors in fungal recognition and crosstalk between intracellular signaling pathways may lead to the final tailored immune responses generated. As an example, some lectin receptors such as dectin-1, galectin-3 and SIGNR1 (a murine C-type lectin homolog of the human DC-SIGN) have been identified as TLR2 co-receptors to design a collaborative recognition or to modulate ligand specificity; similarly, dectin-1 and SIGNR1 may also collaborate with TLR4 in fungal recognition, and dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human macrophages. In some cases, a physical interaction between receptors has been demonstrated, such as galectin-3 and TLR2 or galectin-3 and dectin-1, suggesting that galectin-3 may mediate the cooperation between dectin-1 and TLR2 (Gantner *et al.*, 2003; Ferwerda *et al.*, 2008; Netea *et al.*, 2008; Romani, 2011; Lionakis, 2014; Gil *et al.*, 2016). In addition, this complex network of *C. albicans* sensing receptors allows immune cells to respond to (1) whole fungal cells through interaction between surface PAPMs and PRRs, (2) fungal ligands generated following phagocytosis and fungal destruction (such fungal DNA and RNA), through endosomal PRRs, and (3) cytosolic-located fungal ligands released from phagolysosomes or DAMPs (damage-associated molecular patterns) generated by cellular damage during infection, through NODs/NLRs.

Innate Immune Responses to *C. albicans*

Upon recognition of *C. albicans* PAMPs by PRRs, signaling pathways are triggered to generate effector and secretory antifungal responses by immune cells. Signaling pathways for PRRs are well known is some cases, such as for TLRs and C-type lectins, whereas are still poorly understood in others (Hardison and Brown, 2012; Kawai and Akira, 2009; Kawasaki and Kawai, 2014; Kumar *et al.*, 2011). Briefly, TLR-mediated signaling activates pathways leading to the induction of genes for inflammatory cytokines, as TNF-α, IL-1β, IL-6 and IL-12. Signal transduction starts with the recruitment of a set of intracellular TIR-domain-containing adapters that interacts with the cytoplasmic TIR domain of the TLRs. MyD88 (myeloid differentiation factor 88) is the universal adapter molecule, shared by all TLRs, except TLR3, that triggers inflammatory pathways through activation of the transcription factors NF-κB and AP-1 (JUN), which in

turn induce the expression of inflammatory cytokines. TRIF is critical in the induction of type I interferon genes and interferon inducible genes by TLR3 and TLR4 through the activation of the transcription factor IRF3, whereas the transcription factor IRF7 induces type I interferon genes and type I interferon-inducible genes through TLR7 and TLR9 signaling. TLR-mediated signaling is essential for host protection against candidiasis; MyD88 knockout mice are extremely susceptible to *C. albicans* infections; deficiencies in TLRs, mainly TLR2, and others in a minor extent (TLR4, TLR2 coreceptors, TLR3, TLR7 and TLR9) also cause impaired immune responses to *C. albicans* infection, both in mouse and/or human, although the role of some receptors (as TLR9) may be redundant. Recognition of fungal RNA by TLR7 has a non-redundant role in host defense against candidiasis, as it is partially required for IL-12 production by an IRF1-dependent pathway (Villamón *et al.*, 2004a,b; Biondo *et al.*, 2012; Netea *et al.*, 2008, 2015; Gil *et al.*, 2016).

Dectin-1 induces intracellular signals leading to (1) production of inflammatory cytokines through NF-κB activation (through Syk1/CARD9- or Raf-1-mediated signaling pathways), (2) Syk1-mediated activation of NLRP3 inflammasome that generates bioactive IL-1β and IL-18 following caspase activation, and (3) phagocytosis and production of ROS (reactive-oxygen species, see below). Dectin-1 signaling is only activated by particulate β-glucans that cluster the receptor in a synapse-like structure (phagocytic synapse), which represents a mechanism to distinguish direct microbial contact from detection of soluble ligands. Besides, hyphae induce a less proinflammatory response than yeasts, probably due to masking of glucan exposure by mannan/mannoproteins (Brown and Gordon, 2001; Gantner *et al.*, 2005; Goodridge *et al.*, 2011; Ifrim *et al.*, 2013; Netea *et al.*, 2015). *In vivo* studies with dectin-1 knockout mice showed contradictory results about their susceptibility to systemic candidiasis (probably due to strain-specific differences in glucan exposure), whereas human dectin-1 deficient patients showed clinically mucocutaneous infections but not invasive fungal infections. Interestingly, CARD9 deficiency is associated with susceptibility to invasive candidiasis, both in mouse and humans, probably because CARD9 mediates signal transduction pathways downstream of CLRs other than dectin-1. Dectin-2 deficient mice were more susceptible to systemic candidiasis, and phagocytosis of *Candida* cells by macrophages lacking dectin-2 was moderately decreased (Gross *et al.*, 2006, 2009; Ferwerda *et al.*, 2009; Drummond and Brown, 2011; Drewniak *et al.*, 2013; Ifrim *et al.*, 2016). Mincle, which associates with Fc receptors for IgGs (FcγR) and signals through Syk/CARD9, appears to have a protective role during candidiasis due to cytokine production, but is not involved in phagocytosis (Wells *et al.*, 2008). MR mediates several antifungal activities (such as phagocytosis of yeast by DCs, and IL-17 production by human peripheral blood mononucleated cells), although its role in protection against candidiasis appears to be redundant (Hardison and Brown, 2012). DC-SIGN recognition of *C. albicans* leads to *in vitro* production of cytokines and activation of the respiratory burst, probably in collaboration with dectin-1, although its role *in vivo* during infection has not been reported (Takahara *et al.*, 2011, 2012). Interestingly, chitin sensing by NOD2, TLR9 and MR dampens inflammatory responses through the induction of anti-inflammatory IL-10 production, thus indicating that chitin recognition plays a critical role for immune homeostasis (Wagener *et al.*, 2014).

MBL binding on fungal surface initiates the lectin pathway of complement activation, by cleaving the complement factor 3 (C3) and opsonisation of the microorganism: MBL-dependent deposition of C3b and iC3 fragments promotes phagocytosis of *C. albicans* by neutrophils. Furthermore, MBL may also act as an opsonin directly recognized by complement receptor 1 (CR1) on the surface of neutrophils (Brouwer *et al.*, 2008; Li *et al.*, 2012). In addition, MBL is able to modulate proinflammatory cytokine and chemokine production by modifying the *C. albicans*/TLR-mediated signaling pathways (Wang *et al.*, 2013). In this context, it should be noted that *C. albicans* can induce all three pathways of complement activation (classical, alternative and lectin pathways), that lead to the phagocytosis of the C3b-opsonized microorganism. While alternative and lectin (MBL) pathways are considered as non-specific immune responses, classical complement pathway requires antibodies for activation, and can be considered as a specific immune response. Activated complement is unable to kill *C. albicans* hyphae during infection, probably due to the thick fungal cell wall which may block the formation of the membrane attack complex (C5-C9) and the direct lysis of *C. albicans*. In addition, complement plays a central role in *C. albicans*-induced cytokine production by peripheral blood mononuclear cells. Accordingly, deficiency in complement factors (such as C3 and C5) causes increased susceptibility to disseminated infection in mouse models, since activation of complement by invading fungal cells contributes to opsonisation and a proper cytokine and chemokine production (Mullick *et al.*, 2004; Speth *et al.*, 2008; Tsoni *et al.*, 2009; Ricklin *et al.*, 2010; Cheng *et al.*, 2012).

Mouse models of infection have shown that fungal cells in the bloodstream bind and activate platelets, which in turn produce immune mediators, such as the platelet factor 4 (PF4 or CXCL4) and the CC-chemokine ligand 5 (CCL5 or RANTES), which have antifungal activity; also platelet-enriched plasma causes inhibition of *C. albicans* growth. However, the possible role of platelets in antifungal host defense is far to be deciphered (Robert *et al.*, 2000; Drago *et al.*, 2013).

Different inflammasomes are known to be involved in the response to *C. albicans* (NLRP3, NLRC4, NLRP10). Activation of inflammasomes in immune cells (macrophages, DCs, and others) leads to caspase-1 mediated production of fully mature IL-1β and IL-18 inflammatory cytokines. This production of mature IL-18 and IL-1β can be also produced by non-canonical (caspase-8 and caspase-11) inflammasomes (Gross *et al.*, 2009; Hise *et al.*, 2009; Kumar *et al.*, 2009; Joly and Sutterwala, 2010; Joly *et al.*, 2009, 2012; Tomalka *et al.*, 2011). The production of proinflammatory IL-1β differs from other cytokines and involves two steps: (1) transcriptional upregulation of pro-IL-1β, as an inactive precursor, downstream from PRRs (above cited), and (2) proteolytic cleavage by caspase-1 to release active IL-1β. Activating stimuli induce conformational changes in NLRs leading to inflammasome assembly, caspase activation and IL-1β cleavage. *C. albicans* transition from yeast to hyphae has been shown to be necessary for Nlrp3 inflammasome activation. Furthermore, only hyphae secrete the cytolytic peptide toxin candidalysin, responsible of causing epithelial damage (see below), which has been shown to provide the second signal to activate the NLRP3 inflammasome (Kumar *et al.*, 2009; Joly *et al.*, 2009; Martinon, 2010; Schroeder *et al.*, 2010; Joly and Sutterwala, 2010; Kasper *et al.*, 2018). Mature IL-1β can be generated also in inflammasome-independent manner at the site of infection: (1) by caspase-1 constitutively expressed by monocytes; (2)

by proteinase 3, expressed by neutrophils, and (3) by fungal-derived proteases that can also generate host-derived active IL-1β *in vitro*, and probably during infection, leading to activation of immune system (Beausejour *et al.*, 1998; van de Veerdonk *et al.*, 2009; Netea *et al.*, 2009, 2014; Gross *et al.*, 2012; Gabrielli *et al.*, 2015).

Following recognition of fungal PAMPs, immune cells trigger a variety of effector mechanisms of defense against *C. albicans* infection in order to clear the invading fungi. These mechanisms involve the simultaneous and/or coordinated (spatially and temporally) contribution of different immune and non-immune cell populations.

Epithelial Cells: The First Barrier Against *Candida* Invasion

The first line of defense against microbial infections in mucosal surfaces are epithelial cells (Ross and Herzberg, 2016). An intact epithelium constitutes an effective mechanical barrier against tissue invasion by *C. albicans*. Epithelial cells are able to restrict fungal invasion by a variety of defense mechanisms that appear to be activated depending on the degree of tissue damage and invasion by *C. albicans* (**Fig. 2**). Epithelial cells produce, in the presence of yeast cells, a variety of antimicrobial peptides with potent antifungal activity (β-defensins, cathelicidin, alarmins), suggesting that the uppermost epithelial layers are able to prevent fungal growth and hyphae formation, thus maintaining the commensal status of *C. albicans*, without further activation of immune responses (Lilly *et al.*, 2010; Yano *et al.*, 2012; Naglik *et al.*, 2014).

When *C. albicans* cells overcome this first antifungal barrier, fungal cells can proliferate and invade the epithelial tissues, either by endocytosis (yeast and hyphae) or actively penetrating the epithelial cells (hyphae). Recognition by epithelial receptors, such as the E-cadherin and Her2 (human epidermal growth factor receptor 2), induces endocytosis of fungal cells, promoting tissue invasion and pathogenicity. However, endocytosis does not necessarily contribute to immune activation in a direct manner (Zhu *et al.*, 2012; Villar *et al.*, 2005; Moyes *et al.*, 2010; Naglik *et al.*, 2014; Swidergall and Ernst, 2014). Fungal invasion and tissue damage trigger immune responses, and although PRRs (such as TLRs, C-type Lectins and NOD-like receptors) are expressed by epithelial cells, their role in *C. albicans* recognition and in the consequent activation of immune responses remain to be elucidated in most cases (**Fig. 2**).

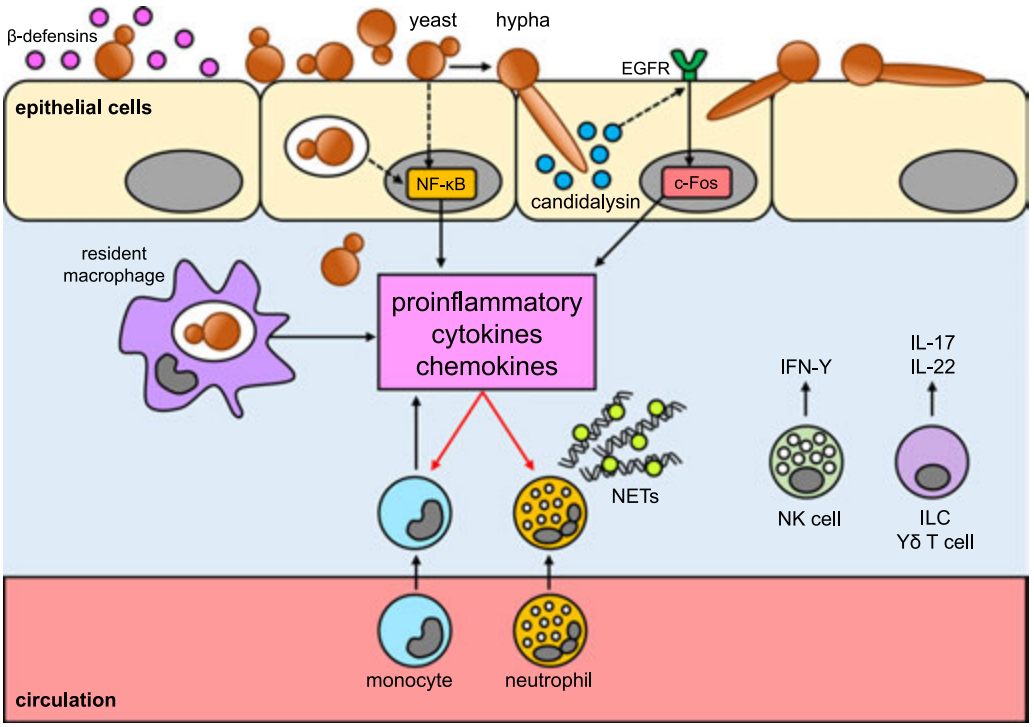

Fig. 2 *Innate defense mechanisms triggered by C. albicans in mucosal surfaces.* The commensal status of *C. albicans* yeast cells can be maintained at the mucosal surface of healthy individuals due to the antimicrobial peptides produced by epithelial cells (β-defensins and others), as well as by interactions with commensal microbiota. Yeast endocytosis by epithelial cells or phagocytosis by resident macrophages induce a small NF-κB-mediated proinflammatory response, enough to restrict fungal proliferation and invasion. Increased fungal proliferation and formation of hyphae results in tissue invasion and damage. Candidalysin produced by invading hyphae activates, via indirect mechanisms, the epidermal growth factor receptor (EGFR), which signaling activates a strong inflammatory response through MAPK/c-Fos mediated signaling. Proinflammatory cytokines and chemokines recruit neutrophils and monocytes to the site of infection (red arrows) to fight against the pathogen either by phagocytosis and intracellular killing or by extracellular mechanisms (NETs). Other innate immune cells, such as NK, ILC and $\Upsilon\delta$ T cells, can contribute to fungal clearance by producing cytokines such as IFN-γ, IL17 and IL22 that in turn activate effector cells, recruit neutrophils and induce secretion of β-defensins.

A mechanism to discriminate between the yeast (commensal) and hyphal (invasive) forms of *C. albicans* has been described in oral epithelial cells (Moyes *et al.*, 2010, 2015; Naglik *et al.*, 2014). These epithelial cells orchestrate an innate response to *C. albicans* via the transcription factor NF-κB (through TLR-mediated signaling pathway) and a biphasic mitogen-activated protein kinase (MAPK) response. Activation of the first MAPK phase is independent of the hyphal formation, and is due to the recognition of fungal cell wall moieties by PRRs (probably TLR4 and others) (Weindl *et al.*, 2007; Netea *et al.*, 2015). Activation of these MAPK pathways, which involve a low level and transient activation of JNK and ERK1/2, leads to the activation of the c-Jun (AP-1) transcription factor (with as-yet-unknown transcriptional effects). This early and low level of NF-κB- and MAPK-mediated proinflammatory responses (production of chemokines and cytokines), and the antifungal effect of resident macrophages (as well as the antimicrobial peptides produced by epithelial cells) may be sufficient to stop tissue invasion and damage, thus maintaining commensalism and low fungal burdens. If this early and transient response fails to avoid fungal proliferation, when a burden threshold of *C. albicans* hyphal cells is reached, a second phase is triggered. The second MAPK phase, which is dependent on fungal burdens and hypha formation, involves a strong activation of MAPK pathways (JNK, EREK1/2 and p38) and the c-Fos transcription factor, leading to immune activation; both NF-κB and c-Fos then up-regulate the production of cytokines, chemokines and other inflammatory mediators. This immune activation and secretion of proinflammatory cytokines (G-CSF, GM-CSF, IL-1α, IL-1β and IL-6), and chemokines (RANTES, IL-8 and CCL20) in oral epithelium induces neutrophil and monocyte recruitment as well as the production of a variety of antimicrobial peptides (as β-defensins or cathelicidin) which favor fungal clearance or reduction of fungal burdens back down below the threshold level of activation, and thus a return to the commensal state (Naglik *et al.*, 2014; Moyes *et al.*, 2015). This biphasic response seems to be a common mechanism enabling different human mucosal tissues to recognize *C. albicans* hyphae and initiate innate immune responses, as a similar (not identical) MAPK-based response is found in vaginal mucosa. Such biphasic response will allow epithelial tissues to remain quiescent under low fungal burdens while responding specifically and strongly to damage-inducing hyphae when burdens increase (**Fig. 2**). Hyphae-secreted candidalysin not only causes epithelial damage during mucosal infection, but also the activation of immune responses by epithelial cells. Candidalysin induces the phosphorylation of the epidermal growth factor receptor (EGFR), not by direct recognition, but via indirect mechanisms. EGFR activation leads to induction of MAPK signaling (via p38, ERK1/2) and the activation of c-Fos (Moyes *et al.*, 2016; Wilson *et al.*, 2016; Ho *et al.*, 2019).

It has been also shown that production of inflammatory IL-1β by mucosal tissues is also mediated by NLRP3 and NLRC4 inflammasomes. Induction of both inflammasomes occurs in mice following oral challenge with *C. albicans*, and deficiency in either NLRP3 or NLRC4 results in a strong reduction of proinflammatory and antimicrobial peptide secretion in the oral cavity. However, NLRP3 and NLRC4 differentially function in the innate response to *C. albicans* infection: NLRC4 plays a prominent role in host protection from mucosal infection, whereas NLRP3 inflammasome plays a predominant role in protection against disseminated infection (Tomalka *et al.*, 2011).

Once inside the tissue, fungal cells first encounter tissue-resident macrophages which phagocytose and kill them, and also secrete proinflammatory cytokines and chemokines that recruit neutrophils. Therefore, clearance of invading fungal cells from mucosal tissues involves various effector mechanisms (**Fig. 2**): phagocytosis and killing by resident macrophages and by recruited neutrophils (see below) and killing by β-defensins and other antimicrobial peptides produced by epithelial cells. Besides, cytokines released by monocyte/macrophages (TNF-α, IL-1β, IL-6 and others), and by adaptive Th1/Th17 cells are also important for host defense against disseminated candidiasis (see below).

Killing of *C. albicans* Cells by Phagocytes: Macrophages and Neutrophils

Effector and secretory responses of phagocytes to *C. albicans* are critical for the development of a protective host response. Macrophages orchestrate innate immunity by phagocytosing fungal cells and coordinating inflammatory responses. Phagocytes use a variety of surface receptors recognizing PAMPs and opsonins on the fungal surface. Major receptors involved in phagocytosis of non-opsonized fungal cells are the glucan receptors CR3 and dectin-1 (phagocytic synapse, above mentioned) and in a minor extent dectin-2, MR and DC-SIGN, whereas opsonized *C. albicans* are recognized by Fc receptors for IgG (FcγR) and complement receptors (CRs). Also, a soluble mannose-binding lectin (MBL) participates in *C. albicans* phagocytosis, either by binding to fungal surface and initiating the pathway of complement activation or by acting as an opsonin recognized by CR1 on neutrophil surface (Brouwer *et al.*, 2008; Tsoni *et al.*, 2009; Li *et al.*, 2012; Miramón *et al.*, 2013) (**Fig. 1**).

Tissue-resident macrophages act as key effector cells for antifungal defense. These macrophages phagocytize *C. albicans* cells and produce inflammatory cytokines and chemokines that recruit and activate other immune cells (monocytes, neutrophils, NK cells) at the site of infection. Early *in vivo* studies showed that macrophage-depleted mice were more susceptible to infection, measured as mortality rate and fungal proliferation in tissues (Bistoni *et al.*, 1986, 1988). Blood monocytes are recruited to the infected tissue and differentiate into proinflammatory macrophages and contribute to clearance of the pathogen; deficiency in chemokine receptors (CX3CR1 and CCR2) leads to increased susceptibility to disseminated candidiasis, both in mouse and human, due to an impaired monocyte recruitment, and therefore an impaired accumulation of monocyte-derived macrophages in infected tissues. (Miramón *et al.*, 2013; Höfs *et al.*, 2016). Phagocytosis of yeast or germ-tubes has distinct effects on the differentiation pathway of human monocytes to dendritic cells, indicating that impairment of this differentiation may constitute a mechanism for hyphae to elude the immune surveillance (Chiani *et al.*, 2000; Torosantucci *et al.*, 2004).

Neutrophils have a crucial role in protection against invasive candidiasis. Release of chemokines by epithelial cells and tissue-resident macrophages upon interaction with *C. albicans* leads to the recruitment of neutrophils to the site of infection.

Neutrophils constitute the most potent immune population cell in killing *C. albicans* and the only immune cells able to inhibit hyphae development from yeast cells; in fact, neutropenia is a major risk factor for disseminated candidiasis in humans, and mouse models of neutropenia have demonstrated an increased susceptibility to disseminated candidiasis (Brown, 2011; Lionakis, 2014; Miramón *et al.*, 2013).

The killing of *C. albicans* by phagocytes involves intra- and extracellular, as well as oxidative and non-oxidative mechanisms. Intracellular mechanisms require internalization of fungal cells into the phagosome. In macrophages, phagosomes are transformed into phagolysosomes (after fusion with lysosomes) with the consequent exposure of fungal cells to acidic pH that favors the activity of hydrolytic enzymes (cathepsin among others) against the pathogen. In contrast, the nascent phagosomes in neutrophils fuse with preformed cytosolic granules without major pH changes. Neutrophil granules contain a variety of antimicrobial proteins (elastase, lysozyme, myeloperoxidase, lactoferrin, gelatinase and defensins, among others), which alternatively can also be released into the extracellular environment to deal with non-phagocytized fungal cells (Brown, 2011; Aratani *et al.*, 2002; Miramón *et al.*, 2013).

Upon phagocytosis, a characteristic production of copious amounts of oxidants does occur. This oxidative burst involves formation, via NADPH oxidase and myeloperoxidase, of ROS reactive oxygen species (ROS: superoxide radical, hydrogen peroxide, hypochlorous acid) with a strong oxidative and damaging properties. These oxidative mechanisms are critical for killing fungal cells after phagocytosis. Besides, phagocytes also express nitric oxide-generating enzymes, as inducible nitric oxide synthase (iNOS) involved in production of reactive nitrogen species (RNS: nitric oxide an peroxynitrite, which is decomposed in nitrogen dioxide and hydroxyl radical), which also contribute to killing of *C. albicans* phagocytized cells (Aratani *et al.*, 2002; Brown, 2011; Miramón *et al.*, 2013).

In addition, neutrophils also use an extracellular mechanism to deal with the pathogen: the release of chromatin containing antimicrobial proteins (such as calprotectin), known as NETs (neutrophil extracellular traps). This DNA-containing fibril structures bind to and neutralize hyphae, providing a mechanism to deal with this fungal morphotype that is too big to be efficiently phagocytosed. Elastase contributes to release of NETs, and NET formation induce the release of antimicrobial substances form the granules of neutrophils (myeloperoxidase, lactoferrin, cathelicidin, and others mentioned above) (Urban *et al.*, 2009; Brown, 2011; Duggan *et al.*, 2015). It has been also demonstrated that NETs cause unmasking of the *C. albicans* hyphal β-glucans and trigger changes in the fungal cell wall architecture that enhance immune recognition by dectin-1 and probably by other host receptors; this remodeling of cell wall architecture enhances host responses but also contributes probably to protect *C. albicans* cells by strengthening the cell wall, and point to the concept that pattern recognition during infection is a dynamic process that depends on the host-pathogen cross-talk (Hopke *et al.*, 2016).

Based on studies with neutrophils from patients with defined genetic defects, it has been shown that human neutrophils have two independent pathways for *C. albicans* killing: (1) a ROS-dependent mechanism, required for clearance of opsonized *C. albicans* cells that depends on the FcγR pathway, whereas (2) a ROS-independent pathway involved in killing of non-opsonized fungal cells involves CR3 engagement and CARD9 ligation. Dectin-1 is dispensable for both killing mechanisms, suggesting differences in the neutrophil cytotoxic response to *C. albicans* between human neutrophils, where glucan is mainly recognized by CR3, and murine neutrophils, where dectin-1 is required to activate CR3 for cytotoxic response to *C. albicans* (van Bruggen *et al.*, 2009; Li *et al.*, 2011; Gazendam *et al.*, 2014).

Innate Lymphoid and $\gamma\delta$ T Cells

Innate lymphoid cells (ILCs) are emerging as important effectors of innate immunity (Spits *et al.*, 2013). Natural killer (NK) cells, a prototypical ILC population that contribute to the rapid innate immune response to invading pathogens, are rapidly recruited at the site of infection. Proinflammatory cytokines and chemokines generated at the site of infection bind to their receptors on NK cells leading to their activation and to a rapid production and secretion of IFN-γ. NK cells also can directly recognize and respond to pathogen components through TLRs by secreting IFN-γ, although whether NK cells are stimulated directly by *C. albicans* cells remains unclear. Therefore, the antifungal immunity mediated by NK cells appears to occur by secretion of cytokines, such as IFN-γ, that activate phagocytic cells and also contribute to the induction of the appropriate adaptive immune response. Human NK cells are also activated following engulfment of fungal cells, leading to degranulation and release of GM-CSF, TNF-α and IFN-γ, and to fungal damage. However, activated NK cells are unable to inhibit filamentation or to kill the engulfed *C. albicans* cells, and direct antifungal activity of NK cells is mainly attributed to secreted perforin. GM-CSF production by activated NK cells in the spleen is also required to boost the *C. albicans* killing capacity of neutrophils in the kidneys and therefore to control the infection. Mechanistically, dectin-1 recognition of *C. albicans* by ccr2-dependent recruited inflammatory monocytes to the spleen, or by splenic dendritic cells, induces type-I IFN dependent-IL-15 production by monocytes, which places a pivotal role in the activation and GM-CSF release by splenic NK cells (Gozalbo *et al.*, 2014; Voigt *et al.*, 2014; Quintin *et al.*, 2014; Domínguez-Andrés *et al.*, 2017) (**Fig. 3**).

It has been also described that inactivated *C. albicans* cells *in vitro* inhibit activation of purified NK cells, and therefore this may be considered as a mechanism of fungal immune evasion or to avoid hyperinflammation in immunocompetent host (Murciano *et al.*, 2006).

Other ILCs are also relevant for controlling mucosal infection, such as IL-17-secreting ILCs (see below), that also secrete IL-22, as both cytokines enhance activation of mucosal antifungal immunity (Gladiator *et al.*, 2013; Netea *et al.*, 2015).

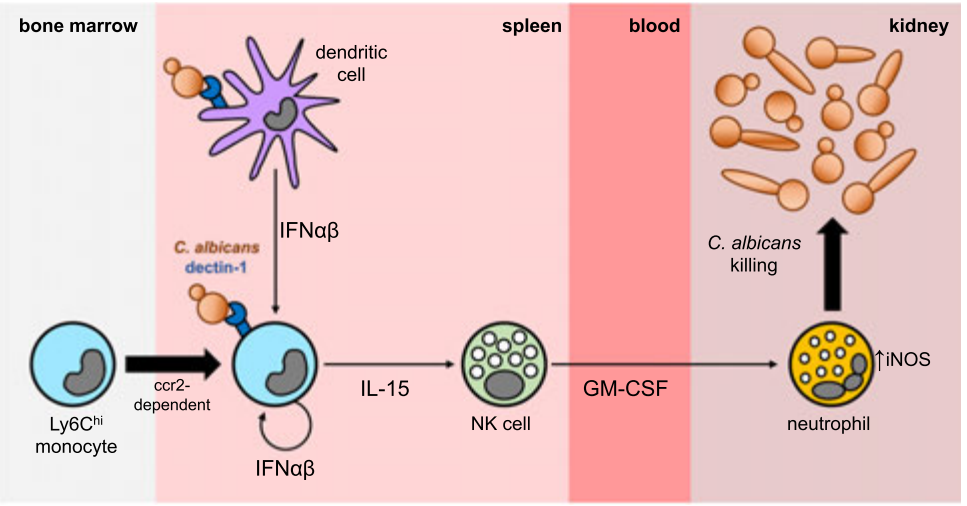

Fig. 3 *Central role for IL-15 in protection against C. albicans.* Systemic *C. albicans* infection in mice induces inflammatory Ly6C^{high} monocytes to leave the bone marrow, in a ccr2-dependent manner, to reach peripheral organs such as the spleen. In the spleen, *C. albicans* can be recognized by resident dendritic cells or by the attracted-monocytes through dectin-1. Dectin-1 signaling induces the production of type I IFNs, whose signaling induces IL-15 production by the recruited Ly6C^{high} monocytes. IL-15 drives splenic NK cell activation and GM-CSF release through the circulation to the kidneys to stimulate iNOS production by neutrophils, which increases their candidacidal potential. This model reinforces the concept that type I IFN production is crucial for defense against systemic candidiasis and gives a critical role to IL-15 in the activation of the NK cell-neutrophil axis in the protection against *C. albicans*.

$\Upsilon\delta$ T cells, other innate-like lymphocyte subpopulation present in mucosal-associated lymphoid tissues, may contribute to immunosurveillance at the body surfaces. $\Upsilon\delta$ T cells can proliferate and produce IFN-γ and IL-17 in response to *C. albicans in vitro*, and therefore may play a role in defense of mucosal tissues against fungal infection. Mice deficient or depleted in these cells are highly susceptible to orogastric candidiasis. Also, natural Th17 (nTh17) cells (another subset of mature Th17 lymphocytes, which are distinguishable from inducible Th17 cells, and possess an intrinsic capacity for immediate activation in naïve host) play a role in immunity to oral candidiasis, probably by IFN-γ production and polarization towards a Th17 adaptive response (see below). Therefore, both $\Upsilon\delta$ T and nTh17 cells are mucosal sentinels that help in the control of mucosal pathogens, including *C. albicans* (Tanaka *et al.*, 2009; Conti *et al.*, 2014; Gozalbo *et al.*, 2014).

Microbiome and Anti-*C. albicans* Defense Mechanisms

The study *sensu stricto* of innate immune response to *C. albicans* in humans is somehow poorly understood, partly because humans are exposed to *C. albicans* early in life and develop adaptive responses. Skin and mucosal surfaces of healthy individuals are colonized by a variety of microorganisms (microbiota or microbiome), including fungal species (mycobiome), being *Candida* spp. (and *C. albicans*) highly prevalent in these surfaces (Cui *et al.*, 2013; Findley *et al.*, 2013; Underhill and Pearlman, 2015; Lagunes and Rello, 2016). This microbiota is part of the first line of antimicrobial defense of epithelial tissues, as members of this microbiota compete with pathogens for nutrients, surfaces and substrates. There is increasing evidence indicating that host microbiota also influences fungal colonization and antifungal immune responses (Oever and Netea, 2014; Romani *et al.*, 2015). As an example, some bacterial species (*Pseudomonas aeruginosa*, *Enterococcus faecalis*) are able to inhibit hypha development of *C. albicans*, and others (lactobacilli) inhibit fungal adhesion and growth, thus protecting gut mucosa from *C. albicans* proliferation. This complex relationship between microbiota and *C. albicans* is clearly deduced by the fact that treatment with antibacterial antibiotics with wide spectrum is a risk factor contributing to *C. albicans* colonization, and disturbances in normal microbiota can lead to mucosal infections in otherwise healthy hosts. Also, patients with chronic mucocutaneous candidiasis showed a decrease in bacterial species that normally colonize the skin (as *Corynebacterium* spp.) and increased presence of other bacteria (Kennedy and Volz, 1985; Oever and Netea, 2014; Smeekens *et al.*, 2014).

Conversely, changes or alterations in the immune system of the host are also associated with changes in microbiota composition, indicating the existence of a delicate balance between microbiota composition and host immune status. Truly life-threatening fungal infections are common only when this balance is disrupted, either by immunosuppression or genetic mutation (Iliev and Underhill, 2013; Oever and Netea, 2014; Romani *et al.*, 2015). Therefore, a three-way interaction between host, fungi and microbiota dictates the types of host-fungus relationship. In this context, it has been shown that members of normal microbiota can interact with epithelial cells inducing the production of cytokines (as IL-22) that favor resistance to *C. albicans* colonization (Zelante *et al.*, 2013); also mice lacking dectin-1 are more susceptible to chemically induced colitis, as a result of an altered response to endogenous fungi, and polymorphism in dectin-1 gene in humans is linked to severe ulcerative colitis (Iliev *et al.*, 2012); commensal *Saccharomyces cerevisiae* strains are able to induce trained immunity in monocytes, probably through

chitin-mediated signaling following phagocytosis of *S. cerevisiae* cells (Rizzetto *et al.*, 2016); TLR2 also modulates gut colonization and dissemination of *C. albicans* in murine models (Prieto *et al.*, 2016), and induces, upon recognition of commensal bacteria, antimicrobial peptides that are involved in murine gut resistance to *C. albicans* (Fan *et al.*, 2015).

In this context, it has been also demonstrated that the commensal microbiota impacts specific immune cell populations and their functions at peripheral sites, such as gut mucosal tissues. Germ-free mice displayed reduced proportions and differentiation potential of specific myeloid cell progenitors, leading to impaired early responses to pathogens. Therefore, gut bacteria direct innate immune cell development via promoting hematopoiesis, further linking microbiota, innate immune responses and hematopoiesis (Khosravi *et al.*, 2014).

Interestingly in an experimental model of evolutionary pressure, in which *C. albicans* was transferred through serial passages into the intestinal tract of antibiotic-treated mice, an accelerated fungal mutation was observed resulting in isolated variants of *C. albicans* with low-virulence phenotypes unable to form hyphae. Moreover, the obtained variants performed better as commensals and worse as blood-borne pathogens. Also, mice colonized with these variants were protected against virulent *C. albicans*, and different fungal and bacterial pathogens. Indicating that adaptive evolution of the fungus in the gut modifies host-pathogen interaction into a mutually advantageous relationship, in which both the microbe and the host gain some benefit from their interaction (Tso *et al.*, 2018).

As a conclusion, commensal microbiota plays an important role in promoting health by modulating host defense toward pathogens, including *C. albicans*, through host microbiota-immune system interactions.

Adaptive Immune Responses to *C. albicans*

In most cases, activation of innate responses by epithelial cells and phagocytes (macrophages and neutrophils) and NK cells is sufficient to restrict fungal tissue invasion from the colonized surface, therefore preventing disseminated infection. In other cases, innate immune mechanisms fail to control fungal infection and activation of adaptive immune responses are required to deal with the pathogen.

Dendritic Cells: Driving CD4+ T Helper Subset Differentiation

T lymphocytes (T cells) constitute an integral component of the host adaptive immunity in response to *C. albicans* infections that provide both direct and indirect means to control fungal proliferation. Activation of both CD8+ (CTL, cytotoxic T lymphocytes) and CD4+ T (T helper cells, Th) is controlled by dendritic cell (DC) populations. DCs patrol tissues where they can detect and phagocytose *C. albicans*. Following exposure to pathogens and/or inflammatory mediators, DCs are transformed into mature DCs which migrate efficiently from peripheral tissues into draining lymph nodes. At this location, DCs activate antigen-specific T lymphocytes, ultimately leading to both T cell expansion and differentiation of effector cells. Despite CTLs can inhibit *in vitro* proliferation of hyphal growth of *C. albicans* (see below), the major mechanism of adaptive immunity to *C. albicans* is the development of Th cell responses. The elevated prevalence of oropharyngeal candidiasis in AIDS/HIV+ patients where CD4+ T cells are depleted clearly shows the paramount importance of Th cell responses (Lee and Iwasaki, 2007; Fidel, 2011; Richardson and Moyes, 2015).

DCs are able to phagocytize and kill fungal cells (although less efficiently than macrophages), but their major role is the activation of Th cell responses through the processing and presentation of fungal antigens to naive CD4+ T cells, which can develop to four different subsets (Th1, Th2, Th17 and Treg) (**Fig. 4**). Development of each specific subset depends on the cytokines and the microenvironment present during CD4+ T cells priming by DCs at lymph nodes. Cytokine milieu drives differentiation to one specific Th subset while inhibits development of the others, and this Th polarization is critical for the outcome of the infection. Differential response to *C. albicans* yeasts and hyphae occurs following phagocytosis by murine DCs, as yeasts induce the production of proinflammatory IL-12 which drives polarization toward the Th1 subset, whereas ingestion of hyphae results in anti-inflammatory IL-4 production and drives Th2 development (d'Ostiani *et al.*, 2000; Montagnoli *et al.*, 2002). TLR/MyD88 mediated signaling (TLR2, TLR4, and endosomal TLR9) is involved in mounting a Th1 response. Despite DCs from MyD88 deficient mice are able to phagocytize fungal cells in a similar manner than wild type DCs, MyD88 is essential for IL-12 production, downregulation of IL-10, and antifungal Th1 priming, although individual TLRs may contribute differentially to these responses (Bellocchio *et al.*, 2004; Miyazato *et al.*, 2009; Gil *et al.*, 2016). Also, recognition of *C. albicans* by CLRs, such as dectin-1, induce production of proinflammatory cytokines through SYK/CARD9-dependent and SYK–independent Raf-1 pathway that converge on NF-κB to drive Th1 and, particularly, Th17 polarization (Gringhuis *et al.*, 2009; Robinson *et al.*, 2009; Richardson and Moyes, 2015). Initial differentiation toward Th17 phenotype is driven by IL-1β, whereas IL-23 signaling is involved in maturation and terminal differentiation of Th17 cells (McGeachy *et al.*, 2009; Chung *et al.*, 2009). IL-23 and IL-6 are produced by DCs following recognition of *C. albicans* mannan, and favor Th17 differentiation (Smeekens *et al.*, 2010; Richardson and Moyes, 2015), and N-linked mannan has been found to be critically involved in DCs interaction with *C. albicans* (Cambi *et al.*, 2008). IL-1β production involves the canonical NLRP3 inflammasome/caspase-1 and also caspase 8 which are activated upon recognition of fungal β-glucan by dectin-1 and CR3 on DCs, and drive protective Th1 and Th17 cellular responses to disseminated candidiasis (van de Veerdonk *et al.*, 2011; Ganesan *et al.*, 2014). Despite Nlrp10 is not involved in innate proinflammatory cytokine production, Nlrp10 deficient mice showed a profound defect in *Candida*-specific adaptive Th1 and Th17 responses, indicating a

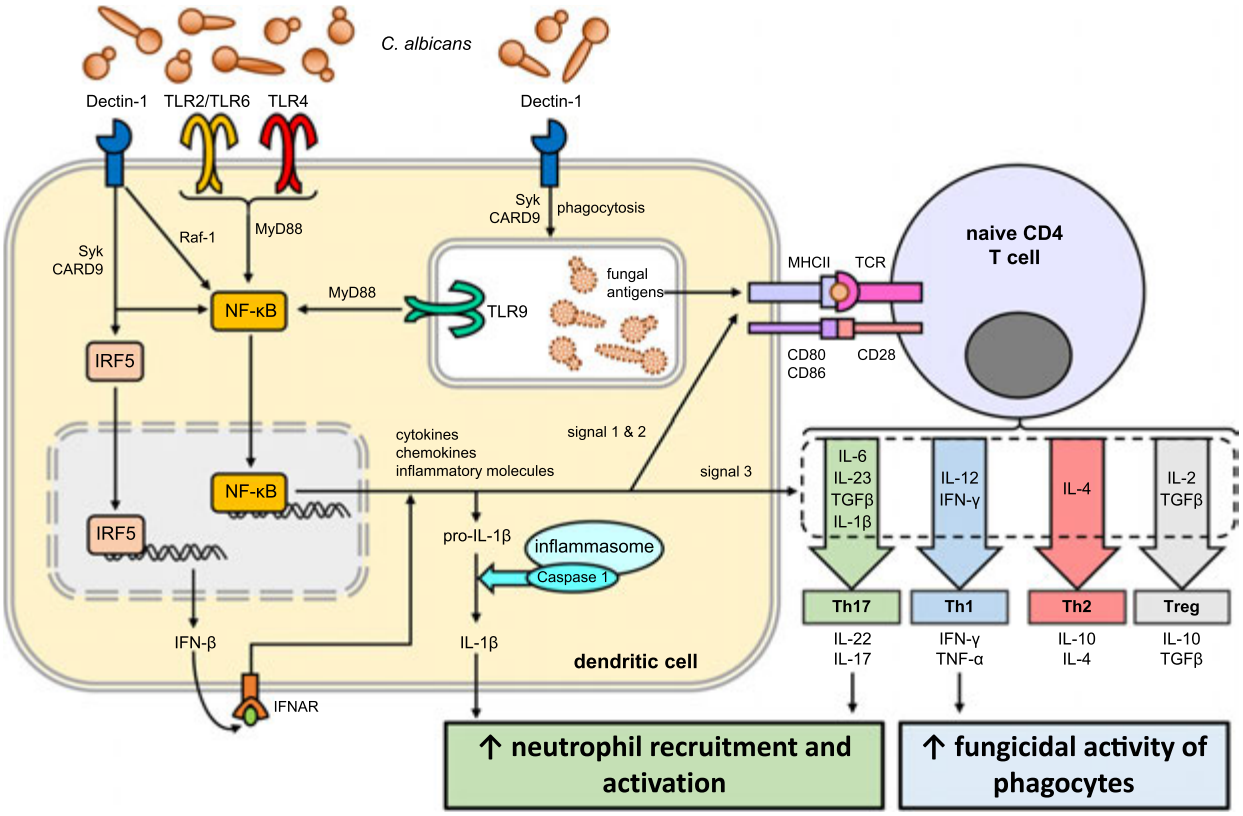

Fig. 4 *Adaptive T-cell responses to C. albicans.* Recognition of fungal cells (yeasts or hyphae) by DCs though PRRs, such as TLRs (both plasma membrane and endosomal receptors, mainly TLR2, TLR4 and TLR9), and dectin-1 trigger MyD88- and SyK/Raf-1-mediated signaling pathways that activate transcription factors (NF-κB, IRF5 and probably IRF1). NF-κB induces expression of signal 1 (MHCII molecules), signal 2 (costimulatory molecules CD80/86) and signal 3 (cytokines) required for antigen presentation, as well as chemokines and inflammatory mediators, whereas IRF5 is involved in IFNβ production, whose autocrine signaling through the IFNAR enhances signals 1, 2 and 3. In addition, these signaling pathways also lead to inflammasome assembly and activation, which causes caspase-1 mediated activation of IL-1β. Simultaneously, DCs phagocytose and kill fungal cells, and processed fungal antigens are presented onto MHC II molecules to naïve CD4+ cells. Recognition of the antigen by CD4+ cells through a T cell receptor (TCR) in the presence of costimulation from CD28 and CD80/86 is followed by cytokine polarization to specific Th subsets. This polarization strongly depends on the pool of cytokines secreted by DCs, which determines the fate of the Th subset polarization. Th1 and Th17 responses are associated to immune protection: Th1 is considered predominant in systemic/invasive infections, whereas Th17 is predominant in mucosal infections. Th1 and Th17 proinflammatory cytokines activate effector mechanisms to clear the pathogen by enhancing the fungicidal activity of phagocytes or recruiting and activating neutrophils respectively. Th2 is considered detrimental for protection, as Th2 cells secrete anti-inflammatory cytokines that favor fungal survival; however, Th2 cytokines are needed to avoid deleterious inflammatory responses and/or to diminish inflammation when infection is decreasing. Treg cells also participate in controlling adaptive responses, although their precise role is still not clearly determined. Production of IFN-β by DCs has been described also to participate in host protection (see text for further details).

role for this inflammasome in the generation of adaptive immune responses to fungal infections (Joly *et al.*, 2012; Eisenbarth *et al.*, 2012).

Therefore, signals triggered upon recognition of different PAMPs are integrated to define particular Th responses. The engagement of distinct receptors leads to disparate downstream signaling events that ultimately determines cytokine production, costimulation and Th responses. Consequently, selective challenge of receptors can be exploited for driving DCs toward a biased protective Th differentiation priming, with important implications in the design of DC vaccine-based strategies (Montagnoli *et al.*, 2002). DCs pulsed with fungal RNA induced protective immunity to *C. albicans* in assays performed to determine their potential role as anti-infective vaccine (Bacci *et al.*, 2002).

Th1 responses have been considered as protective against both mucosal and disseminated infection. Th1 cells secrete IFN-γ (and TNF-α, IL-2, among other proinflammatory cytokines) that has stimulatory effects on the phagocytosis and killing of *C. albicans* by neutrophils and macrophages, and also causes autocrine upregulation of IL-12 receptor, which in turn renders the Th cells more sensitive to IL-12, thus maintaining differentiation to Th1 phenotype (Smeltz *et al.*, 2002; Gozalbo *et al.*, 2014; Richardson and Moyes, 2015).

Th17 lymphocytes secrete numerous cytokines, including IL-17 and IL-22. IL-17 induces neutrophil recruitment and activation, and IL-22 enhances epithelial barrier function with release of β-defensins (Huang *et al.*, 2004; De Luca *et al.*, 2010). *C. albicans*-specific Th17 cells also produce IFN-γ, a cytokine that activates effector antifungal activities of phagocytes, as above mentioned

(Zielinski *et al.*, 2012). Th17 response is critical for protection against *C. albicans* infection at most mucosal surfaces, and chronic mucocutaneous candidiasis often develops in patients with disorders in Th17-mediated antifungal responses, whereas these patients do not show increased susceptibility to invasive candidiasis (Ferwerda *et al.*, 2009; Hernández-Santos and Gaffen, 2012; Hernández-Santos *et al.*, 2013; Smeekens *et al.*, 2013). Interestingly, Th17 responses are less relevant for protection against vaginal candidiasis in mice, as well as in humans, indicating the existence of differences in the anti-fungal responses among mucosal tissues (Yano *et al.*, 2012; Netea *et al.*, 2015). Overall, it is accepted that, while mucosal infections predominantly induce polarization of adaptive immunity to protective Th17 responses, systemic candidiasis is still considered to induce predominantly Th1 responses (Richardson and Moyes, 2015). Besides, another subset of Th cells (Th22) that secrete both IL-22 and TNF-α has been described to be important for cutaneous immunity to *C. albicans* (Eyerich *et al.*, 2011).

Th2 responses are generated in an anti-inflammatory environment (e.g., production of IL-4 by DCs in response to *C. albicans* hyphae). Anti-inflammatory cytokines produced by Th2 cells (IL-10, IL-4) inhibit Th1/Th17 development and deactivate phagocytic effector cells. Consequently, Th2 response has been considered as nonprotective against infections. However, Th2 cells are required for the maintenance of a balanced non-deleterious proinflammatory Th1/Th17 response, and to restore the non-inflammatory status following fungal clearance (Mencacci *et al.*, 2001; Netea *et al.*, 2015).

Treg cells maintain peripheral tolerance and limit the effector responses to control excessive proinflammatory responses leading to immune-mediated tissue damage. The role of Treg cells during candidiasis has been not unequivocally established. The immunosuppressive effects of Treg cells can be blocked by TLR-activated DCs leading to a Th1 response (Lee and Iwasaki, 2007). Besides, Treg cells also express TLRs, and the presence of TLR ligands (e.g., during a *C. albicans* infection) cause temporarily expansion and abrogation of the suppressive phenotype of Treg cells, enabling the enhancement of immune responses (proinflammatory Th1 response); upon pathogen clearance (decrease of fungal ligands), the expanded Treg cells regain their immunosuppressive activity to restore the immune balance (Sutmuller *et al.*, 2006a,b). Th17 and Treg cells are reciprocally regulated during T cell differentiation and can act cooperatively against *C. albicans*, although the final response appears to be dependent on the infection site: Treg cells enhances Th17 protective responses to oropharyngeal candidiasis, while reduces resistance in systemic infections (Whibley and Geffen, 2014a; Whibley *et al.*, 2014b), supporting that Th responses to invasive and mucosal infections are different, as above mentioned.

More recently it has been demonstrated that type I interferons (IFN-α and IFN-β), which are known to inhibit viral replication and mediate protection against viral infection, also play a role in anti-*Candida* host defense. Using mouse models, it has been shown that DCs are able to mount a type I IFN response against *Candida* spp. that requires phagosomal TLR7-mediated IFN-β signaling, and promotes persistence of *C. glabrata* in the host, and that IFN-β production inhibits fungal clearance in mice infected with *C. parapsilosis* (Burgeois *et al.*, 2011; Patin *et al.*, 2016). Similarly, mice lacking a functional IFN-I receptor showed a remarkable protection against invasive *C. albicans* infections and this detrimental role for type I IFN is associated with a reduced recruitment and activation of inflammatory monocytes and neutrophils (Majer *et al.*, 2012; Stifter and Feng, 2015). However, type I IFN has been described also to be beneficial for the immune responses to *C. albicans*; production of IFN-β by DCs, which is largely dependent on dectin-1 and dectin-2 signaling (via Syk and IRF5), is crucial for immunity to *C. albicans* by promoting the mobilization of neutrophils to the kidney (Biondo *et al.*, 2011; Del Fresno *et al.*, 2013).

Recently, it has been shown that type I IFNs, secreted by β-glucan-stimulated DCs via dectin-1, induce the proliferation and activation of CD8+ cells. The type I IFNs act in an autocrine manner via their receptor (IFNAR) to promote the presentation of exogenous antigen on MHC I molecules, surface expression of the co-stimulatory molecules CD40 and CD86, and the release of other cytokines, including IL-12 p70, IL-2, IL-6, and TNF-α (Hassanzadeh-Kiabi *et al.*, 2017). Consequently, these results support a protective role of type I IFNs during candidiasis. Therefore, the role of type I IFN in response to *Candida* infections might differ among fungal species, and the specific roles of type I IFNs in *C. albicans* infections requires further research. During bacterial infections, low level of type I IFNs may be required at an early stage to initiate cell-mediated immune responses, whereas high concentrations have immunosuppressive effects, such as the reduction of responsiveness of macrophages to activation by IFN-γ (Stifter and Feng, 2015). It should be noted that type I IFN genes are also induced through signal transduction pathways initiated by some TLRs (TLR4 and TLR2 via IRF3, and TLR7 and TLR8 via IRF5), although their involvement in development of adaptive responses to *C. albicans* infection remains to be determined. Moreover, findings obtained by integrating transcriptional analysis and functional genomics also indicate that type I IFN pathway is a main signature of *C. albicans*-induced inflammation and plays a crucial role in anti-*Candida* host defense in humans, probably by eliciting antifungal responses of macrophages and NK cells (Smeekens *et al.*, 2013).

CD8+ T Cells

Murine models of infection indicate that CD8+ cells also have a role in protection against candidiasis, both mucosal and disseminated infections. The main antimicrobial effector mechanisms of CD8+ T cells are cytotoxicity (by production and release of cytotoxic granules containing perforins and granzymes) and cytokine secretion (TNF-α and IFN-γ). The role of cytotoxicity in host defense against fungal infections is not well delineated, whereas the activity of cytokines is better understood. The protective effect is most probably due to the production of IFN-γ, whose production is induced by IL-12 (Beno *et al.*, 1995; Ashman *et al.*, 1999; Gozalbo *et al.*, 2014). As above cited, activation of CD8+ cells can be promoted by DCs through autocrine type I IFN signaling upon recognition of fungal β-glucan by dectin-1 (Hassanzadeh-Kiabi *et al.*, 2017).

Humoral Responses: Antibodies

Soluble (humoral) proteins, mainly complement and antibodies, also contribute to defense against candidiasis, as components of the innate and adaptive immune responses, respectively. While cellular adaptive responses play a major role in host defense against *C. albicans* infection, the contribution of adaptive humoral immune mechanisms mediated by antibodies secreted by B cells (B lymphocytes) play relatively minor role in immune protection against the fungus (Netea *et al.*, 2015; Richardson and Moyes, 2015).

Although binding of antibodies to fungal surface antigens triggers activation of the classical complement pathway (above mentioned), the role of anti-*Candida* antibodies, as an important arm of the adaptive immunity, in host defense against candidiasis is paradoxical. Cell wall-associated components (glucans, mannans, mannoproteins) are major *Candida* antigens, as well as secreted enzymes (such aspartyl proteases) or cytosolic fungal proteins (such heat-shock proteins and glycolytic enzymes). Multiple antifungal activities are associated to anti-*Candida* antibodies, depending on the function of their antigens in fungal biology (adhesion to host tissues, germ-tube formation, acquisition of iron, proteolysis, etc.), as specific antibodies interfere with this function and therefore affect the host-pathogen interactions by inhibiting/neutralizing fungal virulence factors (Martínez *et al.*, 1998; Chaffin *et al.*, 1998; Gozalbo *et al.*, 2004). In fact, it has been shown that a variety of anti-*Candida* antibodies (anti-mannan, anti-Als3, anti-Hsp90, anti-SAP, anti-β-glucan), including both mouse and human antibodies, are able to confer some protection in animal models of mucosal and/or disseminated infection, and accordingly, some vaccination assays have shown a decreased susceptibility to infection in animal models (Paulovicovà *et al.*, 2014; Moragues *et al.*, 2014; Wang *et al.*, 2015). Despite these observations, B-cell deficiency in mice does not confer increased susceptibility to *C. albicans* infection, and patients with agammaglobulinemia or hypogammaglobulinemia do not show increased susceptibility to fungal infection, indicating that humoral response during infection has a very modest role in host protection. However, the use of some purified antigens (Als3, Saps, glucan) in vaccination strategies imply presentation of antigens to the immune system in conjunction with suitable carrier proteins and adjuvants capable of eliciting the production of antigen-specific antibodies that, as above cited, may confer limited protection against infection (Moragues *et al.*, 2014; Wang *et al.*, 2015). Therefore, and due to the prevalence of fungal infections and their increase resistance to antifungal therapies, eliciting protective antibodies through vaccination remains as a viable strategy for improving resistance to *C. albicans* infections (Iannitty *et al.*, 2012).

Evasion of Host Defenses by *C. albicans*

In addition to induce the immune responses above described, C. *albicans*, as well as most pathogens, has developed strategies to try to avoid immune responses and to scape clearance from host defenses. These strategies include both (1) evasion of fungal recognition by shielding PAMPs that elicit immune responses, and (2) subversion of the normal immune responses. The main fungal mechanisms for immune evasion are outlined below.

C. albicans can enhance survival to killing by inhibiting phagolysosome maturation and oxide nitric production by macrophages (Collette *et al.*, 2014). In response to *C. albicans*, macrophages switch from an inflammatory phenotype (M1) to less inflammatory macrophages (M2), a change that might contribute to pathogenicity by decreasing immune responses (Reales-Calderon *et al.*, 2014).

The yeast-to-hypha transition is associated with changes both in cell wall composition and PAMPs exposure, as well as with changes in expression of virulence factors (adhesins, proteolytic enzymes, and others), making the hyphal morphology better equipped to resist immune responses. Hyphae induce a less proinflammatory response than yeasts, probably due to masking of glucan exposure by mannan/mannoproteins (Gantner *et al.*, 2005; van der Graaf *et al.*, 2005). In addition, the increased chitin content in hyphae may downregulate protective proinflammatory responses, thus favouring the infectious process (Wagener *et al.*, 2014).

C. albicans yeast and hyphae (germ-tube cells) interfere differentially with human monocyte differentiation into dendritic cells: while phagocytosis of yeasts inhibits their differentiation to DCs, phagocytosis of germ tubes give rise to monocyte-derived DCs which are unable to induce functional polarization of naïve T cells to effector cells. Therefore, phagocytosis of yeast and germ tube forms has profound and distinct effects on the differentiation pathway of human monocytes, indicating that their differentiation into DCs appears to be tuneable and exploitable by *C. albicans* hyphae to elude immune surveillance (Torosantucci *et al.*, 2004). Similarly, *C. albicans* β-glucans also causes the subversion of human monocyte differentiation into dendritic cells (Nisini *et al.*, 2007). As above mentioned, yeasts and hyphae have opposite effects on DCs, as phagocytosis of hyphae by DCs polarizes activation toward a Th2 cell response, subverting the protective Th1/Th17 response, thus enabling fungal persistence within the host (d'Ostiani *et al.*, 2000; Montagnoli *et al.*, 2002).

Induction of IL-10 and an enhanced Treg response (which has immunosuppressive effects), upon PRR (TLR2)-mediated *C. albicans* recognition has also been described to favor the infectious process (Netea *et al.*, 2004; Dillon *et al.*, 2006), despite TLR2 is a key receptor essential for resistance to *C. albicans* infection (Villamón *et al.*, 2004b; Gil *et al.*, 2016). Another probable mechanism of immune evasion are: (1) the production of a fungal soluble factor that *in vitro* reduces production of IL-17 by peripheral blood mononuclear cells, that could lead to dampen Th17 responses (Cheng *et al.*, 2010), (2) the secretion of proteolytic enzymes able to degrade substrates relevant for host protection (as complement components and antibodies) (Naglik *et al.*, 2004), and (3) the inhibition of the activation of NK cells, above mentioned (Murciano *et al.*, 2006).

C. albicans cells are also able to bind to a number of human plasma proteins and complement regulators (Factor H, FHL-1, C4 binding protein and plasminogen) through expression of fungal binding proteins (Gmp1, Pra1, Hgt1p, GpD2, and several plasminogen binding proteins). Those human proteins bound to fungal surface maintain their regulatory activities, and block

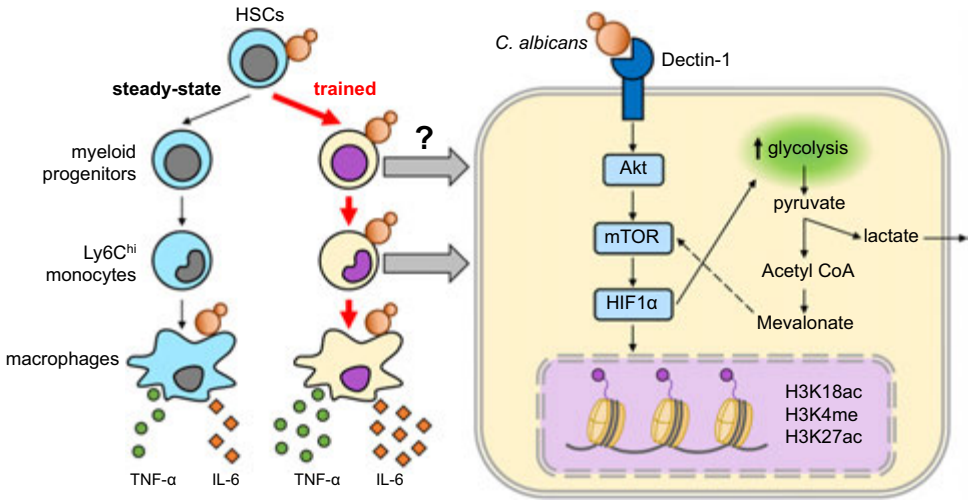

Fig. 5 *Emergency myelopoiesis and trained immunity during candidiasis.* During a systemic candidiasis, hematopoietic stem and progenitor cells (HSPCs) can directly detect *C. albicans* cells. This interaction stimulates HSPCs, through TLR2- and dectin-1-mediated signaling, to proliferate and bias their differentiation towards the myeloid lineage. Dectin-1 signaling in monocytes and in HSPCs induces trained immunity. Macrophages produced by them possess an enhanced ability to produce proinflammatory cytokines. Recognition of fungal β-glucan by dectin-1 in monocytes, and probably by HSPCs, triggers the Akt (protein kinase B)–mTOR (mammalian target of rapamycin)–HIF-1α (hypoxia-inducible factor-1α) pathway, modifications in metabolic pathways, and epigenetic rewiring. Induction of glycolysis resulting in lactate production is one of the central hallmarks in trained immunity metabolism. The cholesterol synthesis pathway appears to be an essential metabolic pathway as well, but not the synthesis of cholesterol itself, rather the metabolite mevalonate mediates training via the activation of mTOR. All these changes result in epigenetic modifications of histone marks H3K4me1, H3K4me3 and H3K27Ac, leading to changes in the expression of proinflammatory cytokine genes. Trained immunity has been shown to be protective against fungal infection or against unrelated pathogens (cross-protection).

complement activation and effector functions, opsonisation and phagocytosis, thus representing immune evasion mechanisms for the fungus to overcome host defense against infection (Meri *et al.*, 2002, 2004; Crowe *et al.*, 2003; Zipfel *et al.*, 2007; Luo *et al.*, 2009; Lesiak-Markowicz *et al.*, 2011).

Emergency Myelopoiesis and Trained Immunity

Myelopoiesis, the production of myeloid cells by bone marrow hematopoietic stem and progenitor cells (HSPCs), is essential during homeostasis to maintain reservoirs of innate immune cells and to replenish short living circulating cells for immune surveillance. During infection HSPCs must be activated to produce large quantities of neutrophils and monocytes to combat the pathogen and to replace the immune cells killed by the invading pathogen. In this context, HSPCs produce myeloid cells at expenses of the lymphoid and erythroid lineages in a process named "emergency myelopoiesis" (Takizawa *et al.*, 2012). It is well known that cytokine production by mature immune cells coordinates enhanced myelopoiesis during infection, but also direct interaction of microorganisms or their ligands, with HSPCs has been reported. Murine and human HSPCs express functional PRRs and their signaling pathways provoke cell cycle entry and myeloid differentiation (Nagai *et al.*, 2006; Sioud *et al.*, 2006). *C. albicans* detection by HSPCs can happen at the infected tissues, by the migratory HSPCs, or at the bone marrow by the circulating microbial components or by invading fungal cells. Direct interaction of *C. albicans* yeasts with HSPCs induces the proliferation and differentiation of HSPCs toward the myeloid lineage *in vitro* and *in vivo* (**Fig. 5**). This response requires signaling through TLR2 and dectin-1 and gives rise to functional phagocytes capable of engulf yeast cells and secrete proinflammatory cytokines (Yáñez *et al.*, 2009, 2010, 2011, 2013; Megías *et al.*, 2012, 2013). Interestingly, the interaction of *C. albicans* with HSPCs not only boosts myelopoiesis, but also influences the functional phenotype of the produced macrophages, following recently described observations of trained immunity for mature monocytes/macrophages. Priming monocytes/macrophages with *C. albicans* or with β-glucans induces trained immunity *in vitro* and *in vivo*, which confers to these cells an enhanced ability to produce proinflammatory cytokines after a second challenge (**Fig. 5**). Trained immunity plays a protective role against a secondary infection, and this effect can last weeks, months and even years. Therefore, trained immunity must take place also in HSPCs, upstream in the hematopoietic system. In fact, HSPCs-interaction with *C. albicans* gives rise to macrophages that are better prepared to deal with the infection, as they produce higher amounts of inflammatory cytokines and also have higher fungicidal capacity (Megías *et al.*, 2016; Martínez *et al.*, 2017, 2018).

Mechanistically, *C. albicans* β-glucan-recognition by dectin-1 induces trained immunity through the AKT, mechanistic target of rapamycin (mTOR) and hypoxia-inducible factor 1α (HIF1α) pathway. The activation of this pathway switches cellular metabolism from oxidative phosphorylation towards aerobic glycolysis, which is associated with a reduced basal respiration rate, increased glucose consumption and higher lactate production. Furthermore, β-glucans activate the cholesterol pathway, which accumulates the intermediate metabolite mevalonate responsible for inducing training via the activation of mTOR. Changes in glucose or lipid metabolism lead to epigenetic modifications underlying the enhanced secretion of pro-inflammatory cytokines (**Fig. 5**). Targeting innate immune

cells to regulate trained immunity could have therapeutic benefits in a range of immune-related diseases, including infectious and inflammatory processes and cancer (Netea *et al.*, 2011; Quintin *et al.*, 2012; van deer Meer *et al.*, 2015).

Final Comments

During the last years much progress has been achieved concerning both innate and adaptive responses to *C. albicans*, as well as on the fungal ligands and host receptors (and signaling pathways) involved in these responses. However, there are still some issues that require further research for a complete and detailed picture of the immune responses to *C. albicans* infections. For instance: (1) how multiple signals from PRRs and cytokine receptors are integrated by immune cells to induce efficient mechanisms for fungal clearance?, (2) how is trained immunity generated and sustained during infection?, (3) how HSPCs behave during infection?, (4) how does mucosal and skin surface discriminate between commensal microbiota and pathogens, and how microbiota influences the infectious process?, (5) is it possible to design protective vaccination strategies or to generate antibodies appropriate for immunotherapy?

The answers to these, and other issues, are probably difficult to be discerned, taken into account that: (1) probably some signaling pathways overlap or are redundant for triggering host immune responses, (2) expression of fungal PAMPs (as well as virulence factors) may depend on *C. albicans* strains, due to the high genetic/phenotypic versatility showed by this species, thus representing an additional source of variability, (3) interpretation of the results obtained in *in vitro* assays, which have been an extremely useful tool for studding immune response to *C. albicans*, cannot be easily evaluated concerning their *in vivo* significance during infection, and (4) PRRs involved in *C. albicans* recognition may have physiological roles other than pathogen recognition, therefore the use of animal models with defective PRRs to study immune responses to *C. albicans* may lead sometime to difficult interpretations.

It should be also stressed that in rodents *C. albicans* is not a commensal, therefore providing an appropriate model to perform studies distinguishing innate and adaptive responses, whereas the study of innate immune response to *C. albicans* in human is more difficult, partly because humans are exposed to *C. albicans* early in life and develop adaptive responses. Furthermore, animal (murine) models have been widely used to study *in vivo* and *in vitro* immune responses to *C. albicans* and have contributed considerably to the present knowledge, although extrapolation to human is also difficult taken into account that murine and human immune systems are similar but no identical. More recently functional genomics (transcriptional analysis and system biology) has emerged as powerful tool that represents an alternative, unbiased approach to study antifungal immunity in humans, allowing identifying host defense mechanism occurring *in vivo*, as well as to establish the relationship between genetic immunodeficiencies and host responses to fungal infection.

Therefore, the study of the immune responses to candidiasis remains still as an exciting challenge for immunologists and microbiologists from both the basic research (immune responses and host-pathogen relationship) as well as from an applied point of view, as patients suffering candidiasis and the immunocompromised expanding population at risk require alternative immunotherapeutic approaches to deal with and/or to prevent infections.

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Infections by *Cryptococcus* species

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Nomenclature

AIDS Acquired immunodeficiency syndrome
CNS Central nervous system
CSF Cerebrospinal fluid
EIA Enzyme immunoassay
GalXM Galactoxylomannan
GXM Glucoronoxylomannan
GXMGal Glucuronoxylomannogalactan
HAART Highly active antiretroviral therapy
HIV Human immunodeficiency virus
IFD Invasive fungal disease
LAT Latex agglutination tests
LFA Lateral flow immunoassay

Glossary

Basidium Filament that is formed during sexual reproduction in basidiomyces and where meiosis occurs.
Capsule Structure that surrounds the cell body.
Free radical Molecular species that contains an unpaired electron in an atomic orbital.
Infection Introduction of a microorganism into a host.
Macrophage Type of leukocyte of immune system that is specialized in phagocytosis. Its function is to recognize, engulf and destroy target cells and foreign particles.
Melanin Dark pigment insoluble in water and resistant to heat, free radicals and acid widely found in nature, from mammals from microorganisms.
Meningoencephalitis Inflammation of the membranes of the brain and the adjoining cerebral tissue.
Phagocytosis Endocytic process that results in the internalization of solid particles inside the cell.
Serotypes Division within the same species that depends on the reactivity of a microbe to specific antibodies.
Titan cell Morphological transition defined as a massive growth a blastoconidia.
Virulence factor Element produced by a pathogen that causes damage in a host.
Yeast Unicellular fungus that divides by budding or fission.

Several fungal species have become a threat for susceptible patients because they can cause serious invasive diseases. Their incidence has increased in the last decades due to the raise in the number of immunosuppressed patients. The main causative agents of invasive fungal diseases (IFD) are yeasts and filamentous fungi (such as *Aspergillus* spp).
The main two yeast genera that can cause invasive diseases in humans are *Candida* spp. and *Cryptococcus* spp. While most *Candida* spp. live with the host as part of its microbiota, *Cryptococcus* spp. are acquired from the environment and infections by these yeasts have particular features that make them unique among fungal pathogens. The present chapter will focus on the importance of *Cryptococcus* spp. as pathogens, epidemiology, and main characteristics of the diseases they cause.

Cryptococcus Neoformans and Cryptococcus Gattii: Main Features

Cryptococcosis is a systemic disease caused by yeasts from the genus *Cryptococcus*. This genus comprises many species, but disease in humans is strongly associated with two of them: *C. neoformans* and *C. gattii*. These yeasts are part of the Phylum basidiomycete and infection occurs through inhalation of infective forms of yeast, the basidiospores, which are found in the environment (Negroni, 2012). Both species are very similar from a genetic and phenotypic point of view, but they present important differences in their epidemiology and virulence. They are both respiratory yeasts, and present an important difference in their cell wall compared to other fungal pathogens, because they have a low content of β-1,3-glucan. However, the main characteristic of *Cryptococcus* spp. is a polysaccharide capsule that surrounds the cell wall, which confers special immunogenic features to these yeasts.

Classification of *Cryptococcus Neoformans/Gattii*

The *C. neoformans/gattii* complex comprises two species that are subdivided and classified in different genotypes. These species have been also divided in serotypes according to different immunological properties of the capsule. *C. neoformans* has been classically divided in two serotypes: A (also denominated variety *grubii*), and serotype D (*C. neoformans* variety *neoformans*). In *C. neoformans*, AD hybrids are also very prevalent (Franzot *et al.*, 1999). *C. gattii* contains serotypes B and C (Boekhout *et al.*, 2001, 1997; Kwon-Chung and Varma, 2006). However, in the last years, several studies using molecular techniques have questioned the use of only two species among *C. neoformans/gattii* complex. Recently, Hagen *et al.* (2015) through phylogenetic and genotyping analysis,

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proposed to recognize the current *C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans* as two different species and, *C. gattii* in five separated species (**Table 1**).

Life Cycle

C. neoformans and *C. gattii* exist in two mating types, MATa and MATα. However, the MATα are predominant in nature and is most frequently isolated from patients (Kwon-Chung *et al.*, 2014; Kidd *et al.*, 2004). Sexual reproduction of these yeast species occurs through the propagation of haploid cells until there is contact between the opposite mating types in response to the production pheromones, a or α (Mcclelland *et al.*, 2004; Stanton *et al.*, 2010; Shapiro *et al.*, 2011). The fusion of a and α cells induces the production of dikaryotic filaments, which eventually leads to the formation of a basidium. Meiosis occurs in this last structure and produces four chains of basidiospores that are ready to be dispersed in the environment (Kwon-Chung, 1975).

C. neoformans can also undergo the transition to a filamentous form during reproduction through another process called monokaryotic fruiting (Lin and Heitman, 2006). In this case, mating occurs when cells from the same mating type cells, a or α, become diploid (α/α or a/a), either by endoduplication or by nuclear fusion following cell fusion between two cells of the same mating type. Similarly to sexual reproduction, blastospores and chlamydospores also are produced during monokaryotic fruiting and meiosis occurs in the basidium (**Fig. 1**; Lin and Heitman, 2006).

Table 1 Current and last names of the species in the *Cryptococcus neoformans* and *Cryptococcus gattii* complex

Past species name	*Molecular types/genotypes*[a]	*Serotype*[b]	*Current species name*[c]
C. neoformans var. *grubii*	AFLP1/VNI AFLP1A and AFLP1B/VNII	A	*C. neoformans*
C. neoformans var. *neoformans*	AFLP2/VNIV	D	*Cryptococcus deneoformans*
C. neoformans hybrid	AFLP3/VNIII	AD	*C. neoformans x C. deneoformans hybrid*
C. gattii	AFLP4/VGI	B	*C. gattii*
	AFLP5/VGIII	C	*Cryptococcus bacillisporus*
	AFLP6/VGII	B	*Cryptococcus deutorogattii*
	AFLP7 and AFLP10/VGIV	B and C	*Cryptococcus tetragattii and Cryptococcus decagattii*

[a]Boekhout *et al.* (2001), Meyer *et al.* (2003, 2009).
[b]Franzot *et al.* (1999), Boekhout *et al.* (1997), Kwon-Chung and Varma (2006).
[c]Hagen *et al.* (2015).

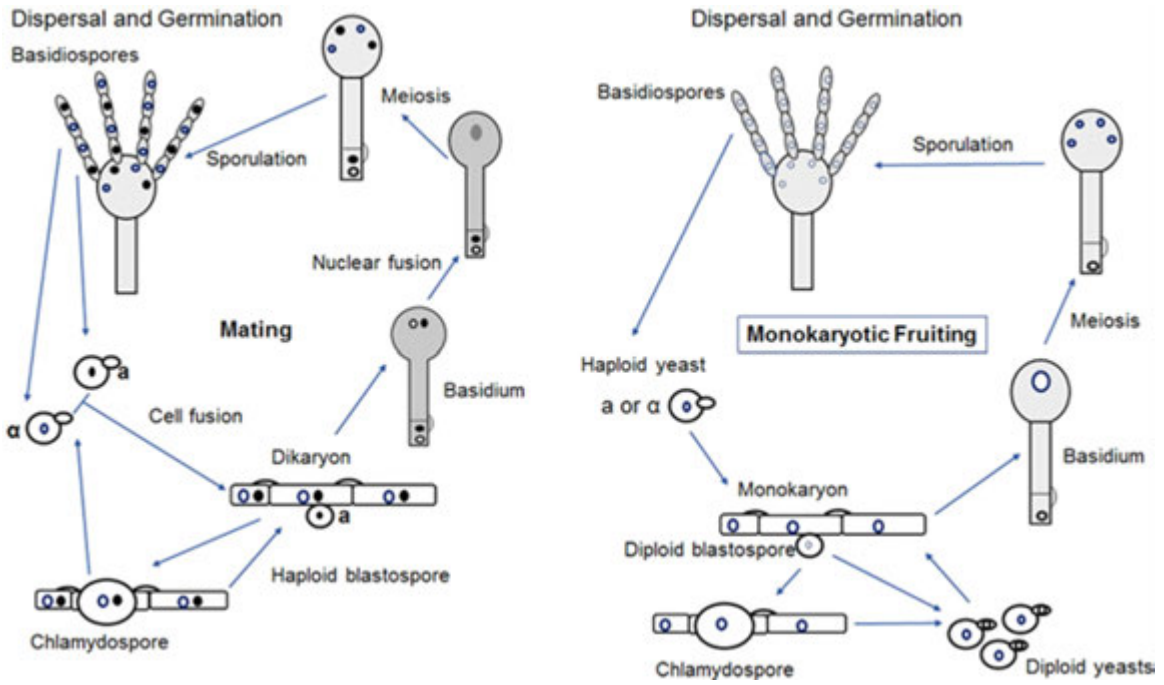

Fig. 1 The *Cryptococcus neoformans* life cycle: mating and monokaryotic fruiting.

Epidemiology of Cryptococcosis

C. neoformans and *C. gattii* differ mainly in their epidemiology where *C. neoformans* affects mainly immunocompromised individuals. The human immunodeficiency virus (HIV) infection is the main risk factor to develop infection by *C. neoformans*. At the end of the 20th century, it was estimated that *C. neoformans* caused disease in around 80% of HIV patients in high-income countries (Pyrgos *et al.*, 2013; Sloan and Parris, 2014). The introduction of the highly active antiretroviral therapy (HAART) reduced the incidence of cryptococcosis in developed areas. However, the incidence and mortality rates associated with cryptococcal meningoencephalitis remain unacceptably high, ranging from 10% to 50%, depending on the clinical situation (Perfect *et al.*, 2010). Nowadays, cryptococcosis continues to have a high prevalence in developing areas, such as countries from Subsaharian Africa and Eastern Asia. In 2009, it was estimated that the number of deaths attributed to criptococcosis was around 600,000 deaths per year (Park *et al.*, 2009). The clinical management of this disease in this countries, including the use diagnostic tools that provide an early diagnosis, has reduced the mortality, but the current estimations is that this disease still causes hundreds of thousands of deaths worldwide (Rajasingham *et al.*, 2017). Even in those regions of Africa where the HAART treatment is available, 20%–60% of patients die from the disease and many individuals develop recurrent disease (Jarvis *et al.*, 2010). In the last few years, other groups of immunocompromised patients have been also identified (Sabiiti and May, 2012), such as patients with hematologic malignancies, organ transplant recipients, and patients affected by autoimmune diseases (Bratton *et al.*, 2013; Henao-Martínez and Beckham, 2015). Infection is acquired by inhalation of spores or dehydrated blastoconidia, such that yeasts first colonize the lungs. In healthy people, the infection is controlled by the immune system. However, it is not known if the yeasts are eliminated from the organism, or if they can remain as latent forms and cause asymptomatic chronic infections. The main risk for disseminated disease occurs in HIV+ patients with low number of CD4 T cells. In these situations, the yeast can disseminate through the blood and invade the central nervous system (CNS), where the blastoconidia can affect the meninges and disseminate through the cerebrospinal fluid (CSF). *C. neoformans* can also cause lesions on the brain mass leading to encephalitis and meningoencephalitis with the develop abscesses (cripotococcomas). The main symptoms associated with these infections are characterized by high fever, photosensitivity, severe headaches and convulsions. Meningoencephalitis is the most typical presentation of this disease and is found in over 70% of cases of cryptococcosis in patients with AIDS.

In contrast to *C. neoformans*, *C. gattii* has been traditionally associated with infections in immunocompetent individuals, although immunosuppressed patients are also at risk of infection (Jobbins *et al.*, 2010). Infections by *C. gattii* have been mainly reported as endemic in regions with tropical and subtropical climates. The infection is acquired from the environment by inhalation, but dissemination to the CNS is not frequent, and approximately 70% of patients suffer from pulmonary disease (Ngamskulrungroj *et al.*, 2012; Chen *et al.*, 2000). *C. gattii* has caused important outbreaks in animals and humans, as happened in the Vancouver Island from the British Columbia in Canada at the beginning of the 20th century (Fraser *et al.*, 2003). This outbreak was had a great impact in the fungal scientific community because it expanded through the North West area of the United States (Byrnes *et al.*, 2010).

Cryptococcus in the Environment

Cryptococcus spp. are isolated from multiple niches in the environment. *C. neoformans* has been associated with pigeon droppings, chickens, turkeys, wood, and hollow tree trunks (Anzai *et al.*, 2014). *C. gattii* is frequently isolated from *Eucalyptus* (Ellis and Pfeiffer, 1990) and other trees, although it can also be recovered from soil, wood debris, and air (Galanis *et al.*, 2010; Springer and Chaturvedi, 2010; Kidd *et al.*, 2004).

In nature, *Cryptococcus* has the ability to infect a wide range of hosts. It has been reported that *C. neoformans* and *C. gattii* can infect unicellular predators, such as amebas, paramecium, flies, Lepidoptera, and mammals (mice, rats, koalas, dogs, and goats among others). To illustrate the variety of animals susceptible to *Cryptococcus*, this yeast can also infect aquatic animals, such as dolphins (Miller *et al.*, 2002), and even plants as *Arabidopsis thaliana* (Warpeha *et al.*, 2013). Infections of these hosts are important to understand the virulence and disease caused in humans, because it is believed that during the interactions with these alternative hosts, *Cryptococcus* has selected virulence traits that are used to infect humans. In this sense, it has been shown that in vitro exposure of *C. neoformans* to amebas increase the virulence of the yeast in mice (Steenbergen *et al.*, 2003).

Virulence Factors

C. neoformans/gattii have several characteristics that allow for adaptation to the host and the development of the disease. First, this fungus can grow at 37°C; that allows thermotolerance and ability to replicate and disseminate at body temperature. In addition, *Cryptococcus* can also produce virulence factors that cause damage in the host. Some of them are lytic enzymes, such as phospholipase B, which contributes to fungal invasion in the host. This fungus also expresses urease, which allows growth in urea, and contributes to invasion of the CNS (Casadevall and Pirofski, 1999, 2003; Kronstad *et al.*, 2011). However, *Cryptococcus* is unique among yeast pathogens because it has two phenotypic characteristics that are the main virulence factors of this yeast: the capsule and melanin accumulation.

Cryptococcal Capsule

The polysaccharide capsule is the most characteristic phenotypic feature of *Cryptococcus*. This structure is easily visualized by suspension of the yeast in India Ink (**Fig. 2**).

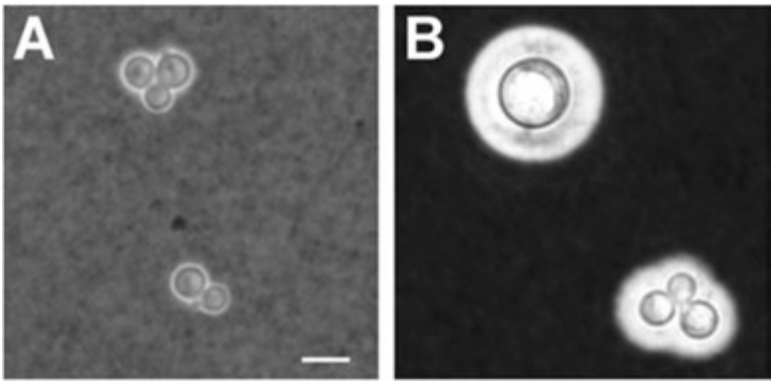

Fig. 2 *Cryptococcus* cells with different capsule sizes. (A) Cells with small capsule and (B) cells with large capsule. Scale bar, 5 μm.

The capsule is composed by two polysaccharides: glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (Zaragoza *et al.*, 2009), although this component also contains traces of glucuronic acid, so it was renamed to glucuronoxylomannogalactan (GXMGal) (Heiss *et al.*, 2009). Furthermore, a small proportion of mannoproteins and chitin-like structures are also present in the capsule. The polysaccharide is organized in fibbers that attach to the α-glucan of the cell wall (Reese and Doering, 2003), although new polysaccharide is bound to the old polysaccharide fibrils through non-covalent interactions (Mcfadden *et al.*, 2006).

The synthesis and attachment of the capsule is a process that includes several steps, as biosynthesis, transport, and maintenance of the polysaccharide on the cell surface (Alspaugh, 2014). The basic components of the capsular polysaccharide are UDP-xylose, UDP-glucuronic acid, GDP-mannose, and glucuronic acid. These components are linked by the action of several enzymes, such as transferases and glycosidases. Furthermore, the polysaccharide fibers undergo other modifications, such acetylation, xylosilation, and mannosylation (O'meara and Alspaugh, 2012). Different genes involved in capsule biosynthesis have been identified. The first one, *CAP59* was described by Chang and Kwon-Chung (1994). In the following years, *CAP60*, *CAP64*, and *CAP10* genes were also identified as participating in the synthesis of capsular polysaccharide of *C. neoformans*. Mutants that do not express any of these genes are avirulent which confirms that the capsule is fully required to cause disease (Chang and Kwon-Chung, 1994, 1998, 1999).

The secretion of the polysaccharide fibers is a process poorly characterized. However, there is increasing evidence that the polysaccharide is exported inside vesicles that cross the cell wall and release its content outside of the cell (Rodrigues *et al.*, 2008). These extracellular vesicles seem to be involved in multiple processes, since they contain virulence factors, such as capsular polysaccharide and melanin (Eisenman *et al.*, 2009).

The capsule is highly dynamic in that it can change its structure and density depending on the environmental conditions and on the age of the cells (Zaragoza, 2011). These changes are of great importance for its virulence because they contribute to immune evasion and inhibit the penetration of proteins from the host immune system. The size of the capsule is not constant, and during the first hours of infection, there is a significant increase in capsule size (Feldmesser *et al.*, 2001). This process can be easily reproduced in vitro, and factors, such as low iron concentration, CO_2, nutrient limitation, and mannitol induce capsule enlargement. This process is important for the virulence because capsule growth inhibits phagocytosis and protects against oxidative stress (Zaragoza *et al.*, 2009).

The capsule is required for the virulence of *C. neoformans* and there is extensive evidence that acapsular strains are not virulent (Bulmer *et al.*, 1967; Fromtling *et al.*, 1982). The capsule confers protection against dehydration and oxidative stress (Aksenov *et al.*, 1973; Zaragoza *et al.*, 2008). Furthermore, it inhibits the phagocytosis of the fungus. However, in addition to its protective role, capsular polysaccharides are secreted and they exert multiple deleterious effects on the host. Among others, the capsular polysaccharides interfere with T-cell function (Feldmesser *et al.*, 2000; Rodrigues and Nimrichter, 2012), induce complement depletion and leukocyte apoptosis, and inhibit leukocyte migration and antibody production. Furthermore, both GXM and GXMGal induce immunomodulation and change the host immune response (Zaragoza *et al.*, 2009).

Melanin

Another important virulence factor described in *Cryptococcus* is melanin (Kwon-Chung *et al.*, 1982), which is a dark pigment widely found in nature, is acid-resistant and water insoluble. One of the main characteriztics of this pigment is its role as antioxidant, so it confers protections against a large number of stresses.

In *Cryptococcus*, melanin is synthesized in the presence of diphenolic compounds (mainly L-DOPA) by the action of a diphenoloxidase encoded by two genes (*LAC1* and *LAC2*, being *LAC1* the most abundantly expressed). Melanin accumulates at the cell wall. In vitro, melanized cells are more protected against a large number of stresses compared to non-melanized cells, such as oxidants, uv light, ionizing radiation, antimicrobial peptides, heat, antifungal drugs, and phagocytosis (Nosanchuk and Casadevall, 2006). For this reason, melanin accumulation confers selective advantage during infection. Mutants unable to melanize have reduced virulence. Cryptococcal melanin contributes to the dissemination from the lungs to other organs (Salas *et al.*, 1996). Melanin precursors such as L-DOPA are abundant in the CNS, so it is believed that this could be one of the reasons for the neurotropism of these yeasts for this organ (Nosanchuk and Casadevall, 2003; van Duin *et al.*, 2004, 2002; Martinez and Casadevall, 2006; Zhu and Williamson, 2004;

Panepinto and Williamson, 2006). Recently, a new role has been attributed to melanin. Since this pigment can absorb energy, it has been shown that melanized cells can also absorb heat more efficiently than non-melanized cells, suggesting a mechanism by which melanized cells confers selective advantage at lower temperatures or in geographical regions far from the Tropics (Cordero *et al.*, 2018).

Cryptococcus as a Facultative Intracellular Pathogen

Although the capsule has antiphagocytic properties, the yeasts can be easily phagocytosed in the presence of opsonins that bind to the capsule, such as circulating antibodies and proteins from the complement system. Once phagocytosed, *Cryptococcus* can survive and replicate inside phagocytic cells (Diamond and Bennett, 1973), so it is considered a facultative intracellular pathogen. There are several mechanisms that allow intracellular survival. Initial studies suggested that *C. neoformans* does not inhibit phagolysosomal acidification, but rather resides and survives in the acidic phagolysosome (Levitz *et al.*, 1999). In agreement, when the pH of the phagolysosome is artificially increased, there is a reduction in the intracellular proliferation of the yeast, suggesting that *C. neoformans* has the ability to divide in acidic pH (Luberto *et al.*, 2001). However, recent studies using fluorescent probes sensitive to changes in pH have demonstrate that acidification of phagolysosomes containing cryptococcal cells is not full, which indicates that *C. neoformans* has the ability to interfere with the phagolysosome maturation. So far, two different mechanisms have been described for the cryptococcal-induced acidification inhibition. It has been shown that the capsule polysaccharide could "buffer" changes in phagolysosomal pH, inhibiting in this way its acidification (De Leon-Rodriguez *et al*, 2018). Furthermore, C. neoformans can modulate phagolysosomal pH through urease and nitrogen metabolism (Fu *et al*, 2018). Some other pathogen factors also contribute to intracellular survival. Capsule enlargement and melanin confer protection against free radicals and lytic peptides produced by the macrophages (Zaragoza *et al.*, 2008; Doering *et al.*, 1999). Melanin production also facilitates cryptococcal survival inside phagocytic cells because it can potentially neutralize cationic antimicrobial peptides and reactive oxygen species.

Cryptococcal survival inside macrophages results in multiple outcomes. Fungal cells can stay as latent forms within the phagolysosome, but they can also duplicate and proliferate. Exaggerate fungal replication can produce macrophage lysis, providing the microorganism an escape route from the intracellular environment (Johnston and May, 2013). However, fungal cells can also be expelled to the extracellular medium through a mechanism that does not involve macrophage death. This phenomenon is denominated non-lytic exocytosis or vomocytosis (Alvarez and Casadevall, 2006; Ma *et al.*, 2006). Non-lytic exocytosis occurs in both *C. neoformans* and *C. gattii* and shows great variation in incidence between strains (Alvarez and Casadevall, 2006; Johnston and May, 2013). Furthermore, this phenomenon also occurs in vivo (Nicola *et al.*, 2011). Finally, yeast cells can also be transferred between macrophages through a mechanism known as lateral transfer (Alvarez and Casadevall, 2006; Ma *et al.*, 2007), although this process remains to be fully characterized.

Formation of Titan Cells

Morphological changes in fungi, such as hypha development, play an important role during virulence because they contribute adhesion, dissemination and to immune evasion (Valero *et al.*, 2016; Coelho *et al.*, 2014; Trevijano-Contador *et al.*, 2016). In the case of *Cryptococcus*, filaments are only formed during sexual reproduction, but they are rarely present in tissues. During infection, most of the cells are found as rounded blastoconidia. However, there is large variation in the size of the fungal cells, that results in the appearance of cells of an abnormal large size denominated as titan cells (**Fig. 3**; Okagaki *et al.*, 2010; Zaragoza *et al.*, 2010; García-Rodas *et al.*, 2011).

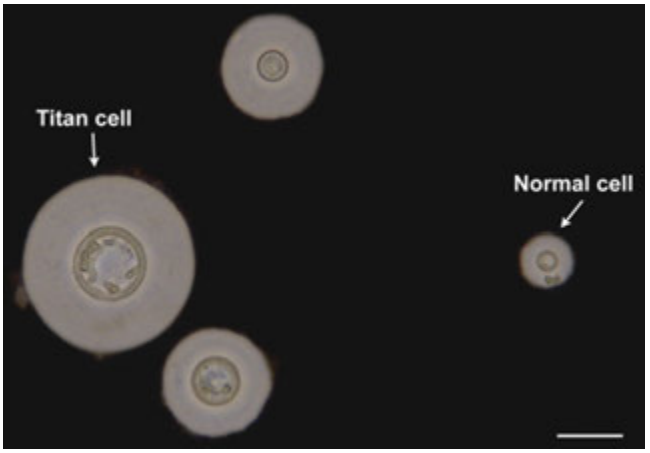

Fig. 3 Image of *Cryptococcus neoformans* cells isolated from the lungs of an infected mouse. Scale bar, 10 μm.

While the size of the cells in vitro is around 5–8 μm, they can reach up to 100 μm when residing in the lungs. These cells are polyploid and have an enlarged cell wall and capsule. Titan cells play a crucial role during cryptococcal infection for several reasons. Due to their size, they cannot be phagocytosed and are more resistant to stress factors, so these cells contribute to the long-term survival of the pathogen in the host. But it has been shown that these cells can also contribute to the disease because they polarize the host immunity to non-protective responses and inhibit the phagocytosis of fungal cells of regular size. Although there are few studies about the role of titan cells in patients with cryptococcosis, it has been shown that these cells were associated with higher CD4 T cell counts and reduced intracranial pressure, which led these authors to hypothesize that titan cells might be involved in the early stages of the infection (Fernandes *et al*, 2018). This is in agreement with mouse experiments which demonstrated that during latent asymptomatic infection with *C. neoformans*, most of the fungal cells presented a "titan" phenotype in the lungs (Zaragoza *et al*., 2010).

The characterization of titan cells has been limited to the fact that they needed to be isolated from animals and they were not efficiently induced in vitro. However, in 2018, three different groups described different laboratory conditions that stimulate cryptococcal growth, resulting in the appearance of cells that resemble titan cells from in vivo (Dambuza *et al*., 2018; Hommel *et al*., 2018; Trevijano-Contador *et al*., 2018). These works highlighted new pathways and factors that regulate titan cell formation, such as mammalian serum, CO_2, iron and oxygen limitation and even the influence of bacterial commensal on this process.

Diagnosis and Treatment

Cryptococcal diagnosis is achieved through various methods. Proved infection is defined after direct examination of the fungus in India Ink suspensions in body fluids, mainly in CSF. However, this method normally provides the diagnosis at the late stage of the disease, so indirect tests that detect the capsular polysaccharides antigens are also available. Classically, two tests have been used: a latex agglutination tests (LAT) assay and enzyme immunoassay (EIA) test (Kambugu *et al*., 2008; Boulware *et al*., 2014). In 2009, a lateral flow immunoassay (LFA) was also developed. When compared to EIA and LAT, LFA provided a good reproducibility and prognosis, so it is now widely used nowadays in low-income countries (Hansen *et al*., 2013; Perfect and Bicanic, 2015).

The treatment of cryptococcosis presents some limitations. There are three main antifungal families to treat invasive fungal infections: polyenes, azoles, and echinocandins, in addition to the nucleic acid inhibitor, 5-flucytosine (5-FC). However, not all antifungal agents available can be used in the treatment of cryptococcosis. *Cryptococcus* is intrinsically resistant to echinocandins and the therapy combined with AmB and 5-FC is not available in low-income countries. So treatment of cryptococcosis is based on an initial therapy with amphotericin B (AmB). However, AmB is toxic and can only be given to the patients for a limited time (around 2 weeks). There are lipid formulations that reduce its toxicity, but its high price limits its use in low-income countries, so treatment on these regions is mainly based on initial administration of conventional amphotericin followed by maintenance therapy with fluconazole, which could take several months to reduce the fungal burden of the patients (Perfect *et al*., 2010).

Conclusions

C. neoformans/gattii are unique fungi due to their ability to infect a large number of hosts. These microorganisms still have a significant incidence in developing countries and mortality rates are high. The treatment is limited for several reasons, such as intrinsic resistance to echinocandins and price of antifungal drugs in developing areas. For these reasons, research on new therapeutic strategies to control cryptococcal infections is warranted.

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Epidemiology of Infections Caused by Molds☆

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Awareness of significant life-threatening fungal infections has increased in recent years. However, given the global burden of these diseases (Bongomin *et al.*, 2017), and the morbidity and mortality associated to them, they are still heavily neglected in public health policy. We know that significant contributing factors to the epidemiology of mold infections, are host factors, geography, nosocomial reservoirs, and antifungal prophylactic strategies. (Dignani, 2014; Cornely, 2008).

There is a profound impact of these infections on population health and economics, and although true estimates of the economic burden of these diseases are lacking, estimates of invasive mold infections per se, has been attempted in a few, well designed studies, with the caveat that they have mostly been confined to restricted geographical areas or classically known at risk individuals (Azie *et al.*, 2012; Steinbach *et al.*, 2012). A recent study, trying to estimate the direct health care cost of fungal infections in the US (Benedict *et al.*, 2019), found that only in 2017, the cost for these infections where 7.2 billion, including hospitalizations and outpatient visits, where *Aspergillus* infections represented more than 25% of fungal related hospitalization costs, and the highest overall costs of any disease. These figures do not take in to account other costs, such as decreased productivity, disability and other indirect costs.

Given the difficulty in establishing invasive mold infections pre-mortem, current estimates are likely underestimating the magnitude of the impact of these diseases. No autopsies studies have established the burden of these diseases throughout different populations and different geographic regions; this is particularly relevant with the growing number of iatrogenic acquired risk factors and the potential of endemic regions as a result of climate change. In general, there are only few autopsies looking at invasive mold infections, thus true estimates are lacking. In a study looking at invasive fungal disease in published autopsies series from single center, multicenter or national studies, found a median prevalence of invasive fungal disease in 8.7 per 100 autopsies, where infections classified as *Aspergillus*-like infections, where the most common overall fungal diseases, followed by *Candida* spp. infections. These series included general population, hemato-oncologic patients, stem cell transplant recipients, and HIV/AIDS patients. In these series, the diagnosis of IFD, was established or suspected pre-mortem in only 30%–50% of cases. (Dignani, 2014).

We know that healthy individuals almost never develop significant mold infections, these are always considered opportunistic infections (Lionakis and Levitz, 2018). They occur either secondary to a disruption of the normal respiratory architecture and/or a decreased ability of the immune system of the host to contain these ubiquitous microorganisms. Invasive mold infections, of which the most prevalent are the ones caused by the *Aspergillus* genus, were traditionally thought to be confined to patients with severe immunosuppression, such as those with hematologic malignancies undergoing induction chemotherapy, hematopoietic stem cell transplant (HSCT) recipients, solid organ transplant (SOT) recipients, or patients with an inherited primary immune defect (PID). Other clinically significant mold infections are those caused by *Fusarium* spp, the agents of mucormycosis, *Lomentospora* spp., *Scedosporium* spp., and the dematiaceous molds. In the severely immunocompromised patient population, mortality for some of these infections often exceeds 50%, despite the availability of newer antifungals. (Seidel *et al.*, 2019; Roden *et al.*, 2005; Kontoyiannis *et al.*, 2010) Recently however, we have improved our recognition of other common risk factors for mold infections that were previously underestimated, and thus their true impact neglected. Some of these include, chronic pulmonary aspergillosis (**Fig. 1**) and invasive disease in prior or concomitant cavitary tuberculosis infections (Denning *et al.*, 2011; Hedayati *et al.*, 2015; Page *et al.*, 2019), in patients with chronic obstructive pulmonary disease (COPD) (Guinea *et al.*, 2010), as well as, undiagnosed invasive infections in non-hematologic critically ill patients.

On the other hand, not only our recognition of these diseases has improved, but there has been a true increase in the number of susceptible hosts. Classic at-risk individuals such as patients with hematologic malignancies live longer, and there is an expanding pool of individuals undergoing HSCT and SOT, worldwide (Gratwohl *et al.*, 2010; Rana and Godfrey, 2019). In addition, newer risk factors continue to be recognized (**Table 1**); precision medicine and the use of small molecules, targeted biologic therapies, and diverse cell therapies, including chimeric antigen receptor (CAR)-engineer T cells, have also expanded the pool of at-risk individuals (Ghez *et al.*, 2018; Varughese *et al.*, 2018; Zarakas *et al.*, 2019; Haidar *et al.*, 2019).

Aspergillosis

Invasive Aspergillosis

Estimates of invasive aspergillosis (IA) are complicated by the fact that most cases of IA in severely immunocompromised individuals are defined as "probable cases", no microbiologic diagnosis exists, and estimates in different studies are not uniformly calculated (incidence, cumulative incidence, etc). Best estimates agree that invasive aspergillosis happens in ∼ 2.6%–13% of patients with hematologic malignancies or marrow failure syndromes (Pagano *et al.*, 2010; Pagano and Caira, 2012; Pagano *et al.*,

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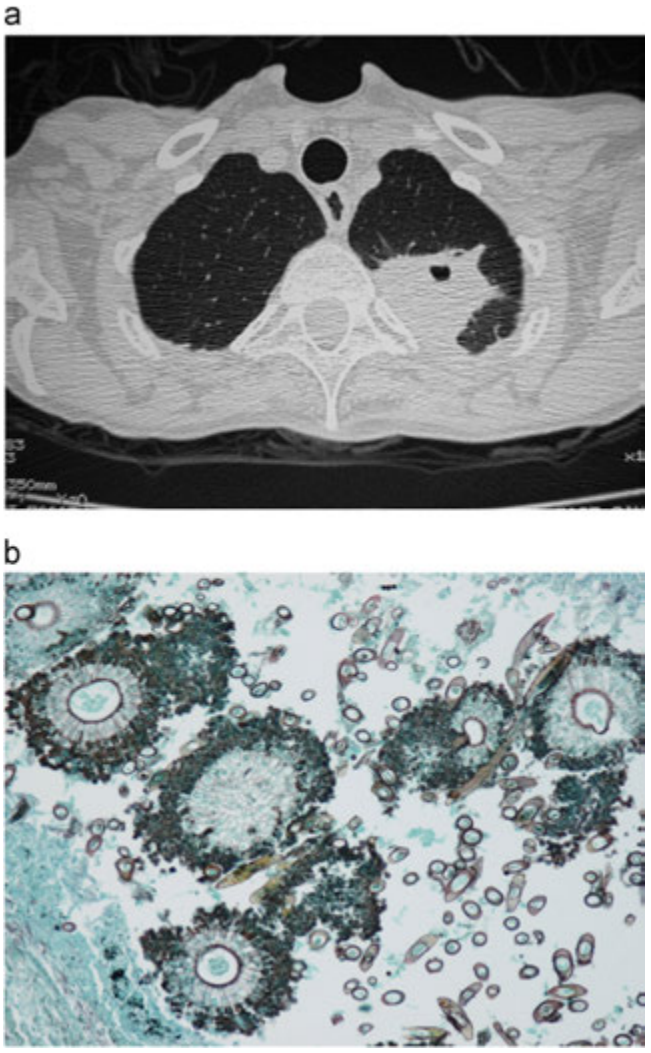

Fig. 1 *Chronic Pulmonary Aspergillosis* (a) CT chest of a 52-year-old Mexican woman with rheumatoid arthritis, weight loss and chronic cough, with an apical cavitary lung lesion and weight loss. Initial diagnostic workup was negative, initially treated for pulmonary tuberculosis, without resolution. Surgical resection of the cavitary lesion showed tissue necrosis and hyphae surrounding the cavity. (b) GMS stain showed Aspergillus-like conidial heads. Culture grew *Aspergillus fumigatus*.

Table 1 Common and emerging risk factors for invasive mold infections

Mold	*High risk*	*Intermediate risk*
Aspergillosis	Chemotherapy Induced Neutropenia, Hematologic Malignancy (AML & MDS), SCT, GVHD, Corticosteroid, Lung Transplant, CGD, Btk-inhibitors	Aplastic Anemia, Liver Transplant, Severe Influenza Infection, Current or Prior Cavitary Tuberculosis, COPD,
Mucormycosis	Uncontrolled DM and Diabetic ketoacidosis, SCT, Lung Transplant.	Voriconazole prophylaxis, Other SOT, Penetrating Trauma, Cavitary Tuberculosis
Fusariosis	SCT, Hematologic Malignancy, Prolonged Neutropenia, GVHD, Treatment of underlying malignancy and/or SCT in Brazil.	Solid Organ Transplantation, Eye Trauma
Other hyaline molds	SCT, graft failure and retransplant. Lung Transplant, CF. Near drowning accidents	Azole prophylaxis

Source: AML, acute myelogenous leukemia; Btk, bruton tyrosine kinase; CGD, chronic Granulomatous Disease; COPD, chronic Obstructive Pulmonary Disease; CF, cystic Fibrosis; GVHD, graft-versus host disease; MDS, myelodysplastic syndrome; SCT, stem cell transplantation; SOT, solid organ transplant.

2006; Nucci *et al.*, 2018); in HSCT recipients in ∼ 1.3%–10% (Atalla *et al.*, 2015; Kontoyiannis *et al.*, 2010; Kojima *et al.*, 2004; Montoro *et al.*, 2019). Allogeneic HSCT recipients have a significant greater risk than autologous HSCT recipients; this increased risk results from the mean duration of neutropenia, and the immunosuppression used to prevent and treat graft versus host disease (GVDH). In addition to prolonged neutropenia, the intensity of the immunosuppression used to treat chronic GVHD is the single most important risk factor, for the development of invasive mold infections. Different surveillance studies have quantified the risk of invasive aspergillosis between ∼1% and 9% in SOT recipients, however recent more accurate estimates, quantify the risk at about 1.6% (Kontoyiannis *et al.*, 2010; Pappas *et al.*, 2010). Given that most invasive aspergillosis is acquired through inhalation of fungal spores; in addition to decreased mucociliary clearance, and intensity of immunosuppression, lung transplant recipients on no anti-mold acting prophylaxis are particularly susceptible to this disease. About ∼9% of lung transplant recipients develop an invasive fungal infection, and approximately 45% of these are due to *Aspergillus* spp. Mortality in this population remains unacceptably high (Seidel *et al.*, 2019; Pappas *et al.*, 2010; Aguilar *et al.*, 2018; Singh *et al.*, 2013).

In inherited immune deficiencies, chronic granulomatous disease represents the strongest risk factor for the development of invasive aspergillosis, and the risk correlates with the level of superoxide production. In large registries of primary immune deficiencies, IA remains a significant cause of mortality, despite best prophylactic strategies (Jones *et al.*, 2008), only in specialized centers has the mortality secondary to *Aspergillus* spp. infection truly decreased (Marciano *et al.*, 2015).

The development of cancer targeted therapies, such as small molecules, have improved the survival of diseases that previously had a dismal prognosis. However, for some of these therapies, there has been an unexpected rise in the number of aspergillus infections. In particular, patients treated with Bruton tyrosine kinase (Btk) inhibitors have been associated to an increased risk of IA. In a trial where ibrutinib was used for the treatment of primary central nervous system (CNS) lymphoma in conjunction to corticosteroids, 39% of patients developed invasive aspergillosis (Zarakas *et al.*, 2019; Ghez *et al.*, 2018). A significant number of other agents in this same pathway are currently under development, as such, the incidence of these infections will continue to rise.

Azole-Resistant Aspergillus Infections

Resistance to itraconazole was documented in the late 90's, possibly as a result of prolonged antifungal exposure (denning, d 1997 Antimicrobial agents and chemo). Slowly over the next 2 decades, resistance to azoles increased, including resistance to voriconazole, these in patients who had not necessarily been directly exposed to azoles. It was noted that this increase in resistance coincided with the widespread use of fungicides in agriculture, and although initial reports seemed to be confined to European countries, specifically the Netherlands (Trovato *et al.*, 2018; Buil *et al.*, 2019); azole-resistant *Aspergillus fumigatus* infections have now been described in all continents (**Fig. 2**; Resendiz Sharpe *et al.*, 2018; Friedman and Schwartz, 2019; Gonzalez-Lara *et al.*, 2019; Alastruey-Izquierdo *et al.*, 2018).

Chronic Pulmonary Aspergillosis (CPA)

Aspergillus fumigatus avidly colonizes structurally abnormal lung, in some patients, it may progress to cause tissue destruction, extensive fibrosis and loss of lung volume. Thus, chronic pulmonary aspergillosis represents a spectrum of infection and disease, that usually

Fig. 2 *Countries where Azole-Resistant Aspergillus fumigatus has been Reported.*

presents in non-immunocompromised individuals with a structural lung abnormality, either due to prior or current pulmonary disease, and present with pulmonary symptoms and elevated inflammatory markers over a prolonged period of time (**Fig. 1**). The most frequent underlying diseases are pulmonary tuberculosis, in which case patients can be misdiagnosed as having recurrent/refractory tuberculosis infection, chronic obstructive pulmonary disease (COPD), both common conditions worldwide, and sarcoidosis. It has been estimated that 10%–25% of patients with treated cavitary tuberculosis will go on to develop CPA, and about 1%–4% of patients without cavities after completion of anti-tuberculosis therapy (Denning *et al.*, 2011; Smith and Denning, 2011). Patients with COPD can develop CPA or invasive disease, invasive disease is more common on those on chronic systemic corticosteroids and broad-spectrum antibiotics (Guinea *et al.*, 2010), and about 20% of patients with COPD in whom aspergillus is isolated, go on to develop invasive disease. This may be un underestimate since there are no good biomarkers for IA in COPD patients, they rarely undergo biopsies and very few autopsies are performed. Recent estimates suggest the burden of CPA to be around 3,000,000 cases worldwide (Bongomin *et al.*, 2017).

Critical Care and Post-Influenza Aspergillosis

In addition to classic neutropenic patients, non-neutropenic patients, are being recognized as susceptible to Aspergillus infections, and a large proportion of these patients end up admitted to critical care units. Recent surveys suggest that clinically relevant aspergillus infections happen in 0.5% to 5.8% of all ICU admissions and it is associated to a very high mortality ($\sim$80%) (Bassetti and Bouza, 2017). It is hypothesized that the biphasic immune response to sepsis or multiorgan failure, includes a late state of immunosuppression, that can lead to invasive aspergillosis. In addition to classic at know risk factors, other recently described risk factors are severe influenza infection, liver cirrhosis, and structurally abnormal lungs, such as COPD (Wauters *et al.*, 2012). In a large retrospective study of patients admitted to the ICU, influenza was an independent risk factor for invasive aspergillosis, and it was associated to a high mortality. In patients admitted to the ICU with influenza and immunocompromised, the incidence of IA, was up to 32%, and in those with influenza but without classically defined immunocompromise, the incidence was 14%, with an overall mortality of $\sim$50% (Schauwvlieghe *et al.*, 2018). Awareness of these associations may lead to an earlier diagnosis, and potentially better outcome.

Mucormycosis

Mucormycosis is a disease caused by fungi in the order of the Mucorales; many species are known to be pathogenic in humans; *Rhizopus* spp is the most commonly globally isolated species. Other frequent pathogenic isolates include *Lichtheimia*, *Apophysomyces*, *Rhizomucor*, *Mucor* and *Cunninghamella* species (Prakash and Chakrabarti, 2019). Mucormycosis is still associated to an unacceptably high mortality rate. Overall all-cause mortality in patients with mucormycosis is around 50%, however this varies widely for different clinical presentation and hosts. Mortality is $\sim$45% in sinus disease, $\sim$75% in pulmonary disease, and $>$95% in disseminated (Roden *et al.*, 2005). Although recently there has been substantial development of newer antifungals targeting these infections, survival has not really improved (Webb *et al.*, 2018; Prakash and Chakrabarti, 2019). One of the main problems in the outcome of this disease is, early diagnosis, and although fungal biomarkers have been helpful in the earlier diagnosis of other more common fungal infections, this has not been the case for infections caused by the agents of mucormycosis. Reports of molecular diagnosis of invasive fungal infections may change this outcome, as well as the more widespread recognition of the need of a multidisciplinary approach to this disease (Cornely *et al.*, 2019). In terms of health care expenditure, average cost per hospitalization is generally higher for mucormycosis, than for any other mold infection (Benedict *et al.*, 2019). Mucormycosis largely predominates in patients with poorly controlled diabetes (mostly in the developing world) (Corzo-Leon *et al.*, 2018; Prakash *et al.*, 2019) and in transplant recipients (Prakash and Chakrabarti, 2019). However as with other mold infections, other risk factors are being recognized; post-pulmonary tuberculosis and alcoholism, were significant risk factors for mucormycosis in a large series from India. Although there is substantial geographic variation, HSCT recipients are at particular high risk, being the third or fourth most common invasive fungal infection in different series. In a North America multicenter cohort of transplant recipients, mucormycosis accounted for 8% of all invasive fungal infections in HSCT (Kontoyiannis *et al.*, 2010), and 2% in SOT (Pappas *et al.*, 2010). Gastrointestinal mucormycosis, is also associated to a very high mortality rate, although it has been described in all HSCT recipients and patients with diabetes, it is more common in pediatric patients with solid organ transplant (Dioverti *et al.*, 2015), very low birth weight and malnutrition (Prakash and Chakrabarti, 2019). Mucormycosis associated to penetrating trauma, carries the best prognosis, likely because it tends to occur in young healthy (non-immunocompromised) individuals (Roden *et al.*, 2005).

Health-care associated outbreaks of mucormycosis have occurred. These have been related to the use of contaminated hospital linens, gauzes, ostomy bags, contaminated steroid injections and construction. These outbreaks have been associated to high morbidity, in some cases mortality and high health care costs (Davoudi *et al.*, 2015). A recent multi-center study of pathogenic molds in freshly laundered hospital linens in 15 cancer and transplant centers in the US, found that linens were contaminated upon arrival in 47% of the centers; at individual centers, mucormycosis was isolated from 0% to 24% of health care linens cultured. Freshly laundered visibly soiled linens were associated to culture positivity (Sundermann *et al.*, 2019). How this translates clinically is difficult to assess, however at these institutions, where a large proportion of susceptible hosts are being hospitalized, more work is needed to secure patient safety.

Fusariosis

Fusarium is another highly pathogenic hyaline mold in immunocompromised individuals. It is found ubiquitously in nature and has a worldwide distribution, but there are areas of regional hyperendemicity. In immunocompetent individuals its responsible for a large number of the global burden of fungal keratitis and blindness, and some onychomycosis. In severe immunocompromise secondary to prolonged neutropenia, mostly patients with hematologic malignancies and HSCT recipients, it causes invasive disease (Nucci *et al.*, 2018; Atalla *et al.*, 2015; Nucci *et al.*, 2015). More recently it has also been recognized as a cause of allergic disease, similar to allergic bronchopulmonary aspergillosis (Chowdhary *et al.*, 2014; Deepak *et al.*, 2019), hypersensitivity pneumonitis and colonization of preexisting cavities, thus it behaves similarly to Aspergillus infections (Dickson and Tankersley, 2015; Lee *et al.*, 2000; Nucci *et al.*, 2015; Ramirez and Jacobs, 2014).

In prospective studies in the United States and Italy, the cumulative incidence of invasive fusariosis was around 0.1%–0.3%, in transplant recipients, and patients with hematologic malignancies, mostly acute myeloid leukemias and HSCT (Kontoyiannis *et al.*, 2010; Girmenia *et al.*, 2014a,b). In contrast, in studies conducted in Brazil, which is one of the regions with the highest reported prevalence of invasive fusariosis, the incidence in patients with hematologic malignancies and HSCT recipients was around 3.8%–5.2% (Atalla *et al.*, 2015; Nucci *et al.*, 2018, 2013). Skin dissemination is common among patients with invasive fusariosis, and fungemia can be documented in about 50% of patients with disseminated disease (Nucci *et al.*, 2003).

Fusarium keratitis is the leading cause of mold associated visual impairment and blindness, usually associated to occupational exposure (farmers), implantation injury (trauma) and contact lens wear (Bongomin *et al.*, 2017; Perez-Balbuena *et al.*, 2009; Hernandez-Camarena *et al.*, 2015; Gopinathan *et al.*, 2002; Das *et al.*, 2015). In underdeveloped countries, these infections can lead to high proportion of enucleation (11%) and visual loss (~50%) (Green *et al.*, 2007).

Other Hyaline Molds

Scedosporium and *Lomentospora* classically known to cause superficial soft tissue infections after trauma, and to be frequent respiratory colonizers in patients with underlying lung pathology (i.e., cystic fibrosis or cavitary lung disease), these molds can be highly pathogenic and cause invasive and disseminated disease in severely immunocompromised individuals, or cause CNS infections in patients with near drowning accidents (Seidel *et al.*, 2019, 2020; Ramirez-Garcia *et al.*, 2018). The majority of reports come from Australia, North America, Europe and Korea. Whether there is a true geographic distribution to these infections, similar to what happens with fusariosis, is possible, however there is probably significant underreporting in many areas of the world (Seidel *et al.*, 2019). Although significantly less common than other mold infections, they have emerged as a cause of severe invasive disease in the face of prophylactic antifungals, in particular, *Lomentospora prolificans*, which similarly to *Fusarium* spp can lead to fungemia. The intrinsic resistance to most currently used antifungals, pose a significant challenge in the treatment of this breakthrough fungal infections, which unless surgically treated are associated to a high mortality rate. As with other mold infections, these are more common in patients with hematologic malignancies, HSCT and lung transplant recipients. Cystic fibrosis patients can frequently be colonized with these molds, and to what extent this colonization contributes to the decline of the pulmonary function is unknown, however is it highly associated to the development of posttransplant invasive pulmonary disease. Lacerating eye lesions and penetrating trauma are significant risk factors in immunocompetent individuals (Seidel *et al.*, 2019, 2020; Ramirez-Garcia *et al.*, 2018).

Conclusions

Although significant advances have been made in understanding the epidemiology and global burden of mold infections (Bongomin *et al.*, 2017), accurate estimates based on surveillance data and not on population assumptions is still lacking for most of the developing world. In the last decade scientists driven efforts have contributed to decreasing the knowledge gap of the risk factors for these diseases, however, estimates of the true burden of these infections is still lacking, and the number of at-risk individuals will continue to change over-time. Prioritizing epidemiologic surveillance of these diseases around the globe, may have a significant public health impact, in the prevention and early treatment of these debilitating diseases.

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Diseases Caused by *Aspergillus fumigatus*

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Glossary

Aspergilloma A clump of fungus in a body cavity, such as the lungs.
Aspergillosis Set of diseases caused by *Aspergillus* spp. which comprises a variety of clinical manifestations.
Aspergillus fumigatus Fungus of the *Aspergillus* genus. One of the most common predominant human-pathogenic mold causing aspergillosis.
Biofilm Multicellular and multilayered hyphae which are embedded in an extracellular matrix.
Colonization Growth in the host without invasive infection. It is defined when there is a positive culture from bronchoalveolar lavage or two sputum cultures positive for the same *Aspergillus* species, in the absence of invasive pulmonary aspergillosis.
Conidia Asexual fungal spores of filamentous fungi.
Conidiophores Highly organized structures of fungi that successively produce conidia on characteristic conidial heads.
Host An animal or plant on or in which a microorganism or commensal organism lives.
Hydrophobins Hydrophobic proteins that are present on the surface of *Aspergillus fumigatus* conidia and are responsible for the typical rodlet configuration of the outer conidial layer.
Saprotrophic fungi Molds species that obtain nourishment by decomposing organic matter, such as dead plants or animals.
Secondary metabolites Organic compounds produced by microorganism metabolism which are not essential for normal growth but can provide advantages for the survival of microbial species.
Siderophore A high-affinity iron-chelating molecule that is secreted by many bacterial and fungal species.
Virulence Ability to infect and degree of damage caused by an organism.

Introduction

Aspergillus species are soil saprophytic molds found ubiquitously worldwide and are associated with decomposing organic biomass playing an important role in carbon and nitrogen recycling in nature. These opportunistic fungi species released abundant conidia into the air, of which at least several hundred will be inhaled by humans every day (Latgé, 1999).

Species of the *Aspergillus* genus are the etiologic agent responsible for several human and animal diseases that are generally called aspergillosis. For most patients, the primary route of infection is the respiratory tract by inhalation of air-borne conidia. While immunocompetent individuals rarely suffer any adverse effect due to the efficient elimination by mucociliary clearance and the innate immune response by macrophages (Hohl and Feldmesser, 2007), in patients with pulmonary disorders, such as asthma or cystic fibrosis, *Aspergillus* can cause allergic bronchopulmonary aspergillosis, and in high-risk immunocompromised individuals, the infection can lead to invasive aspergillosis (Dagenais and Keller, 2009). Apart from the respiratory tract, other sites of infection have been described but are much less common, such as the skin, peritoneum, kidneys, bones, eyes and gastrointestinal tract (Anaissie and Costa, 2001; Latgé, 1999; Walsh, 1998).

Aspergillosis comprises a variety of clinical manifestations. There are several factors that contribute to aspergillosis, including both fungus and host-related factors such as strain virulence and host pulmonary structure and immune status (Almyroudis *et al.*, 2005; Denning, 1998; Latgé, 1999; Singh and Bhalodiya, 2005) (**Fig. 1**). According to the host immunity, clinical presentations of aspergillosis range from asymptomatic colonization, superficial or saprophytic infection, allergy, and acute invasive disease (Latgé, 1999; Walsh *et al.*, 2008). Generally, allergic diseases are caused by a chronic response to mold colonization due to a repeated exposure to *Aspergillus* conidia or antigens, while superficial infections are related to local trauma or fungal overgrowth. In contrast, diseases which involve mycelial growth are more severe, such as allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and invasive aspergillosis (IA) (Latgé, 1999). The last one is a systemic infection that has emerged as the leading cause of morbidity and mortality in immunocompromised hosts (Brown *et al.*, 2012; Denning, 1998). The immunocompromised populations that are most susceptible to invasive aspergillosis include patients with leukemia or genetic immunodeficiency diseases and patients undergoing bone marrow, hematopoietic stem cell, or solid-organ transplants. Mortality rates in these patients can reach up to 90% (Brown *et al.*, 2012; Kontoyiannis *et al.*, 2010; Pappas *et al.*, 2010). Furthermore, during the past few years, it has been increasing evidence for a genetic component that may render individuals more vulnerable to develop aspergillosis diseases (Maskarinec *et al.*, 2016).

Among the *Aspergillus* genus, *A. fumigatus* is the primary and most predominant human-pathogenic species and therefore the most common cause of aspergillosis (Kontoyiannis *et al.*, 2010; Pappas *et al.*, 2010). Besides *A. fumigatus*, there are other species of

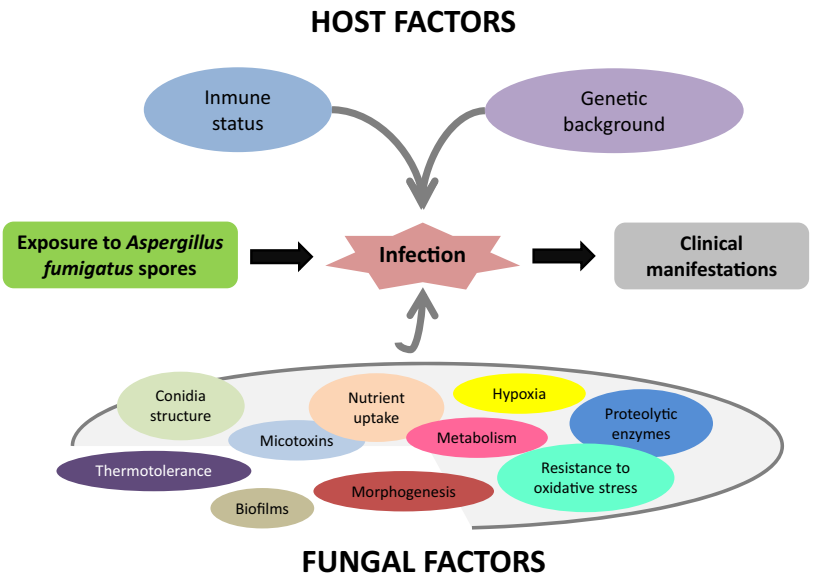

Fig. 1 Schematic view of host and fungal factors contributing to *Aspergillus fumigatus* infections.

this genus that cause human infections, such as, *A. flavus*, *A. terreus*, *A. niger*, *A. tubingensis*, in order of frequency, among many others (Alastruey-Izquierdo *et al.*, 2013).

Currently, the treatment options for aspergillosis are limited to three classes of antifungal drugs that target essential structural components of fungal cells. Azoles and polyenes are aimed at ergosterol, the predominant sterol within the fungal cell plasma membrane, while echinocandins represent the newest class of antifungals and interfere with the fungal cell wall.

Despite the availability of drugs to treat *A. fumigatus* infections, therapy can be challenging for many patients. The reasons for that are associated to the diagnosis difficulties, the late initiation of antifungal therapy, the modest efficacy of existing antifungals and host toxicity. Altogether, they contribute to treatment failure and persistence of the fungus in the human host (Verweij *et al.*, 2016b). Furthermore, *A. fumigatus* azole resistance has emerged as a global health problem and although the amount of aspergillosis caused by azole-resistant isolates is still limited, different resistance mechanisms are arising (Verweij *et al.*, 2016a). Two routes of azole resistance development have been described; acquired during azole treatment and through an environmental exposure due to the use of these compounds as fungicides (Warris, 2015). The percentage of resistance prevalence varies between studies, but some authors reported an overall resistance rate of 5.7% among *A. fumigatus* isolates (Vermeulen *et al.*, 2015). Failing to control antifungal drug resistance, the scientific community has been encouraged to look for novel antifungals compounds which aim to target virulence determinants in *A. fumigatus*.

In this chapter, first we will describe the types of diseases caused by *A. fumigatus* and its symptoms according to the site of infection and the host health condition. Secondly, the molecular bases of *A. fumigatus* pathogenesis are discussed.

Clinical Manifestations of *Aspergillus fumigatus* Infections

Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is a chronic allergic pulmonary disorder caused by hypersensitivity to *Aspergillus* species in response to colonization with the mold (Agarwal, 2009; Agarwal *et al.*, 2013). It is a very difficult syndrome to diagnose, but the classic criteria are chronic asthma, peripheral eosinophilia, skin test reactivity to *Aspergillus* antigen, precipitating *Aspergillus* antibodies, immunoglobulin E elevated in serum, recurrent pulmonary infiltrates, and central bronchiectasis (Agarwal, 2009; Agarwal *et al.*, 2013).

The clinical features are similar to asthma symptoms, including mostly wheezing, bronchial hyperreactivity, hemoptysis, productive cough with brownish black expectoration, and low-grade fever (Agarwal, 2009). Unfortunately, symptoms are not a good guide to predict outcome, as several asymptomatic patients developed eosinophilia and lung consolidation that resulted in chronic lung damage (Stevens *et al.*, 2000).

The prevalence of ABPA is reported to be up to 14% in patients with steroid- dependent asthma (Agarwal, 2009) and between 2% and 15% in patients with cystic fibrosis (CF) (Greenberger, 2003).

Allergic *Aspergillus* Sinusitis

Allergic *Aspergillus* sinusitis is a non-invasive inflammatory sinusitis caused by an allergic response to a local *A. fumigatus* infection (Singh and Bhalodiya, 2005). It is similar to any sinusitis but complicated with *Aspergillus* fungal balls (DeShazo *et al.*, 1997). Management of this infection consists in confirming lack of invasive infection and then treatment centers on aerating the sinus by endoscopic removal of debris and hyperplastic tissue (Schubert, 2004). The symptoms of an allergic *Aspergillus* sinusitis include stuffiness, runny nose, headache, and reduced ability to smell (Singh and Bhalodiya, 2005).

Typically, patients are young (near 30s) and present atopy, nasal polyps, and hypertrophic sinus disease (Schubert, 2004). Unfortunately, the use of antifungal agents or corticosteroids has not been conclusively demonstrated (Walsh *et al.*, 2008).

Aspergilloma

Aspergilloma, also called fungus ball, can be defined as a dense conglomeration of *Aspergillus* hyphae together with fibrin, mucus and cellular debris, within a pulmonary cavity or ectatic bronchus (Fraser *et al.*, 1998), and sometimes may also develop in other body sites, such as maxillary or ethmoid sinus or even in the jaw (Ferguson, 2000). Typically, these fungus balls develop in cavities as a result of pre-existing infections, such as tuberculosis, histoplasmosis, sarcoidosis, or other bullous lung disorders, and in chronically obstructed paranasal sinuses. Fungus balls are separated from the wall of the cavity by an airspace (Latgé, 1999; Stevens *et al.*, 2000).

The diagnosis of aspergilloma is based on chest radiographic features and serum precipitins positive to *Aspergillus* species (Lee *et al.*, 2004; Stevens *et al.*, 2000). Aspergillomas appear on chest radiographs as spherical masses surrounded by a radiolucent crescent (Broderick *et al.*, 1996). Although aspergilloma may be relatively asymptomatic and it usually does not spread to other parts of the body, invasive pulmonary aspergillosis may develop in some patients with aspergilloma.

Hemoptysis is a common clinical symptom of aspergilloma and can lead to fatal asphyxiation. It results from the disruption of blood vessels in the own cavity wall occupied by the fungus or in the bronchial artery supply far away from the aspergilloma (Latgé, 1999). Hemoptysis is the cause of death in up to 26% of patients with aspergilloma (Kauffman, 1996). Other symptoms are cough and shortness of breath.

Chronic Pulmonary Aspergillosis

Chronic pulmonary aspergillosis (CPA) is a progressive, debilitating infection, which progresses slowly, and by convention has to be present at least 3 months before diagnosis (Denning *et al.*, 2003). In this condition, imaging reveals one or more cavities in the lungs, with or without aspergilloma or with solid or cavitating nodules (Muldoon *et al.*, 2016). In addition, pleural thickening is characteristic of CPA but not universal (Denning *et al.*, 2011).

Pulmonary symptoms are productive cough, hemoptysis, chest discomfort and shortness of breath. Also fatigue and weight loss are common features of this disorder (Al-shair *et al.*, 2013).

About 3 million people worldwide have CPA, of whom 1.2 million have previously had pulmonary tuberculosis, therefore they have a history of lung defects but they are not immunocompromised patients (Denning *et al.*, 2011; Smith and Denning, 2011).

Invasive Aspergillosis

Invasive aspergillosis (IA) has become an important cause of death, mainly among hematological patients, such as patients with neutropenia or neutrophil and/or macrophage disorders, also patients exposed to cytotoxic chemotherapy, long-term corticosteroid treatment, bone marrow or organ transplant, or with any immunodeficiency, congenital or acquired (Stevens *et al.*, 2000).

Normally, inhaled airborne conidia invade the lung tissue thanks to the lack of an effective immune response. Afterwards, hyphal invasion into blood vessels is common, which leads to infection of pulmonary or non-pulmonary sites by contiguous or hematogenous spread to the central nervous system or to other organs.

Invasive pulmonary aspergillosis

The main clinical presentation of IA is invasive pulmonary aspergillosis (IPA), which rarely manifests before 10–12 days of profound neutropenia (Patterson, 2011).

As other forms of aspergillosis, the general symptoms of IPA are fever despite therapy with broad-spectrum antibiotics, chest pain, dry cough, dyspnea, and weight loss. Other manifestations include hemoptysis and pneumothorax (Latgé, 1999; Patterson, 2011). Also, when patients develop more severe pulmonary involvement, respiratory failure, hypoxemia with compensatory hyperventilation, hypo apnea, and respiratory alkalosis are characteristic (Patterson, 2011). Other symptoms can develop if the infection spreads from the lungs to other parts of the body.

Since IPA is a devastating infection which progresses very fast and is difficult to diagnose, clinicians often treat the patient empirically before confirming the final diagnosis in order to avoid a greater risk of untreatable IPA due to a too high level of fungal burden (Latgé, 1999). Therefore, a prompt antifungal therapy may be essential for patient survival.

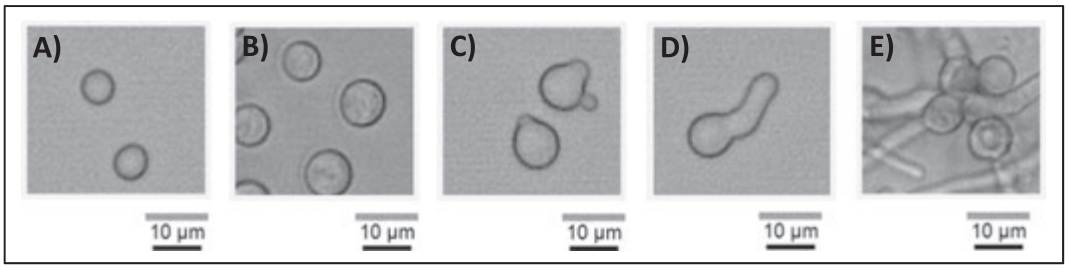

Fig. 2 Morphological stages of *Aspergillus fumigatus* during *in vitro* growth: (A) resting conidia, (B) swollen conidia, (C) starting to germinate conidia, (D) germ tubes, (E) hyphae.

Other aspergillosis invasive syndromes

Non-pulmonary organs, such as the liver, spleen, kidney, skin, bone or heart, can be infected through fungus dissemination towards nearby locations, simply for closeness, or by the own bloodstream (Patterson, 2011). This may result in different invasive infections.

Sinusitis is a relatively common manifestation of IA, developing as an isolated syndrome or within an IPA context. In the case of tracheobronchitis, it is common to develop in AIDS or lung transplantation patients.

Cutaneous aspergillosis can occur as a disseminated hematogenous spread from a primary focus of infection, most often the lungs, in neutropenic or highly immunocompromised patients, or as a local infection associated with intravenous catheter insertion or adhesive dressings for venous access devices contaminated with the fungus (Walsh *et al.*, 2008). The lesions can be single or multiple, most commonly on the extremities, firstly appear as erythematous papules, then become pustular, and subsequently necrotic with a central ulceration and an elevated border covered by a black eschar (Stevens *et al.*, 2000).

Finally, one of the most severe complications is cerebral aspergillosis, which is associated with disseminated disease. It presents very high mortality rates and occurs in 10%–20% of all IA cases (Patterson, 2011).

Molecular Bases of *Aspergillus fumigatus* Virulence

Pathogenicity can be defined as the capacity of a microbe to cause damage in a host while virulence refers to the degree of damage caused by the microbe (Casadevall and Pirofski, 1999). In line, host damage can occur as a result of direct microbial action on tissues, as a result of the immune response to the microbe, or a combination of both. A virulence factor is the microbial component that damages the host (Casadevall and Pirofski, 1999).

Among the 185 species belonging to the genus *Aspergillus*, only a fractional proportion of them are pathogenic for humans. What distinguishes more virulent organisms, such as *A. fumigatus*, from more benign species has been an important research area for many years. In the absence of unique molecules or pathways that have an essential impact on *A. fumigatus* survival it has been assumed that its virulence is multifactorial.

A. fumigatus has one of the fastest growth rates among species of the fungal kingdom that promotes their colonization of multiple niches (van de Veerdonk *et al.*, 2017). The life cycle of *A. fumigatus* involves several distinct morphogenetic transitions. These transitions include the formation of conidial spores through conidiation, the conidial germination, and polarized hyphal growth. These morphogenetic changes can be microscopically observed when *A. fumigatus* is growing in *in vitro* conditions (**Fig. 2**). In a similar manner when conidial spores interact with a susceptible host, they would experience the same morphological transitions until they reach hyphal growth (**Fig. 3**).

According to the life cycle, *A. fumigatus* virulence traits are linked to fungal structures, morphological changes during developmental growth, metabolic requirements that change in order to adapt to the host environment and mechanisms to escape from the host immune responses. These adjustments are classified according to the process in which they are involved, and are described below:

A. fumigatus produces conidia (asexual spores) which are one of the first important pathogenic traits to consider. When comparing to other *Aspergillus* species, such as *A. flavus* or *A. niger*, *A. fumigatus* spores are smaller and much more hydrophobic so they can disperse more quickly in the environment. Altogether, these features facilitate that *A. fumigatus* spores enter easily into the respiratory tract, reaching the human alveoli (Kwon-Chungand Sugui, 2013).

As other human pathogens, *A. fumigatus* grows well at 37°C but it is more resistant than other fungi to changes in temperature, as it can tolerate temperatures ranging from 37 to 50°C (Bhabhra and Askew, 2005; Sales-Campos *et al.*, 2013). The ribosomal biogenesis proteins encoded by *crg*A (Bhabhra *et al.*, 2004), α-mannosyltransferase (*kre*2/*mnt*1) (Wagener *et al.*, 2008), and the endoplasmic reticulum-transmembrane sensor encoded by *ire*A (Feng *et al.*, 2011) are, up to date, the known proteins associated with thermotolerance growth and hypovirulence, when the respective genes were deleted from *A. fumigatus* genome.

The surface of *A. fumigatus* conidia is covered by a thin layer called rodlet layer composed by regularly arranged hydrophobic proteins (hydrophobins) that are covalently bound to the cell wall. This layer contributes to conidial dispersion and soil fixation, but it also prevents conidia from the immune response by masking its recognition (Aimanianda *et al.*, 2009). *A. fumigatus* contains two hydrophobins: RodAp, encoded by the RODA gene, and RodBp, encoded by the RODB gene. Using single and double

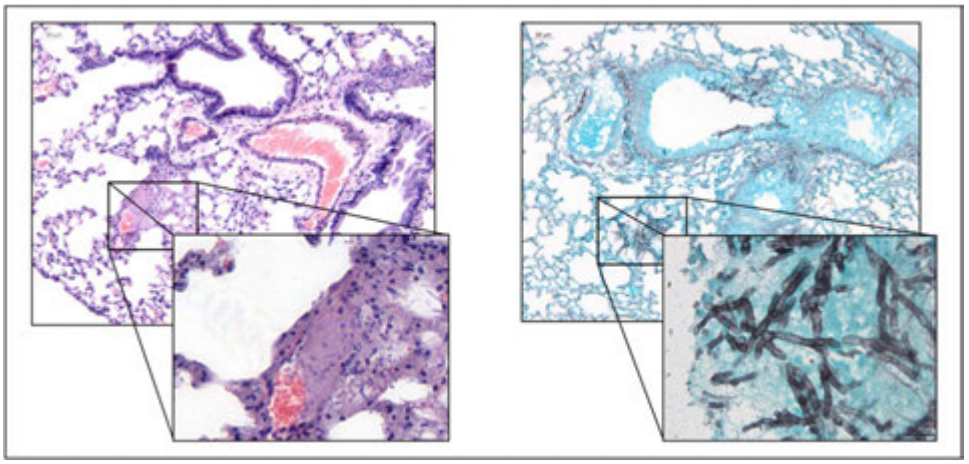

Fig. 3 Histological sections of murine lungs infected with *Aspergillus fumigatus*: (A) Recruitment of immune cells and inflammatory response at the site of infection, (B) In black *A. fumigatus* hyphae can be observed in the murine lung.

mutants it has been shown that RodBp is not involved in rodlet formation neither protects against conidial killing by alveolar macrophages (Aimanianda *et al.*, 2009).

A. fumigatus conidia are grey-green due to the pigment DHN-melanin. The role of melanin has been studied by using pigmentless mutants showing that melanin protects against enzymatic lysis and environmental stress, such as UV light and oxidizing agents. Altogether, these functions contribute to attenuate the host immune response and to promote *A. fumigatus* dissemination (Bayry *et al.*, 2014). As an alternative to melanin, *A. fumigatus* produces a brownish pigment called pyomelanin (Schmaler-Ripcke *et al.*, 2009). Pyomelanin is synthesized via tyrosine degradation pathway and is involved in preserving fungal survival by protecting from host immune defense mechanisms during infection (Keller *et al.*, 2011).

A. fumigatus cell wall represents the first point of contact with the host, playing an important role in pathogenesis. The cell wall is a physical structure that provides protection to the fungal cell and is one of the main targets for antifungal agents. During the life cycle of *A. fumigatus*, the cell wall composition changes continuously with cell cycle progression and in response to environmental changes. The cell wall is mainly composed by polysaccharides: (1,3) glucans, (1,3)/(1,4) glucans, (1,6) glucans, chitins, galactomannans and galactosaminogalactans (Fontaine *et al.*, 2000). The cell wall synthesis can be an example of gene function redundancy found in the *A. fumigatus* genome. For instance the cell wall (1,3) glucan polysaccharide is synthesized by three (1,3) glucan synthases. The three corresponding glucan synthase genes (*ags*1, *ags*2, and *ags*3) have been deleted from the genome. While the *ags*1 mutant displayed the most profound glucan synthetic deficit (Beauvais *et al.*, 2005), the *ags*3 deleted mutant showed enhanced virulence in a murine model of IA (Maubon *et al.*, 2006). In addition, seven chitin synthases have been identified in *A. fumigatus* genome (Mellado *et al.*, 1995). Four genes among them were assessed for virulence profile and only the *chs*G null mutant strain displayed a hypovirulence phenotype (Mellado *et al.*, 1996a,b).

The conidial cell wall also has galactose-containing polysaccharides such as galactomannan (GM) and galactosaminogalactan (GAG) (Gresnigt *et al.*, 2014). GMs are composed by a linear mannan backbone and short chains of (1,5) galactofuranose residues. Galactofuranose biosynthesis starts with the isomerization of UDP galactopyranose to UDP galactofuranose by UDP galactomutase encoded by the *glf*A gene. The absence of UDP galactomutase in *A. fumigatus* led to attenuated virulence in a mouse model of IA. GMs are released during tissue invasion which may activate the innate immune response away from the focus of infection. In addition, GMs are exoantigens and, in fact, are used in commercial kits for the diagnosis of IA (Wheat and Walsh, 2008). GAGs are expressed during conidial germination and hyphal growth and have possible anti-inflammatory effects. GAG induces the anti-inflammatory cytokine interleukin-1 receptor antagonist, turning individuals more susceptible to aspergillosis (Gresnigt *et al.*, 2014).

A. fumigatus cell wall contains at least nine glycosylphosphatidylinositol (GPI) linked proteins connected to the polysaccharide skeleton (Bruneau *et al.*, 2001). These proteins play important roles in the biosynthesis and organization of the fungal cell wall because many proteins, such as cell surface enzymes, receptors, and adhesion molecules, are anchored to the cell membrane by the GPI anchor which in turn may transfer the cell-wall-related information across the cell membrane. In this context, the absence of glucanosyltransferase encoded by *gel*2 gene has been related to hypovirulence of *A. fumigatus* (Mouyna *et al.*, 2005). Another gene related to virulence attenuation in *A. fumigatus* is Afpig-a, which encodes the catalytic subunit of a complex that is responsible for GPI anchor biosynthesis (Li *et al.*, 2007). On the other hand, *ecm*33 gene which encodes a GPI-anchored protein, plays an important role in maintaining fungal cell wall integrity, and the absence of this enzyme enhances the virulence of the fungus. Although Δ*ecm*33 conidia are more resistant to be killed by alveolar macrophages and neutrophils than control cells, Δ*ecm*33 hyphae are more susceptible to neutrophil-dependent killing (Romano *et al.*, 2006; Chabane *et al.*, 2006).

A. fumigatus secretes numerous secondary metabolites which are specially produced during mycelial growth. These secreted products are important for *A. fumigatus* pathogenicity and include mycotoxins, and enzymes such as proteases, catalases, and

phospholipases. Mycotoxins are also produced by different fungal species, can be toxic for both human and animals and are able to cause disease and death. In *A. fumigatus*, five mycotoxins have been identified: gliotoxin, fumagillin, helvolic acid, fumitremorgin A and Asp-hemolysin (Kamei and Watanabe, 2005; Dagenais and Keller, 2009). Gliotoxin is a member of the epipolythiodioxopiperazine class of toxins. It is the most abundantly produced by *A. fumigatus* and due to its relevance in virulence it is the most studied. Gliotoxin can be also detected in human samples from patients with aspergillosis (Lewis *et al.*, 2005) so a special effort has been given in order to use this molecule for diagnostic purposes. Gliotoxin has immunosuppressive properties and modulates the immune response, affects circulating neutrophils, suppresses reactive oxygen species (ROS) production and inhibits phagocytosis of conidia (Scharf *et al.*, 2012). Mutants unable to produce gliotoxin have a dual phenotype in murine models of aspergillosis. While virulence is not affected in a neutropenic model of aspergillosis, it is reduced in a corticosteroid model of infection (Spikes *et al.*, 2008; Sugui *et al.*, 2007). As other secondary metabolites, genes required for gliotoxin biosynthesis are found in clusters in the genome. The gene *lae*A encodes a transcription factor which is a global regulator of these secondary metabolite clusters in *A. fumigatus* (Perrin *et al.*, 2007). The deletion of *lae*A in this fungus reduces the secondary metabolite production, including gliotoxin, and reduces the virulence in a neutropenic murine model.

A. fumigatus secretes proteolytic enzymes such as serine, metallo and aspartic proteases (Bergmann *et al.*, 2009). Although these enzymes are necessary for fungal growth in plants, they appear to be relevant as virulence factors also for its filamentous growth in the mammalian host, as require the breakdown of host tissue for nutrient acquisition and invasion (de Vries and Visser, 2001). This happens in enzymes with elastinolytic activity that acts degrading structural barriers of host tissues. Elastin constitutes nearly 30% of lung tissue and elastinolytic activity has been described to be implicated in the pathogenesis of *Aspergillus* (Blanco *et al.*, 2002; Binder and Lass-Flörl, 2013).

A. fumigatus is able to adapt in response to low nutrient availability in the host and to uptake what it needs to grow from the site of the infection. Among nutrients, the *in vivo* environment iron is essential to begin the infection. To regulate iron availability (iron uptake and iron storage), *A. fumigatus* uses low-molecular mass iron-specific chelators called siderophores. Siderophores are central components of the fungal metabolism as they affect germination, sexual and asexual reproduction, and oxidative stress resistance. In addition, *A. fumigatus* can acquire iron by reductive iron assimilation. While the later mechanism is not essential for *A. fumigatus* virulence, inactivation of siderophore biosynthesis reduces its virulence. Iron also influences processes such as ergosterol biosynthesis, azole drug resistance, hypoxia adaptation, and the interaction with the host immune cells (Haas, 2014).

The iron availability in eukaryotes and cellular response to low oxygen are intimately related (Sales-Campos, 2013). In an infection, *A. fumigatus* is exposed to active changes in the oxygen concentration; in fact the quantity of oxygen at the site of infection is low due to the inflammatory process. The sterol regulatory element-binding protein, SrbA, is critical for coordinating genes involved in iron acquisition and ergosterol biosynthesis under hypoxia (Willger *et al.*, 2008). The *srb*A null mutant is unable of growing in a hypoxic environment and consequently is unsuccessful to cause disease in a murine model of IPA (Willger *et al.*, 2008). In addition, mitochondrial respiration is active during hypoxia. The deletion of the cytochrome C (*cycA*) which is involved in mitochondrial respiration in *A. fumigatus* led to significant impaired conidia germination, and mutant strains displayed attenuated virulence in murine model of IPA (Grahl *et al.*, 2012).

Availability of low levels of zinc can restrict the growth of pathogens. In *A. fumigatus* the transcriptional activator ZafA regulates zinc homeostasis and is essential for its pathogenicity and virulence. The *zaf*A null mutant is avirulent in cortisone acetate-immunosuppressed mice (Moreno *et al.*, 2007). Zinc is also essential for a wide variety of biochemical processes in fungi, for the adequate regulation of gene expression and thus for cellular growth and development. A clear relationship has been shown between zinc homeostasis and virulence of *A. fumigatus*, which requires the zinc transporters ZrfA, ZrfB and ZrfC for growing within the host (Amich and Calera, 2014; Moreno *et al.*, 2007).

Essential metabolic pathways that inhibit fungal metabolism during pathogenesis are also interesting. Fungal species as well as other microorganisms have developed specific pathways for the biosynthesis of all proteinogenic amino acids (Jastrzebowska and Gabriel, 2015). Among the 20 amino acids, nine are essential for humans and therefore several steps of the amino acid biosynthetic pathways are catalyzed by enzymes that are absent in mammals. In *A. fumigatus*, it is known that some biosynthetic pathways of amino acids are crucial determinants for its virulence. For instance, biosynthesis of lysine (Liebmann *et al.*, 2004a,b; Schobel *et al.*, 2010), aromatic amino acids (Schobel *et al.*, 2010), and sulfur-containing amino acids such as methionine and cysteine (Amich *et al.*, 2016), have been evaluated for their role in *A. fumigatus* pathogenesis.

Synthesis of histidine in *A. fumigatus* is encoded by seven genes of which the *his*B was deleted (Δ*his*B) (Dietl *et al.*, 2016). Deletion of this gene function in *A. fumigatus* caused histidine auxotrophy. Further analysis of histidine requirement *in vivo* was performed by testing the pathogenicity of Δ*his*B strain in different models of *A. fumigatus* infection: murine pulmonary infection, murine systemic infection, murine keratitis and the wax moth larvae *Galleria mellonella*. The Δ*his*B strain showed reduced virulence in all murine models (Dietl *et al.*, 2016).

Resistance to oxidative stress. The NADPH oxidase complex produces reactive oxygen species (ROS) in macrophages and polymorphonuclear cells such as neutrophils, and regulates several crucial pathways in host defense against *A. fumigatus*. To fight against these mechanisms, *A. fumigatus* produces specific enzymes for ROS detoxification. ROS detoxification proteins are classified in different categories, of which the catalases peroxidases, Cat1/CatB and Cat2/KatG, are produced by the fungus mycelia. The null mutant of each gene in *A. fumigatus* led to mycelial hydrogen peroxide sensitivity and virulence reduction in the lungs of immunosuppressed rats (Paris *et al.*, 2003; Calera *et al.*, 1997). Another group of genes related to oxidative stress response are the fatty acid oxygenases *ppo*A, *ppo*B, and *ppo*C, which are similar in sequence to specific mammalian prostaglandin synthases, the cyclooxygenases. The *A. fumigatus* fatty acid oxygenases encoding by these genes were tested for virulence, so the triple mutant

strain was produced and it was found to be hypervirulent in an invasive murine model, showing increased tolerance to hydrogen peroxide (Tsitsigiannis *et al.*, 2005).

Another mechanism linked to virulence is the production of biofilms. *A. fumigatus* can grow as a colony characterized by multicellular and multilayered hyphae which are embedded in an extracellular matrix (ECM) defined as biofilms (Loussert *et al.*, 2010). Biofilm growth can be observed in human lung aspergilloma and mice lungs infected with *A. fumigatus*. Composition analysis of *Aspergillus* biofilms *in vivo* shows that the ECM of both lungs contained the polysaccharides GM and GAG. Interestingly, α1,3 glucan polysaccharides and melanin were detected in the ECM of aspergilloma biofilms, while in invasive forms of aspergillosis, the α1,3 glucans were only found in the inner layer of the hyphal cell wall, and melanin could not be determined (Loussert *et al.*, 2010). The MedA protein has been described to be involved in biofilm formation as the Δ*med*A mutant is impaired in biofilm production and adherence to plastic, as well as in adherence to pulmonary epithelial cells, endothelial cells and fibronectin *in vitro*. Consistent with these results, the Δ*med*A strain has reduced virulence in both an invertebrate and a mammalian model of IA as well as reduced capacity to damage pulmonary epithelial cells and stimulate a cytokine response (Gravelat *et al.*, 2010).

In addition, *A. fumigatus* biofilms are more resistant to the antifungal drugs polyenes, azoles, and echinocandins and are reservoirs for microorganisms that may become potential sources of infection for susceptible patients (Beauvais and Latgé, 2015).

A significant relationship exists between *A. fumigatus* morphogenesis and pathogenicity. Proteins that participate in the signal transduction such as the G- proteins, MAP kinases, adenylate cyclases, and protein kinases (PKA) have been associated with virulence control and development of fungal pathogens (Lengeler *et al.*, 2000). Among them, the cAMP-PKA pathway is the best-characterized signaling cascade and is involved in regulating *A. fumigatus* morphogenesis. In this species, the pathway includes a regulatory heterotrimeric G protein (*gpa*B), an adenylate cyclase (*acy*A), and the regulatory (*pka*R) and catalytic (*pka*C1) subunits of PKA (Hohl and Feldmesser, 2007). The malfunction of proteins involved in the cAMP-PKA pathway results in altered germination as well as decreased virulence in murine models (Liebmann *et al.*, 2004a,b; Zhao *et al.*, 2006).

Other signal transduction pathways in fungi involve calcium. Calcium can enter fungal cells in response to external stress and activates the calcium-binding protein calmodulin that in turn activates calcineurin (Sales-Campos *et al.*, 2013). The calcineurin pathway mediates growth, morphology, stress response pathways including survival in the host environment and resistance to antifungal drugs (Kraus and Heitman, 2003). The deletion of the catalytic subunit of calcineurin, *cal*A/*cna*A as well as the downstream effector of calcineurin, *crz*A, in *A. fumigatus*, led to significant defects in conidial germination, hyphal morphology, decreased filamentation, impaired cell wall structure, and virulence attenuation in an experimental neutropenic inhalational murine model of IPA (Cramer *et al.*, 2008; Sales-Campos *et al.*, 2013; Steinbach *et al.*, 2007).

Together with calcineurin, Ras proteins are also involved in the regulation of hyphal and cell wall formation (Sales-Campos *et al.*, 2013). Ras proteins are small monomeric GTPases that transduce signals from outside of the cell to signal pathways inside the cell. The deletion of these Ras proteins encoding genes, Δ*ras*A, Δ*ras*B, and Δ*rhb*A leads to *A. fumigatus* decreased virulence in an IPA murine model (Sales-Campos *et al.*, 2013).

Conclusions

A. fumigatus is ubiquitous in the environment. It is found in soil, decaying vegetation compost and most indoor environments. It also interacts with diverse animal hosts and dominates among microbial communities. In addition, compare to other fungal species even from the same genus, it has an exceptional capacity to reach, develop and invade the human body. *A. fumigatus* is an opportunistic pathogen causing a wide range of infections in humans. Clinical manifestations of aspergillosis are determined by the host's immune response against the fungi and have been classically divided into superficial infections, allergic forms when there is an exaggerated response of the immune system to *Aspergillus* colonization of the airways, and invasive aspergillosis, a systemic infection that affects immunosuppressed patients.

After many years of research, a growing number of virulence factors favoring the pathological process has been continuously described. *A. fumigatus* virulence depends on multiple factors associated to the cell structure, morphogenetic reprogramming, stress response, secretion of enzymes and secondary metabolites, nutrient acquisition and synthesis, and adaptation to host conditions that includes escaping from the immune system.

For the near future, using novel approaches based on whole-genome analysis such as genome sequencing comparison between *A. fumigatus* strains or between different *Aspergillus* species, as well as whole transcriptomics of the fungus growing in host conditions, promise to fuel our understanding of the highly complex nature of *A. fumigatus* pathogenesis and clinical outcomes.

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Mucormycoses

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Introduction

Fungi of the order Mucorales represent a ubiquitous part of the environment, thriving on wet organic materials to cause rot and spoilage. Mucorales fungi are also widely used in food production through their ability to secrete a variety of enzymes (e.g., amylase, lipase and protease), used in fermentation, and production of cheese and soy products (e.g., Tempeh) (Morin-Sardin *et al.*, 2017). The ability to secrete enzymes are also used in the development of several medically and pharmaceutically important compounds such as steroids and terpenoids (Morin-Sardin *et al.*, 2017). In contrast, several species of these thermotolerant/philic Mucorales can cause mucormycosis, a life-threatening infection that mostly occurs in patients with impaired immunity (Ribes *et al.*, 2000; Spellberg *et al.*, 2012; Roden *et al.*, 2005). Humans are frequently exposed to Mucorales spores via inhalation, ingestion or direct inoculation into an abraded skin or an open wound. The genera most commonly implicated in human infections are *Rhizopus*, *Mucor*, *Rhizomucor*, *Lichtheimia*, *Cunninghamella*, *Saksenaea*, and *Apophysomyces*. This review focuses on the medically important Mucorales, elaborating on recent advances in epidemiology, pathophysiology, pathogenesis and molecular diagnosis of mucormycosis. We also present recent treatment strategies and research endeavors to counter the expanding global problem of mucormycosis.

Epidemiology and Burden of Disease

A total of 11 genera and ~27 species under the order Mucorales are known to cause mucormycosis. Amongst these, *Rhizopus*, *Lichtheimia* and *Mucor* represent the most common agents associated with human disease (Jeong *et al.*, 2019).

Rhizopus spp. is frequently associated with the rhinoorbital/cerebral form of the disease, *Cunninghamella* spp. with pulmonary or disseminated disease, while *Apophysomyces* and *Saksenaea* spp. are reportedly isolated from cutaneous mucormycosis (Jeong *et al.*, 2019). Diabetes and ketoacidosis are the primary causes of rhinoorbital/cerebral mucormycosis, whereas corticosteroid usage promotes infection by *Lichtheimia* spp. Lanternier *et al.*, (2005–2007). Of all agents of mucormycosis, *Cunninghamella* spp. causes the highest rate of mortality with ~70% lethality versus ~50% rate of all-cause mortality (Roden *et al.*, 2005; Jeong *et al.*, 2019).

Rhizopus spp. remain the most common cause of mucormycosis with ~ half of the cases seen world-wild attributed to this genus (Jeong *et al.*, 2019). The second most common isolate appears to be geographically-dependent with *Mucor*, *Lichtheimia*, and *Apophysomyces* reported as the second most common in the Americas, Europe and the Indian subcontinent, respectively (Skiada *et al.*, 2018; Nucci *et al.*, 2019; Prakash and Chakrabarti, 2019). Although the worldwide incidence of mucormycosis is difficult to estimate, as it is not a reportable disease, recent data show a striking increase in the number of reported cases of mucormycosis (Gleissner *et al.*, 2004). There has been an alarming rise in the incidence of mucormycosis at major US transplant centers. The number of cases over a 15 year period has more than doubled (Marr *et al.*, 2002; Kontoyiannis *et al.*, 2000). Prevalence rates are up to 8% in autopsied patients with leukemia (Greenberg *et al.*, 2004). A French study showed a 70% increase in mucormycosis cases between 1997 and 2006 (Bitar *et al.*, 2009). The highest incidents of mucormycosis are reported in India, with ~200,000 cases/year (Patel *et al.*, 2017; Bala *et al.*, 2015; Chakrabarti and Singh, 2014). In a more recent retrospective study in the United States using the Premier Perspective™ Comparative Database, with more than 560 participating USA hospitals covering 104 million patients, found the prevalence of mucormycosis-related hospitalizations at 0.12 per 10,000 discharges (Kontoyiannis *et al.*, 2016). Readmission rates were high in this study, with 30% and 37% of patients readmitted within one and three months of discharge, respectively, and the median length of stay was 17 days, with 23% deceased at discharge. The average cost per hospital stay was a staggering: $112,419 (Skiada *et al.*, 2018).

Leading International Fungal Education (LIFE) portal has estimated the global burden of mucormycosis to be ~10,000 cases per year barring the incidents from India. A review of 929 reports published between the years 1885 and 2004 revealed that the common risk factors for mucormycosis were diabetes mellitus (36%), followed by hematologic malignancies (17%) and solid organ or hematopoietic cell transplantations (12%). The majority of patients with malignancy suffered from pulmonary disease, whereas patients with diabetes were afflicted primarily with rhinoorbital/cerebral disease. However, more recent data from developed countries have reported an increase in mucormycosis in hematologic malignancies, especially in patients with graft versus host disease and patients receiving hematopoietic cell transplants (Marr *et al.*, 2002; Bitar *et al.*, 2009; Trifilio *et al.*, 2007; Kontoyiannis *et al.*, 2005; Kauffman and Malani, 2007). A number of factors have promoted the rise in the number of malignancy-related mucormycosis cases including; (1) use of antifungals such as voriconazole or echinocandins that are inactive against Mucorales; (2) severe defects in immune-competency due to use of immunosuppressive agents; (3) a decrease in aspergillosis-related mortality, resulting in the emergence of less common invasive mold infections during the late post-transplant period (Pagano *et al.*, 2009; Nucci and Marr, 2005; Marr *et al.*, 2009); and (4) frequent blood transfusions leading to increased serum

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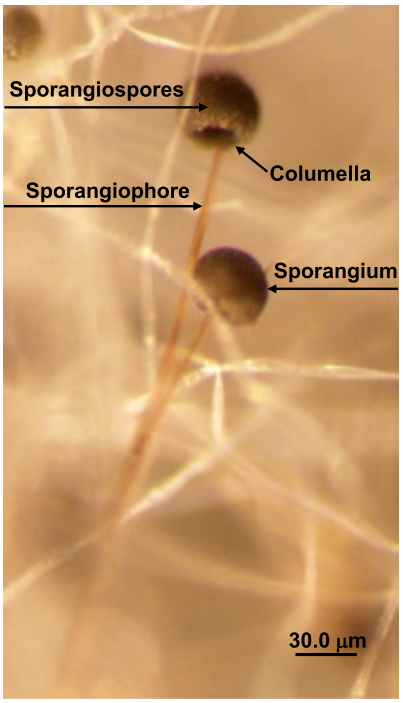

Fig. 1 *Morphology of Rhizopus delemar.* Sporangia form at the apices of sporangiophores and contain the asexual sporangiospores. Columella is a sterile dome shaped structure found at the tip of sporangiophore. Scale bars, 30 μm.

iron levels, a metal that supports the growth of Mucorales fungi (Ibrahim, 2011; Ibrahim *et al.*, 2005b; 2008b). Moreover, a change in epidemiology of mucormycosis has been noted in recent years with emergence of new risk factors and causative agents, worldwide. This is largely driven by advances in molecular diagnostics, and geoclimatic factors influencing causative agents (McCarthy *et al.*, 2017). Post-tuberculosis, chronic renal failure and stay in the intensive care unit are new risk factors for the disease especially in developing countries. *Rhizopus homothallicus*, *Thamnostylum lucknowense*, and *Mucor irregularis* are the newly emerging species (Prakash and Chakrabarti, 2019). This shifting epidemiology warrants an increased need for global reporting and ongoing population-based studies categorized by risk (e.g., diabetes, malignancies) to broaden our understanding of the global burden of the disease.

Infection with Mucorales have also occurred as an aftermath of natural disasters such as tsunamis, tornados or volcanic eruptions (Green and Karras, 2012; Beaumont *et al.*, 1985; Patiño *et al.*, 1991; Neblett Fanfair *et al.*, 2012). For example, following a tornado in Joplin, Missouri, United States in 2011, 13 injured (yet otherwise immunocompetent) people developed necrotizing soft tissue infections by *Apophysomyces trapeziformis* (Green and Karras, 2012). Similar findings were reported following the South-Eastern Asian Tsunami (Andresen *et al.*, 2005). Presumably, the infection occurred after the fungus was introduced into breached skin due to wounds. A more prominent example of trauma-related infections are those reported in military personnel with combat injuries. Between June 2009 and through December 2010, a total of 37 confirmed cases were identified in soldiers who sustained blast injuries during combat in Afghanistan. These patients required frequent debridements or amputation revisions and resulted in a mortality rate of 8% (Warkentien *et al.*, 2012; Tribble and Rodriguez, 2014; Rodriguez *et al.*, 2014a,b; Weintrob *et al.*, 2015; Tribble *et al.*, 2015).

Pathogenesis and Host Immune Response

Mucoralean fungi reproduce both sexually and asexually. The asexual sporangiospores are present on the apex of a globular structure called the sporangium (**Fig. 1**). The sporangiospores can disperse post the disintegration of the sporangium wall, and germinate into hyphae. Most of the pathogenic Mucorales are heterothallic, and their sexual cycle initiates when hyphae of opposite mating types [(−) and (+)] undergo fusion to form zygospores, which later germinate to form a sporangium at the apex culminating in sexual meiospores (Lee *et al.*, 2010). Since the sexual life cycle for sporangiospore production is prolonged, it is anecdotally accepted that asexual sporangiospores may serve as the major source of dissemination and infection.

Rhinoorbial/cerebral and pulmonary mucormycosis, the two major disease forms, are acquired by inhalation of spores from the environment. In immunocompetent individuals, spores are transported to the pharynx and cleared through the gastrointestinal tract. To the contrary, in susceptible individuals, infection begins in the nasal cavity or lungs. Cutaneous mucormycosis represents the third most common manifestation of the disease and usually acquired either through severe trauma or direct inoculation into

abraded skin (Sugar, 2005; Ibrahim *et al.*, 2011). Agents of mucormycosis cause enhanced invasion and destruction of tissues, and are clinically considered to be the most angioinvasive of all of the fungal infections. Mucorales are the cause of the third most common invasive fungal infections among hematologic and allogenic stem cell transplantation patients, trailing only candidiasis and aspergillosis (Petrikkos *et al.*, 2012). In the case of rhinoorbital/cerebral involvement, the disease is manifested as orbital cellulitis, bone rarefaction, erosion of the skull base, cavernous sinus thrombosis, infracts and intra-cranial abscess in the brain, culminating into enhanced facial necrosis and deformation. In pulmonary mucormycosis, the disease is characterized as lung infiltration (58%–96%), pleural effusion (6%–21%), thickly walled cavities (6%–37%), and lymphadenopathy (3.3%) (Sugar, 2005).

Nasal and lung epithelial cells are among the first host cells that encounter inhaled spores. To gain insight into the molecular mechanisms that govern Mucorales-host airway epithelial cell interactions, Watkins *et al.* (2018) employed transcriptome sequencing (RNA-seq) to monitor host transcriptional response during early stages of *R. delemar* infection in a murine model of pulmonary mucormycosis. Network analysis revealed activation of the host's epidermal growth factor receptor (EGFR) signaling. Further studies revealed that EGFR is activated by phosphorylation upon interaction with several genera of Mucorales, and colocalizes with *R. delemar* spores during invasion of alveolar epithelial cells. EGFR inhibitors cetuximab and gefitinib protected airway epithelial cells from invasion and damage by *R. delemar in vitro*. Furthermore, gefitinib significantly prolonged survival of mice with pulmonary mucormycosis, and reduced tissue fungal burden of target organs (Watkins *et al.*, 2018). These results present EGFR as a novel host receptor during invasion of alveolar epithelial cells by *R. delemar*. Importantly, this study shows that the use of the FDA9-approved drug, gefitinib, could prove to be beneficial in treating pulmonary mucormycosis as an adjunctive therapy.

Alveolar macrophages provide the first line of defense against inhaled Mucorales spores (Aberdein *et al.*, 2013; Ibrahim and Voelz, 2017). Studies have shown that macrophages from immunocompetent mice are efficient in taking up Mucorales spores and prevent their germination (Diamond *et al.*, 1982; Waldorf, 1989; Waldorf *et al.*, 1984a,b). However, spore uptake by these macrophages does not translate into cidality, since normal macrophages are inefficient at killing spores. To make matters worse, under diabetic conditions spore germination is enhanced in macrophages, leading to higher mortality in the diabetic mice (Waldorf *et al.*, 1984b). This outcome is in sharp contrast to *Aspergillus* conidia, which can be efficiently cleared by the immune system (Diamond *et al.*, 1982; Waldorf, 1989; Waldorf *et al.*, 1984a,b; Lee *et al.*, 2015). However, macrophages and monocytes can bind to, and damage Mucorales hyphae by release of oxygen radicals without ingesting the hyphae. Unraveling the less understood unique mechanism by which Mucorales persist in alveolar macrophages and resist killing is fundamental to identifying novel approaches to control the infection. In this respect, Andrianaki *et al.* (2018) found that lack of intracellular spore swelling inside alveolar macrophages results in surface retention of melanin. The retention of melanin induces phagosome maturation arrest through inhibition of LC3-associated phagocytosis resulting in persistence of spores within macrophages. On the other hand, studies using transcriptomic, iron-supplementation, and genetic manipulation of iron assimilation genes showed that iron restriction inside macrophages regulates immunity against *Rhizopus* and prevents germination of the fungus (Andrianaki *et al.*, 2018).

The resistance of Mucorales to innate immune cells also extends to neutrophils with hyperglycemia having a detrimental impact on chemotaxis and killing activity of these polymorphonuclear cells (Chinn and Diamond, 1982). Specifically, acidosis disrupts the ability of transferrin to efficiently chelate iron by a proton-mediated mechanism (Ibrahim *et al.*, 2008b; Artis *et al.*, 1982; Gebremariam *et al.*, 2016; Walsh *et al.*, 2012). The released iron is known to be toxic to phagocytes (Cantinieaux *et al.*, 1999; Guo *et al.*, 2002; Omara *et al.*, 1994) and spleen cells from mice fed excess levels of iron secrete less interferon-gamma (IFN-γ) (Omara and Blakley, 1994), a cytokine that upregulates killing of Mucorales fungi by human neutrophils (Gil-Lamaignere *et al.*, 2005). Implementation of IFN-γ and granulocyte-macrophage-colony-stimulating factor (GM-CSF) alone or in combination has been found to augment the ability of neutrophils to damage and kill Mucorales hyphae *ex vivo*, through augmentation of the oxidative burst and TNF-α release (Gil-Lamaignere *et al.*, 2005).

The role of adaptive immunity in mucormycosis patients has not been investigated extensively. Of note, similar to *Aspergillus*, β-glucan exposure during germination of *Rhizopus* triggers dectin-1 signaling in human dendritic cells and results in robust induction of the IL-23/Th-17 responses (Chamilos *et al.*, 2010). Interestingly, only patients with mucormycosis produce Mucorales-specific T-cells, which are adept at damaging hyphal cells (Potenza *et al.*, 2011; Schmidt *et al.*, 2012). In fact, T-cells pulsed with *Rhizopus* extract and activated with IL-2/IL-7 trigger the production of Mucorales-specific T cells that contain $CD4^+$ (Castillo *et al.*, 2018). These cells are capable of producing IFN-γ, IL-5, IL-10, IL-13, and TNF-α and recognize fungal antigens presented by HLA-II molecules rather than through non-specific signaling (Castillo *et al.*, 2018).

Molecular Pathogenesis of Mucorales

A major impediment to the in-depth study of genes and signaling pathways of Mucorales fungi is the availability of only a limited set of tools for genetic manipulation. A whole-genome duplication occurred early in the Mucormycotina lineage and the duplication of genes may have provided new proteins thereby expanding the sensory and signaling pathways (Garcia *et al.*, 2018; Ma *et al.*, 2009; Schwartze *et al.*, 2014). Mucorales are known to be haploid and present zygotic meiosis when sexually reproducing (Morin-Sardin *et al.*, 2017). The only documented Mucorales to be amenable to genetic manipulation are *Mucor circinelloides*, and *Rhizopus delemar*. Even in these two fungi, the genetics are challenging due to lack of dominant selection markers, low efficiency of

transformation, and rarity of chromosomal integration. Thus, instead of commonly manipulating genes by disruption, the most utilized tool for gene manipulation relies on silencing gene expression by RNA interference (RNAi) (Calo *et al.*, 2014; Trieu *et al.*, 2017; Ibrahim *et al.*, 2010; Liu *et al.*, 2015). Gene silencing has enabled identification of genes with roles in virulence and drug resistance. Gene disruption by homologous recombination has also been successfully implemented mainly in *M. circinelloides*, and provided valuable information on the role of the calcineurin pathway in mucormycosis pathogenesis (Lee *et al.*, 2015, 2013). The latest advancement in development of molecular tools is the CRISPR/Cas9 system to preform gene editing of genomic DNA. The first report on using this technology in Mucorales was for disrupting a toxin-encoding gene in *R. delemar* using a single plasmid with *pyrF* as a marker and the biolistic delivery system for transformation (Baldin *et al.*, 2017). Gene disruption was confirmed by Southern blot analysis, abrogation of the toxin expression and significant reduction of *R. delemar* to damage host cells (Baldin *et al.*, 2017). The CRISPR/Cas9 was more recently used to produce *R. delemar pyrF* mutants with single nucleotide deletion at the fourth nucleotide before the protospacer adjacent motif (PAM) sequence, which is consistent with CRISPR-Cas9 induced gene mutation through non-homologous end joining (NHEJ) (Bruni *et al.*, 2019). The CRISPR-Cas9 system has also been successfully used in a plasmid-free method to disrupt two genes in *M. circinelloides*, the *carB* encoding phytoene dehydrogenase and the *hmgR2* encoding 3-hydroxy-3-methylglutaryl-CoA reductase (Nagy *et al.*, 2017). These limited set of tools have been invaluable in understanding the genetic response during host-pathogen interactions, and revealing the fundamental role of several regulatory genes.

Role of Morphogenesis

Mucorales fungi are capable of alternating between yeast, spores and mycelia, in response to environmental conditions (Orlowski, 1991). In *Mucor* spp., anaerobiosis and the presence of a fermentable hexose induce yeast growth, whereas oxygen and nutrient limitation conditions lead to hyphal growth (Wolff *et al.*, 2002). Molecular manipulations including gene deletion constructs have identified genes controlling morphogenesis, and this information has led to promising candidates for drug development. For example, by using homologous recombination of upstream and downstream sequences flanking an auxotrophic marker such as *pyrG*, the calcineurin pathway was determined to regulate yeast-mycelium transition and virulence in *M. circinelloides* (Lee *et al.*, 2015). Disruption of the CnbR gene (or the addition of calcineurin inhibitors) locked the fungus in yeast form making them significantly less virulent in mice. Besides calcineurin, the cyclic AMP (cAMP) and its target protein kinase A (PKA) reportedly play a role in control of morphogenesis. Interestingly, calcineurin negatively regulates PKA, suggesting a relationship between these two regulatory pathways (Lee *et al.*, 2013). Other proteins involved in the regulation of *M. circinelloides* dimorphism, such as heterotrimeric G proteins and the ADP-ribosylation factors (Arfs), were also identified by related gene deletions (Patiño-Medina *et al.*, 2018).

The size of spores is also known to contribute to virulence. In a heterologous wax moth larva mucormycosis model, large multinucleate spores that germinate more rapidly, exhibit higher virulence compared to small mononucleate spores (Li *et al.*, 2011). The larger spores can also germinate inside and overwhelm macrophages, while small spores can be digested more rapidly by the innate immune cells. This phenomenon is not unusual, since another human-pathogenic fungus, *Cryptococcus neoformans*, is also known to similarly produce mononucleate but polyploid giant/titan cells, which are less susceptible to the host immune system (Zaragoza *et al.*, 2010; Okagaki *et al.*, 2010).

Role of Iron uptake

Mucorales possess sophisticated mechanisms to acquire iron from the host, a metal vital for fungal survival (Howard, 1999). In the host, iron is chelated by proteins such as ferritin, lactoferrin and transferrin (Howard, 1999). In patients with hyperglycemia, diabetic ketoacidosis, or other forms of acidosis, elevated blood sugar and low blood pH levels destabilize the ability of host iron chelators, resulting in elevation of the concentration of serum free iron (Artis *et al.*, 1982; Gebremariam *et al.*, 2016; Ibrahim, 2014). Elevated serum free iron increases the possibility of infection with mucormycosis. Mucorales have two different strategies for iron acquisition: a high-affinity iron uptake system and the production of siderophores (Lax *et al.*, 2020; Carroll *et al.*, 2017; Navarro-Mendoza *et al.*, 2018). The high-affinity iron uptake mechanism is composed of a triad of proteins strictly regulated by iron levels: a family of iron reductases (Fre), a ferroxidase (Fet3), and a high affinity iron permease (Ftr1) (Navarro-Mendoza *et al.*, 2018). Low availability of iron triggers the expression of the high-affinity iron uptake mechanism in *R. delemar* (Ibrahim *et al.*, 2010; Liu *et al.*, 2015; Fu *et al.*, 2004), *M. circinelloides* (Navarro-Mendoza *et al.*, 2018), and *L. corymbifera* (Schwartze *et al.*, 2014). Functions of genes associated with iron uptake in Mucorales have been investigated using RNAi gene silencing, especially in the case of *R. delemar* (previously identified as *R. oryzae*) which is less amenable to mutagenesis than *M. circinelloides* (Ibrahim *et al.*, 2010). Reduction of the relative copy number of the *FTR1* gene or the inhibition of its expression by RNAi compromises the ability of *R. delemar* to acquire iron *in vitro* and reduces its virulence in diabetic ketoacidotic mice (Ibrahim *et al.*, 2010). Dialysis patients, who are treated with deferoxamine to alleviate iron-overload toxicity, are uniquely predisposed to a lethal form of mucormycosis (Boelaert *et al.*, 1987, 1989, 1991, 1994). *R. delemar* utilizes iron from ferrioxamine (the iron-rich form of deferoxamine) to boost its growth. Biochemical and genetic analyses (by RNAi-mediated gene silencing) identified two surface proteins/receptors (Fob1 and Fob2) in *R. delemar* that bind ferrioxamine and facilitate iron uptake via a reductase/Ftr1-mediated mechanism (Liu *et al.*, 2015). In *M. circinelloides*, three ferroxidases genes have been characterized, *fet3a, fet3b, and fet3c* (Navarro-Mendoza *et al.*, 2018). All three genes are overexpressed in the lungs of mice intravenously infected with *M. circinelloides* and are regulated by the

availability of iron in the culture media, and the fungal dimorphic state with *fet3a* specifically expressed in yeast growth, while *fet3b* and *fet3c* being expressed in hyphae during aerobic growth (Navarro-Mendoza *et al.*, 2018). Furthermore, gene deletion studies showed a predominant role for *fet3c in vivo* virulence of *M. circinelloides* (Navarro-Mendoza *et al.*, 2018).

In addition to utilizing the bacterial deferoxamine as a xenosiderophore, *Rhizopus* produces rhizoferrin to chelate iron (Carroll *et al.*, 2017; Thieken and Winkelmann, 1992; de Locht *et al.*, 1994). A rhizoferrin synthetase (*rfs*) was identified in *R. delemar* based on homology to the bacterial NRPS-independent siderophore (NIS) protein, SfnaD, and was found to contain a C-terminus conserved ferric iron reductase FhuF-like transporter domain. Furthermore, the *rfs* expression in *R. delemar* was repressed by iron and *E. coli* cells expressing *rfs* were able to synthesis siderophore from citrate and diaminobutane. Finally, site directed mutagenesis of selected conserved residues revealed that H484 is essential for the *rfs* activity (Carroll *et al.*, 2017). However, the role of the *rfs* gene in mucormycosis pathogenesis is yet to be determined.

CotH Proteins and Angioinvasion

To cause angioinvasion, Mucorales actively interact with the endothelium lining blood vessels. CotH family members are kinase-like proteins (Nguyen *et al.*, 2016) that are universally present in Mucorales and absent from any other forms of life (Gebremariam *et al.*, 2014; Chibucos *et al.*, 2016). These are cell-surface proteins that have been reported to act as invasins in invading host tissues via interacting with the endothelium. Copy number of CotH genes correlates with the pathogenic potential of the agents of mucormycosis, with the pathogenic species most isolated from infections (*Rhizopus*, *Mucor*, and *Lichtheimia*) having five to seven copies of the gene (Chibucos *et al.*, 2016). In *R. delemar* which contains six CotH encoding genes, CotH3 protein, and to a lesser extent CotH2 protein, bind to glucose-regulated protein 78 kDa (GRP78) receptor on human umbilical vein endothelial cells (Gebremariam *et al.*, 2014; Liu *et al.*, 2010). GRP78 is a heat-shock protein of the HSP70 family, that localizes to the cell surface of mammalian cells during stress conditions (Ibrahim *et al.*, 2019; Lee, 2007). Moreover, diabetic host factors such as hyperglycemia, elevated available serum iron, and the presence of ketone bodies (e.g., β-hydroxy butyrate), are known to enhance the expression of GRP78 and CotH3 *in vitro* and in the target organs of diabetic ketoacidotic mice (sinus, lungs and brains), resulting in augmentation of fungal invasion of endothelial cells and increased disease severity in mice (Gebremariam *et al.*, 2016; Liu *et al.*, 2010). This increased expression level was further demonstrated by immunohistochemistry of the ethmoidal sinus tissue of a patient with rhinocerebral mucormycosis, revealing GRP78 intense expression on infiltrated endothelial cells and macrophages in necrotic tissues (Shumilov *et al.*, 2018). Consistent with these results, blocking the function of CotH3 proteins either biochemically by using anti-CotH antibodies or genetically by attenuating CotH3 expression, reduces the ability of *R. delemar* to invade and injure endothelial cells *in vitro* and reduces disease severity in mice (Gebremariam *et al.*, 2014). Therefore, the unique enhancement of interaction between CotH3 and GRP78 under hyperglycemic/ketoacedotic conditions explain the specific susceptibility of diabetic ketoacidotic patients to mucormycosis. Of critical importance is the isolation of anti-CotH3 monoclonal antibodies that are partially protective when given alone against murine mucormycosis induced by several Mucorales. A single dose of the most protective monoclonal antibody shows complete protection of diabetic ketoacidotic mice from *R. delemar* lethal infection when used in combination with clinically used antifungal agents (Gebremariam *et al.*, 2019). **Fig. 2**, summarizes the pathogenesis and host immune response during mucormycosis.

The fact that rhinoorbial/cerebral and pulmonary mucormycosis are acquired by inhalation of spores from the environment, yet they specifically afflict diabetic ketoacidosis and immunocompromised patients, respectively, is intriguing. In a recent study, the unique susceptibility of diabetic ketoacidotic subjects to rhinoorbital/cerebral form of the disease was likely found to be due to specific interactions between the nasal epithelial cell GRP78 and fungal CotH3, the expression of which increase in the presence of host factors present in diabetic ketoacidosis (Alqarihi *et al.*, 2020). This results in trapping inhaled spores within the nasal cavity, resulting in damage of adjoining tissues. In contrast, interaction of Mucorales with alveolar epithelial cells was initiated by a different fungal cell surface protein of the CotH family, CotH7, with integrin β1 receptor of the alveolar epithelial cells. Integrin β1 in turn activates EGFR to induce fungal invasion of alveolar epithelial cells (Alqarihi *et al.*, 2020). These results highlight that, Mucorales ligands recognize host receptors unique to individual cell types (i.e., alveolar, nasal, endothelial cells), and that this fungal ligand-host receptor interaction is enhanced by host factors, eventually leading to infections in the respective host niches.

Diagnosis

Delaying amphotericin B-based therapy beyond 5 days of onset of symptoms doubles 12-week mortality in patients with hematological malignancies (Chamilos *et al.*, 2008). Therefore, early diagnosis of mucormycosis is key for timely implementation of appropriate therapy (Spellberg *et al.*, 2012). The conventional definitive diagnosis of mucormycosis relies on culturing the organism from usually sterile body sites and/or tissue histopathology since currently there is no serology test for diagnosis of mucormycosis (Sugar, 2005; Spellberg *et al.*, 2005a; Cornely *et al.*, 2019). Mucorales can be isolated on media such as Sabouraud-dextrose agar incubated at 25–37°C and fungal elements can be stained with Gomori methenamine-silver, hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) or calcofluor white stain to visualize tissue invasion (Lass-Flörl, 2009). However, these techniques are not sensitive enough, and often lead to a misdiagnosis. For example, cultures can lead to false positives owing to the ubiquitous nature of Mucorales fungi. In contrast, lack of growth sometimes can be a sign of

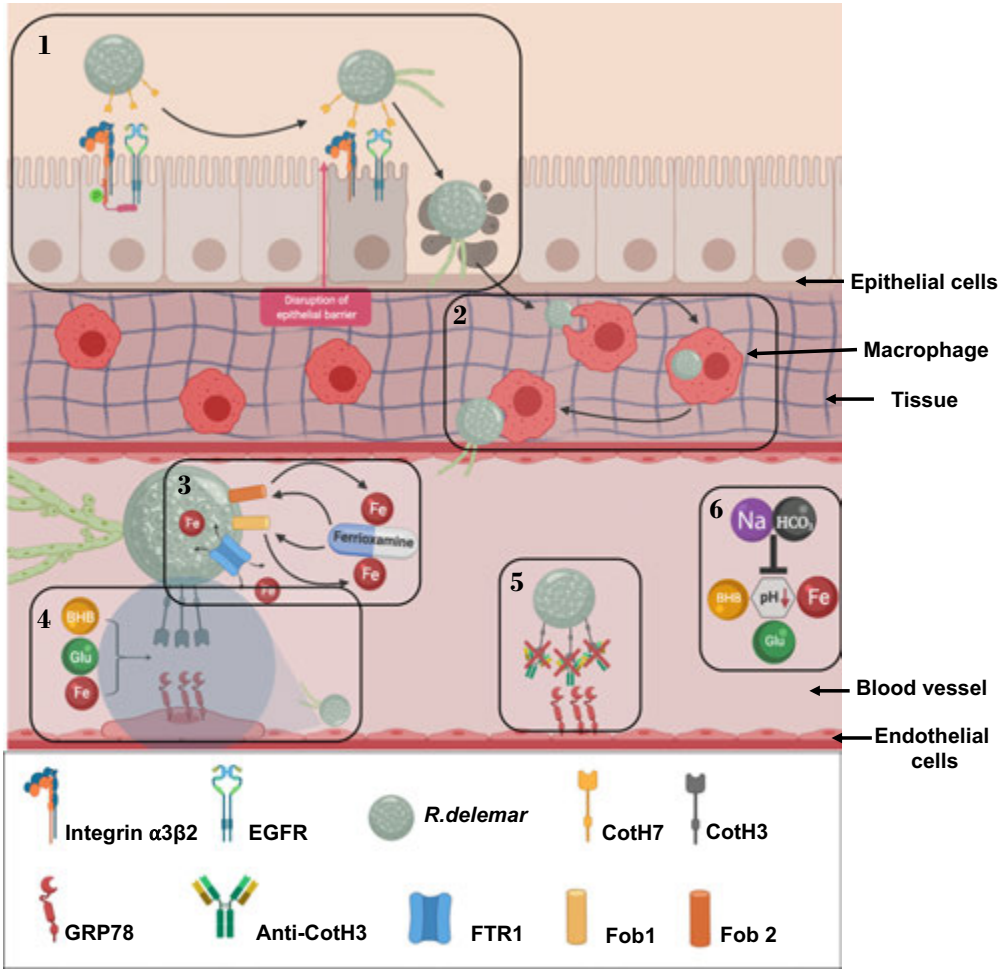

Fig. 2 *Diagram illustrating the interactions of R. delemar 99–880 with epithelial and endothelial cells during invasion and hematogenous dissemination.* (1) *R. delemar* CotH7 ligand interacts with the alveolar cell receptor, integrin $\alpha 3\beta 1$ which activates EGFR leading to invasion and damage of the alveolar epithelium. (2) *R. delemar* is resistant to killing by alveolar macrophages. (3) In deferoxamine-treated patients, ferrioxamine binds to fungal Fob1 and Fob2 leading to internalization of host iron via the high affinity reductase/permease system (Ftr1). (4) Elevated glucose (Glu), iron (Fe), β-hydroxy butyrate (BHB) in the blood of diabetic ketoacidotic patients enhance the expressions of GRP78 and CotH3 leading to invasion and damage of the endothelium and augmentation of fungal growth. (5) Using anti-CotH3 antibodies can block the fungal ligand from interacting with host GRP78 leading to less invasion and damage to the endothelial cells. (6) Sodium bicarbonate ($NaHCO_3$) reverses the effect of acidosis (i.e., elevated BHB and free iron), thereby halting the growth of the fungus and preventing further invasion and hematogenous dissemination.

laboratory mishandling of the specimen (e.g., homogenization can destroy hyphal elements and kill the fungus) resulting in false negatives (Spellberg and Ibrahim, 2015). Definitive diagnosis relying on histopathology can also lead to errors despite the distinctive nature of the board ribbon-like aseptate Mucorales hyphae and often occurs at a late stage of the infection, thereby resulting in little influence on the outcome of therapy (Dadwal and Kontoyiannis, 2018). Computed tomography (CT) is useful for early detection of pulmonary mucormycosis, particularly in patients with cancer. The presence of a reversed halo sign in patients with a hematologic malignancy or neutropenia carries a high predictive value of mucormycosis and along with the absence of airway-invasive features, may help differentiate the condition from invasive pulmonary aspergillosis (Spellberg *et al.*, 2009b; Jung *et al.*, 2015; Legouge *et al.*, 2014). However, radiographic signs may be suggestive of disease, but are rarely diagnostic (Spellberg *et al.*, 2005a).

Recent advances in molecular diagnostics, including the development of polymerase chain reaction (PCR)-based technologies, as well as Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), have the potential to hasten both the diagnosis of mucormycosis and the initiation of appropriate treatment.

Molecular diagnostics

Real-time PCR-based techniques have emerged as a promising new modality to aid in diagnosis of mucormycosis, especially in pulmonary diseases caused by *Lichtheimia*, *Mucor*, *Rhizopus*, and *Rhizomucor* spp. (McCarthy *et al.*, 2017; McCarthy and Walsh,

2016). Amplification of CotH3, a protein specific for Mucorales, has proven to be highly specific and sensitive for diagnosis of mucormycosis (Baldin *et al.*, 2018). CotH3 was shown to be successfully amplified from urine, plasma, and bronchoalveolar lavage retrieved from mice infected with Mucorales fungi but not from mice infected with *Aspergillus fumigatus* (Baldin *et al.*, 2018).

A multiplex real-time PCR (MRT-PCR) platform to detect DNA from the most frequent agents of mucormycosis has also been developed (Nagao *et al.*, 2005; Walsh *et al.*, 2012). Primers and molecular beacon probes have been designed on the basis of the nucleotide sequence of the ITS1 ribosomal DNA region from strains belonging to *R. oryzae*, *R. microsporus* as well as the sequence of the ITS2 region for *Mucor* spp. belonging to *M. circinelloides*, *M. racemosus*, *M. plumbeus* and *M. velutinosus* (Bernal-Martínez *et al.*, 2013).

A semi-nested PCR-based assay amplifying the 18S region of rDNA specific to Mucorales was shown to be more reliable in detecting infection in tissue and serum samples from patients with rhinoorbital/cerebral mucormycosis than using ITS2 PCR (Zaman *et al.*, 2017). Other quantitative PCR methods have evaluated a combination of hydrolysis probes targeting *Mucor*/*Rhizopus*, *Lichtheimia*, and *Rhizomucor* for circulating Mucorales detection in patients with confirmed mucormycosis (Millon *et al.*, 2013). DNA from Mucorales was detected in 9 of 10 serum samples from patients with proven disease, suggesting quantitative PCR could be a useful screening tool in high-risk patients (Caillot *et al.*, 2016).

Other promising strategies such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) have been used to identify Mucorales (Yaman *et al.*, 2012). However, MALDI-TOF MS has not been validated for diagnosis of mucormycosis in clinical samples. Besides, the need for prior culture of fungi before identification, and lack of robust libraries for rare Mucorales limits this diagnostic tool.

Treatment of Mucormycosis

Major steps in treatment of mucormycosis involve surgical debridement of involved tissues, antifungal therapy and implementation of approaches that reverse risk factors like neutropenia, hyperglycemia, or ketoacidosis (Chitasombat and Kontoyiannis, 2016; Kontoyiannis and Lewis, 2011).

Surgery

Surgical debridement has to be conducted as early as possible and should be extensive, involving all necrotic areas often leading to disfigurement, especially in the case of rhinoorbital/cerebral infection (Vironneau *et al.*, 2014). Many uncontrolled studies have documented that local control of the disease in patients with rhinoorbital/cerebral mucormycosis, with wide and repeated surgical debridement is associated with improved outcomes (Gil-Lamaignere *et al.*, 2005; Chamilos *et al.*, 2010; Potenza *et al.*, 2011). Local control of infection was obtained in 90% of the patients after radical surgery versus 41.6% in patients who had limited surgery (Vironneau *et al.*, 2014).

Currently Approved Antifungal Drugs

An early intervention with antifungal drugs is key for improved outcome of infection with mucormycosis. Lipid formulations of amphotericin B (e.g., liposomal amphotericin B [L-AMB]) has been the treatment of choice, since it can be administered in higher doses without the accompanying nephrotoxicity associated with conventional amphotericin B formulations (Cornely *et al.*, 2019; Vehreschild *et al.*, 2013; Fera *et al.*, 2009). Alternative treatment regimen encompass using broad-spectrum oral triazoles such as posaconazole and isavuconazole as a "step-down" treatment after the primary administration of L-AMB (Kontoyiannis and Lewis, 2011; Allen *et al.*, 2015; Marty *et al.*, 2016). Because mucormycosis is a rare disease, there are no randomized trials assessing the efficacy of the antifungal drugs. However, a multicenter open-label single-arm study (VITAL study) including 37 patients with mucormycosis showed that treatment with isavuconazole alone had outcomes comparable to that of L-AMB, or L-AMB followed by posaconazole (Marty *et al.*, 2016). These data are mirrored by comparative efficacy of isavuconazole to L-AMB in treating murine mucormycosis (Luo *et al.*, 2014; Gebremariam *et al.*, 2017b). While any combination studies between isavuconazole and lipid formulation amphotericin are lacking, combination treatment of therapeutic doses of L-AMB and posaconazole have no benefit over L-AMB treatment in experimental models of mucormycosis (Ibrahim *et al.*, 2009).

Mucorales species demonstrate *in vitro* resistance to echinocandins despite harboring the echinocandin-target enzyme FKS required for 1,3-β-glucan synthesis (Ibrahim *et al.*, 2005a). Therefore, echinocandins are not used as stand-alone therapy for mucormycosis (Bassetti and Bouza, 2017). However, animal model investigations have shown evidence of synergism between echinocandins and lipid formulation amphotericin B (Spellberg *et al.*, 2005b; Ibrahim *et al.*, 2008a). Furthermore, a retrospective study of 41 diabetic patients with rhinoorbital/cerebral mucormycosis treated with a combination of lipid formulation amphotericin B and caspofungin acetate had a higher survival outcome than those treated with the polyene alone (Reed *et al.*, 2008). It is assumed that the ability of echinocandins to degrade a small amount of β-glucan on the cell wall of Mucorales, unmasks immune epitopes, thereby facilitating immune recognition and phagocytosis of invading hyphae (Lamaris *et al.*, 2008).

A recent retrospective cohort study of combination L-AmB with posaconazole, L-AmB with echinocandins, and posaconazole with echinocandins showed no differences in 6-weeks mortality between monotherapy and combination treatment in mucormycosis patients with hematologic malignancies and hematopoietic cell transplant recipients (Kyvernitakis *et al.*, 2016). These data

are consistent with the murine data, which showed very marginal benefit for L-AMB + micafungin therapy over L-AMB alone (Ibrahim *et al.*, 2008a). Taken all together, the human retrospective and the murine experimental data suggest that a combination of lipid formulation amphotericin and echinocandins are likely to benefit mucormycosis patients who suffer from diabetic ketoacidosis rather than those with hematologic malignancies and hematopoietic cell transplant recipients. Therefore, any future clinical trials to evaluate the efficacy of polyenes + echinocandins should be conducted in diabetic ketoacidotic patients. While the approach to antifungal therapy remains somewhat controversial (Kyvernitakis *et al.*, 2016), other aspects of care, including reversal of immunosuppression, correction of metabolic deficits, and when appropriate, surgical debridement, remain hallmarks of effective treatment (Cornely *et al.*, 2019; Kyvernitakis *et al.*, 2016).

Investigational Antifungal Drugs

Recently, there has been development of newer investigational drugs in the pipeline, which demonstrate promising activity *in vitro* and in experimental models of mucormycosis. For example, the 1-tetrazole fungal-specific 14 α-lanosterol demethylase (CYP51) inhibitor, VT-1161 showed equal efficacy to high dose L-AMB in delayed therapy model of immunosuppressed mice infected with *R. arrhizus* var. *arrhizus* (Gebremariam *et al.*, 2015). The same drug and posaconazole comparably improved survival and lowered tissue fungal burden of immunosuppressed mice infected with *R. arrhizus* var. *arrhizus* when used in prophylactic therapy. Further, in the continuous therapy, VT-1161 outperformed posaconazole in prolonging mouse survival time (Gebremariam *et al.*, 2017a). Another antifungal drug with promising outcomes against *Rhizopus* species is manogepix (formerly APX001A and E1210). Manogepix has a novel mechanism of action targeting Gwt1 protein which is involved in the conserved glycosylphosphatidylinositol (GPI) post-translational modification pathway (Umemura *et al.*, 2003). In two invasive pulmonary mice models infected with either *R. arrihzus* var. *arrhizus or R. arrihzus* var. *delemar*, treatment with fosmanogepix (the prodrug of manogepix) significantly increased and prolonged median survival time of mice post infection, when compared to placebo. In addition, administration of fosmanogepix resulted in a 1–2 log reduction in both lung and kidney fungal burdens. In fact, tissue clearance and survival were comparable to clinically relevant doses of isavuconazole, which is FDA- and EMA-approved for the treatment of patients with mucormycosis (Gebremariam *et al.*, 2020).

Adjunctive Therapies

As mentioned above, reversal of immunosuppression and inherent risk factors is paramount for control of mucormycosis, along with surgery and appropriate early antifungal agents. Given the relatively poor prognosis associated with the disease, adjunct treatment options have emerged, including iron chelation, hyperbaric oxygen, and immunomodulation (Ibrahim *et al.*, 2008b; Yohai *et al.*, 1994; Walsh *et al.*, 2014).

In patients with diabetic ketoacidosis, reversal of acidemia by administration of sodium bicarbonate partially blocked the ability of *R. delemar* to invade endothelial cells, restored the ability of the host to chelate iron, and improved neutrophil function (**Fig. 2**) (Gebremariam *et al.*, 2016). Additionally, treatment of ketoacidotic mice with sodium bicarbonate specifically protected them from invasive pulmonary infection, suggesting that patients with diabetic ketoacidosis suspected of having mucromycosis might benefit from reversing acidemia by insulin and sodium bicarbonate administration (Gebremariam *et al.*, 2016).

The use of iron chelators that cannot be utilized by Mucorales as siderophores, such as deferasirox and deferiprone, have been shown to reduce available iron, inhibit the fungal growth, and protect diabetic ketoacidosis mice from mucormycosis (Ibrahim *et al.*, 2006; Ibrahim *et al.*, 2007). While case reports suggest a benefit in using iron chelation therapy as an adjunctive treatment in diabetic patients (Spellberg *et al.*, 2009a), a small (20 patient) multi-centered, placebo-controlled, double-blinded study (DEFEAT Mucor) conducted mainly in patients with hematologic malignancies showed an adverse effect of adding deferasirox to L-AMB treatment (Spellberg *et al.*, 2012). Although population imbalances in this small Phase II study make generalizable conclusions difficult, the data do not support a role for initial adjunctive deferasirox therapy for mucormycosis in hematologic malignancies patients.

Another therapy that is likely to be beneficial when combined with surgery and antifungal therapy, particularly in diabetic patients, is the use of hyperbaric oxygen (HBO). HBO administration increases oxygen pressure and improves neutrophil function. In a review of 28 cases, adjunctive HBO was beneficial in diabetic patients (94% survival), but not in patients with hematologic malignancies or bone marrow transplants (33% survival; $p < 0.02$) (Price and Stevens, 1980; John *et al.*, 2005; Barratt *et al.*, 2001; Kajs-Wyllie, 1995; Ferguson *et al.*, 1988). Prolonged courses of HBO were associated with a higher survival (John *et al.*, 2005).

Strategies that augment the immune system, such as administration of granulocyte (macrophage) colony stimulating factor or interferon-γ have been proposed as adjunct therapy, based on limited *in vitro* data and case-reports (Gil-Lamaignere *et al.*, 2005; Abzug and Walsh, 2004). A recent report showed that combination of Interferon-γ with nivolumab (a monoclonal antibody that decreases programmed death-1 (PD-1) expression on T-cells), has proven to be successful in an immunosuppressed patient with intractable mucormycosis (Grimaldi *et al.*, 2017).

Conclusions and Future Perspectives

Mucormycosis caused by Mucorales fungi is a life-threatening infection afflicting mainly immunocompromised patients and less commonly immunocompetent individuals. Global surveillance for mucormycosis is lacking because it is not a reportable disease.

Hence, a public-health based surveillance is essential to improve understanding of their epidemiology and to prioritize research efforts. Such information will not only stratify susceptible patients based on their underlying risk factors, but also open avenues to designing better therapeutic options.

Management of mucormycosis hinges upon correction of underlying conditions such as minimization of the duration of neutropenia (e.g., G-CSF and GM-CSF), prompt correction of hyperglycemia and acidosis, prevention and treatment of thrombocytopenia, and avoiding or tapering down treatment with immunosuppressants. Surgical removal of infected foci whenever possible is also a cornerstone in the successful treatment of mucormycosis. Lipid formulation of amphotericin B remains the first line therapy against mucormycosis, with isavuconazole and posaconazole being alternative options to patients who are refractory or intolerant to polyenes. As a representative of a new class of antifungals targeting GPI-anchored proteins, the investigational drug fosmanogepix, carries heightened promise for adding to the current antifungal agents used to treat mucromycosis. However, management of mucormycosis remains a challenge especially with the lack of rapid, reliable and affordable diagnostic tools.

A thorough understanding of Mucorales virulence factors and their role in interaction with host receptors and the innate immune system is of paramount importance. Identification of these targets carries the promise of designing and implementing novel strategies against the disease. Recent molecular pathogenesis investigations have identified several strategies including the CotH3 invasion protein, the calcineurin-dependent hyphal formation pathway, the implementation of sodium bicarbonate to reverse acidosis in patients suspected of mucormycosis, and the repurposing of FDA-approved anti EGFR signaling inhibitors. Although mucormycosis cases have been steadily increasing in numbers over the last three decades, the disease remains to be rare in many parts of the world and this will complicate future clinical trials to evaluate new modalities of treatment. In the absence of comparative clinical trials, pragmatic studies (such as single-arm open label trials [e.g., VITAL study (Marty *et al.*, 2016)]) aided by comprehensive registries should help overcome publication bias and report both positive and negative outcomes of infections treated with these schemes.

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Epidemiology of Dimorphic Fungi

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Glossary

Arthroconidia Fungal asexual reproductive cell originated from segmentation of hyphae.
Conidia Fungal asexual cell.
Endemic mycosis Mycosis that occur in a specific geographic region.
Etiological agent Causative agent of an infectious disease.
Hematogenous dissemination Dissemination of a pathogen through blood vessels.
Hyphae Fungal elongated multicellular filaments.
Mycelium An aggregate of hyphae cells.
Saprophytic organisms Organisms that inhabit decaying organic matter.
Spores Fungal sexual cell.
Teleomorph Fungal sexual reproductive stage.
Thermal-dimorphic fungi Fungal species that alternate morphology into yeasts and hyphae cells during their lifecycle.
Yeasts Fungal unicellular organisms that reproduce by fission or budding.

Introduction

Dimorphic fungi are organisms that have the ability to switch between two morphologies during their lifecycle: yeast and hyphae (Gauthier, 2015; Klein and Tebbets, 2007). Dimorphism can be induced by several stimuli, such as oxygen concentration, nutrient sources and enzymatic activity (Gauthier, 2015). Thermal-dimorphic fungi are a group of ascomycetes in which morphologic changes are induced by temperature. This group includes species of the genera *Blastomyces*, *Coccidioides*, *Histoplasma*, *Paracoccidioides*, *Sporothrix*, *Talaromyces* and the brand new *Emergomyces* (Boyce and Andrianopoulos, 2015; Gauthier, 2015; Schwartz *et al.*, 2019). These fungi have a very special characteristic: the ability to parasite the mammal host, causing deep mycoses that are usually restricted to specific geographical areas (**Fig. 1**), also called endemic mycosis (Bonifaz *et al.*, 2011; Rappleye and Goldman, 2006). They are often habitants of soil, in temperatures varying between 22 and 25°C, where they grow was a mold. When fungal propagules such as conidia are inoculated in the mammalian host (~37°C), they undergo a very complex process and convert into pathogenic yeasts or spherules (Klein and Tebbets, 2007; Bonifaz *et al.*, 2011; Rappleye and Goldman, 2006). Morphological transition is a key factor for pathogenicity and its failure almost always attenuates fungal virulence (Garber, 2001; Klein and Tebbets, 2007). Thermal-dimorphic fungi are important human pathogens with significant impact on public health, causing substantial morbidity and mortality (Knox and Hage, 2010; López-Martínez and Méndéz-Tovar, 2012; Marques, 2012; Nguyen *et al.*, 2013; Mahajan, 2014; Garber, 2001; Sil and Andrianopoulos, 2015).

Coccidioidomycosis, histoplasmosis, paracoccidioidomycosis, blastomycosis, sporotrichosis, penicilliosis and the recently described emergomycosis are the most common endemic systemic mycoses. Each of these diseases differ in epidemiology, symptoms and prognosis (Bonifaz *et al.*, 2011). Infections are often started in the lungs after inhalation of dormant conidia or spores produced during the differentiation of hyphal cells for asexual or sexual reproduction, from where hematogenous and lymphatic dissemination can occur to the skin and other organs, especially in immunocompromised hosts (Bonifaz *et al.*, 2011; Klein and Tebbets, 2007). Once inside the host, fungal propagules are recognized by host innate immune cells, such as macrophages and neutrophils, inducing a variety of cellular responses (Boyce and Andrianopoulos, 2015; Romani, 2011; Bonifaz *et al.*, 2011). Some of these fungi developed sophisticated strategies to modulate the host immune responses in their favor (Boyce and Andrianopoulos, 2015; Rappleye and Goldman, 2006).

Thermal-dimorphic fungi typically invade immunocompetent hosts. In the United States, it is estimated that *Histoplasma capsulatum* and *Coccidioides spp.* infect 500,000 and 150,000 persons annually, respectively (Gauthier, 2015), and together with *B. dermatitidis*, they are considered to be the most common cause of lung fungal infections in immunocompetent individuals (Sil and Andrianopoulos, 2015). Paracoccidioidomycosis and Emergomycosis are the most prevalent systemic mycosis in Brazil (Coutinho *et al.*, 2015) and South Africa (Schwartz *et al.*, 2019), respectively. However, after the Acquired Immunodeficiency Syndrome (AIDS) pandemic and the developments in modern medicine, such as the immunosuppressive therapy for malignant and autoimmune diseases, and organ transplantation, the incidence of the endemic mycosis has been progressively increasing (Garber, 2001; Mahajan, 2014; Friedman and Schwartz, 2019). In solid organ transplant recipients, thermally dimorphic fungi account for 5% of fungal infections (Gauthier, 2015; Pappas *et al.*, 2010). Life-threatening mycoses occur in both immunocompetent and immunocompromised hosts (Garber, 2001), encouraging the development of new therapies and strategies to overcome these important fungal diseases. In the following sections we will discuss the major characteristics of the main thermal-dimorphic fungal pathogens.

Fig. 1 Aproximate geographic distribution of the major thermal-dimorphic fungal pathogens. *Coccidioides immitis* (●), *C. posadasii* (●), *Histoplasma capsulatum var. capsulatum* (●), *H. capsulatum var. duboisii* (●), *Sporothrix albicans* (●), *S. brasiliensis* (●), *S. mexicana* (●), *S. globosa* (●), *S. schenckii* (●), *Paracoccidioides brasiliensis* (●), *P. americana* (●), *P. restrepiensis* (●), *P. venezuelensis* (●), *P. lutzii* (●), *Blastomyces dermatitidis* (●), *B. percursus* (●), *B. helicus* (●), *Talaromyces marneffei* (●), *Emergomyces pasteurianus* (●), *Es. africanus* (●), *Es. canadensis* (●), *Es. orientalis* (●) and *Es. europaeus* (●). Map by maptive©.

Coccidioides immitis and *C. posadasii*

The first report of *Coccidioides* infection was made in 1892 by Alejandro Posadas and Robert Wernicke, who identified coccidia-like organisms in unusual skin lesion of a soldier in Argentina (Posadas, 1892; Wernicke, 1892; Sil and Andrianopoulos, 2015; Fisher *et al.*, 2002). In 1894, Emmet Rixford described the first case in North America (Rixford, 1894; Sil and Andrianopoulos, 2015) and in 1896 the "protozoal" organism was formally named *Coccidioides immitis* (Rixford and Gilchrist, 1896). Only in 1900, William Ophuls and H.C. Moffit accurately classified it as an ascomycete fungus belonging to the order Onygenales (Sil and Andrianopoulos, 2015; Ophuls and Moffitt, 1900). Over a century later, using molecular tools and phylogenetic analysis, it was proposed that *C. immitis* actually consisted of two non-interbreeding taxa, resulting in its division into two different species: *C. immitis* and *C. posadasii* (Fisher *et al.*, 2002).

Coccidioides spp. grow as saprophytic organisms in soil of arid and semiarid regions, with very low precipitation and extreme temperature variations (0–45°C) (Bonifaz *et al.*, 2011; Fisher *et al.*, 2007; Kollath *et al.*, 2019). In the environment, *Coccidioides* species have a filamentous morphology. During fungal lifecycle, some hyphae cells differentiate and originate arthroconidia (Garber, 2001; Nguyen *et al.*, 2013). Conidia can spread and grow in the environment originating new hyphae. However, *C. immitis* and *C. posadasii* also have the ability to parasite mammal hosts such as horses, dogs and humans (Bonifaz *et al.*, 2011; Sil and Andrianopoulos, 2015; Nguyen *et al.*, 2013). Sexual structures have never been observed in either species, although molecular data suggest the existence of a teleomorphic phase (Nguyen *et al.*, 2013; Mandel *et al.*, 2007).

C. immitis and *C. posadasii* are the etiological agents of Coccidioidomycosis, commonly known as the Valley Fever. The infection usually starts via the respiratory route (98%), when environmental arthroconidia are inhaled, although rare cases of skin inoculation have been reported (Kollath *et al.*, 2019; Bonifaz *et al.*, 2011). Inside the host, these structures undergo a transition into spherules, a unique structure to the genus *Coccidioides*. Spherules are a large specialized cellular structure with a double membrane that contains up to 300 endospores, which can be released and spread to other tissues after the spherule rupture (Nguyen *et al.*, 2013). Coccidioidomycosis, can have a variety of outcomes. Infection can be asymptomatic (60% of cases) or lead to fungal pneumonia 1–3 weeks after exposure to arthroconidia in endemic areas (Valdivia *et al.*, 2006; Stockamp and Thompson, 2016; Smith and Beard, 1946). It is estimated that spontaneously cure occurs in approximately 95% of cases. Depending on host immune status and inoculum size, infection can develop diffuse pneumonia, a more severe form of the disease, with high mortality rates associated due to acute respiratory distress syndrome (ARDS). Dissemination through hematogenic and lymphatic routes often occurs into bones, skin, lymph nodes and joints (Stockamp and Thompson, 2016; Nguyen *et al.*, 2013). However, the most life-threating outcome is central nervous system dissemination, causing coccidioidal meningitis, which is generally fatal when left untreated (Stockamp and Thompson, 2016).

Coccidioides spp. distribution has been mostly obtained by epidemiological data from clinical cases and population skin testing for spherulin or coccidioidin (coccidial antigen preparations from spherules and mycelium, respectively), besides soil isolation (Brown *et al.*, 2013b; Stockamp and Thompson, 2016; Edwards and Palmer, 1957). *C. immitis* and *C. posadasii* species have distinct geographic distribution (**Fig. 1**). *C. immitis* is endemic in central and southern California (especially in the San Joaquin Valley) and Northern Mexico (Bonifaz *et al.*, 2011; Brown *et al.*, 2013b; DiCaudo, 2006). *C. posadasii* has a broader distribution, comprising Arizona, Texas, New Mexico, Nevada and Utah, besides some areas in Guatemala, Honduras, Venezuela, Brazil, Argentina and Paraguay (Sil and Andrianopoulos, 2015; Bonifaz *et al.*, 2011; Brown *et al.*, 2013b; Kollath *et al.*, 2019). Several cases outside endemic areas were reported, the majority of them between travellers or people who temporarily relocated to endemic areas. In the United States, from 2011 to 2017, 95,371 cases of coccidioidomycosis were reported to CDC, where most cases were in Arizona (64.4%) and California (32.5%) (Benedict *et al.*, 2019), and from 1990 to 2008, mortality rate was around 0.59 per million person-years (Huang *et al.*, 2012).

Coccidioides spp. infections have been increasing in recent years and became an important public heath problem in endemic areas. Disease affects all age groups, but the highest incidence rates are between adults above 40 years old (Bonifaz *et al.*, 2011), usually male workers, such as construction and farm workers and people who are frequently exposed to aerosolized arthroconidia through soil manipulation. In 2012, it was described an outbreak among armadillo hunters in Brazil northeastern caused by *C. posadasii* (Brillhante *et al.*, 2012). Epidemics were also reported after windstorms and earthquakes in the USA (Stockamp and Thompson, 2016; Brown *et al.*, 2013b; Schneider *et al.*, 1997; Flynn *et al.*, 1979). African or Filipino descendants are more susceptible to extrapulmonary dissemination (Louie, 1999), suggesting that a host genetic component influences disease outcome. Pregnant women in the third trimester or immediately post-partum are specially vulnerable to severe or disseminated disease. Patients with immunocompromising conditions such as diabetics, AIDS and transplant recipients are also part of the risk-group (Galgiani *et al.*, 2005; Stockamp and Thompson, 2016).

Histoplasma capsulatum

Histoplasma capsulatum was first described by Samuel Darling in 1906 as a protozoan parasite that appeared to have capsules and infect macrophages (Darling, 1906; Sil and Andrianopoulos, 2015; Woods, 2002). In 1948, Chester Emmons isolated it for the first time from soil (Emmons, 1949; Sil and Andrianopoulos, 2015). Nowadays it is clear that *H. capsulatum* have no capsule and is actually an ascomycete fungus that belongs to the order Onygenales (Bonifaz *et al.*, 2011). Two varieties inside the species, *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*, are etiological agents for histoplasmosis in humans, leading to different disease outcomes. Pulmonary and systemic histoplasmosis (classical disease) are associated with *H. capsulatum* var. *capsulatum*, which is found in Americas, Southeast Asia and Africa (**Fig. 1**). *H. capsulatum* var. *duboisii* is found in Western and Central Africa (**Fig. 1**) and causes skin and bone lesions (Retallack and Woods, 1999; Cockshott and Lucas, 1964). Phylogenetic analyses point out to seven distinct phylogenetic species inside *Histoplasma* isolates from six continents (Sil and Andrianopoulos, 2015; Kasuga *et al.*, 2003). Sexual reproduction happens during teleomorphic phase, when they are designated *Ajellomyces capsulatus* (Bonifaz *et al.*, 2011; Muniz *et al.*, 2014).

In the environment, *H. capsulatum* (**Fig. 2**) is a saprobic mold that inhabits rich soil or decaying plant materials associated with large amount of droppings of bat or birds, such as chicken, turkeys or geese (Sil and Andrianopoulos, 2015; Woods, 2002; Bonifaz *et al.*, 2011). High humidity and temperatures between 20 and 30°C are favourable growth conditions (Bonifaz *et al.*, 2011). In São Paulo City (Brazil), isolation of *Histoplasma capsulatum* was reported in up to 34.8% of captured bats (Dias *et al.*, 2011; da Paz *et al.*, 2018; Dos Santos *et al.*, 2018), demonstrating that, due to urban expansion, wild species can reallocate to the city and carry the fungus. This can lead to a relevant impact in public health in big urban areas such as Great São Paulo area, with over 20,000,000 people. There were outbreaks involving cave visitors and people whose house ceilings were contaminated by bats or bird feces (Cury *et al.*, 2001; Vicentini-Moreira *et al.*, 2008; Jülg *et al.*, 2008).

During asexual reproduction, macro and microconidia are produced, which basically differs in size (Sil and Andrianopoulos, 2015; Pine, 1960). When fungal propagules, such as conidia and/or hyphal fragments, are inhaled by the host, they are internalized by phagocytic cells (Bullock, 1993; Sil and Andrianopoulos, 2015), and undergo a transition from mold to budding yeast cells, that is

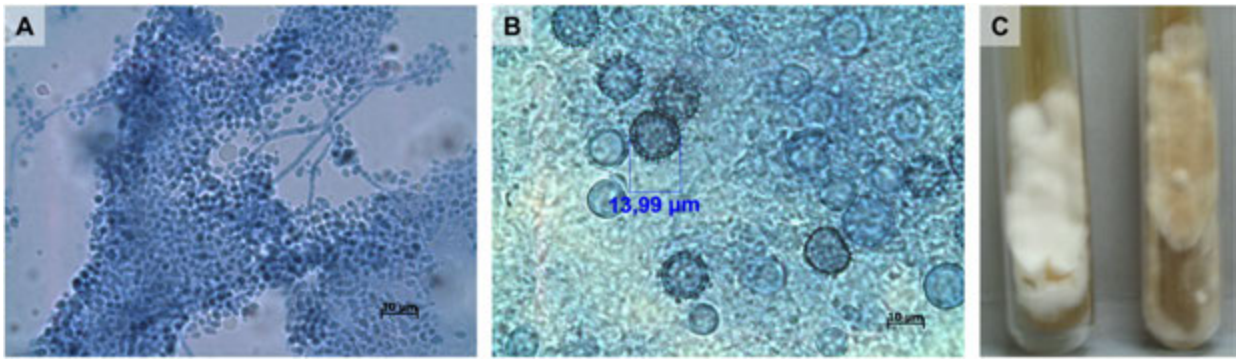

Fig. 2 Micrograph of yeast (A) and hyphae (B) phases of *Histoplasma capsulatum* (40 ×). Macroscopic colonies of *Histoplasma capsulatum* in Sabouraud Agar. Courtesy of Dr. Maria Adelaide Galvão Dias, Centro de Zoonoses do Município de São Paulo, Brazil.

essential for virulence. Yeast cells can evade phagocytic killing, multiply inside alveolar macrophages and use the host cell to spread to other organs such as spleen, liver, lymph nodes and bone marrow through hematogenous dissemination (Rappleye and Goldman, 2006; Woods, 2016). Infection through skin is rare, and usually results from injury during activities such as guano production and cleaning of chicken coops (Bonifaz *et al.*, 2011).

There is a large variety of clinical forms that are influenced by extent of exposure and host immunological status. Skin testing with histoplasmin shows that in more than 99% of cases, primary infections in immunocompetent individuals are mild or asymptomatic (Kauffman, 2007; Bradsher, 1996). Acute disease can occur after high inoculum exposure, especially during activities associated with soil disruption, such as farming and construction, often affecting miners, cave explorers, beekeepers and archaeologists (Bonifaz *et al.*, 2011). Deficiency in cell-mediated immunity increases the risk of disseminated and fatal histoplasmosis (Woods, 2002). Infants, AIDS patients, transplant recipients and patients with other immunological disorders are at particular risk for disseminated histoplasmosis (Sil and Andrianopoulos, 2015; Kauffman, 2007). Chronic cavitary histoplasmosis usually happens concomitantly with pulmonary disorder (Kauffman, 2007; Colombo *et al.*, 2011). Chronic progressive disseminated histoplasmosis is more frequent in elderly even when immunosuppression is not confirmed (Kauffman, 2007; Colombo *et al.*, 2011). In disseminated disease, it is common involvement of the reticuloendothelial system, skin, gastrointestinal tract, gut and adrenal gland (Kauffman, 2007; Colombo *et al.*, 2011). Occasionally, the meninges are affected (Bonifaz *et al.*, 2011). Skin involvement is more frequent in HIV-positive patients. The clinical forms of classical Histoplasmosis listed above are caused by *Histoplasma capsulatum* var. *capsulatum*. *Histoplasma capsulatum* var. *duboisii* causes the African histoplasmosis, which primarily affects skin, lymph nodes and bones, and is limited to equatorial Africa (Bonifaz *et al.*, 2011). In addition to these two varieties, *Histoplasma capsulatum* var. *farciminosum* is a pathogen of horses (Kasuga *et al.*, 2003).

Histoplasmosis is the most common fungal respiratory infection in the world (Bonifaz *et al.*, 2011). Despite its worldwide distribution, *H. capsulatum* var. *capsulatum* is very prevalent in tropical climate zones such as Central and South America, in the eastern USA and also in southern Mexico (Bonifaz *et al.*, 2011). It is highly endemic in the Ohio and Mississippi River Valleys, with over 90% infected individuals. It is estimated that histoplasmosis causes approximately 25,000 life-threatening infections per year in the Midwestern United States (Woods, 2002; Sil and Andrianopoulos, 2015; Brown *et al.*, 2012). Mortality rates due to disseminated disease are high. In a retrospective study, mortality rate was 31% in immunocompromised patients and 17% in immunocompetent patients (Colombo *et al.*, 2011). All age groups can be affected, and although the rates of positive results on skin testing for sensitivity to antigens of *H. capsulatum* are similar in males and females, histoplasmosis has higher incidence in men around 30–40 years old (Bonifaz *et al.*, 2011).

Sporothrix schenckii Species Complex

Sporothrix spp. infection was first described in 1898 by Benjamin Schenck, who identified the fungus in subcutaneous abscesses (Téllez *et al.*, 2014; Sil and Andrianopoulos, 2015; Schenck, 1898). After many years, based in molecular and phenotypic data, it was demonstrated that *S. schenckii* was not a single species, but a complex comprising at least six cryptic species of clinical and epidemiological interest, with significant differences in geographical distribution, biochemical properties, virulence degree, disease patterns and therapy response (Mahajan, 2014; López-Romero *et al.*, 2011). *Sporothrix schenckii* species complex include *S. albicans* and *S. brasiliensis* (in Brazil), *S. mexicana* (in Mexico), *S. globosa* (in United Kingdom, Spain, Italy, China, Japan, USA, and India), and *S. schenckii* sensu stricto (Mahajan, 2014; Marimon *et al.*, 2006; Marimon *et al.*, 2007). *S. schenckii* sensu stricto, *S. brasiliensis*, and *S. globosa* are mainly associated with human infections (Mahajan, 2014; Marimon *et al.*, 2007). There is evidence that *Ophiostoma schenckii* may represent teleomorphic phase. Besides humans, Sporotrichosis can parasite other mammals such as cats, dogs, rats, armadillos and horses (Téllez *et al.*, 2014). These species belong to the order *Ophistomatales*.

S. schenckii species complex (**Fig. 3**) are ascomycetes with worldwide distribution (**Fig. 1**) and associated with warm and humid climate (Téllez *et al.*, 2014). The fungus is found in soil, plant detritus, decaying wood, hay and sphagnum moss, where fungus usually grows as a saprophytic mycelium (Sil and Andrianopoulos, 2015; Mahajan, 2014; Téllez *et al.*, 2014).

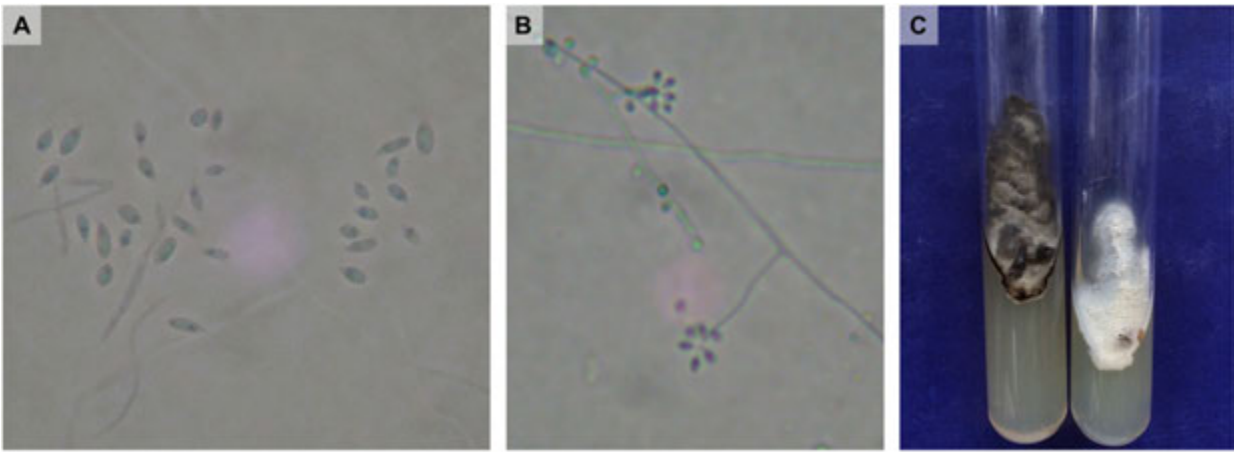

Fig. 3 Micrograph of yeast (A) and hyphal (B) morphology of *Sporothrix* spp. (120 ×). Macroscopic colonies (C) of mycelium (left) and yeasts (right) in Potato Agar.

Mycelium is constituted by septate and thin hyphae, and during asexual reproduction, two categories of conidia are produced: the sympodial conidia and a thick-walled, dark brown individual conidia that show different shapes among *Sporothrix* species (Sil and Andrianopoulos, 2015; López-Romero *et al.*, 2011; Marimon *et al.*, 2007). Yeast cells can also be seen in soil, differently from the other dimorphic fungi discussed herein, indicating that temperature is not a requisite for the morphology transition in these species (Sil and Andrianopoulos, 2015; Brown *et al.*, 2012; López-Romero *et al.*, 2011). Yeast cells, which generally emerge from hyphal cells, divide by budding, and multibudded cells are frequently seen due to slow separation from the mother cell (López-Romero *et al.*, 2011). Both conidia and yeasts can produce melanin, what is variable among different strains of the fungus and is associated to higher virulence (López-Romero *et al.*, 2011). In murine models, *S. brasiliensis* was the most virulent species, while *S. schenckii* and *S. globosa* presented intermediate virulence, and *S. mexicana* and *S. albicans* showed low virulence (Arrillaga-Moncrieff *et al.*, 2009).

Unlike the other dimorphic fungi reviewed herein, infection rarely occurs by inhalation of fungal propagules. Instead, primary route of infection is through superficial injury produced by fungus-contaminated objects of organic material, insect bites (rarely) and cat secretions or scratches (Mahajan, 2014; López-Romero *et al.*, 2011). The average incubation period is 3 weeks (Mahajan, 2014). Inoculum size and location, together with host immune status and strain virulence are important factors in the disease outcome. Professionals handling plant material are at particularly high risk, what explains the reason why men shows much higher frequency of infection (Mahajan, 2014).

Sporotrichosis is characterized by a wide range of clinical illnesses. Primary disease is characterized by cutaneous and subcutaneous infection. In this case, skin and lymphatic vessels surrounding inoculation site are affected, where small papulo-nodules and ulcerative lesions can occur (sporotrichotic chancre) without causing systemic symptoms (Mahajan, 2014). In immunocompromised individuals, particularly AIDS patients, hematogenous spread may lead to extracutaneous infection with visceral and osteoarticular involvement, affecting many parts of the body including the bones, joints, and particularly the central nervous system (López-Romero *et al.*, 2011; Mahajan, 2014; Sil and Andrianopoulos, 2015; Téllez *et al.*, 2014). Another clinical manifestation is pulmonary sporotrichosis, caused by inhalation of conidia (Mahajan, 2014; Téllez *et al.*, 2014).

Although its worldwide distribution, Sporotrichosis incidence is higher in tropical and subtropical climates of America, some Asian countries and Australia, with only a few cases in Europe (López-Romero *et al.*, 2011; Chakrabarti *et al.*, 2014). Sporotrichosis is the most common subcutaneous mycosis in Japan, China, Australia, Central and South America (Mexico, Colombia, and specially Brazil and Peru), and India (along the Sub-Himalayan region) (Mahajan, 2014; Téllez *et al.*, 2014; Sil and Andrianopoulos, 2015; Chakrabarti *et al.*, 2014). Meanwhile, in Europe sporotrichosis is less frequent (Téllez *et al.*, 2014). Epidemic outbreaks were reported in South Africa (with 3000 gold miners), the United States and Western Europe (Téllez *et al.*, 2014; Barros *et al.*, 2011). In Brazil, between 1998 and 2011, sporotrichosis was found in more than 4000 humans and 3.704 cats in Rio de Janeiro, and the most prevalent etiological agent and primary pathogen of feline sporotrichosis was *S. brasiliensis* (Gremião *et al.*, 2015; Pereira *et al.*, 2014; Rodrigues *et al.*, 2013; Silva *et al.*, 2012; Barros *et al.*, 2011).

Paracoccidioides spp.

Paracoccidioidomycosis (PCM) was first described by Adolpho Lutz in 1908 (Lutz, 1945) and for many years its etiological agent was believed to be a single species, *Paracoccidioides brasiliensis*. Almost a century later, phylogenetic studies demonstrated high genetic variability between the isolates, and the taxonomical recognition of five distinct species of the genus *Paracoccidioides* was recently proposed (Turissini *et al.*, 2017). The group is classified in the order Onygenales, where *P. brasiliensis* include isolates from Argentina, Brazil, Peru, Venezuela and Antarctica; *P. americana* (former PS2) includes isolates from Brazil and Venezuela;

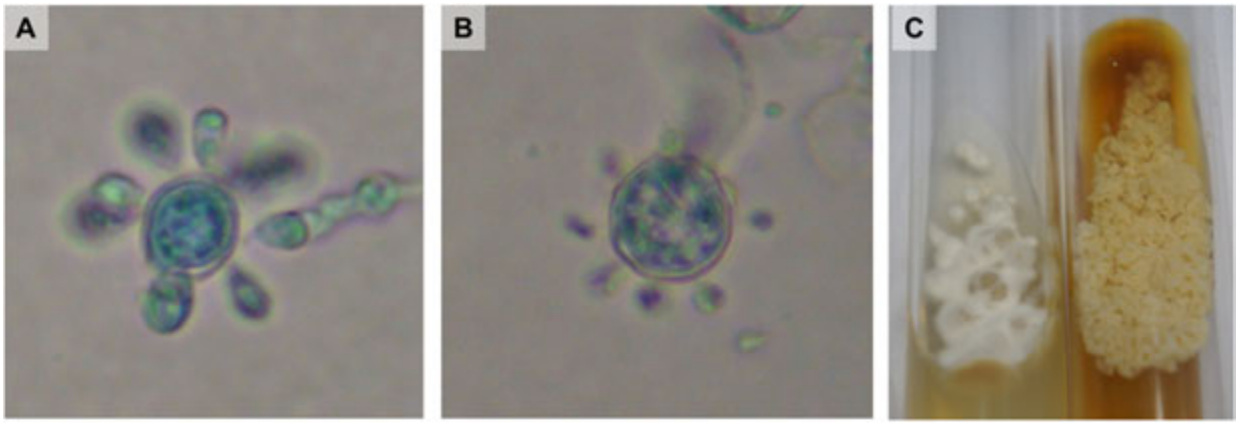

Fig. 4 Micrograph of *P.brasiliensis* (A) and *P. lutzii* (B) yeasts (120x). Macroscopic colonies (C) of mycelium (left) and yeasts (right) in BHI Agar.

P. restrepiensis (former PS3) includes isolates from Colombia; *P. venezuelensis* (former PS4) includes isolates from Venezuela; and *P. lutzii* (Pb01-like group) is endemic in the North and Central-West regions of Brazil and Ecuador (Teixeira *et al.*, 2013; Theodoro *et al.*, 2012; Turissini *et al.*, 2017) (**Fig. 1**). A teleomorphic stage has not been identified yet, although there is evidence for sexual reproduction among some isolates (Sil and Andrianopoulos, 2015; Bocca *et al.*, 2013).

Natural reservoir of *Paracoccidioides* species is not totally determined. However, *Paracoccidioides* spp. have been isolated in soils from Venezuela (de Albornoz, 1971), Argentina (Negroni, 1968) and Brazil (Vergara and Martinez, 1998; Naiff *et al.*, 1986). Besides that, isolates in other mammals such as armadillos, dogs, chickens, pigs, cattle, horses, sheep, goats, bats, rabbits and monkeys have been reported (Nucci *et al.*, 2009; Bagagli *et al.*, 1998; Shikanai-Yasuda *et al.*, 2006; da Paz *et al.*, 2018).It is believed that the fungus is a saprophytic mold that grows in humid soil and decaying organic material, with abundance of vegetation and watercourses, specially from coffee, tobacco and sugar cane plantations (Bonifaz *et al.*, 2011; Bocca *et al.*, 2013; Calle *et al.*, 2001; Barrozo *et al.*, 2009). High altitude (1000–1499 meters) and humidity (rainfall from 2000 to 2999 mm) could lead to a great release of conidia and arthroconidia in the air, with a large risk of exposure, explaining a cluster of acute and subacute cases in a southern region of Brazil after 1982/1983 El Niño Southern Oscillation (Barrozo *et al.*, 2009).

Once inside the host, mycelium cells convert to yeast morphology (**Fig. 4**). Yeast cells divide by budding with slow separation from mother cells, sometimes appearing as multibudded cells (Sil and Andrianopoulos, 2015; Goldani and Sugar, 1995). *Paracoccidioides* yeasts are not predominantly intracellular pathogens; multibudded yeasts are too large to be readily internalized. However, they can infect epithelial and alveolar cells, and this ability to parasite cells may be important for virulence (Mendes-Giannini *et al.*, 2008; Sil and Andrianopoulos, 2015). *Paracoccidioides* infection starts after inhalation of conidia or arthroconidia or less frequently by skin and mucous membranes injury (Sil and Andrianopoulos, 2015; Martinez, 2015; Bocca *et al.*, 2013). Yeast cells can remain latent for years, a condition known as paracoccidioidomycosis-infection. Primary disease is often characterized by asymptomatic lesions of the lung. Disease manifests as two main clinical forms, which depends on host immune status, and its progression is likely to be associated with some kind of immunodeficiency (Sil and Andrianopoulos, 2015; Martinez, 2015). Children and young adults often develop acute and subacute clinical forms of PCM, with disseminated lesions, and it is likely to appear a few weeks or months after infection. Chronic form manifests months to years after *Paracoccidioides* exposure and is more common among adult men, involving lesions in the oral mucosa, the airways and specially lungs. Extrapulmonary paracoccidioidomycosis affects oropharyngeal mucosa, lymph nodes, skin and various organs (Bonifaz *et al.*, 2011; Shikanai-Yasuda *et al.*, 2006; Bocca *et al.*, 2013). Skin test studies show that men and women are equally infected, but disease is highly prevalent in adult men, probably due to a greater exposure to the fungus during professional activities such as farming (Shikanai-Yasuda *et al.*, 2006; Bocca *et al.*, 2013). In addition to that, unlike the other endemic mycosis discussed here, endogenous estrogens have an important influence during infection, inhibiting the conversion of conidia into yeasts and also modulating the cell immune response so that women have more resistance against disease (Martinez, 2015; Bocca *et al.*, 2013; Restrepo *et al.*, 1984).

Paracoccidioidomycosis is highly endemic in South America (Brazil, Colombia, Venezuela, Paraguay), and some regions of Central America and Mexico (Bonifaz *et al.*, 2011; Sil and Andrianopoulos, 2015). Infection occurs exclusively in Latin American countries (Martinez, 2015), and it is believed that cases observed in the United States of America, Canada, Spain and other European countries, the Middle East, Japan and Africa were actually imported paracoccidioidomycosis (Forjaz *et al.*, 1999; Bocca *et al.*, 2013). Majority of the cases are reported in Brazil, followed by Venezuela, Colombia and Argentina. *P. lutzii* endemic area involves especially the Midwest and North regions of Brazil (**Fig. 1**). Between 1930 and 2012, over 15,000 cases of Paracoccidioidomycosis have been reported, but this number only partially reflects the prevalence of the disease in Latin America. Epidemiological surveys using paracoccidioidin skin testing demonstrated positive rates ranging from 2% to 82% in Brazil, up to 77% in rural population of Colombia and 10.2%–19.7% in Venezuela and 1,6%–10,7% in Argentina (Magalhães *et al.*, 2014).

Paracoccidioidomycosis is the most common systemic mycosis in Latin America (Sil and Andrianopoulos, 2015; Coutinho *et al.*, 2002) being responsible for 51,2% of systemic fungal infections and occupying the 10th position among infectious diseases of high mortality during 1996–2006 in Brazil (Prado *et al.*, 2009). It is estimated that annual incidence varies from 10 to 30 cases

per million people and mortality rates range from 0.2 to 2.1 deaths per million inhabitants (Nucci *et al.*, 2009; Santo, 2008; Prado *et al.*, 2009; Martinez, 2017), being the highest cause of death among systemic mycoses in Brazil (Bocca *et al.*, 2013). Higher mortality rates of this mycosis were found in the Central and Southeast States, especially São Paulo, Minas Gerais and Rio de Janeiro (Prado *et al.*, 2009). Colombia has lower and variable incidence, with the highest being 2.4 cases per million inhabitants (Torrado *et al.*, 2000; Colombo *et al.*, 2011). Annual incidences of new cases may vary from 1 to 3.7 new cases per 10^5 inhabitants (Shikanai-Yasuda *et al.*, 2006; Bocca *et al.*, 2013). As so, paracoccidioidomycosis is considered a serious public health problem in endemic areas (Blotta *et al.*, 1999; Shikanai-Yasuda *et al.*, 2006). At the same time, paracoccidioidomycosis is also considered a neglected disease, and its epidemiology is underestimated due to several difficulties, such as the fact that case notification is not obligatory, absence of epidemic outbreaks and poor laboratory diagnostic capacity (Martinez, 2015).

Blastomyces dermatitidis

B. dermatitidis was first described by T.C. Gilchrist as a protozoan parasite that caused skin lesions, in 1894 (Sil and Andrianopoulos, 2015; Gilchrist, 1894). In 1898, Gilchrist isolated and fully described the pathogen as a fungus. These fungi grow in a hyphal form at temperatures below 30°C and as yeasts at 37°C. Teleomorphic phase is called *Ajellomyces dermatitidis*. Environmental niche is likely soil and decaying vegetation, especially in proximity to lakes and rivers, where it grows as mycelium with septate hyphae (Nemecek, 2006; Sil and Andrianopoulos, 2015; Bonifaz *et al.*, 2011; Castillo *et al.*, 2016). Infection occurs after inhalation of conidia or hyphal fragments (rarely through skin injury), and conversion into yeast occurs once inside the host (Castillo *et al.*, 2016; Kauffman, 2007). Yeast cells are characterized by a doubly thick cell wall with the daughter cell often as large as the mother cell before detachment. Inside the host, *B. dermatitidis* grows as extracellular yeasts in microabcesses (Sil and Andrianopoulos, 2015; Kauffman, 2007). Besides humans, *B. dermatitidis* also infects other animals, especially dogs (Castillo *et al.*, 2016; Saccente and Woods, 2010).

Infection is mostly asymptomatic or manifests as a self-limited illness. The disease was considered a localized dermatologic condition for years. However, primary pulmonary blastomycosis is the most important clinical form of disease and can be presented as an acute or chronic infection, with eventual progress to severe lung involvement manifested as acute respiratory distress syndrome (Castillo *et al.*, 2016). High inoculum and defects in cell-mediated immunity can promote a more severe and progressive disease (Bonifaz *et al.*, 2011; Sil and Andrianopoulos, 2015; Castillo *et al.*, 2016). Hematogenous dissemination can involve many organs, specially skin, osteoarticular structures, genitourinary tract and others. Adult men, particularly agricultural workers and farmers, are mostly affected due to higher exposure in the environment (Bonifaz *et al.*, 2011; Sil and Andrianopoulos, 2015; Castillo *et al.*, 2016).

Blastomycosis is one of the three major dimorphic endemic mycoses that occur predominantly in North America (**Fig. 1**). High incidence is found in Canada (especially in provinces of Quebec, Manitoba, and Ontario) and Eastern USA, specially surrounding the Ohio and Mississippi River valleys (Castillo *et al.*, 2016; Sil and Andrianopoulos, 2015). Middle and East Africa are also endemic sites, and sporadic cases were reported in Argentina (Kruse *et al.*, 2010). Due to its similarities with other common diseases, blastomycosis is often misdiagnosed, particularly as malignancy, what impairs epidemiology data (Bradsher, 2014). In addition to that, skin tests are not available and serologic studies have been considered unreliable due to cross reactivity with other endemic infections. Mortality rates range from 4% to 6% (Castillo *et al.*, 2016), and mortality rates associated with CNS blastomycosis are as high as 18%, and 89% in patients with ARDS (Castillo *et al.*, 2016).

The genus *Blastomyces* has been recently revised to include new species. In 2013, *B. gilchristii* was identified as a genetically divergent clade within *Blastomyces dermatitidis* (Brown *et al.*, 2013a). *B. helices*, former formerly classified in the genus *Emmonia* as *Ea. Helica*, differs from *B. dermatitis* because it predominantly affects immunocompromised patients and appears in the western and mountainous regions of North America (Schwartz, Wiederhold, *et al.*, 2019; Jiang *et al.*, 2018). *B. percursus* was isolated from patients in South Africa and Israel, and differently from *B. helicus*, can also cause disease in immunocompetent hosts (Dukik *et al.*, 2017; Friedman and Schwartz, 2019). Two other new species, *B. parvus* (former *Ea. parva*) and *B. silverae* are pathogens of small mammals (Muñoz *et al.*, 2015).

Talaromyces marneffei

T. marneffei (former *Penicillium marneffei*) was originally described in 1956 from a bamboo rat in Vietnam, but only in 1959 the first human infection was reported after an accidental self-inoculation of a researcher (Sil and Andrianopoulos, 2015; Vanittanakom *et al.*, 2006; Segretain, 1959). In 1973, it was registered the first naturally occurring human case, in an American patient with Hodgkin's disease who was living in the southeast Asia. After that, a few number of cases were reported, until AIDS pandemic arrived in Asia, when the number of infections increased substantially, raising *T. marneffei* as an important human pathogen (Vanittanakom *et al.*, 2006; Chan *et al.*, 2016; Sil and Andrianopoulos, 2015). In Southeast Asia, *T. marneffei* represents an "AIDS-defining pathogen" (Sil and Andrianopoulos, 2015; Vanittanakom *et al.*, 2006; Supparatpinyo *et al.*, 1994). *T. marneffei* is classified into *Eurotiales* order.

Unlike all other species in *Talaromyces* genus, *T. marneffei* is a thermal-dimorphic fungi and the only considered to be pathogenic in humans. *T. marneffei* grows as a mycelium at 25°C, with multinucleate and septate hyphae, producing conidiophores and conidia typical of *Talaromyces* genus (Vanittanakom *et al.*, 2006). Once inside the host (37°C), conidia is phagocytized and undergo transition into yeasts, which reside within these cells and divide by fission (Sil and Andrianopoulos, 2015; Andrianopoulos, 2002). *T. marneffei* biologic niche remains uncertain, and there are only a few reports of isolation from environment. Co-existence with some species of

rodents, such as *Rhizomys sinensis*, *Rhizomys pruinosus*, *Rhizomys sumatrensis* and *Cannomys badius*, has been extensively reported, suggesting that the reservoir may be in fact the rat and raising the possibility that infection occurs as a consequence of zoonotic transmission (Sil and Andrianopoulos, 2015; Vanittanakom and Sirisanthana, 1997; Vanittanakom *et al.*, 2006). However, no correlation of geographic distribution between bamboo rats and human infection has been found. On the other hand, age (16–30 years) and agricultural activities were described as factors associated with increased risk of infection (Sil and Andrianopoulos, 2015; Vanittanakom *et al.*, 2006; Chariyalertsak *et al.*, 1997). It is generally accepted that infection is initiated by inhalation of dormant conidia. However, at this moment, it is not clear if it is a consequence of zoonotic or environmental transmission.

T. marneffei infection (penicilliosis) is endemic in tropical Asia, especially Thailand, north-eastern India, China, Hong Kong, Vietnam, and Taiwan (**Fig. 1**). In northern Thailand, disease is the third most common opportunistic infection in AIDS patients (Vanittanakom *et al.*, 2006; Supparatpinyo *et al.*, 1994). Another important characteristic of *T. marneffei* infection is that the disease occurs predominantly in immunocompromised individuals, being considered an opportunistic mycosis. Disease starts as a pulmonary infection followed by hematogenous dissemination to the lymphatic system, liver, spleen and bones (Sil and Andrianopoulos, 2015; Kudeken *et al.*, 1996; Vanittanakom *et al.*, 2006). Systemic disease is often fatal when left untreated. Rare cases of disease in immunocompetent individuals have been described, although immunological status of patients was not adequately tested (Sil and Andrianopoulos, 2015).

Emergomyces spp.

A new genus in the order Onygenales has recently been created in order to accommodate five species of thermodimorphic fungal pathogens: *Emergomyces pasteurianus*, *Es. africanus*, *Es. canadensis*, *Es. orientalis* and *Es. europaeus* (Dukik *et al.*, 2017). They are the causative agents of the recently described emergomycosis, a systemic fungal infection that, unlike other endemic mycosis, is primarily affecting immunocompromised individuals, especially HIV patients (Kenyon *et al.*, 2013; Friedman and Schwartz, 2019; Dukik *et al.*, 2017; Schwartz *et al.*, 2019). Emergomycosis has been reported worldwide (**Fig. 1**): *Emergomyces pasteurianus* in Europe, Asia and Africa; *Es. africanus* in South Africa, *Es. canadensis* in Canada and the USA, *Es. orientalis* in China and *Es. europaeus* in Germany (Schwartz, Maphanga, *et al.*, 2018; Schwartz *et al.*, 2019). *Es. africanus* DNA has been identified in soil (Schwartz *et al.*, 2018) and air (Schwartz *et al.*, 2018) samples in South Africa, and infection is presumably by inhalation of conidia that, once inside the human host, convert into pathogenic yeasts. Emergomycosis has been described mostly as a disseminated infection, and may affect skin, lungs and other internal organs such as liver and lymph nodes (Friedman and Schwartz, 2019; Jiang *et al.*, 2018; Schwartz *et al.*, 2018). *Emergomyces* spp. was first isolated in 1994, and much of its ecology, geographical distribution and pathogenic mechanisms are still to be determined.

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Histoplasma

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Introduction

Histoplasma is an endemic, thermally dimorphic fungus. It is endemic to specific regions on several continents, but epidemiology is difficult to critically assess due to a lack of systematic mandatory reporting. The major factor regulating dimorphism is temperature, with the fungus existing as a mycelium in nature or with cultivation at ~25°C and this mold form undergoes morphogenic transition to a yeast during infection or with culture in rich medium at ~37°C. The mycelial form has hyphal elements that are 1.25–2.0 μm in diameter, which present tuberculate macroconidia (~8–14 μm in diameter) and microconidia (~2–6 μm in diameter), with the microconidia being the purported infectious propagule (Darling, 1909; Helmbright and Larsh, 1956). The yeast cells are typically small, slightly ovoid cells of 1–5 μm in diameter.

The American pathologist Samuel Taylor Darling reported and named histoplasmosis after the autopsy of a carpenter in Panama in 1905 (Darling, 1906, 1909), although he incorrectly determined that the microbe was a protozoan. Darling observed swollen histiocytes (thus "histo") and plasmodium-like organisms (hence "plasma") with what appeared to be a capsule ("capsulatum") leading to the name *Histoplasma capsulatum*. Notably, the yeast does not in fact have a capsule. In 1912, the Brazilian investigator Henrique da Rocha-Lima correctly determined that *Histoplasma* was a fungus (da Rocha-Lima, 1912–1913; da Rocha-Lima, 1912). Recently, genome-wide population analyses have led some researchers to suggest that the genus *Histoplasma* contains at least four distinct species (Sepulveda *et al.*, 2017). However, these taxonomic changes have not been uniformly accepted, although our current chapter will generally refer to this potential group of fungi as "*Histoplasma*."

Acquisition of *Histoplasma* may be asymptomatic and manifestations of disease range from a mild flu-like illness to life-threatening disseminated disease. The severity of this mycosis is closely associated with the quantity of the inoculum and the affected individual's immunological state (Kauffman, 2007), with defects in cellular immunity being closely linked to disease severity. The diagnosis of histoplasmosis remains problematic, frequently resulting in delays of diagnosis. Pharmacological management requires systemic antifungal therapy, typically with a polyene or an azole, depending on disease severity. Therapy is typically several months in duration and individuals with severe immunosuppression may require life-long antifungal treatment.

Epidemiology

Histoplasmosis is reportable in 12 of the 50 states in the USA and it is only sporadically reportable in other areas of the world. *Histoplasma* is responsible for ~500,000 infections annually in the USA, making histoplasmosis the most prevalent fungal pulmonary disease (Teixeira Mde *et al.*, 2016). The areas with the highest rates of infection are in the Ohio and Mississippi River Valleys where up to 90% of individuals have acquired the fungus (Furcolow, 1963; Edwards *et al.*, 1969). A 2018 report from the USA CDC, reviewed hospitalization data from 2011 to 2014 in only 12 states (which excluded Missouri and Tennessee, states with a high incidence of *Histoplasma*) in the USA and identified 3409 cases of histoplasmosis with a 7% mortality rate (Armstrong *et al.*, 2018). The incidence of histoplasmosis was highest in geographic areas adjacent to the Mississippi River. A previous CDC report from 2016 reviewed USA hospitalizations from 2001 to 2012 and found that there were ~5000 admissions annually (Benedict *et al.*, 2016). Analysis of the 2002 Nationwide Inpatient Sample identified 3370 individuals with histoplasmosis with a mortality rate of 7.5% (Chu *et al.*, 2006). Hence, histoplasmosis is a consistently important pathogen in the USA, particularly in specific areas. Notably, *Histoplasma* is also being identified outside of its historic boundaries in the USA (CDC, 2013; Maiga *et al.*, 2018), possibly due to climate change.

Histoplasma is also a major killer in less developed regions of the Americas (Adenis *et al.*, 2014), particularly in Latin American countries including Brazil (Prado *et al.*, 2009; Brilhante *et al.*, 2012), Guatemala (Scheel *et al.*, 2009), and French Guiana, where it is the "first cause of AIDS-related death" (Iriart *et al.*, 2014). Notably, histoplasmosis exceeds tuberculosis in HIV infected individuals as the leading cause of death in certain regions (Adenis *et al.*, 2018; Pasqualotto and Quieroz-Telles, 2018), and it is considered an AIDS defining disease (Adenis *et al.*, 2014). Endemicity is particularly high in specific regions, such as Southeastern portions of Brazil where the prevalence is as high as ~93% (Guimaraes *et al.*, 2006). Although less common, *Histoplasma* is prevalent in the Caribbean, Africa, Australia and Asia (Loulergue *et al.*, 2007; Chakrabarti and Slavin, 2011; McLeod *et al.*, 2011), including newly described areas (Antinori, 2014). Moreover, given the frequency of travel, imported cases of histoplasmosis are globally reported (Bahr *et al.*, 2015).

As noted above, histoplasmosis is highly associated with advanced HIV disease, particularly once CD4+ T cell counts are less than 200/mm^3 (Adenis *et al.*, 2014). Individuals receiving anti-cytokine therapies, particularly inhibition of tumor necrosis factor-α, have a high risk for histoplasmosis (Deepe, 2005). Reactivation disease arising from organ transplants, particularly liver, also may occur (Grim *et al.*, 2012).

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Pathogenesis

The saprophytic mycelial form thrives in soils enriched with organic nitrogen sources, such as areas contaminated with bird or bat dropping. Disturbances of *Histoplasma* contaminated areas result in the aerosolization of conidia (Benedict and Mody, 2016). The inhaled microcondia can reach the alveoli of the lung where they undergo transformation to the yeast form (Medoff *et al.*, 1987; Allendoerfer *et al.*, 1997). Early infection can be arrested by an effective immune response or the yeast cells can be captured by host effector cells and controlled within granuloma. However, during primary infection or in the setting of reactivation, the yeast can disseminate. Notably, splenic granulomas have been found on autopsy in ~70% of individuals with history of any form of histoplasmosis (Straub and Schwarz, 1955).

After recognition of *Histoplasma* by lung resident cells, neutrophils are the first recruited host effector cells to combat *Histoplasma* (Deepe *et al.*, 2008; Maza and Suzuki, 2016). Thereafter, macrophages and dendritic cells primarily engage *Histoplasma* yeast cells, where the pathogen enters into a phagosome. Activated dendritic cells and macrophages can kill *Histoplasma* (Newman *et al.*, 2006). Moreover, activation of cellular immunity is also required for control of histoplasmosis, since severe disease is more frequent in the absence of effective cellular immune responses (Allendorfer *et al.*, 1999). Th1-type cytokines are important for an effective host response, particularly IL-12 and IL-17, tumor necrosis factor-α, granulocyte macrophage colony-stimulating factor [GM-CSF], and interferon-γ (Allendorfer *et al.*, 1999). These cytokines, particularly tumor necrosis factor- α, are vital for the formation and maintenance of granuloma that control histoplasmosis.

Clinical Features

The incubation period from acquisition of *Histoplasma* to disease manifestations typically ranges from 8 to 17 days, but disease may occur within 3 days after heavy exposure (Goodwin *et al.*, 1981). The likelihood of disease is most closely linked to the infectious challenge, with low inoculum infection resulting in approximately only 1% of individuals developing self-limiting disease, whereas subclinical disease occurring in the other 99% (Pfaller and Diekema, 2010). In contrast, symptomatic disease occurs in 50%–100% of high exposures (Wheat, 1997). Fortunately, high inoculum exposures are infrequent. Hence, the vast majority of infected individuals are either asymptomatic or have mild flu-like symptoms. The most common clinical manifestation is pneumonia, although individuals may initially present with rapidly progressive, life-threatening disseminated disease involving virtually any tissue, particularly in individuals with compromised immunity. There are 3 common forms of disease: acute, disseminated and chronic.

Acute Histoplasmosis

If clinical disease manifests, it typically develops one to three weeks after acquisition (Hage *et al.*, 2008). A flu-like illness, with persistent fever, is the most common presentation. Routine laboratory and imaging is typically non-specific, although mediastinal lymphadenopathy with or without bilateral infiltrates can occasionally be observed. Acute progressive pneumonia, particularly following a heavy exposure (Kataria *et al.*, 1981), occurs in ~1 in 2000 adults (Sathapatayavongs *et al.*, 1983). Most patients with acute pulmonary histoplasmosis recover over several weeks without sequalae. Some individuals may have either a single calcified or non-calcified nodule or even a miliary ("buckshot") pattern of calcified granulomas on subsequent chest radiographs, which can be indistinguishable from the miliary appearance of granuloma in recovered tuberculosis patients (Goodwin *et al.*, 1981).

Disseminated Histoplasmosis

In ~0.05% of individuals, *Histoplasma* yeast cells can rapidly disseminate from the lungs (Deepe *et al.*, 2008). This serious disease occurs most frequently in the setting of pre-existing immunosuppression, particularly in individuals with advanced HIV infection or cancer as well as patients on high doses of corticosteroids or cytokine-inhibitors. Subclassifications of this clinical form include acute, juvenile and chronic for respectively infants, adolescents and young adults and advanced age. Although the severity of disease can vary, fever, weight loss, and respiratory symptoms are common. Laboratory testing frequently reveals leukopenia, anemia, thrombocytopenia, abnormal liver function tests and coagulopathies (Wheat and Kauffman, 2003). Diffuse pulmonary infiltrates are present in ~70% of disseminated histoplasmosis patients (McAdams *et al.*, 1995). Disseminated disease is lethal without antifungal treatment.

Chronic Pulmonary Histoplasmosis

This disease occurs in individuals with longstanding lung diseases in which the tissue has been previously damaged. The fungus most frequently causes disease in apical bullae and the infection is characterized by progressive infiltrates, cavitation, and fibrosis (Wheat *et al.*, 1984). Diagnosis is frequently delayed (Hage *et al.*, 2012) and, without antifungal treatment, infection progresses in ~50% of affected individuals (Wheat *et al.*, 1984).

Diagnosis

The diagnosis of histoplasmosis remains problematic. Culture still stands as the gold standard, although this method requires from 1 to 4 weeks for the growth of the fungus. Unfortunately, respiratory cultures from patients with acute pulmonary histoplasmosis are positive in less than 50% of cases (Hage *et al.*, 2011b). Cultures are positive in 65%–85% of patients with chronic pulmonary histoplasmosis. Blood cultures are positive in up to 85% of patients with disseminated disease (Guimaraes *et al.*, 2006); however, yeast are usually present in bone marrow aspirates (Davies *et al.*, 1979) or can occasionally be visualized in peripheral blood (Kurtin *et al.*, 1990).

In the USA, antigen detection is commonly used in diagnosis of histoplasmosis. *Histoplasma* polysaccharide antigen can be detected in the urine of 83% with acute disease, 88% with chronic pulmonary infection and 92% of patients with disseminated histoplasmosis (Hage *et al.*, 2011b). Unfortunately, there is cross reactivity of this assay with polysaccharide of other endemic fungi (Assi *et al.*, 2011). In addition to diagnosis, these tests can be used to determine the efficacy of treatment and patient follow-up (Wheat *et al.*, 2007). In countries where the antigen detection kits are too costly, immunodiffusion tests for detection of antibodies to *Histoplasma* H and M antigens are utilized (Zancopé-Oliveira *et al.*, 1993). Antibodies are detectable by 4 weeks after infection (Williams *et al.*, 1994). Either as an alternative or for confirmation of the immunodiffusion, complement fixation tests using both *Histoplasma* antigens and intact yeast cells can also be applied for diagnosis (Wheat *et al.*, 1982). Unfortunately, these tests are less reliable in immunocompromised patients who may fail to mount antibody responses to the fungus (Bradsher, 1996).

Most recently, a lateral flow assay has been developed that may increase our ability to more widely diagnose histoplasmosis. One lateral flow assay performs similarly to the antigen detection method; however, cross reactivity with *Paracoccidioides* occurred (Caceres *et al.*, 2019).

Treatment

Treatment guidelines are currently based on the consensus of the American Thoracic Society (Limper *et al.*, 2011) and the Infectious Diseases Society of America (Wheat *et al.*, 2007). The majority of individuals who acquire *Histoplasma* do not require antifungal treatment. Unless an individual is significantly immunocompromised, those with mild, self-limited "flu-like" illnesses due to *Histoplasma* should not receive antimicrobial therapy (Wheat *et al.*, 2000). Itraconazole is the typical drug used for mild to moderate disease with the polyene amphotericin B reserved for more severe infections. Patients with chronic histoplasmosis usually require itraconazole and amphotericin B combinations for long periods (LeMonte *et al.*, 2000). Newer azoles can be used in patients not responding to itraconazole prior to the administration of amphotericin B (Perfect *et al.*, 2003; Restrepo *et al.*, 2007; Wheat *et al.*, 2006). Echinocandins should not be used to treat *Histoplasma* (Hage *et al.*, 2011a). Azole drug monitoring is important to confirm effective treatment (Andes *et al.*, 2009; Freifeld *et al.*, 2007). If available, antigen testing should be used to evaluate response to treatment.

Acute Pulmonary Histoplasmosis

Antifungal therapy is appropriate in patients with symptoms lasting more than 3 weeks. Therapy should also be given if a patient has moderate disease. Itraconazole is the recommended drug. Itraconazole should be given as a loading dose of 200 mg thrice daily for 3 days followed by 200 mg twice daily for 6–12 weeks. A new super-bioavailable itraconazole (SUBA-itraconazole) formulation has also been developed and does not have food or acid requirements that somewhat limits standard itraconazole (**Fig. 1**).

Moderately Severe to Severe Acute Pulmonary Histoplasmosis

Liposomal amphotericin is the preferred drug for more severe histoplasmosis (Wheat *et al.*, 2001; Johnson *et al.*, 2002). In patients with hypoxemia or significant respiratory distress, the addition of corticosteroids may be appropriate to reduce side effects in patients at risk for immune reconstitution syndromes (Breton *et al.*, 2006).

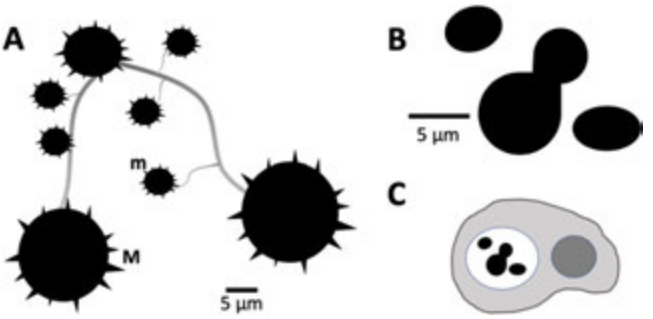

Fig. 1 *Histoplasma*. (A) The environmental form of *Histoplasma* consists of a mold form in which there are macroconidia (M, ~8–14 µm) and microconidia (m, ~2–6 µm). (B) The acquired microconidia undergo morphogenic shifts to the yeast form that reproduce by budding. (C) Yeast forms (~1–5 µm) are typically intracellular, especially in phagosomes of macrophages.

Chronic Cavitary Histoplasmosis

Itraconazole is the most commonly applied antifungal, although amphotericin should be considered for severe disease (Parker *et al.*, 1970).

Disseminated Histoplasmosis

Liposomal amphotericin is the appropriate initial drug, followed by itraconazole. The duration of therapy is typically at least a year long. A caveat is that non-immunosuppressed individuals who present with mild to moderate symptoms and are incidentally found to have disseminated disease may be treated with itraconazole alone.

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Coccidioidomycosis: The Valley Fever

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Introduction

Coccidioidomycosis or valley fever is a caused by *Coccidioides* species, thermally dimorphic fungi found in environmental soil that affect humans and a diversity of vertebrates (Drutz and Catanzaro, 1978a,b; Galgiani *et al.*, 2005; Smith *et al.*, 1961; Stevens, 1995; Stockamp and Thompson, 2016). This typically occurs after the inhalation of coccidioidal arthroconidia released during the perturbation of soil due to natural weather events, agriculture, land development, and recreational activities (Colson *et al.*, 2017; Nicas, 2018). There are two species (spp.) of *Coccidioides* described, *C. immitis* (Ophüls, 1905; Posadas, 1892) and *C. posadasii* (Fisher *et al.*, 2002), the latter was phenotypically described in 2002, more than 100 years after the first known case report of coccidioidomycosis was published in 1892 (Posadas, 1892).

During the 20th century, *Coccidioides* spp. emerged as a public health threat to any region with arid or semiarid conditions that support the growth of the fungus in soil as arthroconidia (Smith, 1955). The fungus is endemic of the United States (US) Southwest, Mexico, and other expansive regions of Latin America, conferring a tremendous burden onto public health systems. Tens of thousands of cases are confirmed and reported every year, with many more unidentified because lack of testing or reporting (McCotter *et al.*, 2019). In California, coccidioidomycosis was responsible for approximately 25,000 hospitalizations and >$2 billion dollars in hospital charges for patients during 2000–2011 (Sondermeyer *et al.*, 2013). In Mexico, the impact of coccidioidomycosis on public health is far more insidious and tragic due to their substantial agricultural workforce that is medically underserved. Upon infection or exposure, people must rely on underdeveloped health systems and lack of testing/reporting.

In the US, *Coccidioides* is reported to be encroaching on greener, more temperate territories it was never before observed in; cases are now appearing out of East Texas (Barker *et al.*, 2019) and as far north as Washington State (Marsden-Haug *et al.*, 2013). The mechanisms behind *Coccidioides*' invasive behavior are, as of yet, mysterious, but climate change might be a likely factor (Casadevall, 2019; Garcia-Solache and Casadevall, 2010). Many leading infectious disease experts have described how increasing global temperatures and the widening of the geographic ranges of thermotolerant fungi might act as a one-way highway for the expansion of fungal etiologies in the environment, and an increase in their virulence in warm human hosts (Casadevall *et al.*, 2019). Consequently, this might be an important factor in *Coccidioides*' dissemination onto newer territories.

This growing threat demands the urgent development of therapeutics and a prophylactic vaccine. Yet regardless of the irrefutable burden that *Coccidioides* causes in the present and the ominous threat it poses to our future, coccidioidomycosis, like other fungi, is relatively underrepresented in research and public attention compared to bacterial and viral agents. However, recent intensified progress has been made in the characterization of *Coccidiodes* pathogenesis and vaccine development. Here, we discuss *Coccidioides* and its history up until the present, with a special emphasis on its public health significance, risk factors, and association with the environment. We then discuss the fungus' virulence factors and pathogenesis during disease. Lastly, we discuss therapeutic options and vaccination efforts.

History

Coccidioidomycosis is a significant threat to the health of millions in the western hemisphere but in 1892, the first known case report of this mycosis was published by Alejandro Posadas, an undergraduate medical student in the University Hospital Clinics (UHC) of Buenos Aires, Argentina (Posadas, 1892). His description of the skin lesions burdening 32-year old Argentine army cavalryman Domingo Ezcurra launched a century of discoveries that epitomized the scientific method and established the fungus' major players during the early 20th century; California, the US ARMY, World War II, prestigious medical institutions, and dust (Deresinski and Mirels, 2019; Hirschmann, 2007).

Early History: A Mistaken Identity

Domingo Ezcurra battled the progressive skin lesions and fevers of his disease for the next 7 years, ultimately succumbing to coccidioidomycosis in 1898. Before his passing, Posadas studied Ezcurra intensely, beginning when Dr. M. Bengolea of Rawson Hospital in San Juan, Argentina first referred Ezcurra to UHC in 1891. Bengolea had diagnosed mycosis fungoides (a cutaneous T cell lymphoma) and ordered administration of mercury and potassium iodide for 20 days, but Ezcurra's lesions were unresponsive to the treatment, thus necessitating the transfer. Previous to that, Ezcurra had been diagnosed with lupus vulgaris at an Argentine military hospital in 1889 after presenting with a verrucous lesion on his right cheek, which he believed to be a spider bite. Treatment with nitric acid in that instance did not lead to improvement and his self-administered tobacco treatment was ineffective as well.

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At UHC under the supervision of Drs. Robert Wernicke and Guillermo Udaondo (Wernicke, 1892), Posadas examined Ezcurra's lesions which were progressively increasing in size and numbers. Most of Ezcurra's right cheek was covered by a fungal mass, his nose was ulcerated, and numerous papules were appearing on his extremities and trunk. Following biopsy, Posadas microscopically observed granular multinucleated giant cells with double-refractile outer envelopes. Following discussion with his mentors, Posadas believed Escurra's pathology had a parasitic etiology similar to microorganisms from the protozoan class *Coccidia*, a conclusion which culminated in his published case report in 1892 (Posadas, 1892).

Contemporaneous with Ezcurra's case in Argentina, the second and third documented cases emerged thousands of miles away in the US (Rixford and Gilchrist, 1896). In July 1893, farm laborer Jose Furtado Silverra was admitted to the City and County Hospital of San Francisco with an enlarging sore that began developing seven years prior in 1886, shortly after he immigrated from the Azores archipelago of Portugal (Hirschmann, 2007). In April 1894, Drs. W. S. Thorne along with Emmett Rixford of Cooper Medical College (soon to became Stanford University School of Medicine) presented Silverra's case before Californian physicians and health officials (Rixford, 1894; Thorne, 1894).

Four months later in August 1894, yet another farm laborer from the Azores, Jose Texeira Pereira, presented to St. Mary's Hospital in San Francisco with coccidioidal granuloma involving the face, where he was admitted by Drs. Thorne and L. Robinson (Hirschmann, 2007). It was now clear that Thorne and Rixford had a novel threat to public health on their hands, and they once again communicated with public health officials when they presented Pereira's case before the California Academy of Medicine. Just a few days later, Jose Texeira Pereira's condition deteriorated rapidly, and he died of coccidioidomycosis. Jose Furtado Silverra's disease progressed relatively slower (considering he first experienced symptoms in 1886) until he also succumbed to the mycosis in January 1895 (Hirschmann, 2007). Autopsy revealed tremendous dissemination as granulomas (containing the same protozoa-like, double-enveloped organisms Posadas had first observed) were found in the lungs, liver, prostate, testes, spleen, lymph nodes, adrenals, and peritoneum (Hirschmann, 2007).

While Silverra was still alive, Rixford investigated the skin as a potential portal of entry by self-inoculating Silverra with the microorganism, to no avail (Hirschmann, 2007). However, he did manage to establish subcutaneous infection in a rabbit and a dog. In collaboration with C.W. Stiles, a prominent parasitologist, Rixford published his findings with T.C. Gilchrist, a pathologist from Johns Hopkins Medical School (Rixford and Gilchrist, 1896). Together they agreed with Posada's previous findings that the etiology in question was indeed a parasite. Thus, they classified the microorganism under the new genus *Coccidioides*, and they named two different species; *immitis* (meaning "not mild") which infected Silverra, and *pyogenes* (meaning "pus-producing") which infected Pereira. Rixford and Gilchrist justified the establishment of two different species after they observed the markedly different course of disease in Silverra and Pereira, along with a difference in inflammatory processes. During their work, Rixford and Gilchrist disregarded a mysterious growth on their culture plates as a mere contaminant "mold".

It was William Ophüls, the future second dean of Stanford Medical School which would correctly establish the identity of Silverra and Pereira's infectious etiology half a decade later (Ophüls, 1905; Ophüls and Moffit, 1900). Once more, a patient with a history of immigration from the Azores and agricultural work presented to the City and County Hospital of San Francisco on January 1900, with severe disseminated coccidioidal infection. The 19-year male patient died, eleven days after admission. Upon examination of a splenic specimen from the patient, Ophüls and his collaborator Dr. Herbert C. Moffitt encountered a mysterious "white mouldy growth" similar to that which Rixford and Gilchrist had discarded during their work (Ophüls and Moffit, 1900). They also believed this to be a contaminant, but eventually proceeded to inoculate material from the Azorean patient into guinea pigs, resulting in severed disease in the animals. Microorganisms from the guinea pigs were then cultured as mycelia, and this used to infect rabbits, which developed coccidioidal nodules. This evidence led to Ophüls making his conclusion; the microorganism was not a two species parasite, but in fact was a dimorphic fungus with mycelial growth in culture that transitions into spherical bodies in mammal host tissues and reproduces by bursting into more spherules. In his publications, Ophüls delineated the life cycle of the fungus, suggested soil as a prospective reservoir, proposed its airborne route of transmission, named the disease "coccidioidal granuloma", and established the singular identity of the fungus; *Coccidioides immitis*.

No history of coccidioidomycosis is complete without mention of Dickson, Chope, Smith, and Gifford who continued to champion discovery through the 20th century. Harold Chope contracted the disease from a culture plate as a medical student in Ernest Dickson's lab, which helped establish respiration as the major route of primary infection. Charles E. Smith and Myrnie Gifford dedicated much of their life to identifying the disease in the US Southwest populations and assisting the US military, which battled coccidioidomycosis on home soil during World War II. Their contributions and humanity are legendary (Hirschmann, 2020; Huntington, 1985). As one of Smith's students wrote to his widowed wife after his passing, "His is the special kind of immortality reserved for those who devote their lives to the teaching of others" (Broughton, 1967). Indeed, this sentiment can be extended to the individuals combating this disease in the past and present.

Ecology and Life Cycle of the Desert Dust Fungus

The first documented cases of coccidioidomycosis occurred in three outdoorsmen; the cavalryman Ezcurra and the agriculturalists Pereira and Silverra. This would prove to be an ominous foreshadowing of this fungus' modus operandi. Today, 17%–29% of community acquired pneumonia cases in endemic regions are attributable to *Coccidiodes* (Thompson, 2011). The environmental persistence of *Coccidioides* as viable arthroconidial spores in arid/semiarid and alkalinic soils is the first critical factor for its status as a medically relevant fungus in these regions (Lacy and Swatek, 1974). The fungus' tolerance of

heat and particular soil chemical characteristics demonstrate that *Coccidioides* is well adapted to regions such as the US Southwest, the Sonoran and Chihuahuan deserts of Northern Mexico, the Motagua Valley in Guatemala, as well as other Latin American arid/semiarid environments in Honduras, Brazil, Argentina, Bolivia, Colombia, Paraguay, and Venezuela (Laniado-Laborin *et al.*, 2019). Thus, the endemic range for this fungus is large, and as previously noted, seems to be expanding (McCotter *et al.*, 2019). *C. immitis* predominates in California, and *C. posadasii* everywhere else, though a few differential characteristics between these species have been established. The second critical factor is the fungus' ease of aerosolization following a disturbance of soil. Were it not for this characteristic, *Coccidioides* would rarely reach the target; a human or other mammals (and occasionally reptiles) (Del Rocio Reyes-Montes *et al.*, 2016). The fungus exists in the saprobic phase in soil as mycelia which germinate to produce barrel shaped arthroconidia that disperse quite easily, possibly in a seasonal manner involving during dry periods (Comrie, 2005; Gorris *et al.*, 2018). Perturbation of soil can result from anthropogenic or natural causes, like weather events and outdoor activities. The third critical factor is the fungus' ability to initiate a morphological and phenotypical switch to spherules upon infection, a characteristic of the thermally dimorphic fungi (Kirkland and Fierer, 2018). This transition is spurred by warm mammalian temperatures and physiological CO_2 tension, and might be enhanced due to contact with host leukocytes (Converse, 1956; Galgiani *et al.*, 1982).

Emergence of Coccidioidomycosis as a Growing Threat

In the US, the range of *Coccidioides* is expanding on multiple fronts and its threat follows right along (Gorris *et al.*, 2019). Greater amounts of cases are being reported eastwardly and valley fever has been diagnosed in workers at Dinosaur National monument, which lies in the far northern reaches of Colorado and Utah (Johnson *et al.*, 2014). Furthermore, recent cases of coccidioidomycosis in humans have been reported in south-central areas of Washington State (Marsden-Haug *et al.*, 2013). In fact, whole genome sequencing has identified highly genetically related *Coccidioides* in soil and human samples even in individuals with no travel history outside of this state (Marsden-Haug *et al.*, 2014; Oltean *et al.*, 2019). In addition, many animals in Washington State have tested positive for coccidioidomycosis (James *et al.*, 2019). This strong evidence indicates that Washington State is in the fungus' endemic range, and nearby states such as Idaho, Montana, Oregon, and Wyoming might soon prove to be as well. Moreover, *Coccidioides* DNA has been isolated from Oregon soil (Benedict *et al.*, 2018). Global warming is the hypothesized driver of fungal proliferation in the environment, as climate change expands arid/semi-arid geography and might put selective pressure on fungi to better survive in regions with warmer temperatures (Casadevall *et al.*, 2019).

Coccidioidomycosis is a Threat to Public Health in the Western Hemisphere

Risk Factors

Ophüls was the first to speculate on *Coccidioides* association with the environment and a century of research since then has given us great insight into the corresponding risk factors. It is now clear that anyone with environmental exposure in endemic areas is potentially at risk, even healthy individuals without immunodeficiency, albeit rarely (Pearson *et al.*, 2019). Involvement in construction (Laws *et al.*, 2018), archeology (Johnson *et al.*, 2014), field expeditions, military field exercises (Crum-Cianflone, 2007), agriculture (Levan and Huntington, 1965), sports (Stern and Galgiani, 2010), hunting (Brillhante *et al.*, 2012), and a multitude of other outdoor/recreational activities can result in infection (Diaz, 2018). Prison populations are of particular concern as very high rates of coccidioidomycosis among incarcerated individuals have been observed on multiple occasions (Lee *et al.*, 2017). In California, the cost in correctional health services due to coccidioidomycosis was $23 million from 2006 to 2010 (Lee *et al.*, 2017).

Travel to endemic regions also presents risks of exposure (Diaz, 2018). US Southwest states are world hubs for tourism and they experience vast amounts of incoming travel, as do many endemic regions in Latin America. This has resulted in a significant incidence of coccidioidomycosis outside of endemic zones (Panackal *et al.*, 2002). Hundreds of cases in Canada, Asia, Europe, and Australia have also been well documented, and it is likely many have remained undiagnosed due to the self-resolving nature of the disease and the multitude of illnesses with similar manifestations. Clinicians around the world should be alert and consider coccidioidomycosis in the differential diagnosis in individuals who present with a travel history to endemic regions.

Non-environmental risk factors have also been characterized. For example, pregnancy is one of the most common risk factors for disseminated coccidioidomycosis (Bercovitch *et al.*, 2011). Men display a predisposition to coccidioidomycosis, and they experience a higher rate of infection and dissemination, as do non-Caucasians such as Hispanics, African Americans, and Filipinos (Louie *et al.*, 1999; Ruddy *et al.*, 2011). Furthermore, Alaskan Natives and American Indians experience high morbidity and hospitalization rates, a racial tendency that is unique to *Coccidioides* (McCotter *et al.*, 2019). The fungus is also opportunistic in HIV/AIDS patients and patients with low $CD4^+$ T-cell population are particularly at risk of disseminated coccidioidomycosis (Kirkland and Fierer, 2018; Ampel *et al.*, 1993). Moreover, individuals with immunosuppression associated to organ transplants, corticosteroid therapy, and treatment with TNF-α antagonists result in greater risk (Bergstrom *et al.*, 2004; Blair and Logan, 2001; Brown *et al.*, 2013). People with chronic conditions such as diabetes and cardiopulmonary disease are also highly susceptible to valley fever (Rempe *et al.*, 2007).

Clinical Manifestations

The incubation period of *Coccidioides* can range from one to four weeks post-infection and 60%–65% of primary infections remain asymptomatic. Individuals with symptomatic infections typically present with usual signs and symptoms caused by respiratory infections such as fever, night sweats, myalgia, arthralgia, headache. These manifestations can be accompanied by pneumonia, in which case the syndrome is classified as community acquired pneumonia. Although not exclusively, pneumonia can resolve in immunocompetent individuals without medical intervention or therapy. Within the first few days of illness, 10%–30% of individuals can also experience non-specific diffuse erythematous rash on the trunk and extremities that typically disappears shortly thereafter (Cox and Magee, 2004).

Radiographic imaging is instrumental in assessing the extent of coccidioidomycosis progression in the lungs. In $<5\%$ of cases, a persistent pulmonary infection develops resulting in progressive pneumonia accompanied by pulmonary nodules and cavities (Cox and Magee, 2004). Further complications can include hemorrhage, secondary infections, and pulmonary lesions. Disseminated disease occurs in $<1\%$ of cases and meningitis is the most serious form of systemic disease. Usually $\sim 90\%$ of individuals with coccidioidal meningitis die within a year and 100% within 2 years (Cox and Magee, 2004). The fungus can also disseminate cutaneously and to other organs. In very rare cases, patients might exhibit primary cutaneous coccidioidomycosis (Shiu *et al.*, 2018), though prognosis for this is excellent and the infection typically resolves with proper treatment.

Coccidioides in the US Southwest

Primary respiratory coccidioidomycosis is colloquially referred to as Valley Fever in some regions of the US Southwest where the disease remains a leading cause of community acquired pneumonia. More than 25% of the US population lives in this region, which puts more than 80 million citizens within the reach of *Coccidioides*. California, Arizona, Nevada, Utah, New Mexico, and Texas are all endemic to varying degrees and, as noted earlier, experts and leading health authorities agree that the fungus' endemic range seems to be expanding. Moreover, millions of older individuals retire to this US region, causing preoccupation about their ability to deal with this infection particularly for those who have never been previously exposed to *Coccidioides* (Benedict *et al.*, 2018). In California, 75% of cases occur in the San Joaquin Central Valley (Dizon *et al.*, 2019) and one estimate placed the average financial burden of Californian patients who sought medical attention at $57,000 (Wilson *et al.*, 2019). Patients with disseminated diseases incurred on average $1,000,000 in costs, while patients with uncomplicated and diffuse pneumonia spent between $22,000 and $132,000, respectively (Wilson *et al.*, 2019). Additionally, 90% of people with disseminated disease lose an average of 90 days of work whereas 10% leave the workforce permanently (Wilson *et al.*, 2019). Arizona also faces similar severe healthcare and economic burden due to coccidioidomycosis (Hector *et al.*, 2011).

Reported cases of coccidioidomycosis in the US decreased steadily from 2011 (22,641 cases) to 2014 (8232 cases), although there has been a recent surge in the number of cases with 15,611 cases documented in 2018 (Anon1). Domesticated and wild animals are also highly susceptible to coccidioidomycosis. For example, dogs are particularly vulnerable with 6%–10% of canine in Arizona's "Valley Fever Corridor" affected, corresponding to 60,000 yearly infections and $1000 per animal in veterinary costs (Anon2; Anon3). Close to 25% of canine coccidioidomycosis becomes complicated and may result in additional costs for owners.

Interestingly, dust storms and natural disasters are important drivers of coccidioidomycosis outbreaks (Hernandez and Martinez, 2018; Tong *et al.*, 2017). In 1977, a dust storm in the San Joaquin Valley was the caused of hundreds of cases across Sacramento, a US Navy air Station in Kings County, and many other Californian counties (Flynn *et al.*, 1979; Williams *et al.*, 1979). In 1994, 204 cases were associated to a 6.7 magnitude earthquake in Northridge, California that produced formidable dust clouds (Schneider *et al.*, 1997). Since the frequency of dust storms and other natural disasters has increased in the past few decades, these have been correlated with increased valley fever cases.

Coccidioides in Latin America

Regions with ecological features conducive to coccidioidal proliferation are widespread in Latin America. Yet, clinical and epidemiological data of coccidioidomycosis is limited due to the underdeveloped heath systems predominating in many areas. Although there have been surveys conducted to assess the prevalence of coccidioidomycosis, much of the data is outdated and unconsolidated, even within individual countries. The lack of knowledge about this infection's true impact is especially worrying in Latin America, since there are so many rural communities whose livelihoods depend on agriculture. Despite the widespread occupational hazard coccidioidomycosis presents, awareness amongst the general public remains low, and diagnostic resources are often unavailable, which exacerbates underreporting and improper cataloging of cases. Furthermore, patients often lack the financial means to access proper treatment.

Coccidioides is particularly well studied in Mexico since the fungus' initial detection in the 1940s (Madrid, 1946, 1948) and majorly endemic of the northern states bordering the US Southwest (Laniado-Laborin *et al.*, 2019; Kirkland and Fierer, 2018). The climate and soil conditions in these states are similar to those in the US Southwest and they provide an adequate ecological niche for the fungus. Up to its dismissal as a reportable disease in 1994, an average of 1,500 cases were reported yearly in Mexico (Laniado-Laborin *et al.*, 2019). Although clinical data has been sparse since then, incidence in Mexico is likely to be the highest amongst Latin American nations as many estimates place statistics on par with the US endemic areas (Ajello, 1967). *Coccidioides*

antigen testing among Mexican populations have consistently demonstrated high infection rates and one particular study from the state of Coahuila revealed a 93% exposure rate within the local residents (Mondragon-Gonzalez *et al.*, 2005).

Central and South America have been less extensively studied and documented autochthonous cases are scarce. However, animal and human cases have been described since the mid 20th century and surveys have shown high reactivity rates in certain regions. In Central America, the majority of data indicates that *Coccidioides* is endemic to some degree in Honduras and Guatemala, specifically in the Motagua Valley as evidenced by a skin test survey of approximately 10,000 individuals (Laniado-Laborin *et al.*, 2019; Mayorga and Espinoza, 1970). The first case in Honduras was described in 1951 (Castro and Trejos, 1951), while Guatemala's first case was reported in 1960 (Garcia Valdes *et al.*, 1960). In South America, less than 1,000 cases have been reported to date in Colombia, Venezuela, Brazil, Paraguay, Bolivia and Argentina (Laniado-Laborin *et al.*, 2019). Over 800 of those cases have been found in Brazil (Giacomazzi *et al.*, 2016), but these statistics are likely confounded by factors such as underreporting, misdiagnosis, lack of access to care, underrepresentation in research, and lack of awareness. Antigen reactivity surveys have demonstrated a significant infection rate in the majority of these South American countries (ranging from 2% to 40%) given that the optimal ecological conditions for the fungus' growth and survival are present. Therefore, it is likely that a combination of the above confounding factors prevents the true scale of this disease in Latin America to be known.

Virulence and Pathogenesis

Respiration is the major avenue of entry into the body for coccidioidal arthroconidia. Once inhaled, arthroconidia (<5 μm in size) differentiate into spherules within the first few days of infection. Spherules are the multinucleated giant cells described by Posadas, Rixford, and Ophüls in their early investigations. The dose of arthroconidia inoculum required to initiate a progressive infection in humans is not well-known, but as few as 10–50 spores have been shown to cause disease in non-human primates (and other animal models) in less than 4–6 weeks post-challenge (Blundell *et al.*, 1961; Converse *et al.*, 1962). Spherules enlarge and segment endogenously until they achieve maturity (15–120 μm diameter) followed by rupture (endosporulation) which releases hundreds of endospores (<10 μm diameter). These endospores grow isotopically and mature themselves into 2nd generation endosporulating spherules thereby repeating the parasitic life cycle and propagating the infection.

As is the case with most foreign bodies in the alveolar space, host innate immune responses, specifically phagocytosis, are critical for clearance. Resident alveolar macrophages are appropriately sized to phagocytize arthroconidia, initial immature spherules, and endospores, but they are much too small to dispose of mature spherules (Castro-Lopez and Hung, 2017; Van Dyke *et al.*, 2019). Upon endosporulation, a chemotactic neutrophil response is mounted by the host and neutrophils are appropriately sized to clear initial spherules and endospores (Drutz and Huppert, 1983; Galgiani *et al.*, 1978). However, phagocytic cells such as macrophages and neutrophils are insufficient to clear the infection alone. *Coccidioides* can escape engulfment (Drutz and Huppert, 1983), prevent phagocytosis (Lee *et al.*, 2015), and inhibit phagolysosomal fusion (Lewis *et al.*, 2015). The role of neutrophils in response to coccidioidal infection is not well understood and appears to be complex and sometimes contradictory in nature. One study revealed similar susceptibility between neutrophil deficient mice and control mice to coccidioidal challenge, indicating that neutrophils play a limited role in the immune response to this fungus (Hung *et al.*, 2014). Further research is needed to fully elucidate neutrophil mechanisms and their effect on coccidioidomycosis, but it is likely that associated inflammatory responses and their modulation during coccidioidal infection are key in determining host outcomes (Van Dyke *et al.*, 2019).

As spherules mature, a membranous layer termed the spherule-outer wall (SOW) develops and sheds (Cole *et al.*, 1988a). A particular glycoprotein within this fraction (SOWgp) has been established as the immunodominant molecule as it exhibits high immunoreactivity to anti-coccidioidal antibodies isolated from patients (Cole *et al.*, 1988b; Hung *et al.*, 2000; Hung *et al.*, 2002). Interestingly, there is evidence that SOWgp is also a coccidioidal adhesin, possibly mediating the fungus' binding to respiratory tissues through ligands like laminin, fibronectin, and/or collagen (Hung *et al.*, 2002). For example, 58% of mice challenged with a SOWgp knockout strain of *C. immitis* survived beyond 40 days post-challenge while none of the mice challenged with wild type *C. immitis* survived longer than 21 days. Furthermore, the SOWgp mutant strain demonstrated a 30%–50% reduction in its ability to bind fibronectin and laminin *in vitro*. SOWgp contains characteristic tandem repeats of proline/aspartate motifs (Hung *et al.*, 2007), and concerted evolution of these sequences is a proposed mechanism for the ability of the fungus to evade host immune responses during release (Johannesson *et al.*, 2005). There is also evidence that proline-rich region are key motifs in other proteins that participate in adhesion of microbes to host tissues, suggesting the role of SOWgp as an adhesin (Hung *et al.*, 2002; Brady *et al.*, 1998; Perfect *et al.*, 1998; Staab *et al.*, 1999).

Coccidioides modulates the expression of SOWgp during different stages of its parasitic life cycle as copious amounts are synthesized and shed during maturation but much less so during endosporulation, when *Coccidioides* is most vulnerable to the host response, especially phagocytosis (Hung *et al.*, 2002). In addition, the fungus releases a metalloproteinase (Mep1) (Hung *et al.*, 2005) that digests SOWgp, lowering opsonization and conferring a higher degree of evasion potential to the endospores during this critical time (Hung *et al.*, 2005). Mice immunized with recombinant SOWgp and challenged with a Mep1 deficient *C. Posadasii* strain exhibited significantly increased survival compared to mice challenged with a wild type strain (Hung *et al.*, 2005).

The extracellular release and accumulation of ammonia during infection is also a proposed *Coccidioides* virulence mechanism, similar to what is observed in *Helicobacter pylori* infection (Mirbod-Donovan *et al.*, 2006; Mobley, 1996; Wise *et al.*, 2013). Endogenous coccidioidal urease and likely ureidoglycolate hydrolase catalyze the formation of ammonia in culture, the environment, and host tissues in order to increase the pH (Mirbod-Donovan *et al.*, 2006; Mirbod *et al.*, 2002; Yu *et al.*, 1997). The

accumulation of ammonia increases the alkalinity of infection microenvironments in the lung, resulting in localized tissue damage and exacerbation of respiratory disease. For instance, a urease deficient *C. posadasii* strain was less virulent to mice than the wild type strain (Mirbod-Donovan *et al.*, 2006; Wise *et al.*, 2013).

Coccidioides can produce melanin (Nosanchuk *et al.*, 2007) and this pigment confers protection against antifungal drugs, UV light, toxic metals, and other environmental stresses (Nosanchuk and Casadevall, 2003). However, limited information on *Coccidioides* melanization is available and further studies are needed.

Treatment and Prevention

The majority of patients with coccidioidomycosis have a subclinical infection and in many cases the disease can resolve on its own. There is no an effective therapy that results in resolution of coccidioidomycosis in all manifestations of the disease. There is also no data arising from randomized, double blinded, placebo controlled clinical trials on the decision to treat primary pulmonary infection. Therefore, current and past practice guidelines suggest a highly individualized treatment plan for patients (Galgiani *et al.*, 2005, 2016a, 2000). Administration of therapeutics is usually not recommended in immunocompetent patients with uncomplicated primary respiratory infection (uncomplicated pneumonia, asymptomatic nodule or cavity). However, patient health education, periodic reassessment of symptoms, and radiographic findings is highly encouraged. Disease related complications (diffuse bilateral pneumonia, symptomatic/ruptured pulmonary cavity, chronic progressive fibrocavitary pneumonia, meningeal/non meningeal dissemination) typically warrants antifungal therapy and possible surgical intervention. Antifungal therapy is typically long (many months to years) and might be indefinite or lifelong varying from case to case basis.

Therapeutics

The pharmacology and treatment of coccidioidomycosis has been comprehensively reviewed (Thompson *et al.*, 2019). Azole and polyene classes of antifungal drugs are the most common therapeutics used against coccidioidomycosis. Due to the variable toxicity and pharmacological profiles of these options, an individualized approach is highly emphasized in every treatment guideline (Thompson *et al.*, 2019). Fluconazole, for example, is the most frequently prescribed drug for coccidioidomycosis. This antifungal drug is low cost, has high bioavailability, is highly tolerable, distributes well into tissues, and can be administered orally or intravenously. This is especially important when treating central nervous system (CNS) disseminated coccidiodomycosis (Arndt *et al.*, 1988; Felton *et al.*, 2014). In contrast, itraconazole becomes highly protein bound in plasma and exhibits extremely low cerebrospinal fluid (CSF) and bone penetration. Despite this, treatment with itraconazole has shown efficacy in animal and clinical coccidioidal meningitis. Moreover, an enhanced response rate in patients treated with itraconazole demonstrated in comparison to fluconazole. Additional clinical data is needed to fully understand whether itraconazole is a more efficacious treatment in the setting of cerebral fungal infection.

Voriconazole is often utilized when patients become intolerant or refractory to fluconazole or itraconazole. The therapeutic exhibits less protein binding than itraconazole and an approximately proportional increase in CSF penetration also demonstrating efficacy in controlling dissemination to the CNS. All azole drugs typically exhibit some degree of hepatotoxicity, however, the concerns for toxicity as well as to drug-drug interactions are elevated for voriconazole (Thompson *et al.*, 2019; Freifeld *et al.*, 2009; Kim *et al.*, 2011). Posaconazole is another option for the treatment of refractory cases, despite its reduced CSF penetration. This drug has demonstrated promising sterilization in animal tissues, although data concerning this therapeutic is limited (Thompson *et al.*, 2019; Gonzalez *et al.*, 2002; Lutz *et al.*, 1997).

Before the advent of the azoles, amphotericin B was widely used in the treatment of coccidioidomycosis. Today and due to its high toxicity (Thompson *et al.*, 2019; Sawaya *et al.*, 1991), it is usually reserved for severe cases or for patients who are refractory or intolerant to other therapies (Thompson *et al.*, 2019; Galgiani *et al.*, 2016b). Amphotericin B also has poor penetration into the CNS when administered intravenously. Intrathecal delivery is an option but requires specialized training which can be unavailable to many patients suffering from coccidioidal meningitis.

Prophylaxis and Vaccination

There is little to no evidence supporting prophylactic therapeutic use to prevent *Coccidiodes* infection. However, a future vaccination option is likely because resolved coccidioidomycosis typically confers lifelong immunity to individuals that recovered from the disease. One of the first vaccine candidates (which proceeded to a phase III trial) was investigated in the 1960s when mice were immunized with formalin-killed spherules (FKS) (Levine *et al.*, 1960). Ultimately, the vaccine did not confer as much protection as anticipated and it induced an over reactive immune response in patients enrolled in a clinical trial (Pappagianis, 1993). Recent attempts at developing live attenuated vaccines have particularly focused on disrupting the chitinase (CTS_2 and CTS_3; ΔT vaccine) (Xue *et al.*, 2009) and CPS_1 (a chitinase homolog; ΔCPS_1 vaccine) genes (Narra *et al.*, 2016) in *Coccidiodes*. Both have shown efficacy in murine models and ΔCPS_1 is currently being developed as a vaccine for dogs (Narra *et al.*, 2016; Shubitz *et al.*, 2018). The ΔT vaccine candidate has also helped to understand the importance of Th1 and Th17 mediated immunity in achieving robust protection against *Coccidioides* (Hung *et al.*, 2011). However, live attenuated strains must display absolute safety in humans

especially in immunocompromised individuals, and there remains much work to be performed before this type, or indeed any vaccine proceeds to be use for human prophylaxis. It is important to note that the US Food and Drug Administration have never approved a live attenuated eukaryotic vaccine.

With the advent of recombinant technology, many vaccine efforts have targeted the use of recombinant spherule-derived antigens. Of these, antigen 2/proline rich antigen (Ag2/PRA), a component of a coccidioidal glycoprotein, has been the most extensively studied (Cox and Magee, 2004). *Coccidioides*-specific antigen (Csa) and peroxisomal matrix protein 1 (Pmp1) have also shown promise (Castro-Lopez and Hung, 2017; Van Dyke *et al.*, 2019). Although they all show individual promise, it has been proposed that a multivalent vaccine containing a number of these recombinant antigens will be necessary to elicit a protective immune response. Leading adjuvant and delivery system candidates are EP67 (Hung *et al.*, 2012), dendritic cells (Awasthi *et al.*, 2005; Awasthi *et al.*, 2019), and glucan particles (Hung *et al.*, 2018; Hurtgen *et al.*, 2012). It is likely that an effective vaccine will utilize such an adjuvant alongside an attenuated strain or multiple recombinant antigen vaccine to elicit a Th1 and Th17 immune response (Castro-Lopez and Hung, 2017; Van Dyke *et al.*, 2019).

Authorship

Both authors contributed to the writing of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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Blastomyces and Blastomycosis

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Overview

The *Blastomyces* species complex belongs to a group of fungi whose dominant biologic feature is thermal dimorphism, which is the ability to switch between hyphae (22–25°C) and yeast (37°C) (Gauthier, 2015). Currently, there are 7 species of Blastomyces: *B. dermatitidis*, *B. gilchristii*, *B. percursus*, *B. helicus*, *B. parvus*, *B. silverae*, and *B. emzantsi*. (Brown *et al.*, 2013; Muñoz *et al.*, 2015; Dukik *et al.*, 2017; Schwartz *et al.*, 2019; Jiang *et al.*, 2018; Maphanga *et al.*, 2020). Infection is acquired via inhalation of aerosolized conidia or hyphal fragments into the lung following spelled soil disruption. Once inhaled into the lungs, these infectious particles convert into pathogenic yeast to cause pneumonia. A subset of patients with pneumonia will develop disseminated infection. Diagnosing *Blastomyces* infection and initiating antifungal therapy in a timely manner can be difficult because blastomycosis mimics other infectious and non-infectious diseases (McBride *et al.*, 2017). A combination of fungal staining, fungal culture, and non-culture based tests are used for diagnosis (Saccente and Woods, 2010). Polyene and triazole antifungals are used for treatment (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019).

Historical Perspective

Blastomycosis was first described as a cutaneous disease in 1894 by Thomas Gilchrist and was initially thought be protozoan infection (Gilchrist, 1894). Additional research by Drs. Gilchrist and Stokes correctly the etiologic agent as a fungus, which was named *Blastomyces dermatitidis* (Gilchrist, 1896; Gilchrist and Stokes, 1896, 1898). The ability for *B. dermatitidis* to switch between mycelia and yeast forms in response to temperature was discovered in 1907 (Hamburger, 1907). In the early 20th century, pulmonary and disseminated blastomycosis was described. Colloquial names for blastomycosis included "Gilchrist Disease", "Chicago Disease", and "North American Blastomycosis". In the early 1950s, the lung was determined to be the primary portal of entry for infection (Schwarz and Baum, 1951). The first recognized outbreak of blastomycosis occurred in Grifton, North Carolina (1953–1954) (Smith *et al.*, 1955). In the mid-1980s, successful culture of *Blastomyces* from the soil in Eagle River, WI and Tomorrow River, WI in association with human outbreaks helped define the incubation period and ecologic niche (Klein *et al.*, 1986a, 1987a). Clinical trials conducted in the 1990s assessed the efficacy of itraconazole and fluconazole for treatment of blastomycosis (Pappas *et al.*, 1995, 1997; Dismukes *et al.*, 1992). Practice guidelines for blastomycosis were first published by the Infectious Diseases Society of American (IDSA) in 2000 and updated in 2008 (Chapman *et al.*, 2008, 2000). In 2011, the American Thoracic Society (ATS) Fungal Working Group published blastomycosis treatment guidelines in 2011 (Limper *et al.*, 2011). The American Society of Transplantation (AST) published their guidelines on the management of blastomycosis in transplant recipients in 2019 (Miller *et al.*, 2019).

Mycology and Phylogenetics

Blastomyces and other thermally dimorphic fungi such as *Emergomyces*, *Histoplasma*, *Coccidioides*, *Paracoccidioides*, *Sporothrix*, and *Talaromyces marneffei* (formerly *Penicillium marneffei*) belong to the Ascomycota phylum (Sil and Andrianopoulos, 2014). *Blastomyces* is a complex of 7 species: *B. dermatitidis*, *B. gilchristii*, *B. percursus*, *B. helicus (formerly Emmonsia helicus)*, *B. parvus (formerly Emmonsia parvus)*, *B. silverae, and B. emzantsi* (Brown *et al.*, 2013; Muñoz *et al.*, 2015; Dukik *et al.*, 2017; Schwartz *et al.*, 2019; Jiang *et al.*, 2018; Maphanga *et al.*, 2020). *B. dermatitidis* and *B. gilchristii* are estimated to have diverged 1.9 million years ago during the Pleistocene epoch (McTaggart *et al.*, 2016). *B. gilchristii* was named in honor of Dr. Gilchrist, who first described blastomycosis in the medical literature (Gilchrist, 1894). *Blastomyces silverae* was named after Eleanor Silver Keeping (née Dowding), a mycologist at the University of Alberta (Jiang *et al.*, 2018). The genomes of *B. dermatitidis*, *B. gilchristii*, and *B. percursus* have been sequenced and are publically available (Muñoz *et al.*, 2015; see "Relevant Website section"). *B. dermatitidis* and *B. gilchristii* contain 9180–10,187 genes and have a genome size of 66.6–75.4 MB, which is 2-fold larger than closely-related fungi such as *H. capsulatum* (Muñoz *et al.*, 2015). *B. percursus* has a similar number of genes but a smaller genome size, 32.3 MB (Dukik *et al.*, 2017). The genomes of *B. helicus*, *B. parvus*, and *B. silverae* have yet to be sequenced. Regardless of species, the defining biologic feature of these fungi is thermal dimorphism, the ability to reversibly convert between hyphal and yeast forms in response to temperature shifts (Gauthier, 2015).

In the environment (22–25°C), *Blastomyces* species grow as septated hyphae (1–2 μm diameter) with conidiophores that each bear a single conidia (4–5 μm) (**Fig. 1(A)**; Winn *et al.*, 2006; Wolf *et al.*, 1975). *B. percursus* conidiophores can bear single or multiple conidia (Dukik *et al.*, 2017). *B. helicus* hyphae have a unique coiled appearance and fail to produce conidia under most in vitro conditions (Schwartz *et al.*, 2019).

At 37°C, *Blastomyces* cells grow as yeast that are typically 8–20 μm in size with a doubly refractile cell wall and an average of 3–4 nuclei per cell (**Fig. 1(B)**; Saccente and Woods, 2010; Winn *et al.*, 2006; Wolf *et al.*, 1975; Clemons *et al.*, 1991). *Blastomyces* yeast

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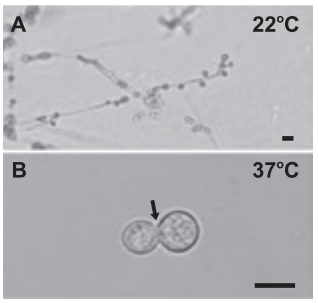

Fig. 1 Hyphal and Yeast forms of *Blastomyces dermatitidis*. (A) hyphal form with conidia at 22°C. (B) Yeast form at 37°C. Arrow points to broad-based budding between mother and daughter cells. Scale bar = 10 μm. Figure was modified from Gauthier, G.M., Klein, B.S., 2020. Chapter 264, Blastomycosis. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, ninth ed., vol. 2. Philadelphia, PA: Elsevier.

exhibit a broad-based pattern of budding between daughter and mother cells. This unique budding pattern allows *Blastomyces* yeast to be distinguished from other fungal pathogens (Saccente and Woods, 2010). In clinical medicine, *B. dermatitidis* and *B. gilchristii* are the 2 most commonly encountered agents of *Blastomyces*. These species are morphologically similar under light microscopy and differentiation requires PCR analysis or DNA sequencing (C for *B. gilchristii* and T for *B. dermatitidis* at base-pair 19 in the untranslated region of ITS2) (Brown *et al.*, 2013; Meece *et al.*, 2010; Frost *et al.*, 2016). *B. helicus* also has a broad-based budding pattern and sequencing of the ITS (internal transcribed spacer) or D1/D2 domains of the long subunit of ribosomal RNA can provide definitive identification (Schwartz *et al.*, 2019).

Although there is an ongoing effort to simplify fungal nomenclature (e.g., one fungus = one name), a dual nomenclature system is used with one name for the asexual form and another for sexual form (Taylor, 2011). *Blastomyces* refers to the asexual form and *Ajellomyces* refers to the sexual form (Li *et al.*, 2013; McDonough and Lewis, 1967). Asexual or clonal reproduction involves hyphal growth, production of asexual spores known as conidia, and yeast cell division. Sexual reproduction only occurs in the hyphal phase in which the hyphae of opposite mating types (+ and −) fuse to form mating structures (cleistothecia) for exchange of genetic material and development of ascospores (Li *et al.*, 2013; McDonough and Lewis, 1967). This is known as heterothallic mating. Homothallic or self mating has not been described. Typically, *B. dermatitidis* mates with *B. dermatitidis* and *B. gilchristii* mates with *B. gilchristii*; however, recent genotyping analyses suggest the potential for mating to occur between *B. dermatitidis* and *B. gilchristii* (Frost *et al.*, 2016; Li *et al.*, 2013). From a genetic perspective, the "+" mating type cells have the α-box gene and "−" mating type cells contain the HMG gene (Li *et al.*, 2013).

Geography and Ecology of *Blastomyces*

Blastomycosis is has been reported from 3 continents including North America, Africa, and Asia (**Fig. 2**). In North America, *Blastomyces* is not uniformly distributed, rather it is restricted to specific ecological niches within the endemic region. *B. dermatitidis* has the widest geographic distribution in North America which includes U.S. states located in watersheds of the Mississippi river, Ohio river, Savannah river, Saint Lawrence river, and areas adjacent to the Great Lakes (**Fig. 2(A)**; McTaggart *et al.*, 2016). In Canada, *B. dermatitis* has been identified in several provinces around the Nelson river and St. Lawrence river watersheds, the Great Lakes, and Saskatchewan (McTaggart *et al.*, 2016). In contrast, *B. gilchristii* has a more restricted distribution and overlaps with *B. dermatitidis* in Canada, Minnesota, Wisconsin and New York (**Fig. 2(A)**; McTaggart *et al.*, 2016). The differences in geographic distribution between *B. dermatitidis* and *B. gilchristii* are hypothesized to be related to differences in glaciation in the Pleistocene epoch, which was when *B. dermatitidis* and *B. gilchristii* diverged, approximately 1.9 million years ago (McTaggart *et al.*, 2016). *B. gilchristii* is restricted to formerly glaciated areas (McTaggart *et al.*, 2016). The ecologic niche inhabited by *B. dermatitidis* and *B. gilchristii* is characterized by sandy soils with an acidic pH and decaying vegetation that are nearby waterways (Klein *et al.*, 1986a,b; Klein et al., 1987a,b; Reed *et al.*, 2008). *B. helicus* has been isolated from humans and animals in the Western United States (California, Montana, Idaho, Colorado, Nebraska, Texas) and Canada (Saskatchewan, Alberta) (**Fig. 2(A)**; Schwartz *et al.*, 2019). *B. helicus* does not appear to overlap with the endemic regions for *B. dermatitidis* or *B. gilchristii* (Schwartz *et al.*, 2019). *B. silverae* isolates have been from the U.S. or Candida; however, the endemic range has yet to be fully elucidated (Jiang *et al.*, 2018). The characteristics of specific ecological niche inhabited by *B. helicus* and *B. silverae* are unknown.

Outside of North America, autochthonous culture-proven cases of blastomycosis have been reported from Africa, India, and Israel (**Fig. 2(B)**). Approximately 100 human cases of blastomycosis have been reported from 18 African countries (Baily *et al.*, 1991; Carman *et al.*, 1989). The majority is isolates were identified before phylogenetic reclassification and have not undergone molecular analysis or sequencing. Thus, the majority of these isolates have been presumptively classified as *B. dermatitidis*; however, a recent study suggests that many African *Blastomyces* isolates may be *B. percursus* or *B. emzantsi* (Maphanga *et al.*, 2020). African *Blastomyces* stains have a decreased capacity to transition to yeast at 37°C, possess unique media requirements for growth, and possess a less antigenically complex cell surface (Klein *et al.*, 1997). *B. percursus* has been cultured from humans in South Africa

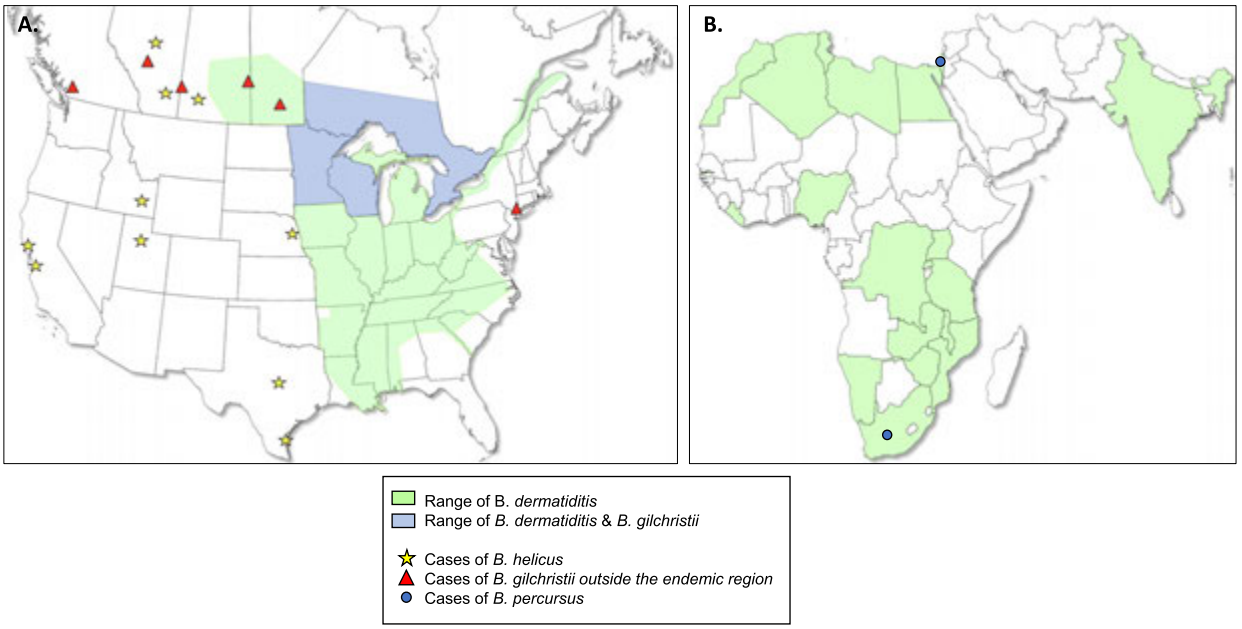

Fig. 2 Geographic distribution of *Blastomyces*. (A) Endemic regions of *B. dermatitidis*, *B. gilchristii*, and *B. helicus* in North America. (B) Geographic range of *Blastomyces* outside of North America. Because the endemic regions for *Blastomyces* spp. in Africa, India, and Israel are unknown, whole countries were shaded for illustrative purposes.

and Israel (Dukik *et al.*, 2017). In India, less than 10 culture-confirmed autochthonous cases of blastomycosis have been reported (Rao *et al.*, 2013; Randhawa *et al.*, 2013). The characteristics that define the ecologic niche for *Blastomyces* species in Africa and Asia is unknown. Blastomyces is not endemic to Central America, South America, Europe, or Australia.

Epidemiology

The absence of reliable skin and serologic tests to investigate *Blastomyces* exposure in populations living in endemic and non-endemic regions has limited the epidemiologic understanding of blastomycosis to persons with clinically apparent infection. Minnesota, Wisconsin, Michigan, Arkansas and Louisiana require mandatory reporting of human blastomycosis. In Canada, blastomycosis is reportable in Manitoba and confirmed human cases in Ontario are monitored by the Northwestern Health Unit jurisdiction. Outside of the United States and Canada, the epidemiology of blastomycosis is unknown.

Within the endemic region for *B. dermatitidis* and *B. gilchristii*, the annual incidence ranges from 0.11 to 2.17 cases/100,000 persons with the highest rates in the Midwest and Southern United states (**Table 1**). Within the endemic region, certain areas are hyperendemic for blastomycosis such as Vilas County in Wisconsin and Kenora in Ontario (Baumgardner *et al.*, 1992; Dwight *et al.*, 2000). Although the majority of blastomycosis cases are sporadic, urban and rural outbreaks have been reported from 7 states and are associated with activities that disrupt soil such as construction or fresh water recreation (**Table 2**). Outbreak investigations have been critical defining the ecologic niche in North America, delineating the risk for symptomatic infection following exposure (approximately 50% develop symptoms), determining the incubation period (3 weeks – 3 months), and understanding environmental risk factors for blastomycosis (**Table 2**; Klein *et al.*, 1986a, 1987a). Outbreaks of blastomycosis in Marathon County, WI in 2006 and 2009–2010 found high rates of symptomatic blastomycosis in persons of Hmong ethnicity (odds ratio 12.1; 95% confidence interval 1.3–611.9; $p = 0.019$) that was unrelated to environmental exposure (Roy *et al.*, 2013). The frequency of disseminated blastomycosis was not increased in persons of Hmong ethnicity during these outbreaks (Roy *et al.*, 2013). This novel and unexpected finding was hypothesized to be related to an immunologic predisposition for infection (Roy *et al.*, 2013). An increased incidence of blastomycosis has also been described for indigenous persons living in Ontario and Manitoba (Crampton *et al.*, 2002; Dalcin and Ahmed, 2015; Kralt *et al.*, 2009).

Pathogenesis, Virulence, and Host Defense

Overview

Relatively few of the estimated 1.5–5.1 million fungal species are known to cause invasive infection in humans or animals (Blackwell, 2011). For the majority of human pathogenic fungi, impairment of host immune defenses is often a prerequisite for

Table 1 Epidemiology of blastomycosis in United States and Canada

State or province	*Years*	*Incidence per 100,000 persons*	*References*
Wisconsin	2004–2016	2.17	Wisconsin Department of Health Services (2018)
Minnesota	2012–2016	0.6	Minnesota Dept. of Health[a]
Illinois	2001–2007	0.4–1.1[b]	Illinois Dept. of Public Health[c]
Michigan	2012–2016	0.14 (0.11–0.20)	Michigan Dept. of Health & Human Services[d]
Arkansas	2007–2016	0.39 (0.2–0.74)	Arkansas Department of Health[e]
Louisiana	1987–2016	0.11	Louisiana Department of Health[f]
Tennessee	1996–2005	1.29	Vasquez *et al.* (1998), Hussein *et al.* (2009)
NW Ontario	1989–2005	17.0	Litvinjenko and Lunny (2017)
Kenora, Ontario	1997–1999	117.2	Dwight *et al.* (2000)
Manitoba	1988–1999	0.62	Crampton *et al.* (2002)

[a]www.health.state.mn.us/divs/idepc/newsletters/dcn/annual.html.
[b]Highest incidence of infection occurred in northeastern Illinois (Cook, Lake, Kane, and Will Counties).
[c]http://www.idph.state.il.us/health/infect.
[d]Personal communication from Kimberly Signs, Michigan Department of Health and Human Services. April 2018.
[e]Personal communication from Dirk Haselow and Haythan Safi, Arkansas Department of Health. November 2017.
[f]Personal communication from Jose Serrano, Louisiana Department of Health. November 2017; Louisiana Office of Public Health – Infectious Disease Epidemiology Section. Blastomycosis Annual Report 2016.
Source: Table 1 was adapted from Gauthier, G.M., Klein, B.S., 2020. Chapter 264, Blastomycosis. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, ninth ed., vol. 2. Philadelphia, PA: Elsevier.

Table 2 Outbreaks of Blastomycosis in the United States

Year	*State*	*No. of cases*	*Rural or urban*	*Source of the outbreak*	*References*
1953–1954	NC	11	Rural	Not identified	Smith *et al.* (1955)
1972	MN	12	Rural	Cabin construction	Tosh *et al.* (1974)
1975–1976	NC	5	Rural	Peanut harvest	Centers for Disease Control (1976)
1974–1975	IL	5	Urban	Construction	Kitchen *et al.* (1977)
1979	WI	8	Rural	Canoeing	Cockerill *et al.* (1984)
1984	WI	48	Rural	Beaver lodge	Klein *et al.* (1986a)
1984	VA	4	Rural	Raccoon hunting	Armstrong *et al.* (1987)
1985	WI	7	Rural	Fishing on river bank	Klein *et al.* (1987a)
1985	WI	7	Rural	Underground fort	Klein *et al.* (1987a)
1988	WI	32	Rural	Hotel construction	Baumgardner and Burdick (1991)
1989	TN	3	Urban	Factory construction	Frye and Seifer (1991)
1989–1990	WI	8	Rural	Not identified	Proctor *et al.* (2002)
1998	CO	2	Rural	Prairie dog relocation	De Groote *et al.* (2000)
1998 – 2000	WI	9	Rural	Likely excavation	Baumgardner *et al.* (2002)
1999	MN	18	Urban	Excavation	Minnesota Dept. of Health[a]
2001–2002	NC	8	Rural	Likely construction or excavation	MacDonald *et al.* (2006)
2005 – 2008	IN	59	Urban	Highway construction	Carlos *et al.* (2010)
2006	WI	21	Urban	Pine needle yard waste	Pfister *et al.* (2011)
2009–2010	WI	55	Urban	Not identified	Roy *et al.* (2013)
2015	WI	90	Rural	Tubing on the river	Koske *et al.* (2015)

[a]http://www.health.state.mn.us/divs/idepc/diseases/blastomycosis/dcn603blasto.html.
Source: Table 2 was modified from Gauthier, G.M., Klein, B.S., 2020. Chapter 264, Blastomycosis. In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, ninth ed., vol. 2. Philadelphia, PA: Elsevier.

invasive infection. However, the thermally dimorphic fungi can infect and cause disease in mammalian hosts with either intact or impaired immune defenses (Gauthier, 2015). Shared virulence traits include yeast growth 37°C (i.e., thermotolerance), intracellular survival, and upregulation of yeast-phase specific virulence factors that subvert host defenses (Gauthier, 2015).

The defining biologic feature of the thermally dimorphic fungi is the temperature-dependent reversible switch between hyphae and yeast which is known as the phase transition. Temperature is the predominant stimulus that drives the morphologic switch. Hyphal growth occurs at 22–25°C, whereas yeast growth occurs at 37°C. In addition to temperature, the uptake of exogenous cysteine is needed to complete the conversion to yeast following a increase in temperature from 22 to 25 to 37°C (Medoff *et al.*, 1987; Maresca *et al.*, 1981). Cysteine uptake induces resumption of mitochondrial respiration during the phase transition (Medoff *et al.*, 1987; Maresca *et al.*, 1981). In contrast to *Coccidioides* and *Paracoccidioides*, exogenous CO_2 tension or estradiol do not influence the morphologic switch of *Blastomyces* (Klotz *et al.*, 1984; Powell *et al.*, 1983; Shankar *et al.*, 2011). In vitro experiments

have demonstrated that engulfment of conidia by macrophages also accelerates the transition to yeast (Sterkel *et al.*, 2015). In vivo during infection, *Blastomyces* yeast undergo intracellular and extracellular replication, which is a conserved feature among the thermally dimorphic fungi (Sterkel *et al.*, 2015).

Molecular Regulation of the Phase Transition to Yeast

The discovery of a group III hybrid histidine kinase encoded by *DRK1* (Dimorphism Regulating Kinase-1) provided definitive proof that the phase transition to yeast was essential for virulence (Nemecek *et al.*, 2006; Lawry *et al.*, 2017). *DRK1* is part of high-osmolarity glycerol (HOG) signaling pathway which is important for adaptation to osmotic and oxidative stresses. Deletion of *DRK1* or reduction in *DRK1* transcript by RNA interference (RNAi) inhibits the conversion of conidia or hyphae to yeast at 37°C (Nemecek *et al.*, 2006). Although *DRK1*Δ cells remain viable at 37°C, they are locked as hyphae, exhibit alterations in the cell wall, fail to upregulate the essential yeast-phase virulence factor *BAD1* (*Blastomyces* adhesion-1, formerly WI-1), and are avirulent in a murine model of pulmonary infection (Nemecek *et al.*, 2006). At room temperature, *DRK1*Δ cells have reduced capacity to produce conidia (Nemecek *et al.*, 2006). The functional role of *DRK1* on the morphologic transition is conserved in *Histoplasma capsulatum* and *Talaromyces marneffei* (formerly *Penicillium marneffei*) (Nemecek *et al.*, 2006; Boyce *et al.*, 2011). *H. capsulatum DRK1* RNAi knockdown strains remain locked as hyphae at 37°C, fail to upregulate the essential virulence factor *CBP1* (Calcium-Binding Protein-1), and are avirulent in a murine model of pulmonary infection (Nemecek *et al.*, 2006). Deletion of *DRK1* impairs the germination of *T. marneffei* conidia to yeast in macrophages (Boyce *et al.*, 2011).

Virulence Factors

In addition to thermotolerance, upregulation of yeast-phase virulence factors is critical for survival within the host. *B. dermatitidis* and *B. gilchristii* yeast upregulate *BAD1*, an essential virulence factor (Brandhorst *et al.*, 1999, 2003, 2013; Beaussart *et al.*, 2015; Finkel-Jimenez *et al.*, 2001, 2002). In vivo transcriptional profiling during a murine model of pulmonary infection demonstrated that *BAD1* was the most highly upregulated gene in the yeast transcriptome (Muñoz *et al.*, 2015). *BAD1* encodes a secreted multifunctional 120 kDa protein (Bad1) that can remain soluble in the extracellular milieu or bind back to coat yeast cells via its interactions with chitin in the cell wall (Brandhorst *et al.*, 1999, 2003, 2013; Beaussart *et al.*, 2015). Bad1 promotes adhesion of yeast cells to host tissues by binding heparin sulfate and adherence to host immune cells by binding with complement receptors (CR3, CD14) (Brandhorst *et al.*, 2013; Beaussart *et al.*, 2015; Finkel-Jimenez *et al.*, 2001). Soluble and bound Bad1 inhibit production of TNF-α by macrophages and neutrophils; however, the mechanisms are different. Bound Bad1 inhibits TNF-α production in a TGF-*β*-dependent manner whereas soluble Bad1 inhibits TNF-α production independent of TGF-*β* (Finkel-Jimenez *et al.*, 2001, 2002). In addition to affecting TNF-α, Bad1 impairs activation of CD4 + T lymphocytes, which in turn, reduces IL-17 and INF-*γ* (Brandhorst *et al.*, 2013). Deletion of *BAD1* renders *Blastomyces* avirulent in a murine model of pulmonary infection (Brandhorst *et al.*, 1999). Thus, *BAD1* is an essential virulence gene. In addition to Bad1 modulating the immune system, yeast cells also secrete dipeptidylpeptidase IVA (DppIVA), a serine protease that impairs the recruitment of innate immune cells to the lungs by inactivating granulocyte-macrophage colony stimulating factor (GM-CSF) (Sterkel *et al.*, 2016).

Other mechanisms that influence *Blastomyces* virulence include alteration in cell wall carbohydrate composition, relative resistance to oxidative and nitrosative killing, and zinc uptake. During the temperature-mediated shift from hyphae-to-yeast, the concentration of *β*-(1,3)-glucan in the cell wall decreases from 40% to <5% (Kanetsuna and Carbonell, 1971). This reduction has the potential to limit the ability of host immune cells to recognize *Blastomyces* yeast via dectin-1 and mannose receptors (Koneti *et al.*, 2008). From a clinical perspective, the paucity of yeast cell wall *β*-(1,3)-glucan renders the echinocandin antifungals ineffective for treatment and makes (1,3)-Beta-D-Glucan blood test unreliable for diagnosis (Nakai *et al.*, 2003; Girouard *et al.*, 2007). During infection, yeast cells upregulate superoxide dismutase and catalase, which may contribute to reduced sensitivity to reactive oxygen species (Muñoz *et al.*, 2015; Brummer *et al.*, 1992). In addition, yeast cells suppressed nitric oxide product by macrophages (Rocco *et al.*, 2011). The mechanism underlying resistance to nitric oxide killing has yet to be fully elucidated but is likely due to inhibition of inducible nitric oxide synthase enzymatic activity (Rocco *et al.*, 2011). During murine pulmonary infection, genes involved with zinc scavenging (*PRA1*) and update (*ZRT1*) are upregulated (Muñoz *et al.*, 2015). *PRA1* encodes a secreted zinc-binding protein that functions as a "zincophore", whereas *ZRT1* encodes a high-affinity zinc transporter (Muñoz *et al.*, 2015). Disruption of *PRA1* and *ZRT1* transcription by CRISPR/Cas9 gene editing results in attenuated virulence during pulmonary infection (Kujoth *et al.*, 2018).

In sharp contrast to histoplasmosis and coccidioidomycosis, disseminated infection with blastomycosis is common in patients with normal and impaired immune defenses (Hage *et al.*, 2015; Odio *et al.*, 2017). Recent data from the Marshfield Clinic in Wisconsin suggests that the propensity for dissemination is species specific (Meece *et al.*, 2010; Frost *et al.*, 2016). In patients infected with *B. dermatitidis*, disseminated infection occurred in 31.4%–33.6%, whereas 7.8%–9.3% with *B. gilchristii* had disseminated disease (Meece *et al.*, 2010; Frost *et al.*, 2016). Thus, *B. dermatitidis* is more likely to disseminate and *B. gilchristii* is more likely to remain localized to the lungs. Single nucleotide polymorphism (SNP) genotyping has identified significant allelic differences in 11 genes including those known to be involved with virulence (*BAD1*) and the temperature-dependent morphologic shift to yeast (*DRK1*) (Frost *et al.*, 2016). Subgroup analysis focusing on *B. dermatitidis* uncovered allelic differences for urease, septin1, and α-1,3-glucan synthase encoding genes (Frost *et al.*, 2016). Collectively, this suggests that

pathogen-related factors may influence dissemination. However, despite advances in understanding pathogenesis, the absence of a model system that mimics human disseminated disease has hindered the ability to delineate mechanism(s) that promote extrapulmonary spread. However, it is likely that multiple mechanisms influence dissemination including, but not limited to, subversion of host immune defenses, ability for intracellular replication in macrophages, suppression of nitric oxide production, and relative resistance to oxidative stress.

In vivo transcriptional profiling during murine infection has identified novel genes in *Blastomyces dermatitidis* that have the potential to influence dissemination (Muñoz *et al.*, 2015). This includes genes involved with nickel transport (*NIC1*), urease (*URE*), and cysteine metabolism (*CDG*, *SSU1*). Nickel is an important cofactor for the urease enzyme, which catalyzes the conversion of urea to ammonia and carbon dioxide. In *Coccidioides*, urease-encoding genes (*URE*) contributes to pathogenesis by enhancing tissue damage (Mirbod-Donovan *et al.*, 2006). In *Cryptococcus neoformans NIC1* and *URE1* promote extrapulmonary dissemination to the central nervous system (Singh *et al.*, 2013). In patients with disseminated blastomycosis, the most common extrapulmonary site of infection is the skin, occurring in 40%–80% (Chapman *et al.*, 2008). There is potential that upregulation of genes involved with cysteine catabolism could influence growth of *B. dermatitidis* in keratinized tissues such as skin. The dermatophyte *Arthoderma benhamiae* exports sulfite to degrade keratin to facilitate growth on keratinized tissues. In this process, cysteine dioxygenase (*CDG*) breaks down cysteine to sulfite which is then exported by a transmembrane sulfite efflux pump (*SSU1*) (Grumbt *et al.*, 2013). During in vivo infection, *B. dermatitidis* upregulates *CDG* and *SSU1* homologs (Muñoz *et al.*, 2015). Functional testing of these candidate genes (*NIC1*, *URE*, *CDG*, *SSU1*) in a model of disseminated infection is needed.

Molecular Regulation of the Phase Transition to Hyphae

Growth as hyphae is essential for genetic diversity via sexual reproduction, production of conidia, and survival in the environment. In *Blastomyces dermatitidis* and *Histoplasma capsulatum*, the conversion from yeast to hyphae following a drop in temperature from 37°C to 22–25°C is regulated in part by GATA transcription factor homologs *SREB* (Siderophore Biosynthesis Repressor in *Blastomyces*) and *SRE1* (Siderophore Repressor-1), respectively (Gauthier *et al.*, 2010; Marty *et al.*, 2015; Hwang *et al.*, 2012). Deletion of *B. dermatitidis SREB* or RNAi knockdown of *H. capsulatum SRE1* impairs the ability of yeast to convert to hyphae and results in excessive of siderophore biosynthesis and iron uptake (Gauthier *et al.*, 2010; Marty *et al.*, 2015; Hwang *et al.*, 2012). Siderophores are iron-gathering molecules produced by fungi to scavenge extracellular iron. The defect in the phase transition to hyphae is independent of alterations in siderophore biosynthesis and iron uptake (Gauthier *et al.*, 2010; Marty *et al.*, 2015). Using a combination of gene expression microarray, chromatin immunoprecipitation with sequencing and quantitative PCR, and functional testing indicated the defect in the morphologic shift in *B. dermatitidis SREB*Δ was due to reduced biosynthesis of neutral lipids and lipid droplets (Marty *et al.*, 2015). The defect in hyphal development at 22°C was temporally associated with reduced transcription of neutral lipid biosynthetic genes, decreased production of triacylglycerol and ergosterol, and a paucity of lipid droplets (Marty *et al.*, 2015). Lipid droplets are intracellular organelles composed of a triacylglycerol and ergosterol core and surrounded by a single phospholipid layer. The morphologic and lipid droplet defects in *SREB*Δ were partially restored when the media was supplemented with exogenous saturated fatty acids (Marty *et al.*, 2015). Supplementation with unsaturated fatty acids did not correct developmental or lipid droplet defects (Marty *et al.*, 2015). Other mechanisms that promote hyphal development include sensing of N-acetylglucosamine (GlcNAc), a component of chitin (Gilmore *et al.*, 2013). Exogenous GlcNAc accelerates *B. dermatitidis* and *H. capsulatum* hyphal development following a shift from 37°C to room temperature. Reduced transcription of GlcNAc transporters *NGT1* and *NGT2* delays hyphal development at room temperature (Gilmore *et al.*, 2013).

Immunology & Vaccine Development

Blastomyces uses several strategies to suppress and evade the immune system, however the host can use innate and adaptive immune cells to defend itself. In a murine model, macrophages and neutrophils kill a large percentage of conidia inhaled into the lungs (Sugar *et al.*, 1995). In immune competent hosts, $CD4^+$ T lymphocytes coordinate the adaptive immune response to control infection by enhancing the fungicidal activity of phagocytes against the yeast (Bradsher *et al.*, 1987; Wüthrich *et al.*, 2011). This process requires TNF-α, INF-γ and IL-17 cytokines (Bradsher *et al.*, 1987; Wüthrich *et al.*, 2011). Cell mediated immunity (CMI) after *Blastomyces* infection can last for at least 2 years (Klein *et al.*, 1990).

The importance of CMI is underscored by recent findings in human populations. Individuals of Hmong ancestry have higher rates of epidemic and endemic pulmonary blastomycosis and have been studied for a genetic predisposition to infection (Roy *et al.*, 2013; Merkhofer *et al.*, 2019). Whole genome sequencing analysis revealed single nucleotide polymorphisms in immune response genes that distinguish individuals of Hmong ancestry from those of European ancestry (Merkhofer *et al.*, 2019). The IL-6 locus in particular displayed multiple polymorphisms that differed between the two groups. Likewise, IL-6 responses to stimuli were found to be significantly lower in the Hmong than in European ancestry individuals (Merkhofer *et al.*, 2019). IL-6 is a cytokine with pleotropic effects on immune cells, but plays a critical role in the development of Th17 cells. The development of anti-fungal Th17 memory cells was correspondingly reduced in Hmong individuals, as compared to individuals of European ancestry (Merkhofer *et al.*, 2019). Finally, a murine model of infection showed similar defects in the development of anti-fungal adaptive immunity and resistance in animals that lacked any IL-6 production (Merkhofer *et al.*, 2019).

Vaccines that induce cell-mediated immune defenses have the potential to prevent invasive fungal infections, including blastomycosis. When *BAD1* null yeast (*BAD1*Δ) are injected subcutaneously in mice, it induces sterilizing immunity, which results in complete protection against lethal pulmonary infection (Wüthrich *et al.*, 2000). Following subcutaneous injection, *BAD1*Δ yeast are transported by inflammatory ($CCR2^+$) monocytes to lymph nodes for antigen transfer and presentation to resident dendritic cells (Ersland *et al.*, 2010). This induces naïve $CD4^+$ T lymphocytes to differentiate into Th17 cells via dectin-2/FcRγ/Syk/Card9, dectin-3, and mannan receptor signaling pathways (Ersland *et al.*, 2010; Wang *et al.*, 2014, 2015, 2016). In response to infection, differentiated Th17 cells migrate to the lungs and secrete IL-17, which in turn, recruits and activates neutrophils and macrophages (Wüthrich *et al.*, 2011). In the absence of CD4+ T cells, *BAD1*Δ vaccinated mice activate IL-17 CD8+ T lymphocytes (Tc17 cells) via Myd88-Akt1-mTOR signaling to mediate vaccine immunity against *Blastomyces* pulmonary infection (Nanjappa *et al.*, 2014, 2015, 2017).

The antigenic component of the *BAD1*Δ vaccine that induces immunity is a 13-amino acid sequence in calnexin (Wüthrich *et al.*, 2015). This antigenic sequence is conserved among ascomycete fungi including *H. capsulatum, Coccidioides posadasii, Aspergillus fumigatus, Fonsecaea pedrosoi, and Pseudogymnoascus destructans* (Wüthrich *et al.*, 2015). This knowledge has led to a calnexin-based vaccine. The addition of *Blastomyces* endogluconase-2 (Bl-Eng2) as an adjuvant greatly enhances the immunogenicity of the calnexin vaccine and protects mice against pneumonia (Wang *et al.*, 2017). Bl-Eng2 not only harbors adjuvant activity, but also contains antigenic epitopes for T-helper cells that mediate strong protective immunity against lethal experimental infection (Dobson *et al.*, 2020).

Clinical Manifestations of Blastomycosis

Overview

Blastomyces infection is typically acquired through inhalation of infectious particles that are aerosolized following disruption of soil. Following inhalation, these particles convert to yeast to cause pneumonia. Once infection is established and host immune defenses are avoided, *Blastomyces* yeast can remain localized to the lung or disseminate to almost any organ. Cutaneous inoculation following trauma is a rare and unusual mechanism of acquisition (Gray and Baddour, 2002; Graham and Callaway, 1982; Gnann *et al.*, 1983; Harris *et al.*, 2011).

Blastomyces infects humans and animals; however, blastomycosis is not considered a zoonotic disease because *Blastomyces* is not transmitted between animal-to-humans. The most common animal infected by *Blastomyces* are dogs, which have a 10-fold higher incidence then humans (Anderson *et al.*, 2014). Although humans and their pets can be infected simultaneously or sequentially, this is due to common source of exposure, not zoonotic transmission (Anderson *et al.*, 2014; Sarosi *et al.*, 1979).

Blastomycosis is known as the "great pretender" because it can mimic bacterial pneumonia, viral pneumonia, pulmonary tuberculosis, lung cancer, laryngeal cancer, skin cancer, pyoderma gangrenosum, and sarcoidosis (Lemos *et al.*, 2002; Bradsher, 2014). Pulmonary blastomycosis is often initially misdiagnosed as a bacterial infection that results in initiation of antibiotics rather than antifungals. In one study, patients received a median of 2.5 courses of antibiotics before blastomycosis was diagnosed (Alpern *et al.*, 2016). Another challenge in recognizing blastomycosis is the long incubation period following environmental exposure, 3 weeks to 3 months (Klein *et al.*, 1986a,b; Klein *et al.*, 1987a,b). Thus, blastomycosis can manifest in any season including the winter.

Human Blastomycosis

Pulmonary Blastomycosis

Pneumonia is the most common clinical manifestation of blastomycosis and occurs in 69%–93% of patients. (Chapman *et al.*, 1997; Vasquez *et al.*, 1998; Hussein *et al.*, 2009; Crampton *et al.*, 2002; Kralt *et al.*, 2009; Lemos *et al.*, 2002; Light *et al.*, 2008; Baumgardner *et al.*, 2004; Azar *et al.*, 2015). Severity ranges from asymptomatic infection, acute pneumonia, acute respiratory distress syndrome (ARDS), to chronic pneumonia (Davies and Sarosi, 1997). There are no pathognomonic clinical or radiographic manifestations to reliably distinguish blastomycosis from other pulmonary diseases. Acute pulmonary blastomycosis often mimics community-acquired pneumonia (CAP) with symptoms of cough, chest pain, fever, chills, decreased appetite, and malaise (Baumgardner *et al.*, 1992; Chapman *et al.*, 1997; Vasquez *et al.*, 1998; Crampton *et al.*, 2002; Kralt *et al.*, 2009; Baumgardner *et al.*, 2004; Azar *et al.*, 2015). Typical radiographic pattern for acute blastomycosis includes lobar consolidation and is indistinguishable from CAP (**Fig. 3(A)**; Fang *et al.*, 2007). Patients with ARDS have involvement of all 5 lobes of the lung and experience rapid decompensation in respiratory status that requires either mechanical ventilation or extracorporeal membrane oxygenation (ECMO). Risk factors for severe pulmonary blastomycosis are multilobar pneumonia, diabetes, and immunosuppression (Kralt *et al.*, 2009; Azar *et al.*, 2015; Schwartz *et al.*, 2016). In chronic pulmonary blastomycosis, patients have slowly progressive symptoms over a period of months that include fever, chills, night sweats, cough, hemoptysis, weight loss, and malaise. Chest radiography often demonstrates dense consolidation or cavity lesions. Other radiographic patterns associated with blastomycosis include masses that mimic lung cancer, nodules (**Fig. 3(B)**), and interstitial infiltrates (Fang *et al.*, 2007). A miliary chest radiographic pattern suggests a high burden of infection, which in turn, increases the risk for respiratory failure if antifungal therapy is not quickly started (Fang *et al.*, 2007). Uncommon chest imaging findings include pleural effusion, empyema, and hilar adenopathy (Fang *et al.*, 2007).

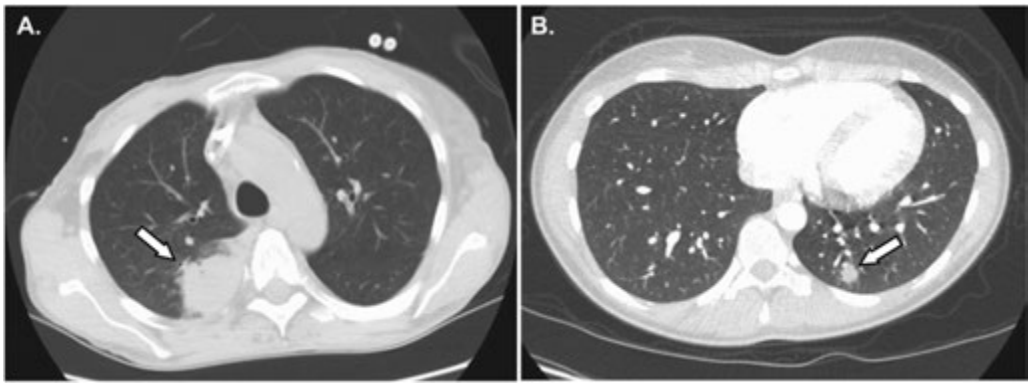

Fig. 3 *Pulmonary manifestations of blastomycosis.* (A) Pulmonary infiltrate in the right lung (arrow). (B) Pulmonary nodule in the left lung (arrow).

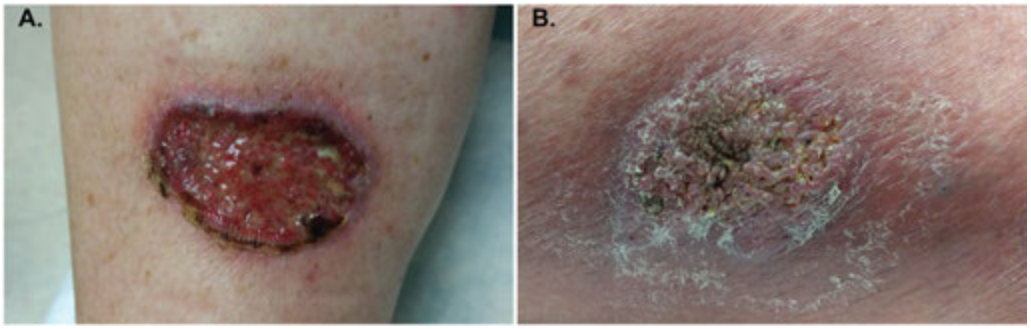

Fig. 4 *Skin lesions of blastomycosis.* (A) Ulcerative skin lesion. (B) Verrucous skin lesion.

Disseminated Blastomycosis

Extrapulmonary dissemination of blastomycosis is estimated to occur in 15%–48% of infected persons and can infection almost any organ. (Baumgardner *et al.*, 1992; Chapman *et al.*, 1997; Vasquez *et al.*, 1998; Hussein *et al.*, 2009; Crampton *et al.*, 2002; Carlos *et al.*, 2010; Kralt *et al.*, 2009; Light *et al.*, 2008; Azar *et al.*, 2015; Lemos *et al.*, 2000). Risk factors for disseminated disease include infection with *B. dermatitidis* (versus *B. gilchristii*) and prolonged duration of pulmonary symptoms (Crampton *et al.*, 2002; Frost *et al.*, 2016). In HIV seropositive patients with a CD4+ T-lymphocyte count <200 cells/mm^3, there is an increased risk for dissemination to the central nervous system (CNS) (Pappas *et al.*, 1992). In contrast, solid organ transplant recipients do not have a predilection CNS blastomycosis and overall dissemination rates range from 33% to 50% (Gauthier *et al.*, 2007; Grim *et al.*, 2012).

The most common sites for disseminated infection are the skin, bone, and prostate. Cutaneous blastomycosis often presents as papule that slowly expands to form ulcerative or verrucous lesions (**Fig. 4**). Any cutaneous region can be involved including the scalp, face, trunk, arms, and legs. Skin lesions can range from small (<1 cm) to large lesions that cause extensive cutaneous damage and heal by scarring.

The second most common site of extrapulmonary infection is the bone, occurring in 15%–44% (Chapman *et al.*, 1997; Vasquez *et al.*, 1998; Hussein *et al.*, 2009; Kralt *et al.*, 2009; Light *et al.*, 2008; Azar *et al.*, 2015; Lemos *et al.*, 2000). Bone infection or osteomyelitis is characterized by pain and soft tissue swelling (Oppenheimer *et al.*, 2007). Most patients will have concomitant pneumonia; however, a subset will not have obvious pneumonia (Oppenheimer *et al.*, 2007). The most frequently sites of osteomyelitis are the vertebrae, long bones (i.e., humerus, femur, tibia), and ribs. Infection in the bone can extend into the soft tissues to cause abscesses, skin ulcers, sinus tracts that drain pus, and septic arthritis (Oppenheimer *et al.*, 2007; Jain *et al.*, 2014). Vertebral osteomyelitis, which typically presents with back pain, can be complicated infection of the intervertebral disks (diskitis), epidural abscesses that compress the spinal cord, paraspinal soft tissue abscesses, and collapse of vertebrae resulting in neurologic impairment (Gehweiler *et al.*, 1970; Saccente *et al.*, 1998). Analogous to pulmonary blastomycosis, there are no radiographic findings that can distinguish bone infection from *Blastomyces* to bacterial osteomyelitis, tumor, or tuberculosis (Gehweiler *et al.*, 1970).

The third most frequent site of dissemination is the genitourinary (GU) system, which occurs in $<10\%$ of patients (Vasquez *et al.*, 1998; Hussein *et al.*, 2009; Crampton *et al.*, 2002; Kralt *et al.*, 2009; Light *et al.*, 2008; Azar *et al.*, 2015; Lemos *et al.*, 2000). Most patients with GU dissemination have concomitant pulmonary or cutaneous blastomycosis (Inoshita *et al.*, 1983). GU blastomycosis results in infection of the prostate (i.e., prostatitis) or epididymis and testicles (epididymoorchitis). Prostatitis causes painful urination (i.e., dysuria), increased frequency of urination at night (i.e., nocturia), suprapubic pain, decreased

strength in urine stream, and bladder outlet obstruction (Inoshita *et al.*, 1983; Seo *et al.*, 1997). Epididymoorchitis results in pain and swelling of the scrotum and testicles (Seo *et al.*, 1997). One case of male-to-female transmission has been reported, which suggests that prostate secretions and semen are potentially infectious (Craig *et al.*, 1970). GU blastomycosis in women is rare and typically results in tubo-ovarian abscess with or without peritonitis (Barocas and Gauthier, 2014).

CNS blastomycosis occurs in $\leq$10% of patients and causes meningitis, intracranial mass, or brain abscess; involvement of the spinal cord is rare (Lemos *et al.*, 2000; Bariola *et al.*, 2010; Gonyea, 1978). In patients with AIDS ($CD4^+$ T-lymphocytes $\leq$200/mm^3), CNS dissemination can occur in up to 40% (Pappas *et al.*, 1992). Isolated CNS blastomycosis is uncommon, the majority of patients (77%–81%) will have blastomycosis involving other organs such as the lung, skin, or bone (Bariola *et al.*, 2010; Gonyea, 1978; Bush *et al.*, 2013; Friedman *et al.*, 2000). The clinical manifestations of CNS blastomycosis are slowly progressive (weeks to months) and include headache, focal neurologic deficits, changes in mental status, and seizures (Bariola *et al.*, 2010; Gonyea, 1978; Bush *et al.*, 2013; Friedman *et al.*, 2000). Cerebral spinal fluid analysis demonstrates an elevated white blood cell count with a neutrophilic or lymphocytic predominance, elevated protein, and low glucose (Bariola *et al.*, 2010; Gonyea, 1978). Diagnosis of CNS blastomycosis can be challenging because the sensitivity of CSF culture is $\leq$45% (Bariola *et al.*, 2010; Gonyea, 1978).

Blastomycosis in Pregnancy

Blastomycosis in pregnancy is rare with only 24 cases reported in the medical literature (Baker *et al.*, 2017; Austin *et al.*, 2019). Patients typically present with either pneumonia (74%) or disseminated disease (48%) in the 2nd or 3rd trimesters (Baker *et al.*, 2017). On the basis of 2 case reports, there is potential for mother-to-child transmission; however, the majority of neonates delivered vaginally to infected mothers are healthy (Baker *et al.*, 2017; Maxson *et al.*, 1992; Watts *et al.*, 1983). The route for maternal-to-child transmission is unclear, but includes intrauterine transmission or aspiration of infected vaginal secretions during birth (Maxson *et al.*, 1992; Watts *et al.*, 1983). *Blastomyces* infection does not increase the risk for congenital malformations; however, treatment with azole antifungals early in pregnancy can result in spontaneous abortion or an Antley-Bixler-like syndrome (cardiac and bone malformations) (Pursley *et al.*, 1996; Cottreau and Barr, 2016).

Blastomycosis in Immunocompromised Persons

Blastomycosis can infect patients immunocompromised by solid organ transplantation, malignancy, high-dose steroids, AIDS, and tumor necrosis factor alpha (TNF-α) inhibitors. In contrast to histoplasmosis and coccidioidomycosis, blastomycosis has been rarely reported in patients with primary immunodeficiency disorders (Sideritis *et al.*, 2010; Spinner *et al.*, 2016; Lee and Lau, 2017).

The incidence of blastomycosis in recipients of solid organ transplants (SOT) in the endemic region is 0.13%–0.14% (Gauthier *et al.*, 2007; Grim *et al.*, 2012). SOT recipients at high risk for respiratory failure, ARDS, and death due to blastomycosis (Gauthier *et al.*, 2007; Grim *et al.*, 2012; Kauffman *et al.*, 2014). A subset can be co-infected with opportunistic pathogens such as cytomegalovirus or *Aspergillus*. Disseminated diseases occurs in 33%–50%, which is similar to the general population (15%–44%) (Gauthier *et al.*, 2007; Grim *et al.*, 2012; Kauffman *et al.*, 2014).

Blastomycosis in patients with AIDS is uncommon, even in highly endemic areas such as Wisconsin. This may be related to preservation of $CD8^+$ T-lymphocytes which provides protective immunity against *B. dermatitidis* (Gulzar and Copeland, 2004; Nanjappa *et al.*, 2014). Most AIDS patients with blastomycosis have pulmonary disease with 53% having disseminated disease ($\geq$3 sites of infection in 33%), and up to 40% can have CNS involvement (Pappas *et al.* (1992)).

Patients with autoimmune disorders such as rheumatoid arthritis or inflammatory bowel disease that are treated with tumor necrosis factor alpha (TNF-α) inhibitors are at increased risk for invasive fungal infections and tuberculosis (Smith and Kauffman, 2009). In a large case-control study, the 2 most common infections for persons treated with TNF-α inhibitors were *Mycobacterium tuberculosis* (60%) and *H. capsulatum* (60%) (Salt *et al.*, 2016). Other infections were less common including blastomycosis (4%) (Salt *et al.*, 2016). There is a paucity of data regarding clinical manifestations and rates of dissemination in persons with blastomycosis who have received TNF-α inhibitors (Smith and Kauffman, 2009; Salt *et al.*, 2016; Smith *et al.*, 2015; Alpern *et al.*, 2016).

Diagnosis of Blastomycosis

The most important "diagnostic test" is considering blastomycosis in the differential diagnosis (**Table 3**). Once blastomycosis is suspected, a combination of culture and non-culture-based diagnostics can be used for diagnosis (Saccente and Woods, 2010). Culture-based diagnostics involve collecting clinical specimens for fungal staining and culture. Non-culture based diagnostics involve serologic or antigen tests.

The most rapid method for diagnosis is staining of clinical specimens to look for broad-based budding yeast (8–20 μm) with a doubly refractile cell wall (Saccente and Woods, 2010). Staining of respiratory specimens, purulent drainage, or skin samples with calcofluor, 10% potassium hydroxide, or Papanicolaou highlights the fungal cell wall (Saccente and Woods, 2010). In contrast, Gram staining, which is used for bacteria, will not result in optimal visualization of the thermally dimorphic fungi. The sensitivity of stained respiratory samples ranges from 50% to 90% and results are usually available within several hours (Saccente and Woods, 2010). Biopsied tissue specimens can be stained with Gomori methenamine silver (GMS) or periodic acid-schiff (PAS)

Table 3 Clinical suspicion of blastomycosis for persons that reside in or visit the endemic region

Risk factors for exposure	Nearby construction of homes, buildings, or roads Home remodeling Landscaping Logging Canoeing or tubing on a river Fishing by riverbanks Beaver dam exploration Use of a community compost pile Work in a microbiology laboratory Concomitant diagnosis in a pet such as a dog or cat[a]
Suggestive clinical findings	Infections of the lung, skin, or bone that do not respond to antibiotic therapy Pneumonia with cutaneous lesions or osteomyelitis CNS disease in setting of pneumonia, skin lesions, or osteomyelitis Acute respiratory distress syndrome (ARDS)[b]

[a]Blastomycosis is not considered a zoonotic infection. Infection in a pet suggests a common source of environmental exposure.
[b]Although there are many causes of ARDS, blastomycosis should be considered as part of the differential diagnosis in any person with ARDS who lives in or has visited the endemic region for blastomycosis.

(Saccente and Woods, 2010). Standard histopathology stains such as hematoxylin and eosin (H&E) poorly visualize *Blastomyces* yeast (Saccente and Woods, 2010). Tissues infected with *Blastomyces* yeast typically demonstrate neutrophil infiltration and granulomas known as pyogranulomatous inflammation (Saccente and Woods, 2010). Histopathology results are usually available within 24–48 h. Morphologic identification of *Blastomyces* yeast on stained clinical specimens correlates with culture results (Patel *et al.*, 2010). Because fungal stains are not routinely performed on clinical specimens, these tests should be specifically ordered by the requesting health care provider.

All clinical specimens obtained for staining should have a paired sample sent to the microbiology lab for fungal culture. *Blastomyces* does not grow well on bacteriologic media and requires specialized media such as brain-heart infusion (BHI), potato dextrose agar (PDA), or Sabouraud dextrose agar. Growth in culture ranges from 5 to 28 days (Saccente and Woods, 2010). Most clinical laboratories incubate fungal cultures at 25–30°C, which will result in growth as hyphae (Saccente and Woods, 2010). Unfortunately, *Blastomyces* hyphae are not distinctive and visually appear similar other fungi. Thus, confirmation by chemiluminescent DNA probe, PCR analysis, or conversion to yeast is required (Saccente and Woods, 2010). The sensitivity for culture typically ranges from 72% to 92% (Chapman *et al.*, 1997; Vasquez *et al.*, 1998; Carlos *et al.*, 2010; Kralt *et al.*, 2009; Azar *et al.*, 2015; Martynowicz and Prakash, 2002). Fungal cultures are not routinely performed and must be specifically ordered by the requesting health care provider.

Non-culture based diagnostics can be used in conjunction with staining and culture. Serologic tests such as complement fixation (CF) and immunodiffusion (ID) suffer from poor sensitivity ($\leq$25% for CF and $\leq$40% for ID) and have been largely replaced by a quantitative antigen test that detects galactomannan shed from the *Blastomyces* yeast cell wall (Vasquez *et al.*, 1998; Martynowicz and Prakash, 2002; Klein *et al.*, 1986b, 1987b; Richer *et al.*, 2014; Durkin *et al.*, 2004; Bariola *et al.*, 2011; Connolly *et al.*, 2012; Frost and Novicki, 2015). The antigen test can be performed on urine, blood, bronchoalveolar fluid, and cerebrospinal fluid. The sensitivity of the urine *Blastomyces* antigen assay is 76.3%–92.9% (Bariola *et al.*, 2011; Connolly *et al.*, 2012; Frost and Novicki, 2015). Data regarding sensitivity of the *Blastomyces* antigen assay on bronchoalveolar and cerebrospinal fluid is limited (Bariola *et al.*, 2011; Akture *et al.*, 2013; Walkty *et al.*, 2017). The underlying burden of *Blastomyces* infection influences the sensitivity of the antigen assay in which patients with a higher infectious burden are more likely to have a positive test (Bariola *et al.*, 2011). Serial measurement of urine antigen levels can be used to monitor response to therapy with a decline in antigen levels indicating a positive response to treatment (Frost and Novicki, 2015).

Overlapping structural similarity in the galactofuranose side chains in galactomannan can results in false positive *Blastomyces* testing in patients infected with *H. capsulatum* (96%–100%), *Paracoccidioides* (100%), and *Talaromyces marneffei* (70%) (Durkin *et al.*, 2004). The galatomannan cross-reaction lowers specificity of the *Blastomyces* antigen test to 76.9–79% (Durkin *et al.*, 2004; Bariola *et al.*, 2011). However, the clinical impact on a false positive test due to cross-reaction is limited because the treatment regimens for *Blastomyces*, *Histoplasma*, *Paracoccidioides*, and *Talaromyces* are similar. Moreover, there is no overlap in the geographic distribution with *Paracoccidioides* spp. (Southern Mexico, Central America, South America) or *T. marneffei* (southeast Asia and China). Rarely, the *Blastomyces* antigen test can cross-react with *Cryptococcus* (2.9%) or *Aspergillus* (1.1%) (Durkin *et al.*, 2004). Recent data suggests that the Platelia™ *Aspergillus* galactomannan assay performed on bronchoalveolar samples can cross-react in patients with pulmonary blastomycosis (Van Der Veer *et al.*, 2012).

The newest non-culture based diagnostic test, serum (1,3)-Beta-D-Glucan, cannot reliably be used for diagnosis because *Blastomyces* yeast contain very little β-(1,3)-glucan (<5%) (Kanetsuna and Carbonell, 1971; Girouard *et al.*, 2007). In contrast, the cell walls of *Histoplasma* yeast and *Coccidioides* sperules contain more β-(1,3)-glucan and the serum 1,3-β-glucan can be positive in patients with these infections (Girouard *et al.*, 2007; Zangeneh *et al.*, 2015).

Basic science discoveries at the bench are beginning to enter the clinical realm with regards to the development of novel diagnostic tests. In addition to being an essential virulence factor, Bad1, is a highly immunogenic protein and results in a detectable antibody response in patients infected with *Blastomyces* (Richer *et al.*, 2014). On the basis of this data, a new serologic assay that detects antibodies against Bad1p has been developed (Richer *et al.*, 2014). The Bad1 antibody assay has a sensitivity of 87% and specificity of 94%–99% (Richer *et al.*, 2014). When the Bad1 antibody and *Blastomyces* antigen tests are combined, the sensitivity improves to 97% (Richer *et al.*, 2014). Because *Blastomyces* is the only known fungus that has the *BAD1* gene in its genome, cross-reactions with other fungi have not been reported (Muñoz *et al.*, 2015; Richer *et al.*, 2014). PCR assays that amplify *BAD1* have been developed for testing of cultures, paraffin-embedded tissues, and soil (Sidamonidze *et al.*, 2012; Burgess *et al.*, 2006). PCR-based soil analysis has been used to investigate human and canine blastomycosis outbreaks (Pfister *et al.*, 2011; Burgess *et al.*, 2006).

Treatment of Blastomycosis

Antifungal therapy is recommended for all patients diagnosed with blastomycosis (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019). Selection of antifungals is influenced by severity of blastomycosis, sites of dissemination, underlying immunocompromise, presence or absence of pregnancy, drug-drug interactions, underlying congestive heart failure, and potential for cardiac arrhythmias due to QT prolongation. Guidelines for the treatment of blastomycosis have been published by the Infectious Diseases Society of America (IDSA), American Thoracic Society (ATS), and American Society of Transplantation (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019).

Antifungals with activity against *Blastomyces* include polyene and azole drug classes. The newest class of antifungals, the echinocandins, have poor activity against *Blastomyces* yeast and should not be used for treatment (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019). Polyene antifungals include amphotericin B deoxycholate and amphotericin B lipid formulations such as liposomal amphotericin B, amphotericin B lipid complex, and amphotericin B colloidal dispersion. Polyene antifungals can only be administered intravenously (IV). Side effects include infusion reactions, renal dysfunction, hypokalemia, and hypomagnesemia. Lipid amphotericin B (AmB) formulations have reduced frequency of infusion reactions and renal dysfunction compared to amphotericin B deoxycholate. Salt loading by administering intravenous 0.9% normal saline before and after AmB infusions can reduce the rate of renal dysfunction. Similarly, IV or oral replacement of potassium and magnesium can be used to correct or minimize AmB-mediated renal wasting of these electrolytes. AmB deoxycholate results in cure in 97% when cumulative dosage is >2 g (Chapman *et al.*, 2008). Lipid formulations are predicted to be equally efficacious as AmB deoxycholate but with lower rates of infusion reactions and renal dysfunction (Chapman *et al.*, 2008). Thus, lipid AmB formulations are preferred over AmB deoxycholate.

Azole antifungal options for treatment of blastomycosis include itraconazole, voriconazole, posaconazole, isavuconazonium sulfate, and fluconazole (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019). Azoles can be administered by IV or oral routes with the exception of itraconazole, which can only be given oral (IV formulation was discontinued). Itraconazole is the preferred azole for treatment of blastomycosis because it has excellent activity against *Blastomyces* and results in a 95% cure rate for patients with non-CNS blastomycosis. However, caution is needed in patients with underlying congestive heart failure due to its negative inotropic effect on cardiac function (Ahmad *et al.*, 2001). In addition, itraconazole can induce hypokalemia. Fluconazole, voriconazole, and posaconazole can prolong the QT interval, which can precipitate cardiac arrhythmias, especially in conjunction with other QT-prolonging medications. In contrast, isavuconazonium sulfate shortens the QT interval and is contraindicated in patients with congenital short QT syndrome (Keirns *et al.*, 2017). Prior to initiation of azole antifungals, a comprehensive medication assessment is needed because azoles have numerous drug interactions. In all patients who have the potential for pregnancy, a pregnancy test is needed before azole initiation because azoles can induce spontaneous abortion and have teratogenic effects on the developing fetus, especially in the first trimester (Pursley *et al.*, 1996; Cottreau and Barr, 2016). Thus, pregnant patients with blastomycosis should be treated with AmB.

Immuncompetent patients with severe pulmonary or severe disseminated blastomycosis require hospitalization and induction therapy with a lipid formulation of amphotericin B (Lipid AmB) for 1–2 weeks. This is followed by step-down therapy with itraconazole for 6–12 months (**Table 4**; Chapman *et al.*, 2008; Limper *et al.*, 2011). For patients with blastomycosis that has disseminated to the CNS, the duration of lipid AmB therapy is 4–6 weeks before starting step-down therapy with voriconazole, fluconazole, or itraconazole (**Table 4**). Voriconazole has become the preferred agent for step-down therapy in patients with CNS blastomycosis because of its excellent CNS penetration and activity against *Blastomyces* (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019). Although fluconazole also has excellent CNS penetration, it is not as potent against *Blastomyces* as other azoles (Chapman *et al.*, 2008; Miller *et al.*, 2019). Despite limited penetration of the blood brain barrier, itraconazole can be used to treat CNS blastomycosis (Bush *et al.*, 2013). In addition, there are reports indicating success of posaconazole for treatment CNS blastomycosis (Miller *et al.*, 2019) For patients with disseminated blastomycosis involving the bone, treatment duration is 12 months (**Table 4**) (Chapman *et al.*, 2008; Limper *et al.*, 2011).

Immunocompetent patients with mild-to-moderate pulmonary or disseminated blastomycosis, azole antifungal therapy can be initiated in the outpatient setting and continued for 6–12 months (**Table 4**; Chapman *et al.*, 2008; Limper *et al.*, 2011). When Itraconazole, voriconazole, posaconazole, or isavuconazonium sulfate are started, loading dosing is recommended before starting maintenance dosing. To optimize serum drug levels for itraconazole capsules, it should be administered with food and an acidic beverage. Itraconazole capsule formulation should not be used in patients taking proton pump inhibitors or histamine-2 blockers

Table 4 Treatment of blastomycosis

Site of infection	*Severity*	*Patient population*	*Recommended therapy*
CNS	Any severity	Non-immunocompromised or immunocompromised	Lipid AmB 5 mg/kg IV daily for 4–6 weeks → Step-down therapy with voriconazole, fluconazole, or itraconazole for at least 12 months of total therapy[a]
Pulmonary	Severe	Non-immunocompromised or immunocompromised	Lipid AmB 3–5 mg/kg IV daily for 1–2 weeks → itraconazole for 6–12 months (12 months for immunocompromised patients)[a]. For patients with ARDS can consider concurrent corticosteroids (40–60 mg prednisone for 1–2 weeks) as well as combination antifungal therapy (lipid AmB plus azole)
	Mild-moderate	Non-immunocompromised	Itraconazole for 6 months
	Mild-moderate	Immunocompromised	Lipid AmB 3–5 mg/kg IV daily for 1–2 weeks → itraconazole for 12 months[b]
Disseminated	Severe	Non-immunocompromised or immunocompromised	Lipid AmB 3–5 mg/kg IV daily for 1–2 weeks → itraconazole for 12 months[a]
	Mild-moderate	Non-immunocompromised	Itraconazole for 6–12 months (≥12 months for osteomyelitis)
	Mild-moderate	Immunocompromised	Lipid AmB 3–5 mg/kg IV daily for 1–2 weeks → itraconazole for 12 months (≥12 months for osteomyelitis)
Any site	Any Severity	Pregnancy	Lipid AmB 3–5 mg/kg IV daily

[a]Can consider combination therapy with Lipid Amphotericin B and azole antifungal.

[b]Although not well-studied, ATS and AST guidelines suggest itraconazole can be used as initial therapy for select immunosuppressed patients with mild-to-moderate blastomycosis; however, azole antifungals are fungistatic against *Blastomyces* spp., whereas AmB is fungicidal.

Source: *Severe infection* requires hospitalization. For *Mild-to-moderate infection*, therapy can be initiated in the outpatient setting. *Immunocompromise* includes but is not limited to HIV/AIDS, high-dose steroids, TNF-alpha inhibitors, solid organ transplantation, solid and hematologic malignancy. *Azole dosing in adults*: Itraconazole 200 mg orally TID × 3 days → 200 mg po BID or QD with dosing based on serum itraconazole levels, Voriconazole 6 mg/kg IV or PO BID × 1 day → 4–6 mg/kg (check dosing) IV or PO BID with dosing based on serum voriconazole trough levels, Fluconazole 800 mg IV or PO daily. *Azole dosing in children*: itraconazole 10 mg/kg per day with maximum of 400 mg daily; voriconazole dosing in children 2-12 years old is 9 mg/kg/dose IV or orally every 12 h for 2 doses, then 8 mg/kg/dose IV every 12 h or 9 mg/kg/dose orally every 12 h (maximum dose 350 mg/dose). *Amphotericin B dosing in adults and children*: Lipid Amphotericin B 3–5 mg/kg IV daily; Amphotericin B deoxycholate 0.7–1.0 mg/kg IV daily.

because they reduce gastric acidity, which in turn, decreases absorption leading to subtherapeutic drug levels. In contrast, itraconazole solution does not require gastric acidity for absorption and should be administered without food or an acidic beverage. Voriconazole should be taken without food, whereas posaconazole delayed release capsules and isavuconazonium sulfate can be administered irrespective of food. Therapeutic drug monitoring (TDM) is recommended when itraconazole, voriconazole, or posaconazole are used for treatment. TDM can be considered for isavuconazonium sulfate. TDM involves measuring serum levels of antifungal drugs to assess if the concentration in a therapeutic range (e.g., 1–5 μg/mL).

Immunosuppressed patients with pulmonary or disseminated blastomycosis should receive 1–2 weeks of AmB followed by 1 year of azole antifungal therapy (**Table 4**; Chapman *et al.*, 2008; Limper *et al.*, 2011). Similar to immunocompetent patients, lipid AmB is preferred over AmB deoxycholate (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019). The IDSA & AST guidelines indicate that some patients with irreversible immunosuppression may require life-long antifungal therapy; however, except for patients with AIDS with persistent CD4+ T-lymphocytopenia (≤ 150–200/μl), there are no specific criteria regarding which patients require indefinite therapy. For solid organ transplant recipients, the risk of relapse is low when treated for 12 months (Gauthier *et al.*, 2007; Grim *et al.*, 2012).

In patients with intracranial abscesses and osteomyelitis including spinal osteomyelitis complicated by vertebral collapse or neurologic impingement, surgical intervention should be considered in conjunction with antifungal therapy. Adjunctive steroids for 1–2 weeks (e.g., prednisone 40–60 mg or equivalent) can be considered in addition to AmB in patients with ARDS (Chapman *et al.*, 2008; Limper *et al.*, 2011; Miller *et al.*, 2019; **Table 4**). Several case reports have suggested a survival benefit; however, a large retrospective study was unable to provide definitive conclusions regarding the benefit of adjuvant steroids (Lahm *et al.*, 2008; Plamondon *et al.*, 2010; Schwartz *et al.*, 2016). Although not well studied, combination therapy with AmB plus an azole can be considered in patients with severe pulmonary blastomycosis or CNS involvement (**Table 4**; Limper *et al.*, 2011).

Outcomes and Mortality

When blastomycosis is recognized early and prompt therapy is initiated, clinical outcomes are excellent. However, in patients with ARDS due to blastomycosis, mortality ranges from 40% to 89% (Vasquez *et al.*, 1998; Azar *et al.*, 2015; Schwartz *et al.*, 2016; Lemos *et al.*, 2001; Meyer *et al.*, 1993). In SOT recipients with ARDS, mortality is 67% (Gauthier *et al.*, 2007). From 1990–2010, the age-adjusted mortality rate for blastomycosis was 0.21 / 1,000,000 person-years resulting in 19,097 years of life lost (Khuu *et al.*, 2014). Highest mortality rates occurred in persons ≥ 65-years-old and in Native Americans (Khuu *et al.*, 2014). As expected the majority of deaths occurred within the endemic region, particularly the midwest and southern United States (Khuu *et al.*, 2014). Early recognition of blastomycosis along with initiation of effective therapy is currently the best approach to reduce mortality. There are no practical methods that can be introduced in a wide-spread manner to reduce environment-to-human transmission. The live-attenuated *Blastomyces* vaccine (*BAD1*Δ strain) is not commercially available and has not been tested in humans.

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Paracoccidioidomycosis

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Introduction

Paracoccidioidomycosis (PCM) is the prevalent systemic mycosis in Latin America, with 80% of cases being registered in Brazil, followed by Colombia, Venezuela and Argentina (Brummer *et al.*, 1993; Martinez, 2015). In Brazil, most cases are concentrated in the southeast region (States of São Paulo, Rio de Janeiro, Minas Gerais and Espírito Santo), west and middle-west (Mato Grosso do Sul and Goiás) and South (Paraná, Santa Catarina and Rio Grande do South). However, other regions have reported cases, especially the amazonian States of Pará, Tocantins, Maranhão and Rondônia (Martinez, 2015). Paracoccidioidomycosis has also been reported in Europe, the USA, Africa and Asia, but all cases are imported since the affected individuals were visitors or had worked in endemic areas in Latin America (Martinez, 2015). The fungal agent *Paracoccidioides* spp. preferably inhabits in a moist environment, with average annual temperature of 18–24°C and rainfall range of 900–1800 nm (Restrepo, 1985).

The disease was described by Adolfo Lutz, a Brazilian physician, in 1908 after examining oral lesions in two patients from São Paulo City (reviewed by Taborda *et al.*, 2016; Moreira, 2008) and originally designated as pseudococcidial hyphoblastomycosis to differentiate from coccidioidomycosis and hyphoblastomycosis (Lacaz *et al.*, 1991). Splendore in 1912, suggested the classification of the agent in the genus *Zymonema*, thus creating the name *Zymonema brasiliense*. The disease was designated as Brazilian blastomycosis, but, since it had been reported in different countries in South America, the disease was assigned as South American blastomycosis or Lutz and Splendore-Almeida disease (Lacaz, 1982). The term Paracoccidioidomycosis was established in 1971 in Medellín, Colombia, during a meeting of the American Continent Mycologists, widely accepted since then (Lacaz, 1982). After systematic studies, in 1930, Floriano Paulo de Almeida officially named the fungal agent as *Paracoccidioides brasiliensis* (Almeida, 1930) and classified by Ajello (Ajello, 1977) as in Kingdom Fungi, Phylum Eumycota, Subdivision Deuteromycotina, class Hyphomycetes, Order Moniales, Moniliaceae Family, Genus and Species *Paracoccidioides brasiliensis*. Based on phylogenetic studies using molecular tools *P. brasiliensis* was positioned along with other dimorphic fungi (*Coccidioides posadasii*, *Coccidioides immitis*, *Blastomyces dermatitidis* and *Histoplasma capsulatum*) in the Kingdom Fungi, Phylum Ascomycota, Plectomycetes class, Order Onygenales, Onygenaceae Family, Genus and Species *Ajellomyces brasiliensis*, then again called *Paracoccidioides brasiliensis* (San-Blas *et al.*, 2002; Arantes *et al.*, 2015).

Paracoccidioidomycosis is a chronic granulomatous systemic mycosis, caused by a fungus of genus *Paracoccidioides*. One of the main characteristics of this genus is the thermal dimorphism, mycelial form at 25°C characterized as saprophytic and yeast as the pathogenic form at 35–37°C (reviewed by (Taborda *et al.*, 2018)). The habitat of *Paracoccidioides* spp. has not yet been determined precisely. Soil samples from different locations in Venezuela, Argentina and Brazil (reviewed by (Franco *et al.*, 2000)) support the hypothesis that the soil is the natural habitat of the fungus. In addition, isolations of *P. brasiliensis* from wild animals such as armadillos (Bagagli *et al.*, 1998), bats (Grose and Tamsitt, 1965) and even dog food (Ferreira *et al.*, 1990) have been reported. Also, genomic analysis of samples from animals living in the soil of endemic areas, such as guinea pig (*Cavia aperea*), raccoon (*Procyon cancrivoros*) and armadillo (*Dasypus septemcinctus*), further indicates acquisition of the infection from the soil in these areas. (Richini-Pereira *et al.*, 2008).

It is important to stress that except for humans, the most frequent host among mammalians is the armadillo ((mainly *Dasypus novemcintus*) but the fungus can occur in other species). *Paracoccidioides* spp. has been isolated ca. 75%–100% from armadillo captured in hyper-endemic areas of PCM, showing a high frequency of infection (Corredor *et al.*, 1999; Hrycyk *et al.*, 2018). The armadillos seem to favor the infection by *Paracoccidioides* spp. yeasts, such as the body temperature (between 32.7 and 35°C), low cellular immunity and constant contact with the soil (Bagagli *et al.*, 2008). It is hypothesized that parasitism in these animals would make it possible to preserve the saprophyte form and contribute to sexual reproduction, by promoting the proximity of mating types in a protected environment, such as the armadillo tissue or organs (Bagagli *et al.*, 2006).

For more than 100 years, only one species was recognized in the Genus: *Paracoccidioides brasiliensis*. Phylogenetic studies have classified *P. brasiliensis* into four cryptic species (S1, PS2, PS3 and PS4) with different evolutionary characteristics and geographic distribution. S1 is considered a recombinant monophyletic group with a wide distribution in South America, being responsible for most cases of PCM. PS2 is also recombinant but paraphyletic, found in Brazil and Venezuela. PS3 is a clonal and monophyletic population, registered only in Colombia. The PS4 group is classified as monophyletic with clinical isolates from Venezuela (Matute *et al.*, 2006; Theodoro *et al.*, 2012). In parallel, a second species was proposed based on phylogenetic and comparative genomic data, recombination analysis, and morphological characteristics and designated as *P. lutzii* (formally known as Pb01-like) (Desjardins *et al.*, 2011; Teixeira *et al.*, 2014). It was estimated that *P. lutzii* monophyletic group was separated from groups S1, PS2 and PS3 approximately 30 million years ago (Teixeira *et al.*, 2014). This species is endemic in the North and Midwest regions of Brazil (mainly in States of Rondônia, Mato Grosso and Goiás) and shares some geographical areas with the S1 group (Bocca *et al.*, 2013; Teixeira *et al.*, 2014). Turissini *et al.* (2017) expanded the phylogenetic studies and showed that despite low levels of genetic difference among the four cryptic species of the *P. brasiliensis*, they are indeed well separated species indicating that cryptic species (S1, PS2, PS3 and

 doi:10.1016/B978-0-12-819990-9.00001-9

PS4) should be elevated to taxonomical species status (Turissini *et al.*, 2017). The authors propose therefore to adopt the names *P. lutzii*, *P. brasiliensis* (S1), *P. americana* (PS2), *P. restrepiensis* (PS3) and *P. venezuelensis* (PS4) (Turissini *et al.*, 2017).

Morphological Aspects

Paracoccidioides spp., are considered dimorphic fungi depending on temperature to change their shape. The fungal species develop as mold at room temperature (19–25°C) and are then known as the saprophytic phase. Otherwise, at temperatures ranging 35–37°C, fungi become unicellular yeasts (**Fig. 1**), consisting in the parasitic phase (reviewed in Taborda *et al.*, 2018).

Depending on the culture medium, nutrients and temperature, colonies assume different characteristics. At room temperature, white fungal colonies grow slowly well adherent to the medium (**Fig. 2(A)**). When examined under the microscope, and using specific culture media, fine, septated mycelial filaments with conidia, arthroconidia, aleurioconidia and arthroconidia are seen (Conant and Howell, 1942; Neves and Bogliolo, 1951; Pollak, 1971). Conidia are small, less than 5 μm, with different shapes and sites on the parental mycelium (Bustamante-Simon *et al.*, 1985). The conidia-to-yeast transition occurs in 96 h at 37°C and colonies now show transition to cream colonies known as cerebriform and yeast-like which appear after 10–20 days (Brummer *et al.*, 1993; **Fig. 2(B)**). The yeast cells are similar to those detected in patients. Spherical cells (3–30 μm but some cells

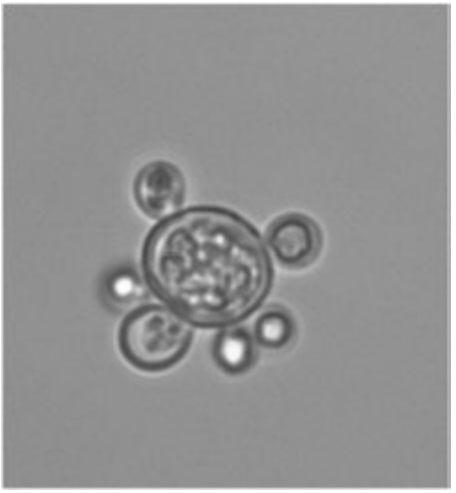


Fig. 1 Micrograph of typical "pilot wheel" yeast cells of *P. brasiliensis* seen on direct examination of culture (120x).

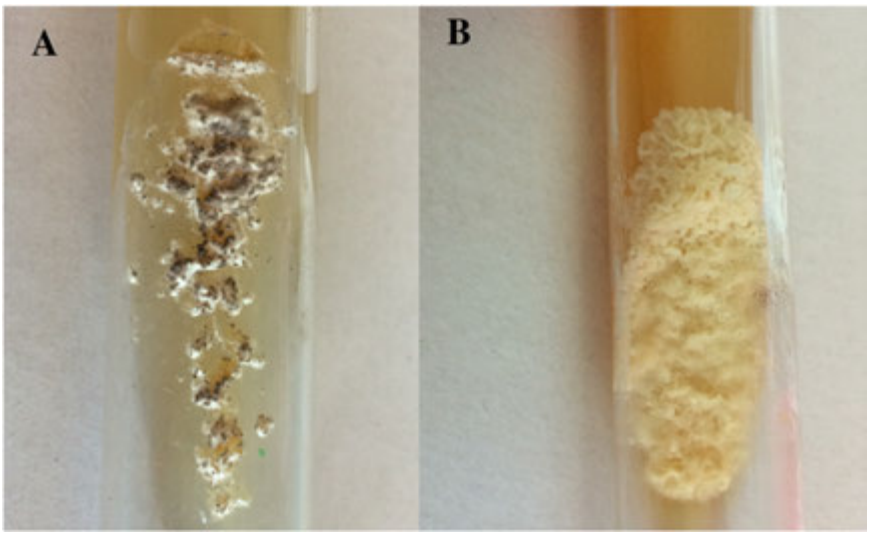


Fig. 2 Macroscopic colonies: (A) of mycelium (room temperature) and (B) yeasts (37°C) in BHI Agar.

may reach 60 μm) with thin and thick walls are seen; along the cell surface single or multiples narrow-necked buds (2–10 μm) (Brummer *et al.*, 1993).

Epidemiological Aspects

The infection is acquired when propagules from the mycelial phase of the fungus are inhaled and, through the respiratory tract, install primarily in the pulmonary alveoli. The transition to the yeast phase starts in the lung and cell transformation is essential for the infection to establish the disease that can spread via hematogenous and or lymphatic routes to any part of the organism (reviewed by Souza and Taborda, 2020). PCM is mostly manifested in male individuals, between 30 and 60 years of age, mostly rural workers in endemic areas. People from urban regions may also have contact with the fungus when visiting endemic areas. Other conditions such as smoking and excessive alcohol consumption are considered risk factors for the establishment of the disease (Shikanai-Yasuda *et al.*, 2017; Souza and Taborda, 2020). The hormone 17-β-estradiol is related to the fungal dimorphism and may protect women against the disease. Studies show that this hormone inhibits the transition from mycelium to yeast in a dose-dependent manner (Aristizábal *et al.*, 2002). It is believed that the Estradiol Binding Protein – PLE – present in the fungal cytoplasm binds to the 17-β-estradiol and blocks the conversion from the infective phase to the pathogenic phase (Shankar *et al.*, 2011). In addition to the hormones, PCM has already been reported in patients undergoing cytotoxic treatment or immunosuppressive therapy against cancer (Marques, Lastória and Marques, 2011), transplant patients (Shikanai-Yasuda *et al.*, 1995) and rheumatoid arthritis (Woyciechowsky *et al.*, 2011). It is assumed that cytotoxic drugs can reactivate a latent lesion, causing an acute lung infection, probably due to the impairment of the immune system due to chemotherapy and radiotherapy.

PCM is the most important systemic mycosis in Latin America and the countries with the highest number of cases are Brazil, Colombia, Venezuela and Argentina. Brazil has 80% of the total cases, and the southeast, south and midwest regions are the most prevalent (Martinez, 2017; Souza and Taborda, 2020). PCM is the eighth cause of mortality from chronic infectious diseases, reaching rates of 1.65 deaths per million inhabitants (Bocca *et al.*, 2013). In the period from 1996 to 2006 there were 517,058 deaths from infectious and parasitic diseases in Brazil. Systemic mycoses were responsible for 3583 deaths, with paracoccidioidomycosis being cited in approximately 51.2% deaths recorded in this period, occupying the tenth position, in this survey, among infectious and parasitic diseases with high mortality (Prado *et al.*, 2009). In 2010, it was estimated that 3360 new cases of PCM per year occur in Brazil alone (Martinez, 2010).

There are two progressive clinical forms of the disease: acute or subacute (juvenile type), which involves 3%–5% of cases, is characterized by having a severe faster course (weeks or months), with involvement of the reticuloendothelial system (spleen, liver, lymph nodes and spinal cord) rather than pulmonary involvement. Active replicating fungi and poor granuloma formation are found in the tissues. The chronic form (adult type) occurs in more than 90% of cases, as the disease takes months or years to establish, being, in most cases, asymptomatic. Pulmonary manifestations are the best indicators of this form of the disease, which can also spread to other organs and / or systems. Patients with the acute form of mycosis have high levels of antibodies depending on the mycosis severity. The reason for this clinical dichotomy is not clear and it is assumed that specific characteristics of the parasite-host relationship contribute to it (Benard, 2008; Benard *et al.*, 2012b; Vidal *et al.*, 2014).

Laboratorial Diagnosis

The laboratory diagnosis is relevant since some clinical aspects are shared with other fungal diseases such as: cryptococcosis, histoplasmosis, chromoblastomycosis, coccidioidomycosis and other diseases including tuberculosis, toxoplasmosis, leishmaniasis, cysticercosis, infectious mononucleosis, leprosy, sarcoidosis, syphilis, lymphoma, leukemia, neoplasms, pneumoconiosis, interstitial pneumonitis and Crohn's disease (Shikanai-Yasuda *et al.*, 2017).

The gold standard diagnosis of PCM is based on visualization of yeast cells in biological specimens and isolation of the fungus in culture (Moreira, 2008; Souza and Taborda, 2020). For isolation of the fungus it is recommended the use of Sabouraud dextrose agar or brain heart infusion agar (BHI) containing chloramphenicol and cycloheximide. The clinical specimens (sputum, bronchoalveolar lavage, scraped injury, ganglion aspirate, biopsy fragment) in culture medium are kept 15–30 days at 25–30°C. In the mycelial form, the fungus grows as white, cottony or glabrous colonies and there are no structures that can be associated to *Paracoccidioides* spp. Reversion to yeast form is achieved by changing the temperature to 36–37°C for at least 15 days resulting in cerebriform colonies. In this phase, it is possible to visualize yeast with multiple peripheral buds characteristic of the fungus (Shikanai-Yasuda *et al.*, 2017; Souza and Taborda, 2020). The clinical specimens treated with 10%–20% of potassium hydroxide (KOH) can be used for yeast cell visualization under an optical microscope (**Fig. 3**). Usually, oval, elliptical cells with 3 μm to 30 μm in diameter with refringent double contour wall and multiple peripheral buds in the typical "pilot wheel" (Shikanai-Yasuda *et al.*, 2017; Souza and Taborda, 2020) are of diagnostic value. Another important alternative as a diagnostic tool is the lesion biopsy. Special stains such as Gomori-Grocott or Schiff's periodic acid can assist in the diagnosis by detecting granulomas and the presence of typical yeast with multiple peripheral buds (**Fig. 4**; Taborda *et al.*, 2018).

Other important tools for diagnosis and follow-up of patients are the serological tests. Specific antibodies to *Paracoccidioides* spp. correlate to the severity of the clinical form, being high in the acute/subacute form and in the disseminated form (Vidal *et al.*, 2014). Although different techniques have been described in the last years, immunoprecipitation methods

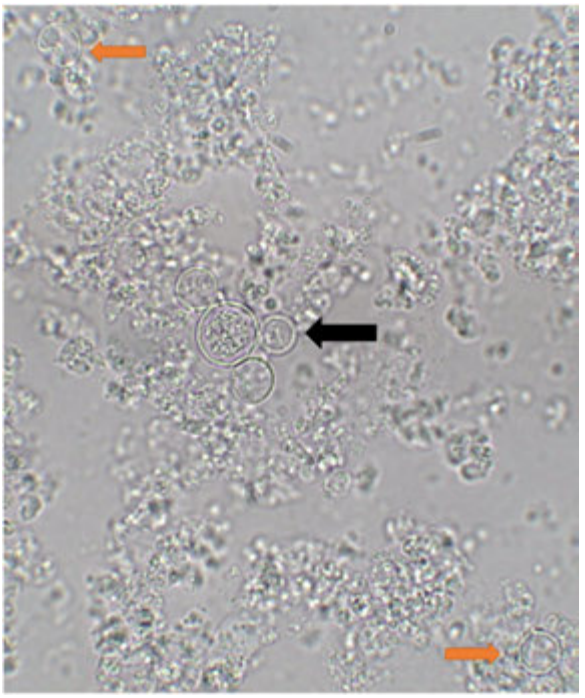


Fig. 3 Smear of a patient's superficial lesion on a KOH mount showing (black arrow) a multi-budding yeast cell, pathognomonic of Paracoccidioides spp. Two isolated yeast cells (red arrows) are also seen, which do not permit the specific diagnosis of PCM.

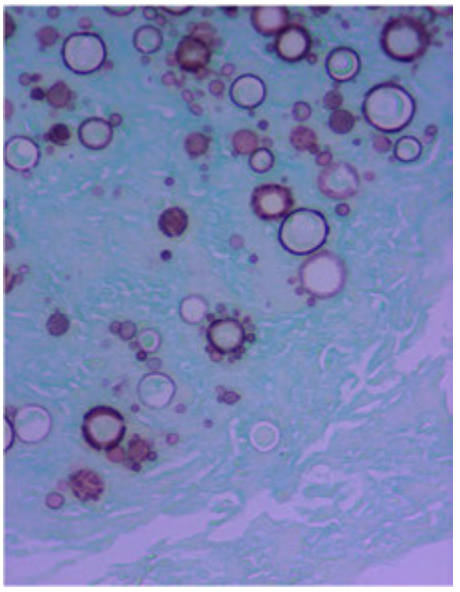


Fig. 4 Lung from infected mice with *P. brasiliensis* and stained by Gomori-Grocott. Multiple budding yeast cells characteristic of genus *Paracoccidioides*.

as in the counterimmunoelectrophoresis and double immunodiffusion still show excellent sensibility and specificity with 80%–95% diagnostic potential (Vidal *et al.*, 2014; Shikanai-Yasuda *et al.*, 2017). Other assays as enzyme linked immunosorbent assay (ELISA) or immunoblot/dot-blot techniques can also be used (de Camargo, 2008; Kamikawa *et al.*, 2017).

The immunoprecipitation methods are widely used in Latin America; however, the lack of standardization of methods and production of antigens sometimes present in the tests used may lead to controversial results (Vidal *et al.*, 2014). False negative results may be obtained in patients with localized lesions or with immunosuppression as in AIDS (Shikanai-Yasuda *et al.*, 2017). False positive results may occur in patients with other fungal infections such as histoplasmosis and aspergillosis or with leishmaniosis (de Camargo, 2008; Shikanai-Yasuda *et al.*, 2017). The main diagnostic antigen of *P. brasiliensis* is the 43 kDa glycoprotein (Puccia *et al.*, 1986) largely used in serological tests (Travassos *et al.*, 2004). The sensibility and specificity of gp43 for

P. brasiliensis and other cryptical isolates is excellent, but not for *P. lutzii* (Shikanai-Yasuda *et al.*, 2017). Although there is no consensus in the serology for *P. lutzii*, Gegembauer *et al.* (2014) developed a serological test with a specific antigen.

Additional diagnostic resources have been developed, such as polymerase chain reaction (PCR) tests, widely used in the diagnosis of infectious diseases. Specifically, in PCM, several authors have described variations in the methods with good results (Bialek *et al.*, 2000; Gomes *et al.*, 2000; Motoyama *et al.*, 2000; Rocha-Silva *et al.*, 2018) however, PCR in the laboratory routine still needs more experimentation. MALDI-TOX MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) has been used for differentiation of isolates of *P. brasiliensis* and *P. lutzii*, previously characterized by molecular techniques, and all isolates were correctly identified (de Almeida *et al.*, 2015).

Pathogenesis and Histopathology

The influence of genetic traits on the mycosis has yet to be elucidated. The observation that only a very small proportion (estimated as less than 2%) of the individuals in endemic areas exposed to the fungus develops the mycosis (Martinez, 2017), points to idea that the host's immunogenetic background plays a role in the susceptibility/resistance to PCM-disease. The occurrence, although rare, of PCM in individuals with inborn errors of the immune system such as defects of the IL-12/IL-23-IFN-γ axis also gives support to this possibility (de Moraes-Vasconcelos *et al.*, 2005; Schimke *et al.*, 2017). PCM has been associated with polymorphisms in some (e.g., IL12RB1, IL4, IL10) but not other (CTLA4, TNF and IFNG) genes related to the immune response (Bozzi *et al.*, 2006; Lozano *et al.*, 2011; Mendonça *et al.*, 2015; Carvalho *et al.*, 2016). The same holds to some class I and class II HLA alleles (Montoya de Restrepo *et al.*, 1983; Lacerda *et al.*, 1988; Goldani *et al.*, 1991). Probably the patient's clinical presentation also bears some relation with his genetic background. A small study suggested that in patients with the unifocal chronic form of the disease (a mild clinical presentation in which lesions are restricted or localized), the HLA allele that was most commonly seen was DRB1*11 (Sadahiro *et al.*, 2007). Clearly, large-scale genetic studies in human PCM are still required to elucidate these aspects.

The role of the host's genetic background in susceptibility to PCM has been more clearly demonstrated in mice models of the mycosis, where resistance and susceptibility varied according to the mouse strain (Singer-Vermfs *et al.*, 2008). Differences not only in adaptive immune response patterns but also in innate immunity (e.g., magnitude of neutrophils influx into the lungs) and type of extracellular matrix composition of the granulomatous lesions were described in these models (Xidieh *et al.*, 1999; Pina, 2006; Sperandio *et al.*, 2015).

However, the difficulty in understanding many of the susceptibility/resistance aspects is largely due to the fact that the events that take place at the initial stages of the infective process are unknown, since patients are diagnosed months to years after the initial exposure to the fungus (Benard, 2008). Data from case-reports, small series of cases, and autopsies studies indicate that the primary PCM lesions occur in the lungs after inhalation of fungal propagules (Brass, 1969; Angulo-Ortega, 1972; Giraldo *et al.*, 1976; Franco *et al.*, 1987; Londero *et al.*, 1996). This has been has also been suggested in experimental models of PCM established through intra-nasal instillation of *P. brasiliensis* conidia (Mcewen *et al.*, 1987). As in other systemic mycoses, the infection initiates when conidia reach the terminal bronchi or alveolar spaces and transform into yeast cells. Most frequently, the local immune response controls this initial inoculum, either fully eradicating the fungus or leaving a few quiescent foci with dormant yeast cells located in the lungs and mediastinal lymph nodes. This has been illustrated by the observation in asymptomatic individuals the presence of residual or regressive pulmonary lesions (called paracoccidioidomas), witnessing the formation of a primary pulmonary complex much similar to that described in the pathogenesis of pulmonary tuberculosis (primary complex of Ghon) (Angulo-Ortega, 1972; Giraldo *et al.*, 1976; Severo *et al.*, 1979; Melo and Londero, 1983; Londero *et al.*, 1996; Santos *et al.*, 1997) Eventually, a few yeast cells may escape this initial local response and disseminate through the lymphohematogeneous route, giving rise to subclinical and quiescent foci in other organs and systems (Angulo-Ortega, 1972; Benard *et al.*, 2013). In most cases this prior initial pulmonary infection tends to pass unnoticed.

Rarely, for yet unknown reasons, the immune response fails to control the initial infection. In these cases, there is lymphohematogeous spread, possibly through phagocytic cells loaded with viable yeast cells, with the patient developing the A/SAF characterized by disseminated involvement of the reticuloendothelial system (Franco *et al.*, 1987). In these patients, the lungs are usually spared, at least clinical and radiologically since autopsy and in vivo studies of A/SAC-like cases showed that the lungs can harbor yeast cells or very small subclinical or radiologically unapparent granulomatous inflammatory foci along the bronchial tree (Angulo-Ortega, 1972; Giraldo *et al.*, 1976; Restrepo *et al.*, 1989; Londero *et al.*, 1996; Buccheri *et al.*, 2015). It has been proposed that, based on the fact that A/SAF patients have profoundly depressed immune response against the fungus and loose granuloma formation, there would be little inflammatory-mediated tissue damage while such granulomas would not mature and evolve to dense fibrosis (Benard, 2008). This would explain the lack of residual fibrosis or other architectural abnormalities in patients with the A/SAF, for whom pulmonary involvement is probably restricted to the initial phase of the disease and remains underdiagnosed because of the absence of clinical or radiological evidence (Campos *et al.*, 1992; Benard *et al.*, 2005). Alternatively, witnessing the wide gamut of clinical presentations of the mycosis, some reports described A/SAF patients with a progressive primary pulmonary PCM much like that seen in tuberculosis: they developed simultaneously clinically overt pulmonary involvement and systemic reticuloendothelial involvement (Ramos *et al.*, 1981; Bittencourt *et al.*, 1986; Martinez and Moya, 2009).

Nevertheless it is roughly estimated that 98% of the infected individuals the quiescent foci, pulmonary or extra-pulmonary, remain so throughout life, making the rate of subclinical infections to outnumber many fold the (low) frequency of PCM disease even in endemic regions (Martinez, 2017). The most common clinical presentation of the disease is the CF, considered to result from fungal reactivation in these foci (Franco *et al.*, 1987). Thus, not infrequently the disease manifests when the patient has left

the endemic area. Of note these cases may manifest decades after leaving the endemic area, occasionally in patients living outside Latin America, when they represent an otherwise diagnostic challenge (Chikamori *et al.*, 1984; Silletti *et al.*, 1996; Horré *et al.*, 2002; Slevogt *et al.*, 2004; Kayser *et al.*, 2019).

In the chronic form, lung involvement frequently occurs. The mycosis is restricted to the lungs in some of these patients, but in most, other organs also are involved due to lymphohematogeneous dissemination from reactivated pulmonary foci or directly from foci at virtually any organ (Angulo-Ortega, 1972; Franco *et al.*, 1987; Benard *et al.*, 2013). Actually, PCM, in the A/SAF and CF, is almost always a disseminated disease, even though clinical manifestations are apparently restricted to a single organ: the degree of dissemination certainly is influenced by the availability of diagnostic procedures (Yamaga *et al.*, 2003; Shikanai-Yasuda *et al.*, 2017). After appropriate therapy, residual fibrotic lesions, remain in significant proportion of patients and represent the actual main challenge in the treatment of PCM (Mendes, 1994; Tabón *et al.*, 1995, 2003; Weber *et al.*, 2006; Costa *et al.*, 2013; Lopera *et al.*, 2015).

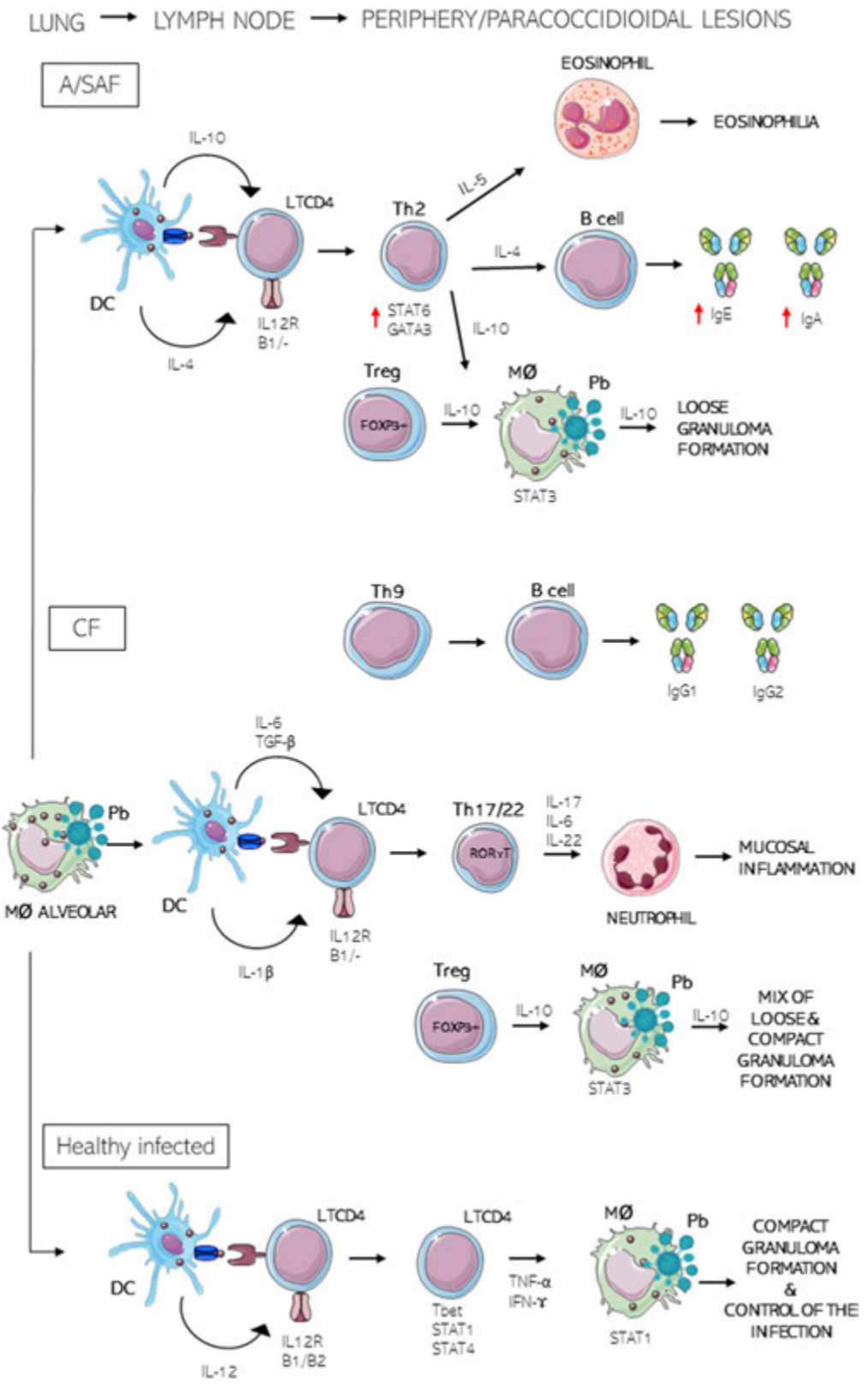
The time between infection and the onset of symptoms in the A/SAF of the disease is not precisely known, being estimated as weeks to many months; it lasted a few months according to the rare case reports with putative known date of infection (Franco *et al.*, 1987; Buccheri *et al.*, 2015). On the other hand, the time elapsed between infection and the onset of symptoms in the CF is estimated as years to decades (Franco *et al.*, 1987).

Histologically, the mycosis is characterized by the formation of compact granulomas containing few fungal elements in most patients; however, A/SAF or CF patients with severe disseminated disease have predominantly loose granuloma formation (Restrepo *et al.*, 1976; Brito and Franco, 1994). Lung involvement is secondary to a chronic lymphangitic process provoked by the fungus itself and the host's response represented by formation of granulomas and fibrosis, the latter of which predominates at the perihilar region (Tuder *et al.*, 1985). This aspect correlates with the butterfly-like (perihilar) micronodular and interstitial infiltration observed on plain films (Funari *et al.*, 1999; Souza *et al.*, 2006). Obstruction and reversal of lymphatic flow would lead to the spread of the inflammatory process throughout the lungs (Tuder *et al.*, 1985). The granulomatous inflammation is associated with a mixed pyogenic component, especially in the case of ulcerated skin lesions or ruptured lymph nodes (Franco *et al.*, 1989). Caseation and central necrosis may be present. In compact granulomas, abundant epithelial cells, Langerhans or foreign-body giant cells, plasmocytes, and lymphocytes are seen; often, phagocytosis of the yeast cells can be observed. $CD4^+$ lymphocytes dominate over $CD8^+$ lymphocytes and appear as peripheral mantles around aggregates of macrophages and histiocytes (Moscardi-Bacchi *et al.*, 1989). In the A/SAF and CF patients with disseminated disease, the inflammatory reaction is loose, with abundance of both mononuclear and yeast cells but sparse formation of compact granulomas (Tuder *et al.*, 1985; Franco *et al.*, 1989; Brito and Franco, 1994). Loose granulomas appear unable to circumscribe fungal antigens, and at their periphery, *Paracoccidioides* spp. antigens may permeate throughout the intercellular space (Sandoval *et al.*, 1996). Skin and mucous membrane lesions usually exhibit pseudoepitheliomatous hyperplasia and intraepithelial microabscesses (Franco *et al.*, 1989; Moscardi-Bacchi *et al.*, 1989). The role of neutrophils in the inflammatory response against paracoccidioides spp. has recently been reinvestigated and it was shown that neutrophil extracellular traps are present in tegumentary lesions of patients (Della Coletta *et al.*, 2015).

An important aspect drawn from the previously described histologic studies is the frequent description of areas of active disease characterized by pyogenic reaction and loose granulomata, rich in budding fungal cells, intermingled with areas with compact granulomas, rare fungal cells, and variable degrees of fibrosis (Tuder *et al.*, 1985). This mixed aspect can be observed in pulmonary, oro-pharyngeal and skin lesions, and even in lymph nodes, suggesting that the disease evolves through localized new bouts of fungal multiplication and tissue invasion, whereas the adjacent older lesions are in their way to fibrotic resolution. CT scans of the lung confirmed this aspect by depicting areas with alveolar condensation along with fibrotic and emphysematous zones and the simultaneous presence of features of more recent and chronic lesions (Funari *et al.*, 1999; Souza *et al.*, 2006).

Tissue reactions are nonspecific; thus, diagnosis depends on finding *P. brasiliensis*. If the parasite is abundant, it may be identified by hematoxylin and eosin stains. Special fungal stains (e.g., Grocott silver methenamine), however, always should be employed, especially when granulomata are examined. The typical multiple budding yeast cells must be found to establish a diagnosis. The presence of fungal cells of different sizes (2–40 μm) suggests the presence of *P. brasiliensis*. In some cases, short chains and cells with single buds also are observed, and in these patients, differentiation of *P. brasiliensis* from *Cryptococcus neoformans*, *Blastomyces dermatitidis*, and even *Histoplasma capsulatum* and *Pneumocystis jirovecii* must be made (Brummer *et al.*, 1993; Silletti *et al.*, 1996). When the disease is chronic, most of the fungal cells are found inside the macrophages, but free yeast cells predominate in disseminated cases. Internalized yeast cells exhibit altered morphology (Restrepo, 2000).

As other chronic granulomatous diseases, PCM is a spectral disease, with the A/SAF characterized by poor cellular immune responses, high fungal burden, high anti-*Paracoccidioides* antibody titers, including those of the IgG4 and IgE subclasses, eosinophilia, all of which typical of a predominance of Th-2 over Th-1 immune responses (Benard, 2008). Conversely, some patients with the chronic form have a more localized disease, low fungal burden, compact granulomas and low antibody titers, suggesting that they are still able to mount Th-1 responses. In between would stay the chronic multifocal patients with deficient Th-1 type responses without marked shift to Th-2 responses (Benard, 2008). However, the simplistic view of a Th-1/Th-2 paradigm of immune response has been challenged by the description of other subset of T cells such as T regulatory cells, Th-17 cells, Th-9 cells and Th-22 cells. The clinical phenotypes presented by PCM patients have been associated with these subsets. A/SAF patients displayed predominantly a mixed Th2/Th9 response while in CF patients there was a balance between Th-1, Th-17 and Th-22 responses depending on the extent of the disease in these patients (de Castro *et al.*, 2013). Recent data demonstrated that the NLRP3 inflammasome was essential for the activation and expansion of Th17 and Th1 cells upon challenge in vitro with *P. brasiliensis* yeast cells, while inhibition of this receptor by DC lead to increased Th2 and Treg frequency (de Castro *et al.*, 2018). **Fig. 5** describes a proposed scheme of the cellular immune response profiles of patients with PCM, based mostly on data from


LUNG → LYMPH NODE → PERIPHERY/PARACOCCIDIOIDAL LESIONS
A/SAF
EOSINOPHIL
EOSINOPHILIA
IL-10
LTCD4
Th2
IL-5
B cell
IL-4
DC
IL12R
B1/-
STAT6
GATA3
IL-10
IgE
IgA
IL-4
Treg
FOXP3+
IL-10
MØ
Pb
IL-10
LOOSE
GRANULOMA
FORMATION
STAT3
CF
Th9
B cell
IgG1
IgG2
IL-6
TGF-β
Pb
LTCD4
Th17/22
IL-17
IL-6
IL-22
RORγT
MUCOSAL
INFLAMMATION
MØ ALVEOLAR
DC
IL12R
B1/-
NEUTROPHIL
IL-1β
Treg
FOXP3+
IL-10
MØ
Pb
IL-10
MIX OF
LOOSE &
COMPACT
GRANULOMA
FORMATION
STAT3
Healthy infected
LTCD4
LTCD4
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Pb
COMPACT
GRANULOMA
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&
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DC
TNF-α
IFN-γ
Tbet
STAT1
STAT4
STAT1
IL12R
B1/B2
IL-12

studies of patients' immune responses, the focus of this review. Apparently, the severity of the disease is driven mainly, on one side, by the host's genetic background and immune response status and, on the other side, by the size of the inoculum, rather than peculiarities of fungus isolate. The immunogenicity and pathogenicity of *P. brasiliensis* samples freshly isolated from patients with PCM were tested in mice to compare with the severity of disease of the isolate's donor but a well defined relationship between pathogenicity of the fungal isolate in mice and the clinical findings of the correspondent patient was not evident (Sadahiro *et al.*, 2007). On the other hand, the mechanisms responsible in vivo for killing of the fungus are not yet known. *In vitro* studies showed that hydrogen peroxide (H_2O_2) released by normal human macrophages activated by Th-1 cytokines, kills yeast cells of *Paracoccidioides* sp. (Carmo *et al.*, 2006). Resting human macrophages are otherwise unable to kill the fungus or to handle the fungal cell wall of phagocytosed yeast cells (Lenzi HL., unpublished data). Others have shown that NK cells are able to recognize and kill in vitro *P. brasiliensis* as well as *P. brasiliensis*-infected monocytes (Longhi *et al.*, 2012). They also found that NK cells from PCM patients exhibit a lower cytotoxic response compared with healthy individuals.

Clinical Manifestations

As yet, there is no differentiation between the clinical manifestations caused by the two *Paracoccidioides* species, *brasiliensis* and *lutzii*, in a same fashion to what is observed with the disease caused by *C. immitis* and *C. posadasii*. PCM is a polymorphic disorder that may affect any system and organ. The current classification of the mycosis relies not only in the organs/systems involved but also in the host's immune status and the natural history of the disease (Franco *et al.*, 1987). The classification comprises a subclinical form (PCM-infection), the PCM-disease, which is subdivided into A/SAF, CF and mixed form, and a residual form. The A/SAF form predominates in children and young adults, though the denomination of juvenile form, while the CF affects adults and aged people, and is thus also named adult form. According to the severity of the disease, the A/SAF may be severe and moderate. The CF may apparently be restricted to the lungs or disseminated. As stated earlier, the degree of dissemination will depend on the availability of diagnostic procedures. Disseminated disease is characterized by involvement of the oral and upper respiratory mucosa, reticuloendothelial system, adrenal glands, skin and central nervous system; and, less frequently, gastrointestinal or genitourinary tract and bones (Giraldo *et al.*, 1976; Restrepo *et al.*, 1976; Mendes, 1994; Bellissimo-Rodrigues *et al.*, 2013). In more than 80% of the disseminated cases there is concomitant lung involvement, probably the initial localization from which distant foci were established through lymphohematogenous route. The CF can be mild, moderate, or severe (Franco *et al.*, 1987). It has been proposed that the disease presented by immunocompromised patients such as those with HIV-infection would correspond to a new, mixed form (Benard and Duarte, 2000). This mixed form was further substantiated by case-control studies (Morejón *et al.*, 2009; Almeida *et al.*, 2017). Finally, a fraction of the patients remains with fibrotic, residual lesions which may compromise their full health recovery, and thus are classified as having the residual form (Franco *et al.*, 1987; Shikanai-Yasuda *et al.*, 2017).

Fig. 5 Proposed schematic view of the immune network that takes place in the main outcomes of the paracoccidioidomycosis host-parasite interaction. Note that the three main outcomes, healthy infected, A/SAF and CF, are also an oversimplification of the polymorphic clinical spectrum of the disease. Infection initiates when inhaled *Paracoccidioides* conidia reach terminal bronchi or alveolar spaces, where they interact with and are phagocytosed by alveolar macrophages. These cells, harboring live and/or dead fungal elements, migrate to draining lymph nodes where transformation into dendritic cells and antigen presentation take place. DC-T cell interaction will then trigger three types, eventually overlapping, immune networks. In HI (lower part), DCs express NLRP3 and secrete predominantly IL-12 which drives activate activated T cells to differentiate into Th-1 cells, expressing the transcriptional factor Tbet and Th-1 cytokine signaling molecules STAT-1 and STAT-4, while expressing the β1 and β2 subunits of the IL-12R. These in turn release IFN-ɣ and TNF-α and activate macrophages to phagocytose and kill the yeast cells. Macrophages in this case express the pro-inflammatory cytokine signaling molecule STAT-1. Overall, this network leads to formation of compact granulomas and control/resolution of the infection. In the A/SAF (upper part), DCs do not express NLRP3 and release predominantly IL-10 and IL-4, which activate Th-2 cells; other T cell subtypes (Treg, Th-9) are also expanded and activated. Gata3 and STAT-6 expressing Th-2 cells induce eosinophilia (via IL-5 release), B-cells production of anti-Paracoccidioides IgE and Ig4 (via IL-4 release), but disarm macrophages via IL-10 release, which upregulate the inhibitory cytokine signaling molecule STAT-3, and as such *Paracoccidioides* yeast cells are not contained and loose granuloma formation ensues. The expanded *Paracoccidioides*-activated Tregs seen in these patients also contribute to the poor cellular immune response and poor granuloma formation. Expanded activated Th-9 cells drive massive *anti-Paracoccidioides* IgG1 and IgG2 isotype production. In the CF (middle part), which comprises patients with mild, localized disease to "localized" but severe lungs involvement to disseminated disease, present immune networks that may or may not be closer to either that of the HF or that of the A/SAF. In CF patients, the dormant yeast cells within clinically silent foci established during the initial infection, for yet unknown reasons, reactivate. DCs at these sites, upon challenge with the fungi, express NLRP3 but secrete predominantly the pro-inflammatory cytokines IL-6, IL1-β and TGF-β, which activates T-cells toward a Th-17 phenotype, the hallmark of the CF, but also Th-22. In parallel, the lack of Th-1 activation, resulting in less well activated (STAT-3 +) macrophages favor further destabilization of the granuloma. Th activated Th cells also lack expression of the β2 subunit of the IL-12R but express the intracellular transcriptional factor RORɣT. Both Th subtypes are responsible for the secretion of several cytokines, among them IL-6, IL-22, IL-17, that induce mucosal inflammation with prominent participation of neutrophils. Tregs and Th-9 are also important participants in the CF immune network. The net result is a variable mix of well-formed granuloma that can limit the infection and loose granuloma that correspond to foci of active disease. Note that the arrows arising from the CF to both the A/SAF and HI outcomes indicate that both Th2 and Th-1 responses may be part of the CF network, depending on the extent and severity of the patient's disease. The factors that dictate the patient's progression to one or another outcome are as yet unknown. Adapted from Benard, G., 2008. An overview of the immunopathology of human paracoccidioidomycosis. Mycopathologia 165, 209–221. doi:10.1007/s11046-007-9065-0. de Castro, L.F., *et al.*, 2013. Characterization of the immune response in human paracoccidioidomycosis. Journal of Infection 67, 470–485. doi:10.1016/j.jinf.2013.07.019, with modifications.

Patients with A/SAF develop signs and symptoms of a wasting process. Fever, malaise, listlessness, weight loss, and emaciation are recorded frequently (Benard *et al.*, 1994; Londero *et al.*, 1996; Nogueira *et al.*, 2006; Benard and Mendes-Giannini, 2019; Romaneli *et al.*, 2019). The A/SAF is a disease of the reticuloendothelial system resulting in damage of the corresponding organs. Superficial lymph node enlargement is the predominant sign in over 80% of these cases (Londero *et al.*, 1996; Benard and Mendes-Giannini, 2019; Romaneli *et al.*, 2019). Cervical and submandibular lymph node chains are involved most commonly, followed by those of the supraclavicular and axillary regions; however, any chain can be affected. Lymph nodes may vary in size from slightly enlarged to large, painful, coalescent masses; some may progress to fistulization draining purulent material rich in yeast cells.

Hepatomegaly and splenomegaly, usually asymptomatic, are the second most common finding in the A/SAF. In a report of 63 cases of PCM in children under age 16 liver involvement was detected in 40%, of whom 68% presented hepatomegaly and 29% jaundice (Pereira *et al.*, 2004). A more recent survey of 142 cases under 15 years-old found slightly lower frequencies of hepatomegaly ($\sim$30%) and splenomegaly (20%) (Romaneli *et al.*, 2019). Liver enzymes, especially alkaline phosphatase and gamma-glutamyl transferase are frequently but not markedly increased (Nogueira *et al.*, 2006). Gross hepatic lesions may not be apparent, but histopathologic examination regularly reveals fungal invasion of this organ in the more severe cases (Benard *et al.*, 1994; Boccalandro and Albuquerque, 1960; Londero *et al.*, 1996), as also revealed by a study of a series of fatal cases (Teixeira *et al.*, 1978). The presence of yeast cells in the liver was associated with a granulomatous tissue response. Splenic lesions are nodular or military. Portal hypertensions a rare occurrence. Importantly, a study showed that liver involvement was associated with younger age, more severe anemia, hypoalbulinemia and malnutrition and eventually higher risk of death (de Melo Braga *et al.*, 2013). Findings and complaints relating to the abdomen and digestive tract, such as presence of abdominal masses, lymph node enlargement, diarrhea, vomiting, abdominal distention or pain, and ascites, also have also been recorded in a sizable proportion of A/SA form patients (Benard *et al.*, 1994; Londero *et al.*, 1996; Pereira *et al.*, 2004; de Melo Braga *et al.*, 2013; Romaneli *et al.*, 2019). Hypertrophied lymph nodes, usually generalized but particularly periaortic, around the hepatic hilum and retroperitoneally, can be detected by US, CT scans or MRI (Martinez *et al.*, 1988). Signs and symptoms of an acute abdomen, caused by masses formed by hypertrophied lymph nodes that may become palpable or perforation, also have been reported. These coalescent masses may result in pathology caused by extrinsic compression of adjacent structures, such as jaundice by compression of the biliary duct, pancreatitis, intestinal occlusion, and blockade of lymphatic drainage followed by ascites (Benard *et al.*, 1994; Londero *et al.*, 1996; Pereira *et al.*, 2004; de Melo Braga *et al.*, 2013; Benard and Mendes-Giannini, 2019; Romaneli *et al.*, 2019). In fact, histopathologic examination of autopsies (likely the more severe cases) showed a specific granulomatous enteritis in 80% (Fonseca and Mignone, 1976). Additionally, in a MRI study of patients mostly with the A/SAF, 48% had abdominal lymph node enlargement, even if no abdominal signs and symptoms had been recorded (Martinez *et al.*, 1979b). This lymph node involvement can cause mesenteric lymphatic stasis and enteric mucosal edema that may progress to fungal enteritis accompanied by abnormal intestinal function such as reduced absorption of fat (Martinez *et al.*, 1979b; Shikanai-Yasuda *et al.*, 1992a,b; Benard *et al.*, 1996; Nogueira *et al.*, 2006; de Melo Braga *et al.*, 2013). Patients may thus develop a disabsorptive syndrome that aggravates the nutritional and immune status. Abdominal complications are still being sporadically reported despite improved diagnosis and treatments (Romaneli *et al.*, 2019). Digestive involvement may eventually be observed in CF patients with severe disseminated disease. The pathogenesis and manifestations are similar to those of the A/SAF, with primary involvement of the lymphatics causing lymph nodes enlargements, hepatosplenomegaly and ulcerative enteritis (Martinez *et al.*, 1979a,b).

Studies also have shown bone marrow infiltration mainly, but not exclusively, in the A/SAF of the disease (Resende *et al.*, 2006). Bone lesions and articular involvement may be important components of the severe forms of the disease, especially in younger children. In these patients, the long bones were frequently affected, with the lytic lesions located at the diaphyseal or metaphyseal-epiphyseal regions (Doria and Taylor, 1997; Pereira *et al.*, 2010; Correa-de-Castro *et al.*, 2012; Monsignore *et al.*, 2012), probably because of their higher vascularization, emphasizing the hematogenous dissemination that typically occurs in this form of the disease. Ribs, skull, phalanges, and vertebral lytic lesions also have been documented. Differently from the painful, motion-restriction joint lesions, the bone lesions can be silent of oligosymptomatic (Doria and Taylor, 1997; Monsignore *et al.*, 2012). A pathologic fracture may occasionally occur (Cimerman *et al.*, 1997).

Skin lesions were noted in 20%–30% of the A/SAF cases, with a tendency toward higher frequency with increasing patient age (**Fig. 6**). Distribution of cutaneous lesions was variable, but face and trunk were involved more frequently Acute-subacute paracoccidioidomycosis: A pediatric cohort of 141 patients, exploring clinical characteristics, laboratorial analysis and developing a non-survival predictor (Londero *et al.*, 1996; Nogueira *et al.*, 2006; Romaneli *et al.*, 2019). Lung abnormalities were recorded in a small proportion of cases. However, even in the absence of clinical and radiologic involvement, colonization of the lung by *Paracoccidioides* spp. can be demonstrated by direct examination and by culture (Restrepo *et al.*, 1989). When chest radiographs (**Fig. 7**) were abnormal, enlarged hilar lymph nodes was the most common abnormality; military or interstitial infiltrates were occasionally seen (Campos *et al.*, 1992; Londero *et al.*, 1996; Benard *et al.*, 2005; Martinez and Moya, 2009). In contrast with the CF, where adrenal involvement is a serious concern, it is uncommon in the A/SAF. A survey of adrenal function before and after treatment in 23 children with the A/SA disease showed normal adrenal function (Pereira *et al.*, 2006).

Anemia, an increased erythrocyte sedimentation rate, severe hypoalbuminemia, and hypergammaglobulinemia with high IgG serum concentrations are found regularly (Londero *et al.*, 1996; Nogueira *et al.*, 2006; Romaneli *et al.*, 2019). Nonetheless, anti–*Paracoccidioides* antibodies may prove undetectable in some patients with localized disease and low fungal burden or due to the production of low avidity antibodies (Neves *et al.*, 2003). Eosinophilia, as well as elevated IgE antibody titers, has been detected in most patients (Yarzábal *et al.*, 1980; Shikanai-Yasuda *et al.*, 1992a,b; Biselli *et al.*, 2001; Mamoni *et al.*, 2002; Nogueira *et al.*, 2006). Recently, it was proposed that the serum albumin level could predict the outcome of children with PCM: an albumin cutoff $\leq$ 2.18 g/dL on admission had a

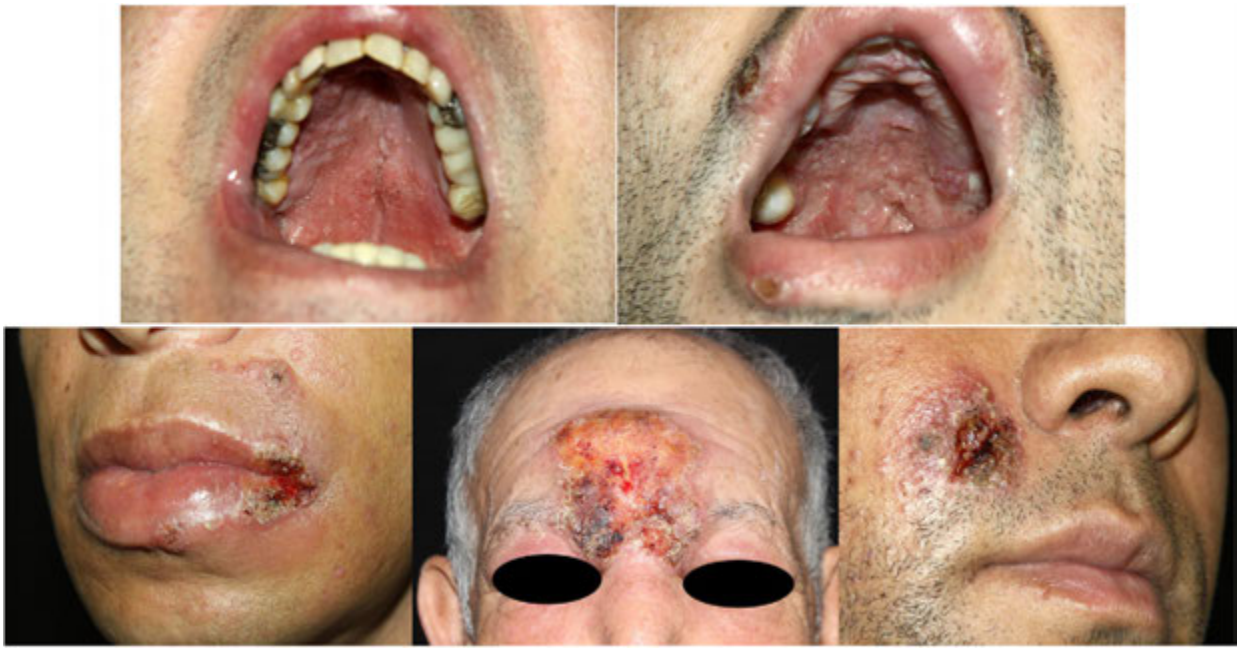

Fig. 6 Patients with the chronic form of the disease presenting characteristic muco-cutaneous lesions: hard palate ulcerated lesions with the typical moriform stomatitis aspect, ulcerated and infiltrative cutaneous facial lesions, and ulcerated lesions of the lips.

85.7% sensitivity and 85.4% specificity for non-survival outcome while values above 2.18 g/dL translated into a 99.1% chance of survival (Romaneli *et al.*, 2019). Studies of other cohorts should be performed to validate this prognostic factor.

The lungs are the target organ in the CF. The respiratory symptoms are nonspecific and, in most cases, evolve slowly, albeit progressively, thus delaying the diagnosis. Many patients attribute their symptoms to the smoking habit, related by ∼90% of the patients with the CF (Costa *et al.*, 2013; Peçanha *et al.*, 2017). Smoking appears to be a risk factor for the development of the CF PCM (dos Santos *et al.*, 2003). Main symptoms are short breathiness, mild dyspnea, dry cough, occasionally with sputum production and, in lesser proportions, hemoptysis. These symptoms may be accompanied by systemic symptoms such as fatigue, weight loss and asthenia. Lung auscultation reveals abnormalities in less than half of the patients; furthermore, at diagnosis over 20% of patients with several alterations on lungs radiological exams exhibit very mild symptoms and no alterations on pulmonary auscultation, a clinic radiological dissociation early observed in this mycosis (Franco *et al.*, 1989; Mendes, 1994; Gomes *et al.*, 2008). Fibrosis is frequently observed already at diagnosis (Campos *et al.*, 2008; Valle *et al.*, 1992; Mendes, 1994; Tobón *et al.*, 2003; Costa *et al.*, 2013) In fact, patients seek medical assistance for oropharyngeal lesions with the pulmonary involvement being diagnosed subsequently through imaging exams. CT scans main findings, according to the Fleischner Society´s Glossary of Terms (Austin *et al.*, 1996), are ground-glass attenuation, small centrilobular nodules, cavitated nodules, large nodules, parenchymal bands, areas of cicatricial emphysema, interlobular septal thickening, and architectural distortion (Funari *et al.*, 1999; Souza *et al.*, 2006; Costa *et al.*, 2013). Cavitations and air-space consolidation are also found (Funari *et al.*, 1999; Souza *et al.*, 2006; Costa *et al.*, 2013). These abnormalities are bilateral and tend to be symmetrical. Hilar lymph node enlargements can be found. The reversed halo sign, defined as central ground-glass opacity surrounded by a crescent or ring of consolidation, is observed in up to 10% of patients with PCM (Gasparetto *et al.*, 2005). Pulmonary function tests show an obstructive pattern affecting mainly the small airways: probably both the fungus and smoking contribute the ventilation/perfusion alterations and alveolo-interstitial destruction (Lemle *et al.*, 1983; Campos *et al.*, 2008; Costa *et al.*, 2013). Smoking alone could probably explain these alterations in some patients (Gomes *et al.*, 2008). However, in 30% of the patients receiving an adequate course of therapy, pulmonary fibrosis will appear de novo or will consolidate if preexisting, such that by the end of the treatment period this sequela will be documented in over 50% of the cases by chest X-ray, or even higher percentages when CT scans are employed (Tobón *et al.*, 2003; Costa *et al.*, 2013).

Lesions in the oropharyngeal and laryngeal mucosa occur frequently in the CF (Mendes, 1994; Sant'Anna *et al.*, 1999; **Fig. 5**). Hoarseness, localized pain, dysphagia, and dyspnea (due to tracheal stenosis) are main complaints. Deaths due to suffocation have been recorded, and immediate laryngoscopic evaluation is highly recommended in patients reporting laryngeal stridor and dyspnea. Urgent tracheostomy may be indicated in these cases (Campos *et al.*, 1986). Lesions in the oral cavity are particularly painfull, and can affect the teeth and gum, which results in difficulties in chewing the aliments and significant weight loss. There is not a uniform or typical aspect of the lesions: they may be infiltrative, ulcerated, nodular, or vegetative, and may mimick squamous cell carcinoma. However, they usually have a granulomatous superficial aspect (Mendes, 1994; Migliari *et al.*, 1998; Sant'Anna *et al.*, 1999): the base of the ulcerated lesions usually is covered by small abscesses (the mulberry-like lesions) that probably represent fungal dissemination through the lymphatic system because they usually are accompanied by regional lymph node involvement (Uribe *et al.*, 1987; Castro *et al.*, 1999). In the A/SAF, mucosal lesions are rather exceptional, but skin

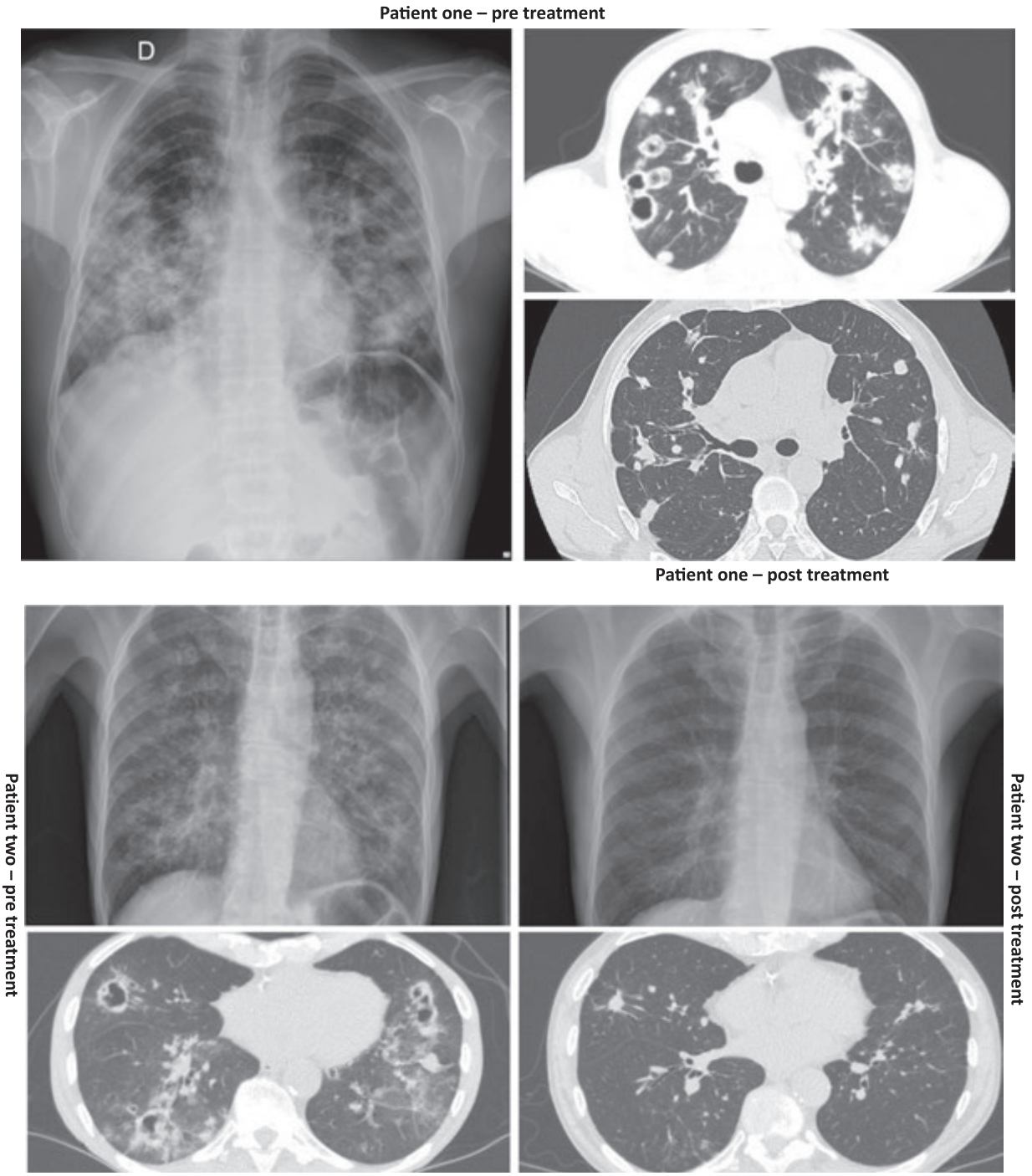

Fig. 7 Typical pulmonary involvement in the chronic form of the disease. Patient one, chest X-ray (left) showing typical bilateral infiltrates on admission, and thoracic CT scans showing the parenchymatous lesions before (upper) and their improvement during antifungal therapy (lower). Patient two, on the left, chest X-ray showing typical bilateral infiltrates and thoracic CT scan showing the parenchymatous lesions on admission; on the right, chest X-ray and CT scan taken at the end of the antifungal therapy: note the presence of residual abnormalities such as small opacities and nodules, fibrosis, bronchial thickening and small area still with ground glass aspect.

involvement occurs more commonly and tends to be multiple, in contrast with the CF (Mendes, 1994; Marques *et al.*, 2007). In the latter, lesions are represented mostly by contiguous involvement of the periorificial mucosal lesions or draining lymph nodes. In both the A/SA and chronic severe disseminated form, they represent hematogenous spread of the fungus. In this case, the lesions may appear as ulcerated or ulcero-vegetative lesions, papules, or crust-covered ulcers and warts, usually at the same stage of

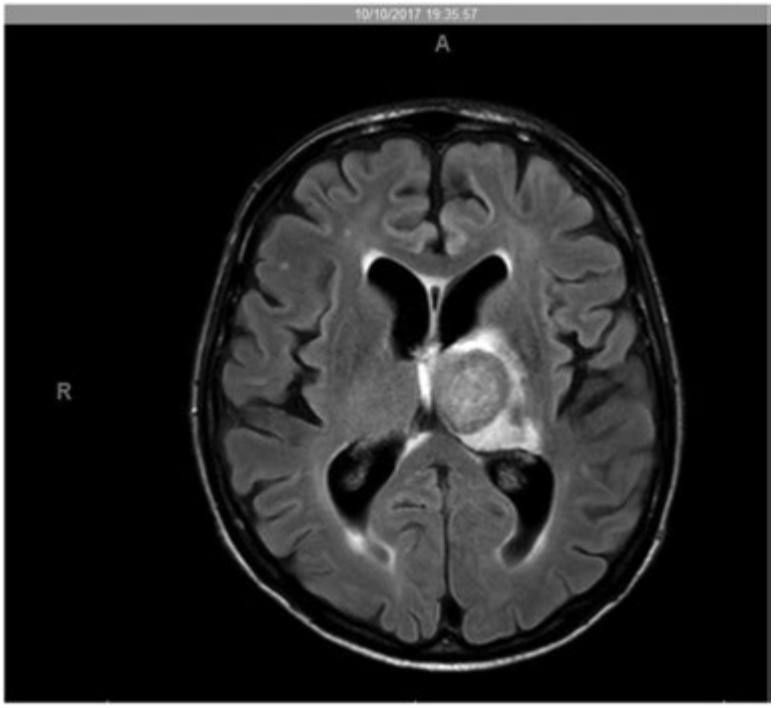

Fig. 8 Brain CT scan showing a pseudotumoral lesion in a patient with the chronic form of the disease.

development (Mendes, 1994; Marques *et al.*, 2007) Skin involvement may rarely present as sarcoidic like lesions (Coelho *et al.*, 2016). Mucosal and skin lesions usually are the first ones to resolve with treatment.

The adrenals often are involved in patients with the chronic form of the mycoses, with a small proportion of the patients evolving to overt adrenal hypofunction or insufficiency (Addison disease). A study showed that adrenal reserve was reduced in 44% of the patients, mostly with the CF (del Negro *et al.*, 1980). Main manifestations of chronic adrenal insufficiency are malaise, fatigue, anorexia, weight loss, arterial hypotension, orthostatic hypotension, hyperpigmentation of the skin, nausea, and reduced libido. The glands contain multiple granulomatous foci, and diffuse necrosis may be seen in the most severe cases. Hyperplasia of the adrenal glands also occurs commonly (Tendrich *et al.*, 1991). The adrenal insufficiency has been documented in patients even after prolonged post-therapy follow up (Tabón *et al.*, 1995), although in a few patients the adrenal function has been reported to fully recover (do Valle *et al.*, 1993).

Reports on the frequency of CNS involvement are quite variable, from 3.4% to 25.45% (Almeida, 2005); it is however more frequent than it has been admitted in the past. NeuroPCM (**Fig. 8**) should always be considered in the differential diagnosis of meningoencephalitis and in expansive processes of the CNS, especially in endemic areas (de Almedia *et al.*, 2004). Most patients with neuroPCM have the CF and involvement of other organs (particularly lungs) at the time SNC symptoms manifest. Involvement of the CNS is considered to be secondary to a primary focus: not infrequently the pulmonary involvement is diagnosed after that of the CNS. Rarely it is the sole localization of the disease (Almeida, 2005). A review showed that while in 21% of the cases the onset of neurological symptoms occurred before the onset of systemic symptoms, in 33% they happened simultaneously and in 46% they appeared after the onset of the systemic symptoms (de Almedia *et al.*, 2004). Pseudotumoral lesions in the brain hemispheres is the most typical presentation, although it can occur in any location of the CNS (Gasparetto *et al.*, 2003; Rosa Junior *et al.*, 2019). Clinical manifestations are non-specific and related to the location of the CNS lesion: according to a review, seizure in 33%, hemiparesis in 25%, cerebellar signs in 25%, headache in 21%, and hydrocephalus in 21%; less frequent symptoms include paresthesia in 13%, confusion in 13%, and bulbar signs in 8% (Almeida, 2005). CT scan images are usually hypodense, with annular or nodular enhancing, surrounded by mild edema after contrast injection. In 65% of the patients, there were multiple mass lesions, and 35% had a single mass lesion (Almeida, 2005). Meningitis associated with pseudo tumoral lesions can also occur. It usually presents as a skull base meningitis, with mild to moderate lymphomononuclear pleocytosis and elevated protein levels, but the finding of yeast cells in the CSF is an exception. Neuro-PCM is a serious condition: in a case series of 24 neuroPCM patients (de Almedia *et al.*, 2004), four patients died while 20 responded to the antifungal treatment, but most remained with residual lesions after treatment, characterized in the CT scan as hyperdense lesions, with irregular contrast enhancement, eventually residual calcified lesions. Neurological sequels are not uncommon in these patients.

Reports of septic shock caused by septicemia by *Paracoccidioides* spp. show that fungemia can occur (Azulay *et al.*, 1988; Londero *et al.*, 1996). However, probably death is not caused by the fungemia per se, but by the severely dysregulated immune response triggered by it which culminates in an Inflammatory cytokine storm and extensive tissue necrosis (Benard *et al.*, 2010, 2012b).

Association between human immunodeficiency virus (HIV) infection and PCM has been reported (Benard and Duarte, 2000; Morejón *et al.*, 2009; Almeida *et al.*, 2017). PCM was also diagnosed occasionally in patients with other immunossupressive conditions such as malignancies, transplant, and immunosuppressive therapies (Shikanai-Yasuda *et al.*, 2008; Ruiz e Resende *et al.*, 2015; de Almeida *et al.*, 2019). The number of cases in HIV-infected patients was much lower than it was initially expected from what was observed with the association of other systemic mycoses with HIV (e.g., histoplasmosis). However the actual number of cases may be underestimated since PCM still is not a compulsory notification disease in most endemic countries. Clinical manifestations of these patients often do not fit into the A/SAF or CF patterns. They frequently present a disseminated disease, with lymph nodes involvement, hepatosplenomegaly and cutaneous lesions, suggestive of the A/SAF, concomitant with pulmonary and/or oropharyngeal mucosal involvement, more frequently seen in the CF (Benard and Duarte, 2000; Morejón *et al.*, 2009; Almeida *et al.*, 2017). The likely explanation is that the disease is due to reactivation of a latent infection as in the CF, but with higher lymphohematogenous dissemination due to the immunosuppression, as seen in the A/SAF. In fact, most patients were living in urban areas when they developed the (Benard and Duarte, 2000; de Almeida *et al.*, 2019). Thus the proposition of the classification as mixed form. Most patients with HIV-PCM had CD4 cell counts below 200 cells/mL (Benard and Duarte, 2000; Morejón *et al.*, 2009; Almeida *et al.*, 2017; de Almeida *et al.*, 2019). Mortality was higher than in non-HIV infected, but it was usually attributed to other HIV-infection related comorbidities than to PCM itself (Morejón *et al.*, 2009; Almeida *et al.*, 2017; de Almeida *et al.*, 2019).

Treatment

Several classes of drugs can be used to treat PCM. Historically sulfonamides were the first class of compounds to be effective in the treatment of PCM (Ribeiro, 1940), followed by the introduction of amphotericin B in 1958 (Lacaz and Sampaio, 1958). Imidazole derivatives were introduced in the end of 1970s' with ketoconazole. This drug is now rarely considered in PCM treatment, having been replaced in the end of the 1980s' by the triazole derivative itraconazole (Negroni *et al.*, 1987; Restrepo *et al.*, 1987). Fluconazole is another option for PCM treatment, but is has less often been used for its lower in vitro activity compared with itraconazole, although it can be indicated for neuro-PCM cases for its low binding to plasma proteins, allowing diffusion into the CSF (Diaz *et al.*, 1992; Shikanai-Yasuda, 2015). Voriconazole, posaconazole, and isavuconazole showed inhibitory activity in vitro against *Paracoccidioides* spp. isolates and, therefore, are potentially useful in the treatment of PCM. These as yet high cost drugs are not easily accessible in the endemic areas. Thus, except for two pilot studies showing voriconazole and isavuconazole effectiveness against PCM, mostly in CF patients without severe disseminated disease, accumulated experience with these drugs is still lacking (Telles *et al.*, 2007; Thompson *et al.*, 2016). Voriconazole was used at 8 mg/Kg/day dose, usually 200 mg PO two times daily, while isavuconazole was used at 200 mg PO 3 times daily for 2 days followed by 200 mg once-daily (Telles *et al.*, 2007; Thompson *et al.*, 2016). Drug interactions and adverse events of prolonged therapy (as required for PCM treatment) may be issues with these drugs.

Until recently there was no consensus on treatment decisions and protocols: these varied considerably among reference centers, for several reasons. Total duration of therapy is quite variable according to the severity of the disease and the patients' clinical, radiological and serologic responses to treatment. Neither the classification of the severity of the disease nor the local availability of antifungal drugs are equal among the reference centers. There are scarce comparative studies on PCM treatment, most of them bearing important limitations (Queiroz-Telles *et al.*, 1992; Shikanai-Yasuda *et al.*, 2002; Borges *et al.*, 2014; Cavalcante *et al.*, 2014). A *quasi-experimental* study of 177 patients showed the superiority of itraconazole over the association of sulfamethoxazole and trimethoprim (SMX-TMP), the most commonly used drugs for PCM treatment in Brazil (the country that contributes with over 80% of the cases worldwide) (Cavalcante *et al.*, 2014). These studies nevertheless support the indication of itraconazole as the first-choice treatment for PCM. However, there are some situations in which SMX-TMP is preferred, including patients with neuro-PCM (itraconazole does not cross the blood brain barrier), and patients with gastrointestinal tract involvement, as itraconazole already has erratic absorption in the gastrointestinal tract (Shikanai-Yasuda, 2015). SMX-TMP may be a viable option for the treatment of severe disseminated life threatening PCM in cases amphotericin deoxycolate is contraindicated or lipid formulations are not accessible. An updated guideline on the management of PCM has recently been published by Brazilian experts (Shikanai-Yasuda *et al.*, 2017).

One of the major issues in the treatment of PCM is compliance for the patients often require at least one year of therapy. This issue is sibling of the other major issue in PCM, the development of sequels after treatment in a sizable proportion of patients, discussed in the next section. A retrospective study of SMX-TMP therapy in a poor resource endemic area showed that only 68.3% of 244 treatment records that fulfilled the study's inclusion criteria reported adequate compliance. However, this proportion is likely an overestimation because from the other 283 patients' records that did not fulfill the inclusion criteria, in 67.8% this was due to the patient's missing more that 50% of the appointments (Nery *et al.*, 2017). In fact, a recent study from another reference center in the same poor resource area reported a compliance rate of only 44.6% (Andrade *et al.*, 2019). The importance of continuous treatment must always be emphasized because relapses and increasing risk to developing sequels occur if the drug is not taken regularly. As the CF is strongly associated with smoking habit and alcohol abuse (Martinez and Moya, 1992; dos Santos *et al.*, 2003), particular care should be provided to these issues, in order to assure better compliance, better response to treatment, and lower risk of developing sequels (especially pulmonary damage).

Itraconazole is administered in 100 mg capsules that should be given short after a meal. The Brazilian guidelines recommend 200 mg/day for low to moderate severity forms (Shikanai-Yasuda *et al.*, 2017). Several groups reported good results with this therapy but relapses may still occur (Marques, 2002; Shikanai-Yasuda, 2015). However, we have now successful experience in treating patients with the more severe forms (either A/SAF and CF) of the disease with higher doses of itraconazole (400–600 mg/daily) provided

there is such an involvement of the digestive tract that could further impair the variable absorption rate of the drug. (amphotericin B, the conventional choice for these severe cases, has been reserved to patients with life threatening conditions). Absorption is also impaired by antacids or inhibitors of gastric acid secretion. Side effects have been few and include transient elevation of hepatic enzymes (Naranjo *et al.*, 1990; Shikanai-Yasuda, 2015). Itraconazole in children is recommended at a dose of 5–10 mg/kg daily, with a maximum dosage of 200 mg twice daily (Shikanai-Yasuda *et al.*, 2017).

SMX-TMP (400 mg/80 mg per tablet) is widely used in Brazil, mainly because of its easier availability within Brazil's public health system than itraconazole (Shikanai-Yasuda *et al.*, 2017). It has also been used in association with amphotericin B (Marques *et al.*, 1985). It has good absorption with predictable serum levels, as well as good tolerability, with myelotoxicity (leukopenia) the main side effect, which can be monitored and controlled by folinic acid administration without modification of the therapeutic regimen (Telles *et al.*, 2007). Interstitial nephritis manifested as hyperkalemia may occasionally occur. The Brazilian guideline recommends 8–10 mg/kg of trimethoprim daily (Shikanai-Yasuda *et al.*, 2017). However, in adults it is given generally at a dose of two tablets PO at 12-h (low to moderate cases) or 8-h (moderate to severe cases) intervals with good results. This combination has the advantage of permitting alternative parenteral administration whenever necessary; in this case it can be administered from 12 to up 6-h intervals. Duration of the treatment with this drug varies in each case, but it usually lasts for no less than nine months. Maintenance treatment can be achieved by using one tablet at 12-h intervals. Development of SMX-TMP resistance is occasionally clinically suspected but has rarely been documented in vitro. There is a single report of resistance of an isolate from an A/SAF patient (Hahn *et al.*, 2003).

Amphotericin B should be reserved for severe disseminated or life threatening cases (Shikanai-Yasuda *et al.*, 2017). It also can be used by patients who relapse during the course of or after treatment with orally administered drug because gastrointestinal involvement may impair drug absorption in these cases. The effectiveness of therapy with azoles has curtailed the need for more aggressive regimens. There is still scarce data regarding effectiveness of the new amphotericin B lipid formulations. However, a series of 28 severe patients treated with amphotericin B lipid complex showed good results in all cases (Peçanha *et al.*, 2016).

Fluconazole is an alternative treatment, although not as effective at least in vitro. High doses, up to 600 mg/day, and longer treatment periods that itraconazole should be considered. Recrudescence and relapse of disease apparently occur more frequently than when itraconazole is used (Diaz *et al.*, 1992). Fluconazole may be useful in severely ill patients who must be treated intravenously (Shikanai-Yasuda, 2015). No experience with the A/SAF has yet been reported.

Aids patients can be treated initially intravenously with amphotericin B or SMX-TMP IV (800/160 mg thrice daily), than moved to itraconazole 400–600 mg/day or SMX-TMP P.O. 800/160 mg thrice daily. Alternatively, patients may also be treated orally with higher doses of itraconazole (400–600 mg/day) or SMX-TMP (800–160 mg PO or IV at 12 8-h intervals). Good therapeutic response is generally observed, achieving control of the PCM. Treatment can be moved to the lower regular doses of both drugs until the TCD4+ counts reach 200 cells/μL (or eventually at least 100 cells/μL), provided the HIV viral load remains undetectable (Shikanai-Yasuda *et al.*, 2017). Pharmacological interaction with antiretroviral drugs is an important issue to be addressed in the treatment of Aids patients. For more details regarding treatment of specific conditions such as HIV-infection, pregnancy, children severe forms, the reader is referred to the Brazilian guidelines (Shikanai-Yasuda *et al.*, 2017).

Many issues remain to be addressed regarding optimal treatment of PCM. As stated earlier, treatment of PCM still faces many challenges such as the low compliance rates and strategies that could curtail the development of sequels. The role of adjunct therapy with immunomodulators, such as peptides derived from the *P. brasiliensis* immunodominant glycoprotein that act by augmenting the Th-1-mediated immune responses, was tested in vivo in experimental models only (Taborda *et al.*, 2015). A few studies demonstrated some in vitro reactivity of patients' T-cells to these peptides (Leo *et al.*, 2007). Further studies are required to demonstrate their utility in promoting protective cell mediated immune responses in the human host. In the opposite direction, some investigators have administrated short courses of corticosteroids as adjunct therapy to severe cases, based on the rationale that some patients suffer from an uncontrolled or excessive inflammatory response (Gryschek *et al.*, 2010; Benard *et al.*, 2012a). In support of this, some fatalities were attributed to the exacerbated inflammatory response (Benard *et al.*, 2010, 2012b). Patients who benefited from adjunct prednisone administration (0.5–1.0 mg/kg) for 1–2 weeks were those with intense inflammation in CNS, obstructive lesions of the larynx or trachea, lung lesions resulting in respiratory insufficiency and unremitting fever and weight loss (Gryschek *et al.*, 2010; Benard *et al.*, 2012a). The clinical and laboratory criteria that allow reduction in the antifungal dose or that could be used to end treatment with the assurance that the patient is cured are not established. Progressive decrease in the titers of the serologic tests currently available is one of the laboratory parameters used most frequently. Newer methods (e.g., antigenemia detection, proteomic biomarkers) still need further standardization and clinical studies and have not yet become widely available (Gómez *et al.*, 1998; Salina *et al.*, 1998; Marques da Silva *et al.*, 2003; Marques Da Silva *et al.*, 2004; Sylvestre *et al.*, 2018). Meanwhile, most specialists agree that treatment decisions need to be tailored according to the patient. **Table 1** includes the first line drugs recommended in the therapy of PCM.

Prognosis

Prognosis depends on the status of the patient at the time of diagnosis. Children and adults in whom fungemia has taken place and who have multiple organ involvement may respond less well to therapy and require critical care. Fatality rate due to PCM is progressively being reduced as a result of earlier diagnoses and better treatments. In a report describing eight deaths in a cohort of 141 pediatric cases, fatality rate was 9.5% in the 1981–2001 period and 2.6% in the 2002–2019 period (Romaneli *et al.*, 2019). As stated

Table 1 First line drugs recommended for therapy of PCM

Drugs	*Administration*	*Treatment schedule*
Itraconazole	Adult: 200 mg/day Children 5–10 mg/kg/day	For $\geq$ 9 months of treatment. In adults and adolescents the 200 mg dose can be reduced to 100 mg/day with clinical/mycological cure and low antibodies titers in sorological tests
SMX-TMP	Adult: SMX 800 mg/TMP 160 mg (PO 8/8 or 12/12hs) children 5MX 40–50 mg/Kg / TMP 8–10 mg/kg (PO 12/12hs)	For $\geq$ 9 months of treatment. In adults and adolescents the dose can be reduced to 400/160 mg/Kg PO 12/12hs with clinical/mycological cure and low antibody titers in sorological tests
Amphotericin B	deoxycholate 0.5 to 0.7 mg/kg/day (IV) lipid formulation 3–5 mg/kg/day (IV)	Attack treatment for few (usually 2–4) weeks then move to itraconazole or SMX-TMP

Note: Adapted with modifications from Shikanai-Yasuda, M.A., *et al.*, 2017. Brazilian guidelines for the clinical management of paracoccidioidomycosis. Revista da Sociedade Brasileira de Medicina Tropical, 50 (5), 715–740. doi:10.1590/0037-8682-0230-2017.

earlier, albumin level on admission could be a predictor factor for death. Other authors indicated that abdominal complications, especially liver involvement, ascites and malnutrition, were associated with the lethal prognosis (Nogueira *et al.*, 2006; de Melo Braga *et al.*, 2013). In the A/SAF, mortality rates are lower. Complete remission is to be expected for most but not all patients with the A/SAF and CF since sequels may develop and certain organ involvements may be definitive (e.g., pulmonary fibrosis, adrenal insufficiency, neurological sequels). Thus, some clinicians consider the term *cure* inappropriate because of the inability to confirm complete eradication of the organism; the term *apparent cure* should instead be used (Shikanai-Yasuda *et al.*, 2017).

PCM persists as a disease with relatively low mortality but considerable morbidity. Complications vary and, like prognosis, their occurrence depends on the extent of fungal invasion. In the A/SAF, early complications that may lead to surgical intervention are intestinal obstruction and jaundice, both of which result from enlarged mesenteric lymph nodes, or emergency tracheostomy due to laryngeal lesions leading to tracheal stenosis. Sequels are more common in adults than children. In general, fibrosis is the cause of serious problems in patients who respond to therapy, particularly when extensive pulmonary infiltrates are present. Despite the newer, very effective therapies, these sequelae preclude, in a proportion of the cases, the complete restoration of the patients' previous health status (Tobón *et al.*, 2003; Lopera *et al.*, 2015). Scarring and fibrosis of the affected nodes and residual pulmonary fibrosis have been noted in earlier studies (Londero *et al.*, 1996). A study showed that almost all recently treated CF patients remain with at least one abnormality on CT scan (Costa *et al.*, 2013), the most frequent of which were architectural distortion, reticulate and septal thickening, centrilobular and paraseptal emphysema and parenchymal bands. These findings likely reflect the pattern of dissemination of the fungi through the lymphatics and subsequent fibrosis previously described in histopathology studies (Tuder *et al.*, 1985). Functionally, patients typically presented with a mild obstructive disorder and a mild reduction in diffusion capacity with preserved exercise capacity. However, one third of patients presented significant oxygen desaturation upon exercise that was associated with respiratory distress (Costa *et al.*, 2013). In addition, centrilobular and paraseptal emphysema were also observed in most patients, reflecting the high tobacco exposure in this population, and emphasizing the importance of tobacco exposure cessation for better treatment results. A recent study of post-treatment patients with a longer follow-up showed persistence of the fibrosis and emphysema abnormalities; all patients were smokers (Pina, 2006). Attempts to reduce post-treatment pulmonary fibrosis have been tested in experimental models only but may be promising (Lopera *et al.*, 2015). Rarely, a patient may remain with significant enough fibrous scarring to result in development of cor pulmonale (Campos *et al.*, 1986). Other important sequels that may be present in the CF are hoarseness, tracheal stenosis leading to definitive tracheostomy, microstomia, which requires surgical reconstruction, adrenal insufficiency, which requires long life hormonal replacement, and neurological sequels (do Valle *et al.*, 1995; Tabón *et al.*, 1995; Londero *et al.*, 1996; Weber *et al.*, 2006; Francesconi *et al.*, 2011; de Pina *et al.*, 2018).

Human PCM has no recognized prophylactic treatment. Vaccine strategies are being developed but are still restricted to experimental models of the disease (Taborda *et al.*, 2015).

Vaccine Development

The antifungal treatment of PCM requires prolonged drug use plagued by relapses and fibrotic sequelae that incapacitate individuals. This lead scientists to look for new tools (reviewed by Taborda *et al.*, 2018; Shikanai-Yasuda *et al.*, 2017). In this sense, the use of prophylactic or therapeutic vaccines would be an option to protect the host or to reduce the treatment time (Travassos and Taborda, 2012).

One of the most promising vaccine studies concerns the P10 peptide, derived from the *P. brasiliensis* 43 kDa glycoprotein (gp43) (Taborda *et al.*, 1998).

The epitope was mapped to a 15-mer peptide (QTLIAIHTLAIRYAN), immunodominant and protective in murine models against a virulent isolate of *P. brasiliensis*. This effect was attributed to a strong cellular immune response mediated by the secretion of IFN-gamma and IL-2 (Travassos and Taborda, 2012). Very importantly, P10 is also immunodominant and promiscuous in PCM patients, being recognized by 9 predominant HLA molecules, thus becoming a promising candidate for an effective vaccine against PCM in humans (Iwai *et al.*, 2003).

An interesting approach was carried out aiming at associating the P10 peptide with antifungal chemotherapy. Treatment of mice (anergic or not) with antifungal drugs such as itraconazole and sulfamethoxazole/trimethoprim plus P10 showed a protective reduction of the fungal load in the lungs, preserving the alveolar structure and preventing the spread of the fungus to the spleen and liver (Travassos and Taborda, 2012). Based on the peptide P10, different vaccine models with anergic or immunocompetent mice were developed involving: dendritic cells (Magalhães *et al.*, 2012; Silva *et al.*, 2017, 2019); hepatitis B virus-derived particle (VLP) as an antigen carrier (Holanda *et al.*, 2017); Different adjuvants such as aluminum hydroxide, flagellin, cationic lipid dioctadecyl-dimethylammonium bromide (DODAB) and complete Freund's adjuvant (CFA) (Mayorga *et al.*, 2012; Braga *et al.*, 2009); P10 incorporated into PLGA (Amaral *et al.*, 2010), etc.

DNA Vaccine

A vaccine against *P. brasiliensis* using plasmid DNA was first tested in 2000 (Pinto *et al.*, 2000) with a mammalian expression vector carrying the full gene of the gp43 under the control of CMV promoter with Freund's adjuvant resulting in the induction of both B and T cell-mediated immune responses, modulated by IFN-γ. This immunization method was protective in mice prior to challenge with virulent *P. brasiliensis*. Similar DNA vaccines were carried out using P10 as the minigene in the plasmid construction (Pinto *et al.*, 2000).

When given prior to or after infection with a *P. brasiliensis* virulent isolate, plasmid-vaccination with P10 successfully reduced the fungal burden in lungs of infected mice. Intramuscular injection of a combination of plasmids expressing P10 and IL-12 given weekly for one month, followed by single injections, restored normal lung architecture and eradicated the fungus in mice infected one month before treatment (Rittner *et al.*, 2012).

Mice immunized with another plasmid carrying the P10 minigene (pcDNA3-*P10*) were examined, before and after infection with *P. brasiliensis*, for the expression of memory $CD4+CD44^{hi}$ T cells together with Foxp3+ Treg cells in the spleens and lungs, post infection. Both T lymphocyte types increased corresponding to a lung histopathology with minimal inflammation. The repeated immunization with the.

DNA-P10 plasmid which generated long-term memory and regulatory T cells, replaced the initially protective pro-inflammatory T effector cells, and were thus effective while preserving the integrity of the infected tissue (de Amorim *et al.*, 2013).

Other researchers have also studied different vaccine models such as: DNA vaccine based on HSP65 from *Mycobacterium leprae*, rPb40 and rPb27 recombinant proteins in association with fluconazole reviewed by (Rossi *et al.*, 2019), radio-attenuated yeast cells (Demicheli *et al.*, 2006).

Antibodies

Clinical studies showed that patients with paracoccidioidomycosis develop antibodies, although only a few of them are protective against the mycosis reviewed by (Boniche *et al.*, 2020). Therapy based on passive transference of monoclonal antibodies showed efficient anti-fungal activity in an experimental model using mice. de Mattos Grosso *et al.* (2003) demonstrated that monoclonal antibodies against a 70 kDa glycoprotein of *P. brasiliensis* protected mice infected with the fungi (de Mattos Grosso *et al.*, 2003). Buissa-Filho *et al.* (2008) showed protective and non-protective monoclonal antibodies against gp43, the main diagnostic antigen of *P. brasiliensis* (Buissa-Filho *et al.*, 2008). Other protective antibodies were described: anti-melanin polyclonal antibodies obtained by immunization of mice with melanin ghosts of *P. brasiliensis* reduced fungal burden (da Silva *et al.*, 2006); monoclonal antibodies generated against heat shock protein 60 from *H. capsulatum* also reduced fungal burden from mice infected with *P. lutzii* (Thomaz *et al.*, 2014), polyclonal antibodies to acidic glycosphingolipids (GSL) purified from *P. brasiliensis* reduced infection (Bueno *et al.*, 2016). The results showed that passive transference of antibodies can be a therapeutic strategy associated or not with antifungal chemotherapy.

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Sporotrichosis

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Introduction

Sporotrichosis is a subcutaneous mycosis with a worldwide distribution, especially in tropical and sub-tropical areas. The infection generally occurs by a traumatic inoculation of an organic matter containing the fungus into the subcutaneous tissue of humans or other susceptible animals. After a few days, lesions appear, commonly affecting the skin and the lymphatic vessels draining to the nearest lymph node (Barros *et al.*, 2011). Apart from fulfilling the criteria to be considered a neglected disease, its emergence in last years has called public health attention in countries of tropical and subtropical regions (Queiroz-Telles *et al.*, 2017).

This chapter will present topics regarding the state of art of the etiological agents of sporotrichosis, including their taxonomic classification, morphology, biology, ecology, and virulence, as well as sporotrichosis epidemiology, clinical presentations, pathogenesis, diagnosis, and treatment.

The Genus *Sporothrix*

The genus *Sporothrix* comprises more than 50 different species of fungi that can cause disease on plants, insects, and animals, including humans (de Beer *et al.*, 2016). All these species are eukaryotic heterotrophic organisms, have no mobility, and present a chitin-containing cell-wall (Barros *et al.*, 2011).

The Human Pathogenic *Sporothrix* Species

Since the first description of human sporotrichosis in the last years of the 19th century (Schenk, 1898) until the beginning of the 21th century, the single species described as an agent of human sporotrichosis was *Sporothrix schenckii* (Lopes-Bezerra *et al.*, 2006). However, after the advent of molecular biology, some studies based on DNA analysis demonstrated a high level of genetic variability among *S. schenckii* strains from different regions and from different clinical presentations of the disease (Liu *et al.*, 2003; Marimon *et al.*, 2006). In 2007, phenotypic and genotypic analyzes revealed that *S. schenckii* should not be considered the only etiologic agent of sporotrichosis (Marimon *et al.*, 2007). Since then, cases of *Sporothrix* infection in humans have been related to at least six species besides *S. schenckii*: *Sporothrix brasiliensis* (Almeida-Paes *et al.*, 2014), *Sporothrix globosa* (Madrid *et al.*, 2009), *Sporothrix luriei* (Marimon *et al.*, 2008), *Sporothrix mexicana* (Dias *et al.*, 2011), *Sporothrix pallida* (Morrison *et al.*, 2013), and *Sporothrix chilensis* (Rodrigues *et al.*, 2016). All these species are classified into the phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, order Ophiostomatales, family Ophiostomataceae (Guarro, 2012).

Morphology and Dimorphism

All *Sporothrix* species that cause sporotrichosis in humans are dimorphic fungi, that is, in the environment or under laboratory conditions at 25–30°C present a mycelial (or filamentous) morphology and during animal infections and when cultivated at 35–37°C in enriched culture media, such as brain heart infusion agar or blood sheep agar a yeast-like morphology is observed (Barros *et al.*, 2011).

Macroscopically, the colonies of these fungi in the mycelial form are initially white and glabrous, but after some days (usually three to ten, depending on the species and strain phenotypic characteristics), they usually develop a wrinkled and membranous aspect, and start to darken, turning brown to black in color (**Fig. 1(A)**). These colonies are composed by hyaline, septate, thin hyphae with sympodial thin-walled hyaline conidia and sessile thick-walled brown-to-black conidia (**Fig. 1(B)**). The color of the colony is related to the amount of dematiaceous conidia produced by the fungus. Thus, some strains that can not produce these structures, remain white, even after months of laboratory cultivation (Almeida-Paes *et al.*, 2015). The dematiaceous conidia may have specific formats depending on the species. For instance, *S. brasiliensis* produce globose to subglobose conidia, whereas *S. schenckii* conidia are triangular to cuneiform (Marimon *et al.*, 2007). However, this is not a specific taxonomic characteristic, since conidial morphology may present some variation among strains from a single species (Oliveira *et al.*, 2012a).

The transition to the mycelial to yeast form is an adaptive process to the host environment that is depending on the activation of some signaling pathways after *Sporothrix* spp. senses the higher temperature and different nutrient conditions of the host (Boyce and Andrianopoulos, 2015). Colonies on enriched media incubated at 35–37°C are white-to-creamy in color and have a smooth to wrinkled texture (**Fig. 2(A)**). The yeast-like cells of the human pathogenic *Sporothrix* species are ovoid with single, double, or in rare occasions, multiple buds (**Fig. 2(B)**). These cells often have a "cigar-shaped" feature, an aspect that is usually used to characterize the genus. (Barros *et al.*, 2011).

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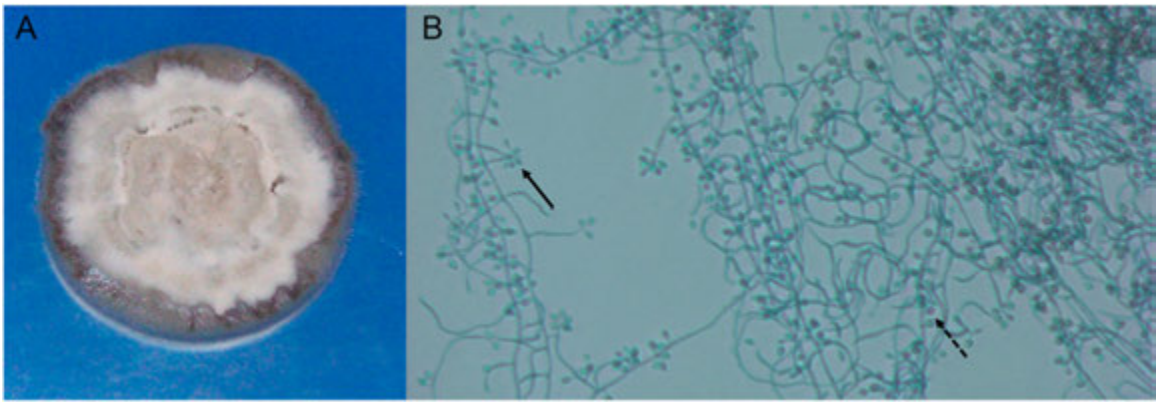

Fig. 1 Morphology of the mycelial form of *Sporothrix* spp. (A) Macroscopic aspect of a *Sporothrix brasiliensis* colony on PDA medium, 20 days of incubation at 30°C; (B) Slide culture of a *S. brasiliensis* strain after 20 days of incubation at 30°C, stained with Lactophenol Cotton Blue. Straight arrow indicates hyaline conidia and dashed arrow indicates dematiaceous conidia.

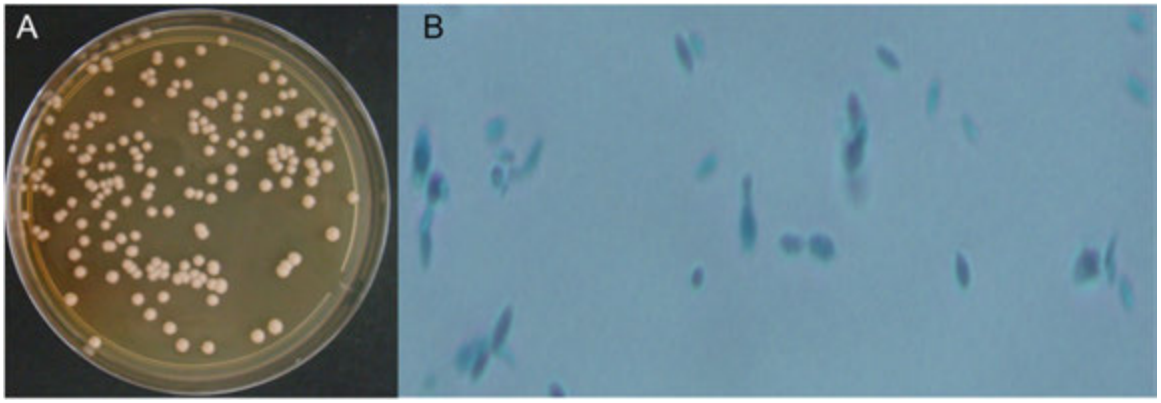

Fig. 2 Morphology of the yeast-like form of *Sporothrix* spp. (A) Macroscopic aspect of *Sporothrix brasiliensis* colonies on BHI agar medium, 7 days of incubation at 37°C; (B) Lactophenol cotton blue stain of *S. brasiliensis* yeasts after 7 days of incubation at 37°C.

Up to now, structures related to sexual reproduction in all *Sporothrix* species that cause infections in humans are unknown. However, genomic analyzes demonstrate that at least *S. schenckii* and *S. brasiliensis* have the potential for sexual reproduction, since they present a MAT locus in the genome, as well as mitogen-activated protein kinase pheromone pathway, pheromone processing enzymes, and meiotic regulators (Teixeira *et al.*, 2015).

Fungal Biology and Ecology

The physiological features of the human pathogenic *Sporothrix* species vary among the different species and morphologies of the fungi. Regarding growth conditions, the mycelial form of the fungus is more tolerant to basic pH, whereas yeasts are more adapted to grow in acidic conditions (Ghosh *et al.*, 2002). Optimum growth temperature is around 20–30°C, with the growth of all species impaired at 40°C. However, despite the ability to cause infections in the human host, the species *S. globosa* has low thermotolerance and the growth of most strains is inhibited at temperatures around 35°C (Marimon *et al.*, 2007). *Sporothrix* spp. have tolerance to both osmotic pressure and high salt concentrations, especially in the parasitic yeast form (Ghosh *et al.*, 2002) and tolerate cycloheximide concentrations up to 0.25% (Barros *et al.*, 2011).

Several carbon sources such as glucose, fructose, glycerol, maltose, sorbitol, threhalose, mannose, xylitol, and cellobiose can be assimilated, but not fermented. Inositol and lactose are not assimilated. Differences among species and even among strains from the same species are observed in the assimilation of sucrose, arabinose, rhamnose, raffinose, starch, and ribitol (Ghosh *et al.*, 2002; Marimon *et al.*, 2007). All species can produce urease at least in one fungal morphology (Almeida-Paes *et al.*, 2015; Ghosh *et al.*, 2002). Iron acquisition, which is indispensable during parasitism, occurs through the reductive pathway (Zarnowski and Woods, 2005) and through hydroxamate siderophore production (Holzberg and Artis, 1983).

As the majority of human pathogenic fungi, the agents of sporotrichosis live in the environment as organic matter decomposers. They release a series of enzymes that break down the decaying material, producing small subunits that can be absorbed by

the fungal cells. Infection in a mammal host is not necessary to *Sporothrix* spp. to complete their life cycle, that is, human parasitism is accidental. The agents of sporotrichosis can be found in cellulose-rich soils with high humidity, plants, water, decaying wood, among other outdoor environments (Barros *et al.*, 2011). The most traditional environmental sources of human pathogenic *Sporothrix* species are rose throns, *Sphagnum* moss, cornstalks, and hay. Despite their association with plants, these species were never described as plant pathogens, growing better on dead wood (Chakrabarti *et al.*, 2015).

Putative Virulence Factors

More than 50 different species are described in the genus *Sporothrix* (de Beer *et al.*, 2016), and the number of new species in this genus is continuously increasing (Ngubane *et al.*, 2018). However, only seven of them have been described as potential agents of disease in humans and other mammals. Moreover, the majority of sporotrichosis cases are caused only by three species: *S. brasiliensis*, *S. globosa*, and *S. schenckii* (Chakrabarti *et al.*, 2015). These species should have evolved differently from others in order to survive when accidentally come into a warm-blooded host.

The most accepted theory to explain the origin of virulence in *S. schenckii* and in the other human pathogenic species is the resistance that they developed against environmental stressors such as extreme pH, temperatures, and water availability, as well as presence of high salinity, radiation, and chemical pollution (Téllez *et al.*, 2014). Moreover, *Sporothrix* environmental interactions with other organisms, such as soil amoeba and bacteria, may have triggered the development of survival strategies that can be also used by the fungus during parasitism (Almeida-Paes *et al.*, 2017, 2019; Steenbergen *et al.*, 2004). As a result of this microbial interaction, the pathogenic species of *Sporothrix* gathered some putative virulence factors that facilitate survival when the fungus accidentally is inoculated into the host. These virulence factors include the dimorphism ability, thermotolerance, cell-surface adhesins, melanin production, and secreted hydrolytic enzymes (Ruiz-Baca *et al.*, 2015).

The Disease

Epidemiology

Epidemiological sporotrichosis data is usually strongly biased because this is not a notifiable disease in most countries, with most information about the disease coming from case reports and case series, which are only a "tip of the iceberg" of the sporotrichosis problematics.

Sporotrichosis usually is a benign disease, although some sporotrichosis-related deaths are reported in the literature. Some countries reporting sporotrichosis as a cause of death in humans include Brazil, the United Kingdom, and the United States of America (Kugblenu and Reeves, 2016; Moreira *et al.*, 2015).

Sporotrichosis had a high incidence in France in the beginning of the 20th century, but nowadays it is an uncommon disease in Europe. Sporotrichosis currently presents a considerable endemicity in Brazil, China, Colombia, India, Japan, Madagascar, Mexico, Peru, South Africa, Uruguay, and Venezuela (Chakrabarti *et al.*, 2015). Differences are also observed in the frequency of the sporotrichosis agents among these countries: while *S. brasiliensis* prevails in Brazil and is emerging in Argentina, *S. schenckii* is the main sporotrichosis agent in other countries of the Americas and Africa, and *S. globosa* is more frequent in Asia (Chakrabarti *et al.*, 2015; Córdoba *et al.*, 2018; Rasamoelina *et al.*, 2019).

All sexes and individuals of any age are equally affected by *Sporothrix* spp. Local peculiarities on sex/age characteristics of sporotrichosis cases are associated to the risk factors for fungal exposure in different regions. In general, sporotrichosis is an occupational disease that mostly occurs in gardeners, miners, and farmers, who are individuals handling or working closely to any organic matter where the fungus can be present. More recently, people handling possible infected animals, such as veterinaries, pet caretakers, hunters, and fishermen are also a high-risk sporotrichosis group (Barros *et al.*, 2011).

Epidemics of sporotrichosis are usually associated with a common source of infection (Bustamante and Campos, 2001). The largest sporotrichosis epidemic related to environmental *Sporothrix* exposure occurred in Witwatersrand, South Africa between 1941 and 1944. In this epidemic, thousands of miners were infected by *S. schenckii* present in the wood logs used in the mines. The miners presented pustular ulcerative, papular, plaque or warty skin lesions. This epidemic ended after the treatment of the mine timbers with a fungicidal spray to kill the fungus present in the surface of the wood (Helm and Bermam, 1947).

The major sporotrichosis epidemic in the United States of America, occurred in 1988, affected 84 patients in 15 states. The environmental source associated to these cases was the *Sphagnum* moss used for reforestation purposes (Coles *et al.*, 1992). A smaller outbreak involved contact of five patients with hay bales in a Halloween haunted house. Other point-source outbreaks of sporotrichosis were reviewed in this work, revealing that hay bales, haystacks, moss and straw were the potential sources of infection, and duration of exposure to these sources varied from a few minutes to six weeks (Dooley *et al.*, 1997).

Some hay-related epidemics have also been reported in Austrália (Conias and Wilson, 1998; Dhingra *et al.*, 2015). In China, outbreaks involving young children with facial lesions are usually associated with decaying cornstalks stacked for cooking and heating during cold seasons (Song *et al.*, 2011). In Peru, where sporotrichosis affects more children than adults, the most common risk factors for this mycosis are the ownership of a cat, activities in crop fields, dirt floor in the house, outdoors work, ceilings made of raw wood, and low socioeconomic status (Lyon *et al.*, 2003).

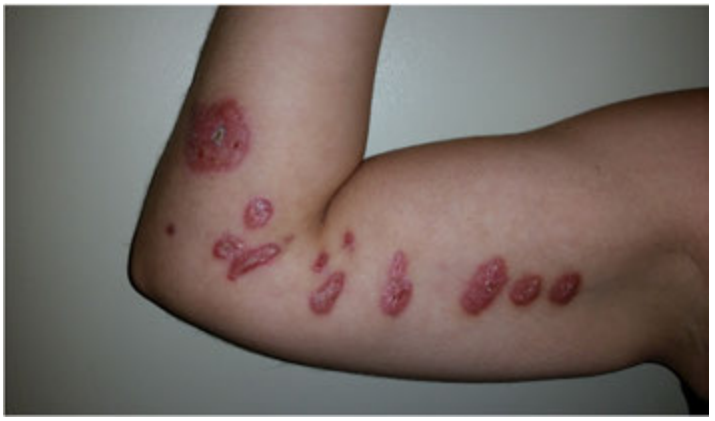

Fig. 3 Lymphocutaneous sporotrichosis in the right arm of a male patient.

In Brazil, since 1998, an increasing number of sporotrichosis cases are observed in human patients infected by the scratch, bites, or contact with naturally *Sporothrix* infected cats (Gremião *et al.*, 2017). In this country, middle-aged homemakers, retired elderly, and young students of deprived social strata are the major at-risk groups, due to their contact with domestic cats. In Rio de Janeiro state, where this zoonotic epidemic started in the late 90′s, the number of cases increased continuously for more than 20 years (Gutierrez-Galhardo *et al.*, 2015), with more than 10,000 human cases diagnosed up to 2019. Other Brazilian states have less cases of this disease, however, a spread of zoonotic sporotrichosis through the Northeast and West parts of the country have been observed in last years (Arantes-Ferreira *et al.*, 2019; Lacerda-Filho *et al.*, 2019).

Zoonotic sporotrichosis occurs in other places, such as Argentina, Australia, Germany, India, Japan, Malaysia, Mexico, Panama Peru, Spain, and the United States of America, but always with a much lower incidence than Brazil (Gremião *et al.*, 2017; Ramírez-Soto, 2016; Rios *et al.*, 2018). In Thailand, sporotrichosis in a cat was also reported, highlighting that zoonotic sporotrichosis may be also occurring in this country (Duangkaew *et al.*, 2019). An important aspect among some of these areas is that *S. brasiliensis* is the major agent in Brazil (Gremião *et al.*, 2017), whereas *S. schenckii* is involved in feline cases from Asia (Han *et al.*, 2017).

Clinical Presentations

The lymphocutaneous form is the most common presentation, accounts for more than 75% of cases and is the easiest form of sporotrichosis to diagnose. The lesions are usually located on the upper extremities. A primary lesion appears at the site of inoculation after two or three weeks, which corresponds to the chancre of inoculation. According to the time of evolution, this lesion can be ulcerated with an infiltrated base or a papular, nodular, nodular-ulcerative, ulcerative-gummy or vegetative plaque. From the initial injury, a chain of painless nodules, known as sporotrichoid spread, forms along the path of the lymph vessels (**Fig. 3**). These nodules may soften and ulcerate with some exudate. Usually, the regional lymph nodes are not involved, and no skin changes are observed between the nodes (Barros *et al.*, 2011).

The fixed cutaneous form is the second most common clinical presentation and accounts for approximately 20% of cases. The lesion remains confined to the inoculation site and the lymph vessels are not involved. It is believed to occur due to prior sensitization of the individual to the fungus which results in better host immune control, thereby limiting the lesion. The lesions are ulcers, verrucous, infiltrative, or ulcero-infiltrative plaques (**Fig. 4**). Small satellite lesions are common. This clinical form tends to be more chronic. The upper and low extremities are the most common sites of lesions, but the face, neck and trunk are affected as well (Barros *et al.*, 2011).

The disseminated cutaneous form is characterized by multiple skin lesions at noncontiguous sites without extracutaneous involvement. They tend to be more extensive than lesions from other cutaneous forms and cystic abscess can also be observed (**Fig. 5**). This form is rare and is caused by hematogenous spread of the fungus, usually associated with immunosuppression (Barros *et al.*, 2011; Moreira *et al.*, 2015). However, disseminated cutaneous sporotrichosis has been more frequently observed (up to 16% of cases) during endemic zoonotic sporotrichosis. In these cases, the involvement of non-immunosuppressed patients has been attributed to multiple inoculations by domestic cats (Barros *et al.*, 2011; Chakrabarti *et al.*, 2015).

In extracutaneous sporotrichosis the lesions may be restricted to a single site (and are only locally progressive) or may be disseminated (multifocal disease). The osteoarticular system is the most frequent site of unifocal disease. It occurs by contiguity with skin lesion and/or by direct inoculation of the fungus. Systemic symptoms are minimal. Osteomyelitis, isolated or with arthritis, may be present with or without skin lesions. The most commonly affected bones are the tibia, the small bones of feet and hands, the radius, the ulna, the skull and the face. Patients may present localized swelling and local sinus tract formation. The knee is the most common affected joint, but hand, elbow, and ankle joints may also be involved. Similarly, tenosynovitis, isolated or associated with osteoarticular sporotrichosis, may appear. Failure to consider the diagnosis leads to a chronic course with sequel (Rex and Okhuysen, 2010; Lederer *et al.*, 2016).

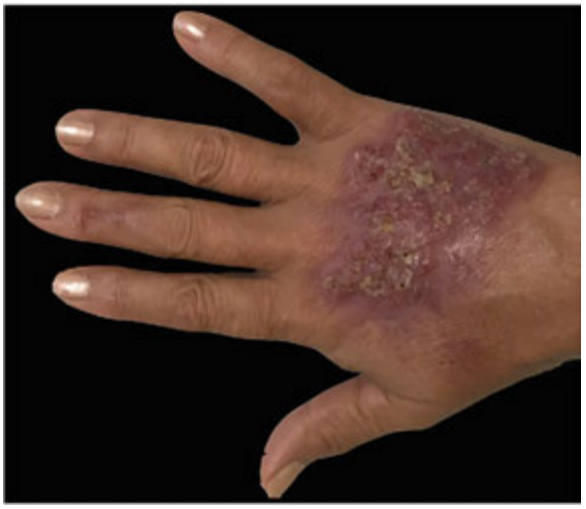

Fig. 4 Fixed cutaneous sporotrichosis in the right hand of a female patient.

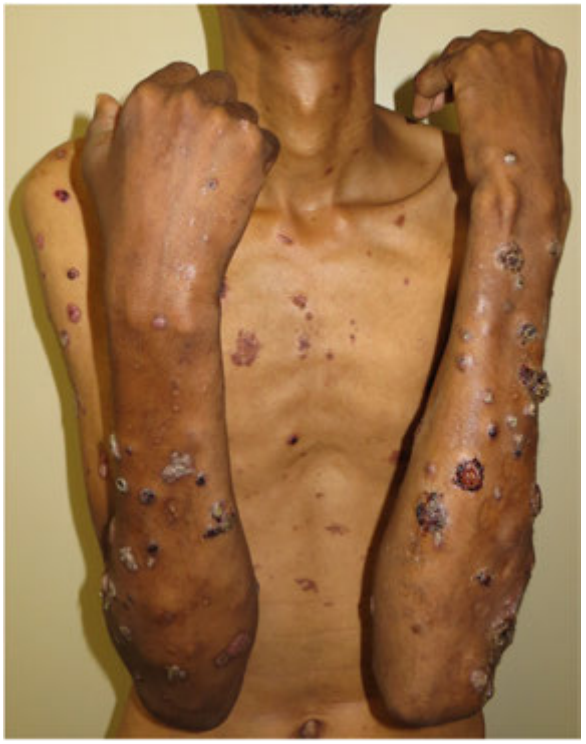

Fig. 5 Disseminated cutaneous sporotrichosis in a male patient.

Primary lung disease occurs after inhalation of *Sporothrix* conidia and is usually associated with chronic obstructive pulmonary disease. The patient may be asymptomatic or have respiratory symptoms, and the radiological pattern of chronic cavitary disease is observed (Rex and Okhuysen, 2010; Aung *et al.*, 2013).

Ocular sporotrichosis is usually related to zoonotic transmission through direct inoculation into the eye of secretions from naturally infected cats. Granulomatous conjunctivitis, eyelid sporotrichosis, and dacryocystitis are the most common presentations (**Fig. 6**). Parinaud oculoglandular syndrome (granulomatous conjunctivitis and satellite adenomegaly) is also frequent. Sequelae such as chronic dacrocystitis and fistulas are observed. Children are particularly affected by ocular sporotrichosis probably due to ludic contact with cats (Barros *et al.*, 2011; Freitas *et al.*, 2014a; Arinelli *et al.*, 2019).

Disseminated extracutaneous (multifocal) disease is associated with immunosuppressive conditions such as HIV infection, use of corticosteroids or other immunosuppressive drugs, diabetes, alcoholism, neoplasms, and organ (especially kidney) transplants.

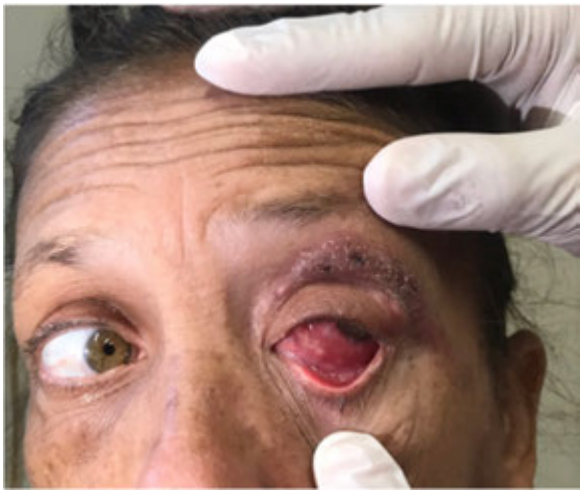

Fig. 6 Ocular sporotrichosis: bulbar conjunctivitis and lesion at the left upper eyelid.

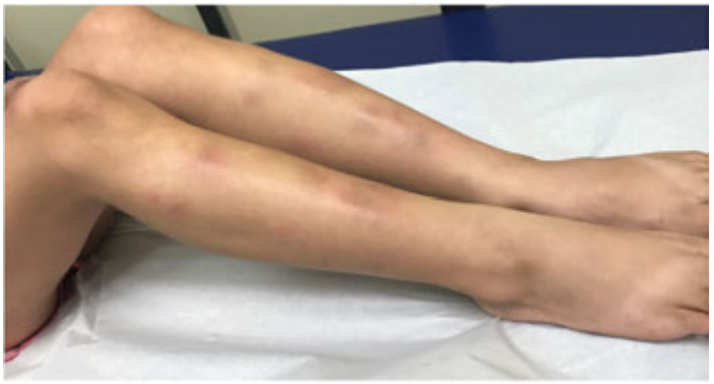

Fig. 7 Erythema nodosum associated with sporotrichosis in a female patient.

This form occurs by hematogenous dissemination of the fungus to any organ or tissue. The symptoms are specific to the involved organ and are followed by fever and general commitment in some cases. In addition to skin involvement, multifocal osteoarticular disease (mainly in the feet and hands), lung, central nervous system, and mucosae (ocular and upper airway), can be affected (Gutierrez-Galhardo *et al.*, 2010; Rex and Okhuysen, 2010). The radiological pattern of pulmonary involvement is diffuse reticulonodular infiltrate, with symptoms similar to those occurring in primary pulmonary disease, except for hemoptysis. Central nervous system involvement is characterized by chronic subacute meningitis or brain abscess, and isolation of *Sporothrix* spp. from the cerebrospinal fluid may be difficult. Hydrocephalus is a common and potentially serious complication in these cases. In intraocular sporotrichosis due to hematogenous dissemination, anterior or posterior uveitis, choroiditis, retinal granuloma and/or endophthalmitis are observed (Gutierrez-Galhardo *et al.*, 2010; Rex and Okhuysen, 2010). The mucosa of the mouth, pharynx, larynx, and nose can be infected by both direct and hematogenous routes. Enanthema, ulceration, suppuration, and vegetation are the clinical manifestations observed. Signs and symptoms may include rhinorrhea, nasal obstruction, odynophagia, and dysphagia. Endocarditis is also reported (Silva-Vergara *et al.*, 2012).

In endemic countries for sporotricosis, disseminated sporotrichosis may be the initial manifestation of HIV/AIDS. In the same way, patients with sporotrichosis who start antiretroviral treatment may experience worsening of pre-existing lesions or the emergence of new lesions characterizing an immune reconstitution syndrome (Freitas *et al.*, 2012a, 2014b).

The recognition of new *Sporothrix* species of clinical interest (Marimon *et al.*, 2007) has raised an important issue, among others, concerning the clinical and therapeutic relevance for the new taxonomy of the sporotrichosis agents. *S. brasiliensis* has been associated with hypersensitivity reactions, specifically in the region of Rio de Janeiro, Brazil, where this species has been highly implicated. These include erythema nodosum (**Fig. 7**) (Gutierrez-Galhardo *et al.*, 2002), erythema multiforme (Gutierrez-Galhardo *et al.*, 2005), Sweet syndrome (Freitas *et al.*, 2012b) and reactive arthritis (Barros *et al.*, 2011; Orofino-Costa *et al.*, 2017). A trend in the association of this species with disseminated disease in patients without an immunosuppressive underlying cause has also been observed, as well with greater severity, causing hospitalizations and deaths especially in aids

patients (Almeida-Paes *et al.*, 2014). Meanwhile, *S. globosa*, the most prevalent etiologic agent of sporotrichosis in China, is more associated with localized forms of the disease (Marimon *et al.*, 2007; Zhao *et al.*, 2017).

Pathogenesis

The broad range of clinical manifestations observed in sporotrichosis is the consequence of interactions between fungal and host cells. The host response depends on the site of infection, the infective *Sporothrix* species, the fungal morphotype (conidia, yeast-like cells), and the immunological status of the host. This response usually begins with an innate recognition of *Sporothrix* spp. by toll-like receptors (TLR) or by an inflammasome-dependent pathway, which modulates the subsequent adaptive immune response that basically consists in a mixed Th1/Th17 pattern (Gonçalves *et al.*, 2017).

Phagocytosis driven by macrophages, dendritic cells, and neutrophils constitute the first line of host defense against *Sporothrix* spp. After phagocytosis, these cells promote fungal antigen processing, production of reactive oxygen and nitrogen species, and antigen presentation, which lead to the inflammatory response. Yeast-like cells of *Sporothrix* spp. have the ability to escape the respiratory burst generated by the phagocytes through ergosterol-peroxide production, by the protection conferred by some components of the fungal cell-wall and by the several types of melanin produced by the fungus (Almeida-Paes *et al.*, 2015; Carlos *et al.*, 2015). More recently, an early role of defense involving natural killer cells in sporotrichosis has been proposed. These cells have a pivotal role in the *in vivo* elimination of *S. schenckii* due to their immunoregulatory role (Ferreira *et al.*, 2018).

Granuloma formation is another process of the host defense and a critical event in the immune response against members of the genus *Sporothrix* (Miranda *et al.*, 2013). Besides intact polymorphonuclear cells, granulomas present in tissues of patients with sporotrichosis normally contain caseous material, cellular debris, giant and epithelioid cell lymphocytes, plasmocytes, fibroblasts, and fungal yeast cells within phagocytes or in the extracellular medium (Barros *et al.*, 2011). The *Sporothrix* melanin has a role in the formation of granulomas. While heavily-melanized strains induce formation of multifocal granulomas, melanin-deficient strains remain restricted to the core of a unifocal granuloma (Almeida-Paes *et al.*, 2015).

Cellular immunity is the main response against *Sporothrix* spp. T CD4 cells were long recognized as pivotal in specific sporotrichosis immunity (Tachibana *et al.*, 1999). More recently, it was demonstrated that during *S. schenckii* systemic experimental mice infection, Th17 and Th1/Th17 mixed cells are developed, leading to an augmented production of IL-17 and IL-22. The Th17 response is necessary for fungal clearance (Ferreira *et al.*, 2015). A differential cellular immunity occurs between different *Sporothrix* species. For instance, *S. brasiliensis* induces a sustained Th17 and regulatory T cell responses, which contribute to a more severe disease than *S. schenckii* (Batista-Duharte *et al.*, 2018).

Specific antibodies against *Sporothrix* spp. are produced after IL-4 stimulation. It occurs in advanced stages of sporotrichosis, usually after four to six weeks post infection. Since patients with different clinical forms of sporotrichosis present similar pattern and amount of specific antibodies against *Sporothrix* spp., these molecules probably do not play a crucial role in sporotrichosis pathogenesis, though being useful for diagnostic purposes (Barros *et al.*, 2011). A role for antibodies in sporotrichosis was proposed, where *S. schenckii* yeast-cells opsonized by specific immunoglobulins are more efficiently killed by macrophages, which produce high TNF-α levels (Franco *et al.*, 2012).

Diagnosis

Although the clinical manifestations of sporotrichosis are well described, the definitive diagnosis of this mycosis cannot be based solely on these manifestations, since they can be mistaken with manifestations of other diseases, and differential diagnosis is mandatory. Lymphocutaneous sporotrichosis is fairly common and can be confidently clinically diagnosed. However, pyoderma, atypical *Mycobacterium* sp. and *Nocardia* infection as well as leishmaniasis must also be considered. The clinical symptoms of the fixed form can be similar to pyoderma, paracoccidioidomycosis, chromoblastomycosis, cutaneous tuberculosis, atypical mycobacteria, tertiary syphilis, leishmaniasis, and even skin cancer. Lesions of the disseminated cutaneous form can be confused with other deep mycosis (paracoccidioidomycosis, cryptococcosis, or histoplasmosis), atypical mycobacteria, or noninfectious granulomatous diseases. The disseminated extracutaneous forms are differentially diagnosed according to the affected organ. Other fungal conjunctivitis, bacterial, viral or fungal meningitis, bacterial osteomyelitis, pulmonary fungal infections, tuberculosis, and sarcoidosis should be considered (Rex and Okhuysen, 2010; Orofino-Costa *et al.*, 2017).

Sporotrichosis is classically diagnosed through the association of clinical, epidemiological, and laboratory data. For the diagnosis of cutaneous forms, the material must be obtained from the most recent lesions. In the evaluation of extracutaneous forms, the approach for isolation of the etiological agent will depend on the affected site. In lesions on conjunctival, nasal and oral mucosa, a swab should be collected for mycological examination. Evaluation of otolaryngologist and ophthalmologist with upper digestive airway endoscopy and ophtalmoscopy, respectively, are important. In cases of pulmonary sporotrichosis, chest X-rays should initially be requested and, if altered, chest CT and sputum examination for fungal screening. In the osteoarticular form, the most indicated exams are radiography, computed tomography (CT) or, if available, magnetic resonance imaging of the affected segment. If sporotrichosis in the central nervous system is suspected, a cranial CT scan should be request to rule out expansive lesions and to guide lumbar puncture for cerebrospinal fluid evaluation (mycological, biochemical and cytological examination). In patients with HIV infection, and CD4 T cell counts lower than 350 cells μL^{-1} should have sputum, urine, blood, and cerebrospinal fluid mycological tests, as well as imaging (chest X-ray, sinus CT), ophtalmoscopy, endoscopy and bone scintigraphy tests.

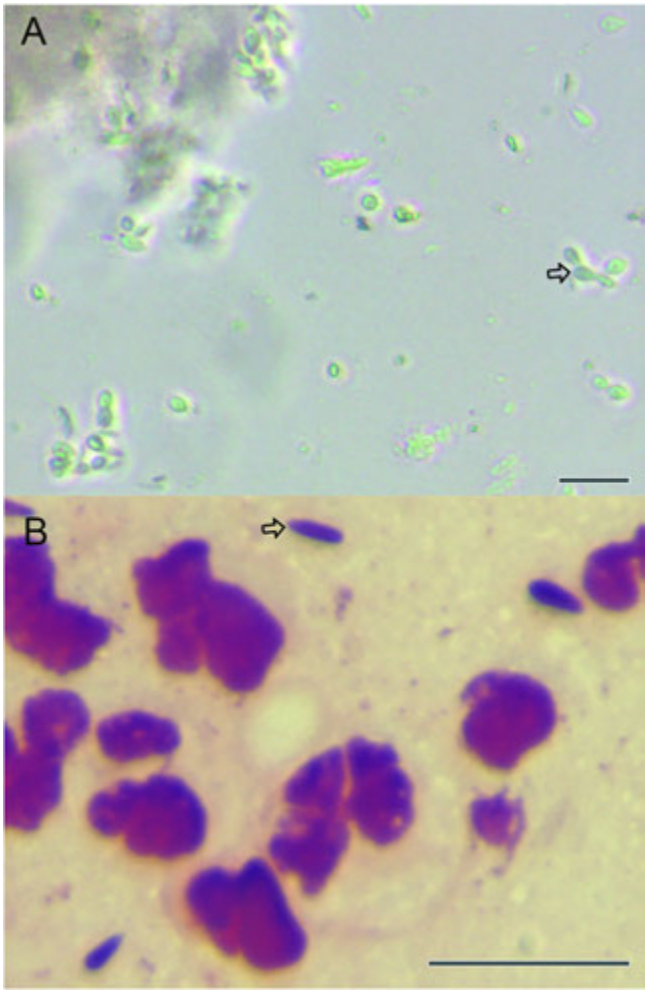

Fig. 8 Direct examination of samples positive for sporotrichosis. (A) Potassium hydroxide slide examination of a skin biopsy from a cat with naturally acquired sporotrichosis. (B) Gram stain of a lesion exudate smear from a human patient with lymphocutaneous sporotrichosis. Arrows in both panels indicate yeast-like fungal cells. Bars: 10 μm.

Mycological examination comprises the direct examination and culture tests. Direct examination is usually performed with potassium or sodium hydroxide, to detect cigar-shaped budding yeast cells of 2–6 μm diameter (**Fig. 8(A)**). These cells are hard to detect in samples from immunocompetent human patients or most animals, but can be found in samples from immunosuppressed patients and cats with sporotrichosis (Barros *et al.*, 2011). Gram and Giemsa stains can help in the visualization of *Sporothrix* yeast-like cells (**Fig. 8(B)**).

The definitive diagnosis of sporotrichosis is based on the isolation and identification of its etiological agent in culture. It is usually performed on Sabouraud Dextrose Agar with chloramphenicol 400 mg L^{-1} to prevent bacterial contamination, and on a cycloheximide-containing culture medium, to inhibit the growth of anemophilous fungi that may grow in the cultures of clinical specimens obtained from non-sterile sites (Orofino-Costa *et al.*, 2017). Cultures should be incubated at 25–30°C, and must be screened at least once a week for a minimum of four weeks to detect the growth of filamentous fungi with a compatible morphology with *Sporothrix* spp. Traditional identification of these colonies are performed through the study of the macroscopic and microscopic characteristics of the colony, previously described in this chapter, both on mycelial and yeast forms. These analyzes, however, do not differentiate the species within the genus. The gold-standard method for *Sporothrix* species identification is the partial sequencing of the calmodulin gene (Marimon *et al.*, 2007). In last years, other methodologies to differentiate the *Sporothrix* species have been described, including qualitative and quantitative polymerase chain reaction (PCR) with specific primers, PCR fingerprinting, rolling circle amplification, random fragment length polymorphism of the calmodulin gene, and matrix-assisted laser desorption/ionization using time-of-flight mass spectrometers (Oliveira *et al.*, 2012b, 2015; Orofino-Costa *et al.*, 2017).

Positive *Sporothrix* cultures provide the definitive evidence for the diagnosis of sporotrichosis, but this procedure has some limitations. It is difficult to obtain a suitable clinical specimen for culture in some disease manifestations, such as *Sporothrix*-induced arthritis (Barros *et al.*, 2011). In addition, cultures often present false-negative results due to low fungal burden, antifungal use, or contamination with bacteria and/or non-pathogenic fungi (Oliveira *et al.*, 2019). To overcome these limitations, non-culture

diagnostic methods have been developed to improve diagnostic sensitivity and quickness. The results of these tests provide a presumptive diagnosis of sporotrichosis and require clinical correlation for the correct assessment and determination of the final diagnosis (Barros *et al.*, 2011).

The most used non-culture diagnostic method for sporotrichosis is the detection of specific anti-*Sporothrix* antibodies in serum samples. Precipitation and agglutination techniques were first used, but these tests lack sensitivity in cases of cutaneous sporotrichosis and do not determine the isotype of immunoglobulins involved in the response (Barros *et al.*, 2011). The use of immunoblot and, more frequently, enzyme-linked immunosorbent assays (ELISA) to substitute these tests has increased in endemic areas in the last years. Together, culture and ELISA tests can vastly diagnose sporotrichosis, with a combined high sensitivity (Oliveira *et al.*, 2019).

So far, few molecular methods have been applied to detect *Sporothrix* DNA from clinical samples. Some PCR-based protocols targeting the large subunit ribosomal ribonucleic acid, chitin synthase, and calmodulin genes show a high level of specificity and are promising tools for sporotrichosis diagnosis (Barros *et al.*, 2011).

Treatment

Although spontaneous regression of lesions can occur in sporotrichosis (Lopes-Bezerra *et al.*, 2006), treatment is generally necessary (Barros *et al.*, 2011). Despite the existence of non-pharmacological treatments such as surgical excision, cryosurgery, thermotherapy, and photodynamic therapy, the use of antifungal drugs is the most widely used therapeutic strategy for this mycosis (Orofino-Costa *et al.*, 2017).

Potassium iodide has traditionally been used to treat sporotrichosis since the beginning of the last century, with satisfactory results. Some authors suggest that the saturated solution of potassium iodide (SSKI) acts on the resolution of granulomas by increasing proteolysis, while others claim that it promotes an increase in phagocytosis. However, the exact mechanism of action remains unknown to this day. The SSKI is still one of the most prescribed drugs for the treatment of cutaneous sporotrichosis, due to its effectiveness and low cost. SSKI is initially administered at five drops, three times daily, and gradually increased, as tolerated, to 40–50 drops, three times daily. The dosage for children is one drop, three times daily, and increased as tolerated up to 1 drop kg^{-1} (maximum of 40–50 drops), three times daily (Kauffman *et al.*, 2007). Recently, lower doses of potassium iodide for the sporotrichosis treatment were proposed, with satisfactory results (Barros *et al.*, 2011; Orofino-Costa *et al.*, 2017).

The azoles act inhibiting ergosterol synthesis by fungal cells. Itraconazole 100–200 mg once a day is currently the treatment of choice for sporotrichosis. This drug has a great *in vitro* activity against the major sporotrichosis agents and a cure rate up to 94% (Barros *et al.*, 2011; Kauffman *et al.*, 2007). Fluconazole is less effective than itraconazole and it is not a good therapeutic option. Ketoconazole, in addition to greater toxicity, has not shown good response in human infection (Kauffman *et al.*, 2007). Voriconazole appears to be modest in *S. schenckii* cases and ineffective against *S. brasiliensis* (Fernández-Silva *et al.*, 2014). Posaconazole is effective against several *Sporothrix* species and has a good therapeutic response, even in cases of disseminated disease (Paixão *et al.*, 2015). Its use in the treatment of sporotrichosis remains to be assessed.

In cases of contraindications to itraconazole, terbinafine (250–500 mg once a day) is the most reliable option, with a good cost-effectiveness. Amphotericin B is reserved for extracutaneous cases and pregnant women who have disseminated disease. Pregnant woman are a difficult group of patients to treat, since potassium iodide, itraconazole, and terbinafine are not indicated. If possible, they should not receive any drug treatment until the end of pregnancy (Kauffman *et al.*, 2007). Thermotherapy and cryosurgery have been used successfully to treat cutaneous forms of sporotrichosis during pregnancy (Ferreira *et al.*, 2012; Fichman *et al.*, 2018).

The antifungal treatment should be offered until the clinical cure of the patient (reepithelization of the lesions, absence of erythema, infiltration, and crusts) that occurs around 12 weeks (Barros *et al.*, 2011; Francesconi *et al.*, 2011). Hypersensitivity reactions should be treated with prednisone (40 mg per day) for short or medium periods.

Conclusions and Perspectives

Sporotrichosis is a disease that meets the criteria to be named as a tropical neglected disease. The advances in the taxonomy of the sporotrichosis agents and the emergence of this mycosis occurring in several countries brought much knowledge on this topic, but progress in some areas are still missing. For instance, there is a lack of rapid, commercially available tests to diagnose the infection. In addition, no vaccines are available and endemic countries do not have public health actions to curb the disease expansion. Translational research is urgently needed to improve the health outcome of this neglected disease.

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Advances in Genomics Research of Pneumocystis Species

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Introduction

Pneumocystis species are harbored in mammalian lungs where they exist in sub-clinical numbers or may cause a lethal pneumonia in mammalian hosts that have weakened immune systems. Once thought to be protozoan parasites or fungi, these organisms were definitively identified as members of the fungal kingdom with the advent of genomic sequencing. The genus *Pneumocystis* belongs to subphylum *Taphrinomycotina* of phylum *Ascomycota* (Redhead *et al.*, 2006, Chabe *et al.*, 2011). These host-specific obligate fungi are typically restricted to the lungs, though reports of extrapulmonary cases have not been infrequent (Ng *et al.*, 1997; Sharma *et al.*, 2019; Kim *et al.*, 2018). The five described species and their mammalian hosts include *P. carinii* and *P. wakefieldiae* in rats; *P. murina* in mice; *P. oryctolagi* in rabbits and *P. jirovecii* in humans. The challenge of Pneumocystis research has been the lack of an *in vitro/ex vivo* growth system, which impacts almost all experimental approaches including gene knockouts to assign function, evaluation of drug efficacy and resistance, and assessment of its full life cycle (Sinai *et al.*, 2012).

The proposed life cycle mostly takes place in the lung, with bi-phasic modes of asexual replication via binary fission by trophic forms (1–4 μm) and a sexual cycle initiated by two mating types resulting in an ascus with eight spores (5–8 μm) (Hauser and Cushion, 2018) (**Fig. 1**). To complete the life cycle, the infection is transmitted via an airborne mode with final deposition of asci in the alveoli of the next host(s). The second extra-pulmonary phase is the release of the propagule (presumably the ascus) from the infected host to complete the life cycle in the next host. Genomic and functional analyzes support primary homothallism as the mechanism for sexual reproduction with each trophic form carrying the genes encoding both mating types (Almeida *et al.*, 2015; Richard *et al.*, 2018) (described in more detail below). Self-fertility being a sound strategy for a host-obligate pathogen.

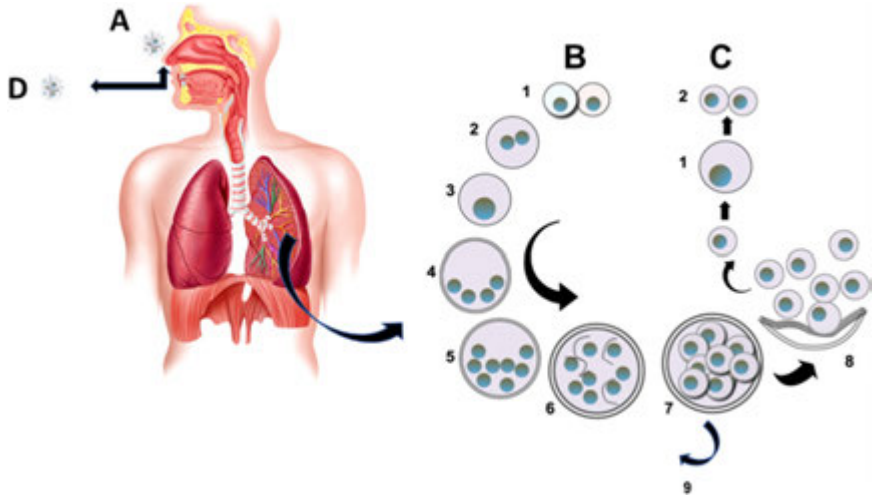


Fig. 1 Putative life cycle of Pneumocystis. (**A**) Airborne asci have been proposed to be the agent of infection. After inhalation, the spores ultimately take residence in the terminal portion of the respiratory tree, the alveoli. Neither the mechanism of migration to the alveoli nor the form in which the organism arrives in the alveoli (intact ascus or individual spores) are known. (**B**) Sexual phase: Two presumptive mating types conjugate (B1), undergo karyogamy (B2), and produce a diploid zygote (B3) that progresses through meiosis to produce four haploid nuclei (B4) followed by an additional mitosis to produce eight nuclei (B5). The nuclei are packaged into spores by invagination of the ascus cell membranes (B6) to produce eight double-membrane spores (B7). The ascus can undergo excystment via a protunicate release by unknown mechanisms (B8) or exit out of the lungs (B9). The spores released in the lung can then undergo asexual (C) or sexual (B) replication with a presumed opposite mating type. (**C**) Asexual phase: Haploid trophic forms are thought to replicate asexually by binary fission, whereby the nuclear content is duplicated (C1) along with cellular contents that divide into two haploid trophic forms (C2). The mechanism of exit out of the lung (**D**) is unknown. The figure of life cycle was adapted with modifications from Cushion, M.T., 2010. Are members of the fungal genus pneumocystis (a) commensals; (b) opportunists; (c) pathogens; or (d) all of the above? PLoS Pathogens 6 (9), e1001009. doi:10.1371/journal.ppat.1001009 and used under the Creative Commons Public Domain declaration; the human image was obtained from Getty Images 165564540 with credit to Leonello Calvetti.

Table 1 Current status of the sequenced Pneumocystis genomes in the NCBI

Organism	*Strain*	*Type of genome*	*Number of genes/ORFs*	*Database accession IDs*
P. carinii	B80	Nuclear	3646	LFVZ01
	N/A	Mitochondrial	17	NC_013660/JX499145
P. murina	B123	Nuclear	3623	AFWA02
	N/A	Mitochondrial	15	NC_020332/JX499144
P. jirovecii	RU7	Nuclear	3761	LFWA01
	SE8	Nuclear	3537	CAKM01
	E2178	Nuclear	N/A	NJFV01
	N/A	Mitochondrial	14	NC_020331/JX499143

Pneumocystis Genomes

With the advent of high-throughput sequencing, such as whole genome shotgun sequencing (WGS), and advanced software tools for assembly, genomes of select *Pneumocystis* species were obtained; for those most relevant for laboratory experiments (rodent models of Pneumocystis pneumonia – *P. carinii* and *P. murina* from rats and mice, respectively) and clinical studies (the species infecting humans – *P. jirovecii*). The mitochondrial genome of *P. carinii* was first reported (Sesterhenn *et al.*, 2010) followed by those of *P. murina*, *P. jirovecii* and a re-sequenced version of *P. carinii* (Ma *et al.*, 2013). Of note, while both rodent pathogens have linear mitochondrial genomes, the human species was found to have a circular structure. Following an initial report of a transcriptional profile of *P. carinii* during fulminant pneumonia (Cushion *et al.*, 2007), more complete assemblies of nuclear genomes and transcriptomes of these fungi were reported (Cisse *et al.*, 2012; Ma *et al.*, 2016). **Table 1** summarizes the current availability of Pneumocystis genomes in the NCBI Genome database.

Ultra-deep pyrosequencing targeting two nuclear genomic regions: ITS2 (internal transcribed spacer 2) and DHFR (dihydrofolate reductase), and one mitochondrial DNA region – mtLSU (the mitochondrial ribosomal RNA large subunit gene) in 25 PCP patients revealed that 92% patients were infected with a mixture of *Pneumocystis* strains (Alanio *et al.*, 2016). This suggests prior therapies used and/or multiple sources of reinfection may dictate the variability of the infecting pathogens, and consequently presents a challenge in defining a treatment regimen while facing various drug susceptibilities of the pathogen populations.

Ploidy

The study of ploidy was reported for *P. carinii* only, based on high-throughput cytometry (Martinez *et al.*, 2011). Most trophic forms contained haploid genomes, but diploid trophic forms were also present though less abundant. The DNA content of cysts (asci) suggests each ascus bears eight spores containing haploid genomes. Of note was the presence of forms with 4C and 3C content, but in much fewer numbers than the 1C and 2C stages. The variability in the overall trophic size and sizes of their nuclei is dramatically illustrated in the ultramicrograph shown in **Fig. 2**. The occurrence of life cycle stages with these nuclear contents may indicate that meiotic replication (4C) is a rapid or relatively rare event while the 3C genomes indicate ploidy variation is not an uncommon event in fungi which could arise from diploid:haploid mating or asymmetric mitotic division (Martinez *et al.*, 2011).

Comparative Genomics

Comparison of the *Pneumocystis* genomes may reveal how the pathogen coevolved with its mammalian hosts, and by comparing these genomes with those from other phylogenetically related but free-living fungi could shed light on metabolic strategies of these fungal pathogens with an obligate parasitic life style. In the past decade, at least three major studies were published where Pneumocystis species were compared among themselves and with *Schizosaccharomyces pombe*, *Taphrina deformans* (both are from the same subphylum as *Pneumocystis spp.*) and a well-studied model fungal organism *Saccharomyces cerevisiae* (Hauser *et al.*, 2010; Porollo *et al.*, 2014; Ma *et al.*, 2016).

Genes Absent From the Genome

Hauser and colleagues noted a dramatic underrepresentation of the enzymes needed for the amino acid biosynthesis in *P. carinii* compared to baker's yeast, suggesting that the pathogen has to scavenge amino acids from the host lung environment (Hauser *et al.*, 2010). While that study supported the notion of the parasitic life style of the pathogen, it did not help cultivate the organism *ex vivo*, as all culture medium formulations used in the field have abundant amounts of all natural amino acids. A biotrophic life style was first proposed in an earlier study of the transcriptome of *P. carinii* (Cushion *et al.*, 2007) and supported by later studies employing comparative genomics (Hauser, 2014; Cisse *et al.*, 2014). Porollo and colleagues expanded comparative genomic analyzes to all three sequenced *Pneumocystis* species and compared them with the genome of the fission yeast, *S. pombe* (Porollo *et al.*, 2014). While confirming the lack of amino acid biosynthesis pathways, as well as a previously noted incomplete ergosterol biosynthesis pathway (Kaneshiro, 2004; Joffrion and Cushion, 2010), they

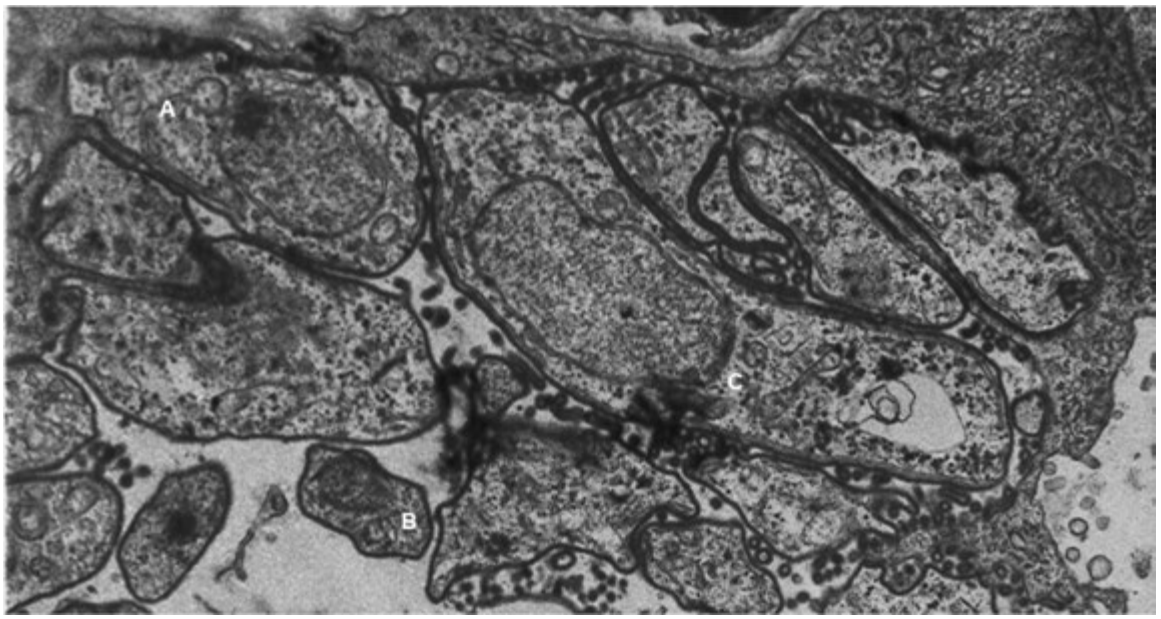


Fig. 2 Variation in nuclear and overall cell size of trophic forms. Transmission electron micrographs of *Pneumocystis carinii* trophic forms in rat lungs. Note the variation in size of mid-sized (A), small (B) and large trophic forms (MTC, personal collection).

also found over-representation of inositol metabolism compared to the fission yeast. Subsequent supplementation of culture media with *myo*-inositol significantly increased viability of *P. carinii* using an *in vitro* short-term cell-free culture compared to un-supplemented controls, but still did not enable continuous growth. Ma and colleagues pushed the field further and made, perhaps, the most comprehensive comparative analysis of the genomes (and partial transcriptomes) of *P. murina*, *P. carinii*, *P. jirovecii*, *T. deformans*, *S. pombe*, and *S. cerevisiae*, by going beyond the analysis of their metabolism (Ma *et al.*, 2016). The latter study outlined major strategies that the Pneumocystis species evolved to adapt to the obligate parasitic life style as well as to evade the host immune system. Overall, the Pneumocystis genomes are much smaller, with reduced gene sets, compared to the free-living fungi.

It should be noted that these results are likely to be further refined. Such comparative studies heavily rely on the sequence homology-based annotation of the genomics sequences. Despite the constant growth of reference databases, *Pneumocystis* species remain challenging in terms of functional annotation of their genes. Only a small fraction of their genes is annotated with high confidence. Moreover, such genome-wide annotations may yield inconsistent results between research groups, depending on what bioinformatics tools and reference sequence databases are used. As an illustration, Ma and colleagues posited that there are no inositol transporters present in the *Pneumocystis* genomes (Ma *et al.*, 2016). Yet, Porollo and colleagues first suggested candidate genes for these transporters shared among all three organisms (Porollo *et al.*, 2014) and subsequently demonstrated their function and ligand specificity (**Fig. 3**; Cushion *et al.*, 2016).

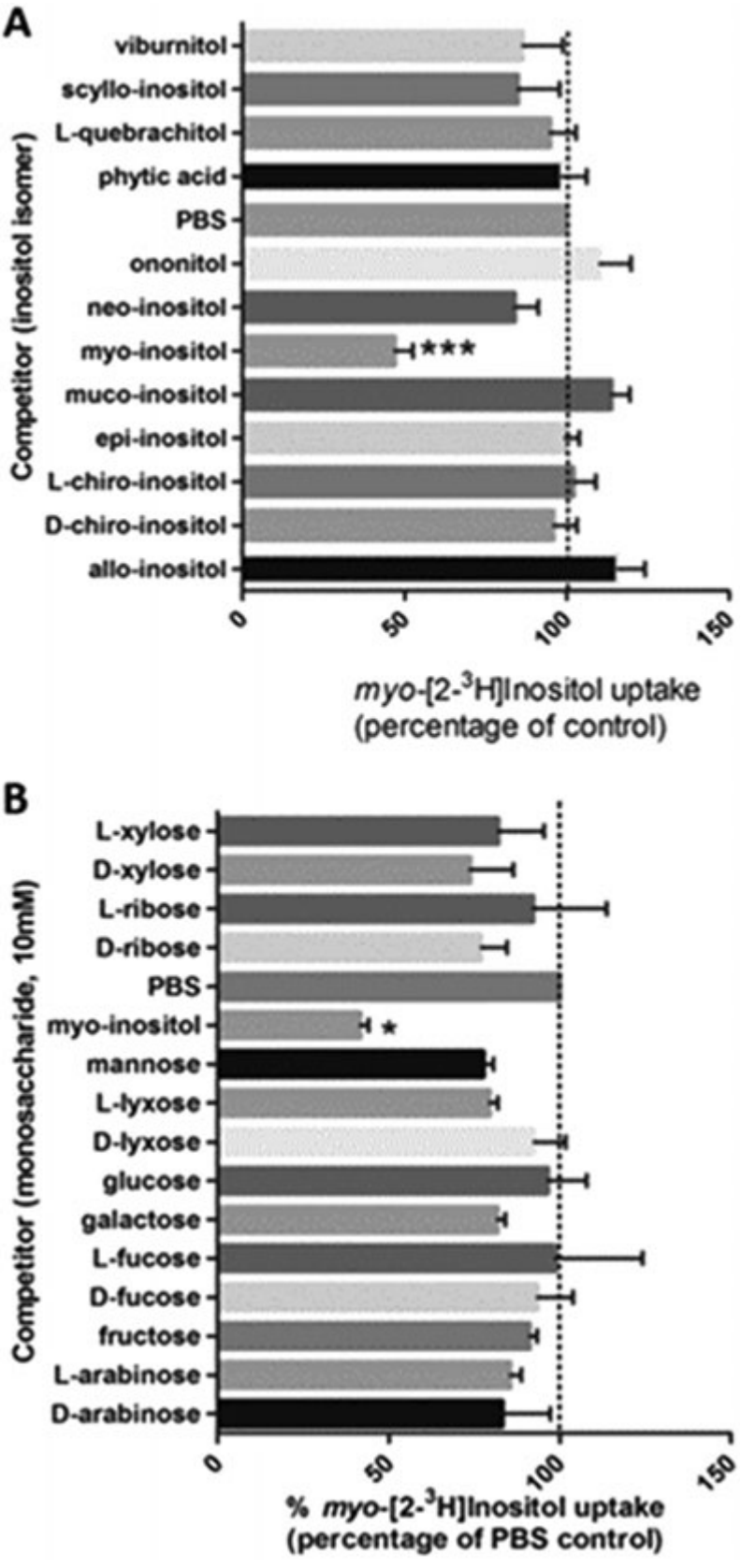
Dramatic reduction or complete loss of enzymes required for *de novo* biosynthesis of many critical nutrients suggests an adaptation of Pneumocystis organisms to the obligate parasitic life style when majority of these compounds are scavenged from the host (Cushion and Stringer, 2010). Such nutrient acquisition strategy assumes a wide range of genes encoding various transporters. In this regard, the published reports are again contradictory. Cisse and colleagues suggest that up to 22% genes in Pneumocystis genomes encode transporters, *e.g.*, amino acid permeases (Cisse *et al.*, 2012). Ma and colleagues, in contrast, report significant reduction of plasma membrane transporters (Ma *et al.*, 2016). As illustrated by the contradictory findings of the *myo*-inositol transporters, we hypothesize that the latter research group might have used very stringent criteria for their sequence homology search that lowered the sensitivity of their bioinformatics approach to gene annotation.

The complexity of experimental annotation of *Pneumocystis* genes impedes a large-scale functional characterization of the pathogen genome, at least until a continuous *ex vivo* growth system is established for these fungal pathogens. Until then, researchers in the field will have to rely on bioinformatics approaches and databases of better annotated organisms. Fortunately, both are being constantly improved.

Select Genomic Features of Pneumocystis

Sexual replication

Even though the life cycle of *Pneumocystis*, including the mechanisms of transmission, are still not fully understood (Skalski *et al.*, 2015), there is mounting evidence that sexual reproduction is an obligate step for the growth and transmission of these fungi (Cushion *et al.*, 2010; Hauser and Cushion, 2018). Moreover, genomic analysis revealed all mating type genes (MAT) located in one conserved region across *Pneumocystis* species on one chromosome thereby suggesting that these organisms use primary homothallism for sexual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neo-inositol
myo-inositol
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epi-inositol
L-chiro-inositol
D-chiro-inositol
allo-inositol
Competitor (inositol isomer)
***
0
50
100
150
myo-[2-3H]Inositol uptake
(percentage of control)
B
L-xylose
D-xylose
L-ribose
D-ribose
PBS
myo-inositol
mannose
L-lyxose
D-lyxose
glucose
galactose
L-fucose
D-fucose
fructose
L-arabinose
D-arabinose
Competitor (monosaccharide, 10mM)
*
0
50
100
150
% myo-[2-3H]Inositol uptake
(percentage of PBS control)

reproduction (Almeida *et al.*, 2015). Homothallism appears to be very common in fungi (Sun *et al.*, 2019). It may help explain the observation of multiple strains coexisting in the same host as noted in the previous section.

Major surface glycoprotein superfamily

While all *Pneumocystis* genomes are dramatically reduced in size compared to free living fungi, there is a notorious enrichment of the major surface glycoproteins (Msg) and related genes. This family of genes share structural similarities, are located at the telomeric ends of all chromosomes, and most use a single site for expression (Cushion and Stringer, 2010). This superfamily of genes are unique to the Pneumocystis species as sequence homology searches have not identified any homologs in other organisms in the NCBI non-redundant protein database (Delaye *et al.*, 2018). With improvements in both sequencing methodology and assembly software, the complexity of this family suggested in earlier studies is being confirmed and expanded (Schmid-Siegert *et al.*, 2017; Delaye *et al.*, 2018; Ma *et al.*, 2016). As an illustration, the genome of a *P. jirovecii* strain assembled from a long-read sequencing approach using a bronchoalveolar lavage fluid (BALF) specimen from a single patient yielded 113 Msg genes (Schmid-Siegert *et al.*, 2017). Another study reported 64–179 unique Msg genes per each sequenced *Pneumocystis* species (Ma *et al.*, 2016). The Msg superfamily in *Pneumocystis spp.* is currently comprised of five families of glycosylphosphatidylinositol-anchored glycoproteins and one family of secreted glycoproteins (Schmid-Siegert *et al.*, 2017). The Msg proteins appear to have conserved domains but otherwise highly variable sequences likely due to gene conversion (Keely and Stringer, 2009; Vink *et al.*, 2011; Delaye *et al.*, 2018). These translocations may be facilitated by the lack or paucity of introns in the Msg genes, repetitive elements or meiotic hot spots comprised of G + C rich regions (Cushion and Stringer, 2010; Delaye *et al.*, 2018). Very few microbes can survive in the lower airways of a healthy host and antigenic variation is a strategy used by fungi and protozoa alike. It was recently suggested that the Msg genes have an accelerated rate of evolution due to their intimate interactions with the host species ("biotic interactions"), fulfilling the "Red-Queen Hypothesis" (RQH) (The RQH has its origins in a statement made by the Red Queen to Alice in Lewis Carroll's Through the Looking-Glass while in Looking-Glass Land ("Now, here, you see, it takes all the running you can do, to keep in the same place") and first used by Leigh Van Valen to describe evolutionary adaptation. Martin, Douglas (October 30, 2010). "Leigh Van Valen, Evolution Revolutionary, Dies at 76". The New York Times).

The RQH contends that as the biotic environment of species is constantly evolving, in this case the mammalian lung, other species within the environment (Pneumocystis) must constantly adapt to the changing environment in a highly dynamic process (Delaye *et al.*, 2018). Besides immunological evasion, the other obvious function for these surface glycoproteins is adherence – both to host cells (predominantly Type I pneumocytes) and to each other. As shown in **Fig. 2**, trophic cells are tightly adherent, forming large clusters akin to biofilms in the alveoli. Not shown in the ultra-micrograph are intermediate life cycle stages and asci, which are also in these aggregates.

Drug Targets, Gene Polymorphism and Drug Resistance

Drug Resistance

Cotrimoxazole (TMP-SMX) is the primary option for the treatment and prophylaxis of PCP. Pneumocystis is required to synthesize its folates as it lacks any salvage genes, and this drug combination targets two enzymes in the folate pathway: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS). Targeted sequencing established significant risks of developing drug resistance by Pneumocystis via mutations in these two enzymes (Kazanjian *et al.*, 2000; Navin *et al.*, 2001; Visconti *et al.*, 2001; Costa *et al.*, 2003; Ponce *et al.*, 2017; Singh *et al.*, 2019a,b; Wang *et al.*, 2019). Heterologous systems based on *S. cerevisiae* expressing the *Pneumocystis* genes allowed for kinetic measurements of reduced inhibition rates in mutants compared to wild type controls (Iliades *et al.*, 2005; Cody *et al.*, 2009; Moukhlis *et al.*, 2010; Cody *et al.*, 2013; Queener *et al.*, 2013). Building on these kinetic data and covariance analysis of protein sequences, Porollo and colleagues proposed a prediction model for drug resistance that correlates well with the experimentally observed data (Baker *et al.*, 2016).

New Drug Targets and Therapies

Adverse reactions to TMP-SMX and concerns of the increasing incidence of drug resistant strains makes the search for new drug targets a priority of the research community. During the past decade, a wide range of potential targets were proposed. Such targets are listed in **Table 2**.

Fig. 3 Isomer and sugar specificity of myo-inositol transport in *P. carinii*. Substrate specificity of *P. carinii* inositol transporter (PcITR1) was tested using the competitive uptake assay. (**A**) Isomer specificity - *myo*-inositol *vs.* 11 other inositol isomers and derivatives. (**B**) Sugar specificity - *myo*-inositol *vs.* 14 common natural sugars. Concentrations of 0.5 μM *myo*-[2–3H] inositol and 100 × the unlabeled inositol isomers and sugars were used. Data are expressed as percentages of the results for the PBS control (no competitor). Data are the average results from 5 separate analyzes. Error bars show standard deviations. Significance was determined by the paired-sample two-tailed *t* test. *P < 0.05; ***P < 0.001. Figure was adapted from Cushion, M.T., Collins, M.S., Sesterhenn, T., *et al.*, 2016. Functional characterization of *Pneumocystis* carinii inositol transporter 1. mBio 7 (6), doi:10.1128/mBio.01851016.

Table 2 New drug targets for anti-PCP treatment

Target	*Function*	*Reference*
AdoMet transport (PET8)	Facilitates scavenging of the host S-adenosylmethionine (AdoMet)	Perez-Leal *et al.* (2011)
Histone acetyltransferase (Rtt109)	Acetylation of histone H3 lysine 56 (H3K56) for maintenance of genome integrity and DNA damage repair; conserved in fungi but absent in humans	Dahlin *et al.* (2014)
myo-inositol transporter (ITR1)	Transport of *myo*-inositol, the only mechanism to obtain this essential molecule	Porollo *et al.* (2014), Cushion *et al.* (2016)
Dihydrofolate synthase (DHFS) and aminodeoxychorismate lyase (ABZ2)	Folate biosynthesis pathway	Luraschi *et al.* (2015)
APL5, COS111, MKK1, and STE2	Sphingolipid glucosylceramide (GlcCer) biosynthesis	Mor *et al.* (2015)
ACS2	acetyl coenzyme A (acetyl-CoA) synthetase	Koselny *et al.* (2016)
GSC1 and KRE6	Essential for 1,3-β- and 1,6-β-glucan biosynthesis; imparts rigidity to asci	Luraschi *et al.* (2017)
GFA1, GNA1, AGM1, and UDP-GlcNAc pyrophosphorylase (UAP1)	N-Acetylglucosamine biosynthesis	Kottom *et al.* (2017)

There have been several reports of therapy with the echincandin, caspofungin, alone or in combination with trimethoprim-sulfamethoxazole or clindamycin (Jin *et al.*, 2019; Yang *et al.*, 2019; Zhang *et al.*, 2018; Li *et al.*, 2016) with inconsistent outcomes. The variability is likely due to a number of factors including co-morbidities, dosing, or severity of the pneumonia. Administration of echinocandin therapy remains controversial for treatment in humans (Luraschi *et al.*, 2019). In a systematic study of 3 echinocandins in a mouse model of PCP it was clear that these anti-fungal agents were effective at dramatically reducing the number of asci to undetectable numbers, but large burdens remained that were comprised of non-glucan expressing *P. murina* (Cushion *et al.*, 2010). Importantly, cessation of the echinocandin treatment after 3 weeks, with the immunosuppressive agent still present, permitted a return of asci to baseline numbers. These studies are a cautionary note to echinocandin monotherapy.

Conclusions and Future Directions

Although there has been substantial progress in understanding the genetic composition and its translation to present or absent metabolic pathways, much work remains to dissect these fungi. There is a notorious lack of strong homology for many *Pneumocystis* genes to sequenced genes in other organisms. It suggests either a strong influence of the mammalian hosts on pathogen to diverge so dramatically from the other members of its phylum, or an underdevelopment of the genome assemblers from the shotgun sequencing data followed by inaccurate gene prediction.

Decades of failing attempts to identify a silver bullet – a major single ingredient missing in the culture medium – to achieve a long-term culture of *Pneumocystis*, even after backing potential supplements with the analysis of metabolic pathways in the organism, may suggest at least two future directions in this area. One is to perform an unbiased analysis of the dynamic changes in a metabolite profile of the extracted pathogen, followed by flux balance analysis (FBA) to optimize the nutrient combination for culture media. Alternatively, cell-based approaches may be required for the *ex vivo* growth of these host obligate fungi and newer approaches such as alveolar epithelial cell- or lung organoid-based system may provide nutrients missing in the cell-free culture systems.

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Subcutaneous Fungal Infections

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Introduction

Subcutaneous fungal infections consist of a group of mycoses involving the skin and the subcutaneous tissues, mainly occurring in tropical and subtropical countries. Some fungi may cause different diseases, according to their aspect in the infected tissue (Verma *et al.*, 2018). For instance, when some dematiaceous fungi appear in the tissue as muriform (or sclerotic) cells, the disease is chromoblastomycosis; when they form hyphae, the diagnosis is phaeohyphomycosis; when grains occur, the classification is eumycetoma. Other diseases are not mycoses but have historically been studied within this group due to former mistaken classifications, such as rhinosporidiosis (not covered here) and actinomycetoma. Since 2017, mycetoma, chromoblastomycosis and other deep mycoses are on the World Health Organization's (WHO) list of neglected diseases (WHO, 2017). Such inclusion brings an important aid to the visibility of these diseases and to the affected patients, implying more resources for their study, diagnosis and treatment optimization. Actions on education, information and prevention may reduce cases, especially considering that most of them are in developing countries. The constant migration around the globe makes the knowledge of this group of diseases fundamental for health care professionals. Sporotrichosis, one of the main mycoses within this group, is covered in another chapter.

Chromoblastomycosis

Also known as chromomycosis or Pedroso and Lane mycosis, chromoblastomycosis (CBM) has a chronic and progressive course. It was first scientifically published by Maximilliano Rudolph, in 1914. This mycosis can be caused by different genera of dematiaceous fungi, i.e., melanin producers. Some agents of CBM also cause other diseases, such as phaeohyphomycosis and eumycetoma (Queiroz-Telles *et al.*, 2017a).

Epidemiology

The overall burden of CBM is unknown, since it is not a notifiable disease, but its highest prevalence occurs in tropical and subtropical regions. CBM rarely occurs in children, and men between 40 and 50 years old are the most involved. The male: female ratio ranges from 5:1 to 9:1. As an occupational disease, it affects agricultural and forestry workers, gardeners and others exposed to contaminated plant material such as construction workers (Queiroz-Telles *et al.*, 2017a). In Brazil, the babassu coconut (*Attalea speciosa*) harvest was closely related to the disease, and in India it is linked to black tea cultivation in Assam and rubber plantations in Kerala and Western Ghats. Madagascar, Brazil, Mexico, Dominican Republic, Venezuela, India, and southern China report most of the cases (Agarwal *et al.*, 2017; Queiroz-Telles *et al.*, 2017a).

The disease is caused by fungi from the soil and plants, as saprobic decomposers of this organic matter. *Fonsecaea pedrosoi* is the most common pathogen in tropical areas. Recently, new species of this genus have been described: *Fonsecaea monophora*, *Fonsecaea nubica* and *Fonsecaea pugnacious* (de Azevedo *et al.*, 2015). In southern China, all reported cases are associated with *F. nubica*. *Phialophora verrucosa* is the second most prevalent fungus. *Cladophialophora carrionii* is the most important agent in dry and desert countries of Australia, South Africa and Cuba. *Rhinocladiella aquaspersa*, *Exophiala dermatitidis*, *Exophiala spinifera* and *Cladophialophora boppii* are uncommon agents. Recently, cases caused by *Cyphellophora ludoviensis* and *Rhinocladiella tropicalis* have been described (Gomes *et al.*, 2016).

There is usually a decrease in the number of cases following changes in agricultural methods, with the use of fungicides and mechanization (Queiroz-Telles *et al.*, 2017a).

Pathophysiology

The accidental contact of humans with the agents contributes to the onset of the mycosis, usually due to skin trauma and fungal inoculation. Within the tissue, the fungus converts its filamentous phase into thick brownish-walled globular structures, called muriform cells. These are rounded structures, and multiply by septation, in one or two planes, stimulating a granulomatous and purulent inflammatory reaction in its surroundings (Queiroz-Telles *et al.*, 2017a).

Clinical Features

The most frequently affected sites are the extremities of the lower limbs, followed by the upper limbs, gluteal region, trunk and face. The initial lesion, usually a papule, arises in the place of the previous trauma, not always noticed by the patient. This lesion can be single or multiple, with a slow growth and polymorphic (Queiroz-Telles *et al.*, 2017a).

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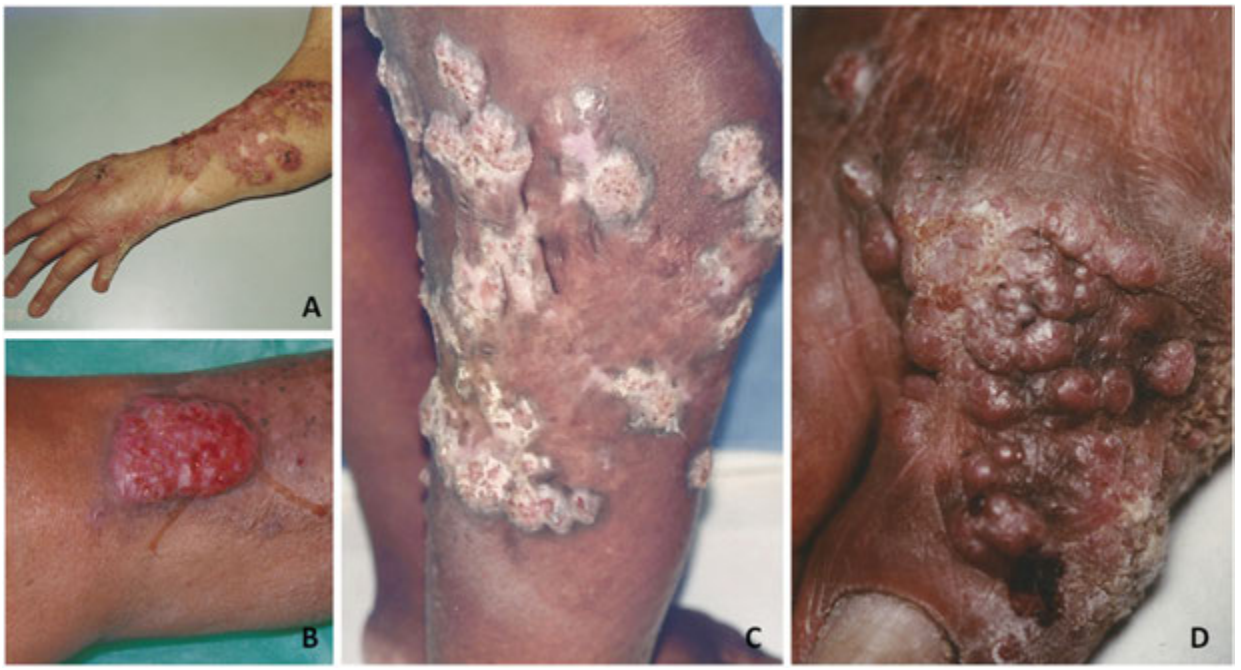

Fig. 1 Chromoblastomycosis. Credits to Antonio Carlos Francesconi do Valle, MD PhD. (A) Verruciform and cicatricial plaques on the forearm of a woman. (B) Tumoral lesion on the leg of a man. (C) Verruciform and cicatricial extensive lesions on the inferior limb of a man. (D) Nodular plaque on the foot of a man.

According to Carrión, the initial lesions may evolve into five distinct clinical types: nodular, plaque, tumoral, verruciform and cicatricial (**Fig. 1**). The lesions are usually unilateral and insidious. The progression of a plaque may present central healing and ulceration. Other factors such as edema or secondary infection may modify the clinical presentation (Carrión, 1950).

The severity of the disease is classified by the number, extent and spread of the lesions. The mild form is represented by a solitary plaque or nodule smaller than 5 cm in diameter; the moderate form includes nodular, verrucous, single or multiple plaque lesions, affecting one or two adjacent regions, presenting less than 15 cm in diameter, and the severe form presents extensive lesions in adjacent or non-adjacent regions, including tumor and cicatricial types (Queiroz-Telles *et al.*, 2017a).

The general clinical condition is not affected, but extensive injuries may interfere with physical capacity. Long-term lesions may have localized pruritus that may be mild or severe. Secondary infection, often frequent and recurrent, can cause pain, strong odor, fibrosis and chronic lymphedema. There may be contiguous spread to bones, causing osteolytic lesions. Neoplastic transformation with the onset of squamous cell carcinoma may occur (Azevedo *et al.*, 2015a).

Differential Diagnosis

Paracoccidioidomycosis, leishmaniasis, sporotrichosis, tuberculosis and tertiary syphilis are the main differential diagnoses. CBM should also be differentiated from squamous cell carcinoma, lacaziosis, keratoacanthoma, psoriasis, sarcoidosis, leprosy and atypical mycobacterioses (Queiroz-Telles *et al.*, 2017a).

Diagnosis

The definitive diagnosis consists in the observation of muriform cells under optical microscopy, by direct examination (10%–40% potassium hydroxide) of the lesion scrape or stained histological sections. Within the lesion, in areas rich in black spots usually contain the muriform cells. CBM agents are not inhibited by cycloheximide or chloramphenicol, allowing the use of selective media to prevent rapid growth of contaminants in culture. Colonies appear after 7–15 days as small, fuzzy black dots, which turn into dark velvety colonies (**Fig. 2(A)**). The fungal micromorphology (**Fig. 2(B)**) has various aspects, and its analysis using the slide culture is crucial for the classification of the genus of the agent (Ameen, 2009).

For species identification, molecular characterization by sequencing of the internal transcribed space region (ITS) of ribosomal DNA (rDNA) is recommended. In addition, taxonomic studies may apply specific genes, such as those encoding the cell division partial cycle gene (*cdc42*), β-tubulin (*BT2*), translation elongation factor 1-α (*TEF-1*α) and actin gene (*ACT1*), among others (de Azevedo *et al.*, 2015).

Histopathological findings of hyperkeratosis, pseudoepitheliomatous hyperplasia of the epidermis, pyogranulomatous reactions and irregular acanthosis alternating with areas of atrophy are the rule. Nevertheless, it is the presence of the muriform

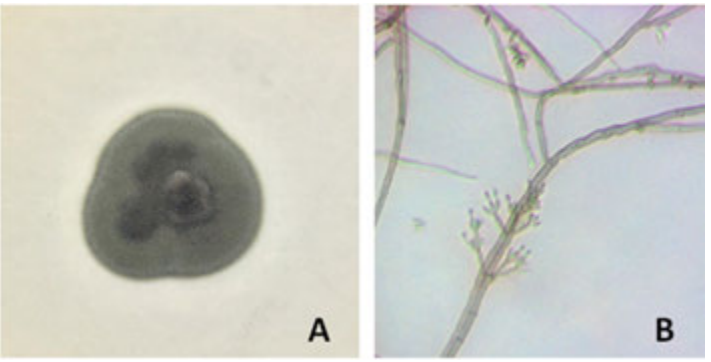

Fig. 2 *Fonsecaea pedrosoi*. Credits to Rowena Alves Coelho, MSc. (A) Dark velvety colony (Potato dextrose agar). (B) Micromorphology – dematiaceous septate hyphae with short-chain *Cladosporium*-type conidiation (400x; Potato dextrose agar 30ºC; 7 days).

cells in the interstitium or within Langhans multinucleated giant cells that seals the diagnosis. These naturally melanized pathognomonic structures of CBM can be easily identified by routine hematoxylin-eosin (H&E) staining. Gomori-Grocott and Fontana-Masson stains may be helpful when the fungal load is low (Uribe *et al.*, 1989).

Treatment

CBM therapy is long lasting and associated with low cure rates and high relapse rates, particularly in chronic and extensive disease. *Fonsecaea pedrosoi* is less susceptible to antifungals when compared to *C. carrionii* or *P. verrucosa*. There is no standard treatment and the association of more than one drug, or the use of physical methods is needed in most cases. Itraconazole 200–400 mg/day and terbinafine 250–500 mg/day are the most used drugs. There are reports of the combination of terbinafine with itraconazole, flucytosine and amphotericin B, flucytosine and itraconazole, fluconazole and itraconazole, which can be used in patients with refractory disease. *In vitro* studies have shown lower values of minimal inhibitory concentration (MIC) with terbinafine and voriconazole, and synergistic interaction of amphotericin B and flucytosine in *F. monophora* isolates, which may point to the use of these combinations. Physical methods such as heat therapy (daily sessions of 2–24 h), cryosurgery (monthly sessions of 30 s to 4 min) or excisional surgery may be used associated with systemic antifungal therapy. Few cases treated with photodynamic therapy and laser therapy had good response (Ameen, 2009; Coelho *et al.*, 2018).

Lobomycosis

Lobomycosis primarily occurs in tropical climates of Latin America. The causative organism is *Lacazia loboi* (formerly *Loboa loboi*), a dimorphic fungus found in soil, vegetation, and water (Paniz-Mondolfi *et al.*, 2012).

Infection occurs through the traumatic implantation of the fungus into the skin, and the most common presentation in humans consists of slow-growing, keloid-like papules, nodules, or plaques (**Fig. 3**). Other clinical presentations are ulcerated, infiltrative, verrucous, gumma-like, multifocal, and disseminated lesions (Woods *et al.*, 2010).

Although lobomycosis is the accepted term, other used names are Jorge Lobo mycosis, Amazonic pseudolepromatous blastomycosis, miraip, or piraip ("burning" in the Tupi language), Caiabi leprosy, and lacaziosis.

Etiology and Transmission

Lacazia loboi is a dimorphic fungus that may exist in a saprophytic phase in soil, vegetation, and water (Paniz-Mondolfi *et al.*, 2012).

Traumatic inoculation into the dermis is the most likely mode for the development of lobomycosis in humans (Borelli, 1962). Also, patients have reported the development of symptoms following snakebites, insect bites, or stingray stings (Campo-Aasen, 1958). The incubation period is estimated to be one to two years (Opromolla and Nogueira, 2000). Lobomycosis does not appear to spread through human to human transmission.

Epidemiology

The overall prevalence of lobomycosis is low. Tropical regions of South and Central America within areas of dense forests with high annual rainfall (>20 cm per year) and warm, humid environments (average temperature $>24^{\circ}$C) are the sites of most significant predilection for the disease (Borelli, 1969).

Lobomycosis is more frequent in men than in women, and most commonly between 40 and 70 years old. In endemic areas, lobomycosis most often occurs as an occupational disease and occasionally as a recreational disease in individuals exposed to harsh outdoor environments, such as forest workers, farmers, hunters, and fishers (Woods *et al.*, 2010).

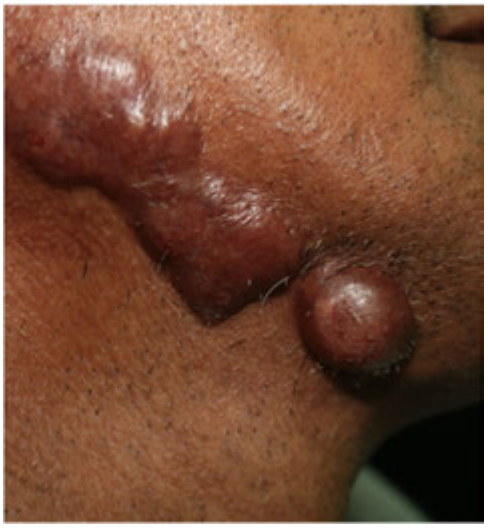

Fig. 3 Lobomycosis – keloid-like nodules on the skin overlying the mandible of a 54-year-old man. Credits to Fábio Francesconi, MD PhD.

Pathophysiology

After inoculation into the dermis, *L. loboi* replicates slowly within macrophages (Pecher *et al.*, 1979). Preserved cellular immunity is probably necessary to hinder the persistence and progression of the disease. Cytokines with suppressive effects on cellular immunity, notably transforming growth factor-beta 1 (TGF-beta 1) and interleukin-10 (IL-10), may play critical roles in the pathogenesis of lobomycosis. Besides, there is an upregulation of regulatory T cell markers in lesions (Azevedo *et al.*, 2015b). Regulatory T cells might inhibit Th-dependent, protective responses to intracellular fungi. The possibility that impaired cellular immunity increases susceptibility to lobomycosis is also suggested by studies that utilized skin tests to assess immunologic function (Pecher and Fuchs, 1988).

Clinical Features

The initial lesion of lobomycosis is a superficial or deep-seated papule, which may appear months to years after inoculation and slowly expands contiguously, leading to the formation of single or multiple monomorphic or polymorphic plaques or nodules that often resemble scars or keloids (**Fig. 3**). The surface of the plaques and nodules is usually smooth, shiny, and intact, and the color usually ranges from skin-colored to red-brown or wine-red. Telangiectasias may be present (Francesconi *et al.*, 2014). Other characteristics may also be seen, including dyschromia, ulceration, and infiltrative, verrucous, or gumma-like lesions (Brito and Quaresma, 2007).

Affected sites usually correspond to exposed skin, like the pinna, upper limbs, and lower limbs. Although localized lesions are the rule, multifocal (multiple sites of involvement on a single limb) and disseminated presentations may also occur. Regional lymph node enlargement, which may represent a lymphatic spread of the fungus, is estimated to occur in 10%–25% of patients (Woods *et al.*, 2010). Skin involvement is usually asymptomatic. However, patients with extensive involvement may experience pruritus or dysesthesia.

Diagnosis

The diagnosis of lobomycosis is based on clinical presentation, by identification of a keloid-like lesion in a person living in an endemic region. Microscopic confirmation is usually recommended, performed by clarification with 10% KOH of a small biopsy sample of the lesion (**Fig. 4**). The fungus does not grow on culture media; thus, this is not a useful method.

The most significant histologic findings of lobomycosis are in the dermis. Although the epidermis is usually normal or atrophic, the transepidermal elimination of the fungus is occasional. The dermis exhibits a dense histiocytic dermal infiltrate with a thin zone of collagen and abundant fungal structures of *L. loboi*, and the fungal structures are round with birefringent membranes and thick walls containing melanin. The fungus reproduces with simple gemmulation without sporulation, resulting in blastoconidia with chain-like or rosary-bead-like configurations (Francesconi *et al.*, 2014).

Treatment

The extent of disease is the main factor in deciding the treatment choice. For patients with localized lobomycosis, wide surgical excision is the preferred treatment. Despite the intent of total removal, recurrences are frequent. Other techniques with reported

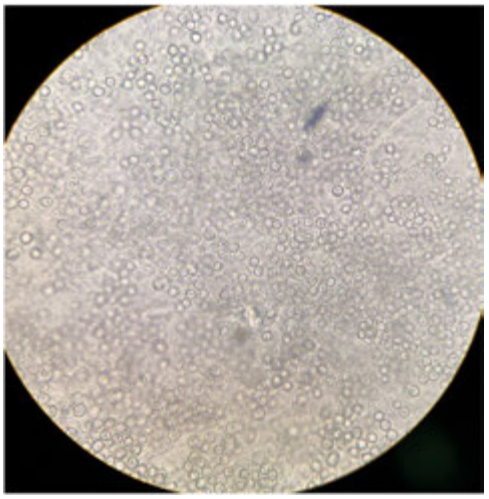

Fig. 4 *Lacazia loboi* – numerous round yeast-like structures with birefringent membranes, some in a chain-like configuration (direct examination with 10% KOH; 100x). The sample was obtained with a 1-mm punch. Credits to Fábio Francesconi, MD PhD.

effectiveness in individual patients include cryosurgery and electrocauterization. There is no reliable effective drug antifungal treatment, and treatment failure is common. Data are primarily limited to case reports. Drugs used are itraconazole, clofazimine, posaconazole, ketoconazole, amphotericin B, sulfa compounds, 5-fluorocytosine, and combination drug regimens. All have showed incomplete or unsatisfactory effect. An incidental finding of favorable responses of lobomycosis among ten patients treated for concomitant leprosy in a dermatology department in Brazil may aid in the search for effective treatments for lobomycosis (Brito and Quaresma, 2007).

If not treated, the disease is usually slowly progressive with a potentially extensive or disseminated skin disease that can cause local tissue destruction and physical disability. Spontaneous resolution is rare. A few case reports document the development of squamous cell carcinoma in sites of longstanding lobomycosis. Long-term follow-up is mandatory since there is a high relapse rate (Nogueira *et al.*, 2013).

Mycetoma

Mycetoma is a chronic subcutaneous disease caused by traumatic inoculation of filamentous fungi (eumycetoma) or aerobic filamentous bacteria (actinomycetoma), which form grains in the tissue (van de Sande, 2013). Usually, there is an indolent clinical evolution, difficulty for laboratory diagnosis and challenging treatment. This disease presents significant medical, occupational and socioeconomic impact, with reduction in the quality of life of affected individuals (Queiroz-Telles *et al.*, 2011; Welsh *et al.*, 2014). Its incidence and prevalence are unknown, since it is not a notifiable disease.

Epidemiology

Mycetoma is also known as 'Madura foot' due to the city of Madurai, in India, where some of the first cases were better described and documented (McGinnis, 1996). Actinomycetoma accounts for 60% of cases worldwide, while the rest is eumycetoma (Welsh *et al.*, 2007).

Most patients are 15–30 years-old, with a gender ratio of 3.7 male:1 female. This male predominance is commonly attributed to a greater risk of occupational exposure, but genetic or immunological factors cannot be ruled out. Mycetoma is seen in most communities in farmers, field laborers and in herdsmen who come in contact with the land, although in endemic areas people from other occupations are affected (Fahal *et al.*, 2018).

Mycetoma occurs worldwide and prevails in tropical and subtropical regions. Although its true incidence is not known, most of the cases occur between latitude 15°S and 30°N, the so-called "mycetoma belt". The endemic countries include Sudan, Somalia, Senegal, India, Yemen, Mexico and Venezuela. In South America, cases have also been reported in Colombia, Brazil, and Argentina (Fahal, 2011; Lichon and Khachemoune, 2006; Zein *et al.*, 2012).

The main agents of eumycetoma are *Madurella mycetomatis*, *Trematosphaeria grisea* (former *Madurella grisea*), *Falciformispora senegalensis* (former *Leptosphaeria senegalensis*), *Acremonium* spp., *Scedosporium apiospermum* and *Curvularia* spp. The most frequent agents of actinomycetoma belong to the genera: *Nocardia*, *Actinomadura* and *Streptomyces* (van de Sande *et al.*, 2018; Zein *et al.*, 2012). In the Americas, *Nocardia brasiliensis* is the leading cause of actinomycetoma (do Valle *et al.*, 2006). Cases by *Nocardia* spp. are more frequent in humid regions, while eumycetoma and actinomycetoma by *Actinomadura* spp. and *Streptomyces* spp. predominate in drier areas (Bonifaz *et al.*, 2014).

Grain color and morphology help to infer the etiological agent. Black grains are caused by dematiaceous fungi, mainly of the genera *Madurella*, *Curvularia* and *Exophiala*. Red grain is characteristic of *Actinomadura pelletieri*. White or yellowish-white grains may be caused by fungi (*S. apiospermum* and *Acremonium* spp.), or by filamentous bacteria (*Nocardia* spp. and *Actinomadura madurae*) (Kloezen *et al.*, 2012; van de Sande, 2013).

Pathophysiology

The main route of infection is by direct traumatic inoculation of the microorganism into the tissue, from thorns, wood, splinters, stones and sharp materials. Once in tissues, adaptative mechanisms such as cell membrane alteration favor survival and progression of infection in the host organism. The grain compounds seem to be partially responsible for the pathogenesis and antimicrobial resistance of the agents. Melanin, found in black grains, also appears to play a protective role against the immune system and antifungal medications (Ibrahim *et al.*, 2013).

Pathogen- host- and environment-related factors play a role in the pathogenesis. The initial nonspecific inflammatory response and neutrophil chemotaxis later become more organized and cellular. T helper 2-like responses [interleukin − 10 and 4] have been found in primary lesions of eumycetoma and in draining lymph nodes (Zijlstra *et al.*, 2016). A polymorphism resulting in decreased human chitotriosidase activity was associated with increased likelihood of eumycetoma (Verwer *et al.*, 2015).

Clinical Features

Mycetoma involves the skin and subcutaneous tissue to form the classic triad of a hard-woody swelling, painless discharging sinuses (**Fig. 5(A)**) and presence of grains, which are colonies of bacteria or fungi and vary in color and size, depending upon the organism (Verma and Jha, 2019). The discharge may be serous, serosanguineous or purulent (Fahal, 2011). Other clinical presentations may occur, but are rare, and include swelling without sinuses, cystic type and verrucous plaque type (Bonifaz *et al.*, 2014). The clinical presentations of eumycetoma and actinomycetoma are almost identical, the first (**Fig. 6**) being more chronic and less inflammatory than the second. The feet are by far the most common sites of involvement, but the disease may present in other parts of the body. Left untreated, the disease spreads through the fascia, bone and muscle, which makes the disease more difficult to treat (McGinnis, 1996; Verma and Jha, 2019). Pain may be produced by the expansion and invasion of the mass, but it is more commonly due to secondary bacterial infection (Fahal, 2011). Mycetoma affecting the back may lead to vertebral compression, causing neurological manifestations. An enlarged regional lymph node is not uncommon, and this may be due to secondary bacterial infection, genuine mycetoma lymphatic spread or may be due to local immune responses to mycetoma. Constitutional symptoms are rare but may occur due to comorbidities. Mycetoma can produce many disabilities, distortion and deformity. It can be fatal especially in cases of cranial mycetoma (Fahal, 2011).

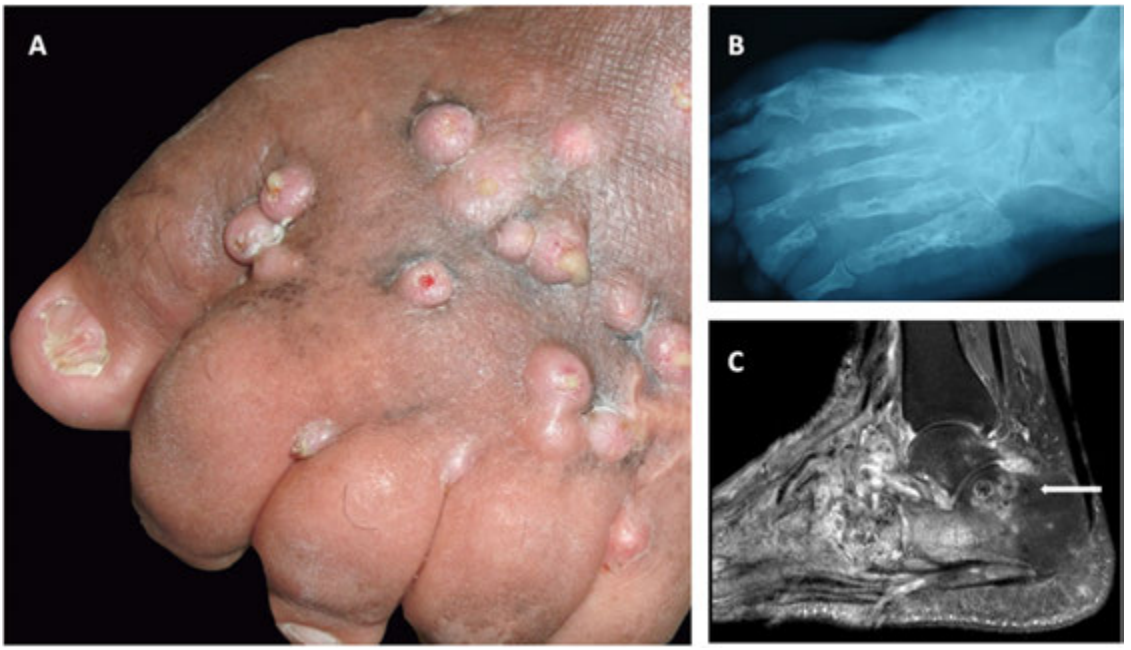

Fig. 5 Actinomycetoma by an unknown agent on the foot of a woman – a 15-year lesion. Credits to Felipe Maurício Soeiro Sampaio, MD MSc. (A) Multiple sinuses with purulent discharge. (B) Radiograph showing osteolytic multiple lesions. (C) Magnetic resonance with the classical "dot-in-circle" signal (arrow).

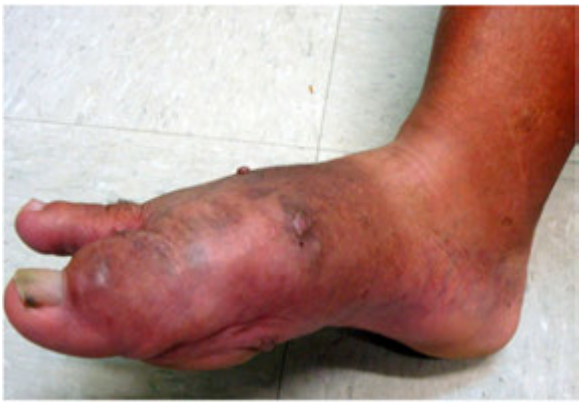

Fig. 6 Eumycetoma by *Scedosporium apiospermum* – foot of a Brazilian male patient, who reported previous trauma. Credits to Antonio Carlos Francesconi do Valle, MD PhD.

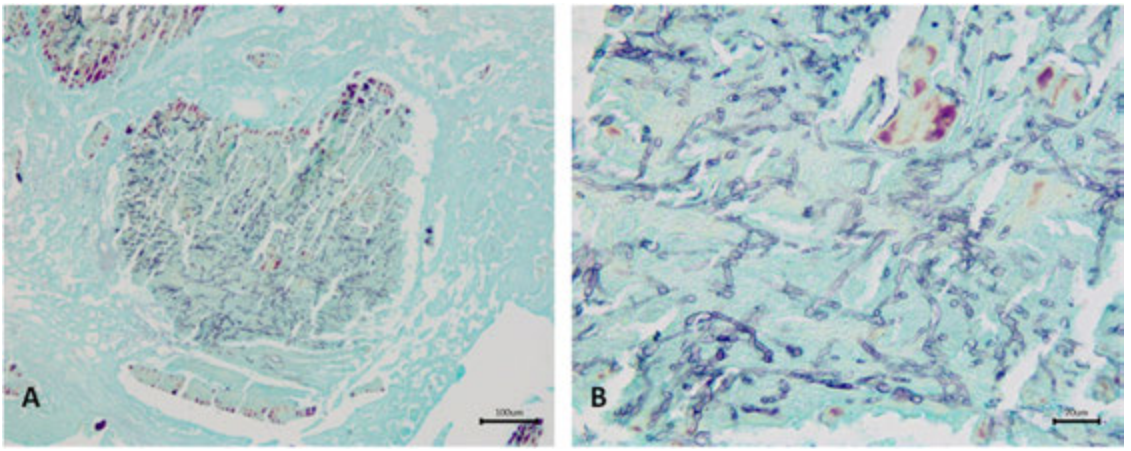

Fig. 7 Eumycetoma grain in Gomori-Grocott methenamine silver impregnation under optical microscopy. Credits to Janice Mery Chicarino de Oliveira Coelho, MD PhD. (A) Grain within the dermis. (B) Thick septate hyphae forming the grain.

Differential Diagnosis

It is important to differentiate mycetoma from botryomycosis, actinomycosis, osteomyelitis with cutaneous manifestations, nocardiosis, fusariosis, chromoblastomycosis and phaeohyphomycosis. Other diseases to consider are: hyalohyphomycosis, atypical mycobacterioses, Kaposi sarcoma, cutaneous tuberculosis, melanoma, foreign body granuloma, osteogenic sarcoma, diabetic foot, among others (Fahal, 2011).

Diagnosis

Clinical samples should be collected at the depth of the lesion (needle puncture or biopsy) for histopathology and direct examination and culture (mycological/bacteriological). Fresh grain analysis can be performed by optical microscopy in 10% KOH (van de Sande *et al.*, 2014).

Grain visualization in histopathology sections is fundamental and can be performed with H&E. However, periodic acid-Schiff staining and Gomori-Grocott methenamine silver impregnation show better results (**Fig. 7(A)**). Fungal grains are composed of 4–5 μm thick septate hyphae (**Fig. 7(B)**), while actinomycetoma grains generally consist of branched and thin filaments or bacillary forms that are only 1 μm thick. Actinomycetoma filaments are Gram-positive and eumycetoma hyphae are Gram-negative (Chufal *et al.*, 2012). The grains are surrounded by polymorphic inflammatory cells (neutrophils, lymphocytes, histiocytes, plasma cells, epithelioid cells and giant cells) that constitute granulomas, and eosinophilic material (Splendore-Hoeppli phenomenon) (do Valle *et al.*, 2006; Fahal, 2011).

Actinomycetoma agents grow on Lowenstein-Jensen medium and eumycetoma on Sabouraud-dextrose agar incubated at 30 and 37°C for about 6–8 weeks. After growth, the species can be identified by macroscopic characteristics of the colony, by micromorphology and by biochemical tests. Agents within the genus *Nocardia* are usually indistinguishable (van de Sande *et al.*, 2014).

Molecular tests, such as DNA sequencing and polymerase chain reaction (PCR) (Ahmed *et al.*, 2014), are often necessary for determining the species of the microorganism. However, they are still expensive or not available, especially in the areas of mycetoma occurrence.

To assess the extent of lesions, radiographs, ultrasounds and magnetic resonance imaging (MRI) are the most useful exams. Radiography is usually the most available and inexpensive method to verify the existence of destruction, periosteal reaction and bone cavitation (**Fig. 5(B)**) (Fahal, 2011). Ultrasonography is also a low-cost, fast and easy-to-obtain exam. It can be performed in both reference centers and point-of-care, but it has the disadvantage of being operator-dependent. When compared to conventional radiography, it is a more specific technique for diagnosis. MRI has higher sensitivity and specificity, but it is expensive. Typical structures with high-signal areas seen on MRI are believed to represent inflammatory granulomata, the low-intensity tissue surrounding these lesions would represent the fibrous matrix, and the small central hypointense foci within the granulomata, the fungal balls or grains. This signal is considered by some authors as pathognomonic of mycetoma and is called "dot-in-circle" (**Fig. 5(C)**) (Sen and Pillay, 2011; Estrada *et al.*, 2012).

Treatment

Actinomycetoma is treated with sulfamethoxazole and trimethoprim at a dose of 800/160 mg twice daily, either alone or in combination with intramuscular amikacin or gentamicin sulfate, 15 mg/kg/day, for three weeks (Welsh *et al.*, 1987). This association with aminoglycoside improves cure rates to 90%. The average treatment is five to ten cycles to achieve clinical cure. Rifampin (10 mg/kg/day) may be used, but in many countries, it may compromise the treatment of tuberculosis and leprosy (Praveen Kumar *et al.*, 2011). Minocycline, amoxicillin/clavulanic acid, moxifloxacin, imipenem and carbapenem are also options (Welsh *et al.*, 2014).

Eumycetoma is treated with azoles, in particular itraconazole at a dose of 200–400 mg/day (Fahal, 2011). Posaconazole and voriconazole are indicated in cases refractory or intolerant to other antifungals, but they are still very expensive. Terbinafine can be used at a dose of 250–1000 mg/day as monotherapy or in combination with itraconazole for 6–12 months (Estrada *et al.*, 2012). However, *in vitro* studies do not support this as a first-line option in eumycetoma (Welsh *et al.*, 2014).

Complete excision is indicated in cases of single lesion or anatomically feasible, alone or associated with pharmacological treatment. Whenever possible, the association of antimicrobials before and after the procedure is pertinent due to possible recurrence. Actinomycetoma responds better to drug therapy when compared to eumycetoma and therefore surgery is less indicated. Cure rates vary from 25.9% for eumycetoma through 90% for actinomycetoma (van de Sande *et al.*, 2014). Amputation is indicated in cases of infection with massive bone destruction and/or unresponsive to drug treatment, secondary bacterial infection unresponsive to drug treatment or serious adverse drug effects. It is important to emphasize the exception character for such conduct, left for extreme cases. Amputated patients should receive early rehabilitation and use of appropriate orthopedic prostheses, mitigating the social impact (Asly *et al.*, 2010; Sampaio *et al.*, 2015).

Criteria for cure include disappearance of the clinical lesion, absence of activity detected by imaging, bone remodeling, and absence of abscesses and grains. The recurrence rate is around 25%–50%. Therefore, follow-up should be prolonged over years, with no time determined by scientific studies (Fahal, 2011; Zein *et al.*, 2012).

Subcutaneous Phaeohyphomycosis

Phaeohyphomycosis is caused by dematiaceous, i.e., darkly pigmented, fungi (Ajello *et al.*, 1974). They are termed for their natural melanin pigment in the wall, an important virulence factor of fungi infectivity. Phaeohyphomycosis has a wide range of clinical presentations: superficial, subcutaneous and disseminated disease. In this topic we focus on subcutaneous presentation.

Epidemiology

The agents of phaeohyphomycosis are ubiquitous in nature, present in soil and vegetation, and distributed worldwide. The number of genera and species of fungi causing phaeohyphomycosis is quite large. *Alternaria* spp. and *Exophiala* spp. are the most common agents in cutaneous phaeohyphomycosis, while other as *Bipolaris* spp., *Cladophialophora* spp., *Curvularia* spp., and *Phialophora* spp. have also been described (Severo *et al.*, 2012). Individuals can acquire the infection in professional or leisure activities. These infections are uncommon and can occur in both immunocompetent and immunocompromised hosts. The most common risk factors for infection are corticosteroid use and solid organ transplantation, mainly the kidney (Revankar *et al.*, 2017). Recently, a case of subcutaneous phaeohyphomycosis with CARD9 mutation was described (Huang *et al.*, 2019).

Pathophysiology

Traumatic implantation is frequently related, and dissemination is rare, but it can occur in immunosuppressed patients. Lung and central nervous system are possible involved sites (Revankar *et al.*, 2017; Schieffelin *et al.*, 2014; Vasikasin *et al.*, 2019).

Clinical Features

The most common presentation of subcutaneous phaeohyphomycosis is an indolent nodule or cyst in exposed areas of the limbs. These lesions tend to be solitary and asymptomatic. Other cutaneous lesions are papules, plaques, pustules, or ulcers, one or multiple (Caviedes *et al.*, 2017; Shields *et al.*, 2019; Tirico *et al.*, 2016).

Differential Diagnosis

Differential diagnoses include sporotrichosis, chromoblastomycosis, cutaneous tuberculosis, American tegumentary leishmaniasis, cutaneous nocardiosis, foreign body granuloma and synovial cysts, among others.

Diagnosis

The diagnosis of cutaneous phaeohyphomycosis is based on fungal culture and histopathology of tissue specimens. The typical histologic appearance is suppurative granulomas, with histiocytes and varying number of giant cells. Brown hyphae are evident in H&E and Fontana-Masson stains (Schieffelin *et al.*, 2014). PCR using ITS 1 region and D1/D2 analyzes are becoming more available for identification of this diverse group of fungi from clinical specimens (Santos *et al.*, 2013).

Treatment

Surgical resection associated with antifungal drugs is essential to cure. Treatment is indicated until the lesions heal. Most of the dematiaceous fungi are susceptible to azoles, making itraconazole, voriconazole and posaconazole the main drugs employed. Prolonged courses are necessary for non-surgery patients. Mortality related to cutaneous infections is rare whereas among patients with disseminated disease it approaches 70% (Revankar *et al.*, 2017).

Entomophthoramycosis

Also called entomophthoromycosis or subcutaneous zygomycosis (former name), this group involves subcutaneous mycoses caused by fungi of the order Entomophthorales, subphylum Entomophthoramycota, which includes Basidiobolomycetes (*Basidiobolus ranarum*), Neozygitomycetes and Entomophthoramycetes (*Conidiobolus coronatus* and *Conidiobolus incongruus*) (Kwon-Chung, 2012).

Epidemiology

These fungi are part of the soil microbiota, and have been isolated from insects, decaying vegetables, intestines of some reptiles, amphibians, and domestic and wild mammals. Although their prevalence is highest in tropical and subtropical zones in Africa, Asia and Latin America, the burden of basidiobolomycosis (*Basidiobolus ranarum*) might be expanding, with cases reported in the United States, Australia, Iran and Saudi Arabia (Roden *et al.*, 2005; Shaikh *et al.*, 2016).

Pathophysiology

These organisms are believed to exert low virulence in human hosts. It has been proposed that infected patients may acquire the organism during a state of transient immunosuppression or as the result of repeated exposure. *Basidiobolus ranarum* may enter human hosts via insect bites or via minor trauma and subsequent inoculation due to contact with soil and vegetation with the agent. *Conidiobolus* spp. may be acquired via the inhalation of spores or from minor trauma (Shaikh *et al.*, 2016).

Clinical Features

Entomophthoramycosis may be classified as mucocutaneous, subcutaneous and deep visceral disease. Conidiobolomycosis mainly affects the mucocutaneous facial structures (rhinoentomophthoramycosis). It progresses slowly, with edema, disfigurement and sequelae (Gugnani, 1992; Roden *et al.*, 2005). Rhinoentomophthoramycosis may cause intracranial impairment, resulting in a rhino-orbitocerebral infection (Shaikh *et al.*, 2016). Basidiobolomycosis is more prevalent in men than in women, and it also affects children. It starts with erythematous nodular lesions on the trunk and limbs that may converge and become necrotic, affecting muscles, bones, regional lymph nodes, and adjacent organs. Involvement of the gastrointestinal system has been reported, mimicking inflammatory bowel disease, especially Crohn's disease, and malignancy (Gugnani, 1999). Although affecting primarily immunocompetent patients, immunocompromised ones may be involved, with greater severity.

Differential Diagnosis

The differential diagnosis includes chromoblastomycosis, sporotrichosis, paracoccidioidomycosis, blastomycosis, coccidioidomycosis, pythiosis, rhinosporidiosis, mucocutaneous tuberculosis, atypical mycobacterioses, nocardiosis, tularemia and cat-scratch disease. Among noninfectious processes, differentiation from squamous cell carcinoma and cutaneous T-cell lymphoma is important (Cameroon, 1990).

Diagnosis

The mycological direct examination and culture of lesion specimens, along with the histopathology seal the diagnosis. Hyphae are broad, distorted, with very few septations, and show right-angle branching. Histopathology shows a chronic granulomatous inflammatory infiltrate composed by macrophages, epithelioid and multinucleate giant cells, plasma cells, lymphocytes and eosinophils, typically with Splendore-Hoeppli phenomenon.

When cultured, *B. ranarum* initially displays a gray to beige colony that is glabrous or waxy and becomes radially furrowed and covered with aerial hyphae over time. It grows moderately rapid at 30°C and slower at 37°C. The microscopy reveals hyphae with occasional septa, sporangiophores with terminal sporangiospore, club-shaped spores with a knoblike tip and distinctive intercalary zygospores with a beaklike appendage (Ribes *et al.*, 2000).

Conidiobolus spp. grow rapidly within 3–5 days at 37°C on conventional media as glabrous, flat, beige to brown colonies and develop scattered white aerial hyphae. The hyphae of *Conidiobolus* spp. display occasional septa. The sporangiospores of *Conidiobolus* have a distinct papilla and sporangiola are also formed. The spores may display hair-like structures called villi (Ribes *et al.*, 2000).

Molecular identification can be performed from paraffin embedded tissues. Different methods with specific DNA probes and panfungal primers, as well as real time PCR, are used.

Computed tomography or MRI are important to define the extent of the lesions, plan possible surgeries and monitor therapeutic response. In suspected cases of gastrointestinal disease, colonoscopy is useful for observing the lesions and for obtaining biopsies (Shaikh *et al.*, 2016).

Treatment

No controlled trials exist, but itraconazole is the therapy of choice (Queiroz-Telles *et al.*, 2017b), although for conidiobolomycosis the response tends to be disappointing. Potassium iodide has been used as monotherapy or in combination with itraconazole (Kamalam and Thambiah, 1979). Reports of terbinafine as an associated option also exist (Yang *et al.*, 2010). Surgical resection has its role, especially in initial conidiobolomycosis and in basidiobolomycosis. Voriconazole might be preferable for gastrointestinal basidiobolomycosis (Albaradi *et al.*, 2014). For severe and disseminated disease, amphotericin B formulations are indicated. Nevertheless, the MIC often signal a frustrating response (Guarro *et al.*, 1999).

Prevention

Protective measures are needed, like proper footwear, gloves, body protection and tools when dealing with soil and plants. Such measures are useful not only for those at occupational risk, but also for those who, for leisure, are in contact with the environment (Queiroz-Telles *et al.*, 2017a). Proper and feasible local behavior changes might be helpful.

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Superficial Infections of the Skin and Nails

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Introduction

Superficial and cutaneous mycoses are diseases caused by fungi of the skin microbiome and environmental fungi, occurring either in the soil or colonizing animals. Clinically, there is a superficial involvement of the skin, usually limited to the *stratum corneum* and the naked hair shaft. In cutaneous mycoses, the host immune response is typically triggered, leading to an inflammatory process, usually absent or mildly seen in superficial mycoses.

Superficial Mycoses

Pityriasis Versicolor

It is the most prevalent superficial mycosis, caused by yeasts belonging to the genus *Malassezia*. To date, 14 species of *Malassezia* have been identified. *Malassezia furfur* (former *Pityrosporum ovale*), *Malassezia globosa*, and *Malassezia sympodialis* are responsible for the largest number of cases of pityriasis versicolor (Karray and McKinney, 2019). Other species described are *Malassezia pachydermatis*, *Malassezia obtusa*, *Malassezia restricta*, *Malassezia slooffiae*, *Malassezia dermatis*, *Malassezia yamatoensis*, *Malassezia nana* and *Malassezia japonica*.

P. versicolor has universal distribution being most prevalent in the tropics, where it can affect up to 40% of the population (Schwartz, 2004). There is no gender or race pattern but young adults are usually more affected due to hormonal issues. *Malassezia* yeasts are part of the microbiome of human skin. Pathogenesis involves a genetic predisposition and an imbalance in the host-fungus interaction (Sparber and LeibundGut-Landmann, 2017). As *Malassezia* yeasts are generally lipophilic, the disease is usually related to activities that cause intense sweating and the use of oily cosmetics. Other risk factors are diabetes, Cushing's disease, prolonged corticosteroid therapy, oral contraceptive use and pregnancy. Prematurity, malnutrition, parenteral nutrition, central venous catheter and immunodeficiencies can lead to an invasive and severe presentation, not discussed here (Pedrosa *et al.*, 2018).

Clinically, p. versicolor is characterized by multiple round, sometimes confluent, well demarcated hypochromic macules (hypochromic variety), occurring predominantly in seborrheic regions such as face, shoulders, upper trunk, and arms. A thin scaling indicates disease activity (**Fig. 1**). The "evoked-scale" sign (Zireli's sign) corresponds to the scaling observed when the skin is stretched (Hudson *et al.*, 2018). Some fungal metabolites have been linked to the clinical presentation of p. versicolor such as melanin, azelaic acid, and other products of skin lipid peroxidation. The hypothesis that cutaneous hypopigmentation is related to a competitive inhibition of the tyrosinase activity caused by *Malassezia*-produced azelaic acid is reported to be probably not relevant as this dicarboxylic acid cannot be synthesized in biologically significant quantities on diseased skin (Gaitanis *et al.*, 2012). Lesions may also present brownish (hyperchromic or hyperpigmented variety) to erythematous colors. In case of atypical presentation, the Wood lamp may help revealing yellowish, gold or silver fluorescence related to the fungal production of coproporphyrin (Veasey *et al.*, 2017).

The diagnosis is based on clinical picture; however, the skin scraping direct mycological examination with 10% potassium hydroxide (KOH) may help in the most atypical cases. Short fragments of curved and thick-walled hyphae usually without branches are observed on the skin scales (**Fig. 2**). Spherical thick-walled blastoconidia, alone or in groups, next to the hyphae, are also seen, some of them with phialide budding, typical of the *Malassezia* genus. *Malassezia* spp. do not grow in traditional media used in medical mycology because they are lipophilic fungi, requiring specific media such as Dixon's agar or Dixon's agar plus glycerol (Bonifaz *et al.*, 2010).

Treatment is usually topical and indicated for limited or acute cases with keratolytic agents such as 20%–40% sodium hyposulfite, 2.5% selenium sulfide, 2% zinc pyrithione and antifungal imidazole compounds such as 2% ketoconazole, for 30 days. Shampoo or spray should be preferred as they are not oily. To prevent relapses, it is important to treat the scalp and all affected area, even the healthy skin. Associated systemic treatment is indicated for extensive, relapsing or chronic cases with itraconazole 200 mg/day, 5–10 days or fluconazole 450 mg as a single dose (Bonifaz *et al.*, 2010). Sun exposure post-treatment is important to induce skin repigmentation. Narrow-band UVB was reported to be an effective alternative tool for extensive and recurrent cases unresponsive to conventional treatments, probably due to its immunomodulatory and inhibitory effect on *Malassezia* growth (Balevi *et al.*, 2018). Prevention includes control of risk factors.

Tinea Nigra

Tinea nigra is a chronic superficial mycosis caused by the geophilic halotolerant dematiaceous fungus *Hortaea werneckii*. It occurs in tropical and sub-tropical regions and often affects young people, mainly children, who get in contact with soil. The first clinical

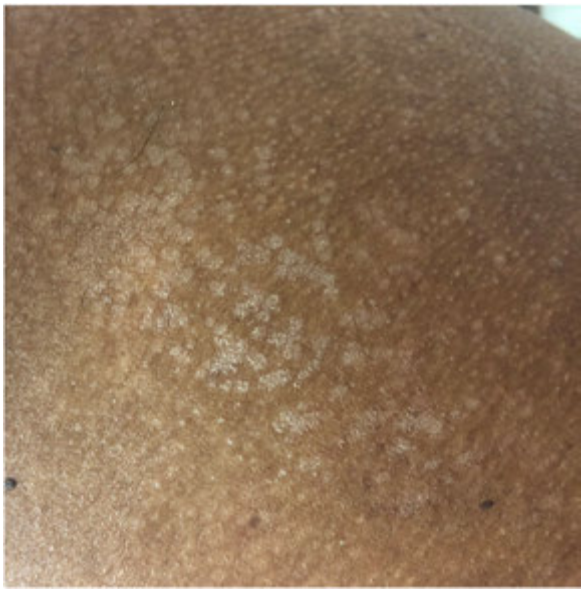

Fig. 1 Pityriasis versicolor – Multiple small round hypochromic macules with thin scaling on the upper trunk of a young man.

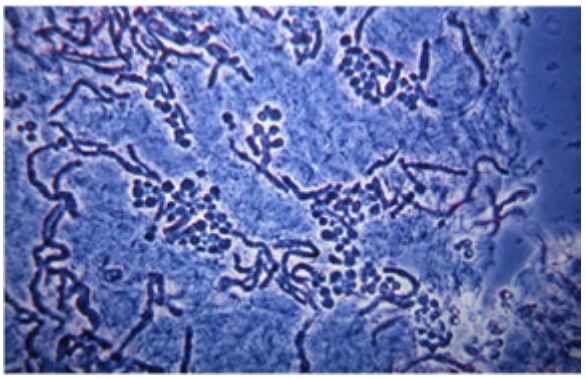

Fig. 2 *Malassezia* sp. on direct mycological examination showing short fragments of curved and thick-walled hyphae along with spherical thick-walled grouped blastoconidia. Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*).

report was provided by Alexandre Cerqueira in 1891, in Bahia (Brazil). In the literature, there are 155 reported cases of *tinea nigra* in Latin America between 1966 and 2017 (Giordano *et al.*, 2018).

Clinically, non-scaly smooth brown-black asymptomatic macules on the palms of hands and soles of feet are observed. Dermoscopy is a useful tool to differentiate *tinea nigra* from nevi, and especially melanoma (Maia Abinader *et al.*, 2016).

In direct mycological examination of the cutaneous scales, *H. werneckii* is seen as branched and septate hyphae (**Fig. 3(A)**). The apical part of the hyphae is hyaline, has parallel walls and regular septation. The older parts have a brownish color, irregular cell wall and typical irregularly distributed septa. On Agar Sabouraud, colony is slow growing, initially yeast-like and shiny black. Over time, it develops abundant aerial mycelia (**Fig. 3(B)**). Microscopically, we observe numerous groups of two-celled brown yeasts with prominent darkly pigmented septa that taper towards the extremities forming an annellide (**Fig. 3(C)**). Also, brown to dark septate hyphae are present with one to two-celled conidia.

Treatment is topical with keratolytic and antifungal agents such as imidazoles, ciclopirox, terbinafine and butenafine with a good clinical response. Spontaneous cure can rarely occur (Rossetto and Cruz, 2012).

White *Piedra*

Trichosporon spp. are part of the skin microbiome (oral mucosa and inguinal regions) and are widely distributed in the environment (Schwartz, 2004). They can eventually cause soft nodules, known as white *piedra*, and are also responsible for opportunistic infections in immunosuppressed patients, mainly those with hematologic neoplasia.

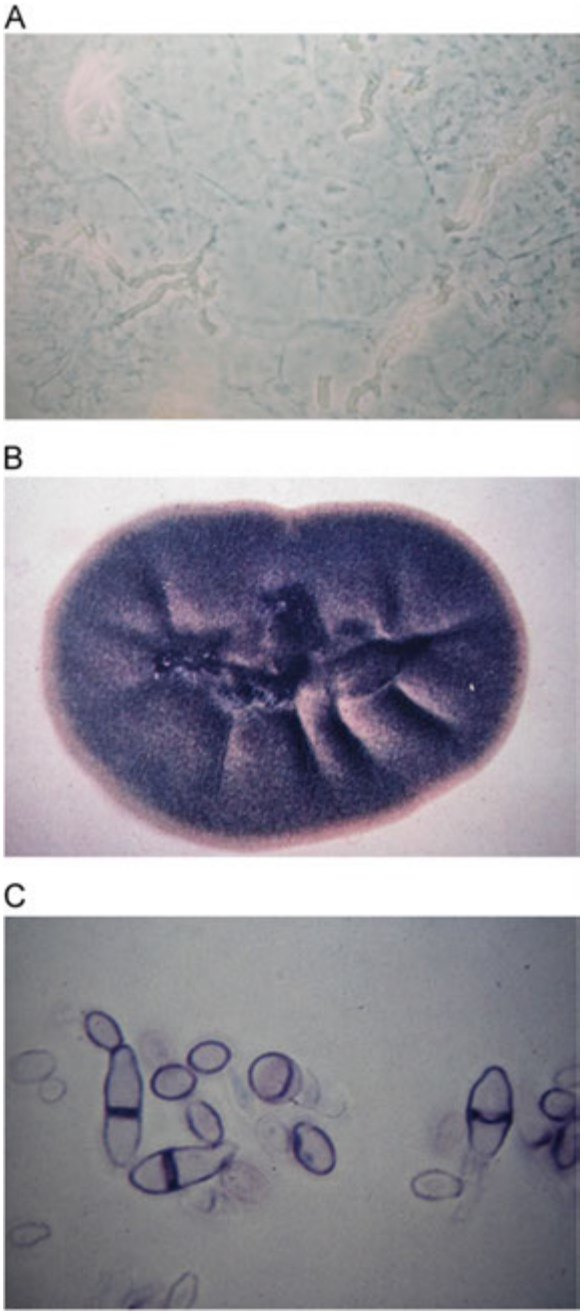

Fig. 3 *Hortae werneckii* – Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*). (A) Direct mycological examination showing branched and septate black hyphae (10 ×). (B) Macroscopic aspect of the colony on Agar Sabouraud, showing black velvety aerial mycelia folded in the center and with radial grooves. (C) Microscopy of the colony showing two-celled brown yeasts with prominent darkly pigmented septa.

A recent systematic review reported 106 cases of white *piedra* in the Americas, being 73.58% in South America, where the most endemic countries are Brazil, Colombia, and Venezuela. Mexico and the US had most of the cases from North America (Ramírez-Soto *et al.*, 2019).

Pathogenesis of white *piedra* is unknown. Clinically, it presents with clear, soft and asymptomatic nodules in the naked hair shaft of the pubic hair, axillary hair, beards, mustaches, eyebrows, eyelashes and scalp hair (**Fig. 4**).

In direct mycological examination with 10% KOH, the nodule of white *piedra* consists in a web of septate branched hyaline hyphae (**Fig. 5(A)**). Hyphae can fragment into oval or rectangular arthroconidia (**Fig. 5(B)**). There are no fungi parasitizing the inner layers of the affected hair. Fast-growing colonies on Sabouraud Agar are light beige in color, creamy or glabrous and sometimes develop radial furrows on their surface (**Fig. 5(C)**). Microscopically, they are composed by septate and branched hyaline hyphae that fragment into arthroconidia. Blastoconidia are also seen.

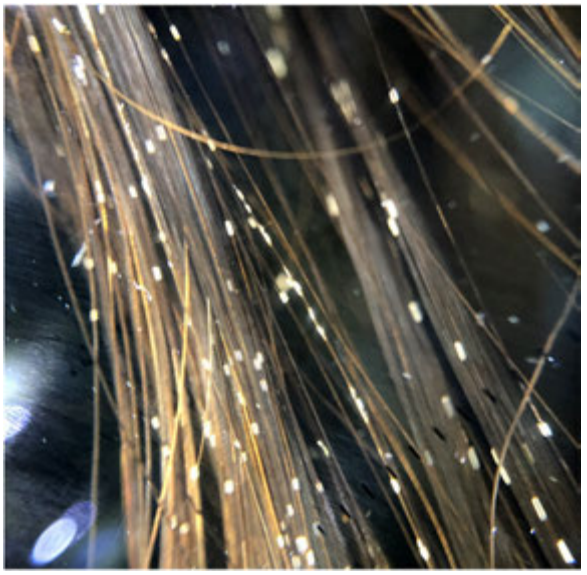

Fig. 4 White *piedra* – Trichoscopy showing clear and soft yellowish nodules on the hair shaft of the scalp. Credits to Mariana de Almeida Seigneur d'Albuquerque, MD.

Treatment includes clipping or shaving hair in the affected area but recurrent cases are frequent, especially in endemic regions, where topical antifungals are indicated. Antifungal treatment is based on 2% ketoconazole shampoo for 1–2 months but oral itraconazole as well as keratolytics such as 1% salicylic acid preparations can be necessary in some cases (Bonifaz *et al.*, 2019).

Black *Piedra*

It is a rare superficial mycosis affecting the naked hair shaft caused by the geophilic dematiaceous filamentous fungus named *Piedraia hortae* (da Fonseca and de Arêa Leão, 1928). It occurs in the soil and water of riverbanks of tropical forests such as Amazon rainforest.

The fungus is acquired by the contact with humid soil, causing asymptomatic black and hard nodules, strongly adhered to the hair shaft of primates and humans.

On direct mycological examination with 10% KOH, nodules are composed by coiled dark hyphae (**Fig. 6(A)**). Large nodules constitute an ascostroma with some oval spaces between these hyphae containing ascos, each one with eight ascospores. On Sabouraud Agar media, slow-growing black glabrous colonies are observed, usually raised in the center and flat in the periphery (**Fig. 6(B)**). Microscopically, a web of septate, branched brown hyphae, sometimes with irregular cells and chlamydoconidia are seen.

Shaving or cutting the hair is the best treatment for black *piedra* as the nodules are strongly adhered (Schwartz, 2004). Additionally, topical antifungals and oral terbinafine can be used (Drake *et al.*, 1996). Due to cultural issues, treatment is not indicated for Brazilian Indians, as the nodules are considered beauty ornaments by some groups.

Cutaneous Mycoses

Dermatophytosis

These are cutaneous fungal infections of the hair, skin and nails, caused by specific filamentous fungi named dermatophytes. Historically, three anamorphic genera of dermatophytes were described: *Trichophyton*, *Microsporum* and *Epidermophyton*, while the known teleomorphs were distributed in the genera *Arthroderma* and *Nannizzia* (Lacaz *et al.*, 2002). However, the advent of polyphasic taxonomy, using methodologies of phylogeny and molecular biology, allowed the description of nine holomorphic genera: *Trichophyton*, *Epidermophyton*, *Nannizzia*, *Paraphyton*, *Lophophyton*, *Microsporum*, *Arthroderma*, *Ctenomyces* and *Guarromyces*, the first seven species being capable to cause infection in humans and/or other animals (de Hoog *et al.*, 2017a).

Dermatophytes are keratinophilic fungi, able to invade and degrade keratinized tissues, causing a variable inflammatory response. Anthropophilic dermatophyte species are more adapted to human tissues and usually trigger a milder inflammation when compared with zoophilic and geophilic species (de Hoog *et al.*, 2017b).

Dermatophytoses are common cutaneous infections, universally distributed, affecting individuals of all ages and both sexes leading to serious chronic morbidity. Changes in frequency and epidemiologic features of the disease have been increasing due to population mobility, changes in human lifestyle, and advents of antifungal drugs (Zhan and Liu, 2017). Transmission occurs

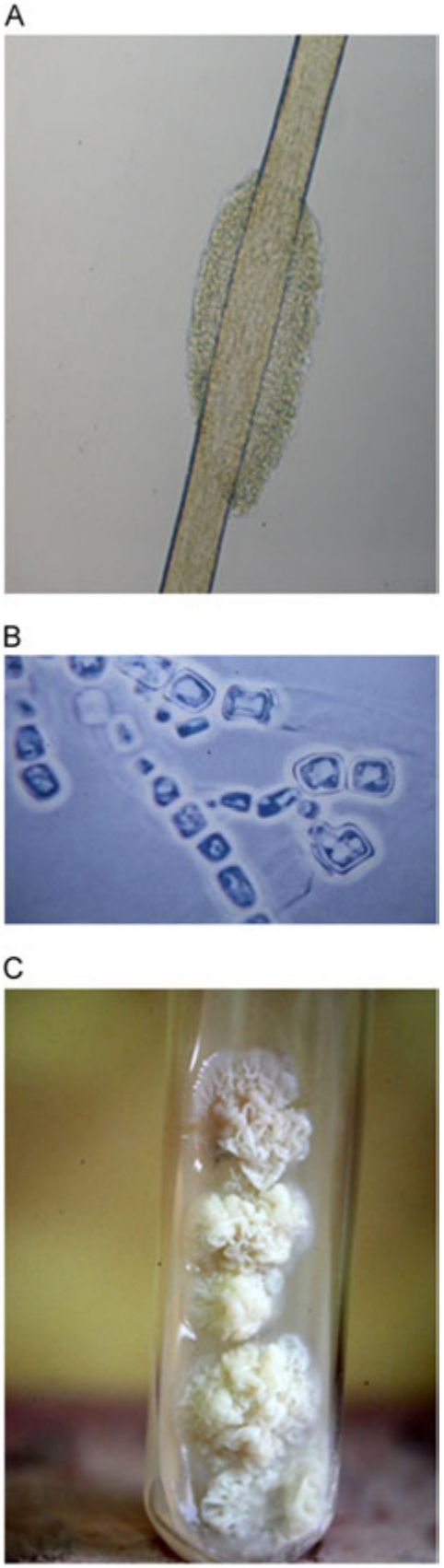

Fig. 5 *Trichosporon* sp. – Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*). (A) Direct mycological examination with 10% KOH showing a nodule of white *piedra* (10 ×). (B) Microscopic aspect of the colony showing branched hyaline hyphae fragmented into rectangular arthroconidia (100 ×). (C) Macroscopic aspect of the colony on Agar Sabouraud showing a light beige creamy colony with radial furrows in its surface.

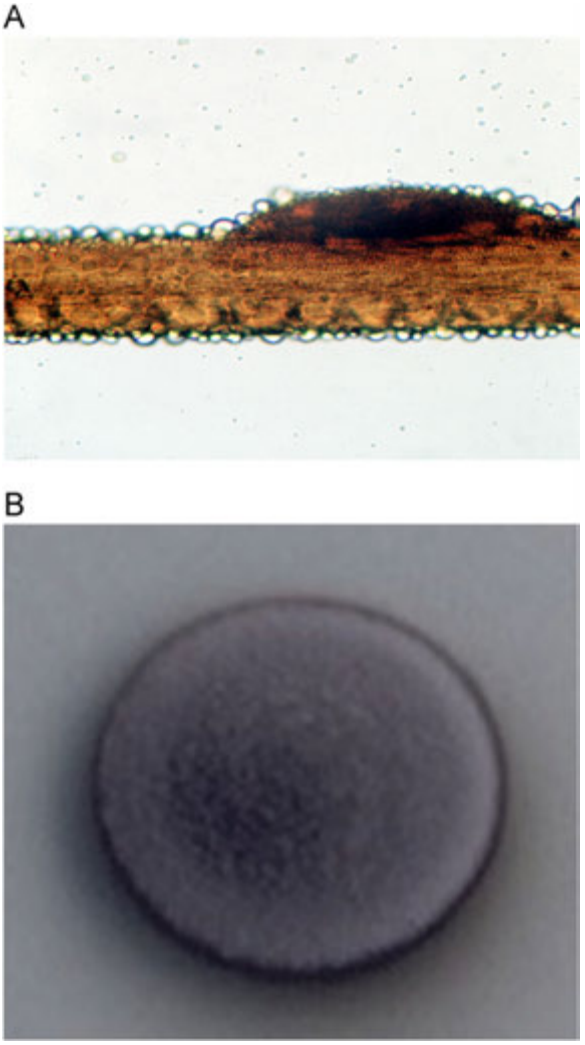

Fig. 6 Black *piedra* – Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*). (A) Direct mycological examination showing a nodule of black *piedra* (10 ×). (B) Macroscopic aspect of the colony on Agar Sabouraud, showing black velvety aerial mycelia colony raised in the center and flat in the periphery.

through human contact with infected individuals, animals, soil, and fomites. Epidemiology and clinical presentation varies according to the affected body site and the species of dermatophyte involved.

Dermatophytosis of the body (*tinea corporis*) affects the glabrous skin and is commonly known as ringworm. The most common agents are anthropophilic fungi, being *Trichophyton rubrum* the most common, followed by *Epidermophyton floccosum* and *Trichophyton mentagrophytes*. Clinically, the lesions present as annular plaques with central clearing, leading scale, and a ring-shape appearance (**Fig. 7**). They occur mainly on the trunk, limbs, and may be single or multiple, of varying sizes, and may coalesce (Kovitwanichkanont and Chong, 2019).

Dermatophytosis of the face and the beard (*tinea faciei*, *tinea barbae*) are less common clinical forms. Zoophilic species are mostly implicated such as *T. mentagrophytes* but anthropophilic dermatophytes can also be involved. Usually, there is an inflammatory pustular eruption with crusts resembling bacterial folliculitis, kerion presentation can also occur (Moriarty *et al.*, 2012).

Dermatophytosis of the scalp (*tinea capitis*) is almost exclusively a disease of childhood. It occurs in poor socioeconomic conditions and involves mainly the anthropophilic species *Trichophyton tonsurans* and the zoophilic *Microsporum canis*. The former usually presents with multiple scaly alopecic patches and poor or absent inflammatory signs (**Fig. 8**). The latter usually occurs as a large single alopecic patch and can be associated with a more intense inflammatory reaction. Diffuse scaling, black dots and diffuse pustular lesions can also be present (Moriarty *et al.*, 2012). Kerion Celsi is a clinical variant characterized by an exacerbated inflammatory response, usually misdiagnosed as bacterial infection. Late diagnosis of Kerion Celsi can lead to permanent scarring alopecia.

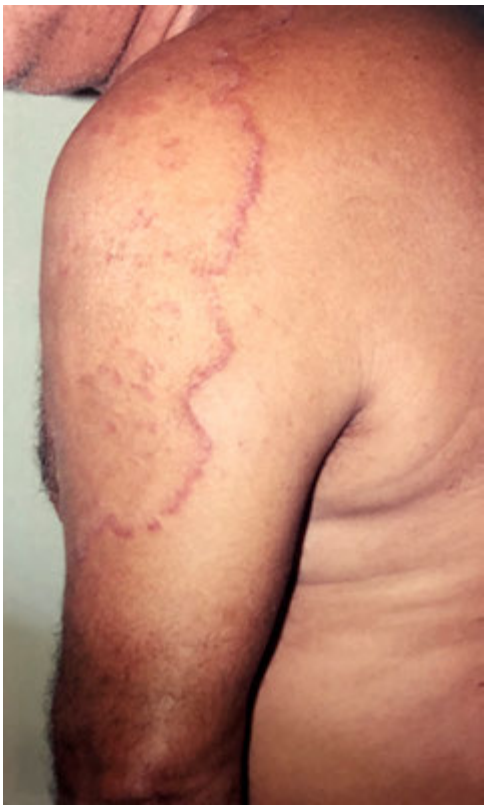

Fig. 7 *Tinea corporis*: single scaly ring-shaped patch on the left arm of a patient. Credits to Antonio Carlos Francesconi do Valle, MD PhD.

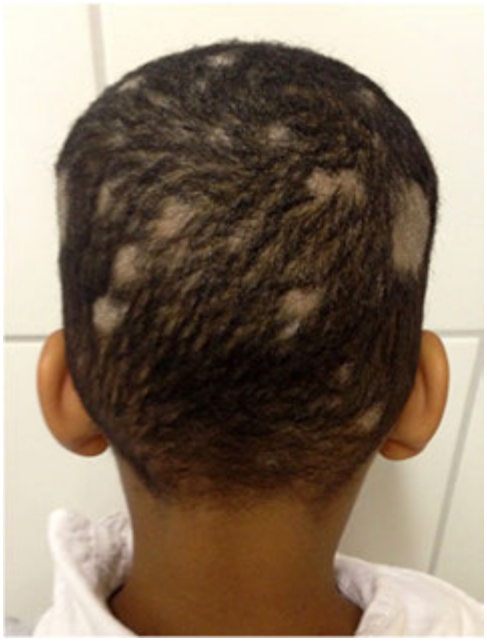

Fig. 8 Multiple round scaly alopecic patches without inflammatory signs.

Dermatophytosis of the hands (*tinea manuum*) affects the palmar surface and is usually unilateral. When it presents bilaterally, it must be distinguished from contact eczema and psoriasis for correct treatment. *Tinea manuum* is quite rare. On the contrary, dermatophytosis of the feet (*tinea pedis*) is very common. The main species involved in *tinea pedis* are *T. rubrum*, *T. mentagrophytes*, and *E. floccosum*. They may affect the plantar and interdigital areas causing erythema, and thin or laminar scaling. There are three

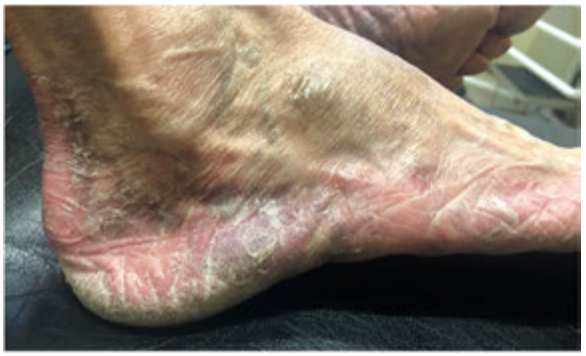

Fig. 9 Chronic squamous *tinea pedis* showing erythema and laminar scaling.

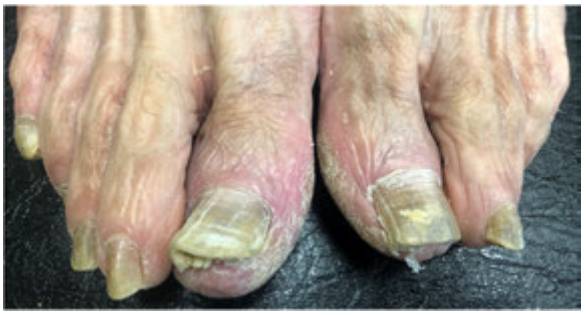

Fig. 10 *Tinea unguium* showing severe subungual keratosis.

main clinical variants: the interdigital form, the vesicobullous form and the chronic squamous form (Kovitwanichkanont and Chong, 2019). The interdigital form is the most common and presents with maceration or scales between toes, intense itching and eventually erythema and painful fissures. The chronic squamous form (moccasin-type) is clinically characterized by plantar erythema with scaling involving the lateral (**Fig. 9**) and plantar surfaces of the foot, and usually occurs due to anthropophilic fungi, mainly *T. rubrum*. The vesicobullous is an inflammatory, less common form, usually related to zoophilic and geophilic species.

Nail dermatophytosis (*tinea unguium*) is the most common nail infectious disorder and may have several clinical presentations according to the type of fungal invasion of the nail plate. Five major presentations are described: distal and lateral subungual, proximal subungual, white superficial, endonyx, and total dystrophic onychomycosis. The Distal and Lateral Subungual Onychomycosis (DLSO) is usually associated with *tinea pedis* because fungi reach the nail through the hyponychium invading the undersurface of the nail plate and spreading proximally. It usually affects one or both great toenails and it is usually caused by *T. rubrum*. The White Superficial Onychomycosis is usually caused by *T. mentagrophytes*, which invades the dorsal nail plate forming white opaque lesions, easily scraped away. The Proximal Subungual Onychomycosis (PSO) typically occurs in the ventral nail plate, producing a proximal leukonychia and it is rarely related to dermatophytes, being more observed in immunocompromised patients or related to non-dermatophyte infection. Endonyx Onychomycosis is very rare, caused by *T. soudanense* or *T. violaceum*, and is characterized by massive nail plate invasion without nail bed involvement. Total Dystrophic Onychomycosis occurs after long-term DLSO or PSO presenting severe subungual keratosis (**Fig. 10**), thickening, and dystrophy of the total nail unit plate (Piraccini and Alessandrini, 2015).

Dermatophytosis of the inguinal region (*tinea cruris*) is more frequent between adult men and presents as annular lesions with erythematous-scaly border on the groin. The main fungal agents involved are the same agents of *tinea corporis*.

Some special clinical presentations of dermatophytosis must be highlighted due to their specific characteristics. Majocchi granuloma is a deeper dermatophyte infection with dermic and follicular involvement and greater inflammatory reaction. *Tinea incognita* corresponds to a dermatophytosis lesion modified after topical or oral corticosteroids use, reducing inflammatory signs, making diagnosis difficult. *Tinea imbricata* is caused by *Trichophyton concentricum* (named after the concentric aspect of the skin lesions), occurring in the southwest Polynesia, Melanesia, Southeast Asia, India, and Central America (James *et al.*, 2006). *Tinea corporis gladiatorum* can present in athletes (classically wrestlers) with extensive direct skin-to-skin contact (Yee and Al Aboud, 2019). In immunocompromised and susceptible patients, dermatophytosis may be a deep, extensive, severe and invasive disease (Nazarian *et al.*, 2019).

A dermatophytid is a secondary, distant, hypersensitivity reaction that can occur as immunological response to a dermatophyte infection. The essential criteria for diagnosis are: (1) a proven focus of dermatophyte infection confirmed by fungal isolation in

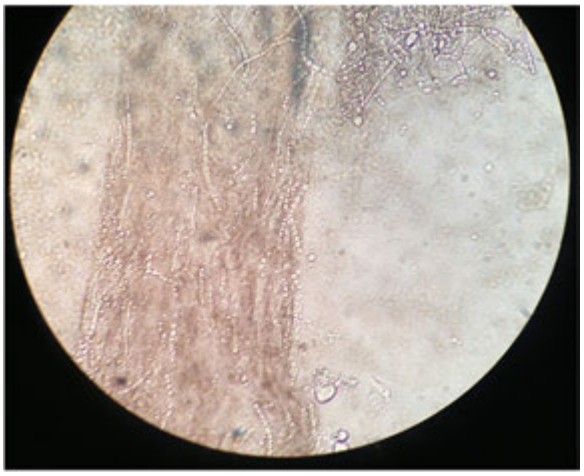

Fig. 11 Direct mycological examination of the hair showing hyphae and arthroconidia distributed around and inside the tonsured hair shaft.

Table 1 Macroscopic and microscopic characteristics of most clinically important dermatophytes

Dermatophyte	*Macromorphology*	*Micromorphology*
T. rubrum	White colony with downy to velvety texture, and red reverse pigment	Delicate pyriform to claviform microconidia and cigar-shaped macroconidia (**Fig. 12(A)**)
T. mentagrophytes	White to beige colony with powdery surface, brown reverse pigment	Clustered round microconidia, spiral hyphae, and claviform macroconidia (**Fig. 12(B)**)
T. tonsurans	Beige colony with velvety to powdery texture, radial grooves, and reddish-brown reverse pigment	Long claviform to broad pyriform microconidia, cigar-shaped macroconidia, and clamidoconidia (**Fig. 12(C)**)
E. floccosum	Beige velvety colony raised and folded in the center, yellowish-brown reverse pigment	Thin-walled macroconidia growing directly from the hyphae and numerous clamidoconidia (**Fig. 12(D)**)
M. canis	White colony with fuzzy texture, bright golden yellow reverse pigment	Spindle-shaped, thick-walled macroconidia with more than 6 cells, and few pyriform to claviform microconidia (**Fig. 12(E)**)
Nannizzia gypsea	Beige colony with granular texture, brown reverse pigment	Ellipsoidal, thin-walled, four to six-celled macroconidia, and numerous claviform microconidia (**Fig. 12(F)**)

culture; (2) absence of dermatophyte isolation from the dermatophytid lesion, and (3) clearing of dermatophytid lesion after dermatophyte has been eradicated (Al Aboud *et al.*, 2003).

Diagnosis of dermatophytoses involves the direct microscopic analyses of the skin and nail scales, where any dermatophyte presents as septate, branched hyaline hyphae that can disarticulate, resulting in arthroconidia. In hair, dermatophytes also appear as hyphae or arthroconidia chains being classified in three types of parasitism: (1) *ectothrix*, when hyphae and arthroconidia are distributed around and inside the tonsured hair shaft (**Fig. 11**); (2) *endothrix*, when hyphae and arthroconidia are distributed inside the tonsured hair shaft; and (3) favic, when hyphae and arthroconidia are distributed inside the hair shaft, without tonsure. Dermatophytes grow well in Sabouraud and Mycosel Agar after incubation at 25°C for 10 days on average. All dermatophytes are resistant to cycloheximide present in Mycosel Agar, which helps in their isolation in this media. The species are routinely identified by macroscopic (texture and pigment of the colonies) and microscopic (morphology and abundance of macroconidia and microconidia) analyses of fungi isolated in these media. Identification can also be provided by Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) (L'Ollivier and Ranque, 2017). **Table 1** summarizes macroscopic and microscopic (**Fig. 12**) characteristics of most clinically important dermatophytes.

Treatment of dermatophytoses usually requires topical antifungals although oral drugs (itraconazole, terbinafine) should be prescribed in case of extensive lesions, failed topical treatment, immunocompromised host, *tinea capitis*, and frequently for *tinea unguium* (Kovitwanichkanont and Chong, 2019). The time of treatment depends on the site of infection. Palm-plantar, hair and nail infections require longer time treatment. For prevention purposes, do not share objects of personal use, wear shoes in public collective environments, wash and dry feet and intertriginous areas well, avoid close contact with animals, fomites, and environmental sources as soil. In case of zoophilic transmission, it is important to take the domestic animal to veterinary evaluation and treatment, when indicated. When the agent involved is anthropophilic, it is recommended to examine all domiciliary contacts. Once treatment is started, the patient can return to his/her labor activities.

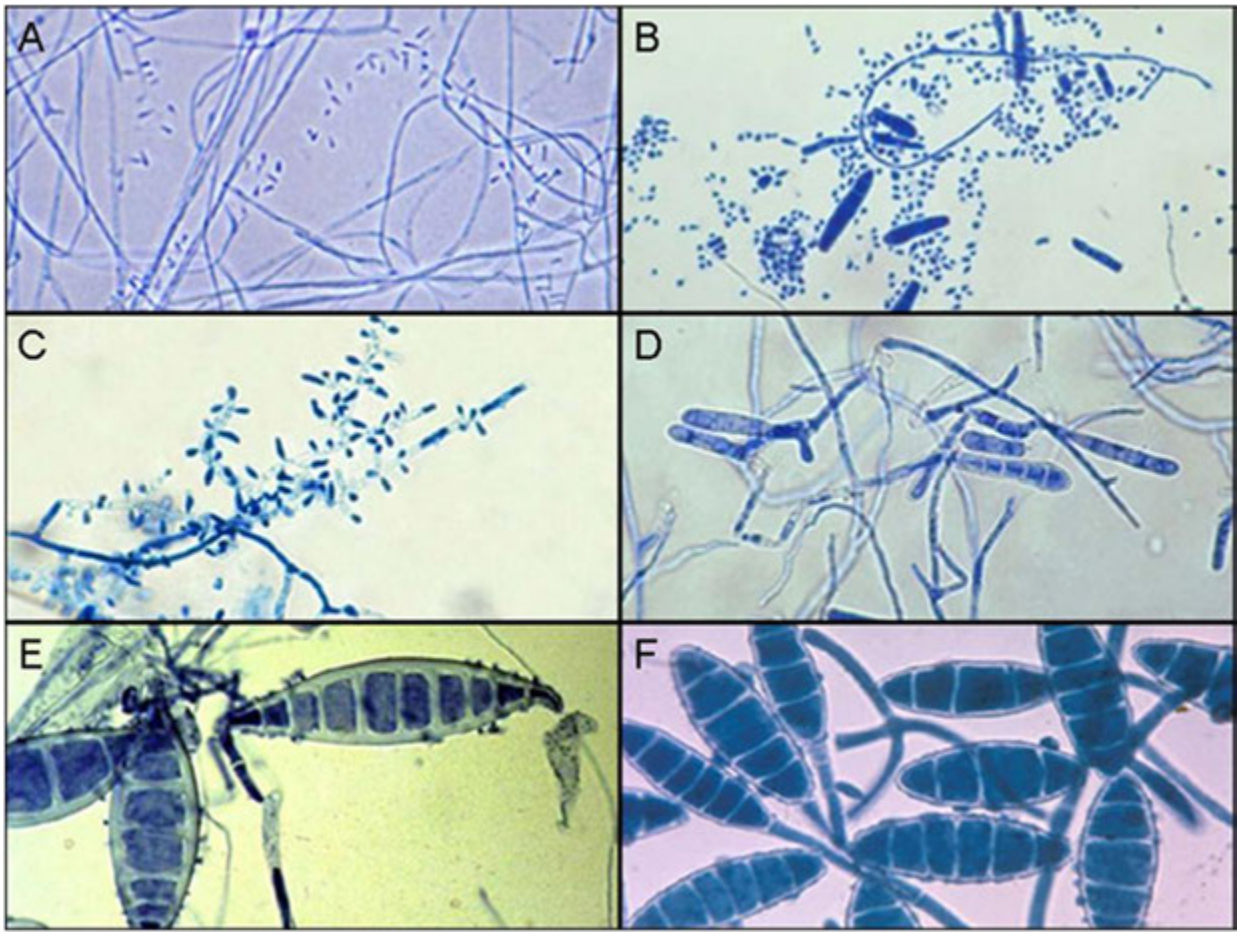

Fig. 12 Microscopic characteristics of most clinically important dermatophytes – Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*). (A) *Trichophyton rubrum.* (B) *Trichophyton mentagrophytes.* (C) *Trichophyton tonsurans.* (D) *Epydermophyton floccosum.* (E) *Microsporum canis.* (F) *Nannizzia gypsea.*

Candidiasis

Yeasts of the genus *Candida* are part of the oral, intestinal, vaginal and skin human microbiome although they can cause diseases when there is a host immune imbalance, favoring opportunistic invasion of the fungal agent. We herein briefly discuss some aspects of cutaneous candidiasis, as there will be a specific chapter for *Candida* infections.

The disease can occur in any gender or age group, prevailing in individuals with risk factors such as diabetes, antibiotic treatment, oral contraceptives use, pregnancy, as well as local conditions like heat, humidity and friction that lead to skin barrier impairment (Taudorf *et al.*, 2019). Some special clinical presentations must be highlighted due to their specific characteristics.

Candidiasis in the diaper area usually occurs in newborns between the third and fourth months, but it may also be present in adult patients requiring continuous use of diapers. Clinically, it presents shiny erythematous macules in the inguinal and diaper contact areas. Typical satellite papule-pustules are observed in the border of the lesion. The most important risk factors are the prolonged contact with urine and feces, or allergies to a diaper component.

Paronychia and onychomycosis due to *Candida* spp. usually affect the hand nails and cuticles. The main risk factor is the frequent contact with water, detergents and other chemical products. The inflammatory process of the nail fold is called paronychia and is acutely characterized by erythema, edema and pain. Chronic inflammation due to persistent exposure to risk factors may lead to a secondary involvement of the nail matrix, which may cause nail dystrophy, often misdiagnosed as onychomycosis. Onychomycosis is clinically characterized by detachment and thickening of the nail plate unit and may also lead to nail dystrophy.

Diagnosis involves clinical aspects and is reinforced by the presence of risk factors. Laboratory diagnosis involves direct examination with 10% KOH, where delicate septate hyaline hyphae, pseudohyphae and blastoconidia are observed (**Fig. 13(A)**). All *Candida* species grow on Sabouraud Agar, and some species are inhibited on Mycosel Agar. In general, the growth of white to cream yeast colonies (**Fig. 13(B)**) occurs in 3–5 days and the identification of the species can be done through morphological (clamidoconidia and germ tube production), biochemical (sugar assimilation and fermentation), MALDI-TOF and molecular tests (ITS region sequencing) (Aydin *et al.*, 2019).

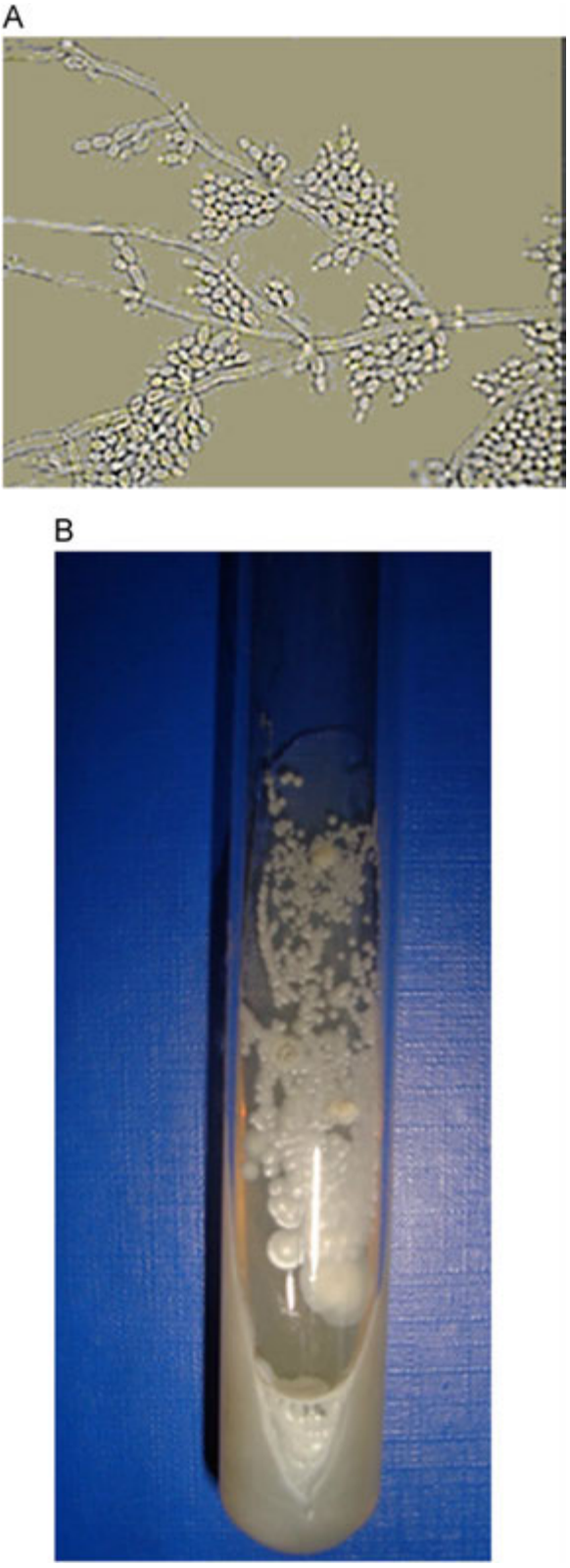

Fig. 13 *Candida* sp. – Credits to Paulo Cezar Fialho Monteiro, MD (*in memoriam*). (A) Microscopy showing septate hyaline hyphae and pseudohyphae. (B) Macroscopy of the isolate showing a beige, white to creamy yeast colony.

Treatment of cutaneous candidiasis requires the use of azoles, which may be used topically (clotrimazole, nystatin and miconazole) or orally (fluconazole, itraconazole) depending on the extent of the lesion and the immune status of the host. Combination of antifungals with topical corticosteroids may be necessary if the inflammatory process is intense unless a concomitant underlying skin disease is present (Taudorf *et al.*, 2019). Control and, if possible, elimination of associated risk factors is important for the treatment and prevention of relapses.

Dermatomycoses

These are cutaneous mycoses caused by non-dermatophyte filamentous fungi (*Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Acremonium* spp., *Scopulariopsis* spp., *Neoscytalidium* spp. etc.), which occur naturally in the environment or as plant pathogens. In humans, they behave as opportunistic agents (except *Neoscytalidium*), causing skin and nail disease (Cursi *et al.*, 2011).

They predominate in elderly patients, mostly affecting the palm-plantar regions, the toenails (Gupta *et al.*, 2016), and are clinically indistinguishable from dermatophytosis. They rarely affect the glabrous skin and the hair.

Diagnosis is based on the following criteria: (1) presence of dermatophytosis-like clinical lesion (especially in the plantar region and toenails); (2) direct microscopic examination with 10% KOH presenting hyphae similar to dermatophytes; (3) isolation of non-dermatophyte filamentous fungi from the lesion at least three different times to rule out the possibility of contamination (except *Neoscytalidium*); (4) fail to isolate dermatophytes; (5) growth absence of other contaminating fungi; (6) thermotolerance by growing agent at 37°C.

Treatment is challenging due to the host characteristics and to poor antifungal susceptibility profile, with high relapse rate and high tendency to chronicity (Gupta *et al.*, 2019). Some options are nail abrasion, nail laser therapy, ciclopirox or amorolfine nail lacquer, and oral terbinafine or itraconazole, with variable responses.

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Genitourinary Fungal Infections (Other Than Vaginal Candidiasis)

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Introduction

Most fungal urinary tract infections are caused by Candida species. Infrequently urinary tract infections can also be caused by other pathogenic fungi including *Aspergillus*, *Cryptococcus*, *Histoplasmosis*, *Blastomyces* species and other rare fungal species. Candiduria is a common clinical finding that does not necessarily imply disease. Candida albicans are part of the human mycobiome and readily colonizes the skin, as well as mucosal surfaces of gastrointestinal and genital tracts (Bradford and Ravel, 2017; Neville *et al.*, 2015). Increasing numbers of patients with candiduria can be expected as the number of immunocompromised patients is steadily increasing with more aggressive chemotherapy in cancer patients and rising usage of therapeutics that alter the immune response either to prevent graft rejection during organ transplantation or to treat other autoimmune diseases (Kauffman *et al.*, 2000; Safdar *et al.*, 2005; Tsiodras *et al.*, 2008). Distinguishing when candiduria reflects a state of disease resulting in damage to the host is important because Candida can be recovered in up to 90% of hospitalized patients with indwelling urinary catheters (Fisher *et al.*, 2011a; Datta *et al.*, 2018; Padawer *et al.*, 2015; Yashavanth *et al.*, 2013) from urine specimens.

Definition and Diagnosis of Urinary Tract Diseases Caused by Fungi

Most clinical studies on funguria focus on candiduria, which is diagnosed by culture of urine. Yeast cells can also be seen on microscopic analysis of the urine and are reported as yeast cells per ml urine after growth. The threshold for the reported colony forming units (CFU) in candiduria varies between 10^3–10^5 yeast cells per ml urine (Kauffman, 2005; Achkar and Fries, 2010). It has been reported that standard urine culture methods on blood agar and Mac-Conkey plates, do not support the growth of all Candida species (Chaturvedi *et al.*, 2017). In addition, fungal UTIs can be a result of hematogenous spread from disseminated fungal infections especially in immunocompromised hosts (Kauffman, 2005). Diagnosis of genitourinary infections secondary to Aspergillus, Cryptococcus, Histoplasma or Blastomyces species has also been made by analysis of tissue stained with special fungal stains derived from biopsy specimens of a fungal ball, prostate abscess or transplant kidney (Guarner and Brandt, 2011). Furthermore, over the years several fungal antigen tests (summarized in **Table 1**) have been developed to detect diverse fungal antigens in the urine. However, these tests do not specifically signify genitourinary infection but rather detect shed cell wall derived antigens that stem from systemic infection and were filtered through the glomerular membrane.

Epidemiology of Candiduria

Reported incidence and prevalence of candiduria depend on the setting and the populations studied. Different urine culture methods can also affect candiduria prevalence and outcomes. The standard urine culture methods using Mac-Conkey and blood agar may not exhibit the same sensitivity to recover all Candida species and thus could result in underestimating the incidence of candiduria (Achkar and Fries, 2010). Contamination during collection is also common and complicates the matter further. Candiduria is rarely found in healthy individuals but is very common in hospitalized patients (Kauffman *et al.*, 2000; Alvarez-Lerma *et al.*, 2003). Multiple studies indicate that 10%–40% of nosocomial UTIs are caused by Candida species (Lundstrom and Sobel, 2001; Richards *et al.*, 2000; Shay and Miller, 2004; Toya *et al.*, 2007).

In the hospital setting, candiduria is regularly diagnosed in intensive care units (ICUs) as well as oncology and bone marrow transplant units (Kauffman, 2005; Richards *et al.*, 2000). Among the intensive care units, higher incidences are reported in burn units when compared to medical and surgical ICUs (Bougnoux *et al.*, 2008).

Common predisposing factors for candiduria in hospitalized patients include indwelling urinary tract catheterization, urinary tract anatomical abnormalities, prior surgical procedures, recent antimicrobial administration, prolonged hospital stay, older ages, female gender, diabetes mellitus, total parenteral nutrition (TPN) use, and immunosuppressive therapy (Kauffman *et al.*, 2000; Richards *et al.*, 2000; Kauffman, 2005; Bougnoux *et al.*, 2008; Lundstrom and Sobel, 2001; Harris *et al.*, 1999; Yang *et al.*, 2013; Febré *et al.*, 1999; Hamory and Wenzel, 1978; Paul *et al.*, 2007; Simpson *et al.*, 2004). Several studies demonstrate increased mortality in patients with candiduria (Kauffman *et al.*, 2000; Alvarez-Lerma *et al.*, 2003; Paul *et al.*, 2007). Factors associated with death in candiduric patients are the use of urinary diversion devices, prior antibiotic use ($\geq$ 2 classes), ICU admission, and renal failure (Paul *et al.*, 2007). However, many of these factors may reflect the severity of underlying illness, rather than direct pathogenic effects of candida. Compared to hospital-acquired candiduria, patients with

Table 1 Urine Fungal Antigen Tests

	Method	*Sensitivity (%)*	*Specificity (%)*	*References*
Aspergillus sp	LFA	80	98	(Marr *et al.*, 2018)
Blastomyces sp	EIA	73	80	(Durkin *et al.*, 2004)
Coccidiodes sp	EIA	71	98	(Durkin *et al.*, 2008)
Cryptococcus sp	LFA	85	ND	(Huang *et al.*, 2015)
Histoplasma sp	ELISA	80	98	(Fandino-Devia *et al.*, 2016)
Penicillium sp	ELISA	97	98	(Desakorn *et al.*, 1999)
Paracoccidiodes sp	EIA	75	100	(Salina *et al.*, 1998)

Abbreviations: EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; LFA, lateral flow assay; ND, No data.

community-acquired candiduria tend to be younger and include a higher percentage of pregnant women. Similar to nosocomial candiduria, *C. albicans* is the most prevalent species in community-acquired candiduria, followed by *C. glabrata* and *C. tropicalis* respectively (Colodner *et al.*, 2008).

The percentages of concomitant candidemia in patients with candiduria ranges from 1% to 10% (Kauffman *et al.*, 2000; Bougnoux *et al.*, 2008; Paul *et al.*, 2007; Ang *et al.*, 1993; Storfer *et al.*, 1994). However, blood cultures are not consistently performed on candiduric patients and therefore the incidence of candidemia might be underestimated. Urinary tract obstruction is found to be a major risk factor for candidemia in patients with candiduria (Ang *et al.*, 1993).

Candiduria and candidemia are commonly diagnosed in neonatal and pediatric ICUs especially in premature newborns (Shay and Miller, 2004; Jarvis *et al.*, 1991; Triolo *et al.*, 2002). It is noteworthy that concomitant candidemia in neonates diagnosed with candida UTIs ranges between 33% and 52%, and is more common than in older children and adults (Phillips and Karlowicz, 1997; Bryant *et al.*, 1999). As a result, fungal balls in the collecting system are common findings in candida UTIs of critically ill neonates (Visser *et al.*, 1998). Although *C. albicans* is the also most common pathogen in candiduria in pediatric patients, *C. parapsilosis* is an emerging etiology of candiduria among neonates (Robinson *et al.*, 2009).

Microbiology

The distribution of candida species in urine is widely variable in different geographic regions. In most studies, Candida albicans continues to be the most common species isolated causing 50%–70% of the infections (Kauffman *et al.*, 2000; Alvarez-Lerma *et al.*, 2003; Simpson *et al.*, 2004; Gajdács *et al.*, 2019). In more recent years, studies have reported candiduria can also be caused by non-albicans Candida species (Paul *et al.*, 2004; Ozhak-Baysan *et al.*, 2012). *C. glabrata* and *C. tropicalis* are the predominant non-albicans Candida species reported (Paul *et al.*, 2007; Ang *et al.*, 1993; Gajdács *et al.*, 2019; Ozhak-Baysan *et al.*, 2012). Specifically, prior Fluconazole use is associated with an increased risk of *C. glabrata* isolation from the urine (Harris *et al.*, 1999). In immunocompromised patient populations non-albicans strains are becoming the dominant pathogens. For example, *C. glabrata* is a more commonly isolated fungal species (3% versus 35%) in renal transplant patients with candiduria than *C. albicans* (Safdar *et al.*, 2005). Mixed Candida species, mostly *C. glabrata* and *C. albicans* were co-isolated in 5%–10% of patients with hospital-acquired candiduria (Kauffman *et al.*, 2000; Bryant *et al.*, 1999). Molecular typing of sequential Candida isolates from patients with candiduria showed that recurrent infections occur due to the same strains (Jain *et al.*, 2007). The most recently, emerging multidrug-resistant species *C. auris* has also been reported as a cause of chronic candiduria with recurrent candidemia (Biagi *et al.*, 2019). Antifungal resistance profile of Candida isolates in urine depends on the infecting strains. Non-albicans strains are more commonly resistant to fluconazole (Toner *et al.*, 2016). Reported fluconazole resistance of *C. albicans* is between 1%–4% in contrast to 15%–25% for *C. glabrata* (Achkar and Fries, 2010).

Pathogenesis

Our understanding of pathogenesis of Candidiasis including UTIs has been informed by data from experimental rodent and rabbit models. Candiduria in these animal models is induced by intravenous infection of yeast which does not adequately mimic the ascending route in human infection (Kauffman, 2005). In rodent infection models, once candida invades from the renal artery into the kidney cortex; microabscesses form throughout parenchyma, followed by penetration of yeasts into glomeruli, renal tubules before they are shed into the urine (Kauffman, 2005). Candida species express several virulence factors to accomplish the commensal colonization and invade the tissue during infection, including adhesion, biofilm production, hyphal formation, phenotypic switching, and production of hydrolytic enzymes.

Normal urine flow is an important protective factor against microbial colonization or infection of the urinary tract. Adherence of Candida species to the host mucosal cells or the surfaces of indwelling urinary catheters is an essential virulent attribute to overcome the flow condition. It is the first step of colonization or infection. Adhesin facilitates adhesion of yeast cells to host tissue and formation of biofilms. In *C. albicans*, the agglutinin-like sequence (Als) proteins and the hypha associated GPI-linked proteins

(Hwp1) are the major adhesins (Hoyer *et al.*, 2008; Mayer *et al.*, 2013). Tamm-Horsfall glycoprotein, urine protein produced exclusively by kidneys, is found to prevent C. albicans colonization of bladder epithelium by specifically blocking Als3 protein (Coady *et al.*, 2018). Analagous, Epithelial adhesion proteins (Epa) and adhesin-like cell wall proteins (Awp) are principal adhesins in *C. glabrata* (Timmermans *et al.*, 2018) which exhibit a similar function as the Als proteins in Candida albicans.

Candida biofilms can form on urinary catheters. They are composed of mixed yeast and filamentous forms embedded in the extracellular matrix attached to the mucosal or artificial surfaces such as urinary catheters. They promote fungal growth and protect Candida species against host immune defenses (Wall *et al.*, 2019). Importantly, the organisms within the biofilms develop increased resistant levels to antifungal therapy especially azoles and polyenes (Hawser and Douglas, 1994; Pierce *et al.*, 2013). The Candida biofilms are intrinsically resistant to azole antifungal agents including fluconazole (Pierce *et al.*, 2013). Therefore, antifungal therapy is not likely to eradicate candiduria if the indwelling urinary catheters are not removed. Echinocandins are found to have excellent activity against *C. albicans* biofilms (Kuhn *et al.*, 2002). However, due to their poor kidney penetration and excretion, their use for treatment of fungal UTIs are limited (Groll and Walsh, 2000). In patients with chronic indwelling urinary catheters, candida biofilms on the catheters are likely the sources of candiduria rather than an infection of other components of the urinary tract (Fisher *et al.*, 2011a).

C. albicans can reversibly convert from budding yeasts to pseudohyphal and hyphal forms under different conditions. This morphological transition plays an important role in invasive candida infection and biofilm development. The yeast form is generally associated with dissemination whereas the filamentous or hyphal form facilitates the adhesion and invasion of host mucosal surfaces (Whiteway and Bachewich, 2007). During candida biofilm maturation, the yeast cells convert to hyphal or pseudohyphal cells forming a complicated network inside the biofilm (Wall *et al.*, 2019). In urinary tracts, certain conditions such as low pH or proteinuria can affect the morphological transition (Fisher *et al.*, 2011a).

Phenotypical switching is important for host colonization and contributes to virulence. In a rodent model, the *C. albicans* WO-1 strain can switch reversibly from white to opaque colony-forming phenotypes. The opaque-phase cells have greater cutaneous colonization capacity than the opaque-phase counterpart (Kvaal *et al.*, 1999). *C. tropicalis* can switch between five distinct phenotypes. Switching to crepe and rough phenotypes is associated with higher biofilm formation. Besides, certain switch phenotypes of *C. tropicalis* exhibit changes in susceptibility to itraconazole (Moralez *et al.*, 2014).

The extracellular hydrolytic enzymes are important in Candida virulence (Calderone and Fonzi, 2001). These enzymes can facilitate adherence, invasion of mucosal surface and blood vessels, and evasion of host immune defenses. Secreted aspartyl proteinases (SAPs) and phospholipases are key hydrolytic enzymes. SAPs hydrolyze structural proteins such as collagens, keratin as well as antibodies, complements, and cytokines. At least 10 SAP genes are identified in *C. albicans*. Different groups of SAPs are secreted at different stages of infection, such as colonization or symptomatic disease (Achkar and Fries, 2010). Phospholipases break down glycerophospholipids on cell membranes and promote tissue adherence, colonization and invasion (Ghannoum, 2000). *C. albicans* produce all phospholipases A-D. Phospholipase B is directly associated with pathogenicity (Ghannoum, 2000).

Candida species require multiple virulence factors discussed above to cause infection of the urinary tract. Different virulence factors may be predominant at different steps of UTI pathogenesis. In humans, Candida cells can reach the urinary tract either by hematogenous spread or by ascending from the vagina or perineum. Successful colonization of the urethra is the first step required for ascending infection. Interestingly, co-infection with enteric bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* is found to increase susceptibility of the urinary tract to *C. albicans* infection in an animal model (Fisher *et al.*, 2011a). However, a recent in vitro study demonstrates that *E. coli* significantly suppresses Candida biofilm growth in a Foley catheter infection model (Samaranayake *et al.*, 2014).

Sodium-glucose cotransporter-2 (SGLT2) inhibitors (canagliflozin, dapagliflozin, and empagliflozin), which are a new class of antidiabetic agents, reduce blood glucose through increased glucosuria. Safety studies of these SGLT2 inhibitors have revealed slightly increased incidence of symptomatic UTI (Vasilakou *et al.*, 2013; Usiskin *et al.*, 2014). A tendency toward increased prevalence of candiduria in patients treated with SGLT2 inhibitors has been suggested (Nicolle *et al.*, 2012). A murine model of candida UTI demonstrated dapagliflozin-induced glucosuria significantly increased candida CFU in the kidney as well as UTI (Suzuki *et al.*, 2014). This clinical data indicates treatment with the SGLT2 inhibitors could be a risk factor for candiduria in diabetic patients.

Clinical Presentation and Diagnosis

Candiduria can be categorized into an asymptomatic and symptomatic infection. The majority of patients with candiduria have an asymptomatic infection (Kauffman *et al.*, 2000), a benign condition that reflects colonization without disease. In one large prospective multicenter study, only 4% of patients with candiduria have clinical UTIs (Kauffman *et al.*, 2000). Candida UTIs can involve any part of the genitourinary tract and thus present as cystitis, epididymo-orchitis, prostatitis, pyelonephritis or renal parenchymal abscess. In some cases, the latter can also present in an orthotopic transplant kidney. Diagnosis of candida UTIs can be challenging as standardized methods to differentiate colonization from true infection due to Candida are lacking (Kauffman, 2005; Lundstrom and Sobel, 2001). The first step in evaluating candiduria is to rule out contamination and colonization. Contamination can be excluded by obtaining a new urine sample either by mid-stream urine or a catheterized urine sample to

confirm candiduria (Kauffman *et al.*, 2011). In the case of a true UTI, it is important to determine what part of the urinary tract is affected (upper or lower).

Symptomatic candiduria can result in cystitis and/or pyelonephritis similar to bacterial UTIs. Their symptoms can be indistinguishable. The majority of patients with candiduria do not have associated candidemia. Candiduric patients with cystitis may present with dysuria, urinary urgency, frequency or suprapubic tenderness and occasional fever. In pyelonephritis, the primary clinical manifestation includes fever, chills, and flank or costovertebral angle tenderness. However, patients with indwelling urinary catheters may not have urinary symptoms and only complain of suprapubic or flank pain (Tambyah and Maki, 2000). Candiduria in the presence of urinary tract obstruction can result in candidemia (Ang *et al.*, 1993; Gross *et al.*, 1998). In addition, candiduria in critically ill patients can be a potential indicator for invasive candidiasis or candidemia (Kauffman *et al.*, 2011). Therefore, blood cultures and skin and fundoscopic examination may be warranted in these patients especially if there is a concern that candidemia was present for a prolonged time.

Although a rare presentation, Candida prostatitis is usually secondary to systemic fungal infection and can result in candiduria (Haas *et al.*, 1998; Wise and Shteynshlyuger, 2006). Symptoms and clinical findings are similar to bacterial prostatitis. Patients can present with fever, irritating voiding symptoms or possibly urinary retention, lower abdominal or perineal pain, pressure at the symphysis pubis. Bladder outlet obstruction such as benign prostatic hypertrophy can lead to formation of a prostatic abscess (Lentino *et al.*, 1984). The digital rectal examination usually reveals focal tenderness and fluctuance of prostate glands (Collado *et al.*, 1999). Candida epididymo-orchitis can also lead to candiduria. These patients present with testicular tenderness and enlargement or scrotal masses (Kauffman *et al.*, 2011).

Currently, there are no reliable diagnostic tests that can accurately distinguish candiduria due to colonization from infection. Although urinalysis of patients with candida UTIs can exhibit pyuria and yeasts, these constitute non-specific findings as they can be found also in patients with asymptomatic candiduria or indwelling urinary catheters. Data from rabbit models, suggests that detection of Candida casts may be a diagnostic marker for candida pyelonephritis but it is not a very sensitive finding (Navarro *et al.*, 1994; Argyle *et al.*, 1984). In contrast to bacterial UTIs, quantification of yeasts in urine has limited value for the diagnosis of candida UTIs (Kauffman, 2005; Kauffman *et al.*, 2011; Sobel, 2002). Higher colony counts of yeasts do not always indicate candida UTIs and CFUs as low as 10,000 CFU/mL of Candida can be found in patients with UTI (Kauffman, 2005).

Imaging studies can be useful modalities for confirming candida UTIs as well as evaluating the anatomical level of infection and complications such as urinary obstruction, perinephric abscess or fungal balls. Ultrasonography is the preferred initial study especially in patients with impaired renal function as it does not require intravenous contrast (Kauffman *et al.*, 2011). It can demonstrate hypoechoic kidney lesions as well as hydronephrosis. A presence of gas in kidneys suggests emphysematous pyelonephritis [58], which requires immediate evaluation by surgery as these complicated infections are associated with high mortality. Ultrasonography is also a diagnostic study of choice for epididymo-orchitis. Computed tomography scan is superior to ultrasonography in delineating the extent of pyelonephritis and perinephric abscess (Kauffman *et al.*, 2011). Transrectal ultrasound is a reliable imaging study to confirm prostatic abscess (Hildebrand *et al.*, 1999). It usually shows ill-defined hypoechoic lesions within an enlarged prostate gland.

Recent studies indicate an elevation of inflammatory cytokine, IL-17, in the urine of candiduric patients compared to non-candiduric patients (Ahmadikia *et al.*, 2018; Helbig *et al.*, 2013). The immunological biomarker may be a useful tool in differentiating colonization from Candida UTIs requiring treatment. A large prospective study will be necessary to further elucidate the role of biomarkers.

Treatment

A presence of candida in urine requires careful clinical evaluation to determine if antifungal therapy is needed. Even though candiduria is confirmed, antifungal therapy may not be warranted. Asymptomatic candiduria generally represent colonization and rarely lead to invasive disease (Kauffman *et al.*, 2000). It can resolve spontaneously or with the removal of indwelling urinary catheters alone. Hence candiduria is commonly overtreated with antifungal therapy (Jacobs *et al.*, 2018). Unnecessary exposure to antifungals can increase the risk of emergence of antifungal resistance in colonizing Candida species. The largest randomized controlled trial of treatment of asymptomatic candiduria demonstrated that 47% of candiduria was eradicated by the sole removal of indwelling catheters (Sobel *et al.*, 2000; Pappas *et al.*, 2016). Most importantly, although candiduria is associated with increased mortality, its treatment did not improve morbidity and mortality outcomes (Simpson *et al.*, 2004; Safdar *et al.*, 2005; Jain *et al.*, 2007). Therefore, antifungal therapy is not recommended in asymptomatic patients except for those with high-risk of dissemination such as neutropenic patients and newborns with very low birth weight. In addition, patients undergoing urologic procedures are at increased risk of candidemia during instrumentation and antifungal treatment before and after the procedure is required (Pappas *et al.*, 2016). Once a decision is made to treat candiduria, unnecessary antibiotics should be discontinued as well as eliminating predisposing factors when feasible (Pappas *et al.*, 2016; Fisher *et al.*, 2011b).

Fluconazole is considered the drug of choice for candida UTIs for most Candida species except for *C. glabrata* and *C. krusei* because of concern for preexisting or inducible resistance (Brammer *et al.*, 1990). Oral Fluconazole has > 90% bioavailability and 80% is excreted in urine as an active drug (Fisher *et al.*, 2011b). A dose of 200–400 mg daily for 2 weeks is recommended (Brammer *et al.*, 1990; Fisher *et al.*, 2011b). Other azoles including itraconazole, voriconazole as well as posaconazole are

minimally excreted as active drugs into the urine (Pappas *et al.*, 2016; Johnson and Kauffman, 2003; Herbrecht, 2004). Therefore, their usefulness for fungal UTI treatment is very limited.

The echinocandins (caspofungin, micafungin, and anidulafungin) are also poorly excreted in urine and hence are of little value for the treatment of candida cystitis (Boucher *et al.*, 2004). Although very limited clinical data, series of case reports have shown both success and failure of caspofungin and micafungin for treatment of candiduria (Sobel *et al.*, 2007; Kane and Muzevich, 2016; Malani, 2010).

Flucytosine, at 25 mg/kg orally four times daily, is another alternative drug for candida UTIs (Brammer *et al.*, 1990; Pappas *et al.*, 2016). It requires a dose adjustment in patients with renal insufficiency. When used alone for an extended period, treatment-associated resistance emerges quickly. Furthermore, high cost, bone marrow suppression, hepatoxicity, and severe gastrointestinal side effects due to flucytosine have limited its utility (Pappas *et al.*, 2016).

Although associated with significant renal toxicity, amphotericin B (AmB) (Tambyah and Maki) deoxycholate, 0.5–0.7 mg/kg daily, is efficacious and can be useful in selected patients such as those who are critically ill. It is recommended for fluconazole-resistant *C. glabrata* and *C. krusei* (Pappas *et al.*, 2016). A single intravenous AmB dose is found to be effective for Candida UTIs due to prolonged urinary excretion (Wise *et al.*, 1982). However, the lipid formulations of AmB do not achieve adequate levels in the kidneys or urine (Pappas *et al.*, 2016). Bladder irrigation with a suspension of AmB has been used but its therapeutic value is not clear (Drew *et al.*, 2005). High relapse rates have been reported (Fisher *et al.*, 2003).

For upper UTIs, imaging studies to exclude urinary obstruction, papillary necrosis or persistent fungal ball is recommended (Pappas *et al.*, 2016). Candida UTIs refractory to medical treatment as well as complicated emphysematous UTIs and perinephric abscess often need surgical drainage and/or nephrectomy (High and Quagliarello, 1992; Kamaliah *et al.*, 2005; Ronald and Ludwig, 2001).

Due to the rarity of candida prostatitis and epididymo-orchitis, their treatment recommendation is based on anecdotal reports and expert opinions. Fluconazole is the agent of choice for fluconazole-susceptible organisms. AmB is another antifungal agent commonly used. Combination with surgical drainage or debridement is often required, especially for prostate abscess (Wise and Shteynshlyuger, 2006; Bartkowski and Lanesky, 1988).

Other Fungal Infection of Genitourinary Tract

Genitourinary tract infections caused by other fungal organisms are infrequent. Most cases have been reported in severely immunocompromised hosts. *Trichosporon asahii* is an emerging fungus causing systemic infections as well as localized UTIs in hospitalized patients. It is frequently associated with indwelling medical devices including urinary catheters (Urs *et al.*, 2018; Walsh *et al.*, 2004). *Blastoschizomyces capitatus*, another non-Candida yeast, has been reported as a cause of nosocomial UTIs (Krcmery *et al.*, 1999).

Cryptococcuria has been documented in patients with AIDS as well as in patients with other causes of immunocompromised state. It can be an early marker of disseminated disease such as meningitis and indicates a poor prognosis (Butler *et al.*, 1964; Severo *et al.*, 2011). In males, the prostate gland can serve as a reservoir of *Cryptococcus neoformans* and become a source of cryptococcuria and relapsed infection (Larsen *et al.*, 1989). Cryptococcal prostatitis is usually asymptomatic. Cryptococcal pyelonephritis is another source of cryptococcuria especially in renal transplant patients (Hellman *et al.*, 1981).

Genitourinary tract infection is a rare manifestation of disseminated Coccidiodomycosis likely from hematogenous spread. The sites of infection involve kidneys, prostate glands, epididymis, and testes (Halsey *et al.*, 2005; Sohail *et al.*, 2005; Yurkanin *et al.*, 2006). Fifteen to 30% of patients with systemic blastomycosis have genitourinary tract involvement. It frequently affects the prostate gland, epididymis and testes (Eickenberg *et al.*, 1975; Inoshita *et al.*, 1983). Most patients with prostatic involvement eventually have cutaneous lesions of blastomycosis (Inoshita *et al.*, 1983).

Aspergillus infection of the urinary tract can manifest as a perinephric or parenchymal abscess or aspergilloma in the renal pelvis (Bibler and Gianis, 1987; Lisson *et al.*, 2002). The latter can cause ureteropelvic obstruction.

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Oropharyngeal and Vulvovaginal Candidiasis

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Introduction

Although not always severe, oral and vulvovaginal *Candida* spp. infections represent some of the most common human infections. Since the fungus was first described by Bernard von Langenbeck in 1839, our understanding of *Candida* spp. as both a human commensal organism and a pathogen has continued to expand (Gow and Yadav, 2017; Lionakis and Edwards, 2019). Since the 1970s, the incidence of *Candida* infections has particularly accelerated as a result of widespread antibiotic use (Lionakis and Edwards, 2019). In addition, advances in antiretroviral therapy, malignancy treatment, and solid organ transplant has led to an increasing population of immunocompromised patients, many of whom are at increased risk for oral candidiasis, the most common opportunistic yeast infection (Martins *et al.*, 2014).

Candida albicans, the most common cause of oral and vulvovaginal candidiasis, accounts for 50%–90% of human candidiasis (Martins *et al.*, 2014). The names *Candida* and *albicans* are both derived from Latin words for white, referring to the typical white plaques of pseudomembranous candidiasis (Gow and Yadav, 2017). The yeast is a small pathogen, at 4–6 μm in size, and ovoid in shape, with thin walls (Lionakis and Edwards, 2019; Stoopler and Sollecito, 2014). *Candida* is a commensal organism present on the skin and in the gastrointestinal and vaginal tracts of healthy people (Lionakis and Edwards, 2019; Stoopler and Sollecito, 2014). *Candida* spp. can exist in yeast and hyphal forms, and reproduce by budding (Lionakis and Edwards, 2019; Stoopler and Sollecito, 2014).

Candidiasis is often diagnosed clinically, but can also be identified by the visualization of hyphae and pseudohyphae with 10% potassium hydroxide (KOH) smears or culture on Sabouraud dextrose agar (Lionakis and Edwards, 2019). Treatment of oral and vaginal candidiasis is often accomplished through topical polyenes or azoles, or with short courses of systemic azole therapy (Pappas *et al.*, 2016; Lionakis and Edwards, 2019). However, the rising incidence of non-*albicans Candida* species in human infection has been associated with increasing antifungal resistance for mucosal candidiasis (Sobel, 2016; Lionakis and Edwards, 2019).

Epidemiology

As a normal commensal organism of humans, *Candida* is frequently found in hospital and home environments; it has also been found in soil, animals, and food products (Lionakis and Edwards, 2019). Most infections occur endogenously, though human to human transmission is possible, as in cases of maternal-fetal transmission or from female genital tract to male balanitis (Lionakis and Edwards, 2019).

It is estimated that at least 75% of women will have an episode of Candida vaginitis during their lifetime, and 50% will have at least one recurrence (Lionakis and Edwards, 2019). Vulvovaginal candidiasis is especially common among pregnant and diabetic women, and in women undergoing antibiotic treatment (Martins *et al.*, 2014). Some women develop recurrent vulvovaginal candidiasis (RVVC), a syndrome that has only recently been widely recognized. RVVC is defined as three to four symptomatic episodes of vulvovaginal candidiasis per year, with asymptomatic periods in between (Sobel, 2016). RVVC is most common among women between ages 25–34, and represents a significant economic burden and negative impact on quality of life (Denning *et al.*, 2018). Rates of RVVC are estimated between 5%–9% of the female population (Sobel, 2016; Martins *et al.*, 2014; Lionakis and Edwards, 2019; Matheson and Mazza, 2017).

Oral and esophageal candidiasis predominantly affect neonates and adults with immunocompromising conditions such as HIV or malignancy, or individuals on steroid therapy (Pappas *et al.*, 2016). However, *Candida* is present in the normal oral flora of 45%–65% of healthy infants and 30%–55% of healthy adults (Millsop and Fazel, 2016). Patients using steroid inhalers, dentures, or suffering from xerostomia are at particular risk for oral candidiasis (Pappas *et al.*, 2016; Stoopler and Sollecito, 2014; Vazquez and Sobel, 2002).

Chronic mucocutaneous candidiasis is a rare condition that may be related to primary genetic mutations such as CARD9 deficiency, or associated with broader syndromes such as autoimmune polyendocrinopathy syndrome type 1 (also known as APECED) or thymoma-associated chronic candidiasis (Pappas *et al.*, 2016; Kirkpatrick and Windhorst, 1979; Glocker *et al.*, 2009). IL-17 deficiency, which impairs the host's innate immune response to *Candida*, may be the result of a primary genetic mutation or acquired from targeted destruction by immunotherapy (Netea *et al.*, 2015).

Pathogenesis

Our understanding of the process by which *Candida* changes from commensal, normal flora to problematic, opportunistic pathogen is still evolving. A failure of some aspect of the host's natural defense (such as incomplete immune response or break in

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the epithelial barrier) is likely necessary to allow virulence factors of the fungus (including adhesion to epithelia, secretion of hydrolytic enzymes, and phenotypic switching) to prevail (Jayatilake, 2011).

A normal host mucosal immune response to candidiasis involves Th17 helper T cell activation, leading to production of cytokines (including IL-17 and IFNγ) that stimulate neutrophils and macrophages to kill the fungus (Netea *et al.*, 2015). Mucosal epithelial cells and platelets are also part of this complex immune response that involves adaptive, innate, and local host immune defenses (Netea *et al.*, 2015).

Interestingly, oral candidiasis is typically associated with immunocompromising conditions (such as HIV or malignancy) which do not predispose individuals to vulvovaginal candidiasis (which often occurs in healthy women) (Denning *et al.*, 2018). Additionally, some genetic mutations associated with mucosal candidiasis are not associated with invasive candidiasis, emphasizing the role of local immune response in the pathogenesis of these infections (Netea *et al.*, 2015).

Clinical Manifestations

Oral Candidiasis

Oral candidiasis most commonly presents with white lesions covering the dorsal tongue and buccal mucosa, as well as occasionally the soft or hard palates (Millsop and Fazel, 2016; Lionakis and Edwards, 2019). This classic presentation, called pseudomembranous candidiasis, manifests with confluent plaque-like lesions that can be easily scraped off to reveal irritated mucosa (Millsop and Fazel, 2016). Patients are sometimes without symptoms, or may complain of dysgeusia or burning sensation in the mouth. It is important to recognize atypical presentations of oral candidiasis as well, including "denture stomatitis", which commonly presents with edematous erythematous gums, and "hyperplastic candidiasis", which must be distinguished from leukoplakia on the buccal mucosa (Millsop and Fazel, 2016; Stoopler and Sollecito, 2014). *C. albicans* may also be implicated in angular cheilitis, presenting with painful fissures at the corners of the mouth, and central papillary atrophy of the tongue (Millsop and Fazel, 2016; Stoopler and Sollecito, 2014).

Esophageal Candidiasis

When *Candida* infects the epithelial lining of the esophagus, patients often complain of odynophagia or dysphagia. *Candida* esophagitis often occurs in conjunction with oral candidiasis, but esophagitis can occur without oral lesions (Lionakis and Edwards, 2019). Other less common symptoms of esophageal candidiasis may include chest pain, obstructive symptoms, nausea, epigastric pain, and vomiting (Telles *et al.*, 2017; Lionakis and Edwards, 2019; Vazquez and Sobel, 2002). If left untreated, as the yeast pseudomembrane extends and becomes invasive over time, the esophagus can become denervated, and patients may have less pain (Lionakis and Edwards, 2019). Esophageal candidiasis may appear similar to or occur simultaneously with viral (herpes simplex or cytomegalovirus) esophagitis, especially when there are both ulcers and pseudomembranes present (Lionakis and Edwards, 2019).

Vulvovaginal Candidiasis

Vulvovaginal candidiasis classically presents with symptoms of intense vaginal itching or discomfort associated with a thick, clumpy discharge and vulvar edema (Lionakis and Edwards, 2019; Sobel, 2007). Dyspareunia and dysuria may also be reported (Gonçalves *et al.*, 2016; Sobel, 2007). Differential diagnoses include bacterial vaginosis, trichomoniasis, and gonorrhea (Gonçalves *et al.*, 2016). As previously noted, recurrent vulvovaginal candidiasis involves several episodes of these symptoms associated with mycologic diagnosis within one year (Sobel, 2016).

Syndromes Involving Candidiasis

Chronic mucocutaneous candidiasis (CMC) is a disease characterized by recurrent oral and skin candidiasis that is commonly associated with immune dysfunction (Millsop and Fazel, 2016). CMC may occur independently in relation to an isolated genetic mutation, or may be part of a larger syndrome such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which may present in conjunction with hypoparathyroidism or adrenal insufficiency (Ferre *et al.*, 2016; Glocker *et al.*, 2009; Netea *et al.*, 2015).

Diagnosis

The gold standard for diagnosing all mucosal *Candida* infections involves microscopic identification of yeast forms from the site of suspected infection (Vazquez and Sobel, 2002; Telles *et al.*, 2017). Because *Candida albicans* is part of the normal flora of the gastrointestinal and female genital tract, it is imperative that symptoms or signs suggestive of infection be present in order to make the diagnosis.

Practically, candidiasis is often diagnosed presumptively in patients with risk factors for infection, classic symptoms, and typical signs on exam. Diagnosis is confirmed when these individuals respond quickly to antifungal therapy. However, in patients with atypical, recurrent, or refractory presentation, a suspected lesion can be swabbed or biopsied. Hyphae of *Candida* can be then be visualized under the microscope with samples that have undergone Gram staining, 10% KOH preparation, or periodic acid-Schiff staining (Telles *et al.*, 2017). Fungal culture on Sabouraud agar may be performed to determine antifungal susceptibilities in cases that recur or persist despite usual treatment. However, fungal culture for mucosal candidiasis is not routinely necessary and cannot distinguish between colonization and infection (Vazquez and Sobel, 2002).

For vaginal candidiasis, the vaginal pH is almost always normal (Sobel, 2016). Simple saline wet mount preparation of vaginal secretions under the microscope are about 40%–50% sensitive for the diagnosis of vulvovaginal candidiasis (Vazquez and Sobel, 2002); KOH microscopy examinations from vaginal samples are about 40%–70% sensitive (Sobel, 2016). Alternative diagnoses should be considered and fungal cultures obtained if the microscopic examination is negative (Sobel, 2016).

In esophageal candidiasis, endoscopic visualization with biopsy should be pursued if a patient presenting with classic symptoms does not respond to standard therapy (Lionakis and Edwards, 2019). Radiographic findings, such as filling defects or irregularities on barium contrast studies, may be suggestive of *Candida* esophageal infection, but are nonspecific (Vazquez and Sobel, 2002).

Diagnosis of chronic mucocutaneous candidiasis, endocrinopathies, or other primary immune deficiencies manifesting with recurrent oral, esophageal, or vaginal candidiasis requires a high degree of clinical suspicion and specific tests for genetic mutations (such as Th-17 deficiency) and immune function (Telles *et al.*, 2017; Ferre *et al.*, 2016).

Treatment

Because *Candida albicans* causes the majority of oral, esophageal, and vulvovaginal candidiasis, the treatment for different forms of mucosal candidiasis are often similar. Fortunately, *Candida albicans* tends to be fairly susceptible to many of the available antifungal agents, with only rare instances of antifungal resistance in heavily-antifungal-exposed patients.

Treatment of oral candidiasis consists of containing an overgrowth of yeast as well as removing the underlying risk factor, if possible. For mild oral candidiasis, topical antifungals such as clotrimazole troches or nystatin suspension for seven days is often adequate (Pappas *et al.*, 2016). Nystatin is a polyene antifungal medication available in many topical forms that inactivates yeast by binding an enzyme called ergosterol in the fungal cell membrane (Millsop and Fazel, 2016). For more severe oropharyngeal candidiasis, oral fluconazole tablets (200–400 mg daily) is recommended, and courses may be one to two weeks depending on clinical improvement (Pappas *et al.*, 2016). Azole medications disrupt fungal cell membranes by inhibiting ergosterol synthesis (Millsop and Fazel, 2016). For patients with oral candidiasis related to denture use (denture stomatitis), washing the dentures regularly in an antifungal solution may reduce recurrences (Pappas *et al.*, 2016). Individuals using steroid inhalers should be educated on rinsing out the mouth following inhaler use to reduce recurrence. Where possible, treatment of an underlying immune deficiency predisposing to oral candidiasis will also help resolve the infection and prevent recurrences, as in the case of starting antiretroviral therapy for HIV/AIDS (Millsop and Fazel, 2016; Pappas *et al.*, 2016). For patients with chronic recurrent oral candidiasis, prophylaxis with 150 mg fluconazole three times weekly has been suggested as a safe and effective regimen (Pappas *et al.*, 2016).

Topical agents are not effective for *Candida* esophagitis (Vazquez and Sobel, 2002). Rather, the first line treatment is oral fluconazole (at a higher dose such as 400 mg daily) for fourteen days (Pappas *et al.*, 2016). Intravenous fluconazole at the same dose may be used instead in patients without parenteral access (Vazquez and Sobel, 2002; Pappas *et al.*, 2016). Other oral triazole agents (posaconazole and itraconazole) are likely as effective as fluconazole in patients who have developed resistance to or intolerance of fluconazole (Vazquez and Sobel, 2002). Micafungin, an intravenous echinocandin antifungal, may be used in patients with esophageal candidiasis who cannot tolerate azoles, though the dose should be 150 mg daily (Pappas *et al.*, 2016). Intravenous liposomal amphotericin B has largely fallen out of favor for the treatment of mucosal candidiasis, but may be used for patients with azole-refractory esophagitis (Vazquez and Sobel, 2002). Treatment duration for any of these regimens may be extended to 21 days in patients with severe disease (Pappas *et al.*, 2016).

First line treatment of uncomplicated vulvovaginal candidiasis is topical antifungal agents (such as clotrimazole cream, miconazole suppository, or nystatin cream) or a single dose of 150 mg oral fluconazole tablet (Vazquez and Sobel, 2002; Pappas *et al.*, 2016). Topical agents are effective in about 80%–85% of uncomplicated cases (Gonçalves *et al.*, 2016). For severe acute vulvovaginal candidiasis, three doses of 150 mg fluconazole given every 72 h may be used (Pappas *et al.*, 2016). Although topical agents have fewer risk of systemic side effects, oral azole therapy is often preferred for convenience and ease of use (Vazquez and Sobel, 2002; Gonçalves *et al.*, 2016). Pregnant women should be treated with topical agents, as oral azoles are not recommended due to the risk of fetal toxicity (Pappas *et al.*, 2016). For recurrent vulvovaginal candidiasis, treatment begins with an induction course of one to two weeks of 150 mg oral fluconazole every 72 h followed by a maintenance treatment of fluconazole 150 mg weekly for six months (Pappas *et al.*, 2016; Sobel, 2016; Matheson and Mazza, 2017). For vulvovaginal candidiasis due to non-azole susceptible *Candida* such as *Candida glabrata*, vaginal boric acid or flucytosine may be attempted (Sobel, 2016).

It has been suggested that probiotics may have a beneficial effect in reducing oral candidiasis recurrence by lowering the quantity of *Candida* in the oral flora, but evidence for this remains limited (Mundula *et al.*, 2019). Similarly, probiotics have been suggested for recurrent vulvovaginal candidiasis on the basis of increasing the proportion of *Lactobacillus* present in the bacterial

microbiome, but there is not yet enough evidence to definitively recommend this (Xie *et al.*, 2017; Sobel, 2016). Novel antifungal therapies currently being studied for vulvovaginal candidiasis include TOL-463, a vaginal insert that targets the fungal biofilm, and VT-1161, an oral medication which inhibits the fungal lanosterol demethylase enzyme (Marrazzo *et al.*, 2019; Brand *et al.*, 2018).

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Fungal Infections of Human Mammary Gland During Lactation

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Breastfeeding

Breastfeeding is the optimal way of infant feeding. The World Health Organization recommends exclusive breastfeeding from birth to the age of six months. Then it recommends to continue breastfeeding, while expanding the baby's diet, at least until the age of two (World Health Organization, 2001). Numerous studies indicate the irreplaceable role of breastmilk in optimal growth and development of a child's body. The breastmilk is perfectly matched to the needs of a developing organism providing all the necessary nutrients. The milk composition varies with the age of the breastfed child (Andreas *et al.*, 2015). Breastmilk is considered a functional food due to the fact that in addition to nutrients, it also contains elements that support development and maturation. Particular importance is attributed to the influence of natural feeding on the proper development of the immune system (Silvers *et al.*, 2012). Bioactive components of human milk support the innate and acquired specific immunity of the body (Ballard, 2013). Feeding with human milk, due to its beneficial effect on immunity, reduces susceptibility to gastrointestinal and respiratory tract infections, urinary tract infections (Maldonado *et al.*, 2012) and in the case of illness it contributes to a milder disease course. Feeding children at early stage of development with breastmilk brings not only short-term benefits, such as: protection against infectious diarrhea, respiratory tract infections, otitis media, but it is also an investment for the future (Hoddinott *et al.*, 2008). A diet consisting of breastmilk contributes to reduced risk of civilization diseases development in later life, including type II diabetes mellitus, obesity, hypertension or hypercholesterolemia (Dieterich *et al.*, 2013; McCrory and Layte, 2012). Breastmilk is an extremely important source of biologically valuable ingredients, including probiotic microflora (Fernández *et al.*, 2013; Donnet-Hughes *et al.*, 2010). Human breastmilk is an abundant source of microorganisms belonging to the probiotic microflora (Latuga *et al.*, 2014). Colonization by microorganisms occurs during the child passage through the mother's birth canal, during skin to skin contact and with breastmilk consumption. Breastmilk is one of the main factors shaping the microbiological balance of the newborn's digestive tract (Martín *et al.*, 2003). Benefits of microbial homeostasis are both short and long-term. Composition of the microflora stabilizes between 2 and 7 years of age. Adequate supply of probiotic microorganisms supports the correct microbiological profile formation and ensures maximum benefits from the gastrointestinal tract microbiological homeostasis. Probiotic microorganisms affect the maturation and immune system development (Toscano *et al.*, 2017). Breastmilk contains not only probiotic microorganisms that affect the composition of the intestinal microflora, but also factors supporting its growth, such as oligosaccharides, considered to be prebiotics (Marcobal *et al.*, 2011). Breastfeeding has also a beneficial effect on the mother's health. Scientific research confirms that breastfeeding contributes to reduce the incidence of women with such diseases as breast cancer, hypertension, diabetes, hyperlipidaemia, and cardiovascular disease (Newcomb *et al.*, 1994; Dieterich *et al.*, 2013). Although breastfeeding is a natural activity for the human species, it often causes many difficulties, especially at the initial stage of lactation. During this period, the support of a young mother is particularly important (Govoni *et al.*, 2019). Both psychological and physiological factors can affect the breastfeeding failure. The discomfort experienced during breastfeeding can be caused by the anatomical conditions or infections (Amir, 2003). Pain felt during breastfeeding is a common cause of premature weaning (Schwartz *et al.*, 2002). One of the causes of breast inflammation is fungal infections. There are no definitive data on the incidence of fungal infections among breastfeeding women, however, the problem is significant due to the susceptibility of this population group to infections and the consequences it causes (including premature weaning and termination of lactation). Breastfeeding women are a group susceptible to bacterial and fungal infections due to the resulting environment, conducive to the growth and development of pathogenic microorganisms. Lack of medical intervention and the spread of the disease may be the reason for worsening of the disease state, feeling of discomfort and, as a consequence, premature resignation from breastfeeding, which is unfavorable from the point of view of both the child and the mother (Kent *et al.*, 2015). Early diagnosis helps to control the problem and return to the optimal way of feeding child in comfort.

Structure of the Mammary Gland

The mammary glands are made of glandular tissue – the breast body (corpus mammae) and adipose tissue – the fat body (corpus adiposum mammae). In addition, the structure of the gland also forms a network of blood and lymphatic vessels, as well as nerves (Jesinger, 2014). On the skin, in the middle or slightly below the center of the gland, there is a dark colored areola of the nipple (areola mammae) with centrally located nipple (papilla mammae). The papilla is equipped with muscle fibers, which at the base pass to the areola and radiate there. At the top of the nipple, there are the outlets of the milk ducts (ductus lactiferi), which form a branched network in the gland. Their role is to remove the produced milk. The papilla (areola mammae) is equipped with radially arranged smooth muscle fibers. On its surface, there are skin glands: sebaceous, sweat and Montgomery's apocrine glands (Montgomery glans). The breast glandular tissue is made of lobes (lobi glandulae mammariae) and smaller structures – lobules (lobuli glandulae mammariae). Their number varies individually. Ultrasound studies allowed for verification of previous knowledge about the internal structure of the mammary gland. Previously, it was thought that glandular tissue in the breast forms

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from 15 to even 25 lobes. There are currently around 9 (Ramsay *et al.*, 2004; Kent *et al.*, 2005; Ramsay *et al.*, 2005). There is often an additional lobe of the mammary gland, located partly in the armpit, called Spence's tail. Alveoli (the smallest structures) are surrounded by myoepithelial cells, smooth muscle cells, the work of which depends on hormonal changes (Jesinger, 2014). The size of a woman's breast is mainly determined by the amount of fat, which does not affect the breast productivity. Fatty tissue in the breast, in the largest amount, is located under the gland and around it at the base. It is also found in smaller amounts between breast structures (Geddes, 2007). The gland is surrounded by a capsule of fibrous connective tissue that penetrates the gland's parenchyma forming its stroma in the form of Cooper ligaments (Cardoso *et al.*, 2015).

Pathogenesis of Fungal Breast Infections

Nipple and breast mycosis is an infection caused by fungal microorganisms *Candida albicans*. *Candida* spp. are commensal organisms. Their presence does not always mean infection (Amir *et al.*, 2013). *Candida albicans* is a physiological microflora of the gastrointestinal tract, genitourinary system, respiratory system and skin. In healthy people, it does not lead to infection. Under certain conditions, for example reduced immunity, disturbance of the natural balance in the species composition of the human microflora, excessive proliferation occurs, which results in the appearance of the disease state. In the non-lactation period, female breasts do not show high susceptibility to fungal infections (Tanguay *et al.*, 1994). *Candida albicans* prefers a moist environment. Dry, hairless and intact breast skin does not create favorable conditions for excessive fungi multiplication. The situation changes during lactation. Breastfeeding increases the likelihood of excessive moisture and skin damage, which are the gate to infection (Tanguay *et al.*, 1994). Increasing the humidity of the skin of the mammary gland may be favored by the incorrect use of lactation pads. Breastfeeding women often use them, especially in the first period of lactation. Their proper use consists in frequent replacement of inserts for new ones, which allows the breast environment to be kept clean. Replacing the pads too rarely results in an increase in the humidity around the nipple and promotes the microorganism's growth (Amir, 1991). A factor that significantly increases the risk of developing a fungal infection is the use of antibiotic therapy (Tanguay *et al.*, 1994). Antibiotics lead to the elimination of pathogenic microorganisms but at the same time adversely affects the general condition of human microflora, also destroying beneficial probiotic bacteria. The situation worsens if during and after antibiotic therapy no cover is used in the form of supply of probiotic bacteria. Disorders of the natural balance of microorganisms in the human body result in a number of adverse phenomena, including a decrease in immunity and thus an increase in sensitivity to opportunistic and pathogenic microorganisms. Under conditions of disturbed homeostasis, the commensal microorganisms can multiply and lead to disease symptoms (Sullivan *et al.*, 2001). Factors that increase the likelihood of a fungal infection are also all the other elements that reduce the immunity of the human body. A woman, who has recently given birth to a child and is struggling with many emerging problems, is exposed to a large dose of stress, which is destructive to the body's defenses. Chronic sleep deprivation, anxiety, and an unbalanced diet can significantly contribute to the failure of the immune system. Because the nursing mother and breast-feeding baby stay in close contact with each other, any infection can easily spread between them (Johnstone and Marciniak, 1990). Studies confirm the correlation between the incidence of maternal fungal inflammation and the incidence of oral fungal infections in a child. Thrush in a child, caused by the yeast *Candida albicans*, can become a cause of fungal infection of the mother's breast. On the other hand the mother can becomes the source of the child infection. By taking food from the breast, the child is exposed to the transmission of microorganisms along with milk or directly from the surface of the nipple. Thrush is manifested by white coatings occurring on the oral mucosa. The observed changes cause pain and thus impede sucking. Cutaneous candidiasis in the diaper area often coexists or is a consequence of a child's oral thrush (Harris, 1958). A serious problem during lactation are mechanical injuries to the nipple caused, among others, by incorrect attachment of the baby to the breast (Tanguay *et al.*, 1994). They arise most often at the beginning of breastfeeding. They can be the gate to infection for microorganisms. In addition, the strong discomfort they cause may discourage women and become a reason for giving up the breastfeeding. A factor that promotes fungal infections of the breast may also be inflammation of the breast caused by factors other than fungal microorganisms. A positive correlation between the occurrence of vaginal fungal infections and fungal breast infections is also observed (Hanna and Cruz, 2011). In case of difficulties with breastfeeding or need to have more time spent separately from a child, a lot of mothers are opting for an alternative form of feeding with breast milk using breast pump. Mothers express breast milk for a variety of reasons (Labiner-Wolfe *et al.*, 2008). The benefits of breastfeeding for both child and mother are still preserved, but this way of feeding presents many difficulties. It is a time-consuming process, which is particularly troublesome at the initial stage of lactation and the child's life, when feeding sessions are long and feeding intervals are relatively short. In this case, the woman must schedule regular breast milk removal between feedings. Unfortunately, it has been observed that breast pumps can become a serious source of microbial contamination, including fungi such as *Candida albicans* (Jiménez *et al.*, 2017). If it is necessary to use this type of equipment, it is important to maintain the highest hygiene standards. It is of key importance to maintain high hygiene in the process of expressing milk from the breast and keep the breast pump clean. It has been proved that symptoms appearing after a fungal infection of the mammary gland can also appear as a result of infections caused by other microorganisms such as staphylococci, streptococci (Amir *et al.*, 2011; Kvist *et al.*, 2008) and corynebacteria. It happens that such cases are mistakenly diagnosed as candidiasis of the mammary gland (Delgado *et al.*, 2008; Mediano *et al.*, 2017; Hale *et al.*, 2009; Thomassen *et al.*, 1998). Treatment of incorrectly diagnosed women reporting symptoms of breast pain leads to unnecessary pharmacological burden on the body. In addition, desired effects of treatment in the form of improvement in the patient's condition are not observed and the risk of infection becoming chronic.

Candida albicans

Candida albicans – a microorganism that is part of the human saprophytic microflora. Under normal conditions, it lives in the digestive tract, on the skin or in the external organs of the genitourinary system (Bernhard and Knoke, 1997). Under favorable conditions for *Candida* spp. and in the situation of reduced immunity of the human body, uncontrolled multiplication of these microorganisms may occur, resulting in an imbalance between the host organism and the fungus and the appearance of the disease process (Hube, 2004). It can cause surface fungal infections of the skin but also life-threatening systemic fungal infections. The virulence factors are complex and are being learned all the time. Among them, there are molecules that mediate adhesion to and invasion into host cells, the secretion of hydrolases, the yeast-to-hypha transition, contact sensing and thigmotropism, biofilm formation, phenotypic switching (Mayer *et al.*, 2013; Calderone and Fonzi, 2001). *Candida albicans* is a polymorphic fungus that can appear as single cells or form hyphae that are more (Liu, 2002). This dimorphism depends on external factors such as environmental pH, presence of CO_2, serum, N-acetylglucosamine or availability of nutrients. *Candida albicans* can enter the host cells in two ways. One of them is induced endocytosis. Invasive binding ligands of the host (Als3, Ssa1) are of major importance in this process. The second way is active penetration, the molecular mechanisms of which are not yet fully understood. The hydrolases secreted by *Candida albicans* (proteases, phospholipases and lipases) participate in this process. *Candida albicans* has the ability to adapt to a wide environmental pH. In addition, it also has the ability to affect the pH value of the environment by its alkalization. This process promotes the formation of hyphae. The source of nutrients for *Candida albicans* can be glucose, proteins, amino acids or lipids. The use of specific ingredients depends on the environment in which it is located. The ability to adapt the fungus in terms of metabolism is extremely important. Changing environmental conditions determine the cycle of metabolic changes. *Candida albicans* is able to absorb nutrients in the form of glucose and metabolism by glycolysis. In the event of entry into the phagocytic cells of the host's immune system, the metabolic pathway is switched to gluconeogenesis and lipids and amino acids become the source of nutrients. A feature that helps colonize both abiotic and biotic surfaces is the ability to create a biofilm (Nobile *et al.*, 2009; Xie *et al.*, 2012). Biofilm is a structure formed by microorganisms that increases their resistance to external factors (Taff *et al.*, 2012; Robbins *et al.*, 2011). The extracellular matrix produced by microorganisms has a protective effect (Finkel and Mitchell, 2011; Uppuluri *et al.*, 2010). In the case of fungal infections of the breast during lactation, it is important to remember about high hygiene of the breast pump components. On individual parts of this device, fungi can form coatings in the form of a biofilm being a source of pathogenic microorganisms release. *Candida albicans* is a cause of vulvovaginitis that affects most women during the life. Factors conducive to the occurrence of this disease are a state of reduced immunity, concomitant diabetes, use of antibiotics, oral contraception, pregnancy and hormone therapy (Mayer *et al.*, 2013). *Candida albicans* is widely believed to be the cause of pain problems among breastfeeding women. However, results of the tests carried out up to date are not conclusive. Some researchers recognize the dominant role of *Candida albicans* in the pathogenesis of breast pain in breastfeeding women. Others are of the opinion that the problem is much more complex and indicate a number of factors underlying the ailments discussed (Kaski and Kvist, 2018). *Candida albicans* cannot always be cultured from samples taken from women reporting symptoms, on the other hand *Candida* spp. cannot be excluded.

Symptoms of Fungal Breast Infections

Fungal infection can lead to pain that gets worse during breastfeeding. Felt pain is a stinging pain that can radiate. It covers one or both breasts. It often gets worse after feeding a baby. Pain in fungal infections is a prolonged pain of varying severity. The use of lactation pads, breastfeeding or warm compresses does not bring a relief (Amir *et al.*, 2013). Itching and redness of the nipple may appear, as well as damage to the skin surface in the form of cracks or flaking (Heinig *et al.*, 1999). In addition, increased breast tenderness is observed, which hinders normal functioning. Discomfort is felt with the slightest nipple irritation. Fungal infection may be complicated by a bacterial infection. In these cases, the symptoms overlap. As a result of the disease process, milk production in the mammary gland may decrease. The cause of this condition is the infectious process itself but also the limitation of the baby's attachment to the breast caused by the mother's breast pain during breastfeeding. The mother's body perceives the reduced frequency of attaching a baby as a signal for reduced food demand, which results in reduced milk production (Kent, 2007). Symptoms associated with fungal infection are nonspecific and are difficult to correct diagnose. The causes of nipple pain during lactation are very diverse. Differential diagnosis is very important so that the applied treatment is effective. Pain that occurs exactly during breastfeeding may indicate mechanical injury to the nipple. When applying warm compress brings relief, the cause of breast pain may be vasoconstriction (Amir *et al.*, 2013). Fungal infection of the mother's mammary gland can easily spread to the baby's body and occur in the form of oral thrush. In this case, the child experiences severe discomfort when taking food. White round deposits appear on the mucous membrane of the mouth. These layers are easy to separate from the mucosa surface. Scrapped reveal red, bleeding spots. A thick white coating on the surface of the tongue is also observed. The presence of these symptoms may indicate the ongoing disease process in the mother and the need for treatment. It can also be a reason for the development of fungal infection in the mother's breast (Tanguay *et al.*, 1994).

Diagnosis of Breast Fungal Infections

Early recognition of disease symptoms enables quick and effective therapy. It allows to return to feeding child in comfort, which indirectly reduces the risk of preterm weaning. Both clinical history and visible symptoms are important in diagnostics. An external

evaluation of the appearance of the breast can help to recognize the ongoing disease process. This may be indicated by skin discoloration from red to purple, swelling or tightening of the breast skin (Francis-Morrill *et al.*, 2004). As a result of excessive tension on the skin of swollen breasts, radiating stretch marks may appear. A history of stabbing pain and increased nipple tendency dominate. If symptoms indicating a fungal infection occur in the mother or child, both the child and the mother should be diagnosed. Transmission of infectious agents between mother and child is very easy and treatment of only one from the tandem mother-child can lead to recurrence of pathological symptoms and lack of success in treatment. In the child's examination, white deposits can be observed on the surface of the oral mucosa and on the tongue. An additional symptom may be a red diaper rash (Seifi *et al.*, 2017). The pain felt by the child affected by thrush causes his reluctance to feed. During feeding, a characteristic flexing of the baby can be observed, which indicates the discomfort felt during sucking. Diagnosis of fungal infections is routinely based on the clinical picture and history. Many scientific studies indicate the ambiguous cause of pain reported by breastfeeding women (Kaski and Kvist, 2018). It is important to exclude technical difficulties during breastfeeding. Many problems in breastfeeding arise from the wrong technique of attaching the baby to the breast or abnormalities regarding the process of suckling. In addition, anatomical conditions of the child may affect the occurrence of pain during breastfeeding (Hogan *et al.*, 2005; Ghaheri *et al.*, 2017) and cause a misdiagnosis. Additional tests, such as milk microscopy and skin scraping do not give a precise answer regarding the sources of observed symptoms. This is due to the fact that *Candida albicans* constitute human commensal microflora. In addition, microbiological testing of milk not always proved the presence of *Candida albicans* in milk samples (Hale *et al.*, 2009). This makes these studies not in the widespread use, although they could limit the cases of unjustified use of antifungal drugs in the case of misdiagnosed breast candidiasis and precisely target the patient's treatment (Jiménez *et al.*, 2017). Pain that occurs at the beginning of lactation is often caused by physiological hypersensitivity of nipples. Sometimes, it occurs at the beginning of lactation. The pain occurs at the beginning of breastfeeding and after a while passes. In this case, the best solution is to wait out this period. It is not recommended to temper the nipples during pregnancy. Such behavior is sometimes propagated among women expecting a child, while the use of these techniques can lead to unnecessary injuries and is not medically justified. Raynaud's syndrome should be included in the differential diagnosis. Severe pain for breastfeeding women with Raynaud's syndrome is caused by the vasoconstriction of the nipple tip (Wu *et al.*, 2012). Often the trigger factor for vasoconstriction, and thus the occurrence of a pain reaction, is a sudden change in temperature. In the case of Raynaud's syndrome, the symptoms usually relate only to temporary changes in the color of the nipple and areola, as well as pain during breastfeeding. No skin changes in the form of injuries, dry or flaky skin surface are observed. Symptoms will also not appear in a breastfed child.

Treatment

Treatment of fungal infections of the mammary gland and thrush in a child using appropriate antifungal preparations is not complicated. Both mother and child should be treated because of the ease of transmitting the pathogen (Brent, 2001). Topical antifungal drugs are used in the therapy. Oral antifungal drugs may also be used (Riordan and Wambach, 2010). It should be remembered that oral medications may pass into breast milk. It is recommended to check active substances ability to pass into breastmilk. For example, therapy with fluconazole is possible (Bodley and Powers, 1997; Chetwynd *et al.*, 2002). The dose that a breastfed child takes is lower than that used in systemic treatment (Kaplan *et al.*, 2015). For this reason, the active substance fluconazole is considered to be a substance with very low risk for breastfeeding. When choosing the right treatment it should consider severity of symptoms and their duration. The duration of treatment usually lasts for a week after which the symptoms disappear. If symptoms of oral thrush occur in the child's mouth, antifungal drugs are applied after each feeding (Kent *et al.*, 2015). The use of these medicaments must be carried out very carefully, especially in case of children under six months of age. There is a risk of choking. The best form of applying medicaments on the surface of oral mucosa is lubrication by finger. In the treatment of a child whose mother has been diagnosed with breast mycosis, probiotic therapy may be used as a protection (Matsubara *et al.*, 2016) however, any supplements must be consulted with the pediatrician. Child may also need treatment for genital and anal fungal infections (Scheinfeld, 2005).

Prevention of Fungal Breast Infections

In the prevention of fungal infections, it is important to prevent situations that promote the multiplication of microorganisms. A high level of personal hygiene should be maintained and the nipples should be kept dry. Frequent replacement of breast pads or complete abandonment of their use is helpful. Attention should also be paid to the quality of underwear and clothing in contact with the breasts. Fabrics that facilitate air access to the skin's surface are preferred (Riordan and Wambach, 2010). It is also important to avoid mechanical injuries to the nipple during breastfeeding. Emphasis should be placed on a solid preparation of women expecting a baby for breastfeeding. Education is of great preventive importance (Miracle and Fredland, 2007). Correct attachment of baby to the breast will help to avoid dangerous cuts (Cox, 2007). In the case of problems with attaching a baby to the breast, it is worth use the help of certified lactation counsellors, who will help diagnose and solve the problem. They have experience in capturing abnormalities that are the cause of incorrect milk intake by the child, which may result in nipple injuries. There are many positions that mother and baby can take during breastfeeding. The appropriate settings are selected individually, depending on the problem that arises. It is also recommended to ventilate the breast for several minutes a day. Exposing breasts to fresh air helps maintain proper conditions and allows the skin to rest in some way (Riordan and Wambach, 2010). When using

baby teats or lactation pads, care should be taken to keep them clean and replace them regularly with new ones. These kind of accessories are personal items for the child. Regular washing and disinfecting helps maintain an appropriate level of microbiological purity and prevents from infections, including fungal infections (Sachdeva *et al.*, 2016). Also, the use of equipment that enables the milk collection, such as breast pumps, creates an additional potential source of contamination, including fungi (Jiménez *et al.*, 2017). The basis for maintaining safety in this case is also maintaining the highest hygiene of the process and the breast pump itself, which should be thoroughly washed and dried after each use. Prevention is of the highest importance. Mothers should take care of the immunological resistance, preventing from chronic exhaustion, stress and providing all necessary nutrients in the diet. Women after childbirth are exposed to a high dose of stress, including that connected with lactation problems. In this situation, it is important to ensure support for mothers with breastfeeding problems (Labarere *et al.*, 2005). Early reporting of observed changes and ailments is important to stop pathological state spreading. Any abnormalities found at an early stage of development are easier to solve and the mothers can come back to breastfeeding in comfort (Kent *et al.*, 2015). It has been shown that breast milk is also a source of fungi (Heisel *et al.*, 2019). Research results indicate the dominant share of fungi *Malassesia*, *Candida* and *Saccharomyces*. These fungi have been found in human milk samples of healthy women, which may indicate the involvement of fungi in shaping the correct newborn's microbiome (Boix-Amorós *et al.*, 2017). The mechanism of fungi entering the breast milk environment is not fully understood. The entero-mammary path is taken into account (Heisel *et al.*, 2019).

Conclusions

Fungal infections of the breast are a disease that may contribute to the resignation of mothers from breastfeeding. They are the reason for felt discomfort and pain. In addition, they can become a cause of a fungal infection in a breastfed child, which affects the sensation of pain when feeding and unwillingness to suck. Precise problem identification of the problem and accurate diagnosis is not easy, but the quick implementation of appropriate treatment and compliance with hygiene rules allows for recovery and breastfeeding in comfort.

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Fungal Infections of the Central Nervous System

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Central Nervous System Fungal Infections

Over the last few decades, cases of life-threatening fungal infections of the central nervous system (CNS) have significantly increased. Specifically, immunocompromised populations, the number of which has expanded in recent years, suffer from high mortality rates due to these infections (Murthy and Sundaram, 2014). The expansion of the immunocompromised population is due to increase in advanced transplant procedures, the use of immunosuppressive therapies, and the pandemic spread of HIV (Raman Sharma, 2010). The increased prevalence of these infections challenges physicians and researchers across the globe to develop accurate and rapid diagnostics associated with effective management strategies (Panackal and Williamson, 2015; Raman Sharma, 2010). In fact, research on fungal brain infections has produced impressive publication records over the last 20 years (**Fig. 1**).

Pathogenic fungi comprise approximately 300 species among highly diverse fungi, of which an estimated 1.5 million species are found living as saprophytic organisms that colonize distinct environmental niches, including some in animals and human hosts. Among the species able to colonize and cause disease in mammalian hosts, only approximately 10%–15% are able to colonize the CNS, where they cause severe infection (Murthy and Sundaram, 2014; Goralska *et al.*, 2018).

Upon exposure to fungi, immunocompetent individuals are generally able to defend against and avoid infection, having effective clearance. However, an immunocompromised host who is not able to fight the infection develops disease that generally begins in the lungs and skin and can be disseminated elsewhere in the organism, including the CNS, through hematogenous spread (Murthy and Sundaram, 2014; Black and Baden, 2007; Rauchway *et al.*, 2010).

Brain colonization is not an easy task for fungal pathogens, since the CNS is surrounded by a relatively impermeable blood–brain barrier (BBB). This barrier makes the CNS an immunologically privileged site, where few immunological cells, mainly activated T-lymphocytes, are able to penetrate. However, in immunocompromised hosts, the BBB permeability is increased, which facilitates fungal penetration into the CNS (Raman Sharma, 2010).

Fig. 1 Genus-based distribution of documents resulting from research on fungal brain infections from 1998 to 2018. Google Scholar (https://scholar.google.com.br) searches combining each fungal genus with the term "brain" indicated *Candida*, *Aspergillus*, *Cryptococcus*, *Fusarium*, *Pneumocystis*, *Histoplasma*, *Rhizopus*, *Blastomyces*, *Mucor* and *Coccidioides* as the top 10 fungal genera producing research documents. In combination, these 10 genera produced more than 200,000 research documents.

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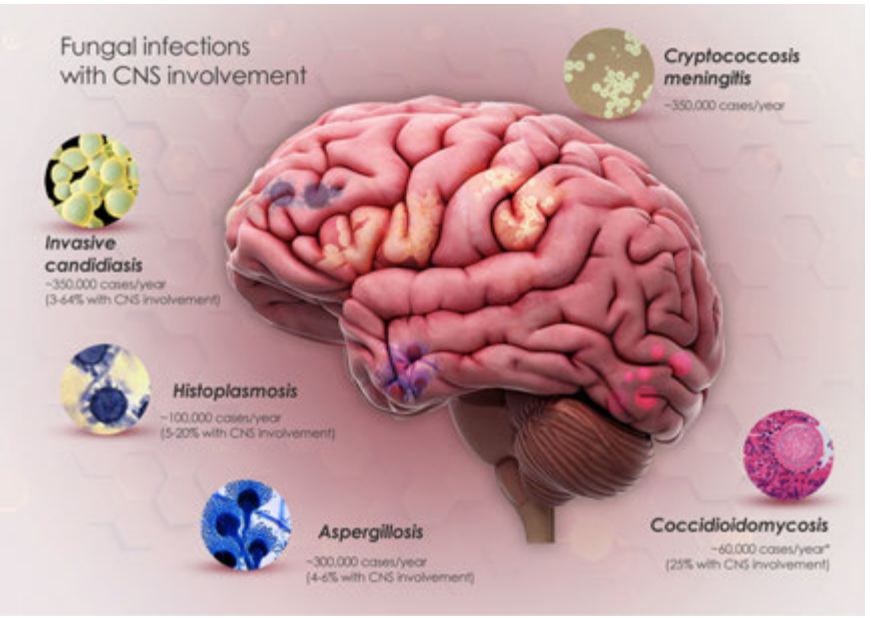

Fig. 2 Brain diseases caused by fungi and their incidence in humans. Data was collected from Goralska, K., Blaszkowska, J., Dzikowiec, M., 2018. Neuroinfections caused by fungi. Infection 46, 443–459 and the Global action Fund for Fungal Infections (GAFFI, https://www.gaffi.org). Asterisks indicate epidemiological data available in the US only. Image credit: Wagner Nagib (Instituto Carlos Chagas, Fiocruz).

Although the host's immunological status has an important role in brain colonization, fungi have also evolved strategies to modulate and evade the host immune system. **Fig. 2** presents an overview of the most common fungi that causes CNS infections, represented by yeasts (*Cryptococcus* spp. and *Candida* spp.), filamentous (*Aspergillus* spp.) and dimorphic (*Histoplasma capsulatum* and *Coccidioides* spp.) species, together with the number of cases of each type of mycosis that is diagnosed per year and the frequency with which these mycoses can disseminate to brain tissue to cause lethal CNS diseases.

Candida species are known for their capacity to cause an invasive disease that can affect the CNS. Invasive candidiasis presents with a high incidence rate of CNS involvement (Panackal and Williamson, 2015), and has a mortality of 10%–70%, which is increased to 90% for patients in which the CNS is involved (Goralska *et al.*, 2018; McCarthy *et al.*, 2017; Pana *et al.*, 2017). The most common manifestation of *Candida* spp. CNS infection is meningitis, which mostly affects newborns and neurosurgical and immunocompromised patients (Panackal and Williamson, 2015; Murthy and Sundaram, 2014); however, it can occasionally affect healthy patients (Borha *et al.*, 2009).

Aspergillosis can also affect the brain and is associated with mortality rates of more than 90% (Baddley *et al.*, 2010; Deigendesch *et al.*, 2017). In addition to its ability to cause meningitis, the most common CNS infection by *Aspergillus* affects intra-axial brain structures (Deigendesch *et al.*, 2017). Immunocompromised patients are the individuals most commonly affected by invasive aspergillosis, with bone marrow transplantation, hematological diseases, AIDS, pulmonary diseases, chronic granulomatous disease, and solid organ transplantation among the most common risk factors (Ruhnke *et al.*, 2007; Singh and Paterson, 2005; Potluri *et al.*, 2014).

Coccidioidomycosis is an endemic mycosis caused by the dimorphic fungi *Coccidioides immitis* and *C. posadasii* that, in addition to being commonly asymptomatic, can evolve to a disease that is disseminated with CNS involvement, leading to meningitis, which is the most serious form of the disease (Galgiani *et al.*, 2005; Johnson *et al.*, 2018). Although able to affect immunocompetent individuals, *Coccidioides* meningitis development depends, to some extent, on the immunological status of the patients, with immunocompromised patients being the most susceptible (Brown *et al.*, 2013; Twarog and Thompson, 2015; Jackson *et al.*, 2019).

Histoplasma capsulatum is the etiological agent of another endemic mycosis, histoplasmosis. In addition to being rare, the dissemination of *Histoplasma* to the CNS generally occurs in patients with AIDS and other immunosuppression disorders, leading to severe cases of meningitis (Riddell and Wheat, 2019) that are associated with high morbidity and mortality rates (Wheat *et al.*, 2018); however, immunocompetent patients can be affected (Schestatsky *et al.*, 2006). In addition to meningitis, CNS histoplasmosis causes parenchymal mass lesions in the brains or spinal cord, stroke due to cerebral emboli and diffuse encephalitis (Saccente, 2008; Wheat *et al.*, 1990).

The main species responsible for fungal meningitis worldwide is *Cryptococcus neoformans* (Srikanta *et al.*, 2014). In cryptococcal infections, meningitis is the clinical manifestation that has the greatest effect on patients. Even when properly treated, in most cases, this meningitis cannot be ameliorated, and the mortality is approximately 70%, depending to the condition of the patient's immune system and the geographical location (Rajasingham *et al.*, 2017).

On the basis of the highest indices of mortality deriving from brain infection, in this essay, we mostly address the mechanisms used by *Cryptococcus* to disseminate in the body and invade the CNS and discuss some virulence factors associated with the infection of the CNS, elucidating interesting studies that have helped to unravel the fascinating predilection of this fungus for the CNS.

The *Cryptococcus* Genus

The *Cryptococcus* genus includes two important species, *C. gattii* and *C. neoformans*, that infect humans, causing cryptococcosis, an important systemic mycosis with high rates of morbidity and mortality worldwide (Maziarz and Perfect, 2016; Zaragoza, 2019).

Cryptococcus neoformans and *C. gattii* are spherical yeast with buds that each have a polysaccharide capsule that can vary in size among distinct strains (Voelz *et al.*, 2010; Rodrigues *et al.*, 1999; Negroni, 2012). Under the capsule, a rigid and dynamic cell wall consists of pigments, proteins, and polysaccharides that determine cell shape, interaction with other matrix cells, osmotic control, and other important biological processes, such as permeability (Rodrigues *et al.*, 2007, 2008). In nature, these species inhabit different niches. *C. neoformans* can be found in the soil and bird excreta, while *C. gattii* is more likely to be found in eucalyptus trees (Mitchell and Perfect, 1995), as shown in **Fig. 3**.

Both *Cryptococcus* species are etiological agents of cryptococcosis, a systemic worldwide mycosis able to affect both human and animal individuals (Rodrigues *et al.*, 1999) and to affect both healthy and immunocompromised individuals (Bandalizadeh *et al.*, 2019), as shown in **Fig. 3**. *C. neoformans* is the major causative agent of cryptococcosis, infecting mainly immunocompromised individuals (Voelz *et al.*, 2010; Rodrigues *et al.*, 1999), displaying a predilection for infecting the CNS, leading to the most severe manifestation of cryptococcosis, cryptococcal meningitis (Rodrigues *et al.*, 2007; May *et al.*, 2016; Bielska and May, 2016). In immunocompetent individuals, *C. gattii* shows the ability to establish cryptococcal infection with a predilection for infecting the lungs, showing elevated pathogenicity that is difficult to treat and is associated with high mortality rates (Brouwer *et al.*, 2007; Voelz *et al.*, 2010; Bielska and May, 2016).

Cryptococcosis starts with the inhalation of *Cryptococcus* spores or small, desiccated and less-encapsulated yeast cells spread in the environment. These are the infectious propagules of an ideal size for alveolar deposition and interaction with resident macrophages. In this first contact with the host, the host defenses will determine the development of the infection (Bielska and May, 2016; Gottfredsson and Perfect, 2000; Rodrigues *et al.*, 1999; Velagapudi *et al.*, 2009).

To evade host defenses and cause systemic mycosis, in addition to producing different virulence factors, *Cryptococci* undergo substantial morphological changes that drive their interaction with hosts (Rodrigues *et al.*, 1999, 2008). Morphological changes are common among fungi; however, *Cryptococcus* morphological changes are unique, as they permit the fungi to significantly increase its size by increasing its capsule, its body or both, becoming so-called titan cells (cells with bodies larger than 15 μm, excluding the capsule) (Zaragoza and Nielsen, 2013). Titan cells are polyploid cells with a thickened cell wall and a dense capsule, and they are important virulence factors because they impair phagocytosis and induce the polarization of the host immune response to promote successful infection (Zaragoza *et al.*, 2008; García-Rodas *et al.*, 2018; Zaragoza *et al.*, 2009).

Cryptococcus interactions with the host are critically influenced by polysaccharides that are produced and mounted into a network composed mainly of glucuronoxylomannan (GXM), glucuronoxylomannogalactan (GXMGal), and mannoproteins that form a capsule surrounding the cell (Rodrigues *et al.*, 1999; Bielska and May, 2016; Rodrigues *et al.*, 2015; Larocque-de-Freitas *et al.*, 2018). The capsule is a distinctive feature of cryptococcal cells that protect the fungus against host immunological defenses

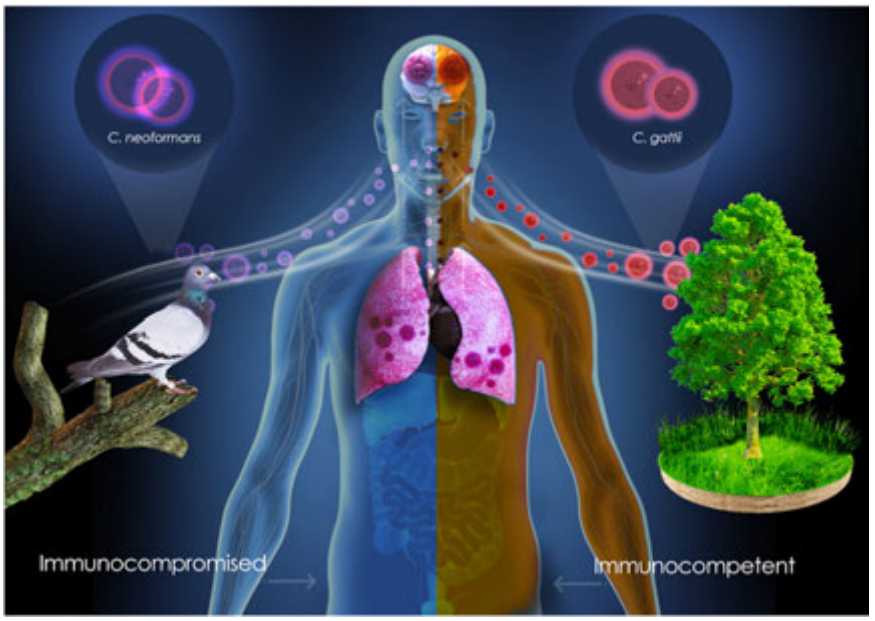

Fig. 3 Transmission of cryptococcal propagules from environmental niches to the human host. *C. neoformans* preferentially affects immunocompromised hosts, while *C. gattii* is an efficient causer of human disease in immunocompetent individuals. For details about the environmental niches of each species, see text. Image credit: Wagner Nagib (Instituto Carlos Chagas, Fiocruz).

during infection (Zaragoza *et al.*, 2006). The *Cryptococcus* capsule can be visualized by microscopy, and **Fig. 4** shows different techniques to visualize this structure, such as India-ink staining, immunofluorescence, and electron microscopy.

Capsule components are synthesized in the cytoplasm and released to the extracellular space by extracellular vesicles (EVs) (Bielska and May, 2016). These particles are efficient carriers of molecules, such as lipids, pigments, polysaccharides, proteins,

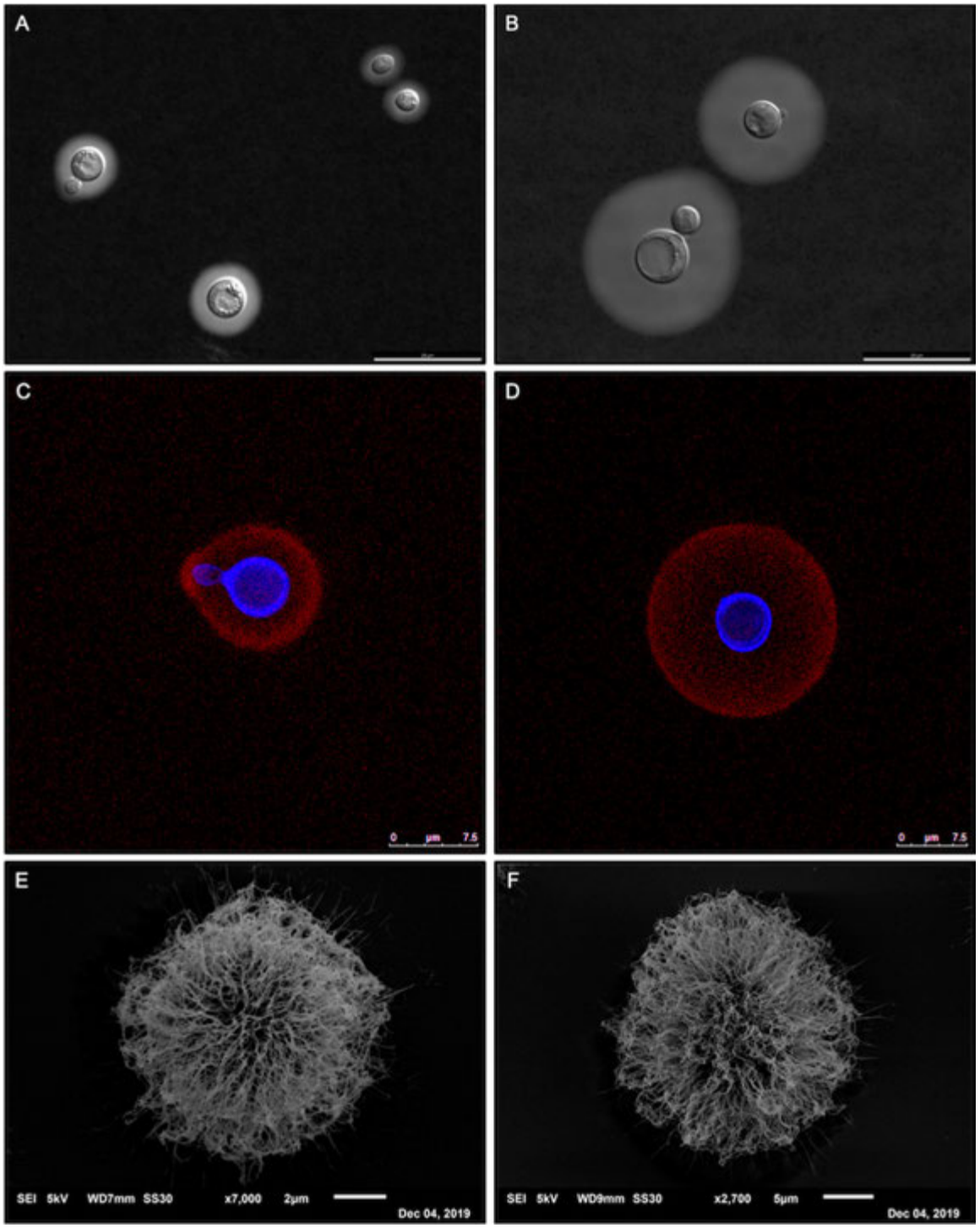

Fig. 4 Microscopic examination of the cryptococcal capsule. Panels A, C and E illustrate *C. neoformans* cells, while panels B, D and F illustrate *C. gattii*. Capsular structures can be evidenced by counterstaining with India ink (A–B), immunofluorescence (C–D, where the blue fluorescence denotes the cell wall after staining with calcofluor-white and the red fluorescence represents the capsule after antibody staining) and scanning electron microscopy (E–F).

toxins, nucleic acids, and other virulence factors, to the extracellular environment (Herkert *et al.*, 2019; Coelho and Casadevall, 2019). EV size and cargo may also affect the fungi–host interactions by delivering toxins to the host and affecting antimicrobial resistance (Coelho and Casadevall, 2019; Herkert *et al.*, 2019).

In addition to their morphological changes, other important virulence factors must be considered, such as melanin deposition into the cell wall, lipids, phospholipase, urease, and laccase, in addition to the ability to grow at mammalian temperatures (Rodrigues *et al.*, 1999, 2007; Bielska and May, 2016; Janbon, 2004). Altogether, these factors result in a great ability to manipulate the host immune system and cause life-threatening CNS infection both in immunocompetent and immunocompromised individuals, making it an important worldwide health and economic problem.

Cryptococcosis Epidemiology

Epidemiological data show that the majority of cryptococcal disease cases occur among immunocompromised individuals (Nyazika *et al.*, 2018), with HIV infection presenting as the main risk factor and accounting for 95% of the cases in middle- and low-income countries and 80% of the cases in high-income countries. Individuals taking immunosuppressive drugs constitute most of the remaining cases, although immunocompetent patients can also be affected in some settings (Sloan and Parris, 2014; de Azambuja *et al.*, 2018). Given the differences in HIV endemic status and geographic and ethnic factors, cryptococcosis numbers can vary greatly between different countries and regions, ranging from 6.6 cases per million in Australia to 15.6 per 100,000 in the Gauteng Province of South Africa (Fang *et al.*, 2015).

At the peak of the HIV pandemic, more than 600,000 deaths were estimated to have occurred globally as a result of cryptococcosis (Park *et al.*, 2009). France reported a fivefold increase in cryptococcosis from 1985 to 1993, while the number of cases in patients not infected with HIV remained stable. Of the 517 reported cases in New York City in 1991, 96% were related to HIV (Sloan and Parris, 2014). Although this number was greatly reduced as access to effective antiretroviral therapy expanded in developed countries, cryptococcosis continued to have a great impact on immunocompromised individuals, mainly in sub-Saharan Africa and low- and medium-income countries of middle Southeast Asia (Nissapatorn, 2008) and Latin America (Leimann and Koifman, 2008), where mortality rates could be as high as 70% (Leimann and Koifman, 2008).

C. gattii was previously prevalent in subtropical and tropical regions, spreading to temperate zones and affecting the Vancouver Island, British Columbia (218 cases during 1999–2007) (Galanis *et al.*, 2010), and the northwestern US states of Washington and Oregon (96 cases reported to the CDC between December 2004 and July 2011) (Harris *et al.*, 2011). Evidence suggests that the outbreak is expanding along the US West Coast (Applen Clancey *et al.*, 2019). Data from studies conducted in Australia, Papua New Guinea, British Columbia, Canada, and the US Pacific Northwest point to a mortality rate ranging from 13 to 33%, depending on differences in treatment, time of follow-up and patient characteristics (see "Relevant Websites section").

In addition to outbreaks in previously unaffected regions, another important fact to consider is the epidemiological change currently observed in medium- and high-income countries, attributed to the effectiveness of HIV diagnosis and treatment, skewing cryptococcosis susceptibility data and highlighting other susceptible populations. For example, between 1992 and 2007, in Atlanta and Houston in the United States, diabetes was found to be the most common underlying condition associated with cryptococcal infection. In Ottawa, Canada, data from 2005 to 2017 revealed that the most common underlying condition associated with cryptococcosis was hematological malignancies and, considering the fatality rates, solid organ transplantation was also considered an important risk factor (Patel *et al.*, 2019).

Cryptococcosis represents approximately 8% of the invasive fungal infection cases among solid organ transplantation recipients, being the third most common fungal infection in these patients, with an overall incidence ranging from 0.2% to 5%. Among the types of organs transplanted, the lung is associated with an increased risk of cryptococcosis (Baddley *et al.*, 2019). In patients with malignancies, lymphomas were the most common predisposing conditions (66%), followed by leukemias (29%) and myelomas (4%) (Schmalzle *et al.*, 2016).

Sex also seems to have had a role in the epidemiology of cryptococcosis. Curiously, men are affected by cryptococcosis more frequently than women with the disparity present both in HIV^+ ($\sim$8 M:2F) and HIV^- ($\sim$3 M:1F) subjects. In addition to being male as a predisposing risk factor, men affected by cryptococcosis also have more severe symptoms and poorer outcomes (Guess *et al.*, 2018). Although the mechanisms involved in this trend have not been completely elucidated, it seems that hormones may play a role in influencing both the immune response and *Cryptococcus* virulence (McClelland *et al.*, 2013).

With respect to CNS infection, cryptococcal meningitis (CM) is the most common cause of adult meningitis in large parts of the world with high rates of HIV infection. Cryptococcosis is considered, after tuberculosis, the most common cause of illness in people living with HIV/AIDS, being responsible for 15% of the AIDS-related deaths globally. Approximately 200,000 cases are estimated to occur worldwide every year (see "Relevant Websites section"), culminating in 181,000 deaths, of which 73% occur in sub-Saharan Africa (Rajasingham *et al.*, 2017). The management of CM is complicated by the fact that, in several locations, one-half of the patients present with a medical history of antiretroviral therapy (ART) combined with persistently low CD4+ T cell counts due to loss of follow-up, treatment non-adherence or development of resistance to therapy (Williamson *et al.*, 2017).

Although globally associated with HIV cases in developed countries, the prevalence of CM is also increasing in populations not infected with HIV, such as solid organ transplantation recipients, patients with rheumatic diseases, and those receiving immunosuppressive therapies (Beardsley *et al.*, 2019).

Regarding the CM 1-year mortality rate worldwide, the numbers generally reflect country income level. In low-income patients, mortality ranges from 70% for patients in care to 100% for patients not in care. In the medium income category, mortality is

approximately 40% and 60% of all in-care and not-in-care patients, respectively; specifically, in Europe, it is 30 and 45%, and in North America, it is 20% and 30% (de Azambuja *et al.*, 2018; Rajasingham *et al.*, 2017).

Cryptococcus "Travels" to the Brain

Cryptococcus can disseminate and reach the CNS due a specific tropism for this site (Santiago-Tirado *et al.*, 2017). The CNS environment confers an immune privilege condition to the fungi that is related to the lack of a robust inflammatory response against antigenic changes (Santiago-Tirado and Doering, 2017; Vestweber, 2015). The first step required to reach and invade the CNS is to interact with endothelial cells and then pass through the BBB that protects the host against invaders or toxic compounds (Weiss *et al.*, 2009; Kim, 2008).

The structure of the BBB is composed of an endothelial cell layer with tight junctions and microcapillary blood vessels, representing the interface between the peripheral circulation and CNS. The BBB is highly selective for drug and microorganism permeation due to the action of efflux transporters, restrictive passive diffusion, absence of gaps and paracellular permeation (Daneman and Prat, 2015). To reach the CNS, *Cryptococcus* uses three routes, known to occur concomitantly: (1) inside infected phagocytes and possibly neutrophils (Trojan horse model) (Santangelo *et al.*, 2004; Shi *et al.*, 2010; Charlier *et al.*, 2009), (2) penetration between the cell barriers with or without disruption of the BBB endothelial cell tight junctions (paracellular model), and (3) crossing through the microvasculature as free fungi (transcellular model). **Fig. 5** illustrates each mechanism.

Trojan Horse Model

In the Trojan horse mechanism, *Cryptococcus* parasitizes phagocytes and survives within them, using these cells as vehicles to reach the brain (Zaragoza, 2019; Casadevall, 2010). Evidence that *Cryptococcus* can disseminate to the CNS through Trojan horse mechanisms was obtained from macrophage depletion studies in which the results showed a reduction in BBB invasion (Cheng *et al.*, 2009). Additionally, several studies have pointed to the occurrence of this phenomenon in vivo. First, monocyte depletion in mice before *Cryptococcus* infection resulted in a reduction in disease severity and in fungal burden (Charlier *et al.*, 2009). Second, the transfer of macrophages and monocytes from mice infected with *Cryptococcus* to healthy animals caused cerebral cryptococcosis. Finally, intravenous infection of mice with macrophages infected with ingested cryptococcal cells led to cryptococcal meningoencephalitis (Santangelo *et al.*, 2004; Charlier *et al.*, 2005). Indeed, Santiago-Tirado *et al.* (2017) demonstrated that cryptococcal cells inside phagocytes cross the BBB. Once in the brain, *Cryptococcus* can be expelled from the phagocytes through a mechanism called vomocytosis, a nonlytic process that represents the end of the Trojan horse route (Taylor-Smith and May, 2016), spreading the cells to the CNS.

Paracellular Model

This route of brain infection involves crossing the BBB through intercellular spaces with or without tight junction damage. Olszewski *et al.* (2004) demonstrated that *C. neoformans* had the ability to cross the BBB, showing fungal colocalization in

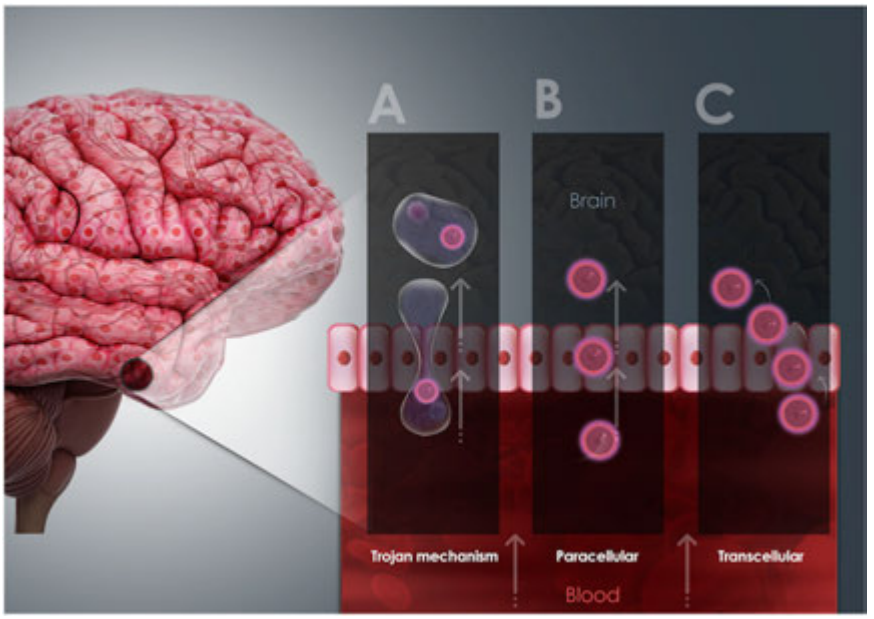

Fig. 5 Brain invasion by cryptococci. The three major mechanisms illustrating how cryptococci leave the blood to cross the endothelial layer and colonize the brain tissue are illustrated. For details, see related text. Image credit: Wagner Nagib (Instituto Carlos Chagas, Fiocruz).

capillaries and damaged endothelial cells. The same paracellular entry was verified by Charlier *et al.* (2005). Additionally, differences related to the capsule and size of the yeast could be involved in the damage to the endothelium. During infection, *Cryptococcus* induces host cell cytoskeleton changes; fungal adhesion promotes the dephosphorylation of cofilin and occludin, two proteins involved in cytoskeleton integrity, resulting in actin rearrangement and alterations in tight junction integrity that can facilitate the paracellular route of entry into the CNS (Chen *et al.*, 2003). Another possible route of paracellular entry by *Cryptococcus* relates to its ability to subvert host plasminogen by converting it into plasmin, a serine protease with proteolytic activity that can promote disruptions to the host membrane and extracellular matrix, thus weakening tight junctions and facilitating fungal invasion of the CNS (Stie *et al.*, 2009; Stie and Fox, 2012).

Transcellular Model

CNS invasion by transcytosis involves the endocytosis processes and requires that cryptococci adhere to endothelial cells before entering into brain tissue. Chang *et al.* (2004) demonstrated that, during the interaction of *Cryptococcus* with human brain microvascular endothelial cells (HBMECs) and after fungal adhesion to host cells, endothelial cells form microvillus-like projections that engulf the yeast cell; additionally, they verified the presence of free yeast inside the brain parenchyma near the capillaries, demonstrating the transcellular passage of *Cryptococcus* through the BBB. Charlier *et al.* (2005) reported the presence of yeast at cortical capillaries without the involvement of the choroid plexus at early stages after *Cryptococcus* infection and detected the damage to endothelial layers (Charlier *et al.*, 2005); in addition, the yeast crossed the HBMECs without affecting HBMEC integrity.

The association of hyaluronic acid (HA), produced by *Cryptococcus*, with CD44, the receptor in lipid rafts of endothelial cells, was described as important to the entry of the fungus at HBMEC membrane rafts since it can induce protein kinase alpha (PKc-α), which contributes to host cytoskeleton reorganization and phagocytosis (Chang *et al.*, 2006; Jong *et al.*, 2008).

CD44, an HBMEC cell surface protein, has been in fact identified as a *C. neoformans* receptor. The association of (HA), with CD44 increased the presence of this molecule in lipid rafts of HBMEC during fungal engagement. This recruitment generated docking sites for a subsequent yeast engulfment. The association of *C. neoformans* with HBMEC was followed by the activation of protein kinase alpha (PKc-α), which contributed to host cytoskeleton reorganization and phagocytosis (Chang *et al.*, 2006; Jong *et al.*, 2008). Indeed, the interaction of *Cryptococcus* with HBMECs triggered the activation of other host signaling proteins relevant to cytoskeleton rearrangement, impacting fungal transmigration. Among the host proteins activated, Rho GTPases can phosphorylate other host proteins, such as FAK, PKCα and ezrin (Kim *et al.*, 2012), promoting cytoskeleton remodeling tight junctions modulation. Another signaling pathway that likely contributes to cryptococcal transmigration is that involving the EPH-EphrinA1 (EphA2) tyrosine kinase receptor, which, when activated via CD44, triggers changes in membrane permeability, promoting the weakening of tight junctions through the activity of Rho, Rac and Cdc42. Inhibition of EphA2 resulted in the prevention of transmigration; in contrast, endothelial cell treatment with an EphA2 agonist, a ligand of EphA2, resulted in enhanced *C. neoformans* transmigration (Aaron *et al.*, 2018).

Cryptococcus "Passport" to Brain Travel

Both fungal and host factors have been identified to be involved in CNS invasion. Some virulence factors are described as being important to *Cryptococcus* infection and are shared by both pathogenic species, such as phospholipase B1 (Plb1) and laccase, which are related to fungal egress at the initial site of infection (lungs) and dissemination into the CNS (Noverr *et al.*, 2004; Santangelo *et al.*, 2004), and Plb1 and urease in the crossing of the blood–brain barrier (Santangelo *et al.*, 2004; Shi *et al.*, 2010).

Inositol

Cryptococcus CNS predilection is not yet fully understood. However, some evidence points to inositol involvement. Inositol is a carbocyclic sugar that is found at higher levels in the brain, where it acts as an osmolyte. *Cryptococcus* has also developed ways to acquire inositol, using it as a carbon source, thus influencing brain infection. The availability of inositol to fungal cells can be influenced by the induction of *Cryptococcus* alterations in the tight junctions between endothelial cells, which leads to an increase in inositol permeability and increasing levels of inositol that enhance *Cryptococcus* transmigration in a species-specific way; inositol does not influence the transmigration of *C. albicans* (Liu *et al.*, 2013).

Inositol acquisition occurs through a specific system comprised of the transporter gene (*ITR*) family, mainly the Itr1a and Itr3c transporters. Mutations in these transporters led to a significant reduction (50%) in BBB transmigration in vitro when compared to the wild-type strain. In vivo, defects in inositol acquisition correlated with reduced fungal burden and lesions in the brain (Liu *et al.*, 2013). Exposure of *Cryptococcus* to inositol leads to an increase in the expression of a hyaluronic acid synthase that can associate with CD44 in endothelial cell membrane lipid rafts to enhance the invasive potential of *Cryptococcus* (Jong *et al.*, 2012; Liu *et al.*, 2013). The inhibition of inositol transporters reduced the inositol uptake and impaired the crossing of *Cryptococcus* through the HBMEC monolayer (Liu *et al.*, 2013). Finally, strains lacking ITR1a and ITR3c inositol transporters evoked a more

pronounced protective immune response and had reduced levels of GXM secretion in the presence of inositol as carbon source (Liu *et al.*, 2014).

Urease

Urease is a metalloenzyme evenly distributed in the human body, including in the CNS, responsible for catalyzing the hydrolysis of urea into carbon dioxide and ammonia, the latter of which can be protonated to form ammonium, resulting in an increase in pH with consequences to pathogen-host interaction (Rutherford, 2014).

Urease improves the ability of *Cryptococcus* to invade the CNS. Mice infected with a mutant strain lacking urease showed a reduced brain fungal burden when compared to the load in the wild type mice (Olszewski *et al.*, 2004). Urease can directly affect the *Cryptococcus*–host BBB interaction. The ammonia generated due to urea hydrolysis can elicit both direct toxic effects in endothelial cells, promoting BBB disruption ("opening" the endothelial cell junctions) and resulting in increased permeability to cells, which facilitates brain invasion (Stamatovic *et al.*, 2008) and the induction of adhesion molecules at the endothelial surface (Olszewski *et al.*, 2004).

Fu *et al.* (2018) infected mice with macrophages containing urease-positive and urease-negative strains and compared the fungal burden in the lungs and brain. Differences were observed only in fungal recovery from the brain with a reduced number of viable urease-negative strains. Knowing that *Cryptococcus* can cross the BBB through the Trojan horse mechanism or transcytosis of endothelial cells, the authors suggested that cryptococcal urease enabled the fungus to persist in the phagocytes and thus be transported to the brain, where it is likely released through the induction of nonlytic exocytosis.

Phospholipase B1

Phospholipase B (PLB1) facilitates *C. neoformans* adhesion to host cells, replication, and survival within macrophages (Ganendren *et al.*, 2006; Santangelo *et al.*, 2004; Noverr *et al.*, 2003). Because of its hydrolytic activity on ester linkages in glycerophospholipids found abundantly in the host cell membrane and in pulmonary surfactants (Djordjevic, 2010), PLB1 contributes to the invasion and dissemination processes of fungus (Cox *et al.*, 2001; Noverr *et al.*, 2003) by breaking down host membranes and contributing to fungi escape from phagolysosomes (Wright *et al.*, 2007).

Rearrangement of host cell actin is related to the internalization process for several pathogens (Munoz-Duarte *et al.*, 2016; Mendes-Giannini *et al.*, 2004). During *Cryptococcus* transmigration to the brain, remodeling of the host endothelial cell cytoskeleton occurs, with fingerlike projections (microvilli) of actin surrounding intracellular yeast (Maruvada *et al.*, 2012) without altering host cell integrity (Chang *et al.*, 2004). *Cryptococcus* PLB1 participates in the activation of an important molecule involved in actin cytoskeleton dynamics, Rac1, which belongs to the small Rho GTPase family (Sepp and Auld, 2003; Noverr *et al.*, 2003). *Cryptococcus* mutant strains lacking PLB1 showed lower Rac1 activation levels in endothelial cells, leading to a significant reduction in *Cryptococcus* transmigration and fungal burden in the brain (Noverr *et al.*, 2003).

Laccase

The functions of laccase in *C. neoformans* include protection against macrophage antifungal activity through the production of melanin, iron oxidation products, and prostaglandin E2 (PGE2) (Liu *et al.*, 1999a; Eisenman *et al.*, 2007). Laccase can oxidize catecholamine substrates (dopamine, norepinephrine, and epinephrine) to generate melanin during infection (Liu *et al.*, 1999b), and melanin has the potential to alter host cell defense (Montine *et al.*, 1997). Melanins are polyphenolic and/or polyindolic pigments that can be produced by several pathogens and are frequently related to the pathogenicity (Lee *et al.*, 2019). When comparing melanized and nonmelanized yeast strains, several studies have concluded that melanin can protect the fungus against oxidant agents, microbicidal peptides, phagocytosis, and killing by macrophages and that melanin confers particular resistance to antifungals (Wang *et al.*, 1995; Liu *et al.*, 1999a; Wang and Casadevall, 1994; Jacobson and Tinnell, 1993), acting as a free radical scavenger (Porebska-Budny *et al.*, 1992). The presence of fungal melanin in vivo was first detected in tissues from patients with AIDS and cryptococcal meningoencephalitis (Nosanchuk *et al.*, 2000). The ability of the fungus to produce melanin from catecholamines was associated with the neurotropism of *Cryptococcus* (Polacheck *et al.*, 1982) Qiu *et al.* (2012). A mutant strain lacking laccase was unable to disseminate from the lungs to the CNS, leading to a nonprotective polarization of the host immune response.

Metalloprotease 1 (Mrp1)

Mrp1 belongs to the M36 peptidase class. It is upregulated during CNS infection and secreted by *C. neoformans* during the infectious process (Steen *et al.*, 2003; Vu *et al.*, 2014). Mutant strains lacking Mrp1 had a decreased ability to adhere to endothelial cells and to transmigrate through the host BBB (Vu *et al.*, 2014). In addition, in mice infected with a mutant yeast strain lacking Mrp1 the brain fungal burden was significantly reduced, suggesting the involvement of Mpr1 in the BBB transmigration process (Vu *et al.*, 2014). Interestingly, Na Pombejra *et al.* (2017) showed that *Cryptococcus* Mrp1 expressed in *Saccharomyces cerevisiae* led to nonpathogenic fungi crossing the BBB model in vitro without any damage to tight junctions. They also found that *Cryptococcus*

Mrp1 interacts with several endothelial cell surface proteins, most of which were related to membrane and cytoskeleton rearrangement.

Antifungal Alternatives for Treating Cryptococcal Meningitis

The treatment of fungal infections continues to be a challenge. The limited antifungal arsenal is related to the similarity between fungal and human cells, leading to serious toxicity and difficulty in the search for new targets (Perfect, 2017). Another obstacles for mycosis treatment are the high cost, narrow spectrum of action and low bioavailability of the currently available antifungals (Denning and Bromley, 2015). Currently, the available antifungal drugs are classified into four classes: (1) inhibitors of membrane ergosterol biosynthesis (azoles, triazoles, and allylamine); (2) compounds affecting membrane stability (polyenes); (3) inhibitors of cell wall and β-glucan synthesis (echinocandins); and (4) anti-metabolite action (5-fluorocytosine, 5-FC).

The recommended treatment regimen for cryptococcosis in HIV patients is composed of three steps: induction therapy, consisting of the combination of amphotericin B (AmB) and 5-FC for 2 weeks; next, this treatment set is consolidated with fluconazole (8 weeks); and finally, maintenance therapy consists of fluconazole (FLZ) (until the immunodeficiency is reverted). Alternatives to this treatment regimen are also described in **Fig. 6** (Perfect *et al.*, 2010).

AmB is a fungicidal compound belonging to the polyene class, and its mechanism of action is the formation of pores in the fungus cell membrane causing the loss of ions and sugars and, as a consequence, osmotic imbalance; however, it has been proposed that the mechanism of action involves the production of reactive oxygen species and immune response modulation (Walsh *et al.*, 2008; Sangalli-Leite *et al.*, 2011; Mesa-Arango *et al.*, 2012; Perfect, 2017). *Cryptococcus* resistance to AmB is rare (Kelly *et al.*, 1994; Nolte *et al.*, 1997); however, this antifungal agent has high toxic effects, especially nephrotoxicity (de Souza *et al.*, 2016).

5-Fluorocytosine (5-FC) inhibits nucleic acid (DNA and RNA) synthesis by incorporating 5-FC metabolites, which have a molecular structure analogous to pyrimidines (Odds *et al.*, 2003; Perfect, 2017). 5-FC treatment is recommended during CM in combination with AmB. The use of this combination decreases the risk of CM relapse and promotes a faster clearance of *Cryptococcus* spp. from the cerebrospinal fluid compared to AmB alone (Day *et al.*, 2013).

Belonging to the triazole class, fluconazole (FLZ) has as mechanism of action that involves the inhibition of the 14-alpha demethylase enzyme (encoded by the *ERG11* gene), which is critical for converting lanosterol to ergosterol (Perfect, 2017). Different studies reported the resistance of *Cryptococcus.* spp. to FLZ, with efflux pumps and *ERG11* gene mutations responsible for this phenotype. In special cases, such as intolerance to FLZ, other triazoles and itraconazole can be used during consolidation therapy; however, these alternative antifungal agents have poor aqueous solubility and poor oral bioavailability, which means that their levels must be monitored in the serum (Perfect *et al.*, 2010; Kale and Johnson, 2005).

Cryptococcus spp. cause meningitis mainly in HIV-infected individuals in an advanced stage (CD4 cell count <200 cells/mm^3), and the introduction of antiretroviral therapy (ART) was an important milestone for the reduced mortality incidence rate of this mycosis in

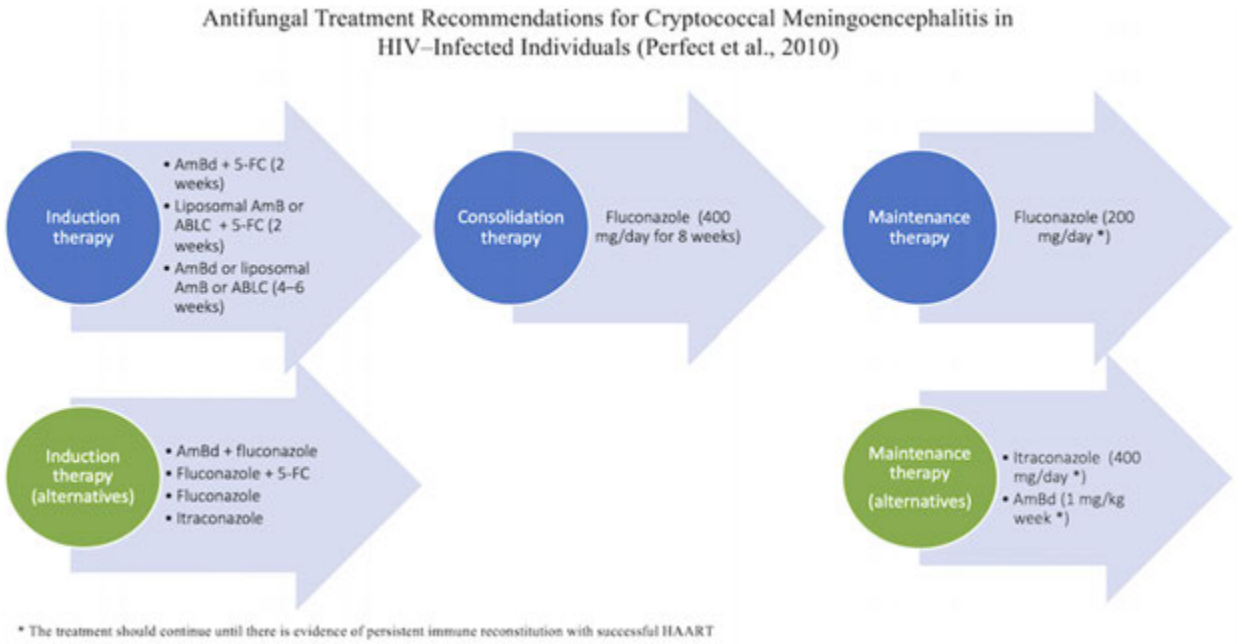

Fig. 6 Therapeutic protocols for fighting cryptococcal disease. AmBd, deoxycholate amphotericin B; 5-FC, fluorocytosine; ABLC, amphotericin B lipid complex.

developed countries (Mirza *et al.*, 2003; Antinori *et al.*, 2009). However, in limited-resource counties where late HIV diagnosis and limited access to treatment is prevalent, the number of deaths caused by cryptococcal meningitis are still alarming (Rajasingham *et al.*, 2017). Moreover, recommended induction treatment (AmB plus 5-FC) is not available in most African clinical centers, and FLZ is often the only drug available (Roemer and Krysan, 2014).

With the unavailability of the induction treatment in resource-limited settings, only monotherapy intervention can be performed, and the use of FLZ at 400 mg/kg as monotherapy is related to inefficacy (Lightowler *et al.*, 2010). However, good outcomes have been described for FLZ ranging from 800 to 2000 mg/daily (Milefchik *et al.*, 2008). To control residual infection, high-dose FLZ (800 mg/day) was also described as an option during consolidation therapy and has been recommended as an alternative by the World Health Organization HIV management guidelines (after short-course induction with amphotericin for 5–7 days) (Murphy *et al.*, 2018). A recent study demonstrated that the use of high fluconazole doses can kill a susceptible population of yeast but can also cause emerging fungal resistance, which is associated with the development of aneuploidy (Hope *et al.*, 2019).

Aiming for treatment optimization, a review of clinical trials reported the evidence of effectiveness treatment for *Cryptococcus* meningitis by comparing the following antifungal regimens: FLZ to FLZ with 5-FC; AmB to AmB with 5-FC; AmB given alone to AmB given with 5-FC and FLZ alone; high to standard dose AmB with 5-FC; and AmB to liposomal AmB; however, no evidence was found to consider one treatment superior to any other (Sloan *et al.*, 2008). Recently, in a meta-analysis study that investigated 13 trials with a total of 2426 participants and compared 21 antifungal therapy interventions (July 2014 to July 2018), the authors concluded that the combination of AmB deoxycholate and 5-FC therapy for one week is a promising treatment alternative for the treatment of cryptococcal meningitis in HIV patients in limited-resource conditions. Moreover, the combination of 5FC and FLZ for two weeks, administered orally, may be an alternative for AmB deoxycholate or when intravenous therapy is unavailable (Tenforde *et al.*, 2018).

CM also affects HIV-negative immunocompromised patients (e.g., solid organ transplantation). In these cases, the treatment regimen is similar to the recommended regimen for HIV-infected individuals. Moreover, important considerations, such as the immunocompromised state, antifungal response, presence of neurological complications, therapy cost and toxicity, should be taken into account (Henao-Martinez *et al.*, 2018). An alternative treatment for CM in patients not infected with HIV but with refractory infection or intolerance to other antifungal drugs is therapy with fluconazole 800 mg/day; however, the data on this therapeutic regimen are scarce, and this treatment needs further investigation (Zhao *et al.*, 2018).

Alternatives to CM treatment also have been studied. The antidepressant sertraline (selective serotonin reuptake inhibitor) is one example. The in vitro and in vivo antifungal activity and synergistic activity of sertraline with other antifungals have been described (Trevino-Rangel Rde *et al.*, 2016; Rossato *et al.*, 2016; Zhai *et al.*, 2012). However, a phase 3 clinical trial did not find benefit for the use of sertraline as a therapeutic component, and this trial was terminated (Rhein *et al.*, 2019). Other treatment strategies include use of natural product derivatives (Li *et al.*, 2019a,b), administration of vaccines (Hester *et al.*, 2019) and extracorporeal filtration of yeast from cerebrospinal fluid to reduce the fungal burden (neurapheresis therapy) (Smilnak *et al.*, 2018).

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https://www.cdc.gov/fungal/diseases/cryptococcosis-gattii/statistics.html
C. gattii Infection Statistics.
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C. neoformans Infection Statistics.

Fungal Cardiac Infections

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Candida Species

Candida is a genus of yeasts that are part of the normal skin and gut flora, and are common colonizers of the genitourinary tract. They can be detected on the mucosal surfaces of 50%–70% of healthy individuals. *Candida* are common sources of nosocomial infections. While the genus encompasses at least 15 pathogenic species, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* are responsible for ∼ 95% of invasive diseases. Candidemia occurs when the host is immunocompromised, has a breach of the protective mucosal barriers, or has had prolonged antibiotic use (McCarty and Pappas, 2016; Pappas *et al.*, 2018). *C. albicans* remains the most common pathogenic *Candida* spp. Otherwise, the second most common pathogenic species varies widely between regions, medical centers, and intensive care units with *C. glabrata* or *C. parapsilosis* being the most frequently encountered species. In the United states and northwestern Europe, *C. glabrata* is the second most common species isolated (Pfaller *et al.*, 2011). We will be focusing on *C. albicans* and briefly touch on other relevant *Candida* species.

Pathogenesis

As mentioned above, there are three disposing factors for candidemia. Immunosuppression, particularly neutropenia, is a major risk factor for disease as is the bypassing of mucosal barriers. The mechanisms for prolonged antibiotic use increasing risk is through the removal commensal gut microbiota species that secrete protective factors that suppress *Candida* overgrowth (Fan *et al.*, 2015). *C. albicans* undergoes morphogenic transition between a unicellular yeast form and a multicellular, filamentous pseudo-hyphae and/or hyphae form. This dimorphism helps the organism evade the immune system as different states have distinct cell surface and wall compositions. Strains that are locked to one form have impaired virulence in mice model (Lo *et al.*, 1997). *C. albicans* also effectively adheres to endothelial and epithelial cells through the expression of adhesins along with the secretion of proteases and phospholipases, which also help with biofilm formation and tissue invasion (Schmidt *et al.*, 2012; Felk *et al.*, 2002). Neutrophils play the most important role in controlling invasive candidiasis. While monocytes and macrophages have mildly less *candida* killing capacity, they confer lymphocyte-independent memory that provides protection from future infections (Lionakis *et al.*, 2013).

Endocarditis

Candida endovascular infections usually manifests as endocarditis, which is an infection of the inner lining of the heart chambers and heart valves, or infection of a implanted device such as a pacemaker (McCarty and Pappas, 2016). *Candida* endocarditis is the most common form of fungal endocarditis with *C. albicans* being the most prevalent (24%) and the non-albicans *Candida* combining to represent the second most common etiology (28%) (Ellis *et al.*, 2001). In an observational cohort study of 70 confirmed *Candida* endocarditis cases, the most common species isolated were *C. albicans* and *C. parapsilosis*, where the latter tended to develop in diabetic patients and were community acquired (Arnold *et al.*, 2015). Overall, half the infections were hospital acquired, and about a quarter were community acquired. The biggest risks factor for endocarditis was the presence of a prosthetic heart valve (46%) in which there was a 36% in-hospital and a 59% 1-year mortality rate. In the same study, congestive heart failure, embolization, and intracardiac abscess were the most common complications (Arnold *et al.*, 2015).

The clinical presentation of *Candida* endocarditis typically consists of fever, major peripheral emboli (i.e., ischemic limb), and cardiac murmur (Ellis *et al.*, 2001). Both the Infectious Diseases Society of America (IDSA) 2016 candidiasis guideline and American Heart Association 2015 infective endocarditis guideline recommend combined antifungal and valve replacement therapies (Pappas *et al.*, 2015; Baddour *et al.*, 2015). Notably, some studies have found no statistically significant mortality benefit between medical vs medical and surgical treatment (Rivoisy *et al.*, 2017; Arnold *et al.*, 2015).

Myocarditis

Since the routine use of immunosuppressive medications, *Candida* have been the most prevalent cause of fungal myocarditis, an infection of the heart muscles, which occurs in 10%–60% in patients with disseminated candidiasis (Frenkel, 1962; Gruhn and Sanson, 1963; Walsh *et al.*, 1980). In 1968, a cardiac autopsy report of 420 acute leukemia patients identified a prevalence of *candida* myocarditis of 1.4%, while there was only one identified *Candida* (named "Torulosis") myocarditis in the 1402 cases during American military autopsies performed over the course of World War II (Gore and Saphir, 1947; Roberts *et al.*, 1968). In *Candida* myocarditis, histological findings primarily show myocardial abscesses with random distribution, which is consistent with hematogenous dissemination patterns (Franklin *et al.*, 1976). *C. albicans* is the most common *candida* species causing myocarditis, followed by *C. parapsilosis*, which is frequently associated with endocarditis (Pfaller *et al.*, 1998). Of note, pathogenesis of

myocarditis in the setting of *Candida* endocarditis is not an extension of the endocarditis vegetation itself, but myocardial abscess can progress to involve the endocardium (Atkinson *et al.*, 1984a,b).

Risk factors identified from multiple autopsy reviews include prolonged antibiotic therapy, corticosteroid administration, malignancy, autoimmune diseases, history major operations, central venous catheters, and intravenous (IV) drug use (Walsh *et al.*, 1980; Gaines and Remington, 1972; Franklin *et al.*, 1976; Hofman *et al.*, 1992). Cooccurrence rates with endocarditis range from 16% to 71% (Rubinstein and Lang, 1995; Walsh *et al.*, 1980; Parker, 1980; Atkinson *et al.*, 1984a). Franklin *et al.*'s case series from 1959 to 1974 of 31 confirmed post-mortem revealed that 59% of the cases had T-wave abnormalities, 23% with supraventricular arrhythmia, 14% with atrial-ventricular block, and only 1 case had normal ECG. Diagnosis can be difficult as blood cultures were only positive in 61% of confirmed *candida* myocarditis cases. Hypotension or shock occurred in 42% of the myocarditis cases. *Candida* is not a frequent invader of the blood vessels, but case reports have documented angioinvasion resulting in myocardial infarction (Franklin *et al.*, 1976; Van Kirk *et al.*, 1974; Atkinson *et al.*, 1984a). It is hard to pinpoint the mortality rate of *candida* myocarditis as the vast majority of reports are postmortem.

Aspergillus Species

Overview

This is a genus of mold that encompasses hundreds of species. *Aspergillus fumigatus* and *Aspergillus flavus* are the most common species to cause invasive diseases, primarily in immunocompromised individuals. *Aspergillus* endocarditis is second to *Candida* endocarditis with a ~20% prevalence (Ellis *et al.*, 2001; Pierrotti and Baddour, 2002). The overall rising trend in invasive aspergillosis (IA), along with invasive candidiasis, correlates with the increased use of immunosuppressive medications, antibiotics and invasive procedures (Nosanchuk, 2002).

Pathogenesis

Aspergillus inhabits decaying materials in the environment. The primary mode of infection is through inhalation of its asexual spores, the conidia. These spores are usually cleared by alveolar macrophages and neutrophils, but they may successfully evade killing and germinate into hyphae. Neutrophils are the dominant cells that eliminate hyphae from tissue. Indeed, neutropenia and corticosteroid-induced immunosuppression are the predisposing factors responsible for IA. IA in neutropenia is characterized by angioinvasion from unchecked hyphae growth that leads to thrombosis and hemorrhage (Stergiopoulou *et al.*, 2007). Corticosteroid-induced immunosuppression more often results in non-angioinvasion due to intact neutrophil presence, despite corticosteroid's many effects on phagocytosis and cytokine production, while lung tissue injuries are from local inflammation (Lionakis and Kontoyiannis, 2003; Dagenais and Keller, 2009).

Endocarditis

Aspergillus endocarditis (AE) is typically associated with underlying cardiac abnormalities and cardiac procedures such as prosthetic valve placements (Pierrotti and Baddour, 2002). Clinical presentation typically consists of fever, major peripheral emboli (i.e., ischemic limb), and cardiac murmur (McCormack and Pollard, 2011). Osler's nodes or Janeway lesions are not usually seen (Rubinstein and Lang, 1995). Meshaal *et al* observed in a series of 374 AE cases that a lack of fever, acute limb ischemia at presentation, aortic abscess, and prosthetic valve vegetation were strong predictors for AE (Meshaal *et al.*, 2018). Peripheral emboli often turn into mycotic aneurysms due to the angioinvasive nature of *Aspergillus* spp.

Diagnosis for *Aspergillus* endocarditis has been difficult as blood cultures are usually negative, and many cases are diagnosed post-mortem (Antinori *et al.*, 2014). *A. fumigatus* prosthetic valve endocarditis is usually presumptively diagnosed by positive serum (1, 3)-β-D-glucan (BDG) and galactomannan (GM) testing (McCormack and Pollard, 2011). A Cochrane review concluded serum galactomannan sensitivity and specificity at 0.78 and 0.85, respectively, for AE (de Heer *et al.*, 2019; Cruciani *et al.*, 2015). Meanwhile, PCR sensitivity and specificity were comparable at 80.5% and 78.5%, respectively (Cruciani *et al.*, 2015). Mortality rates for AE vary with reports of 68%–100% (McCormack and Pollard, 2011; El-Hamamsy *et al.*, 2005) with average survival length of 11 days (Ellis *et al.*, 2001).

The first line treatment for AE is voriconazole. For patients who cannot tolerate voriconazole, lipid formulation of amphotericin B or isavuconazole can be used (Marr *et al.*, 2015). For monotherapy resistant diseases, there is evidence that echinocandin and amphotericin combination can improve survival (Kontoyiannis *et al.*, 2003). Valve replacement is recommended as adjunct therapy, but surgical results are disappointing.

Myocarditis

In 1984, Atkinson *et al.* reviewed 3801 autopsies of immunocompromised patients and found 8 patients with *Aspergillus* myocarditis. In a following autopsy of just cardiac fungal infections in 60 patients, the author discovered another 8 cases of

Aspergillus myocarditis (Atkinson *et al.*, 1984a,b). Xie *et al* found *A. fumigatus* was the most common species (44%), followed by *A. flavus* (38%) and *A. terreus* (5%) (Xie *et al.*, 2005).

Risk factors for aspergillosis included chemotherapy, broad-spectrum antibiotics, corticosteroids, cardiac surgery, and AIDS (47, 170). Pathogenesis of *Aspergillus* myocarditis differs from *candida* myocarditis as it occurs with *Aspergillus* invading the pulmonary vessels followed by ascending infection into the endocardium and myocardium, direct invasion from the endocarditis vegetation, or hematogenous seeding (Young *et al.*, 1970; Zimmerman, 1950; Walsh and Hutchins, 1979; Buchbinder and Roberts, 1972; Welsh and Buchness, 1955). Because of angioinvasion, *Aspergillus* myocarditis usually presents with myocardial infarction in which approximately 15% of infarctions have accompanied ECG changes (Williams, 1974). Complications include arrhythmias, cardiac tamponade, heart block, and congestive heart failures (Carbone *et al.*, 1964; Nosanchuk, 2002). Diagnosis is often delayed due to negative blood cultures, with Xie *et al* observing that 12% of cases had positive blood cultures. Mortality is high, as most literatures are autopsy reviews. Mortality among AIDS patients is 100% (Xie *et al.*, 2005). Medical therapy for myocarditis is the same as for AE.

Histoplasma Capsulatum

Histoplasma capsulatum is one of the endemic dimorphic fungi. It is the most common cause of fungal pneumonia, but it is an infrequent cause of endovascular infections including endocarditis. This fungus exists as a mold in the soil, and, at 37°C and in tissues, it converts into yeast form. The fungus is endemic in Mississippi and the Ohio River valleys as well as in Latin America (Cano and Hajjeh, 2001; Kauffman, 2007).

Pathogenesis

In endemic regions, as much as 80% of young adults were previously infected (Edwards *et al.*, 1969). The most common route of infection is through inhalation of conidia followed by conversion to yeast form. The initial host response includes neutrophils, macrophages and dendritic cells. Notably, the fungus can replicate within macrophages travel to other organs via blood or lymph. Infection can be asymptomatic or lead to life-threatening disease. If the host effectively controls the infection, reactivation disease can occur if the cell-mediated immune system is subsequently suppressed or damaged by factors such as HIV, chemotherapy, immunomodulation medications, or cancer (Mihu and Nosanchuk, 2012; Luckett *et al.*, 2014; Woods, 2016).

Endocarditis

Two reviews indicate that *H. capsulatum* accounts for 1%–6% of the fungal endocarditis, behind *Aspergillus* (Ellis *et al.*, 2001; Pierrotti and Baddour, 2002). However, in a review of 21 patients with fungal prosthetic valve endocarditis in an endemic region, 20 patients were immunocompetent and histoplasma accounted for 14% of the cases (Boland *et al.*, 2011). Risk factors for histoplasma endocarditis includes the male sex, rheumatic heart disease and prosthetic valves with complicated perioperative course, but there are also cases involving bicuspid aortic valves, atrial myxoma, and luetic aortic valvulitis. Interestingly, Riddell *et al.*'s case series of 12 patients gathered from 2004 to 2012 described only one patient with HIV and two patients with malignancies. Six patients were in other immunosuppressed states such as congestive heart failure, liver cirrhosis, and chronic kidney diseases (Blair and Raymond, 1981; Riddell *et al.*, 2014).

Across multiple case series, the most consistent clinical symptoms are fever (92–98%), heart murmur (75%–76%), and major artery embolization (19%–32%). Blair described leukopenia (50%) and hepatomegaly or splenomegaly (48%), while Riddell *et al* described heart failure (38%) and splenomegaly (23%). Timing to diagnosis has been consistently prolonged on a scale of weeks to months. Aortic valve involvement was the most common (47%–58%) followed by mitral (19%–31%), and then tricuspid (7%–11%) (Blair and Raymond, 1981; Bhatti *et al.*, 2005; Boland *et al.*, 2011; Riddell *et al.*, 2014).

Diagnosis is usually delayed with negative blood cultures, and the most recent reviews estimating 16%–34% of the patients were serum culture positive and direct tissue cultures were 40%–73% positive. Antigen testing also lack sensitivity as the urine antigen tests was initially positive in 64%, while serum antigen was 50% (Riddell *et al.*, 2014; Bhatti *et al.*, 2005). Morality rate is 13% overall, and for prosthetic valve endocarditis, morality is generally reported as 50%–57% (Boland *et al.*, 2011; Bhatti *et al.*, 2005). However, Riddell *et al.*'s 14-case series included 10 patients with prosthetic valves who had a mortality rate of only 20%.

Myocarditis

There are reports of *H. capsulatum* myocarditis in patients with malignancy or AIDS. Myocarditis can occur by hematogenous dissemination (Merchant *et al.*, 1958). There has been one report of histoplasma myocarditis in an immunocompetent teenager after a mud run with suspected inhalation of heavy inoculum (Scott *et al.*, 2018). Within the tissue, histoplasma granulomas with numerous macrophages filled with yeast cells are usually present, but patients with AIDS may have minimal inflammation or granuloma formation (Binford, 1955; Altieri *et al.*, 1994; Anderson *et al.*, 1988). *H. capsulatum* myocarditis is usually clinically silent; however, sudden cardiac death, heart failure, tamponade, ventricular tachycardia, nonspecific ECG changes and

cardiomegaly can occur (Crawford *et al.*, 1961; Lewis *et al.*, 1985; Scott *et al.*, 2018). Amphotericin and flucytosine is the recommended treatment, and symptoms usually resolve after successful treatment (Kinney *et al.*, 1989).

Blastomyces Dermatitidis

Blastomyces dermatitidis is another of the endemic dimorphic fungi in the United States that primarily occurs in areas surrounding the Great Lakes, Mississippi and Ohio River valleys, and around the Saint Lawrence Seaway (Castillo *et al.*, 2016). Within the endemic area, *B. dermatitidis* likely resides in decaying vegetation around surroundings of lakes or other bodies of water (Baumgardner *et al.*, 1992).

Pathogenesis

Most infections are acquired via inhalation of mold forms with subsequent conversion to yeast phase in the lungs (Saccente and Woods, 2010). Once the mold reaches the lung, alveolar macrophages, neutrophils, and monocytes are the main responders (Drutz and Frey, 1985). The conversion to yeast form facilitates immune evasion (Smith and Gauthier, 2015). Blastomycosis typically induces a characteristic pyogranulomatous response. The elimination of the fungi is primarily dependent on the induction of cell-mediated immunity (Wüthrich *et al.*, 2016).

Cardiac infections are an exceedingly rare consequence of disseminated blastomycosis. As of 2013, Alhaji and Sadikot stated that there are only 5 case reports in the known literature with the oldest case reported back in 1914, which described multiple myocardial abscesses (Alhaji and Sadikot, 2013; Hurley, 1916). One case described in 1951 details a patient with primary infection of a lung tumor that progressed to endocarditis with septic thrombi resulting in major arterial occlusions to organs and lower extremities (Pond and Humphreys, 1952). Interestingly, disseminated blastomycosis is quite rare in patients with solid organ and bone marrow transplants when compared to other fungi such as the *Candida* spp., *Aspergillus* spp., and *C. neoformans* (Kauffman *et al.*, 2014). Clinical presentations are non-specific, and they include dyspnea with sputum production, fever, night sweats, tachycardia, leukocytosis. Skin lesions were not readily described in these case reports.

Disseminated blastomycosis is often delayed in its diagnosis due to its low prevalence, but blastomycosis should be considered in patients with pulmonary symptoms and skin involvement, which can increase the specificity to 64% in an endemic area (Chapman *et al.*, 1997). Blood cultures are generally negative. There is an urine antigen detection assay with a positive predictive value of 92.9% in patient blastomycosis, and 89.3% in cases of disseminated disease, but there seem to be strong false positives in patients with histoplasmosis and paracoccidioomycosis (Saccente and Woods, 2010). Moreover, its utility in cardiac infections has not been established. The optimal treatment approach for cardiac blastomycosis has not been determined, but amphotericin B is recommended for severe, disseminated disease (Chapman *et al.*, 2008). Morality of disseminated blastomycosis with cardiac involvement from the 5 case reports is 100%.

Coccidioides

Coccidiomycosis is caused by *Coccidioides* species, which are endemic fungi in the southwestern United States and Latin America. Most primary infections are asymptomatic, while symptomatic patients usually present with flu-like symptoms to pneumonia. Extrapulmonary presentation accounts for $<1\%$ of the cases (Saubolle *et al.*, 2007).

Pathogenesis

Pathogenesis is not completely understood, but inhalation of arthroconidia of this fungus undergoes morphogenic transition into a spherule, a multinucleated spherical structure. The spherule can subsequently release 800–1000 endospores into the nearby tissue, which than form into new spherules (Saubolle *et al.*, 2007). T cell-mediated immune response plays a crucial role in eradication, but how the response is regulated is largely unknown (Galgiani, 1993). The endospores can disseminate via lymphatics and blood (Saubolle *et al.*, 2007).

Endocarditis

Cardiac involvement may occur with disseminated disease, where the risk factors include the male sex, African or Filipino ancestry, pregnant women in their 2nd and 3rd trimester, and immunosuppression from medication or HIV (Saubolle *et al.*, 2007). Prolonged coccidioidomycosis increases risk for cardiac disease (Reuss *et al.*, 2004). However, cardiac involvement is very rare. Most common symptoms include cough, chest pain, and dyspnea. Infections have occurred on both prosthetic and native aortic or mitral valves. Blood cultures are rarely positive, and serological titers can range from 1:1 to1:2048 for endocarditis. In contrast to other fungal vegetations that are frequently large and bulky, *Coccidioides* vegetations may be small such that they can be missed by

transesophageal echocardiogram (Chan *et al.*, 2016). The recommended treatment is amphotericin B with surgery, which, in one study, resulted in 4 out of 6 patients surviving (Horng *et al.*, 2015; Chan *et al.*, 2016).

Myocarditis

From autopsy case series, myocarditis occurs at a rate between 9% to 28% in patients with disseminated coccidioidomycosis. Microscopically, spherules are dispersed within the myocardium, with abscesses characterized by central necrosis but lacks. Granulomas may be present but are frequently absent in individuals with AIDS (Gore and Saphir, 1947; Bloor, 1978; Chapman and Kaplan, 1957; Forbus and Besterbreutje, 1946). Patients with AIDS are at high risk for hematogenous disseminated infection (Minamoto and Rosenberg, 1997). Most cardiac lesions in patients with AIDS are clinically latent or may be masked by respiratory findings (Hofman *et al.*, 1992). Since the literature is largely autopsy reports, there is limited information on clinical symptoms of *Coccidioides* myocarditis. Chapman and Kaplan reported on six patients with cardiac infection that all had dyspnea and orthopnea. Of note, half of these patients also had pericarditis who also had chest pain with EKG showing S-T segment elevation and T wave abnormalities. Pericarditis can occur from myocardial abscesses rupturing into the pericardial space (Chapman and Kaplan, 1957; Chan *et al.*, 2016; Schwartz *et al.*, 1976; Forbus and Besterbreutje, 1946).

Cryptococcus Neoformans

Cryptococcus neoformans is the main causative pathogen of cryptococcosis where it is estimated to cause 80% of cryptococcosis cases worldwide. *Cryptococcus gattii* is responsible for most of the other cases (Heitman *et al.*, 2010). *C. neoformans* has a global distribution, and it is particularly abundant in soil rich in pigeon guano (Emmons, 1951).

Pathogenesis

Infection begins with inhalation of airborne yeast cells or basidiospores. In the lung, alveolar macrophages phagocytose the pathogens and then trigger CD4 + T Helper type 1 dependent immunity, which is crucial for anti-cryptococcal defense (Olszewski *et al.*, 2010). *Cryptococcus* has characteristic polysaccharide capsule that serves as its main source of virulence (Fromtling *et al.*, 1982). This capsule hinders phagocytosis and antibody binding, while promotes intracellular survival within phagosomes. Additionally, capsular component glucuronoxylomannan and galactoxylomannan are anti-inflammatory. Interestingly, the fungus has adapted to increase its capsule size after infecting its host (Granger *et al.*, 1985; Kozel *et al.*, 1977; Kozel, 1993; Alvarez *et al.*, 2008). *C. neoformans* also has a variety of other mechanisms to increase its survival within the host (Olszewski *et al.*, 2010), with melanin being especially important (Nosanchuk *et al.*, 2000; Nosanchuk and Casadevall, 2006). The fungus is also able to remain dormant within granulomas and can reactivate and disseminate in patients with HIV or other immunosuppressive conditions such as corticosteroid treatment. *C. neoformans* primarily presents as meningoencephalitis, but it can involve every organ, although cardiac involvement is rare (Kwon-Chung *et al.*, 2014).

Endocarditis

Cryptococcal endocarditis is a very rare entity. According to Roy *et al.*, there around 10 reported confirmed cases (Banerjee *et al.*, 1997; Boden *et al.*, 1983; Colmers *et al.*, 1967; Lombardo *et al.*, 1957; Alhaji and Sadikot, 2011; Blanc *et al.*, 1996; Harford, 1974; Roy *et al.*, 2018; Nakajima *et al.*, 2019; Kowatari *et al.*, 2019). Comorbidities that predispose these patients include rheumatic heart disease or mitral stenosis, prosthetic aortic or mitral valve, diabetes mellitus, chronic kidney disease, acute lymphocytic leukemia, and substance misuse. Clinical symptoms include dyspnea, weight loss, fever, splenomegaly, hepatomegaly, 1st degree atrial-ventricular block, and thromboembolic events. Diagnosis was delayed in all the cases due to initially negative blood cultures, but the fungus eventually grew in 66% of the cases. Serum cryptococcal antigen was detected in most cases. For treatment, Amphotericin-B is the initial drug and concomitant flucytosine should be considered. Infected prosthetic valves needed replacement, but native valve endocarditis may be treated with medical therapy alone with success. Overall, estimated overall mortality is ~44% (Nakajima *et al.*, 2019).

Myocarditis

Up until 1983, cryptococcal myocarditis was a rare opportunistic infection in patients with immunocompromised conditions such as cirrhosis, malignancies, or steroid use (Bergman *et al.*, 1967; Wilson *et al.*, 1970; Zimmerman and Rappaport, 1954). Since then, cryptococcus myocarditis, although still rare, has been more associated with AIDS. Cryptococcal myocarditis may have abscess, and histologically has yeast cells diffusely infiltrating the myocardium with minimal acute or chronic inflammation. Clinical symptoms are non-specific; although they can include heart failure with ECG abnormalities have been described (Lafont *et al.*, 1987; Nosanchuk, 2002).

Zygomycetes (Phycomycetes)

Zygomycetes is a class of molds where fungi within orders *Mucorales* and *Entomophthorales* are pathogenic (Chayakulkeeree *et al.*, 2006).

According to a review of 929 case reports, genera *Rhizopus* and *Mucor* are two most common culprits of zygomycosis. The male sex, diabetes and malignancy are the biggest risk factors at 65%, 36% and 17%, respectively. Solid organ transplant, bone marrow transplants, and HIV infection have lower associations at 7%, 5%, and 2%, respectively. Zygomycosis mainly involve rhino-orbital-cerebral infection. However, cardiac involvement is a rare but serious complication of disseminated disease, which has mortality approaching 100% (Roden *et al.*, 2005). Cardiac zygomycosis is thought to be secondary to primary pulmonary disease with angioinvasion, and it can result in pericarditis, myocarditis, myocardial infarction due to fungal thrombus, endocarditis, chordae tendineae rupture, arrythmias, and congestive heart failure (Tuder, 1985; Jackman and Simonsen, 1992). Standard therapy involves high dose Amphotericin B and aggressive surgery (Riley *et al.*, 2016).

Of the *Entomophthorales*, *Conidiobolus* spp. and *Basidiobolus ranarum* are the most commonly encountered, but they are usually located in tropical regions and are not angioinvasive, so they are not often associated with dissemination or cardiac infections (Ribes *et al.*, 2000). One team reported *Conidiobolus* endocarditis in a cocaine user where the organism demonstrated angioinvasion (Jaffey *et al.*, 1990). A patient with a renal transplant with disseminated *Conidiobolus coronatus* developed a pericardial effusion due to the fungus (Walsh *et al.*, 1994).

Miscellaneous Fungi

The following fungi are extremely rare causes of cardiac infections, with only one or a few case reports to date. These include *Fusarium soleni*, *Trichosporon beigelii*, *Blastoschizomyces capitatus* (Ghosh *et al.*, 2018; Ramos *et al.*, 2004; Polacheck *et al.*, 1992). Disseminated Phaeohyphomycosis can also lead to endocarditis or myocarditis, but this disease describes a group of melanin-pigmented dematiaceous fungi that include *Scedosporium prolificans*, *Myceliophthora thermophilia*, *Exophiala jeanselmei*, *Bipolaris spicifera*, and *Bipolaris* species (Nosanchuk, 2002). Cardiac involvement happens in disseminated stage of the disease especially in hosts with prosthetic valves or with immunocompromising conditions with AIDS, malignancy, end-stage renal disease, solid organ or bone marrow transplants.

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Fungal Ophthalmological Infections

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Introduction

Tremendous advances in medicine including immunosuppressive agents for organ transplants and autoimmune diseases, new chemotherapies to combat cancer, and the discovery of highly-active antiretroviral therapy to treat HIV/AIDS have saved lives and improved quality of life. With these advances, however, the incidence of observed local and systemic fungal infections has increased. Although fungal infections are significantly less common than bacterial infections of the human eye, there are a variety of fungal infections that ophthalmologists encounter in clinical practice.

Candida, *Fusarium*, and *Aspergillus* are common culprits of fungal keratitis, or infection of the cornea. Another term for fungal keratitis is keratomycosis. These infections occur most commonly following trauma to the eye with exposure to soil, organic, or vegetable matter, but can result from contact lens wear as well. *Candida*, *Aspergillus*, *Cryptococcus*, *Coccidioides*, and *Fusarium* are the most common causes of fungal endophthalmitis, or infection of the intraocular cavities (i.e., aqueous and vitreous humor), and most commonly reach the eye via hematogenous dissemination from a systemic infection. *Histoplasma capsulatum* can cause ocular histoplasmosis syndrome, a multifocal infection affecting the choroid and retina after hematogenous dissemination from inhalation of spores into the lungs. *Cryptococcus neoformans* can cause disease of the brain and optic nerve in an immunocompromised state, resulting in optic neuropathy. Ocular rhinosporidiosis from exposure to *Rhinosporidium seeberi* can present as sessile or pedunculated lesions in the conjunctiva. Exposure to *Blastomyces* and *Sporothrix* can result in focal granulomas or dermatitis affecting the eyelid and/or conjunctiva. Lastly, nonseptate filamentous fungi such as *Mucor* and *Rhizopus* can cause life-threatening infections of the paranasal sinuses, brain, and orbit in immunocompromised patients (American Academy of Ophthalmology, 2017a).

This chapter will focus on the two most common fungal infections of the eye: fungal keratitis and fungal endophthalmitis. For each infection, its epidemiology, signs and symptoms, diagnostic workup, and treatment will be described.

Fungal Keratitis

Overview

Infectious keratitis, or infection of the cornea from a viral, bacterial, fungal, or parasitic agent, can result in severe visual impairment. When infectious keratitis is associated with a corneal epithelial defect (or opening of the corneal surface), the infection is commonly described as a corneal ulcer. Fungal keratitis is much less common than bacterial keratitis, representing less than 10% of cases in developed countries and roughly 40% of cases in the developing world. The most common cause of fungal keratitis is trauma to the cornea from plant, organic, or vegetable matter. Other risk factors include contact lens wear, topical corticosteroid use, immunocompromised status, previous corneal surgery, chronic keratitis (particularly from herpes simplex virus), and other chronic erosions and/or pathology of the cornea.

Epidemiology and Risk Factors

In the United States, less than 10% of microbial keratitis cases are from fungal infections, although the prevalence is much higher in tropical climates. In southern Florida, fungal keratitis is estimated to be 35% of microbial keratitis cases, compared to 17% in Texas and 1% in New York (Asbell and Stenson, 1982). Worldwide, this relationship with climate is also evident; a study in Bangkok, Thailand found that 38% of ulcerative keratitis cases were from a fungal etiology, and the most common organism isolated was *Fusarium* species (Sirikul *et al.*, 2008).

Multiple studies have demonstrated that fungal keratitis is more likely than bacterial keratitis to be associated with ocular trauma, particularly from vegetable, organic, or plant matter. An investigation of fungal keratitis in South India demonstrated that 54.4% of cases were associated with a history of recent trauma to the affected eye. The object causing the trauma was not reported in 37.5% of cases, injury with plant material was reported in 13.9% of cases, injury with animal material was reported in 2.1% of cases, and a history of dust going into the eye was reported in 11.4% of cases. A significant percentage (48.3%) of patients were engaged in outdoor activities for their occupation (Gopinathan *et al.*, 2002).

Fungal keratitis is also less likely to be associated with contact lens wear than bacterial keratitis. However, in 2006, an outbreak of *Fusarium* keratitis was associated with use of ReNu with MoistureLoc (Bausch & Lomb, Rochester, NY, USA) contact lens solution, which was subsequently removed from the market. A retrospective case series performed during this time period demonstrated that among fungal keratitis cases, 22% of cultures grew yeasts, 72% grew filamentous fungi (of which 54% were *Fusarium* culture positive), 3% grew nonfilamentous fungi, and 3% grew fungi not further identified. Prior to and following this

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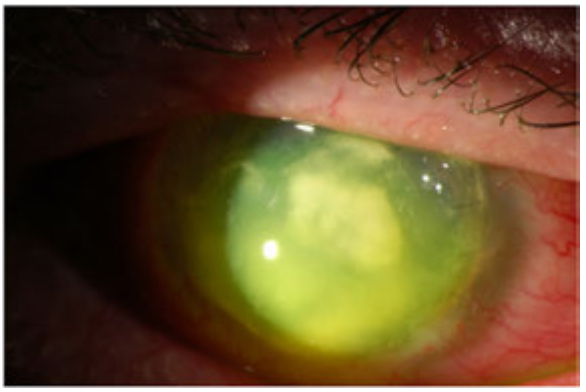

Fig. 1 Fungal keratitis secondary to *Fusarium.*

outbreak, fungal keratitis has been reported to comprise less than 5% of microbial keratitis cases among contact lens wearers overall (Chang *et al.*, 2006).

Other risk factors for fungal keratitis include systemic and/or topical corticosteroid use, immunocompromised status, previous corneal surgery, chronic keratitis, and chronic pathology of the cornea (American Academy of Ophthalmology, 2017a). Topical and systemic corticosteroids decrease the cornea's immune response and can increase the virulence of fungal organisms. Immunocompromised status can make fungal infection more likely as the body cannot mount an adequate immune response for defense against these organisms. Previous corneal surgery, chronic keratitis, or corneal pathology can lead to irregularities or erosions of the corneal surface, known as the epithelium, disrupting the physical protective barrier that it provides for the eye. This allows virulent organisms to penetrate the deeper corneal layers and infect the cornea.

The most common fungal species associated with fungal keratitis are *Fusarium* **Fig. 1**, *Aspergillus*, and *Candida*. In the Mycotic Ulcer Treatment Trial, a randomized clinical trial comparing antifungal medications to treat fungal keratitis in South India, microbial culture results demonstrated *Fusarium* growth in 39% of fungal cultures, *Aspergillus* in 16%, and *Curvularia* in 6% (Prajna *et al.*, 2013).

Signs and Symptoms

Symptoms of fungal keratitis include severely decreased visual acuity, foreign body sensation, tearing, discharge, and photophobia. A characteristic finding is eye pain that is severe and out of proportion to the amount of inflammation. On examination, patients with fungal keratitis often exhibit fewer signs of eye inflammation compared to patients with bacterial keratitis (i.e., minimal injection of the conjunctiva). The exam typically demonstrates a corneal infiltrate, or feathery, gray-white opacification of the cornea **Fig. 2**. The infiltrate may contain filamentous margins and/or multifocal satellite lesions. In severe cases, a plaque may be present along the most posterior layer of the cornea, known as the corneal endothelium, consisting of infectious material and indicating that the infection has progressed through the cornea and may penetrate into the anterior chamber of the eye. A hypopyon, or layering of inflammatory and white blood cells in the anterior chamber of the eye, may be present as well. Significant anterior chamber infection may lead to fungal invasion of the iris, resulting in elevated intraocular pressure. Invasion into the sclera can result in scleritis, which can be complicated by scleral thinning **Fig. 3** and/or nodules **Fig. 4**. Fungal invasion of the posterior chamber of the eye results in fungal endophthalmitis.

Diagnostic Workup

In clinical practice, a patient with suspected fungal keratitis requires a slit lamp examination, which involves the use of an upright microscope to examine the eye by an ophthalmologist. If fungal keratitis is suspected or observed, a culture is collected. To perform this procedure, a swab is scraped against the cornea to collect a sample from the infiltrate. This sample is then placed on agar plates with culture media and sent to a microbiology laboratory for analysis. Multiple culture media for fungi can be utilized, such as Sabouraud dextrose agar and brain heart infusion agar, and fungal hyphae and yeast cells are readily labeled with stains that include Gomori methanamine silver, Calcoflour white, and Periodic acid-Schiff (American Academy of Ophthalmology, 2017a).

In addition to laboratory evaluation, confocal microscopy can be useful to differentiate fungal from bacterial, viral, or parasitic keratitis. Confocal microscopy is an optical imaging technique that can capture multiple, two-dimensional images to create a reconstructed, three-dimensional image. In cases of suspected fungal keratitis, it can provide visualization of branching filaments, individual septa, and other fungal characteristics to aid in prompt diagnosis. Confocal microscopy may be able to differentiate fungal from bacterial keratitis while the clinician awaits culture results from the laboratory.

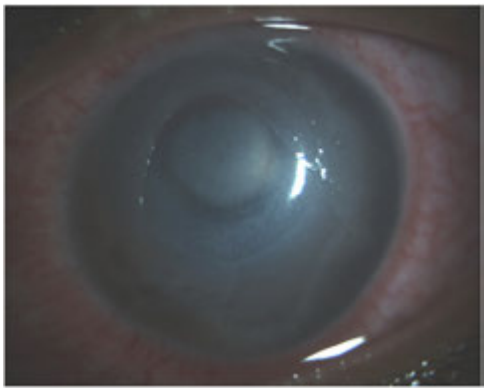

Fig. 2 A corneal infiltrate secondary to yeast infection. Note the white central opacification of the cornea.

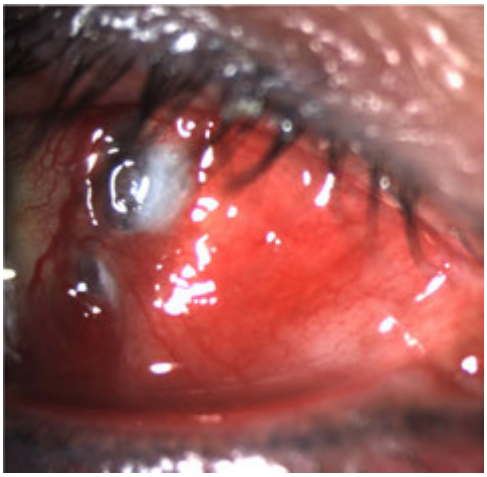

Fig. 3 Severe scleral thinning with visible uveal tissue. This patient developed a fungal keratitis with extension into the sclera secondary to *Aspergillus*.

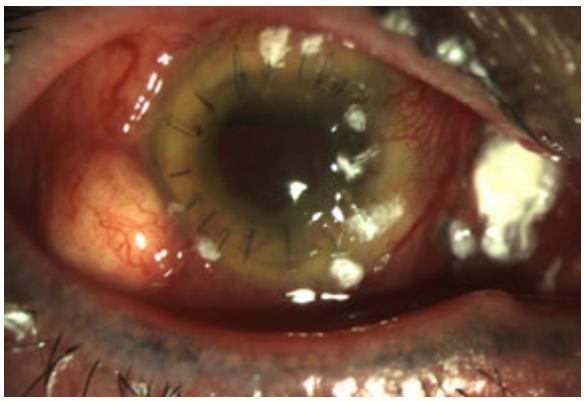

Fig. 4 Large nodule in *Aspergillus* scleritis. This patient previously received a tectonic penetrating keratoplasty secondary to severe *Aspergillus* keratitis.

Treatment

The initial treatment for fungal keratitis consists of topical antifungal medications. Natamycin 5% suspension is the gold-standard antifungal agent used for filamentous fungal keratitis, including *Fusarium* species. The Mycotic Ulcer Treatment Trial evaluated topical natamycin 5% and topical voriconazole 1% for the treatment of filamentous fungal keratitis, and found that natamycin treatment was associated with significantly better clinical and microbiological outcomes than voriconazole treatment, particularly in *Fusarium* cases (Prajna *et al.*, 2013). Topical amphotericin B (0.15%–0.30%) is the medication of choice in treating yeast keratitis, including *Candida* species, however it serves as an additional option for the treatment of filamentous keratitis caused by *Aspergillus* species.

Oral antifungal agents are frequently used in addition to topical agents to treat more severe cases of fungal keratitis. Among the azoles, ketoconazole, fluconazole, and itraconazole can be utilized. However, oral voriconazole and posaconazole have superior intraocular penetration and are often the preferred oral agents for fungal keratitis (and any fungal eye infection for which oral antifungal agents are considered). However, evidence in the medical literature is lacking to support the use of these oral agents in the treatment of fungal keratitis. The Mycotic Ulcer Treatment Trial II evaluated the use of oral voriconazole in addition to topical agents in the treatment of filamentous fungal keratitis and found no benefit to adding oral voriconazole (Prajna *et al.*, 2016). The study also showed a significant increase in adverse events in the group that received topical antifungals with oral voriconazole compared to the group that received topical antifungals alone (Prajna *et al.*, 2016).

In severe cases of fungal keratitis, a corneal transplant may be necessary to preserve corneal integrity or prevent scleral or intraocular extension if the infection progresses on maximal topical and/or oral antifungal therapy. This is known as a tectonic penetrating keratoplasty. If the infection clears solely with medical management, the patient may be left with a visually significant corneal scar. These patients may undergo routine penetrating keratoplasty to improve visual acuity.

Fungal Endophthalmitis

Overview

Endophthalmitis is inflammation of both the anterior and posterior chambers of the eye from bacterial or fungal infection. Endophthalmitis is generally subdivided into two types: exogenous and endogenous. Exogenous endophthalmitis occurs when bacteria or fungi are introduced into the eye from an external source, such as trauma, intraocular surgery, or progressive keratitis. Endogenous endophthalmitis results when bacteria or fungi are hematogenously disseminated into the eye.

Epidemiology

A retrospective study in Miami, Florida published in 2008 evaluated all culture-positive cases of exogenous fungal endophthalmitis over a sixteen year period and found that 44% of cases were associated with fungal keratitis, 24% of cases occurred after penetrating intraocular trauma, and 32% of cases occurred following intraocular surgery. Filamentous fungi accounted for 85% of cases and *Candida* species accounted for 15% of cases (Wykoff *et al.*, 2008).

Postoperative endophthalmitis can be classified as acute or chronic. Acute postoperative endophthalmitis typically occurs 48–72 hours after surgery, has an incidence between 0.07% and 0.1%, and is most commonly bacterial in etiology. Chronic postoperative endophthalmitis can occur as early as 1–2 weeks after surgery, however can present months and sometimes years later. The incidence of chronic postoperative endophthalmitis is not well-documented in the medical literature due to its insidious nature and delay in diagnosis, however the disease is known to be of both fungal or bacterial etiologies. Common fungal agents that can cause chronic postoperative fungal endophthalmitis include *Candida parapsilosis*, *Aspergillus flavus*, *Candida glabrata*, *Paecilomyces lilacinus*, and *Verticillium* species (American Academy of Ophthalmology, 2017b).

Risk factors for exogenous endophthalmitis following ocular trauma include retained intraocular foreign body, rural setting of injury, disruption of the crystalline lens during the trauma, and delay in primary wound closure. The incidence of exogenous endophthalmitis (bacterial and fungal) following open-globe injuries is approximately 1%, compared to the rate of 0.1% following intraocular surgery (Ahmed *et al.*, 2012). Fungal etiologies are less common than bacterial, but injury with vegetable, soil, or organic matter increases the likelihood of fungal infection. Common fungal agents include *Candida*, *Aspergillus*, *Paecilomyces*, *Fusarium*, and *Dematacious* species.

Endogenous endophthalmitis typically occurs following fungal infection of the bloodstream in severely immunocompromised individuals, intravenous drug users, those with prior use of indwelling catheters, or long-term antibiotic use. The most common fungal agents implicated in endogenous endophthalmitis include *Candida*, *Aspergillus*, and *Fusarium* (American Academy of Ophthalmology, 2017b).

Among cases of endogenous endophthalmitis secondary to *Candida* species, a history of recent gastrointestinal surgery, hyperalimentation, diabetes mellitus, and organ transplantation are implicated as risk factors (Shah *et al.*, 2008). *Candida albicans* is the most common pathogen, although other species such as *Candida parapsilosis*, *Candida tropicalis*, and *Candida glabrata* have also been identified as causative agents (Krishna *et al.*, 2000).

The incidence of endogenous endophthalmitis is considered rare, however reported incidences vary. Studies in the medical literature report that the incidence of endophthalmitis in patients with a positive fungal blood culture for *Candida* ranges from 0% to 52%; however, in 1994 a classification for ocular candidiasis was published and reported incidences for endophthalmitis subsequently declined. A 2019 systematic review demonstrated that the cumulative incidence rate among studies across years was 2.3% (Breazzano *et al.*, 2019).

Signs and Symptoms

Symptoms of fungal endophthalmitis include blurred vision, photosensitivity, floaters, and eye pain. In cases following ocular surgery, patients may present with delayed-onset, progressive inflammation that is unresponsive to or even worsens after corticosteroid therapy. Ocular examination may demonstrate decreased visual acuity, periorbital and eyelid edema, conjunctival

injection, corneal haze, anterior chamber cell or inflammation, hypopyon, vitreous haze, retinal hemorrhages, retinal exudates, cotton wool spots, vascular sheathing, and areas of retinal whitening. Ocular involvement is typically unilateral in exogenous endophthalmitis as the etiology is frequently from surgery or trauma. Endogenous endophthalmitis is also typically unilateral, however both eyes can be affected simultaneously.

Diagnostic Workup

Any patient with ocular signs and symptoms concerning for endophthalmitis must seek ophthalmologic evaluation and receive a dilated eye exam. A thorough history is crucial to determine a possible etiology, which will guide subsequent workup.

If trauma has occurred and an intraocular foreign body is suspected, diagnostic imaging may aid in its detection. Axial computed tomography (CT) scans with fine cuts through the orbit of 0.5–1.0 mm are recommended for visualization of a metallic foreign body of 0.5 mm or larger. CT has limited ability to visualize materials such as wood, ceramics, and plastics, thus magnetic resonance imaging (MRI) can be utilized to localize a foreign body not appreciated on CT. However, MRI employs a strong magnetic field that can attract metal, thus a metallic intraocular foreign body must be definitively excluded first (Ahmed *et al.*, 2012).

If exogenous endophthalmitis is suspected, then aerobic bacterial, anaerobic bacterial, and fungal cultures of the aqueous and vitreous humor must be obtained. An anterior chamber paracentesis is performed to collect an aqueous humor sample and vitreous aspiration is performed to collect a vitreous humor sample; these procedures can be performed in an ophthalmologist's office. If the decision is made to proceed to surgery for both diagnosis and treatment, a pars plana vitrectomy is performed in an operating room to gain access to the vitreous cavity for vitreous aspiration.

Recommended studies from aqueous and/or vitreous samples include Gram stain, Giemsa stain, and polymerase chain reaction (PCR) of suspected fungi. Cultures must be promptly sent off to a microbiology laboratory. Fungal organisms can be slow-growing and insidious in nature, thus cultures should be retained by the laboratory for at least 2 weeks to ensure adequate time for organism growth.

If endophthalmitis is discovered due to ocular symptoms in the absence of recent trauma, surgery, or previous eye infection, the etiology is presumed to be endogenous. The patient's medical history and any active, systemic infections or diseases must be considered. Consultation with an infectious disease specialist is recommended to evaluate for systemic disease and aid in management.

Screening for endogenous endophthalmitis is performed routinely in clinical practice; the Infectious Diseases Society of America recommends that all patients with positive *Candida* blood cultures undergo a dilated ophthalmologic examination, regardless of ocular symptoms or clinical status (Pappas *et al.*, 2016). These patients are typically monitored closely for the development of endophthalmitis and receive a repeat dilated ophthalmologic examination 2 weeks after the initial examination. However, a recent systematic review found that most patients captured by routine ophthalmologic screening will have no associated ocular findings, and those who do will respond without changing systemic management, thus the potential harm of examination and possible invasive treatment may outweigh the perceived benefit of screening asymptomatic patients (Breazzano *et al.*, 2019).

Treatment

In cases of exogenous endophthalmitis associated with ocular trauma, prompt evaluation by an ophthalmologist is crucial to ensure that the integrity of the eye is preserved after injury; if trauma results in a full-thickness wound and intraocular contents are exposed, this is an ophthalmologic emergency called a ruptured globe or an open-globe injury. Immediate surgery by an ophthalmologist to restore the integrity of the globe is necessary. If the injury involves soil material, the patient may require tetanus immunization depending on prior vaccination history (Ahmed *et al.*, 2012).

If fungal exogenous endophthalmitis is suspected, injection of intravitreal antifungals can be performed at the time of vitreous culture. Frequently in clinical practice, both antibiotics and antifungals are injected concurrently if the etiology of the infection is unknown. If pars plana vitrectomy is performed, intravitreal antifungals are injected at the time of surgery. The intravitreal antifungals of choice in the treatment of fungal endophthalmitis are amphotericin for yeast coverage and voriconazole for filamentous fungal coverage. These injections may be supplemented with systemic antifungals, although the role of systemic therapy is not well established or proven.

In cases of endogenous fungal endophthalmitis secondary to *Candida* species, treatment is often successful with systemic medications alone; frequently azole antifungals are used as they can achieve therapeutic intravitreal levels and have broad-spectrum anti-fungal activity. If treatment is ineffective with systemic antibiotics, pars plana vitrectomy with injection of intravitreal antifungals may be effective in de-bulking the pathogen load (American Academy of Ophthalmology, 2017b).

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AIDS-Related Mycoses

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Candidiasis

Epidemiology

Oropharyngeal candidiasis (OPC) and esophageal candidiasis (EC) are the most common HIV-associated fungal opportunistic infections and are an important cause of morbidity in people living with HIV (Lortholary *et al.*, 2012; Low *et al.*, 2016). The incidence of OPC is ~16%–40% in antiretroviral therapy (ART)-naive patients (Attia *et al.*, 2001; Wolff *et al.*, 2005; Desakorn *et al.*, 2011), and EC is the second most common AIDS-defining illness (Grabar *et al.*, 2008). There is a clear correlation between worsening immunodeficiency and the occurrence of OPC and EC, with a CD4+ cell count of <200 cells/μL a risk factor for both (Mönkemüller *et al.*, 2005; Lortholary and Dupont, 2011). Depletion of the IL-17 producing T-helper-17 cells, a subset of the CD4 cell lineage, allows Candida to overcome epithelial defenses and is key to the pathogenesis of mucosal candidiasis (Cassone and Cauda, 2012).

Candida albicans is the most common causative pathogen, however there is significant geographical variation in the prevalence of non-albicans species (~10%–60%) such as *C. glabrata, C. parapsilosis, C tropicalis and C. krusei* (Mushi *et al.*, 2017; Patil *et al.*, 2018). The widespread introduction of ART has significantly lowered the incidence of OPC and EC (Schwarcz *et al.*, 2013; Low *et al.*, 2016). However, mucocutaneous candidiasis remains an important cause of morbidity due to lack of access to healthcare services, increasing prevalence of azole-resistant Candida species, and in people on ART with poor CD4+ T cell recovery (Patil *et al.*, 2018).

The impact of HIV on vulvovaginal candidiasis (VVC) is uncertain; some studies suggest a higher incidence, while others suggest higher rates of colonization without vaginitis only (Duerr *et al.*, 1997; Ohmit *et al.*, 2003). Multiple other risk factors contribute (antibiotic use, diabetes, vaginal dysbiosis and pH) and VVC is common in women without HIV. Furthermore, HIV is not a risk factor for invasive candidiasis (Kullberg and Arendrup, 2015; Pappas *et al.*, 2018), so this chapter will focus on OPC and EC in HIV.

Clinical Presentation

OPC has different clinical forms. Pseudomembranous candidiasis appears as creamy-white plaques which can be removed with a swab to reveal erythematous or bleeding mucosa, whereas in hyperplastic candidiasis there are homogenous adherent or nodular lesions (Akpan and Morgan, 2002; Patil *et al.*, 2015). In erythematous candidiasis, there are localized patches of erythema, often on the dorsum of the tongue or palate (Akpan and Morgan, 2002) and angular cheilitis appears as fissuring at the corners of the mouth. The pseudomembranous and erythematous variants are the most common in people living with HIV. OPC is often asymptomatic, but may present with pain (particularly with erythematous candidiasis or angular cheilitis) or taste disturbance (Darouiche, 1998).

EC often presents with odynophagia, dysphagia, and/or retrosternal pain (Darouiche, 1998). While concurrent oropharyngeal lesions are common, EC can occur in isolation (Darouiche, 1998; Vazquez, 2010). Symptoms may result in reduced oral intake and weight loss. Important differential diagnoses include other infectious causes of esophagitis (e.g., cytomegalovirus, herpes simplex virus), gastroesophageal reflux disease, strictures, esophageal carcinoma and Kaposi's sarcoma.

Diagnosis

OPC is a clinical diagnosis, based on the appearance of oral lesions. A swab for fungal culture should be performed in those who fail to respond to initial therapy or have recurrent episodes to assess for azole resistance. In patients with oropharyngeal lesions and symptoms of EC, a diagnostic trial of antifungal therapy is appropriate before investigation with endoscopy (Lortholary *et al.*, 2012; Pappas *et al.*, 2015; Dockrell *et al.*, 2019). Endoscopy should be performed in patients with lack of clinical response, those without oropharyngeal lesions, or to exclude an alternative diagnosis (Dockrell *et al.*, 2019). The diagnosis can be confirmed by endoscopic visualization of lesions, alongside tissue biopsies demonstrating microbiological and histopathological evidence of candidiasis.

Management

Prophylaxis

ART initiation is key to preventing OPC and EC and reducing relapses through immune restoration. Primary prophylaxis with fluconazole has been shown to be effective (particularly when CD4 count <50 cells/uL) (Powderly *et al.*, 1995; Schuman *et al.*, 1997; Lortholary *et al.*, 2012) but is not recommended as it propels resistance emergence (Fichtenbaum *et al.*, 2000). Secondary prophylaxis to prevent relapses is also generally not recommended. For multiple relapses, daily or weekly therapy reduces relapse frequency without increasing the risk of developing fluconazole-refractory disease, compared to episodic fluconazole therapy administered for each relapse (Goldman *et al.*, 2005). Concomitant risk factors and predisposing co-morbidities (inhaled corticosteroids, dentures, oral hygiene, diabetes) should also be optimized.

Treatment

Fluconazole is the recommended first-line therapy for both OPC and EC in HIV patients given its efficacy, availability and low cost (Lortholary *et al.*, 2012; Pappas *et al.*, 2015; Dockrell *et al.*, 2019). It has excellent oral bioavailability and good penetration into gastric tissue and saliva (DeMuria *et al.*, 1993; Felton *et al.*, 2014). Fluconazole 100–200 mg/day for 7–14 days is recommended treatment of OPC, and 200–400 mg/day for 14–21 days in EC (Lortholary *et al.*, 2012; Pappas *et al.*, 2015; Dockrell *et al.*, 2019): the higher dose and duration is indicated for severe or relapsed disease. Fluconazole can be administered orally (tablet or liquid formulation) or parenterally in patients with odynophagia or dysphagia. Studies examining the efficacy of fluconazole have reported clinical cure rates of ~85%–90% of HIV patients with OPC (Pons *et al.*, 1993, 1997; Sangeorzan *et al.*, 1994; Flynn *et al.*, 1995) and EC (Phillips *et al.*, 1998; Villanueva *et al.*, 2002).

For milder cases of OPC, topical therapies such as nystatin (100,000 units/mL, 5 mL four times daily for 7–14 days), clotrimazole trouches (10 mg trouche, five times per day 7–14 days) or miconazole buccal adhesive tablets may be considered (Pons *et al.*, 1993; Flynn *et al.*, 1995; Vazquez *et al.*, 2010). Nystatin and clotrimazole are less efficacious, with higher rates of relapse when compared to fluconazole (Pons *et al.*, 1993, 1997; Powderly *et al.*, 1995).

Refractory OPC or EC is defined as persistent symptoms despite ≥14 days of ≥200 mg/day of fluconazole (Lortholary *et al.*, 2012). Prior azole exposure and persistent immunodeficiency (particularly CD4 count <50 cells/μL) are risk factors. In such cases, Candida speciation and susceptibility testing are advised to tailor further therapy. While cross resistance to triazoles can occur, itraconazole is effective in 55%–65% of fluconazole-resistant cases (Phillips *et al.*, 1996; Saag *et al.*, 1999), and posaconazole in 74% of itraconazole- and fluconazole-resistant isolates (Skiest *et al.*, 2007). Itraconazole oral solution has better bioavailability than capsules, with similar clinical and mycological cure rates as oral fluconazole (Wilcox *et al.*, 1997; Phillips *et al.*, 1998). Echinocandins have a similar efficacy to fluconazole, but higher rates of relapse and can only be administered intravenously (Villanueva *et al.*, 2002; Krause *et al.*, 2004). Hence, echinocandins should be reserved for use in pan-azole resistant cases or where there is a contraindication to azole administration. Amphotericin B deoxycholate (0.5 mg/kg/day) is less well tolerated compared to echinocandin therapy (Villanueva *et al.*, 2001; Arathoon *et al.*, 2002) and should be reserved for use when there are no suitable alternatives. With all therapies, but particularly the triazoles which are potent CYP450 enzyme inhibitors, it is crucial to consider drug-drug interactions prior to prescribing.

Cryptococcosis

Epidemiology

Cryptococcosis is the most common AIDS-related opportunistic mycosis globally, with an estimated 223,000 infections occurring each year causing 180,000 deaths, accounting for 15% of AIDS-related deaths (Rajasingham *et al.*, 2017). Exposure to the causative pathogens, the ubiquitous environmental saprophytes *Cryptococcus neoformans* or *Cryptococcus gattii*, is via inhalation (Perfect and Casadevall, 2002; May *et al.*, 2016). In the context of advanced HIV infection with depressed CD4 T-cell count (<100 cells/μL), dissemination from the lungs occurs either following acute exposure or reactivation of latent infection, spreading hematogenously to most organs, with a predilection for the central nervous system resulting in cryptococcal meningoencephalitis (CM) (Mitchell and Perfect, 1995; Williamson *et al.*, 2016).

Disease burden is greatest in sub-Saharan Africa (Rajasingham *et al.*, 2017) where despite excellent ART coverage, CM incidence remains high (Osler *et al.*, 2018; Tenforde *et al.*, 2019) and access to the best antifungal drugs is lacking (Loyse *et al.*, 2013). In the HIV-infected patient, CM can present as an AIDS-defining opportunistic infection in the ART-naïve patient, as unmasking of latent infection through immune restoration within the first few months of starting ART, or as a manifestation of defaulted or failed ART, with the latter now responsible for >50% of CM presenting in sub-Saharan Africa (Molloy *et al.*, 2018; Osler *et al.*, 2018; Rhein *et al.*, 2018).

Clinical Presentation

CM usually presents sub-acutely, presenting most commonly as headache, sometimes accompanied by confusion, drowsiness, seizures, visual or hearing loss. In advanced HIV, clinical signs may be absent but can include reduced conscious level, meningism,

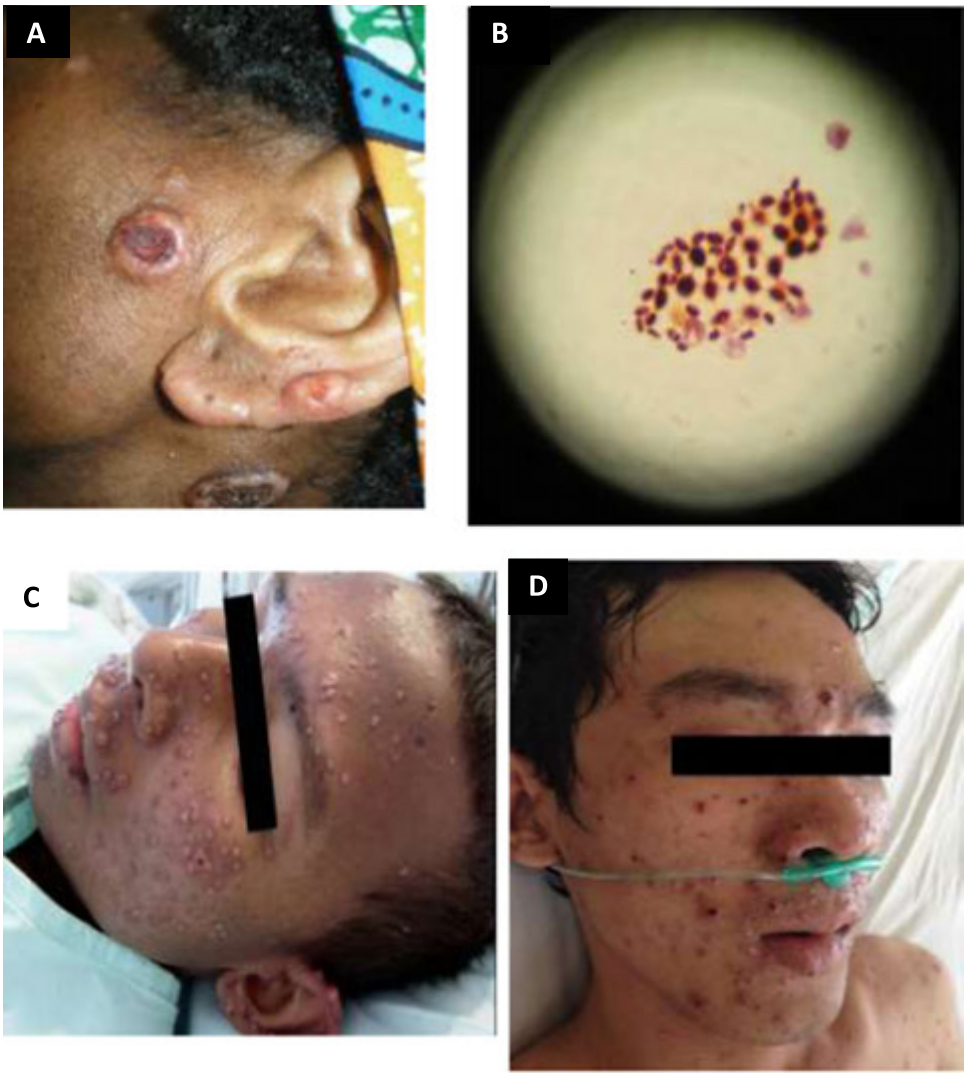

Fig. 1 *Cutaneous lesions associated with disseminated mycoses in HIV/AIDS*. A. Ulcerated nodules in South African female with unmasking disseminated cryptococcosis (serum CRAG >1:2048) characterized as diarrhea, weight loss and shortness of breath developing one month after commencing ART. B. Gram stain of aspirate from facial lesion in A. revealed budding yeasts (*C neoformans*). C. Progressive disseminated histoplasmosis in Colombian male with multiple facial papules, some showing ulceration Disseminated talaromycosis in a Vietnamese male presenting with 1 month of fever, fatigue, and diarrhea and 4 days of characteristic central-necrotic skin lesions skin lesions that began on the face and spread to his trunk and extremities.

papilledema and cranial nerve palsies – most commonly VI nerve palsy secondary to raised intracranial pressure (CSF opening pressure >20 cm), (Graybill *et al.*, 2000; Bicanic *et al.*, 2009; Rolfes *et al.*, 2014). Altered mental status and elevated baseline CSF fungal burden (cryptococcal antigen/CRAG titers>1024 or quantitative cryptococcal culture) are adverse prognostic markers (Saag *et al.*, 1992; Jarvis *et al.*, 2014). Cryptococcomas can occur following immune restoration through ART, presenting as focal neurological signs (Haddow *et al.*, 2010).

Pulmonary cryptococcosis can have myriad manifestations from asymptomatic to cough and dyspnea with focal or diffuse pulmonary infiltrates, which can be misdiagnosed as pulmonary tuberculosis (Jarvis and Harrison, 2008). Other common manifestations in HIV-infected patients include skin lesions (a manifestation of disseminated disease)- most frequently papules with a soft or ulcerated center (**Fig. 1(A)**). Other infection sites include bone and lymph nodes (Mitchell and Perfect, 1995).

Diagnosis

A lumbar puncture (LP) is the investigation of choice in HIV-infected patients presenting with a CD4 count <100 cells/μL and headache. Typical CSF findings include lymphocytosis (can be absent in advanced HIV), mildly elevated protein (0.5–1 g/dL) and low CSF glucose. The India ink test, showing the characteristic yeasts with a surrounding halo, is a simple and cheap test which is 70%–90% sensitive in patients with HIV/AIDS (Bicanic and Harrison, 2004), who have higher fungal burdens compared to

immunocompetent patients. The detection of capsular polysaccharide antigen (CRAG) in CSF or serum is >95% sensitive and specific for diagnosis of CM (WHO, 2019). CRAG titers correlate with CSF fungal burden by quantitative culture: although titers fall with successful treatment, the kinetics of the decline are very slow and cannot be used to monitor response to therapy nor in deciding when to stop maintenance therapy (Brouwer *et al.*, 2005; Perfect *et al.*, 2010).

A cheap CRAG lateral flow device is now widely available at $2/ test (sub-Saharan Africa) and greatly facilitates diagnosis, including of early dissemination via a serum CRAG (Jarvis *et al.*, 2009; Lindsley *et al.*, 2011). The pooled prevalence of cryptococcal antigenemia is 6.5% in patients with advanced HIV and CD4 count <100 cells/μL (Ford *et al.*, 2018). A serum CRAG titer >1:160 is predictive of CNS infection and mandates an LP even in asymptomatic patients (Wake *et al.*, 2018). Blood cultures may be positive in patients with disseminated infection, including those presenting with CM. Cryptococci can also be visualized in skin or lymph node biopsies using specialized fungal stains such as GMS, PAS or mucicarmine (capsule stain) (Bicanic and Harrison, 2004).

Primary antifungal resistance to amphotericin B, fluconazole and flucytosine is rarely observed in global isolate collections (Pfaller *et al.*, 2005), however Cryptococcus has an intrinsic heteroresistance to fluconazole which will be missed by conventional MIC testing (Sionov *et al.*, 2009), but will be selected for and predominate upon exposure to fluconazole, resulting in relapse with secondary resistance (Stone *et al.*, 2019). Fluconazole MIC testing is recommended for patients with a culture-positive relapse during the consolidation or maintenance phase of treatment (Govender *et al.*, 2019).

Management

Drug therapy

Antifungal therapy for CM in HIV/AIDS is some of the best studied for invasive mycoses, with evidence from phase II/ III trials incorporating both fungal clearance and mortality supporting a 1–2-week induction regimen using Amphotericin B deoxycholate (AmBd, 1.0 mg/kg/day) plus flucytosine (5FC, 100 mg/kg/day) as the gold standard for HIV-CM (Van Der Horst *et al.*, 1997; Brouwer *et al.*, 2004; Day *et al.*, 2013; Molloy *et al.*, 2018), followed by consolidation with 8 weeks' fluconazole at 400–800 mg/d, then maintenance therapy with 200 mg until immune restoration with ART to CD4 count >100 cells/μL and a fully suppressed HIV viral load (Perfect *et al.*, 2010; WHO, 2019).

For settings where intravenous AmBd is unavailable or cannot be safely administered, an all-oral regimen of fluconazole plus 5FC was non-inferior to AmB-based regimens in a recent large African randomised trial, ACTA (Molloy *et al.*, 2018), as well as cost effective compared to fluconazole monotherapy (Shiri *et al.*, 2020). Liposomal amphotericin B (L-AmB) is less toxic compared to AmBd but far more expensive: at 3–6 mg/kg/d for 2 weeks, outcomes were no better compared to AmBd based regimens (Hamill *et al.*, 2010). L-AmB pharmacokinetics facilitate higher intermittent dosing − a single dose of 10 mg/kg on a backbone of 2 weeks' fluconazole plus 5FC is being compared to the standard 2-week induction regimen in the AmBition African phase III trial, with results expected in 2021 (Lawrence *et al.*, 2018; Jarvis *et al.*, 2019). In terms of adjunctive immunotherapy in HIV-CM, dexamethasone was associated with worse fungal clearance and outcome in a phase III trial (Beardsley *et al.*, 2016), whilst IFN-γ enhanced fungal clearance in two phase II trials (Jarvis *et al.*, 2012). 10-week mortality with the optimized antifungal induction regimen in clinical trial settings is 20%–40% (Day *et al.*, 2013; Molloy *et al.*, 2018; Boulware *et al.*, 2020). In the real-world setting in sub-Saharan Africa, where fluconazole monotherapy is marred by poorer fungal clearance and clinical failure due to treatment-emergent resistance(Hope *et al.*, 2019; Stone *et al.*, 2019), this figure approaches 50%–70% (Longley *et al.*, 2008; Rothe *et al.*, 2013; Gaskell *et al.*, 2014). Several novel antifungals with anti-cryptococcal activity are in the pipeline, whilst repurposed agents sertraline and tamoxifen have been disappointing (Beardsley *et al.*, 2016; Boulware *et al.*, 2020).

Raised intracranial pressure (ICP)

Raised ICP must be managed aggressively by CSF drainage through serial lumbar punctures, shown to mitigate its impact on mortality (Rolfes *et al.*, 2014). Temporary drains can be inserted for refractory ICP in CM (Macsween *et al.*, 2005). Almost half of HIV-CM survivors suffer neurological sequelae, including cognitive impairment, visual or hearing loss (Goldberg *et al.*, 2018).

Cryptococcal antigen screening

To prevent CM in advanced HIV, the WHO recommends a serum CRAG-based screening strategy for Persons living with HIV (PLHIV) with CD4 counts <100 cells/mm^3, and fluconazole pre-emptive treatment in those testing positive, until immune reconstitution (WHO, 2019). Even with this approach, CRAG-positive patients suffer excess 6-month mortality compared to CRAG negatives (Mfinanga *et al.*, 2015; Wake *et al.*, 2020): ongoing trials (ACACIA NCT03945448; EFFECT) are exploring addition of either L-AmB or flucytosine to fluconazole to treat cryptococcal antigenemia.

Immune restoration and ART timing

Immune restoration through ART is essential for all ART-naïve patients with AIDS-related mycoses: if ART has not already started, initiating ART after 4–6 weeks of optimal antifungal therapy is recommended in CM (Boulware *et al.*, 2014). IRIS (immune response inflammatory syndrome) can occur either as unmasking (CSF culture positive) or paradoxical (CSF usually culture negative) (Haddow *et al.*, 2010): the latter occurs in 10%–30% of CM patients, usually in the early months following start of ART: CSF cultures are usually sterile, and management includes control of raised ICP and short-course steroids.

Histoplasmosis

Epidemiology

Histoplasmosis, caused by the dimorphic fungus *Histoplasma capsulatum*, is the most frequent cause of fungal respiratory infection and has a broad spectrum of clinical manifestations ranging from a self-limited, acute, influenza-like illness to life-threatening progressive disseminated infection (Deepe, 2020; Thompson and Gómez, 2019). Histoplasmosis is highly endemic in regions of North America, Central America, and South America, as well as being reported in parts of Asia and Africa (World Health Organization/Pan American Health Organization, 2020; Nacher *et al.*, 2016; Bahr *et al.*, 2015). The risk of histoplasmosis is greatest in those with $CD4^+$ counts of <200 cells/μL. Despite advances in the treatment of HIV infection, incidence and mortality associated with *H. capsulatum* infection remains high: with an estimated 15,600 new cases and 4500 deaths in the Americas each year (Adenis *et al.*, 2018), where it is estimated to be responsible for 5%–15% of AIDS-related deaths (World Health Organization/Pan American Health Organization, 2020).

The environmental niche for *H. capsulatum* is soil enriched in nitrogen contained in bird and bat guano. Infection begins when small mycelial fragments and microconidia of *H. capsulatum* are inhaled into the alveoli, following which they bind to the CD11-CD18 family of integrins and are engulfed by neutrophils and macrophages (Newman, 1999). After transformation of conidia into yeasts in the lungs, yeasts migrate to local draining lymph nodes and subsequently to distant organs rich in mononuclear phagocytes (e.g., liver, spleen).

The extent of disease is determined both by the number of conidia inhaled and the immune response of the host. Although all primary infections can be considered disseminated because yeast cells migrate from the lungs to organs rich in mononuclear phagocytes, the term progressive disseminated histoplasmosis (PDH) refers to the relentless growth of the organism in multiple organ systems (Deepe, 2020; Thompson and Gómez, 2019). Following exposure, histoplasmosis has been reported to reactivate and cause disease following later immunocompromise, often many years after leaving an endemic area (Miceli *et al.*, 2016).

Clinical Presentation

PDH is the most frequent clinical presentation of histoplasmosis in HIV/AIDS. Symptoms of PDH are non-specific and may be indistinguishable from those of other infectious diseases, especially tuberculosis, complicating diagnosis and treatment (Deepe, 2020; World Health Organization/Pan American Health Organization, 2020; Adenis *et al.*, 2014). Common clinical findings, reported in $>90\%$ of patients with AIDS and PDH, include fever, fatigue, weight loss, and respiratory symptoms (Hage *et al.*, 2015; Deepe, 2020). The most common sites of extrapulmonary dissemination are the liver, spleen, gastrointestinal tract and bone marrow (Hage *et al.*, 2015). In severe PDH in HIV-infected patients, defined as the presence of symptoms/ signs involving >1 vital organ and functional impairment (WHO performance status score >2) (World Health Organization/Pan American Health Organization, 2020), gastro-intestinal manifestations were most common (84%), followed by pulmonary manifestations (76%), lymphadenopathy (58%) and skin lesions (49%) (Caceres *et al.*, 2016) (**Fig. 1(C)**). Laboratory findings include abnormal liver enzyme values and pancytopenia (Deepe, 2020; Caceres *et al.*, 2016). Up to 20% of patients with PDH in one case series were reported to have central nervous system (CNS) involvement (Wheat *et al.*, 1990a,b).

Diagnosis

Culture of *H. capsulatum* is highly specific and remains the gold standard diagnostic test for histoplasmosis, but takes several weeks. Studies show variable sensitivity when cultures are not properly processed (e.g. when blood cultures are not processed by lysis centrifugation), with overall sensitivity of 77% (range 60%–90%) from blood cultures in HIV patients, with much poorer performance from respiratory sample cultures (0%–60%) (Caceres *et al.*, 2019). Where available, histopathological examination of involved tissues reveals distinctive 2–4 μm, oval yeasts with a single narrow-based bud (Deepe, 2020; Thompson and Gómez, 2019; Miceli *et al.*, 2016). Other diagnostic methods include detection of specific host antibodies against *Histoplasma* antigens, detection of circulating *Histoplasma* antigens and detection of fungal DNA.

Antibody tests have high specificity, but low sensitivity (38%–70%) in HIV, likely due to the degree of immunosuppression (World Health Organization/Pan American Health Organization, 2020; Caceres *et al.*, 2019; Hage *et al.*, 2015). Antigen detection assays are the diagnostic modality of choice for PDH in HIV, with overall sensitivity of 95% and specificity of 97% (Connolly *et al.*, 2007; Cáceres *et al.*, 2018; Caceres *et al.*, 2019; World Health Organization/Pan American Health Organization, 2020). Histoplasma antigen levels in urine and serum typically decrease after initiation of antifungal therapy, so serial antigen tests are useful to monitor response to therapy (Hage *et al.*, 2011; Caceres *et al.*, 2014). Antigen detection kits have recently become commercially available (World Health Organization/Pan American Health Organization, 2020; Cáceres *et al.*, 2018), facilitating implementation in clinical laboratories and reducing technical issues related to reproducibility and quality control. Settings implementing *Histoplasma* antigen testing have seen a significant increase in the number of diagnosed cases (Samayoa *et al.*, 2017; Caceres *et al.*, 2015; Bansal *et al.*, 2019; Falci *et al.*, 2019). The recent development of a histoplasma antigen lateral flow assay holds promise of a simple and reliable testing platform (sensitivity 96%, specificity 90%) (Cáceres *et al.*, 2020), but it is not yet commercially available.

PCR-based assays have reported sensitivity of 95% and specificity of 99% (Caceres *et al.*, 2019), but the lack of methodological consensus and unavailability of commercial kits means these assays are not yet in widespread clinical use (World Health Organization/Pan American Health Organization, 2020; Thompson and Gómez, 2019).

Treatment

Amphotericin B deoxycholate has long been the recommended induction therapy of histoplasmosis (Wheat *et al.*, 1990a,b) but is associated with considerable toxicities, including infusion reactions, renal failure, electrolyte abnormalities, and anemia (Johnson *et al.*, 2002; Wheat *et al.*, 2007). A single small randomized trial (n = 80) comparing the efficacy of amphotericin B preparations for induction therapy for moderately severe to severe disseminated histoplasmosis among people living with HIV was suggestive of higher clinical success rate and lower risk of death and nephrotoxicity with the liposomal compared to the deoxycholate preparation (Johnson *et al.*, 2002). To date, no randomized trials have compared amphotericin B formulations with triazole antifungal agents for treatment of PDH.

For treatment of severe or moderately severe histoplasmosis in HIV-infected patients, guidelines recommend liposomal amphotericin B (L-AmB) at 3 mg/kg/d for two weeks, with higher doses and duration (3–5 mg/kg/day for 4–6 weeks) used for those with meningitis. In settings where L-AmB is not available, Amphotericin B deoxycholate (AmBd) 0.7–1.0 mg/kg/d is recommended, with measures to prevent and monitor toxicity (Deepe, 2020; World Health Organization/Pan American Health Organization, 2020). Following symptom resolution, therapy is stepped down to itraconazole (200 mg three times daily for 3 days, then 200 mg twice daily) for a total duration of 12 months and/or until clinically stable with immune restoration and HIV viral load suppression on ART (Deepe, 2020; World Health Organization/Pan American Health Organization, 2020). Mild-to-moderate histoplasmosis in HIV can be treated with itraconazole alone for 12 months (Deepe, 2020; World Health Organization/Pan American Health Organization, 2020), with amphotericin B for those failing to improve on or intolerant of azole therapy.

ART should be initiated as soon as possible in HIV-infected patients with disseminated histoplasmosis without CNS involvement (World Health Organization/Pan American Health Organization, 2020); drug-drug interactions are common with triazoles and ART, specifically protease inhibitors and NNRTIs (discussed below). Immune reconstitution inflammatory syndrome (IRIS) associated with histoplasmosis is unusual, occurring at about 0.74 cases per 1000 person-years (Melzani *et al.*, 2020). The features of histoplasmosis-associated IRIS are non-specific and resemble disseminated histoplasmosis. Typically, antifungal therapy does not need to be adjusted. Short-course oral steroid therapy can be considered if there are life-threatening complications (1–2 mg/kg per day of prednisone or equivalent for 1–2 weeks followed by dose-tapering for two weeks) (World Health Organization/Pan American Health Organization, 2020).

Pneumocystis Pneumonia (PCP)

Epidemiology

The dominant opportunistic infection at the beginning of the AIDS epidemic, HIV-associated PCP incidence has declined dramatically in high resource countries over the past three decades due to the introduction of potent combination ART and effective chemoprophylaxis (Maini *et al.*, 2013; Buchacz *et al.*, 2016; Kelley *et al.*, 2009). Despite this decline, PCP remains an important AIDS-defining event (Buchacz *et al.*, 2016), particularly amongst people unaware of their HIV infection or not on ART. Overall mortality of HIV-associated PCP is consistently 10%–13% (Walzer *et al.*, 2008; Schmidt *et al.*, 2018; Roembke *et al.*, 2014), even in cohorts after the widespread introduction of ART (Walzer *et al.*, 2008). Survival is much lower amongst patients with respiratory failure, usually below 70% (Curtis *et al.*, 2000; Fei *et al.*, 2009; Monnet *et al.*, 2008), and outcomes are particularly poor if mechanical ventilation is required. PCP is increasingly recognized as an important cause of pneumonia amongst HIV-infected people in resource-limited countries (de Armas Rodriguez *et al.*, 2011). A recent systematic review found a PCP prevalence over 20% amongst HIV-infected inpatients presenting with respiratory symptoms in sub-Saharan Africa (Wasserman *et al.*, 2016). Outcomes are poor in these settings, with case fatality rates of 15%–30% (Wasserman *et al.*, 2016), reaching 60% in those admitted to intensive care at one center (Chiliza *et al.*, 2018).

Pathophysiology and Clinical Presentation

Absolute and functional CD4 + T cell depletion in HIV-infection results in uncontrolled organism replication and high fungal burden, triggering immune responses that lead to lung damage and characterize PCP pathophysiology. Incidence of HIV-associated PCP is inversely related to CD4 count and most cases occur at CD4 counts below 200 cells/μL (Hoover *et al.*, 1993; Stansell *et al.*, 1997). The typical clinical presentation is a sub-acute respiratory illness with progressive dyspnea, cough (which may be productive), and fever (Thomas and Limper, 2004). Constitutional symptoms including weight loss and sweats may also be present (Chiliza *et al.*, 2018). Although onset of respiratory symptoms is usually gradual, over 2–3 weeks, a subset of patients can present with rapidly progressive symptoms over days (Chiliza *et al.*, 2018). Unmasking (ART-associated) or paradoxical immune reconstitution inflammatory syndrome (IRIS) is well-described in HIV-associated PCP, presenting similarly to *de novo* infection (Barry *et al.*, 2002; Koval *et al.*, 2002; Wislez *et al.*, 2001).

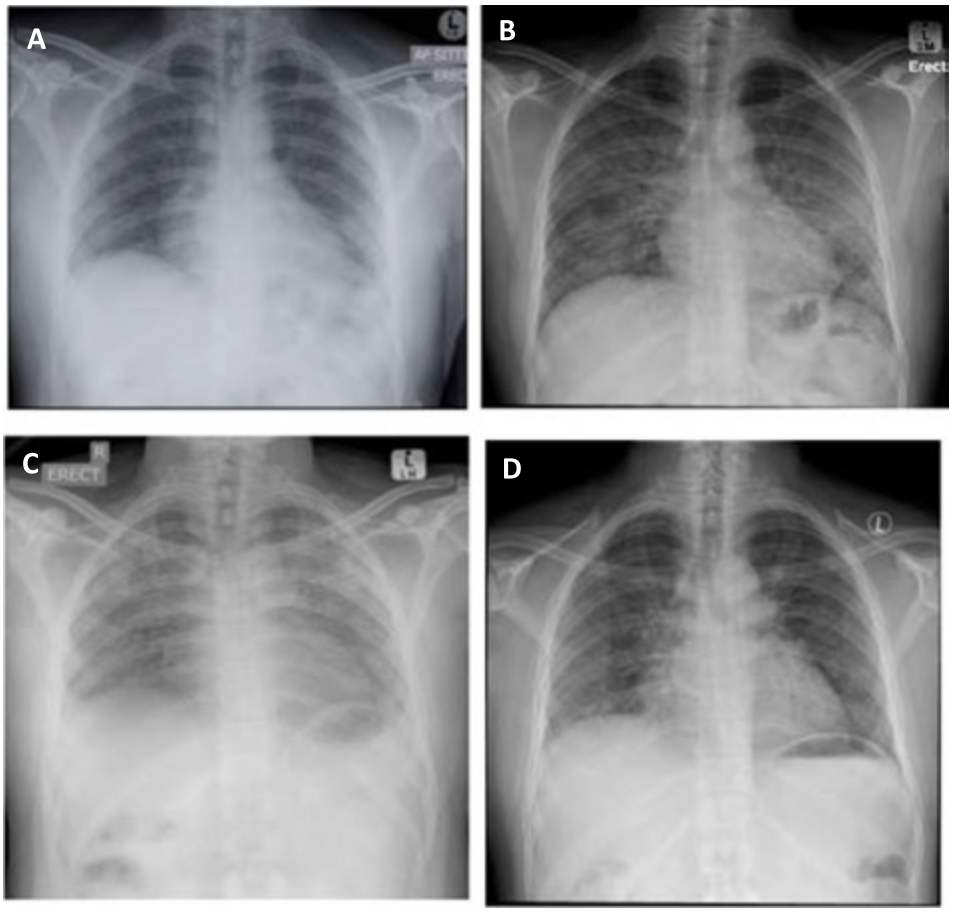

Fig. 2 *Radiographic features of HIV-associated PCP.* 36-year-old man, presented with progressive respiratory symptoms and newly diagnosed with HIV, CD4 cell count 41 cells/mm3. He was unable to produce sputum and *P. jirovecii* testing was not performed. Chest X-ray showed bilateral interstitial and ground glass opacification on presentation (A), becoming more confluent after 5 days (B) along with worsening hypoxia despite high dose cotrimoxazole and corticosteroids. Therapy was switched to clindamycin and primaquine after 2 weeks due to persistent respiratory failure and extensive alveolar-interstitial infiltrates on chest x-ray suggestive of acute respiratory distress syndrome (C). Hypoxia persisted a month after presentation with residual bilateral ground-glass opacification and cyst formation (D).

Exercise-induced oxygen desaturation on pulse oximetry occurs in >90% of PCP cases (Smith *et al.*, 1988, 1992; Stover *et al.*, 1989), and together with compatible clinical presentation and radiology is strongly supportive of the diagnosis. Serum LDH, reflecting lung injury (Quist and Hill, 1995), is elevated in most confirmed PCP cases and has been associated with worse outcome (Chiliza *et al.*, 2018; Zaman and White, 1988; Benson *et al.*, 1991) but does not distinguish PCP from other severe opportunistic infections, including disseminated TB (Butt *et al.*, 2002). Other non-specific inflammatory markers, such as C-reactive protein and procalcitonin, also have poor discriminatory value (Mendelson *et al.*, 2018). In contrast, serum beta-1,3-D-glucan is frequently elevated (Sax *et al.*, 2011) and is a useful diagnostic biomarker for HIV-associated PCP.

Chest X-ray typically shows diffuse bilateral interstitial infiltrates, usually with both central and peripheral distribution, becoming confluent with disease progression (**Fig. 2**). Unilateral involvement occurs infrequently (DeLorenzo *et al.*, 1987). High resolution computed tomography (HRCT) can detect subtle ground-glass opacification missed on plain radiography, and although non-specific, has high sensitivity and negative predictive value for HIV-associated PCP (Hidalgo *et al.*, 2003).

Diagnosis

Bronchoscopy with BAL is the gold standard diagnostic test but is not widely available in high HIV burden areas and is rarely used in the routine management of patients with suspected PCP in these settings. Immunofluorescent or histochemical staining will identify *P. jirovecii* on induced sputum. In a meta-analysis including 322 HIV-infected patients, the sensitivity and specificity of induced sputum Pneumocystis immunofluorescence was 67% and 96% respectively compared to BAL as the reference test. Polymerase chain reaction (PCR) has excellent diagnostic performance in BAL fluid (Fan *et al.*, 2013) and is a good rule-out test on induced sputum (NPV 96%) (Moodley *et al.*, 2017) and even oral wash samples (NPV 91%) (Helweg-Larsen *et al.*, 1998). However, performance varies between sample type, molecular target, and PCR method. The World Health Organization definition

of probable PCP incorporates clinical and radiological features only which are non-specific, especially in the context of COVID-19 (World Health Organization, 2007). A research priority is to derive and validate PCP prediction algorithms, especially in low resource populations with high burden of tuberculosis.

Management

Antimicrobial therapy

Trimethoprim-sulfamethoxazole (TMP-SMX) is the preferred first-line treatment for HIV-associated PCP, regardless of severity. Dosing and duration of therapy is the same as for HIV-negative disease (TMP-SMX 80/400 mg (single strength) 12–16 tabs/day for 21 days), followed by secondary prophylaxis with TMP-SMX two single strength tablets daily until CD4 count >200 cells/μL. Treatment failure on TMP-SMX occurs in approximately 10%; the most frequent cause is TMP-SMX toxicity (predominantly rash and hepatitis), but a subgroup of patients experience progressive disease despite tolerating TMP-SMX (Benfield *et al.*, 2008; Helweg-Larsen *et al.*, 2009). Clindamycin-primaquine is superior to other agents when used as second-line therapy after failing TMP-SMX (Smego *et al.*, 2001; Benfield *et al.*, 2008). Alternative agents for mild disease include trimethoprim-dapsone (Safrin *et al.*, 1996) or atovaquone (for patents with glucose-6-phosphate dehydrogenase (G6PD) deficiency) (Falloon *et al.*, 1991). Intravenous pentamidine is poorly tolerated and associated with increased mortality (Helweg-Larsen *et al.*, 1999). There is interest in adjunctive echinocandin therapy based on promising data from animal models (Cushion *et al.*, 2010; Lobo *et al.*, 2013) but currently insufficient evidence exists to recommend routine use.

Adjunctive therapy

Corticosteroids provide consistent survival benefit (44% relative reduction in mortality) and reduction in mechanical ventilation (60% lower rates) for patients with HIV-associated PCP and respiratory failure (Ewald *et al.*, 2015). International guidelines recommend early initiation of prednisone (or equivalent) for all cases with severe PCP or associated hypoxia (paO_2 <70 mmHg (9.3 kPa)). Optimal dose and duration are unknown, but the standard regimen is prednisone 40 mg 12-hourly for 5 days, followed by 40 mg daily for 5 days and 20 mg daily for 11 days. Tapering can be delayed in very severe or unresponsive disease, or when IRIS is suspected.

Antiretroviral therapy

ART initiation within 2 weeks of PCP diagnosis reduced risk of death and was not associated with increased toxicity or IRIS in a randomized controlled trial (Zolopa *et al.*, 2009). All patients with PCP should be initiated on standard ART as soon as tolerating oral anti-pneumocystis treatment. Clinical-radiological deterioration after starting ART should prompt evaluation to exclude other causes before attributing to paradoxical IRIS.

Ventilatory support

Patients with respiratory failure who are awake and able to maintain their airway benefit from continuous positive airway pressure (CPAP) by face mask (Gregg *et al.*, 1990). Intubation and mechanical ventilation is reserved for patients that do not improve on CPAP (Boix *et al.*, 1995), and should be delivered with low tidal volumes and plateau pressures (Davis *et al.*, 2008). Extracorporeal membrane oxygenation (ECMO) has been used successfully for refractory ARDS (Ali *et al.*, 2016; Cawcutt *et al.*, 2014).

Emergomycosis

Epidemiology

Emergomycosis is a severe multisystem HIV-associated mycosis caused by a novel thermally dimorphic fungus, *Emergomyces* sp. Initially described as an *Emmonsia*-like fungus among patients with advanced HIV and systemic mycosis in South Africa (Kenyon *et al.*, 2013), subsequent morphological and phylogenetic observations led to reclassification as *Emergomyces africanus* (Dukik *et al.*, 2017), which is now the most frequently diagnosed cause of dimorphic infection in South Africa with an estimated 100 cases annually (Maphanga *et al.*, 2017; Schwartz *et al.*, 2018a). Other similar species have been reported worldwide but appear to be less important (Schwartz *et al.*, 2019). The ecological niche is unknown but based on molecular detection the mycelial form of *Es. africanus* may have a reservoir in soil (Schwartz *et al.*, 2018b). Transmission and pathophysiology are thought to be similar to that of other pathogens in the family Ajellomycetaceae.

Clinical Presentation

Emergomycosis occurs almost exclusively in advanced HIV. In the largest published case series median CD4 cell count was 16 cells/μL (interquartile range 6–60) and the majority of patients were either ART-naïve or failing ART; recent initiation (within 4 weeks of presentation) of ART has been associated with emergomycosis suggesting possible unmasking IRIS. Clinical presentation may be indistinguishable from other opportunistic diseases, including other dimorphic fungal infections, creating diagnostic challenges. Fever and cutaneous lesions are almost universal, and most patients have subclinical pulmonary involvement

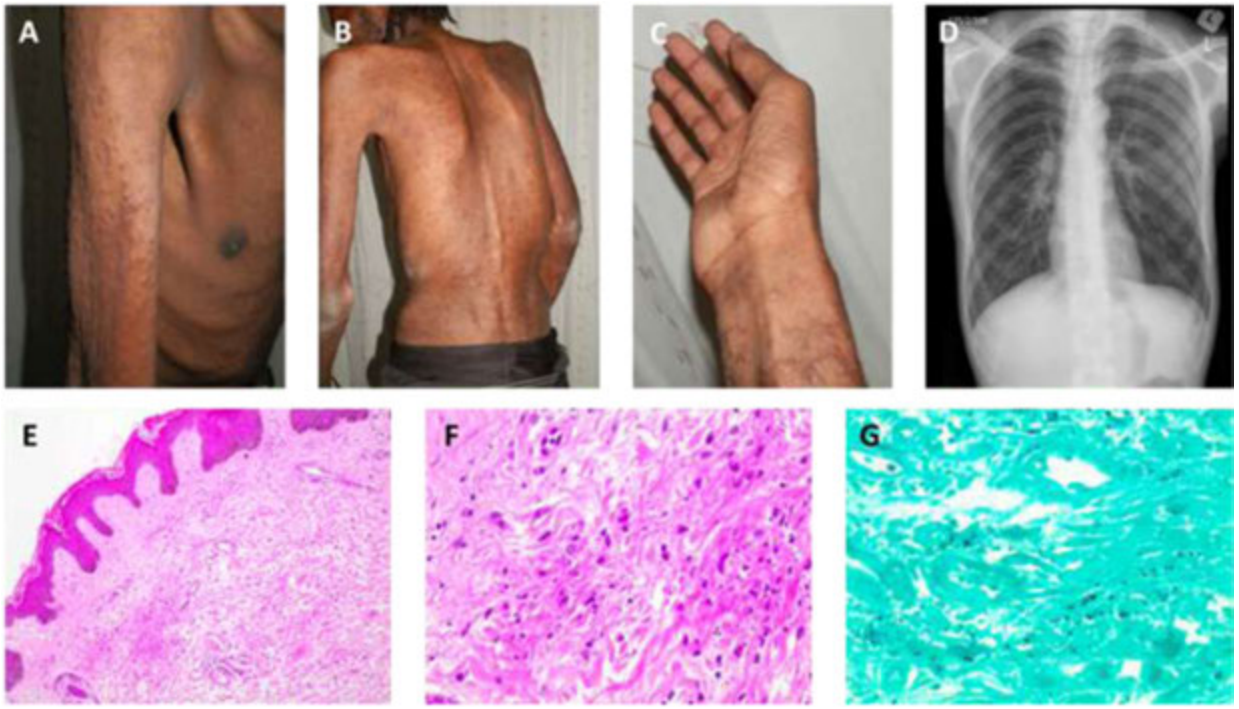

Fig. 3 *Clinical, radiological, and histological appearance of disseminated emergomycosis.* 34-year-old man with HIV and CD4 cell count 6 cells/mm3 after interrupting ART, presented with fever, rash, weight loss, and cholestatic hepatitis. On examination there were widespread papular erythematous lesions (Figs. A – C). CXR showed subtle bilateral nodular infiltrates (Figure D). Biopsy of a skin lesion demonstrated granulomatous inflammation and intracellular yeasts on H&E (Figs. E and F) and PAS (Figure G) staining.

evidenced by radiographic abnormalities on chest X-ray (diffuse and focal infiltrates, lobar atelectasis, and hilar adenopathy) (Schwartz *et al.*, 2017; Kenyon *et al.*, 2013). Skin involvement is usually generalized with heterogeneous morphological features including papules, plaques, nodules, ulcers, and hyperpigmented patches (Schwartz *et al.*, 2015, 2017) (**Fig. 3(A–D)**). Hepatomegaly and intraabdominal lymphadenopathy are common (Schwartz *et al.*, 2017). Paradoxical IRIS has been described with worsening of cutaneous lesions approximately 4 months after initiating effective ART (Crombie *et al.*, 2018).

Diagnosis

Emergomycosis should be considered in all patients from southern Africa presenting with advanced HIV, cutaneous lesions, and fever. Skin biopsy for histopathological examination and fungal culture is the diagnostic procedure of choice, supplemented by fungal blood cultures (fungaemia occurs in up to 50% of cases) (Schwartz *et al.*, 2017; Kenyon *et al.*, 2013). Bone marrow aspiration and biopsy also has good diagnostic yield (Schwartz *et al.*, 2015). The finding of PAS-positive intracellular yeasts with granulomatous or suppurative dermal inflammatory infiltrate on tissue biopsy is supportive but does not distinguish emergomycosis from other HIV-associated dimorphic fungal infections. In tissue, *Es. africanus* yeasts are small globose or oval measuring 2–7 μm in diameter, often exhibiting single narrow-based budding; inflammation may be absent in severely immunocompromised patients (Govender and Grayson, 2019). Definitive diagnosis requires molecular identification from a clinical sample or positive culture. Indirect diagnostic tests such as 1,3-β-D-glucan and *Histoplasma* galactomannan urine antigen assay may be positive but appear to have poor discriminatory value (Schwartz *et al.*, 2015). It is important to screen for tuberculosis in patients with suspected emergomycosis, as these conditions are difficult to distinguish and may co-exist.

Management

The case fatality of emergomycosis is up to 40% even with antifungal therapy (Schwartz *et al.*, 2017; Kenyon *et al.*, 2013; Maphanga *et al.*, 2017). The recommended antifungal treatment is similar to other endemic mycoses in immunocompromised patients: amphotericin B deoxycholate dosed 1 mg/kg daily or, where possible, liposomal amphotericin B dosed at 3 mg/kg daily for 10–14 days, followed by oral itraconazole for at least 1 year. Voriconazole and posaconazole have in vitro activity against *Es. africanus* and are alternative options for consolidation/maintenance therapy (Maphanga *et al.*, 2017). Fluconazole is slightly less active in vitro but may be considered if other triazole agents are unavailable or contraindicated, preferably after confirmation of susceptibility.

ART should be initiated within 2 weeks. There are important pharmacokinetic drug-drug interactions between itraconazole and antiretrovirals: non-nucleoside reverse transcriptase inhibitors should be avoided, and itraconazole dose should be reduced when co-administered with boosted protease inhibitors (Schwartz and Wasserman, 2017). Dolutegravir is unlikely to result in drug interactions and is an alternative for patients who require itraconazole.

Talaromycosis

Epidemiology

Talaromycosis, caused by the dimorphic fungus *Talaromyces marneffei* (Tm), is a leading cause of AIDS-related mycoses in Southeast Asia (Limper *et al.*, 2017), with an estimated 17,300 new infections causing 4900 deaths each year (Ning *et al.*, 2020). In highly-endemic regions in northern Thailand, Vietnam, and southern China, talaromycosis is a leading cause of AIDS-related opportunistic infections, accounting for 4%–20% of HIV admissions (Jiang *et al.*, 2019; Le *et al.*, 2011; Hu *et al.*, 2012; Supparatpinyo *et al.*, 1992), with up to 50% mortality in patients with and without HIV (Jiang *et al.*, 2019; Le *et al.*, 2011; Kawila *et al.*, 2013). Advanced HIV disease (CD4 count <100 cells/μL) is the main driver of talaromycosis incidence (Le *et al.*, 2011; Hu *et al.*, 2012; Supparatpinyo *et al.*, 1992), both of which remain prevalent in the ART era. Talaromycosis is increasingly reported in persons with a primary immunodeficiency condition due to idiopathic CD4 lymphopenia, anti-interferon-γ autoantibody, mutations in CYBB, CD40L, or gain-of-function mutation in the STAT1/STAT3 pathways (Chan *et al.*, 2016), and is emerging in the general population due to increased incidence of malignancies and increased use of immunosuppressive therapy and organ transplantation (Chan *et al.*, 2016). To date, talaromycosis has been reported in 20 countries spanning 6 continents outside of the endemic region due to travel and immigration (Ning *et al.*, 2020; Antinori *et al.*, 2006; Cristofaro and Mileno, 2006).

Tm has been cultured from the soil and in internal organs of wild bamboo rat species living in the highlands of Southeast Asia (Cao *et al.*, 2011; Huang *et al.*, 2015). Human infection occurs via inhalation of fungal spores and is associated with occupation in cultivation of plants and livestock (Chariyalertsak *et al.*, 1997; Le *et al.*, 2015) and residence in or travel to highland regions of Southeast Asia (Le *et al.*, 2015). Incidence increases by 30%–50% during the rainy and humid months (Le *et al.*, 2011; Chariyalertsak *et al.*, 1996), suggesting that humidity facilitates expansion of the environmental reservoir, resulting in increased exposure and new infections. Reactivation of latent infection can occur up to 7 years after exposure (Cristofaro and Mileno, 2006; Antinori *et al.*, 2006; De Monte *et al.*, 2014).

Clinical Presentation

Disseminated infection involving the reticuloendothelial system, lungs, gastrointestinal tract, blood stream, and skin is the most common manifestation of talaromycosis in patients with advanced HIV disease (Limper *et al.*, 2017). The subacute onset and generalized symptoms of fever, weight loss, and fatigue are indistinguishable from HIV wasting syndrome, tuberculosis, salmonellosis, and other systemic mycoses. The characteristic central-necrotic skin lesions on the face, trunk, and extremities, seen in 40%–70% of patients, are the hallmark yet late manifestations of talaromycosis (**Fig. 1(D)**) (Le *et al.*, 2011; Chen *et al.*, 2017).

Hepatosplenomegaly is present in 70% of patients. An acute pulmonary syndrome of cough, dyspnea, and infiltrates on chest X-ray is present in 40% of patients and is associated with a more rapid disease progression and a higher risk of death (Le *et al.*, 2011, 2017). Acute CNS infection was seen in 21 of 677 (3%) of patients in one case series, with a mortality of 80% (Le *et al.*, 2010). Common laboratory abnormalities include anemia, thrombocytopenia, and transaminitis.

Localized infections are more commonly seen in non-HIV infected patients. These include chronic pulmonary cavity lesions, obstructive tracheal lesions, pericarditis with pericardial effusion, osteoarticular lesions, abdominal abscess, genital chancroid, and cutaneous ulcers (De Monte *et al.*, 2014; Saadiah *et al.*, 1999; Annam *et al.*, 2007; George *et al.*, 2008; Louthrenoo *et al.*, 1994; Luo *et al.*, 2011; Pun and Fang, 2000).

Diagnosis

The diagnosis of talaromycosis is established by cultures of Tm from blood or clinical specimens, which takes 4–14 days and requires demonstration of temperature dimorphism (**Fig. 4**). An early presumptive diagnosis can be made by histopathological examination of skin lesions or other biopsy materials. Identification of extracellular and intra-macrophage oval-round yeast cells that divide by binary fission, resulting in a midline septum, discriminate Tm from other budding yeasts (**Fig. 4**). Tm can also be identified by microscopic examination of a Wright's-stained peripheral blood smear (Supparatpinyo and Sirisanthana, 1994). Culture yield is highest in the bone marrow (100%), followed by skin lesions (90%), and blood (70%) (Le *et al.*, 2011; Supparatpinyo *et al.*, 1992). The Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) method has been used to identify Tm from cultured specimens.

Real-time PCR assays targeting the fungal ribosome and *MP1* genes have a high specificity (100%) but limited sensitivity (60%–70%) (Hien *et al.*, 2016; Li *et al.*, 2020; Lu *et al.*, 2016; Pornprasert *et al.*, 2009). Antigen-detection enzyme immunoassays (EIAs) using the Mp1p and 4D1 monoclonal antibodies are promising rapid diagnostics (Thu *et al.*, 2020; Prakit *et al.*, 2016). In a

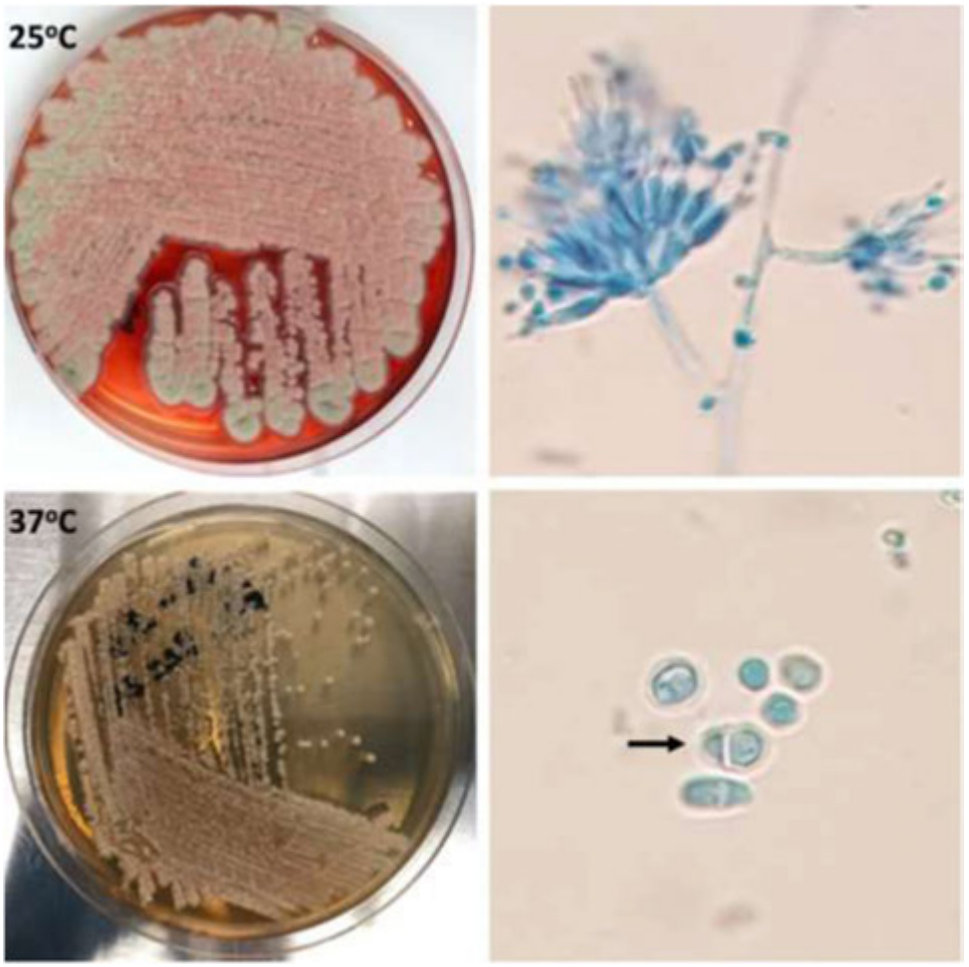

Fig. 4 *Talaromyces marneffei subcultures from the bone marrow of a HIV-infected patient with talaromycosis.* At 25°C, *T. marneffei* produces powdery greenish mold colonies and a bright red pigment that diffuses into the Sabouraud Dextrose Agar medium. Tape preparation of the mold colonies shows septate hyphae with conidiophores bearing phialides and round conidia under the microscope. At 37°C, *T. marneffei* produces white yeast colonies without the red pigmentation. Microscopic examination shows oval yeast cells, one with a central septum (arrow).

large validation study (372 culture-confirmed cases, 517 controls), the M1p1 EIA was more sensitive than blood culture (86.3% vs. 72.8%) with a specificity of 98% (Thu *et al.*, 2020). Antigen is detected in urine as well as in plasma samples and can detect occult culture-negative infections that are subsequently confirmed by bone marrow cultures (Ly *et al.*, 2020). The Mp1p EIA is being evaluated prospectively as a rapid diagnostic test and as a screening tool for pre-clinical talaromycosis (clinicaltrials.gov: NCT04033120). Point-of-care platforms are being developed which will enable testing and screening for talaromycosis in clinic and community settings.

Management

Treating disease

Induction therapy with amphotericin B deoxycholate (AmBd) at 0.7 mg/kg/day for 2 weeks has been shown in a small, non-comparative study (n = 74) in Thailand to have a clinical and mycologic success rate of 97% (Sirisanthana *et al.*, 1998). In a multi-center randomized controlled trial in Vietnam (IVAP, n = 440), induction therapy with AmBd was superior to itraconazole with respect to six-month mortality (11.3% versus 21.0%), fungal clearance from blood (99% clearance by day 7 versus 67%), rates of disease relapse and IRIS (Le *et al.*, 2017). These studies provide high-quality evidence for international guidelines recommending induction therapy (2 weeks) with amphotericin B (liposomal or deoxycholate, depending on availability) regardless of disease severity. Induction therapy is followed by consolidation (10 weeks) and maintenance therapy with itraconazole (or voriconazole) until CD4 count is above 100 cells/mm^3 for 6 months (The National Institute of Health, and the HIV Medicine Association of the Infectious Diseases Society of America, 2019). The IVAP trial also suggested that 7 days' amphotericin may be adequate for induction therapy: a phase II trial is being planned in Vietnam.

Primary and secondary prophylaxis

Primary prophylaxis with itraconazole has been shown to reduce the incidence of talaromycosis and other invasive fungal infections in a small randomized trial (n = 63) (Chariyalertsak *et al.*, 2002). The study was not powered to detect a mortality benefit and was conducted prior to the ART era. For these limitations and for concerns of long-term toxicity, drug-drug interactions, and cost, primary prophylaxis is not recommended (see footnote).

Secondary prophylaxis with itraconazole 200 mg daily has been shown in a double-blinded placebo-controlled trial (n = 71) to reduce relapse rate from 57% to 0% (Supparatpinyo *et al.*, 1998). Optimal time to stop secondary prophylaxis is unknown, but a retrospective study (n = 33) reported no relapse in patients with a CD4 count > 100 cells/mm^3 on ART (Chaiwarith *et al.*, 2007).

Timing of ART

The optimal time to start ART is unknown. In the IVAP trial, the median time to ART initiation was 3 weeks (range 1–5 weeks). Paradoxical IRIS events occurred only in the itraconazole arm (11.4%), suggesting that ART can be safety initiated as early as 1 week after effective induction therapy with amphotericin (Le *et al.*, 2017).

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Fungal Infections in Transplant Recipients

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Introduction

Fungi are for the most part soil dwelling organisms that are pathogens of plants, insects and ectothermic vertebrates, but not mammals. The fact that even pathogenic fungi are generally not spread from human to human argues against any substantial selection for virulence in humans. Rather, the virulence displayed is largely an "accidental" byproduct of selection for survival and replication inside natural predators such as amoebae (Casadevall *et al.*, 2019). An impaired immune system alters the balance of forces, however, and permits the establishment of opportunistic fungal infections in hosts where this would not otherwise be possible. Thus, transplant recipients are at substantially greater risk of almost all fungal infections compared with immunocompetent hosts.

Transplant recipients can be broadly divided into two main groups: hematopoietic stem cell transplant (HSCT) recipients and solid organ transplant (SOT) recipients. Although there is substantial overlap, differences exist in the relative incidences of specific fungal infections between the two groups, reflecting differing immune deficits and immunosuppression regimens, as well as factors such as the presence or absence of myeloablation. In SOT recipients, invasive candidiasis accounts for $>50\%$ of invasive fungal infections, followed by invasive aspergillosis (19%), cryptococcosis (8%), non-*Aspergillus* molds (8%), endemic fungi (5%) and mucormycosis (2%) (Pappas *et al.*, 2010). By contrast, HSCT recipients suffer from more invasive aspergillosis (43%) and mucormycosis (8%) than SOT recipients, but proportionately less candidiasis (28%), and cryptococcosis is extremely rare (Kontoyiannis *et al.*, 2010; Sun *et al.*, 2009).

The various fungal infections also differ by their median time to infection after transplant. In SOT recipients, the vast majority of early invasive fungal disease (≤ 90 days) is comprised of candidiasis and invasive aspergillosis, whereas the median times to infection for endemic mycoses, non-Aspergillus molds and cryptococcosis are all a year or more (Pappas *et al.*, 2010). Invasive fungal infections in HSCT recipients tend to occur earlier, perhaps as a result of the greater initial immunosuppression inherent in HSCT compared to most SOT. In one large-scale study, the median times of all invasive fungal infections was less than 180 days in HSCT recipients (Kyvernitakis *et al.*, 2016; Kontoyiannis *et al.*, 2010).

A major goal of care following transplantation is the prevention of fungal infections. To this end, most transplant recipients receive some form of antifungal prophylaxis in the immediate post-transplant period. The particular prophylaxis regimen is tailored to the patient, the organ transplanted and the immunosuppressive regimen given.

In this chapter, we review the major fungal infections in transplant recipients.

Candidiasis

Candida species are a normal constituent of the human gastrointestinal tract's flora. Estimates of asymptomatic *Candida albicans* intestinal carriage in humans range from 30% to 60%, and other *Candida* species such as *C. parapsilosis*, *C. glabrata* and *C. tropicalis* are also frequently found in the gastrointestinal tract in healthy individuals (Hallen-Adams and Suhr, 2017). To cause invasive disease *Candida* must either traverse the gastrointestinal mucosa or enter the body via a defect in one of the body's anatomical barriers, such as via central venous catheters (Koh *et al.*, 2008).

The cells of the innate immune system, particularly neutrophils, are required to prevent invasive candidiasis (Gazendam *et al.*, 2014; Fradin *et al.*, 2005; Koh *et al.*, 2008). Thus, neutropenia is among the strongest risk factors for candidemia and other forms of invasive disease. By contrast, humoral immunity appears to play no significant protective role in this regard, and a Th17-based immunity is important in controlling only mucosal candidiasis (Gazendam *et al.*, 2014). Thus, patients with impaired Th17 responses due to HIV may develop esophageal candidiasis, but HIV does not directly increase rates of invasive candidiasis like neutropenia does.

Candida blood stream infection is the predominant form of invasive candidiasis. Once in the blood stream, the organism may disseminate to other organs such as the eye, heart, skin, brain or bones. Hepatosplenic candidiasis is a rare syndrome particular to neutropenic hosts that occurs as their neutrophil counts begin to recover. In essence, hepatosplenic candidiasis is a form of Candida immune reconstitution inflammatory syndrome (IRIS). A fever that persists after neutrophil recovery should prompt consideration of this syndrome, which frequently involves the liver and spleen, though other organs such as they kidney may also be affected. Abdominal imaging may reveal small abscesses in the involved organs, but blood cultures are frequently negative at the time of diagnosis (Pagano *et al.*, 2002).

The species responsible for candidemia are been well described, though considerable shifts have taken place worldwide over the past two decades (Lamoth *et al.*, 2018). Although historically the most common species was *C. albicans*, this species now comprises less than 50% of Candida infections in many countries, including the United States. Correspondingly, a higher proportion of non-*albicans* is now seen, and in the USA, much of this is due to *C. glabrata* and *C. parapsilosis* (Lamoth *et al.*, 2018). Of specific concern is the worldwide emergence of *C. auris*. This organism has several adaptations that make it a fearsome nosocomial pathogen, including its ability to colonize the skin (unlike other *Candida* species), the production of biofilms,

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resistance to several chemical disinfectants, and the ability to survive for prolonged periods in the immediate environment around infected or colonized patients (Cadnum *et al.*, 2017; Sherry *et al.*, 2017; Eyre *et al.*, 2018). *C. auris* is frequently resistant to one or more antifungals and has been shown to be responsible for several recent nosocomial outbreaks.

Diagnosis

Mucocutaneous candidiasis can usually be identified clinically. The classic appearance is the so-called pseudomembranous form, characterized by white curd-like plaques that can be easily scraped away to reveal a red, inflamed mucosal surface beneath. The signs and symptoms of invasive candidiasis depend somewhat on whether the transplant patient is neutropenic or not. Symptoms and signs of acute infection in severely neutropenic patients are notoriously insensitive. Redness, pain, swelling, fluctuance and local heat are all less prevalent in hosts with neutropenia (Valdivieso *et al.*, 1977). Laboratory and radiological tests may also be much less sensitive. For instance, in one study of neutropenic patients with cancer who had a UTI, only 11% had pyuria (Sickles *et al.*, 1975). In another study of neutropenic patients with pneumonia, 40% had normal radiograph, and just 8% had neutrophils on sputum Gram stain (Valdivieso *et al.*, 1977). Thus, a high index of suspicion may be required to diagnose *Candida* infections in the neutropenic host.

Blood cultures appear to be ~75% sensitive for candidemia, and are recommended whenever the condition is suspected (Clancy and Nguyen, 2013). Importantly, candidiasis of a particular organ may not be associated with candidemia at the time of presentation. The sensitivity of blood cultures for invasive candidiasis as a whole is probably closer to 50% (Clancy and Nguyen, 2013).

Nonculture methods include ß-D-glucan (BDG), polymerase chain reaction (PCR), or T2 magnetic resonance technology. A recent meta-analysis pegged the sensitivity of BDG for invasive candidiasis at 75% (Karageorgopoulos *et al.*, 2011). In cases of deep-seated *Candida* infection, BDG may be positive days before blood cultures are (Tissot *et al.*, 2013). False positives are common however, and can result from things as diverse as bacteremia, surgical gauze, mucositis and some intravenous antibiotics, such as amoxicillin-clavulanate.

PCR assays on blood samples show great promise, with a recent meta-analysis reporting a sensitivity of 95% and specificity of 92% for invasive candidiasis (Avni *et al.*, 2011). A further advantage of PCR is its ability to rapidly provide species-level identification though cultures are still needed to determine drug susceptibilities.

T2 magnetic resonance diagnostics combine molecular amplification methods with T2 magnetic resonance measurement and are able to provide accurate results within 3–5 h. The assay can be run directly off whole blood. One study showed a sensitivity of 91% and a specificity of 99.4% when compared to blood cultures (Mylonakis *et al.*, 2015).

Treatment

Prompt antifungal therapy is vital in treating invasive candidiasis in general. A delay in administering appropriate antifungal therapy within 12–24 h has persistently been found to be an independent risk factor for mortality in candidemia (Morrell *et al.*, 2005; Kollef *et al.*, 2012; Garey *et al.*, 2006). In neutropenic patients in particular, candidemia is almost always life-threatening, particularly if the neutropenia is prolonged such as following induction therapy for hematological malignancies (Pappas *et al.*, 2016).

Oral candidiasis can be treated with a topical antifungal agent (e.g., nystatin swish and swallow). Severe cases of oral disease or nonresponsive cases can be given oral fluconazole. By contrast, patients thought to have invasive candidiasis should be commenced empirically on an echinocandin. The particular echinocandin selected usually depends primarily on cost and availability. Azole-resistant *Candida* species are too frequent for fluconazole to be recommended as initial therapy in most centers, particularly amongst neutropenic patients. In institutions with low rates of fluconazole resistance however, it may be a reasonable alternative in patients who are not critically ill and have had no prior azole exposure (Pappas *et al.*, 2016). An alternative to echinocandins as empiric therapy is amphotericin B. However, even the liposomal form of amphotericin B is tolerated far less well than the echinocandins are, precluding its use as a front-line drug.

If fluconazole susceptibility of the *Candida* species is demonstrated, the patient can be stepped down to fluconazole therapy when clinically stable. In most patients, intravenous fluconazole therapy is unnecessary, since the oral bioavailability of fluconazole is excellent.

Where possible, central venous catheters should be removed. The evidence for catheter removal in neutropenic patients is less compelling than for non-neutropenic patients, however (Pappas *et al.*, 2016). One likely reason is that the venous catheter is less likely to be the source of the candidemia in neutropenic patients, in whom gut translocation is a more common source. Nonetheless, any indwelling central catheters can become secondarily colonized during candidemic episodes and thereby act as a nidus for relapse after completion of therapy.

Endophthalmitis is an important complication that should be actively sought out in all cases of candidemia by means of a dilated fundoscopic examination (Pappas *et al.*, 2016; Kullberg *et al.*, 2011). Amongst studies from the 1990s onwards, intraocular candidiasis was found in approximately 7% of candidemic patients (Vinikoor *et al.*, 2013). Signs of endophthalmitis are usually minimal during neutropenic episodes, and so fundoscopy should be performed only once the neutrophil count has recovered (Pappas *et al.*, 2016).

The optimal duration of antifungal therapy for candidemia is unknown, but in the absence of randomized control trial data, a duration of 14 days after clearance of blood cultures is usually recommended (Pappas *et al.*, 2016). For those patients who are still neutropenic by day 14, antifungal therapy should generally be continued until neutrophil recovery occurs. Efforts to shorten the anticipated period of neutropenia, by granulocyte colony-stimulating factor (G-CSF) administration, or G-CSF-mobilized granulocyte transfusions can be considered in individual cases where appropriate, although the evidence base supporting these interventions is extremely limited.

The duration of therapy for other forms of invasive candidiasis depends on the site but is generally substantially longer than 14 days. Cases requiring special consideration include:

(1) *Candida endophthalmitis* – Echinocandins do not penetrate the eye well, and so fluconazole or voriconazole are usually used orally or intravenously. Amphotericin B can be used as an alternative in resistant cases. Endophthalmitis needs a minimum of 4–6 weeks of therapy and may sometimes require intravitreal antifungals and/or a vitrectomy additionally. Co-management with an ophthalmologist is strongly recommended.
(2) *Disease involving the central nervous system (CNS)* – Echinocandins have poor CNS penetration, and probably do not achieve high enough drug levels with conventional doses to treat CNS infections reliably (Pappas *et al.*, 2016; Hope *et al.*, 2008). Thus, for CNS disease, amphotericin B is given initially, sometimes with flucytosine in addition. Once the patient shows a response to this regimen, they may be switched to fluconazole (if susceptibilities permit) and continue this regimen until signs, symptoms and radiological abnormalities have all resolved.
(3) *Urinary tract infections* – Attempting to distinguish a urinary tract infection from colonization by means of urinary white cells is futile in neutropenic patients, who cannot mount a local neutrophil response (Sickles *et al.*, 1975). In those patients requiring treatment, echinocandins do not penetrate the urinary tract well, and thus either fluconazole or, in fluconazole-resistant cases, amphotericin B, should be used. The urinary catheter should be removed in all cases where this is feasible. Amphotericin B bladder washes are a treatment of last resort for *Candida* cystitis when neither fluconazole nor systemic amphotericin B can be used.
(4) *Hepatosplenic candidiasis* – this rare but important syndrome requires prolonged therapy until all lesions have resolved radiographically. This often takes several months. An echinocandin or liposomal amphotericin B should be used as initial therapy, stepping down to fluconazole (if susceptibilities permit) once the patient shows an initial response. Because this is essentially an IRIS response, some patients have been reported to show a symptomatic improvement with the addition of corticosteroids or nonsteroidal anti-inflammatory drugs. However, the evidence for anti-inflammatory agents is too limited for them to be routinely recommended.

Prophylaxis

Neutropenia from cancer-related immunosuppression warrants antifungal prophylaxis if it is profound ($<0.1 \times 10^9$/L) and protracted (≥ 7 days). Either an oral azole or an echinocandin is recommended. For *Candida* prophylaxis, fluconazole is sufficient (in contrast to empiric treatment regimens). However, in practice, a mold-active triazole such as posaconazole, voriconazole, or isavuconazole is often used for broader antifungal prophylaxis. Neutropenia from causes other than cancer-related immunosuppression does not generally warrant prophylaxis.

Aspergillosis

Aspergillus species account for the majority of mold infections in transplant recipients, making up 76% and 81% of mold infections in HSCT recipients and SOT recipients respectively (Pappas *et al.*, 2010). *Aspergillus fumigatus* is the species most commonly associated with disease in transplant patients, but *A. flavus*, *A. niger*, and *A. terreus* are also frequently identified (Vazquez *et al.*, 2013).

Aspergillus is a ubiquitous mold, and infection is acquired in most cases through inhalation of its conidia. Infection can more rarely be acquired by consumption of contaminated food products, or by direct cutaneous inoculation following a break in the skin barrier (as seen in burn or trauma victims for instance) (Patterson *et al.*, 2016). Nosocomial transmission has also been described (Pegues *et al.*, 2001). The risk of aspergillosis is higher in HSCT recipients than SOT recipients, probably as a result of performing HLA-mismatched or unrelated HSCTs, in whom more intense immunosuppression is required (Vazquez *et al.*, 2013). Within SOT recipients, lung recipients are at particular risk of contracting aspergillosis due to both the chronic exposure of the transplanted lung to the external environment and the transplanted lung's anatomical and functional abnormalities (such as anastomotic sites) (Shoham and Marr, 2012).

Aspergillus spp. may colonize the airways without tissue invasion. Colonization is particularly frequent and important in lung transplant recipients, in whom it predisposes to subsequent invasive disease (Silveira and Husain, 2007). Invasive aspergillosis (IA) refers to invasion of tissue due by hyphae due to *Aspergillus* species (Kosmidis and Denning, 2015). The most common site of involvement is the lung and, in lung transplant recipients, the tracheobronchial tree (at the anastomotic site). Neutropenia is the classic risk factor for IA, and such patients usually have the most aggressive form of IA, where visible disease progression may be noticeable over the course of several days (Kosmidis and Denning, 2015). Angioinvasion is common,

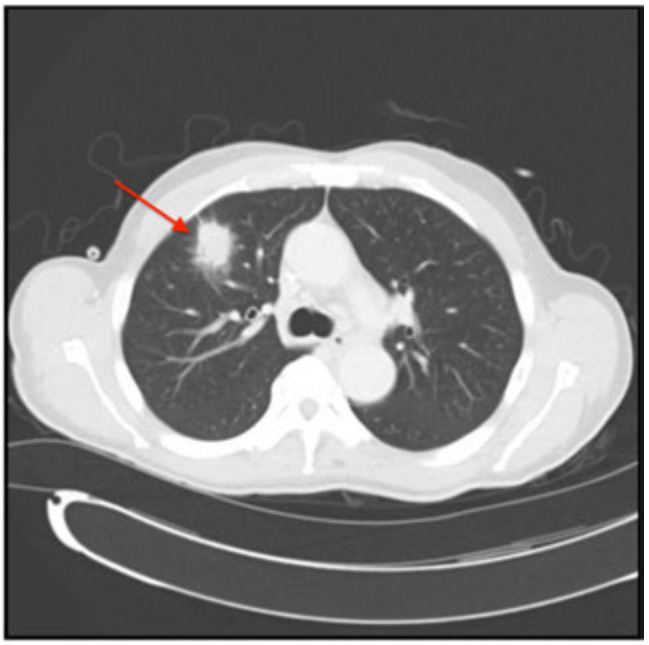

Fig. 1 Halo sign (arrow) – a central nodule surrounded by a hazy ground glass infiltrate. The halo sign represents an area of pulmonary infarction (the nodule) with surrounding hemorrhage (the ground glass infiltrate). Although somewhat nonspecific, in the neutropenic host its strongest association is with invasive aspergillosis.

facilitating disease dissemination to distant sites such as the skin, brain, or eyes. Non-neutropenic patients can also develop IA, though the course is often more subacute (over weeks to months), usually without angioinvasion or fever. Risk factors for this include non-neutropenic HCST and SOT recipients, HIV, COPD, corticosteroid use, critically ill patients in the ICU, and severe influenza infection (Vanderbeke *et al.*, 2018).

Diagnosis

The diagnosis of IA can be extremely challenging. In HSCT recipients a characteristic radiological finding is the "halo sign" (**Fig. 1**), but this has a low sensitivity in SOT recipients (Singh and Husain, 2003). Despite angioinvasion, blood cultures are almost always negative. Hyphal invasion of tissue together with culture of *Aspergillus* from the same tissue is the gold standard, but obtaining these samples in critically ill patients may be challenging (Silveira and Husain, 2007). Given that the lung is the most frequently involved organ, bronchoalveolar lavage (BAL) and/or lung biopsy often provides the highest diagnostic yield, though any tissue may be sampled when involved (e.g., skin). Aspergillus grows readily on most media, but the yield is increased if plated on to dedicated fungal growth media (Shoham and Marr, 2012). Aspergillus-specific galactomannan assays can be used on both BAL and serum specimens, but BAL specimens generally demonstrate a higher sensitivity than serum ones. The sensitivity of the galactomannan test ranges from 60% to 93%, depending on the cut-off value used (higher sensitivities are seen with a cutoff of 0.5) (Kosmidis and Denning, 2015; Zou *et al.*, 2012). Serum ß-D-glucan assays show comparable sensitivity, though they are much less specific. PCR offers great promise, but as with BAL cultures, it lacks the ability to distinguish colonization from infection.

Treatment

Treatment with voriconazole has been shown to be superior to amphotericin B with respect to both treatment success and 12-week survival (Herbrecht *et al.*, 2002). Recently, the SECURE trial demonstrated that isavuconazole was non-inferior to voriconazole for the treatment of *Aspergillus* and other filamentous molds (Maertens *et al.*, 2016b). Treatment is continued for a minimum of 6–12 weeks, depending on the patient's immunosuppressive regimen, the site of disease and evidence of improvement. (Patterson *et al.*, 2016) Mortality from IA remains high however, with fewer than half of transplant recipients being alive 12 months after diagnosis. (Pappas *et al.*, 2010).

Prophylaxis

Prophylaxis is recommended for patients at high risk of aspergillosis, including patients with prolonged neutropenia, patients with graft-versus-host disease and lung transplant patients (Patterson *et al.*, 2016). Other SOT recipients may also be given *Aspergillus*

prophylaxis depending on institutional policy and individual patient risk factors. A mold-active azole such as voriconazole, posaconazole or itraconazole is usually chosen. In lung transplant recipients inhaled amphotericin B is sometimes used instead of or in addition to a systemic triazole.

Mucormycosis

Mucormycosis (previously known as zygomycosis), is an invasive fungal disease caused by fungi of the Mucorales order. The most commonly implicated species are *Rhizopus*, *Lichtheimia*, *Rhizomucor*, *Mucor* and *Cunninghamella*. These fungi are ubiquitous and found in decaying organic materials such as bread, fruit, vegetable matter, soil, compost and animal excreta. The usual mode of acquisition is by inhalation of the conidia, though direct inoculation following breaks in the skin barrier, or ingestion in patients with breaks in the gastrointestinal tract mucosa, are also described. The first line of defense are the host's macrophages and neutrophils, and impairment of this during periods of neutropenia or glucocorticoid use is a major risk factor for disease acquisition (Ibrahim *et al.*, 2012). Overall, the disease is more common in HSCT recipients than SOT recipients, and comprises 8% and 2% of invasive fungal infections in these two populations respectively (Armellino *et al.*, 2012).

Once established, mucormycosis runs an aggressive course marked by angioinvasion and consequent tissue infarction and dissemination. The main forms of disease that occur are pulmonary, rhinocerebral, sinus, cutaneous and gastrointestinal mucormycosis. The frequency of presenting site vary somewhat by transplant type. In HSCT recipients, pulmonary disease accounts for over half of all mucormycosis seen, whereas it makes up just over a third of cases seen in SOT recipients, in whom cutaneous and rhinocerebral disease makes up a proportionally greater percentage of cases (Roden *et al.*, 2005). When skin involvement is present, it usually represents the site of inoculation rather than haematogenous spread, unlike the case for many other filamentous fungi (Roden *et al.*, 2005).

Diagnosis

Infarction of involved tissues is the usual clinical finding (**Fig. 2**), with skin areas often appearing as eschars. Other clinical findings include pleuritic pain (lung involvement) or proptosis (extension into the orbital cavity). Though these finding are not specific for mucormycosis, the disease must remain high on the differential diagnosis in transplant recipients. Histopathologic specimens show characteristic broad hyphae with right-angle branching and few or no septae. There are no biochemical or serological biomarkers to aid in the diagnosis, and the culture yield is suboptimal (50%–71%) (Katragkou *et al.*, 2014). Overall, the diagnosis is confirmed ante mortem in fewer than half of cases.

Treatment

Key treatment principles are (1) aggressive surgical debridement of all necrotic tissue, (2) prompt antifungal therapy and (3) reversal of any predisposing factors if possible. Amphotericin B is the mainstay of the antifungal therapy. Combination therapy with an azole or an echinocandin is controversial but is often given in clinical practice (Reed *et al.*, 2008; Kyvernitakis *et al.*, 2016). Repeat surgical debridements are usually required, since there is a delay between fungal angioinvasion and visible necrosis.

Mortality from mucormycosis is exceedingly high, even in cases that are treated promptly and aggressively. In one large review of cases, 91% of HSCT recipients and of 48% of SOT recipients died during the course of their mucormycosis (Roden *et al.*, 2005).

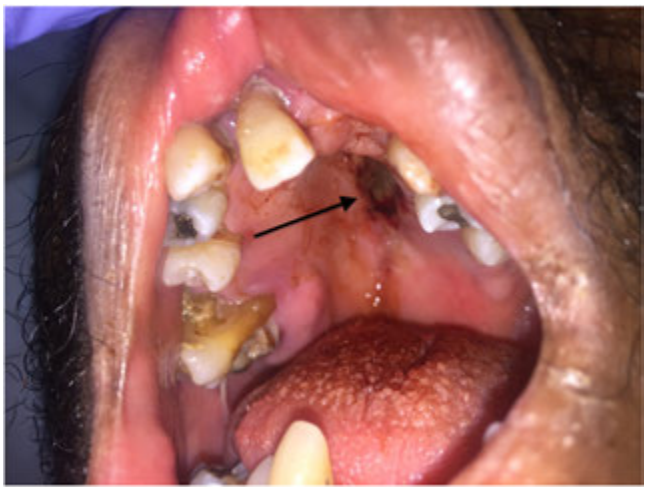

Fig. 2 Area of palatal infarction (arrow) as a result of extension of rhinocerebral mucormycosis.

Cryptococcosis

Cryptococcosis is a relatively common cause of invasive fungal disease in SOT recipients, with an incidence of 0.3%–5% (Silveira and Husain, 2007). Interestingly, cryptococcal infection is exceedingly rare in HSCT recipients – a review in 2009 found only nine reported cases in the literature (Sun *et al.*, 2009). *Cryptococcus neoformans* accounts for the vast majority of cases of cryptococcosis. Its natural habitat appears to be soil contaminated with pigeon and other bird droppings as well as the decaying wood from certain trees, such as *Eucalyptus* trees, mopane trees and the baobab trees (Cogliati, 2013). Exposures and infections occur worldwide.

As with most other fungal infections in transplant recipients, the lungs are the usual portal of entry as well as a frequent site of disease. Disease can occur shortly after exposure or result from reactivation of an exposure many years previously. Cryptococcosis has also been transmitted from donor organs on rare occasions (Shoham and Marr, 2012). The usual radiographic changes are nodules, with or without cavitation, though a variety of other manifestations are also seen including pleural effusions, and even ARDS. The fungus has a particular tropism for the central nervous system, where it may cause a chronic meningitis, intracerebral cryptococcomas, or both. Headache, fever and mental status changes are frequent findings, though meningism is found in fewer than a third of patients (Silveira and Husain, 2007). In the Transplant-Associated Infection Surveillance Network's review of cases from 2001 to 2006, central nervous system involvement was seen in 45% of cases and disease limited to the lungs was seen in 39%. Interestingly, calcineurin inhibitors such as tacrolimus exhibit anti-cryptococcal activity, and a decreased incidence of CNS disease is seen in patients on these agents compared to transplant recipients on alternative immunosuppressant regimens (Silveira and Husain, 2007). After the lungs and CNS, the next most common site of involvement is the skin, where skin-colored papules and plaques can occur. Cutaneous lesions usually have a degree of central umbilication, appearing as a larger version of molluscum contagiosum. (**Fig. 3**) *Cryptococcus* may also cause cellulitis that is clinically similar to bacterial cellulitis. This entity should be suspected in transplant recipients with cellulitis that is unresponsive to antibacterials.

Diagnosis

Disseminated and CNS cryptococcosis in immunocompromised patients is usually detected by a serum cryptococcal antigen test, which has a sensitivity of >95% for both conditions. The diagnosis of central nervous system involvement can similarly be established by performing the antigen test on a CSF sample. The sensitivity in isolated pulmonary disease is lower, however, and a negative result does not rule the condition out (Aberg *et al.*, 1999). The fungus is also readily cultured from appropriate samples such as sputum, BAL fluid, or CSF. Of note, all transplant recipients with suspected or proven cryptococcosis outside the CNS should have a lumbar puncture to exclude occult CNS disease.

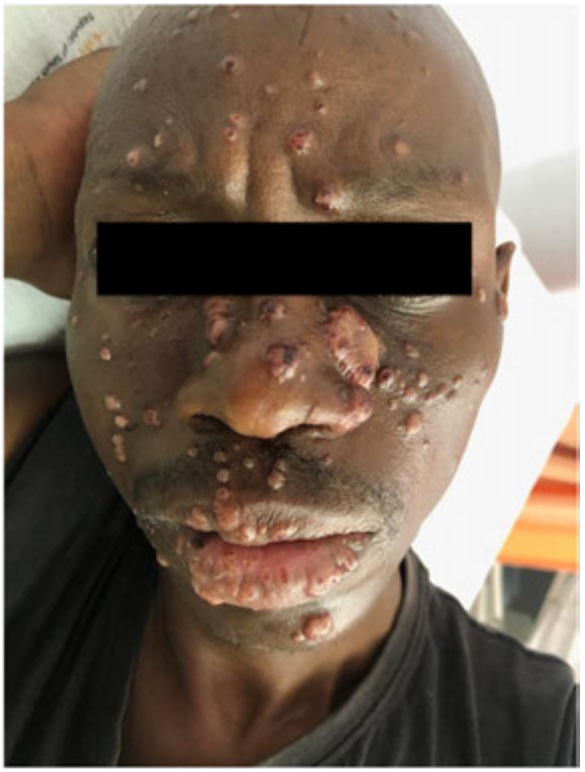

Fig. 3 Multiple umbilicated papules and plaques typical of cutaneous cryptococcosis.

Treatment

Treatment of cryptococcal meningitis in transplant recipients consists of induction therapy with a combination of amphotericin B and flucytosine for at least two weeks (Perfect *et al.*, 2010). This is followed by maintenance and consolidation therapy with fluconazole, the latter typically given for at least 6–12 months. The treatment duration may be extended if the patient's net state of immunosuppression is still high at that point. Mild cases of pulmonary disease can be treated with fluconazole alone, but more severe should be treated as for CNS disease (Perfect *et al.*, 2010).

Although case fatality rates of ~40% were historically described, mortality rates in more recent cohorts have roughly halved (Sun *et al.*, 2009). In addition to prompt antifungal therapy, a key component to managing cryptococcal meningitis successfully is to relieve elevated CSF pressures with repeated therapeutic lumbar punctures and/or temporary CSF shunts.

Endemic Fungal Infections

The endemic mycoses are a group of thermally dimorphic fungi that occupy discrete geographic niches. The principal diseases in this group are histoplasmosis, blastomycosis, coccidioidomycosis, sporotrichosis, paracoccidioidomycosis, talaromycosis and emergomycosis. The first four of these are found in North America. In the environment, the dimorphic fungi exist as molds, but at body temperature they transform into yeasts (or in the case of coccidiodomycosis, into a specialized structure called a spherule).

The incidence of all the dimorphic fungi is increased in transplant recipients. The usual way infection is acquired is by the inhalation of aerosolized conidia, often following disturbance of the soil or organic matter where the fungi are residing. In the lung, the host defenses frequently either clear or control the nascent infection, but this ability is attenuated in transplant recipients. Importantly, infection may be contained subclinically for many years, and only escape host immune control when the patient's immune system is depleted by solid organ transplantation, hematologic stem cell transplantation, immunosuppressant medications, old age, HIV or other immunocompromising conditions. Rarely, the infection may be inadvertently transmitted by an infected allograft. Donor-derived infection has been demonstrated in the case of histoplasmosis and coccidioidomycosis.

Detailed descriptions of each of these diseases are beyond the scope of this chapter, but the three most common dimorphic fungi seen in transplant recipients – histoplasmosis, blastomycosis and coccidioidomycosis – are summarized below.

Histoplasmosis

The preferred soil environment for *Histoplasma capsulatum* within the USA appears to be the Ohio, Mississippi and Missouri River basins. Parts of India, South East Asia, Australia and Africa are also endemic for the disease. Soil that has been enriched with bat and bird guano is a particularly rich environment for *Histoplasma* growth, and exposure has frequently been linked to spelunking, cleaning of attics, cleaning bird coops, and renovating buildings.

The Transplant-Associated Infection Surveillance Network (TRANSNET) reported 48 cases of histoplasmosis in SOT recipients and 4 in HCT recipients from 15 surveillance sites over 5 years from 2001 to 2006, making histoplasmosis the most frequent dimorphic pathogen in transplant recipients in the US (Kauffman *et al.*, 2014). The cumulative incidence in SOT recipients was 0.1% for the 12-month period following their transplants, and 0.02% for HSCT recipients.

In immune competent individuals, disease manifestations are largely confined to the lungs, which are the primary site of infection. Acute, subacute, or chronic pneumonias are common, and less frequently manifestations such as acute respiratory distress syndrome, broncholithiasis, and fibrosing mediastinitis can also be seen. However, in transplant recipients, diseases frequently disseminates widely to other sites in addition. These may include lymph nodes, the liver, spleen, bone marrow, skin, and gastrointestinal tract (Cuellar-Rodriguez *et al.*, 2009; Freifeld *et al.*, 2005). Symptoms are correspondingly attributable to organ enlargement, cytopenias from marrow infiltration or haemophagocytic lymphohistiocytosis, and the effects of cytokine production, such as fever, anorexia, loss of weight, and malaise.

The diagnostic test with the highest sensitivity for disseminated disease is the urine histoplasma antigen, with an estimated 93% sensitivity in SOT recipients (Nel *et al.*, 2018). Antigen testing may also be performed on the blood, BAL fluid, or CSF with variable sensitivity. False-positive results due to *Aspergillus* infection have been reported in SOT recipients (Vergidis *et al.*, 2012). Cultures from blood or tissues from affected sites have moderate sensitivity, but typically take 2–4 weeks to turn positive. Histologic specimens can provide a presumptive diagnosis by revealing typical intracellular clustering of small yeast-like organisms within macrophages and other phagocytes (Guarner and Brandt, 2011). *Histoplasma* may also appear to have a capsule on histopathologic specimens. PCR assays appear to be a promising diagnostic modality, but experience and availability with molecular testing is limited to date.

Treatment in disseminated histoplasmosis is typically with a lipid formulation amphotericin B for 1–2 weeks initially. When the patient demonstrates clinical improvement, the amphotericin can be switched to itraconazole for a duration of at least 12 months (Wheat *et al.*, 2007). Histoplasma antigen levels may be monitored serially to assess response to therapy, since a failure of the antigen level to decline is associated with treatment failure. In addition, antigen levels should be negative at the end of treatment, though some patients with persistent low-level antigenuria without evidence of active disease have had good clinical outcomes when their treatment was stopped (Wheat *et al.*, 2007; Goldman *et al.*, 2004). Histoplasmosis can relapse after treatment, and so urine antigen levels should also be checked periodically for the 6–12 months following treatment completion.

Blastomycosis

Blastomycosis in the USA is caused by one of two species, *Blastomyces dermatitidis* and *Blastomyces gilchristii*. The natural habitat of *Blastomyces* is largely unknown, though recent phylogeographic work has demonstrated a strong association with freshwater basins. In North America, endemic areas are the Ohio, Mississippi, Tennessee and Nelson River basins, as well as a small area surrounding the St Lawrence River. As with histoplasmosis, risk factors for blastomycosis are activities that disturbs the soil in these regions, such as construction, fishing, or cutting trees or brush.

Blastomycosis is less common than histoplasmosis amongst transplant recipients – just 9 cases were described over a 5 year period in 15 surveillance sites in the USA (Kauffman *et al.*, 2014; Pappas *et al.*, 2010). While *Blastomyces* infection in immunocompetent patients is asymptomatic in approximately half of cases, transplant recipients develop symptomatic pulmonary disease in 80%–100% of infections (Grim *et al.*, 2012; Gauthier *et al.*, 2007). *Blastomyces* pneumonia may progress to acute respiratory distress syndrome (ARDS) and result in substantial residual lung damage. Disseminated disease is also frequent in transplant recipients. The skin (verrucous lesions, pustules, ulcers), bone (lytic lesions), genitourinary system (prostatitis, epididymitis) and central nervous system (meningitis or cerebral abscesses) are the most frequent extra-pulmonary sites (Kauffman and Miceli, 2015).

Blastomyces can be presumptively identified on tissue specimens by its large size, double-contoured walls and characteristic broad-based budding (Guarner and Brandt, 2011). *Blastomyces* is also readily cultured from sputum, BAL fluid and skin when these sites are involved. Cultures may take several weeks to turn positive however (Smith and Kauffman, 2010). A urine antigen test has a sensitivity of 76%–93%, though cross-reaction with other dimorphic fungi is frequent (Nel *et al.*, 2018).

As with histoplasmosis, initial therapy with amphotericin B is recommended for all immunocompromised patients, followed by itraconazole for at least 12 months.

Coccidioidomycosis

Two species of *Coccidioides* are responsible for human coccidioidomycosis: *C. immitis* and *C. posadasii*. The fungus is endemic in the hot, dry, arid and semiarid zones of California, Arizona, New Mexico and Texas, together with northern Mexico and smaller areas in South America. Sandstorms, construction work and military exercises are risk factors for acquiring coccidioidomycosis (Hage *et al.*, 2012).

9 cases of coccidioidomycosis were described in a 5 year period from the 15 surveillance sites in the Transplant-associated Infection Surveillance Network (TRANSNET) (Pappas *et al.*, 2010). Compared to immunocompetent patients, transplant recipients have a much higher rate of both symptomatic pulmonary disease and disseminated disease. Pulmonary disease mimics a community-acquired pneumonia, and the diagnosis is sometimes only suspected when the patient fails to improve on conventional antibiotics. The disease can progress to ARDS in severe cases. Common extra-pulmonary sites in disseminated disease include the CNS, liver, spleen, kidney, skin and joints (Blair and Logan, 2001).

Unlike other dimorphic fungi, the inhaled conidia transform not into yeasts but rather into a specialized structure called a spherule. Visualization of the spherule on tissue samples is therefore pathognomonic (Guarner and Brandt, 2011). Cultures from involved sites also grow more readily than most other dimorphic fungi, often within a week (Blair and Logan, 2001). The sensitivity of serology is lessened in transplant recipients, but a positive *Coccidiodes* antibody result indicates active or recent disease since antibody levels become negative again after successful treatment (Galgiani *et al.*, 2016). Urine and serum antigen testing is moderately sensitive, though cross-reaction with antigens from other endemic mycoses occurs (Durkin *et al.*, 2008).

Mild cases of coccidioidomycosis can be treated with an azole alone, but most cases in transplant recipients require initial therapy with amphotericin B. Once the patient shows clinical improvement, the amphotericin can be transitioned to azole therapy for approximately 12 months. Fluconazole and itraconazole are the most commonly used azoles, with fluconazole often being favored due to its predictable pharmacokinetics and its less troublesome drug-drug interactions (Galgiani *et al.*, 2016).

Coccidioidomycosis has a strong propensity to relapse in transplant recipients, and so suppressive azole therapy (e.g., fluconazole 200–400 mg daily) is usually given indefinitely following completion of the treatment course in HSCT and SOT recipients, or at least until all immunosuppressant medication is withdrawn (Galgiani *et al.*, 2016). Similarly, life-long suppressive therapy is probably indicated for all transplant recipients with a prior history of coccidioidomycosis, or changes consistent with prior coccidiodomycosis on a chest radiograph of patients who live in endemic areas, or who are seropositive. Finally, transplant recipients living in an endemic area who have no evidence of active or prior coccidioidomycosis, a low-dose azole (e.g., fluconazole 200 mg daily) is recommended for 6–12 months to prevent de novo infection (Galgiani *et al.*, 2016).

Pneumocystis Pneumonia

Pneumocystis jirovecii is the causative agent for *Pneumocystis* pneumonia (PCP) in humans. A ubiquitous fungus, infection is acquired through inhalation. Most cases of PCP occur as a result of reactivation of latent infection, but human-to-human transmission has been demonstrated to occur amongst transplant recipients (Vindrios *et al.*, 2017; de Boer *et al.*, 2007). The incidence in SOT recipients is approximately 0.3%–2.6%, and approaches 0% in HSCT recipients provided appropriate prophylaxis is taken (Iriart *et al.*, 2015; Cordonnier *et al.*, 2016).

PCP in transplant recipients presents much more acutely than it does in patients with HIV. (Iriart *et al.*, 2015) Disease is almost always confined entirely to the lungs, with dyspnea, hypoxemia, non-productive cough and fever being the predominant symptoms. Chest auscultation is often unremarkable even in the face of extensive pulmonary infiltration. Plain films may be normal in ~10% of cases, but computed tomography (CT) scans of the lung almost always shows abnormalities. The typical changes are of bilateral patchy areas of ground glass infiltration often abutting normal areas of lung, with a mid- to lower-zone predominance (Kanne *et al.*, 2012). Pleural effusions and lymphadenopathy are unusual in PCP since the fungus adheres to type I pneumocytes in the alveoli rather than invading into the parenchyma.

Diagnosis

Pneumocystis jirovecii cannot be cultured on standard laboratory media and thus diagnosis rests on visualization of the organism. Giemsa stains, silver stains, or immunofluorescent stains are required for this. BAL fluid is the optimal sample to test, with a sensitivity of 80%–97% using any of the staining methods. Induced sputum is less invasive alternative, but one with a correspondingly lower sensitivity (30%–55%) (Rodriguez and Fishman, 2004). An alternative to direct visualization is PCR. PCR assays have extremely good sensitivity and a correspondingly high negative predictive value. However, since *Pneumocystis jirovecii* frequently colonizes human airways without causing disease, false positive results are a concern (Iriart *et al.*, 2015). Using single-copy gene targets may allow for a better distinction between colonization and infection (Wilson *et al.*, 2011). Serum ß-D-glucan assays are more readily available than BAL fluid and are sensitive (>90%) but not specific for PCP.

Treatment

Trimethoprim-sulfamethoxazole (TMP-SMX) is the agent of choice to treat PCP. Alternatives for patients who cannot tolerate TMP-SMX include intravenous pentamidine or a combination of primaquine and clindamycin (Martin *et al.*, 2013). Patients who are significantly hypoxemic ($pAO_2 < 70$ mmHg) should receive adjunctive corticosteroids, since PCP symptoms may otherwise tend to worsen when treatment is initiated.

Prophylaxis

Prophylactic doses of TMP-SMX are extremely effective at preventing PCP, such that almost all infections in transplant recipients occur only after TMP-SMX is stopped (whether per institutional protocol or due to patient nonadherence) (Overgaard and Helweg-Larsen, 2007). The optimal duration of TMP-SMX prophylaxis is much debated, and probably varies by transplant type and immunosuppression regimen. For most SOT recipients, 6–12 months of prophylactic TMP-SMX is recommended, though lung transplant recipients in particular may require lifelong prophylaxis (Martin *et al.*, 2013). Allogeneic HSCT recipients should receive TMP-SMX for a minimum of 6 months starting from engraftment or as long as their immunosuppression is ongoing, whichever is longer. (Maertens *et al.*, 2016a).

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Fungal Infections in Cancer Patients

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Introduction

Fungi are a diverse group of organisms that cause a wide array of disease in humans. Fungi are associated with superficial infections of the skin or subcutaneous tissues, but may also cause invasive disease, even in immunocompetent hosts. In immunocompetent patients, a large inoculum of endemic fungi (e.g., Coccidioides) may cause severe invasive infection. In heavily immunocompromised hosts, such as patients with hematologic malignancies, even a low inoculum of opportunistic fungi (e.g., *Fusarium* species) results in severe disease. In the USA, fungal diseases were estimated to cost $7.2 billion in 2017 (Benedict *et al.*, 2019b). This is divided between 75,033 hospitalizations costing $4.5 billion and nearly 9 million outpatient visits, costing $2.6 billion. Notably, opportunistic mycoses, such as aspergillosis, invasive candidiasis, pneumocystosis, and mucormycosis, are associated with the largest economic burden (Benedict *et al.*, 2019b).

The increased risk of invasive fungal infection in cancer patients is attributable to impairment of the immune system from the cancer itself, such as those with hematologic malignancies, or with structural lung damage from tumor invasion, but also due to the detrimental effects of various cancer treatments on the immune response. Patients who are severely immunocompromised by cancer treatment or stem cell transplant could develop uncommon opportunistic fungal infections. In addition, cancer patients may develop endemic fungal infections to which immunocompetent hosts are also susceptible, such as coccidioidomycosis or histoplasmosis.

Effects of Cancer Treatment on Immune Response to Fungal Infection

Cancer therapy is constantly changing. Traditional modalities, such as radiation, surgery, and cytotoxic chemotherapy have led to the revolution of therapeutics in cancer with monoclonal antibodies targeted against surface molecules on cancer cells and therapies which harness the immune response against cancer. These include checkpoint inhibitors (CPIs), which enhance the cellular immune response, targeting cancer cells, but may also result in immune-mediated injury to several organs (Fishman *et al.*, 2019). The impact of CPIs on the risk of fungal infection is a matter of debate, with animal models of histoplasmosis, candidiasis, aspergillosis, and cryptococcosis suggesting a benefit from PD-1 and PDL-1 inhibitors (Abers *et al.*, 2019). A recent study examined the impact of PD-1 inhibitor alone, and in combination with caspofungin, on a mouse model of invasive pulmonary aspergillosis (Wurster *et al.*, 2020). The PD-1 inhibitor alone improved survival, decreased tissue fungal burden, and also reduced markers of pulmonary inflammation. In addition, the combination of PD-1 inhibitor with immunomodulatory antifungal caspofungin led to an additional benefit in mortality, compared to either agent alone (Wurster *et al.*, 2020). CPIs may be of particular benefit in patients with chronic fungal infections refractory to therapy, such as invasive candidiasis, reversing "immune exhaustion" that is a common barrier to overcoming such infections. Insufficient clinical data, however, are available regarding impact of CPIs on opportunistic mold infection.

Ibrutinib and other kinase inhibitors are another class of agents used to treat cancers, including chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and primary central nervous system (CNS) lymphoma. Although randomized clinical trials with these agents failed to demonstrate an increased risk of invasive fungal infections (IFI) (Chamilos *et al.*, 2018), several ibrutinib-related IFIs have been reported with the "real life" experience in CLL, especially CNS invasive aspergillosis, *Pneumocystis jirovecii* pneumonia, and cryptococcosis (Ruchlemer *et al.*, 2019).

Other novel cancer treatments include immunotherapy platforms, such as CAR-T cell and, more recently CAR-NK cell therapies. The conditioning therapy used to prepare patients with malignancy for these cell-based therapies, results in significant acute impairment of the immune response. Depending on the type and intensity of conditioning therapy and the underlying cancer, impairment of several elements of the immune system may be seen, lasting 6 months to 1 year. The impact of these therapies on risk of fungal infection is unclear, with current efforts to optimize prophylaxis, based on evolving understanding of risk stratification, including influence of the underlying cancer, prior cancer treatment, co-morbidities as well as the exposure to conditioning therapy in preparation for administration of CAR-T cells (Haidar *et al.*, 2019; Lewis and Kontoyiannis, 2020). In patients with hematologic malignancies, at highest risk of invasive fungal infection (IFI), efforts are ongoing to develop models to predict incidence of IFI (Stanzani *et al.*, 2019b). Future efforts to predict the patients at highest risk for severe IFI will provide guidance for which patients need prophylaxis and which patients benefit from surveillance with biomarkers without prophylaxis.

Opportunistic Fungal Infections

Invasive candidiasis (IC) remains the most common invasive fungal infection in patients with cancer, with *Candida* bloodstream infection the 2nd most common etiology of health-care associated bloodstream infection overall (Magill *et al.*, 2018). Candidiasis affects patients with compromise in skin and mucosal barriers. These barriers may be compromised by direct physical injury with

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Table 1 Fungal infections in cancer patients

Organism	*Clinical presentation*	*Treatment comments*
Candida spp.	Thrush Esophageal candidiasis Urinary tract infection Candidemia Disseminated candidiasis	• Remove CVC within 24 h of diagnosis for CVC-associated invasive candidiasis • Eye exam (await neutrophil recovery to improve yield) • Echocardiogram in patients with persistent Candidemia • Caspofungin for severe illness, may stepdown to fluconazole
Aspergillus spp.	Pulmonary Sinusitis Skin infection Disseminated	• Voriconazole, isavuconazole, Amphotericin B • Measure azole drug levels, especially voriconazole levels • CT sinus→if sinusitis present • Serial CT chest and serum Galactomannan are helpful in assessing response to treatment
Cryptococcus spp.	Pneumonia CNS Disseminated	• Serum Cryptococcus antigen • Amphotericin B 1st line for severe infection, with 5-flucytosine for meningitis • Fluconazole as initial therapy for less severe infection, may also be used for stepdown • Lumbar puncture, with repeat LP, if elevated CSF pressure
Fusarium spp.	Sinus infection Pneumonia Skin infection Dissemination	• Voriconazole 1st line treatment • Posaconazole, Amphotericin B also options • Echinocandins, isavuconazole are not effective • Difficult to treat without recovery from neutropenia
Mucorales spp.	Rhinocerebral Pulmonary Gastrointestinal Cutaneous	• Amphotericin B, posaconazole, isavuconazole, are effective • Surgical debridement and hyperbaric oxygen • Difficult to treat without recovery from neutropenia

indwelling vascular catheters and surgery (Tsay *et al.*, 2020). Cancer treatment, particularly cytotoxic cancer therapy, not only damages gastrointestinal mucosa, leading to translocation of organisms, but also leads to neutropenia, which impairs the immune response to invading organisms, including *Candida* species (Kullberg and Arendrup, 2015). Patients with invasive candidiasis develop persistent fever despite broad spectrum antibacterials, in context of neutropenia. As all other IFIs, a high index of suspicion is necessary to identify invasive candidiasis.

Several *Candida* species cause infection. *Candida albicans* remains the most common species, with high virulence, but low resistance. *Candida glabrata* is increasing in frequency, particularly in oncology patients, and is frequently resistant or tolerant to fluconazole (Farmakiotis and Kontoyiannis, 2017). The landscape of IC in patients with hematologic cancers on azole prophylaxis has changed dramatically in the last 20–30 years. Among leukemia patients with candidemia, at our tertiary center, approximately 1% of cases are attributable to *Candida albicans*, with *Candida parapsilosis* (32%) the most commonly isolated species, followed by *Candida tropicalis* (23%) and *Candida glabrata* (20%) (Wang *et al.*, 2015). This change in species has implications regarding empiric selection of antifungals, with 20.5% of isolates of *Candida glabrata* resistant to fluconazole, and 6.8% resistant to multiple classes of antifungals (Farmakiotis *et al.*, 2014).

Multiple clinical presentations are associated with candidiasis, from superficial infections to candidemia (Pappas *et al.*, 2016) (**Table 1**). *Superficial infections* include oropharyngeal candidiasis, esophagitis, and urinary tract infection (Pappas *et al.*, 2016). Oropharyngeal candidiasis, known as "thrush", is the most common of superficial fungal infections associated with candidiasis, classically occurring in patients undergoing chemoradiation (Samonis *et al.*, 1998). The diagnosis is clinical, with identification of whitish plaques on the buccal mucosa, palate, or tongue that may be scraped off, revealing an erythematous base (Pappas *et al.*, 2016). Treatment consists of clotrimazole 10 mg troches or nystatin suspension for 7–14 days (Pappas *et al.*, 2016).

Esophageal candidiasis should be considered in cancer patients with dysphagia, retrosternal pain, and odynophagia, especially if they have concomitant candidiasis (Samonis *et al.*, 1998). Given that endoscopy for direct identification may be problematic due to severe thrombocytopenia, esophageal candidiasis is often a clinical diagnosis (Pappas *et al.*, 2016). Treatment is longer, compared with oropharyngeal candidiasis, extending to 14–21 days. Therapy consists of fluconazole 200–400 mg (3–6 mg/kg) daily, with parenteral echinocandins, such as caspofungin, being an option for those patients who fail to tolerate, or fail therapy with, fluconazole (Pappas *et al.*, 2016).

Urinary tract infections with *Candida* species are typically associated with device infection, with Foley catheters and nephrostomy tubes serving as a nidus of these infections. In many cancer patients, these organisms may be associated with colonization of plastic surfaces and do not require treatment. In patients with neutropenic fever, however, candiduria may suggest occult disseminated candidiasis. Whether reflecting infection or colonization, an important first step is removal, if possible, or exchange of infected devices, such as nephrostomy tubes and Foley catheters (Pappas *et al.*, 2016). The most recent guidelines for management of candidiasis recommend 14 days of therapy with fluconazole 400 mg (approximately 6 mg/kg) daily. If a resistant *Candida* species is known or suspected, treatment may be changed to IV amphotericin B formulations, or amphotericin B bladder irrigation (Pappas *et al.*, 2016).

Invasive candidiasis, in contrast to superficial candidiasis, is a systemic infection and always requires systemic antifungal treatment. The infection still is associated with high mortality in cancer patients. A cohort study from Europe pointed to a 12 week crude

overall mortality in cancer patients of 49% (Cornely *et al.*, 2015). Historically, *Candida albicans* was the most common species associated with cancer, particularly in patients with less than 7 days of exposure to antifungals (Kullberg and Arendrup, 2015). However, in the era of widespread and prolonged antifungal prophylaxis, breakthrough candidemia with *Candida glabrata* or *Candida krusei* has been increasingly reported (Kullberg and Arendrup, 2015). *Candida parapsilosis* is associated with central venous catheter (CVC) infections (Horn *et al.*, 2009), but has also been noted, along with *Candida krusei*, in breakthrough infections in patients receiving micafungin for prophylaxis (Yacoub *et al.*, 2016). Candidemia is the most common manifestation of invasive candidiasis, with disseminated or single organ candidiasis now very rare, given the frequent use of prophylaxis for at-risk patients.

Diagnosis of invasive candidiasis routinely relies on blood culture, but given that cultures are negative in approximately 50% of cases of invasive candidiasis, non-culture based approaches have been developed (Pappas *et al.*, 2016; Clancy and Nguyen, 2018; Clancy *et al.*, 2018). These evolving tools include detection of 1,3-beta-D-glucan, T2 Candida, mannan antigen and mannan antibody, and various PCR-based approaches (Pappas *et al.*, 2016; Clancy and Nguyen, 2018). These molecular assays have a sensitivity ranging from 58% to 91% and specificity 80%–98% (Johnson *et al.*, 2020). Concerns with the molecular approaches include cross-reactivity with other organisms that result in poor specificity, variable sensitivity, and concern that PCR testing, in particular, may identify organisms that are no longer viable. Therefore, blood culture remains the "gold standard" for diagnosis of invasive candidiasis.

Treatment of invasive candidiasis requires knowledge of the local epidemiology, as the frequency and resistance profile of *Candida* species vary in different centers, and the prior exposure of the particular patient to antifungals. In addition to appropriate selection of antifungals, various other elements are important in reducing mortality in patients with candidemia (Farmakiotis *et al.*, 2015). These elements have been developed into recommended bundles of care (Mejia-Chew *et al.*, 2019; Johnson *et al.*, 2020). Bundle elements that are associated with lower mortality include central line removal, echocardiography to examine for evidence of endocarditis (especially in patients with persistent candidemia), ophthalmologic examination, and longer duration of antifungal therapy (Mejia-Chew *et al.*, 2019). In neutropenic cancer patients, some elements of the bundle might be different. For example, the yield of ophthalmologic examination is considered very low in neutropenic patients, until at least one week after neutrophil recovery (**Table 1**) (Pappas *et al.*, 2016; Breazzano *et al.*, 2019). Risk factors associated with the rare complication of candidemia, *Candida* endocarditis, include prosthetic heart valves and presence of CVCs (Pappas *et al.*, 2016). Similarly, a recent study, however, showed that hematologic malignancy was associated with decreased risk for *Candida* endocarditis (aOR 0.09 [95% CI, 0.04–0.69]) (Foong *et al.*, 2020). This finding on multivariate logistic regression was attributed to thrombocytopenia, which reduces the formation of the fibrin-platelet complex and subsequent formation of valvular vegetations. Another proposed explanation for the low frequency of *Candida* endocarditis is that patients with hematologic malignancy are more aggressively monitored and treated, resulting in earlier identification and treatment of candidemia (Foong *et al.*, 2020).

Candida species identification and possibly in vitro susceptibility inform the selection of appropriate antifungal agents. Classes of antifungal agents used to treat invasive candidiasis include azoles (e.g., fluconazole, voriconazole), echinocandins (e.g., caspofungin), and polyenes (e.g., amphotericin B formulations) (**Table 2**) (Pappas *et al.*, 2016). If patients are stable clinically, without prior exposure to azole therapy, fluconazole is the 1st line agent recommended for treatment of invasive candidiasis (Pappas *et al.*, 2016). If patients are critically ill or neutropenic, echinocandins are recommended (**Table 1**) (Pappas *et al.*, 2016). Amphotericin B formulations are recommended for treatment of *Candida glabrata* (2nd line treatment to an echinocandin), *Candida parapsilosis* (2nd line treatment), and *Candida krusei*, with use limited due to nephrotoxicity (Pappas *et al.*, 2016). Several conundrums in treatment of candidiasis in neutropenic cancer patients exist (Colombo *et al.*, 2020).

Aspergillus spp. are the most common causes of invasive mold infections in cancer patients, affecting those with impaired immune systems, particularly those with hematologic malignancies. Similar to the endemic fungi, these organisms are inhaled, with conidia migrating to the distal alveolar space, where they germinate. At these sites, the immune system responds, with mononuclear cells and polymorphonuclear leukocytes attacking these spores as they germinate into hyphae. *Aspergillus* spp. are angioinvasive, occasionally resulting in catastrophic hemorrhagic episodes. The angioinvasive nature of *Aspergillus* spp. can be appreciated by tissue injury in the lungs seen on CT chest. A recent study suggested that enhanced CT imaging, utilizing angiography, may be particularly helpful in identifying infection by angioinvasive molds (Stanzani *et al.*, 2019a). Aspergillosis also affects sinuses, with acute sinusitis occurring in 15%–20% of neutropenic patients (Kosmidis and Denning, 2015; Andes *et al.*, 2016). In fact, sinus discharge with headache and facial swelling and ocular pain associated with sinus infection may be the initial clue to diagnosis of invasive aspergillosis (Fung *et al.*, 2019).

Risk factors for invasive aspergillosis include prolonged and significant cytopenias, high dose corticosteroids and active hematologic malignancy. Recent reports of invasive aspergillosis after respiratory viral infection, however, suggest a need for vigilance in less immunocompromised cancer patients with these viral infections. In a recent multicenter study, 19% of influenza patients admitted to the ICU were diagnosed with pulmonary aspergillosis (Schauwvlieghe *et al.*, 2018). In the midst of the SARS-CoV-2 pandemic, up to 27% of patients admitted to an ICU and mechanically ventilated were diagnosed with probable COVID-19 associated pulmonary aspergillosis (CAPA) (Bartoletti *et al.*, 2020). Suboptimal diagnostics make it difficult to define the role of SARS-CoV-2 infection in increasing risk for pulmonary aspergillosis, since SARS-CoV-2 patients do not typically undergo bronchoscopy with bronchoalveolar lavage (BAL).

The diagnosis of invasive aspergillosis infection relies on a combination of clinical suspicion, assessment of radiologic abnormalities on chest and sinus CT, culture and cytology from respiratory, sinus, or other specimens, β-D-glucan, and serum galactomannan (Marr *et al.*, 2004; Andes *et al.*, 2016). Other mold infections of the lung may appear similar, with a single center study suggesting that invasive pulmonary aspergillosis, in contrast with mucormycosis, is less likely to exhibit less than

Table 2 Antifungal characteristics

Drug	*Spectrum of activity*	*Daily dose*	*Route*	*Side effects*
D-AMB	Broad yeasts, molds	1–1.5 mg/kg	IV only	Nephrotoxicity, hypokalemia, hypomagnesemia
Lipid AMB	Broad yeasts, molds	3–5 mg/kg	IV only	Nephrotoxicity < D-AMB, hypokalemia, hypomagnesemia
Fluconazole	Yeasts, including *Cryptococcus*	400–800 mg (6 mg/kg/day)	IV, Oral	Headache, rare hepatotoxicity
Isavuconazole	Broad yeasts, molds, not *Fusarium*	372 mg	IV, Oral	Diarrhea, headache
Itraconazole IV	Endemic molds, some *Candida* spp.	200 mg	IV/oral	Nausea, vomiting, headache, rare hepatotoxicity, drug interactions (oral with osmotic diarrhea)
SUBA-itraconazole	Endemic molds, *Aspergillus* spp., some *Candida* spp.	130 mg (130 mg q12hrs Aspergillosis)	Oral	Nausea, vomiting, headache, rare hepatotoxicity, pulmonary edema, drug interactions
Posaconazole tabs	Broad yeasts, molds	300 mg	Oral	Nausea, vomiting, hepatotoxicity
Posaconazole IV	Broad yeasts, molds	300 mg	IV	Nausea, vomiting, hepatotoxicity
Posaconazole (susp)	Broad yeasts, molds	200 mg every 6 h	Oral	Nausea, vomiting, hepatotoxicity
Voriconazole IV	Yeasts, molds, not *Mucorales* spp.	4 mg/kg every 12 h	IV	Visual abnormalities, rash, nausea, vomiting, headache, hepatotoxicity, drug interactions, skin cancer, bone health issues, neuropathy
Voriconazole tabs	Yeasts, molds, not *Mucorales* spp.	200 mg every 12 h (>40 kg)	Oral	Visual abnormalities, rash, nausea, vomiting, headache, hepatotoxicity, drug interactions
Caspofungin	Broad yeasts, Aspergillus, not endemic, other molds	50 mg	IV	Fever, nausea, flushing; rash; some drug interactions; phlebitis
Micafungin	Broad yeasts, Aspergillus, not endemic, other molds	150 mg	IV	Fever, nausea, flushing; rash; some drug interactions; phlebitis
Anidulafungin	Broad yeasts, Aspergillus, not endemic, other molds	100 mg	IV	Fever, nausea, flushing; rash; some drug interactions; phlebitis

Note: D-AMB, amphotericin B deoxycholate; IV, intravenous; susp, suspension; tabs, tablets.

10 pulmonary nodules or pleural effusion (Chamilos *et al.*, 2005). Given the challenge of identifying specific causes of invasive pulmonary mold infections, early bronchoscopy significantly increases likelihood of identifying a specific mold. Bronchoscopy performed less than 4 days after presentation increases the yield from as low as 22% to as high as 78% (Shannon *et al.*, 2010).

Treatment of aspergillosis consists of various options. Mold active triazoles, such as voriconazole, isavuconazole, and posaconazole are the mainstay of treatment, while lipid formulations of amphotericin B are considered second line in view of nephrotoxicity (**Table 1**). Echinocandins have a limited role as monotherapy for treatment of IA (**Table 1**). Mortality depends on the resolution of neutropenia with invasive aspergillosis, with mortality of 45% resulting from treatment with either leading option of voriconazole or isavuconazole, in the setting of persistent neutropenia (Kontoyiannis *et al.*, 2018). General principles of management of invasive aspergillosis include efforts at early diagnosis, attention to drug-drug interactions, therapeutic drug monitoring, discontinuation of unnecessary immunosuppressive medications, and appropriate follow up to assure resolution of signs and symptoms attributable to infection, to include imaging and/or biomarkers (Patterson *et al.*, 2016; Fernandez-Cruz *et al.*, 2020).

Cryptococci are encapsulated yeasts that emerged as important pathogens affecting HIV patients in the 1980s. Among the Cryptococci, *Cryptococcus neoformans* is the most common species infecting humans (Perfect *et al.*, 2010). This organism is found in pigeon secretions, and when inhaled, it could cause disease in patients with lymphopenia or lymphocyte dysfunction, associated with chemotherapy and corticosteroids (Kontoyiannis *et al.*, 2001). Ibrutinib and other kinase inhibitors, as mentioned earlier, have been associated with excess risk for cryptococcosis, as well as other invasive fungal infections (Chamilos *et al.*, 2018). Patients may have various presentations of cryptococcal infection, including pneumonia, central nervous system (CNS) infection, and disseminated cryptococcosis. Diagnosis of pneumonia is confirmed by fine-needle aspiration, BAL, and/or open lung biopsy, with 90% yield on culture, in a single cancer center study (Kontoyiannis *et al.*, 2001). CNS infection may manifest with fever and headache, dizziness, somnolence, confusion, and obtundation (Perfect *et al.*, 2010). Painless skin lesions are often a visible manifestation of disseminated cryptococcosis, which also affect the liver, prostate, eyes, and bone (Perfect *et al.*, 2010). Biopsy of the lesions with fungal culture may identify these yeasts, but serum cryptococcal antigen has a sensitivity and specificity over 90% for invasive disease (Perfect *et al.*, 2010).

Treatment of cryptococcal infection depends on the affected site (**Table 1**). In addition to patient with CNS manifestations of cryptococcal infection, other high risk groups for CNS infection should undergo routine diagnostic lumbar puncture. For example, patients with proven disseminated Cryptococcus, severe pulmonary cryptococcal infection, HIV, and otherwise immunocompromised hosts with proven infection, should undergo routine lumbar puncture. For patients with severe pulmonary or CNS infection, amphotericin B formulations (e.g., Liposomal amphotericin B 5 mg/kg/day) are typically combined with 5-flucytosine, as an induction therapy. 5-flucytosine levels should be measured, to avoid toxicity. Patients with suspicion of CNS infection

should undergo lumbar puncture with measurement of baseline CSF pressure. If the CSF pressure is ≥ 25 cm H_20, then the patient should undergo daily lumbar puncture until the CSF pressure and symptoms remain stable for more than 2 days (Perfect *et al.*, 2010). After establishing control of infection, typically after at least 2 weeks, there is a transition to consolidation therapy with fluconazole 6 mg/kg/day, to be followed by maintenance therapy 200 mg daily for a period of at least 6–12 months. Patients with mild to moderate pulmonary infection may be treated with fluconazole 6 mg/kg/day alone, not requiring the more toxic amphotericin B formulation with 5-flucytosine (Perfect *et al.*, 2010). Given the complexity of management, infectious diseases consultation has been shown to reduce mortality (Spec *et al.*, 2017).

Fusarium spp. are opportunistic molds derived from air and soil, causing a spectrum of infections, from superficial skin and keratitis, due to direct inoculation, to locally invasive disease following trauma, to severe sino-pulmonary or disseminated infection. The *Fusarium solani* complex of species is associated with half of cases (Campo *et al.*, 2010). The invasive form of these infections affects profoundly immunosuppressed patients with uncontrolled hematologic malignancy or stem cell transplant (Campo *et al.*, 2010). These cases are often associated with fungemia, with blood cultures positive in 50%–70% of cases (Lionakis and Kontoyiannis, 2004). In these neutropenic patients, dissemination may occur as often as 75% of cases, classically with papules with central necrosis or eschar (Bodey *et al.*, 2002). Unfortunately, outcomes remain poor, with mortality ranging from 50% to 94% (Lionakis and Kontoyiannis, 2004; Campo *et al.*, 2010; Lamoth and Kontoyiannis, 2019), since the underlying disease is the major driver of mortality. Treatment is challenging, with limited effectiveness of various agents, with voriconazole and posaconazole used as alternatives to amphotericin B formulations, depending on the *Fusarium* spp. (**Tables 1** and **2**).

Mucormycosis is caused by various *Mucorales* spp., with exposure by inhalation of these mold spores from the environment. This mold, similar to *Aspergillus* spp., is highly angioinvasive, resulting in thrombosis and tissue infarction. Cancer patients affected include those with acute leukemia and HSCT recipients, but may also include those with high corticosteroid use, diabetic ketoacidosis or iron overload (Kontoyiannis and Lewis, 2014). Prior exposure to voriconazole is also a significant risk factor for mucormycosis (Chamilos *et al.*, 2005). Mucormycosis in cancer patients typically presents as sinusitis, sinopulmonary disease, less often as rhino-orbital infection (typically seen in patients with diabetic ketoacidosis) (Roden *et al.*, 2005). Disseminated infection, manifesting as cutaneous, GI, or other single organ mucormycosis, can be encountered in severely immunocompromised cancer patients. As with *Fusarium* spp., treatment can be difficult, with an overall mortality rate of 44%–71% (Kontoyiannis and Lewis, 2014), although neutropenic patients with disseminated disease have an abysmal 90% mortality rate, especially if treatment is delayed (Chamilos *et al.*, 2008). Therapy for mucormycosis consist of amphotericin B lipid formulations, with posaconazole and isavuconazole used for stepdown or intolerance to amphotericin B formulations in less severe disease (**Table 1**) (Roden *et al.*, 2005; Spellberg *et al.*, 2009; Miceli and Kauffman, 2015).

Endemic Fungal Infections

In the USA, there are three common endemic fungal infections, which are caused by dimorphic fungi. These include coccidioidomycosis, histoplasmosis, and blastomycosis, with a wide geographic distribution (**Fig. 1**). The organisms are dimorphic fungi that share inhalation as the most common mode of acquisition of infection, but differ in their geography and associated climate. These endemic fungal infections share the predisposition to cause pulmonary disease, with lung nodules that may at times be confused with malignancy (Kontoyiannis, 2017). In patients with known primary or metastatic lung disease, pulmonary imaging may show overall improvement of oncologic lung disease, but with development of new lung lesions. Given suspicion of cancer, patients undergo biopsy, with fungal organisms present on pathology, rather than the anticipated cancer. In such a scenario, cultures are often not sent, leaving pathologists with the challenge of attempting diagnosis based on morphology. Fungal antibody and/or urine antigen testing may also fail to reveal the endemic fungal infection, leaving healthcare providers with the task of selecting empiric treatment, based on limited information. In such cases, other features noted on careful history, examination, and assessment of available laboratory and radiologic data, help to identify the specific endemic mold infection.

Coccidioidomycosis, otherwise known as "Valley fever", is a fungal infection associated with the inhalation of single celled arthroconidia, with a wide distribution in the southwest USA (**Fig. 1**) (Donovan *et al.*, 2019). The two species that cause disease in the USA are *Coccidioides immitis* and *Coccidioides posadasii* (Van Dyke *et al.*, 2019), with the distribution in the arid regions of the southwestern USA, although with endemic cases reported in Eastern Washington (Van Dyke *et al.*, 2019), as well as Mexico, Central and South America (Donovan *et al.*, 2019). Approximately 60% of the estimated 350,000 patients infected annually develop asymptomatic or mildly symptomatic infections (Donovan *et al.*, 2019; Van Dyke *et al.*, 2019). Some patients develop a syndrome suggestive of community-acquired pneumonia, with nonproductive cough, chest pain, and shortness of breath (Valdivia *et al.*, 2006).

Other patients develop more severe symptoms, including arthralgias, fever, night sweats, and weight loss, with dissemination to bones and joints, meninges, central nervous system (meningitis), and/or skin estimated to occur in $<1\%$ of patients (Galgiani *et al.*, 2016; Van Dyke *et al.*, 2019). Approximately 16% of patients with disseminated disease develop central nervous system infection (typically meningitis), which is almost universally fatal, if untreated. A recent study showed immunosuppression increased risk for dissemination, estimated to occur in 30%–50% of immunocompromised hosts, but only 13% of those in the study were cancer patients (Odio *et al.*, 2017). Diagnosis of coccidioidomycosis consists of biopsy with culture, serologic testing, antigen testing, and PCR (Nguyen *et al.*, 2013). On biopsy, a spherule may be seen, which contains endospores, recognized as consistent with coccidioidomycosis, if seen in this form.

Treatment of coccidioidomycosis depends on the presentation of the patient and risk factors (Galgiani *et al.*, 2016). Recent expert commentary suggested that treatment is not necessary for patients with self-limiting disease, stable observed manifestations of

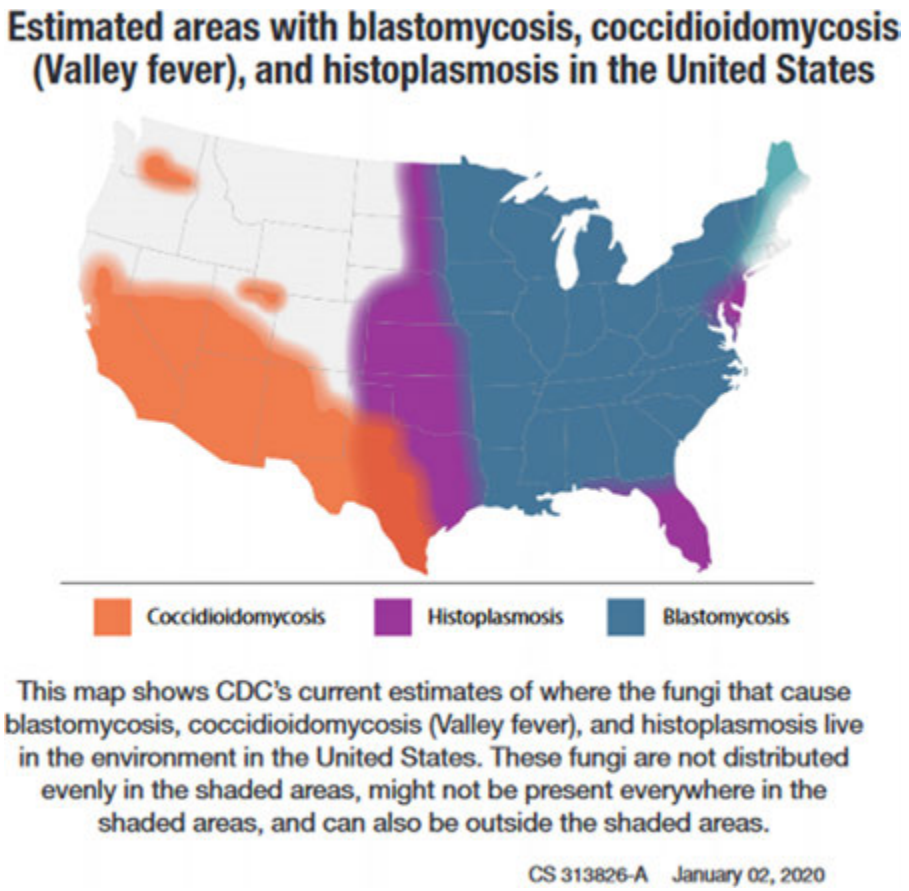


Fig. 1 Geographic distribution of endemic fungal infections. Available at: https://www.cdc.gov/ncezid/dfwed/PDFs/fungal-factsheet-508c.pdf.

disease, such as lung nodules, but noting that those with "major cellular immunodeficiency" are predisposed to progressive disease, so should proceed with treatment (Galgiani *et al.*, 2019). To reflect the challenge of the scenario of an immunosuppressed cancer patient with asymptomatic coccidioides lung nodules, the suggestion is to evaluate for occult extrapulmonary findings with PET/CT (Foerter *et al.*, 2016) and proceed with treatment trial with an oral azole (e.g., fluconazole) for 3 months, with close monitoring (Kontoyiannis, 2017). Depending on the risk for other mold infections, such as hematologic malignancy or allogeneic transplant, a more broad mold active agent, such as voriconazole or posaconazole may be considered (**Table 2**) (Kontoyiannis, 2017).

Histoplasmosis is classically seen in the Ohio and Mississippi River Valleys, in areas of high humidity and moderate temperature (**Fig. 1**). Patients with histoplasmosis may share a recent history of cave exploration ("spelunking"), work or live near chickens or birds, chop wood, live or frequent wooded areas, or be involved in construction, renovation, or cleaning of buildings with bird or rat droppings. Patients typically acquire this fungal infection by inhalation, with the formation of granulomatous lesions in the lung and other organs (Benedict *et al.*, 2019a). Patients predominantly have asymptomatic or self-limited infection, but one out of every two thousand patients develops chronic pulmonary infection, and some severely immunocompromised patients develop disseminated histoplasmosis. A recent surveillance study conducted by the CDC found a 67% hospitalization rate with 7% mortality (Armstrong *et al.*, 2018). A 2012 study that provided greater detail regarding immunocompromised patients showed a distribution of HIV (17%), diabetes mellitus (21%), and autoimmune diseases (10%), but only 7% of patients with hematologic malignancy or transplant (Benedict *et al.*, 2016).

Diagnosis of histoplasmosis, as with coccidioidomycosis, can be challenging. Epidemiology provides important initial clues, in patients with lung nodules. As with other fungal infections, the optimal approach is direct visualization of the fungus from tissue, ideally with culture to confirm the diagnosis. Chest imaging commonly demonstrates not only pulmonary nodules, but also mediastinal lymphadenopathy. A study of an enzyme immunoassay to detect IgG and IgM antibodies to optimize sensitivity in acute pulmonary histoplasmosis showed a combined sensitivity of 76.7% (Richer *et al.*, 2016). In the study, complement fixation showed 66.7% sensitivity and urine histoplasmosis antigen sensitivity of 53.8%, but increased to 76.7% when combined with serum antigen (Richer *et al.*, 2016). More recently developed tests include lateral flow and isothermal amplification assays (Sanguinetti *et al.*, 2019). A high index of suspicion is necessary to consider appropriate testing, with many options available for diagnosis.

Treatment is generally deferred in patients with incidental finding of an asymptomatic pulmonary nodule, but cancer type and underlying degree of immune deficiency are considered in assessing risk for progression without treatment. Treatment of

histoplasmosis depends on severity of infection, in addition to underlying immunocompromised status, with Amphotericin B formulations recommended for patients with severe pulmonary or disseminated infection (**Table 2**) (Wheat *et al.*, 2007). Itraconazole continues to be the recommended 1st line oral therapy, per guidelines (Wheat *et al.*, 2007), but other options include posaconazole, voriconazole, and isavuconazole (**Table 2**) (Wheat *et al.*, 2016). In addition, SUBA-itraconazole is a new formulation of itraconazole with significantly improved bioavailability (**Table 2**) (Abuhelwa *et al.*, 2015). Fluconazole is another option, but has a high failure rate, so it is generally avoided (Wheat *et al.*, 2007; Wheat *et al.*, 2016). Treatment should be individualized, with multiple factors considered, including underlying immunodeficiency from cancer and from the treatment for cancer, comorbidities, underlying lung disease, potential for drug-drug interactions with ongoing cancer treatment, absorption, compliance, and feasibility for follow up.

Blastomycosis is the 3rd most endemic invasive fungal infection, and can be encountered in both immunocompetent, and immunocompromised hosts, including cancer patients. As with histoplasmosis and coccidioidomycosis, blastomycosis is a dimorphic fungus present in soil, with conidia inhaled into the lungs, where they convert to yeast form. Similar to coccidioidomycosis and histoplasmosis, approximately 50% of patients develop subclinical infection, with up to 75% of cases remaining confined to the lungs (Castillo *et al.*, 2016). Blastomycosis shares a similar geographic distribution to histoplasmosis, with highest incidence in the Mississippi and Ohio River valleys, but is seen further to the north, particularly the Great Lakes area into Canada, and the southeast (**Fig. 1**).

Blastomycosis may present as an incidental finding on chest imaging during the initial diagnostic workup for cancer or during radiologic surveillance of cancer patients. Patients may also develop fever, chills, cough, and occasionally hemoptysis. A study from Indiana showed a 12% mortality with diabetes mellitus, multilobar pneumonia, and immunosuppression (16% of the cohort, with 56% of those either with solid or hematologic malignancy) associated with increased risk of ICU admission (Azar *et al.*, 2015).

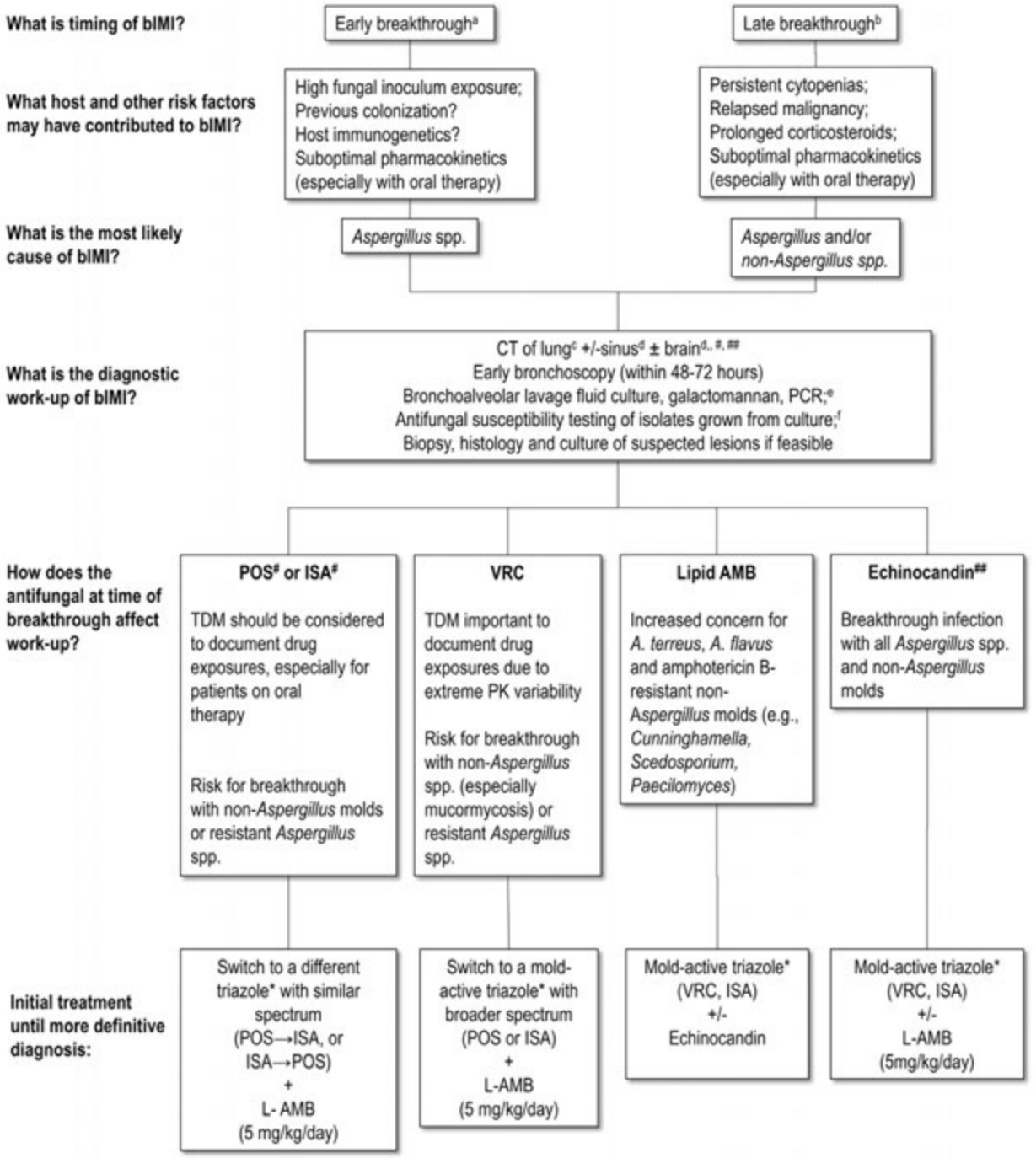
Diagnosis of blastomycosis consists of direct visualization of the organism on biopsy of skin or pulmonary nodules and yield of culture from bronchoscopic specimens has been reported as high as 92% (Martynowicz and Prakash, 2002). A differentiating feature of blastomycosis is that rather than formation of pulmonary nodules, lung consolidation is more common (McBride *et al.*, 2017). An antigen assay that targets the galactomannan component of the cell wall may be used on urine, serum, cerebrospinal fluid, and bronchoalveolar lavage specimens. Sensitivity in urine is 76%–93%, with specificity of 79%, but there is cross-reactivity with other endemic fungi such as histoplasmosis and coccidioidomycosis. Of note, the antigen concentration can be used to monitor response to treatment (Frost and Novicki, 2015).

Guidelines for the treatment of blastomycosis exist (Chapman *et al.*, 2008). The decision to treat and the selection of agent depends on the site of infection, as well as the severity of immunosuppression of the host. In contrast to other endemic fungal infections, the recommendation is to routinely proceed with treatment, except in cases with significant clinical improvement before confirmation of the diagnosis (Chapman *et al.*, 2008). Given the concern for development of extrapulmonary infection, particularly in immunocompromised hosts, the preference is to proceed with oral itraconazole, based on available guidelines for asymptomatic cases, as well as those with mild to moderate pulmonary infection or disseminated infection (Chapman *et al.*, 2008; McBride *et al.*, 2017). For patients with moderately severe disease, the recommendation is to start with a lipid formulation of amphotericin B. Patients who are immunocompromised, as well as those who have CNS disease, should also be given amphotericin B formulations, regardless of manifestation of infection. The duration of the amphotericin B-based intravenous regimen, is typically limited to 1–2 weeks, or until the patient improves clinically and/or radiologically. Itraconazole poses challenges of absorption and toxicity. The new formulation of itraconazole, SUBA-itraconazole, addresses the challenge of absorption, dramatically increasing bioavailability (**Table 2**) (Abuhelwa *et al.*, 2015). Newer antifungals, such as voriconazole, posaconazole, and isavuconazole (**Table 2**), however, exhibit activity against blastomycosis. These medications have less drug-drug interactions than itraconazole, and achieve target concentrations with less difficulty, particularly in cancer patients, in whom drug-drug interactions and requirements for absorption of eating or drinking certain foods are challenging (McBride *et al.*, 2017).

In summary, the endemic mycoses, including coccidioidomycosis, histoplasmosis, and blastomycosis, often manifest with lung nodules and constitutional symptoms, which may initially be mistaken for lung cancer or lung metastases of another primary cancer. All these endemic mycoses result from environmental exposures to spores, which can transform after inhalation or inoculation into different forms. Endemic mycoses are typically associated with mild disease, but may cause severe disease in both immunocompromised hosts with cancer. A high index of suspicion and knowledge of epidemiological range of these organisms help to guide diagnosis. Once the diagnosis is established, careful consideration is essential, beginning with "staging" of infection, to determine if dissemination has occurred from the lungs and the extent of the seeding of infection. This "staging" of infection will impact the selection of the appropriate antifungal, route of therapy, and duration of antifungal therapy.

Prevention of Fungal Infections in Cancer Patients

The prevention of fungal infections in cancer patients requires a baseline assessment of risk, based on knowledge of host factors as well as the impact of cancer treatment on immune responses to fungal infection. Among recommendations to prevent fungal infection in cancer patients, an important note is to consider environmental exposures (Ariza-Heredia and Kontoyiannis, 2014). This encompasses exposure to soil excavation, construction, landscaping, farming, and gardening. These activities may lead to inhalation of mold spores or direct inoculation of the organisms into the skin. Other environmental exposures of concern include



[a] Primary prophylaxis during initial remission-induction chemotherapy; [b] Prophylaxis during re-induction for relapsed leukemia, prolonged corticosteroid treatment for graft-versus-host disease; [c] CT pulmonary angiography used in some centers to identify angioinvasive process (vessel occlusion sign) inside nodular infiltrates or consolidations; highly suggestive of invasive mold disease and not affected by antifungal prophylaxis; [d] If suggestive signs of involvement; [e] PCR test for *Aspergillus* spp. with or without detection of L98H, tandom repeat 34, T289A, and Y1212F triazole resistance mutations. Not approved in U.S. or available in most centers; [f] Susceptibility testing is not available in many centers and clinical breakpoints are not established for non-*Aspergillus* molds; # although ISA and POS achieve lower concentrations in the CNS compared to VRC, it is unclear whether more CNS bIMIs are seen with the first 2 triazoles; ## echinocandins are considered to achieve suboptimal CNS concentrations; * starting with an IV formulation is favored, especially for VRC. Less convincing data exist that IV POS results in better levels than POS DR, tablets, no data exist regarding ISA.
AMB, amphotericin B; CNS, central nervous system; CT, computed tomography; ISA, isavuconazole; IV, intravenous; PCR, polymerase chain reaction; POS, posaconazole; PK, pharmacokinetic; TDM, therapeutic drug monitoring; VRC, voriconazole.

Fig. 2 Management of breakthrough fungal infections.

smoking, especially marijuana, and consumption of certain foods, particularly for patients who are neutropenic. These include fresh fruits, smoked foods, moldy cheeses, and spices and nuts that can be contaminated with fungi.

In patients with cancers, such as ALL, MDS, AML, prophylaxis with antifungals is often recommended to prevent fungal infection. Prophylaxis with fluconazole is associated with high risk of breakthrough mold infection (**Fig. 2**) (Halpern *et al.*, 2015; Lionakis *et al.*, 2018). Echinocandins (e.g., micafungin) provide activity against *Candida* spp., as well as some *Aspergillus* spp. isolates, but do not provide protection against non-Aspergillus molds, such as *Fusarium* or *Mucorales* spp., and are not favored for prophylaxis in very high risk patients (**Table 1**) (Gomes *et al.*, 2014). Voriconazole provides excellent activity against *Candida* spp., *Aspergillus* spp., and *Fusarium* spp. (**Table 1**) (Halpern *et al.*, 2015), but is associated with excess cases of infections with *Mucorales* species (**Fig. 2**) (Chamilos *et al.*, 2005; Lionakis *et al.*, 2018). Posaconazole has very broad activity against yeasts and many mold isolates, including *Mucorales* species (**Table 1**), showing an impact on survival in patients undergoing induction therapy for AML (Halpern *et al.*, 2015). The class of polyenes (e.g., amphotericin B) also has broad activity, but exhibits unacceptably high rates of nephrotoxicity and is reserved for treatment, not prophylaxis.

Mycobiome

In contrast to the study of the microbiome, the "mycobiome" has not been well characterized in cancer patients. Increasing work, however shows the impact of cancer treatment on the mycobiome and the impact of the mycobiome on cancer (Aykut *et al.*, 2019, Robinson *et al.*, 2020). In a recently described cohort of patients with acute myelogenous leukemia (AML) undergoing remission induction chemotherapy (RIC), the change in mycobiome diversity did not appear to be impacted by antibacterial therapy, but was decreased in patient undergoing high-intensity RIC. In addition, the relative abundance of *Fusarium* species increased with amphotericin B exposure (Robinson *et al.*, 2020). Of interest, relative abundance of particular fungi at particular time points during RIC was associated with risk of bacterial infection, adding to the evidence that there are interkingdom interactions between fungi and bacteria that likely have an impact on patient outcomes (Robinson *et al.*, 2020). A fascinating, yet concerning, finding is that an approach to addressing refractory *Clostridioides difficile* infection, using fecal microbiota transplantation (FMT), is negatively impacted by *Candida* colonization of the GI tract (Galloway-Pena and Kontoyiannis, 2020). This is attributed to the impact of *Candida* colonization on attempts to reconstitute gut flora.

A change in the mycobiome not only impacts the risk for bacterial infection, but a recent study suggests that fungi may migrate from the intestinal lumen to the pancreas in an animal model of pancreatic ductal adenocarcinoma. This migration results in tumor growth driven by binding of the glycan surface of the cell wall to mannose-binding lectin, driving the complement cascade to "accelerated" oncogenesis (Aykut *et al.*, 2019). Of interest, the oncogenesis, even in the setting of inflammation, was not driven by all fungal species, but specifically by *Malassezia* species (Aykut *et al.*, 2019). Similarly, changes in the mycobiome, with increasing *Malassezia* and decreased *Saccharomyces* species, have also been associated with colorectal cancer (Galloway-Pena and Kontoyiannis, 2020).

Future Directions

In summary, fungi are a diverse group of organisms that may affect cancer patients with manifestations, ranging from superficial skin infection to pneumonia with disseminated disease. Some virulent fungi, such as Histoplasma, infect patients with intact immune systems. However, most fungal infections in cancer patients are caused by opportunistic fungi, such as *Candida* or *Aspergillus* spp., which are typically harmless colonizers on mucosal surfaces in immunocompetent hosts. The impact of new cancer treatments, including immunomodulatory therapies, on the risk of fungal infection is an exciting area of study (Chamilos *et al.*, 2018; Abers and Lionakis, 2020). Novel diagnostics and new antifungals with novel mechanisms of action are being developed, with emphasis on varied routes of administration and decreased drug-drug interactions.

With improved outcomes of cancer treatment resulting in a 27% decrease in cancer death rate from 1991 to 2016, the role of fungal infection will become increasingly important not only in patients undergoing active cancer treatment, but also in cancer survivors. The number of cancer survivors in the USA is expected to grow from 16.9 million cancer survivors on 2019 to a projected 22.1 million by 2030 (Siegel *et al.*, 2019). An improved understanding of how fungi interact with the host and other organisms will guide optimal outcomes for these often underappreciated organisms in cancer care.

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Fungal Infections in the Setting of Biological Therapies (in the Non-Transplant Host)

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Introduction

In the past few decades, the AIDS epidemic and major advances in modern medicine associated with the introduction of a variety of immunomodulatory and chemotherapeutic interventions for the treatment of autoimmune and neoplastic diseases and the expansion of the clinical indications and applications of hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT) have markedly expanded the populations of individuals who are at risk for development of mucosal and/or invasive fungal infections (IFIs) (Brown *et al.*, 2012; Lionakis *et al.*, 2017b; Lionakis and Levitz, 2018). Indeed, opportunistic fungal infections have emerged as significant causes of morbidity and mortality in acutely ill and immunosuppressed patients. For example, invasive candidiasis is among the three most common causes of nosocomial bloodstream infections in intensive care units in the United States with mortality that exceeds 30%–40% despite administration of antifungal therapy (Magill *et al.*, 2014, 2018; Strollo *et al.*, 2016). The recent emergence of drug-resistant *Candida* species including *Candida glabrata* and *Candida auris* raises significant public health concerns (Alexander *et al.*, 2013; Vallabhaneni *et al.*, 2019). *Candida* species also cause mucosal infections, which occur in the form of oral and esophageal disease in the vast majority of AIDS patients when they are not treated with antiretroviral therapy and in the form of recurrent vulvovaginal candidiasis in over 100 million women annually worldwide (Denning *et al.*, 2018). Invasive pulmonary aspergillosis (IPA) is a leading cause of infection-related mortality in neutropenic and/or corticosteroid-treated patients with hematological malignancies and HSCT (Lionakis and Kontoyiannis, 2003; Segal, 2009). Cryptococcosis is a major opportunistic infection in AIDS patients that is responsible for $\sim$200,000 deaths per year worldwide (Rajasingham *et al.*, 2017), while *Pneumocystis jirovecii* pneumonia (PJP), the most common opportunistic respiratory infection in AIDS patients (Siegel *et al.*, 2016), is estimated to cause $>$400,000 life-threatening infections per year worldwide (Brown *et al.*, 2012).

Besides these "traditional" risk factors that predispose to opportunistic fungal disease, a recent surge in the discovery and clinical use of mechanism-based biological therapies in the form of either monoclonal antibodies (mAbs) (and associated biosimilars) or small molecule kinase inhibitors (SMKIs) has revolutionized the management of neoplastic and autoimmune disorders (Grilo and Mantalaris, 2019; Wu *et al.*, 2016). The biologic agent that changed everything in the landscape of clinical applications for such therapies is the SMKI imatinib, which in the early 2000s showed remarkable efficacy and safety for the treatment of Philadelphia chromosome-positive chronic myelogenous leukemia and gastrointestinal stromal tumors via inhibition of the BCR-ABL tyrosine kinase and the KIT receptor tyrosine kinase, respectively (Demetri *et al.*, 2002; Kantarjian *et al.*, 2002; Longo, 2017).

Since then, an unprecedented rate of discovery and clinical use of biological therapies that inhibit a variety of extracellular and intracellular immune-related molecules has underscored the critical contribution of some of these targeted molecules and pathways in mounting effective innate and adaptive antifungal immune responses in humans (Chamilos *et al.*, 2018; Zarakas *et al.*, 2019). Indeed, opportunistic mucosal and/or systemic fungal infections have emerged in the setting of treatment with some of these biological therapies (Chamilos *et al.*, 2018; Ghez *et al.*, 2018; Lionakis *et al.*, 2017a; Varughese *et al.*, 2018). In this chapter, I highlight key biological therapies, both mAbs and SMKIs, that have been associated with an increased risk of opportunistic fungal disease in patients with malignant and autoimmune diseases. The risk of fungal disease associated with such biological therapies in the context of transplantation, either HSCT or SOT, is beyond the scope of this chapter. Before outlining key biological therapies as novel risk factors for human fungal disease, I briefly summarize the cellular and molecular immune factors that are required for effective host defense against mucosal and/or invasive infection by different pathogenic fungi, as this outline will provide a useful roadmap for understanding the pathogenesis of opportunistic fungal diseases that arise in the context of biological therapies.

Immune Cells and Signaling Pathways That Promote Fungus-Specific Host Defense

The immune cell subsets and signaling pathways that mediate protective antifungal immunity vary greatly depending on the infecting fungus and anatomical site (Lionakis *et al.*, 2017b; Lionakis and Levitz, 2018). Hence, understanding the cellular and molecular basis of fungus-specific and tissue-specific host defense is critical for recognizing and predicting the fungal infection risk conferred by biological therapies that target the corresponding immune cells and signaling pathways.

Mucosal Candidiasis

Lymphoid cells and IL-17 receptor (IL-17R) signaling play a crucial role in protection against oropharyngeal candidiasis in mice and humans. Studies in mice have shown that the IL-17 cytokines IL-17A and IL-17F are rapidly produced at the mucosal surface upon *Candida* infection by proliferating $\alpha\beta$ T cells termed natural Th17 cells, by $\gamma\delta$ T cells, and by innate lymphoid cells type-3 (ILC3) (Conti *et al.*, 2009, 2014, 2016; Trautwein-Weidner *et al.*, 2015; Gladiator *et al.*, 2013). IL-17A and IL-17F then bind to IL-17RA and IL-17RC on the epithelial cell surface within the keratin 13-expressing suprabasal epithelial layer and drive the production of antifungal antimicrobial peptides such as S100a8, S100a9, β-defensin 1 and β-defensin 3 that are thought to curtail

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Candida invasion within the mucosa. Concordant to these mouse findings, patients with inherited immune deficiencies caused by mutations in *IL17F*, its receptors *IL17RA* and *IL17RC*, and the IL-17R adapter protein ACT1 (which is encoded by *TRAF3IP2*) are susceptible to chronic mucocutaneous candidiasis (CMC), which is characterized clinically by recurrent, treatment-refractory, and/or severe *Candida* infections that involve mucocutaneous anatomical sites (i.e., oral cavity, esophagus, vagina, skin, nails) but without fungal systemic dissemination (Boisson *et al.*, 2013; Puel *et al.*, 2011, 2012; Levy *et al.*, 2016; Ling *et al.*, 2015). Similarly, several other monogenic disorders that manifest with CMC such as those caused by mutations in *STAT1*, *STAT3*, *AIRE*, *RORC*, *IRF8*, *DOCK8*, or *CARD9* feature various defects in IL-17R–associated immune responses (Constantine and Lionakis, 2019; Corvilain *et al.*, 2018; Hambleton *et al.*, 2011; Lionakis *et al.*, 2014; Liu *et al.*, 2011; Milner *et al.*, 2008; Okada *et al.*, 2015; Puel *et al.*, 2010; van de Veerdonk *et al.*, 2011; Keles *et al.*, 2016; Kisand *et al.*, 2010).

IFIs by *Candida* and Inhaled Molds

Although lymphoid cells and IL-17R signaling critically promote immunity against *Candida* at the mucosal level, they are dispensable for host defense against invasive candidiasis (and invasive mold infections [IMIs]). Indeed, patients with AIDS are susceptible to mucosal candidiasis but are not at high risk for developing invasive candidiasis or IMIs.

Instead, protective immunity against invasive infections by *Candida* and inhaled molds relies on neutrophils and monocytes/macrophages, which traffic to the site of fungal infection in response to fungus-specific and tissue-specific molecular cues and become activated locally to exert direct fungal killing via both oxidative burst- and non-oxidative burst-dependent molecular mechanisms [reviewed in detail elsewhere (Lionakis and Levitz, 2018; Netea *et al.*, 2015)]. Indeed, patients with chemotherapy-induced profound and prolonged neutropenia and those with iatrogenic phagocyte defects associated with corticosteroid use are at high risk for developing invasive candidiasis and IMIs. In addition, defective phagocyte oxidative burst in patients with chronic granulomatous disease (CGD) significantly increases the risk of invasive mold (and to a lesser extent *Candida*) infections (Lionakis, 2012; Winkelstein *et al.*, 2000). In addition, defective neutrophil recruitment and non-oxidative burst-mediated fungal killing in the setting of inherited deficiency of the C-type lectin receptor (CLR) adapter protein CARD9 (Caspase recruitment domain-containing protein 9) drives susceptibility to invasive candidiasis and IMIs with a propensity to involve the central nervous system (CNS) (Drewniak *et al.*, 2013; Drummond *et al.*, 2015, 2019; Rieber *et al.*, 2016; Corvilain *et al.*, 2018; Lanternier *et al.*, 2015b).

Infections by Intracellular Fungi (*Cryptococcus*, Endemic Dimorphic Fungi and PJP)

Effective immunity against AIDS-associated intracellular fungal pathogens such as *Cryptococcus* species, PJP, and endemic dimorphic fungi (i.e., *Histoplasma*, *Coccidioides*, *Blastomyces*, *Talaromyces* species) depends on establishment of an efficient cross-talk between IFN-γ-producing lymphocytes (i.e., CD4 T cells and NK cells) and activated mononuclear phagocytes (Lionakis *et al.*, 2014; Rosain *et al.*, 2019). Specifically, upon engulfment of fungal elements, mononuclear phagocytes produce IL-12, which binds on its receptor (IL-12R, consisting of β1 and β2 subunits) on the surface of CD4 T and NK cells. IL-12 engagement in these lymphocytes activates STAT4 that facilitates the production of IFN-γ, which binds on its receptor (IFN-γR, consisting of β1 and β2 subunits) on the surface of macrophages. IFN-γR engagement activates JAK1-JAK2-STAT1 signaling, which leads to STAT1 nuclear translocation and induction of the transcription of IFN-γ–related genes, which result in intracellular fungal killing (Milner and Holland, 2013; Rosain *et al.*, 2019; O'Shea *et al.*, 2013). Besides IFN-γ, two other Th1-polarized cytokines, TNF-α and GM-CSF, are also critically important in inducing an effective lymphocyte-macrophage cross-talk that drives intracellular fungal clearance (Lionakis and Levitz, 2018). In agreement with the role of the aforementioned IFN-γ/IL-12 signaling cascade in host defense against intracellular fungi, patients with inherited immune deficiencies caused by mutations in *IL12*, *IL12RB1*, *IL12RB2*, *STAT4*, *IFNGR1*, *IFNGR2* and *STAT1* and individuals with acquired immune deficiencies caused by neutralizing autoantibodies against IFN-γ, IL-12 or GM-CSF manifest with disseminated infections by intracellular fungal (and mycobacterial) pathogens (Browne *et al.*, 2012; Lionakis and Levitz, 2018; Saijo *et al.*, 2014; Rosain *et al.*, 2019; Rosen *et al.*, 2013).

Fungal Infections in the Setting of Biological Therapies

In the following sections, the epidemiology and pathogenesis of fungal infections that arise in the setting of mAbs and SMKIs that target various extracellular or intracellular immune molecules will be outlined (**Table 1**).

Lymphocyte-Depleting Mabs

Alemtuzumab, a humanized mAb that targets cell-surface CD52, causes profound lymphocyte depletion via complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). While B cell depletion is short-lived, CD4 T cell depletion is prolonged and may last up to 60 months (Cox *et al.*, 2005). Not surprisingly, alemtuzumab-induced lymphocyte depletion predisposes to opportunistic AIDS-defining fungal infections. These include mucosal candidiasis, driven by alemtuzumab-mediated depletion of IL-17-producing Th17 cells and $\gamma\delta$ T cells (Martin *et al.*, 2013), PJP, and, less often, cryptococcosis. Teplizumab, a humanized anti-CD3 mAb, was recently reported to prevent type-1 diabetes in high-risk individuals and

Table 1 Biological therapies associated with fungal infection susceptibility in humans

Biological agent	*Drug target*	*FDA-approved indication(s)*	*Fungal infection susceptibility*	*Mechanism(s) of fungal infection susceptibility (when known)*
Alemtuzumab	CD52	CLL, MS	Mucosal candidiasis, PJP, cryptococcosis	Profound and prolonged T cell lymphopenia
Rituximab	CD20	CLL, NHL, RA, microscopic polyangiitis, Wegener's granulomatosis	PJP	B cell lymphopenia, impaired T cell responses
Tocilizumab	IL-6R	RA, JIA, giant cell arteritis, cytokine release syndrome (CAR T cell therapy)	Invasive candidiasis, PJP, cryptococcosis, coccidioidomycosis	Impaired phagocyte recruitment and function (*Candida*)
Secukinumab Ixekizumab	IL-17A	Psoriasis, psoriatic arthritis, AS	Mucosal candidiasis	Impaired IL-17 cellular responses
Bimekizumab	IL-17A/IL-17F	N/A		
Brodalumab	IL-17RA	Psoriasis		
Ustekinumab	IL-12p40	Psoriasis, psoriatic arthritis, CD, UC		
Guselkumab Risankizumab Tildrakizumab	IL-23p19	Psoriasis CD		
Infliximab Adalimumab Etanercept Certolizumab pegol Golimumab	TNF-α	Psoriasis, psoriatic arthritis, RA, JIA, AS, CD, UC	IPA, invasive candidiasis, PJP, histoplasmosis, blastomycosis, coccidioidomycosis	Impaired IFN-γ production and granuloma formation (endemic fungi); impaired phagocyte recruitment and activation (*Candida* and *Aspergillus*)
Emapalumab	IFN-γ	HLH	PJP, coccidioidomycosis	Impaired IFN-γ cellular responses
Eculizumab	C5a	PNH, HUS	IPA, invasive candidiasis	Impaired phagocyte function
Ibrutinib Acalabrutinib	BTK	CLL/SLL, MCL, MZL, WM, GvHD	IPA*, mucormycosis, fusariosis, cryptococcosis, PJP, histoplasmosis, blastomycosis	Impaired macrophage activation (*Aspergillus*); impaired macrophage uptake and IgM production (*Cryptococcus*)
Ruxolitinib Baricitinib, Tofacitinib Upadacitinib	JAK1/2/3	Myelofibrosis, polycythemia vera, GvHD, RA, psoriatic arthritis, UC	IPA, PJP, cryptococcosis, talaromycosis, histoplasmosis	Impaired IFN-γ/STAT1 signaling and lymphopenia (endemic fungi); impaired IFN-λ signaling (*Aspergillus*)
Fostamatinib	Syk	ITP	Mucosal candidiasis, skin fungal infection	Impaired IL-17 cellular responses
Sorafenib	B-RAF C-Raf	HCC, RCC	IPA, mucosal candidiasis, *Rhodotorula* skin infection	Impaired ERK signaling (*Aspergillus*); impaired T cell responses (*Candida, Rhodotorula*)
Dasatinib	BCR-ABL	CML	PJP	Impaired T cell activation
Idelalisib	PI3K (p100δ)	CLL/SLL, NHL	PJP	Impaired T cell activation
Abatacept	CTLA4	RA, JIA	IPA, invasive candidiasis, PJP, histoplasmosis	Impaired T cell activation (PJP, histoplasmosis)
Natalizumab	α_4 integrin	MS	Cryptococcosis	T cell trafficking

Abbreviations: AS, ankylosing spondylitis; BTK, Bruton's tyrosine kinase; CML, chronic myelogenous leukemia; CTLA4, cytotoxic T-lymphocyte associated protein 4; CAR, chimeric antigen receptor; CD, Crohn's disease; GvHD, graft-versus-host disease; HLH, hemophagocytic lymphohistiocytosis; HUS, hemolytic uremic syndrome; HCC, hepatocellular carcinoma; ITP, idiopathic thrombocytopenic purpura; JIA, juvenile idiopathic arthritis; JAK, Janus kinase; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; MS, multiple sclerosis; NHL, Non-Hogdkin's lymphoma; N/A, not available.PJP; Pneumocystis jirovecii pneumonia; CLL, chronic lymphocytic leukemia; PNH, paroxysmal nocturnal hemoglobinuria; PI3K, phosphoinositide 3-kinase;RAF, rapidly accelerated fibrosarcoma; RCC, renal cell carcinoma; RA, rheumatoid arthritis, SLL, small lymphocytic leukemia; UC, ulcerative colitis; WM, Waldenström macroglobulinemia.

is being evaluated in additional clinical trials of patients with diabetes and psoriasis. Whether teplizumab-treated patients are at risk of developing opportunistic fungal (and non-fungal) infections associated with T cell lymphopenia remains unknown. Importantly, the initial studies in teplizumab-treated patients demonstrated only transient (i.e., <2 weeks) and modest T cell lymphopenia after therapy (Herold *et al.*, 2019), suggestive of a much lower infection risk compared to alemtuzumab.

B cell depletion ensues via complement-dependent cytotoxicity and ADCC with rituximab, a CD20-targeting mouse-human chimeric mAb used to treat a variety of hematological and autoimmune conditions. Although bacterial infections and hepatitis B reactivation are the most common infectious complications following rituximab, several studies have also inferred an increased susceptibility to PJP (Gea-Banacloche, 2010). Besides B cell depletion, mouse studies showed that anti-CD20 treatment-associated

PJP predisposition may relate to impaired anti-*Pneumocystis* CD4 T cell responses (Elsegeiny *et al.*, 2015). Whether the newer-generation B cell-depleting and plasma cell-depleting mAbs also confer PJP susceptibility remains to be determined.

Biologics That Target Cytokine-Mediated Pathways

In recent years, several biologics that target cytokines and/or their receptors have become available for clinical use in a variety of autoimmune and inflammatory conditions (**Table 1**). Biologic-mediated inhibition of cytokine pathways phenocopies the fungal infection susceptibility (or resistance) profile of the corresponding inherited immune deficiencies of cytokine signaling (Lionakis and Levitz, 2018).

Biologics that target IL-1 receptor (IL-1R) and IL-6 receptor (IL-6R) signaling

Blockade of IL-1β or its receptor IL-1R by canakinumab or anakinra, respectively, has been successfully used to treat rheumatological disorders without promoting patients' fungal infection susceptibility. Absence of fungal infections in patients treated with IL-1R signaling-targeted biologics is consonant with the lack of fungal infection risk in patients with inborn errors of MyD88 immunity who have defective IL-1R signaling (Picard *et al.*, 2011).

The IL-6R–targeting mAb tocilizumab has proven an effective treatment for rheumatological conditions and for cytokine release syndrome in the setting of chimeric antigen receptor (CAR) T cell therapy. Tocilizumab is well-tolerated from a fungal infection risk standpoint in the vast majority of patients, in agreement with two recent reports of a small number of patients with inherited IL-6R deficiency who developed bacterial, but not fungal, infections (Nahum *et al.*, 2019; Spencer *et al.*, 2019). However, a few sporadic cases of systemic candidiasis, coccidioidomycosis, PJP and cryptoccocosis have been reported in tocilizumab-treated individuals, particularly when tocilizumab was combined with other immunomodulatory therapies (Kivitz *et al.*, 2018; Kyriakidis *et al.*, 2017; Nishioka *et al.*, 2018).

mAbs that target IL-17R signaling

Several biologics that inhibit cytokines and/or receptors that are crucial for the effective induction of IL-17R-dependent cellular responses have recently been introduced in the management of psoriasis, psoriatic arthritis, ankylosing spondylitis, and inflammatory bowel diseases (IBD) (Frieder *et al.*, 2018). These biologics inhibit IL-17 cytokines or their receptor directly such as with IL-17A–targeting secukinumab and ixekizumab, IL-17A/IL-17F–targeting bimekizumab, and IL-17RA–targeting brodalumab, or indirectly by inhibiting critical cytokines upstream of IL-17 production such as with IL-12p40–targeting ustekinumab, and IL-23p19–targeting guselkumab, risankizumab, and tildrakizumab. As observed with patients suffering from inborn errors of IL-17R-dependent signaling (Puel *et al.*, 2011, 2012), patients treated with IL-17–targeted biologics are also at risk for refractory mucocutaneous candidiasis, but not systemic candidiasis or other IFIs, further attesting to the fundamental role of IL-17R signaling in antifungal mucosal immunity in humans. However, despite the $\sim$80%–90% inhibition of IL-17R cellular responses that is achieved at the mucocutaneous barrier by the aforementioned IL-17R–targeted biologics (Krueger *et al.*, 2012, 2019; Tomalin *et al.*, 2019), only a small proportion of treated patients (i.e., 2%–5%) develop mucosal fungal disease, with a greater infection frequency observed in patients receiving IL-17RA– and IL-23p19–targeted molecules relative to those receiving IL-17A–, IL-17A/IL-17F–, and IL-12p40–targeted molecules (Saunte *et al.*, 2017). These observations indicate that humans can tolerate a $\sim$80%–90% inhibition of IL-17-dependent cellular responses without significant mucosal fungal infection consequences, and that a deeper, $>$90%–95% inhibition is required to induce mucosal fungal disease, as seen with the inherited, complete, IL-17R/ACT1 deficiencies.

TNF-α–targeting biologics

Infliximab, a humanized mAb against TNF-α, was the first biologic that was linked to susceptibility to opportunistic infection in 2001, namely tuberculosis, including severe extrapulmonary and/or disseminated disease (Keane *et al.*, 2001). Beyond tuberculous and non-tuberculous mycobacterial disease, TNF-α blockade, which is now widely used for the treatment of IBD and rheumatoid arthritis (RA), also predisposes to infections by other intra-macrophage pathogens such as PJP and the endemic dimorphic fungi *Histoplasma*, *Blastomyces*, and *Coccidioides*. Mechanistically, TNF-α inhibition abrogates IFN-γ production and IFN-γ-mediated macrophage activation, and impairs granuloma formation, which are all detrimental for intracellular pathogen control. The risk of opportunistic fungal (and mycobacterial) infection upon TNF-α blockade is greater with the anti-TNF-α mAbs infliximab and adalimumab as compared to the soluble TNF-α receptor etanercept. This differential infection risk is accounted for by fact that the anti-TNF-α mAbs mediate complement-induced lysis of TNF-α-expressing cells and inhibit both cell-associated and soluble TNF-α, as opposed to etanercept that only inhibits soluble TNF-α. In addition, TNF-α inhibitors have been associated with the development of invasive candidiasis and IPA; mechanistically, impaired phagocyte recruitment and activation in the setting of TNF-α blockage may underlie such susceptibility (Lionakis and Levitz, 2018).

IFN-γ–targeting mAb

Emapalumab is a humanized mAb against IFN-γ, which received FDA approval in 2018 for the treatment of pediatric and adult patients with refractory primary hemophagocytic lymphohistiocytosis (HLH), an IFN-γ–driven immune dysregulation condition (Burn *et al.*, 2019; Lounder *et al.*, 2019; Vallurupalli and Berliner, 2019). In addition, emapalumab is currently being evaluated for

the management of secondary HLH in the setting of systemic juvenile idiopathic arthritis. Given the central role of IFN-γ in orchestrating effective host defense against intracellular pathogens including viruses, mycobacteria and fungi (Lionakis and Levitz, 2018), emapalumab-treated patients are at risk for such infections. Indeed, fungal infections including cases of disseminated coccidioidomycosis and PJP have been reported in 9% of emapalumab-treated patients based on the drug's FDA package insert.

GM-CSF receptor (GM-CSFR)-targeting mAb

Mavrilimumab is a humanized monoclonal antibody that targets GM-CSFR. The drug has exhibited clinical efficacy in patients with rheumatoid arthritis in early clinical trials and is currently also being evaluated for the treatment of giant cell arteritis. No significant infection risk has been revealed in the relatively small number of patients that have been enrolled in the early clinical trials thus far, except for a small percent of patients who were reported to develop pneumonia, for which no information about the pathogen was provided (Burmester *et al.*, 2017, 2018; Takeuchi *et al.*, 2015; Weinblatt *et al.*, 2018). Given the importance of GM-CSF in host defense against cryptococcosis in humans (Rosen *et al.*, 2013; Saijo *et al.*, 2014) and the indispensable role of GM-CSF in anti-*Aspergillus* innate immunity in mice (Kasahara *et al.*, 2016), surveillance will be required in mavrilimumab-treated individuals to determine their potential risk of developing such opportunistic fungal infections.

Complement-Targeting Biologics

Terminal complement factor deficiency in the form of inherited C5a deficiency is known to predispose to severe infections by encapsulated bacteria including pneumococcus, *Haemophilus* and *Neisseria*; mucosal candidiasis has also rarely been reported in these patients (Rosenfeld *et al.*, 1976). Eculizumab is a humanized C5-targeting mAb that causes iatrogenic terminal complement deficiency, it has been successfully used to treat patients with a variety of hemolytic disorders, and it carries a significant risk for infection by encapsulated bacteria. In 2018, the FDA updated eculizumab's package insert to also alert for the potential risk of IFIs, including IPA, especially in patients with low neutrophil counts and/or those receiving other immunomodulatory medications. Post-marketing surveillance studies have indeed uncovered cases of IPA and invasive candidiasis in eculizumab-treated patients (Socie *et al.*, 2019). Moreover, in a small study of eculizumab-treated allogeneic HSCT recipients with transplant-associated thrombotic microangiography (TA-TMA), a striking 20% incidence of IMIs despite receipt of mold-active antifungal prophylaxis was reported as opposed to no IMIs developing in HSCT recipients with TA-TMA who did not receive eculizumab (Bohl *et al.*, 2017). Mechanistically, C5 genetic deficiency in mice results in significantly greater mortality following systemic infection with *Candida* and *Aspergillus* and C5 is important for the antifungal effector function of mouse and human myeloid phagocytes (Ashman *et al.*, 1993; Hunniger *et al.*, 2015). Therefore, more clinical studies are needed to precisely define the relative contribution of eculizumab, used alone or in combination with other immune modulators, in promoting opportunistic fungal disease. Notably, the advent of additional biologics that target the complement axis in loci that have been shown to be crucial for antifungal surveillance in mice such as the C3 inhibitor APL-2 and the C5AR1 antagonist avacopan, which recently showed clinical efficacy in steroid-refractory ANCA-positive vasculitis (Jayne *et al.*, 2017; Tsoni *et al.*, 2009; Zipfel *et al.*, 2019), will require clinical surveillance to determine whether they may confer fungal infection susceptibility.

mAbs That Target Checkpoint Pathways

The development of mAbs that block the checkpoint molecules programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) has revolutionized the treatment of a wide array of malignancies. These checkpoint inhibitors (CPIs) do not predispose to fungal disease; instead CPIs show promise as potential adjunct immunotherapy for certain fungal infections. However, immune-related adverse events (irAEs) occur often following CPI treatment and are major causes of morbidity and, in some instances, mortality. The management of irAEs requires immunomodulatory intervention with TNF-α inhibitors and/or corticosteroids, which themselves predispose to opportunistic fungal (and other) infections [reviewed in detail elsewhere (Abers *et al.*, 2019)].

SMKI

SMKIs that inhibit various intracellular molecules have emerged as effective therapeutic modalities for several neoplastic and inflammatory diseases (**Table 1**). The inhibition of certain signaling pathways by such SMKIs has resulted in either expected or unexpected fungal infection susceptibility profiles in treated patients, while other SMKIs that are currently being evaluated in early clinical trials are predicted to enhance fungal infection risk based on the indispensable role of the corresponding pathway that they inhibit in antifungal host defense. In this section, the epidemiology and pathogenesis of fungal infections that have developed or are expected to develop in the setting of key SMKIs is discussed.

SMKIs targeting BTK signaling

Ibrutinib is an irreversible inhibitor of the Bruton's tyrosine kinase (BTK) that blocks B cell receptor signaling activation and impairs B cell development and survival in hematologic malignancies. Indeed, ibrutinib has become a game-changing therapeutic modality in chronic lymphocytic leukemia (CLL), and has also shown clinical efficacy against other B cell-targeted hematologic

malignancies such as diffuse large B cell lymphoma, mantle cell lymphoma, marginal zone lymphoma, Waldenström macroglobulinemia, and primary CNS lymphoma (PCNSL), as well as graft-versus-host disease (GvHD) following HSCT (Chamilos *et al.*, 2018; Zarakas *et al.*, 2019).

The development of IFIs during ibrutinib-based therapy has been a somewhat surprising clinical observation, because (a) patients with X-linked agammaglobulinemia caused by germline *BTK* mutations seldom develop fungal disease (Kanegane *et al.*, 2009; Nishi *et al.*, 2018), and (b) B cells, the primary cellular target of ibrutinib-induced BTK inhibition, are considered less critical for fungal host defense relative to T cells and myeloid phagocytes (Lionakis and Levitz, 2018). Taken together, the clinical experience indicates that ibrutinib-associated IFIs most often consist of IMIs (IPA much more often than mucormycosis and fusariosis), followed by cryptococcosis, followed by PJP, followed by endemic fungal disease (i.e., histoplasmosis, blastomycosis). In patients traditionally considered low-risk for development of IFIs such as those with CLL or non-Hogdkin's lymphoma (NHL) (incidence, $<1\%$) (Pagano *et al.*, 2006), the incidence of IFIs associated with ibrutinib monotherapy is estimated at 2.4%–4.2% (Rogers *et al.*, 2019; Ruchlemer *et al.*, 2019; Varughese *et al.*, 2018). When ibrutinib is used in combination with corticosteroids, the incidence of IFIs is estimated at 5%–11% (Grommes *et al.*, 2017; Lakshmanan and Byrd, 2017). When ibrutinib is used in combination with corticosteroids and chemotherapeutic or other biological therapies, the incidence of IFIs can be as high as 39%, as shown in PCNSL patients (Lionakis *et al.*, 2017a).

A few clinical observations that relate to ibrutinib-associated IFIs are worthwhile briefly highlighting. Firstly, the majority of such IFIs develop within the first 2–3 months after initiation of ibrutinib and often occur in the absence of traditional risk factors for IFIs such as neutropenia (for IMIs) or lymphopenia (for cryptococcosis or PJP) (Ghez *et al.*, 2018; Ruchlemer *et al.*, 2019; Varughese *et al.*, 2018). Secondly, ibrutinib-associated IFIs feature rapid clinical progression unless ibrutinib is promptly discontinued, and they carry a remarkably high frequency of dissemination including CNS involvement (up to $\sim$45%) and high mortality (30%–70%) (Lionakis *et al.*, 2017a; Ghez *et al.*, 2018).

Mechanistically, BTK is thought to protect from IPA via its expression on macrophages, whereas it acts downstream of TLR9- and Dectin-1-mediated *Aspergillus* recognition to promote NFAT/NFκB-dependent TNF-α secretion upon *Aspergillus* stimulation (Bercusson *et al.*, 2018; Herbst *et al.*, 2015). In agreement, Btk-deficient mice and myeloid phagocyte-specific conditional Btk-knockout mice are susceptible to IPA and exhibit defects in phagocyte anti-*Aspergillus* effector function (Lionakis et al., 2017a), (Lionakis lab, unpublished). In addition, BTK is thought to protect from cryptococcosis via promoting phagocytosis of *Cryptococcus* yeasts by alveolar macrophages and generation of protective anti-*Cryptococcus* IgM (Szymczak *et al.*, 2013). The mechanisms by which BTK orchestrates macrophage-T cell cross-talk to protect against PJP remain unknown thus far.

SMKIs targeting JAK-STAT signaling

The JAK inhibitors ruxolitinib, baricitinib, tofacitinib and upadacitinib inhibit JAK/STAT signaling and, as a result, inhibit the activation of mononuclear phagocytes and down-regulate the production of a wide array of inflammatory mediators including common γ-chain cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21), type-I interferons (IFN-α, IFN-β), type-II interferon (IFN-γ), type-III interferons (e.g., IFN-λ), as well as IL-6, IL-10, IL-12, IL-22, IL-23 and GM-CSF [reviewed in detail elsewhere (Bechman *et al.*, 2019b; O'Shea *et al.*, 2013; Moodley *et al.*, 2016)].

JAK/STAT inhibitors are now widely used in the treatment of a broad spectrum of inflammatory and neoplastic conditions including myelofibrosis, polycythemia vera, steroid-refractory GvHD, rheumatoid arthritis, psoriatic arthritis, and ulcerative colitis, and opportunistic viral, mycobacterial and fungal disease has been reported in some patients (Bechman *et al.*, 2019a). Given the critical contribution of the lymphocyte-macrophage cross-talk in host defense against intracellular pathogens, JAK/STAT inhibitors have been associated with development of cryptococcosis, histoplasmosis, talaromycosis and PJP, via inducing lymphopenia and abrogating IFN-γ/STAT1 signaling within macrophages (Chan *et al.*, 2015; Dioverti *et al.*, 2018; Prakash and Richman, 2019). In addition, the recent recognition that IFN-λ/IFNLR1 signaling is critical for neutrophil oxidative burst and *Aspergillus* killing in mice indicates that aspergillosis and other IMIs may occur in the setting of JAK/STAT inhibition in humans (Espinosa *et al.*, 2014); indeed, a few such IPA cases are now emerging in the literature (Moruno-Rodriguez *et al.*, 2019; Polverelli *et al.*, 2017).

SMKIs targeting CLR-Spleen tyrosine kinase (Syk)-CARD9 signaling

Fostamatinib is a Syk inhibitor that was recently FDA-approved for the treatment of immune thrombocytopenic purpura, and is currently being evaluated in a variety of other inflammatory and neoplastic conditions including acute myelogenous leukemia, NHL, GvHD, renal transplant rejection, systemic lupus erythematosus, RA, autoimmune hemolytic anemia, and IgA nephropathy (See "Relevant Website section").

Syk is an important intracellular kinase that relays fungal-sensing signals downstream of the CLR family of pattern recognition receptors through the adaptor CARD9 for induction of protective innate and adaptive immune responses (Gross *et al.*, 2006; Drummond *et al.*, 2018). Given the profound fungal-specific infection susceptibility of patients with inherited CARD9 deficiency, it is expected that iatrogenic, fostamatinib-induced Syk/CARD9 signaling inhibition will heighten the risk of opportunistic fungal disease in fostamatinib-treated patients. The infection susceptibility phenotype of CARD9-deficient maps to 4 fungal diseases: (a) mucosal candidiasis, associated with IL-17R signaling defects (Glocker *et al.*, 2009; Lanternier *et al.*, 2015b; LeibundGut-Landmann *et al.*, 2007), (b) CNS candidiasis, associated with impaired neutrophil *Candida* killing and impaired microglial responses that lead to defective neutrophil recruitment to the fungal-infected CNS (Drewniak *et al.*, 2013; Drummond *et al.*, 2015, 2019), (c) extrapulmonary aspergillosis, associated with impaired neutrophil recruitment to infected tissues while neutrophil *Aspergillus* killing remains intact (De Bruyne *et al.*, 2018; Rieber *et al.*, 2016), (d) severe subcutaneous dermatophytosis and phaeohyphomycosis (some of which disseminate in deep tissues including the CNS), typically

following traumatic fungal inoculation, via mechanisms that remain to be elucidated (Lanternier *et al.*, 2015a, 2013). Therefore, surveillance of fostamatinib-treated patients for the development of the aforementioned fungal diseases is warranted. In fact, a case of vaginal yeast infection has been described in a fostamatinib-treated woman with hematological malignancy (Friedberg *et al.*, 2010) and a case of skin fungal infection has been described in one of the 18 fostamatinib-treated patients with T-cell lymphoma (incidence 5.6%) (clinicaltrials.gov, NCT00798096).

SMKIs targeting MAPK and other intracellular pathways

The emergence of several other SMKIs that target key intracellular pathways of antifungal host surveillance has been accompanied by development of opportunistic fungal disease. Some such examples are worthwhile briefly mentioning. For example, sorafenib inhibits multiple cell-surface and intracellular kinases within the RAS/MAPK signaling cascade and blocks the function of BRAF, Raf-1, RAS/ERK (extracellular signal-regulated kinase), and MEK (mitogen-activated kinase/ERK) pathway (Wilhelm *et al.*, 2004). Sorafenib-treated patients have been reported to develop IPA in the absence of other iatrogenic risk factors (Bazaz and Denning, 2018) and mucocutaneous fungal infections by *Rhodotorula* and *Candida* (Chen *et al.*, 2016; Coppola *et al.*, 2015). Mechanistically, sorafenib-induced inhibition of ERK signaling, which is critical for phagocyte *Aspergillus* killing, may underlie susceptibility to IPA (Dubourdeau *et al.*, 2006), while sorafenib-induced inhibition of NF-kB and PI3 (phosphoinositide 3-kinase), which is important for impaired T cell responses via affecting dendritic cell-dependent antigen presentation (Hipp *et al.*, 2008), may underlie mucosal fungal infection susceptibility.

Furthermore, the BCR/ABL tyrosine kinase dasatinib and the PI3K inhibitor idelalisib have been associated with susceptibility to PJP (Chang *et al.*, 2014; Hipp *et al.*, 2008), the CTLA4 modulator abatacept has been associated with development of PJP, IPA, histoplasmosis and invasive candidiasis (Buch *et al.*, 2008; Jain *et al.*, 2018; Kyriakidis *et al.*, 2017), and natalizumab that targets α_4 integrin and inhibits lymphocyte trafficking to the CNS in multiple sclerosis patients has been associated with development of cryptococcosis (Valenzuela *et al.*, 2014).

Conclusion

The recent explosion in the use of mAbs and SMKIs for the treatment of malignant and autoimmune conditions has been associated with the development of mucosal and/or life-threatening IFIs driven by complex iatrogenic immunodeficiency states in treated patients and has uncovered the critical contribution of certain immune-related molecules and pathways in human antifungal host defense. Moving forward, increased awareness among clinicians, improved surveillance and reporting of opportunistic infections during clinical trials, and research to better understand the epidemiology of fungal infections associated with biological therapies and the effects of these molecules on immune responses should help devise better strategies for the diagnosis, prophylaxis and treatment of such opportunistic infections and improve patient outcomes.

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Uncommon Yeasts and Molds Causing Human Disease

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Uncommon Yeast-Like Pathogens

Malassezia Species

Introduction

Members of the *Malassezia* genus are lipophilic, basidiomycetous yeasts belonging to the subphylum Ustilaginomycotina and the class Malasseziamycetes. There are currently 18 recognized species: *M. furfur*, *M. globosa*, *M. restricta*, *M. obtusa*, *M. sympodialis*, *M. pachydermatis*, *M. japonica*, *M. yamatoensis*, *M. dermatis*, *M. nana*, *M. caprae*, *M. equina*, *M. slooffiae*, *M. cuniculi*, *M. brasilensis*, *M. psittaci*, *M. arunalokei*, *and M. verspertilionis* (Lorch *et al.*, 2018; Wang *et al.*, 2014). Unique among *Malassezia* species, *M. pachydermatis* is the only species not dependent on lipids for growth. *Malassezia* species are predominantly plant and animal pathogens, but do comprise an estimated 50%–80% of the human skin mycobiome, with *M. globosa*, *M. restricta*, and *M. sympodialis* most frequently isolated (Findley *et al.*, 2013). Recently, *Malassezia* species within the gut microbiome have been linked to the progression of pancreatic cancer through interaction with the complement cascade (Aykut *et al.*, 2019).

Clinical presentation

Malassezia species are associated with several cutaneous diseases, namely seborrheic dermatitis, pityriasis (tinea) versicolor, folliculitis, atopic dermatitis and, occasionally, onychomycosis. Systemic infections most frequently occur in premature neonates receiving lipid-rich total parenteral nutrition, immunocompromised children, and adults with indwelling central venous catheters (Arendrup *et al.*, 2009; Shparago *et al.*, 1995). Additional risk factors for invasive disease include hematologic malignancy and peritoneal dialysis. Almost all reported systemic human infections are caused by *M. furfur* and *M. pachydermatis*, with the latter considered zoonotic, passing to immunosuppressed patients from pets. While fungemia is the most commonly reported site of invasive infection, *Malassezia* has also been implicated in catheter-associated peritonitis (Johnson *et al.*, 1996), meningitis (Rosales *et al.*, 2004), and sinopulmonary infection (Redline and Dahms, 1981). Furthermore, nosocomial outbreaks of *Malassezia* fungemia and folliculitis have been reported in intensive care units, with the hypothesized transmission of *M. pachydermatis* from the hands of healthcare workers that owned pet dogs (Archer-Dubon *et al.*, 1999; Chang *et al.*, 1998).

Diagnosis and identification

The diagnosis of invasive *Malassezia* infection is made by isolation in laboratory culture from a sterile site. This is often challenging, as most culture media are not enriched with lipid nutrients required to support *Malassezia* growth. Thus, any clinical specimen with positive staining for yeast forms and negative culture growth should prompt consideration of *Malassezia*. Use of modified Dixon agar, Leeming-Notman agar, CHROMagar, and Sabouraud's agar with sterile olive oil will allow for recovery of *Malassezia* from clinical samples (Iatta *et al.*, 2018). *Malassezia pachydermatis* is the exception, and does not require lipid supplementation for growth.

Once present in culture, *Malassezia* colonies grow rapidly over 5 days at 30–37°C. Colonies appear creamy yellow to brown, initially smooth and raised, and wrinkle over time. Microscopically, *Malassezia* species form small, elongated blastoconidia which demonstrate unipolar budding (Kaneko *et al.*, 2007) and yeast cells may have the appearance of a wine flask structure.

Distinction between species is achieved by a variety of methods. Previously, species identification was made by a combination of morphology and biochemical testing, including Tween test, esculine splitting test, and catalase and urease reactions (Kaneko *et al.*, 2007; Marcon and Powell, 1987). However, these methods are complex, laborious, and occasionally inaccurate. As a result, they have been replaced largely by PCR based methods. Species-level identification can be made through sequence analysis of the internal transcribed spacers (ITS) and D1/D2 domains of the large ribosomal subunit (Ilahi *et al.*, 2017; Makimura *et al.*, 2000). More recently, several studies have shown that matrix-associated laser desorption/ionization-time of flight (MALDI-TOF) is a promising modality for identification of *Malassezia* at the species level (Denis *et al.*, 2017; Honnavar *et al.*, 2018; Kolecka *et al.*, 2014).

Treatment and outcomes

Susceptibility testing is primarily reserved for strains causing invasive infection, and therefore, mostly restricted to *Malassezia furfur* and *Malassezia pachydermatis*. However, there are no standardized clinical breakpoints for *Malassezia* species. Various methods have been used for susceptibility testing, including modified microdilution and E-test (Cafarchia *et al.*, 2012; Velegraki *et al.*, 2004). Modified disk diffusion testing has shown poorer correlation with microdilution testing, and its clinical application remains uncertain (Rojas *et al.*, 2017). Minimum inhibitory concentrations for itraconazole and posaconazole are lower than those for fluconazole and voriconazole (Cafarchia *et al.*, 2015; Cafarchia *et al.*, 2012; Iatta *et al.*, 2014; Schmidt and Ruhl-Horster, 1996). Susceptibility testing for amphotericin B demonstrates lower MIC values for the liposomal formulation, which is likely due to the lipophilic nature of these fungi (Alvarez-Perez *et al.*, 2014; Iatta *et al.*, 2015). *Malassezia* species are uniformly resistant to echinocandins and flucytosine, and these antifungal agents should not be used to treat invasive disease. Currently, no rigorous clinical

studies exist to inform optimal medical therapy. Liposomal amphotericin B is the agent of choice, which is occasionally combined with a second agent, usually an azole. Treatment duration is not standardized and has ranged from anywhere from three days to fifty days in the medical literature (Pedrosa *et al.*, 2018). Removal of indwelling central venous catheters and discontinuation of lipid-rich total parenteral nutrition are critical for rapid treatment of *Malassezia*. This is so important that the exact antifungal agent used has less impact on outcome for these fungi which are not facile at producing clinical disease.

Pseudozyma Species

Introduction

Pseudozyma species (recently renamed as *Moesziomyces*) are basidiomycetous, true smut fungi belonging to the Ustilaginales order. Predominantly plant pathogens, the first cases of invasive human disease were reported in 2003 (Sugita *et al.*, 2003). There are currently over twenty species within the genus. Members implicated in human disease include *P. aphidis*, *P. antarctica*, *P. parantarctica*, *P. thailandica*, *P. alboarmeniaca*, *P. crassa*, and *P. siamensis* (Mekha *et al.*, 2014; Sugita *et al.*, 2003). *P. aphidis* is responsible for the vast majority of reported infections.

Clinical presentation

The majority of human disease is caused by either *P. aphidis* or undifferentiated *Pseudozyma* species. Infections have been reported in patients with hematologic malignancy, neutropenia, advanced age or prematurity, underlying gastrointestinal illness, or indwelling central venous catheters (Pande *et al.*, 2017; Prakash *et al.*, 2014). Of the eleven published cases, fungemia is most commonly observed. However, brain abscess, post-surgical endophthalmitis, mycetoma, and pneumonia have also been reported (Hwang *et al.*, 2010; Joo *et al.*, 2016; Parahym *et al.*, 2013; Voon *et al.*, 2019). Clinical signs and symptoms are protean, and depend upon location of infection.

Diagnosis and identification

Diagnosis is made by culture of *Pseudozyma* from a sterile site. These fungi will grow readily on Sabouraud dextrose agar at 30–37°C. When isolated, rapidly expanding, orange-white, wrinkled colonies develop. Microscopically, *Pseudozyma* demonstrate fusiform yeasts budding from short stalks, and form elongated, ellipsoidal blastoconidia on slide culture (Sugita *et al.*, 2003).

Species identifications through morphological and biochemical testing are difficult, time-intensive and unreliable. *P. aphidis*, the primary isolated pathogen, assimilates D-galactose, lactose, and melibiose but not ethanol, which may help distinguish it from other members of the *Pseudozyma* genus (Hwang *et al.*, 2010). However, identification is most often made by molecular probing of the ITS DNA between ribosomal domains and D1/D2 domain of the 26S ribosomal subunit (Siddiqui *et al.*, 2014). Unfortunately, MALDI-TOF at present cannot reliably distinguish *Pseudozyma* at the genus or species level.

Therapy and outcomes

Given the rarity of infection, no standardized susceptibility testing for *Pseudozyma* species currently exists. Low MICs to amphotericin B, voriconazole, posaconazole, and isavuconazole have been reported, but most clinically isolated specimens have high MICs to fluconazole (Joo *et al.*, 2016). Echinocandin and flucytosine resistance is intrinsic. Amphotericin B or voriconazole are recommended as first-line therapy, and suggested by the European Society of Clinical Microbiology and Infectious Diseases and European Confederation of Medical Mycology guidelines (Arendrup *et al.*, 2014). Most reported cases have been treated with a two-week course of therapy and central venous catheter removal (Pande *et al.*, 2017). Of the eleven cases reported in literature, outcomes have been generally good, with only one fatality reported in a patient with a *Pseudozyma* brain abscess (Hwang *et al.*, 2010).

Rhodotorula Species

Introduction

Originally believed to be a non-pathogenic red yeast, the first case of *Rhodotorula* infection in humans was reported in 1960 (Louria *et al.*, 1960). Since then, well over 240 cases have been reported in the medical literature. Fungi belonging to the *Rhodotorula* genus are imperfect basidiomycetes within the Sporidiobolaceae family (Urbina and Aime, 2018). They are widely disseminated in nature and have been cultured from air, soil, saltwater, plants and household fixtures (Galan-Sanchez *et al.*, 1999). *Rhodotorula* species have been isolated from the skin, respiratory, alimentary, and urinary tracts of humans and form a small, but important, part of the human mycobiome (Monteiro-da-Silva *et al.*, 2014). While there are many *Rhodotorula* species, only three are reported to cause disease in humans. These are *R. mucilaginosa* (formerly *R. rubra*), *R. glutinis*, and *R. minuta*. Of these, *R. mucilaginosa* accounts for the vast majority of human infections.

Clinical presentation

Fungemia is the most frequent syndrome witnessed in humans, representing approximately 60% of all infections caused by *Rhodotorula* species, which comprise 0.5%–2.3% of bloodstream infections caused by all fungal pathogens (Ioannou *et al.*, 2019). Most disease occurs in patients with indwelling central venous catheters with underlying hematologic or solid organ malignancies (Garcia-Suarez *et al.*, 2011). Other risk factors include chronic kidney disease, continuous ambulatory peritoneal dialysis, cirrhosis,

total parenteral nutrition, and solid organ or stem cell transplantation (Lunardi *et al.*, 2006; Wirth and Goldani, 2012). Of note, breakthrough cases of fungemia have been described in patients on fluconazole and posaconazole prophylaxis. Endocarditis, while extremely rare, complicates around 5% of all *Rhodotorula* fungemia, and should be considered as a potential diagnosis in fungemic patients (Simon *et al.*, 2014).

It is clear that *Rhodotorula* displays neurotropism, with a propensity to cause meningitis, often in patients with AIDS or underlying autoimmunity (Menon *et al.*, 2014; Mohd Nor *et al.*, 2015). However, cases have been reported in patients without known immunocompromise. Symptoms of meningitis may be acute, subacute, or chronic. Importantly, *Rhodotorula* meningitis may be mistaken for cryptococcal meningitis, which is a much more commonly encountered central nervous system infection. Appropriate identification is imperative to ensure correct clinical management as treatment of *Cryptococcus* and *Rhodotorula* differs.

Ocular infections and catheter-associated peritonitis make up most of the remaining reported cases of *Rhodotorula* infection. Keratitis and scleritis are often preceded by orbital surgery or trauma, while endophthalmitis occurs endogenously in patients with AIDS or intravenous drug use (Luong *et al.*, 2017; Merkur and Hodge, 2002). Other less frequently observed infections include skin infections, joint infections, intraabdominal and urinary tract infections (Diktas *et al.*, 2013; Savini *et al.*, 2008; Seyfarth *et al.*, 2012).

Diagnosis and identification

As part of the human mycobiome, *Rhodotorula* is a common cause of either laboratory contamination or colonization. Therefore, patients with positive cultures for *Rhodotorula* should have accompanying signs and symptoms consistent with infection for diagnosis (i.e., fever, tachycardia, hypotension, leukocytosis, etc.). When isolated, these fungi are non-fastidious and grow rapidly on most media. On Sabouraud agar they form characteristic coral red colonies, although orange and pink colonies may also be seen. Microscopically, they appear as round or ovoid budding yeasts without formation of pseudohyphae. They may be mistaken for *Cryptococcus* species, but can be easily distinguished by their inability to assimilate inositol. *R. mucilaginosa* can be differentiated from *R. glutinis* by its inability to assimilate nitrate. While the pathogenic species of *Rhodotorula* can be discriminated by morphology and biochemical testing, PCR and mass spectrometry (MALDI-TOF) techniques also provide reliable identification at a species level (Lohmann *et al.*, 2013; Saracli *et al.*, 2003b).

Therapy and outcomes

Treatment of *Rhodotorula* infections is managed with prompt initiation of antifungal agents, removal of indwelling catheters, and restoration of host immunity. As with other rare fungal pathogens, there are no standardized *in vitro* antifungal drug susceptibility breakpoints, but multiple studies have demonstrated both amphotericin B and flucytosine possess excellent *in vitro* activity (Falces-Romero *et al.*, 2018; Nunes *et al.*, 2013). Importantly, *Rhodotorula* species are intrinsically resistant to fluconazole. Reported MICs are consistently greater than 32 μg/ml, and thus, fluconazole cannot be recommended for therapy. As *Rhodotorula* species are most often mistaken with *Cryptococcus* species, which are fluconazole susceptible, proper identification is imperative. Itraconazole, voriconazole and posaconazole all appear to have some activity against *Rhodotorula*, while studies suggest isavuconazole may be an alternative to first-line therapy (Gomez-Lopez *et al.*, 2005). Echinocandins should not be used for treatment of *Rhodotorula* infections. Research into combination therapy in the treatment of *Rhodotorula* is sparse and cannot form the basis for any specific clinical recommendations at this time.

Clinical outcomes range widely depending upon the nature of the underlying infection. Mortality attributable to infection in patients with fungemia approximates 10%, while the attributable mortality in *Rhodotorula* meningitis is around 40% (Ioannou *et al.*, 2019). Ocular infections have not been associated with mortality, but often result in significant morbidity with visual loss and enucleation reported. Duration of therapy varies widely in published case reports.

Saccharomyces Cerevisiae (Var *Boulardii*)

Introduction

Saccharomyces cerevisiae and its variant *Saccharomyces boulardii*, otherwise known as "baker's yeast" (or "brewer's yeast"), are ubiquitous in nature, cultivated from soil, plants, and fruit, and are occasionally isolated from the human genitourinary and gastrointestinal tracts (Hallen-Adams and Suhr, 2017; Rose and Kurup, 1977). It is a major model yeast for study of molecular biology, genetics, and biochemistry. Taxonomically, they are ascomycetous fungi belonging to the class Hemiascomycetes, order Saccharomycetales, and family Saccharomycetaceae. Previously thought to be a separate species, *Saccharomyces boulardii* is now considered a variant of *Saccharomyces cerevisiae* on the basis of molecular testing (McCullough *et al.*, 1998). *Saccharomyces* is frequently utilized in the preparation of probiotics to treat and prevent a variety of gastrointestinal illnesses, including *Clostridioides difficile* infection. The first case of invasive *Saccharomyces* disease was reported in 1970 in a woman with prosthetic valve endocarditis (Stein *et al.*, 1970). Since that time, it has been implicated in various clinical syndromes.

Clinical presentation

Saccharomyces has been implicated in a number of infections in humans including empyema, liver abscess, esophagitis, peritonitis and urinary tract infection (Aucott *et al.*, 1990; Chertow *et al.*, 1991; Snyder, 1992). However, the most commonly encountered syndrome is fungemia (Enache-Angoulvant and Hennequin, 2005). Infection occurs in both immunocompromised and immunocompetent individuals, with the former group at higher risk of dissemination. Premature neonates, persons receiving parenteral

nutrition, critically-ill patients and those with intravenous catheters appear to be at highest risk. Exposure to Saccharomyces-containing probiotics constitutes a primary risk factor for disease, and nosocomial transmission of infection from healthcare workers has also been reported (Cassone *et al.*, 2003; Lherm *et al.*, 2002). It is recommended that probiotics containing *Saccharomyces* not be used in seriously ill, immunocompromised patients. Infection is theorized to occur by either translocation across disrupted mucosal barriers, or through decreased skin integrity (i.e., venous catheters). These species are capable of forming biofilms to allow persistence on catheters (Helmuth, 2001). In a small proportion of women, *Saccharomyces* can also cause recurrent, difficult-to-treat azole-resistant vulvovaginitis (Sobel *et al.*, 1993). Vaginitis caused by *Saccharomyces* species is clinically indistinguishable from that caused by more common *Candida* species.

Diagnosis and identification

Diagnosis of infection is made by isolating this yeast from a sterile body site. As *Saccharomyces* are only intermittent colonizers of humans, positive cultures from the bloodstream should lead to strong consideration of invasive disease. Identification is readily made by morphological evaluation and biochemical testing. On Sabouraud agar, smooth, moist, cream-colored colonies grow rapidly in 3 days. On CHROMagar these colonies appear dark pink. They are able to ferment carbohydrates and assimilate raffinose, but cannot assimilate nitrate. Microscopically, yeast cells are round or ovoid, measure 5-10 μ, and are slightly larger than *Candida glabrata*. *Saccharomyces* species do not form hyphae, but may form psuedohyphae when starved for nitrogen. On nutrient-depleted media, *S. cerevisiae* can sporulate to form one to four ascopores per ascus. β-1,3-D-glucan has been detected both *in vitro* and *in vivo* from clinical specimens, however the clinical utility of serologic testing has not been evaluated (Odabasi *et al.*, 2006; Yoshida *et al.*, 1997).

Molecular methods allow for identification at the species level through PCR based techniques targeting the ITS2 region or the 26S rRNA gene. MALDI-TOF-MS has also demonstrated capability to discriminate *Saccharomyces* at the species level (Marklein *et al.*, 2009).

Therapy and outcomes

MIC testing against *Saccharomyces* isolates may be performed by micro- or macro-dilution and disk diffusion methods. While no standardized breakpoints exist, CLSI guidelines for *Candida* species were previously used for interpretation. After breakpoints for *Candida* were made species-specific in 2012, *Saccharomyces* isolates appear to closely mirror *in vitro C. glabrata* susceptibilities. Amphotericin B, flucytosine, and echinocandins seem to have reliable *in vitro* efficacy against *Saccharomyces* species, while MICs for fluconazole show wide variability. Extended spectrum azoles likely have excellent *in vitro* activity (Thompson *et al.*, 2009). Amphotericin B or fluconazole are most commonly used clinically with reasonable clinical success—occasionally in contrast to the *in vitro* data – which speaks to the low pathogenicity and virulence of *Saccharomyces*. Clinical experience with echinocandin therapy is scarce, but has been effective in at least two cases (Lolis *et al.*, 2008; Roy *et al.*, 2017) and probably is a first-line treatment agent. Combination therapy has been previously described for recurrent disease, but its role remains inconclusive (Davies *et al.*, 2019). As with most invasive fungal infections, removal of central venous catheters is an important aspect of therapy, due to the ability of *Saccharomyces* to form well-developed biofilms. All-cause mortality rates for invasive *Saccharomyces* infection are estimated around 25%, but this is thought to reflect underlying host morbidity rather than pathogenicity of *Saccharomyces*.

Saprochaete Species

Introduction

Saprochaete capitata (formerly *Geotrichum capitatum* or *Blastoschizomyces capitatus*) and *Saprochaete clavata* (previously *Geotrichum clavatum*) are non-fermentative, urease-negative, ascomycetous yeasts. The teleomorphic form of *S. capitata* is identified as *Magnusiomyces capitatus*. Currently, they are classified in the subphylum Saccharomycotina, class Saccharomycetes, order Saccharomycetales, and family Dipodascaceae (de Hoog, 2004). Specimens have been isolated from environmental sources and from human respiratory and gastrointestinal tracts. Rarely pathogenic in humans, *S. capitata* infection appears to occur more frequently than *S. clavata*. However, it is unclear if this observation is due to differences in pathogenicity or difficulty in discriminating at the species level.

Clinical presentation

The first case of invasive *S. capitata* was described in 1989 in a patient with acute myeloid leukemia (Mahul *et al.*, 1989). Since that time, hematologic malignancy and prolonged neutropenia have been recognized as the primary risk factors for disseminated Saprocheate infection (Bansal *et al.*, 2018; Ozdemir *et al.*, 2017; Trovato *et al.*, 2018). Upwards of 90% of infections occur in neutropenic patients with underlying hematologic malignancy. In one multicenter epidemiologic study, *Saprochaete* accounted for 1.7% of invasive fungal infections in immunocompromised hosts (Cornely *et al.*, 2015). Interestingly, most infections have occurred in Europe, but whether this is a result of true geographical distribution or bias in reporting is unknown. Fungemia is commonly reported, and is often associated with deep space infection involving the lungs, liver, spleen, central nervous system, or kidneys. Occasionally, skin lesions may manifest in a manner similar to disseminated candidiasis (Duran Graeff *et al.*, 2017). Both *S. capitata* and *S. clavata* have been associated with nosocomial disease. For instance, a nosocomial outbreak of *S. capitata* was described in association with contaminated vacuum flasks for milk delivery in a Spanish hospital (Gurgui *et al.*, 2011).

While most commonly afflicting those with hematologic malignancy, cases of fungemia, meningitis, endocarditis, and pneumonia have been reported in immunocompetent individuals (Cavanna *et al.*, 2017; Tanabe and Patel, 2018).

Diagnosis and identification

Invasive disease is diagnosed by recovery of *Saprochaete* species from blood or another sterile body fluid. The fungi forms colonies rapidly with maturation in culture over five days. Colonies appear white to cream colored, with formation of short aerial hyphae. Microscopically, true hyphae, pseudohyphae, annelloconidia, arthroconidia, and blastoconidia can be seen (Martino *et al.*, 1990). Careful attention must be made to avoid morphologic confusion with *Trichosporon species* or *Candida krusei* which may appear similarly, but have different susceptibility profiles. While the genus can be distinguished from *Trichosoporon* by its urease negativity, biochemical testing is not reliable for species identification. Both MALDI-TOF-MS and sequencing of the internal transcribed spacer can discriminate *S. capitata* and *S. clavata* (de Almeida *et al.*, 2016; Desnos-Ollivier *et al.*, 2014). Distinction at species level is important, as *S. capitata* and *S. clavata* have differing *in vitro* susceptibility profiles. Cross-reactivity may occur with the *Aspergillus* galactomannan antigen and with β-1,3-D-glucan testing, but clinical experience with serologic testing is negligible and negative results should not be used to exclude disease (Giacchino *et al.*, 2006; Odabasi *et al.*, 2006).

Therapy and outcomes

There are no standardized interpretive criteria for determining antifungal susceptibility for *Saprochaete* infections. European guidelines currently recommend amphotericin B alone or in combination with flucytosine as first-line therapy, with voriconazole as an acceptable alternative (Arendrup *et al.*, 2014). This is based upon *in vitro* susceptibility data along with limited clinical experience from case reports and case series. *In vitro*, amphotericin B MICs are moderate, while flucytosine MICs are consistently low. However, as most infected patients are neutropenic, clinicians may be reluctant to use flucytosine due to risk of bone marrow toxicity. S. *capitata* MICs to itraconazole, posaconazole, and voriconazole are low. Various studies of MIC testing for isavuconazole are disparate with MICs ranging widely. In contrast, fluconazole MICs are consistently elevated (Guinea *et al.*, 2010). While *Saprochaete* are intrinsically resistant to echinocandins, combination therapy with voriconazole has been utilized due to a potential *in vitro* synergy (Etienne *et al.*, 2008; Fianchi *et al.*, 2008). Breakthrough infections have unsurprisingly occurred on echinocandin prophylaxis, but have been reported on prophylaxis with fluconazole, posaconazole, and amphotericin B as well (Bansal *et al.*, 2018; Chittick *et al.*, 2009; Schuermans *et al.*, 2011).

In addition to antifungal therapy, early catheter removal may improve survival. Granulocyte infusions and interferon-gamma administration in combination with antifungal therapy has also been described with success in one neutropenic patient (Favre *et al.*, 2016).

Outcomes in disseminated *Saprochaete* disease are generally poor, with thirty-day mortality ranging from 39% to 90% in various retrospective reviews of neutropenic patients (Vaux *et al.*, 2014). In immunocompetent patients, mortality rates are significantly lower, suggesting survival depends significantly on recovery of the host's immune function.

Trichosporon and Cutaneotrichosporon Species

Introduction

In contrast to members of the *Saprochaete* genus, *Trichosporon species* are anamorphic, non-fermentative, urease-positive basidiomycetous yeasts (Agaricomycotina, Tremellomycetes, Tremellales, Trichosporonaceae). They are ubiquitous throughout temperate and tropical environments, and are estimated to colonize mucosal surfaces of around 1%–10% of the healthy human population (Haupt *et al.*, 1983; Messias Silvestre *et al.*, 2010). First identified as a cause of superficial mycosis in the 1860s, *Trichosporon species* form a complex group of fungi, previously all classified as *T. beigelii*. Advanced biochemical and molecular techniques have shed light on the heterogeneity of the genus, resulting in several revisions of the taxonomic classification (Liu *et al.*, 2015), with some members now classified under the genus Cutaneotrichosporon. Currently, over 50 species of Trichosporon and Cutaneotrichosporon have been identified, with six significant species causing disease in humans: *T. asahii*, *T. asteroides*, *T. ovoides*, *C. mucoides*, *C. inkin*, and *C. cutaneum*. A seventh species, *Cutaneotrichosporon mycotoxinivorans* has been increasingly reported in association with pulmonary colonization and infection in patients with cystic fibrosis (Hickey *et al.*, 2009).

Clinical presentation

Infections caused by members of the *Trichosporon* and *Cutaneotrichosporon* genera range from superficial infection to invasive and disseminated disease, often depending upon underlying host immunity.

Superficial infection, occasionally referred to as trichosporosis or white piedra, occurs as infection of the hair follicles. The scalp, eyebrows, mustache, beard, axillary and genital hairs may all be involved. White piedra disproportionately affects those of all ages living in tropical climates, although cases in temperate climates may occur. Socioeconomic status and hygiene are not known risk factors. *C. inkin*, *C. cutaneum*, and *T. ovoides* are the predominant pathogens causing superficial infections (Bonifaz *et al.*, 2019; Douchet *et al.*, 1994; Shivaprakash *et al.*, 2011).

Conversely, *T. asahii*, *T. asteroides*, and *C. mucoides* are most frequently implicated in systemic or disseminated infections. Predominant underlying patient risk factors are the presence of hematologic malignancy and neutropenia. Other risk factors include prior corticosteroid use, organ transplantation, HIV/AIDS, and intravenous drug use. Disseminated trichosporonosis

was first reported in 1970 in a patient with metastatic lung adenocarcinoma (Watson and Kallichurum, 1970). Since then, over 300 cases have been documented in the medical literature including fungemia, pneumonia (Ruan *et al.*, 2009), peritonitis, endophthalmitis (Chakrabarti *et al.*, 2008; Scofield-Kaplan *et al.*, 2018), meningitis (Kumar *et al.*, 2015), esophagitis (Lo Passo *et al.*, 2001; Macedo *et al.*, 2011), and hepatosplenic abscesses. In mouse models, *Trichosporon* disseminates quickly with a propensity to infect the brain, heart, kidneys, lungs and liver (Hospenthal *et al.*, 1993; Montoya *et al.*, 2018). Dissemination is believed to occur by translocation across disrupted mucosal barriers during periods of mucositis and neutropenia. Virulence factors and pathogenicity among *Trichosporon* species causing invasive disease remains unknown (Colombo *et al.*, 2011).

Interestingly, *C. cutaneum* has been associated with summer-type hypersensitivity pneumonitis. However, this phenomenon has been identified only in Japan, and not observed elsewhere (Shimazu *et al.*, 1984; Yoshida *et al.*, 1989; Yoshida *et al.*, 1988). *T. mycotoxinivorans* is unique among *Trichosporon* species in causing pulmonary disease in patients with cystic fibrosis. Initially reported in 2009 as a case of fatal pneumonia (Hickey *et al.*, 2009), it has since been described in eight additional cases with variable clinical courses (Dabas *et al.*, 2017; Shah *et al.*, 2014a). However, the clinical significance of a positive respiratory culture for this yeast from cystic fibrosis patients remains unclear.

Diagnosis and identification

Trichosporon species grow easily on standard bacterial culture media and Sabouraud agar, and isolation in culture from a sterile body site or visualization on tissue histopathology is required for diagnosis. Isolation from mucosal sites may occur, and culture from these sites should prompt thorough clinical evaluation to distinguish colonization from infection. Rapidly growing, colonies initially appear smooth and cream-colored, but wrinkle and gray with age. Since the yeasts transform to mold *in vitro*, the presence of septate, hyaline hyphae with production of arthroconidia should prompt consideration of *Trichosporon*. While *Saprochaete* species also form arthroconidia, the two genera can be distinguished by urease testing and the absence or presence of annelloconidia. Biochemical testing can determine *Trichosporon* to the genus level, but is unreliable in species identification. Both genetic sequencing of the ITS region, and increasingly, MALDI-TOF-MS are useful modalities for determining species level identity (de Almeida Junior *et al.*, 2014; Kolecka *et al.*, 2013; Li *et al.*, 2005; Marklein *et al.*, 2009).

Cross reactivity with the cryptococcal polysaccharide latex agglutination assay has been reported in cases of trichosporonosis, due to presence of glucuronoxylomannan in the cell wall of *Trichosporon species* (Lyman *et al.*, 1995; Melcher *et al.*, 1991). However, this finding is not sensitive, and should not be used to exclude diagnosis of invasive disease.

Therapy and outcomes

Treatment of *Trichosporon* infection depends upon the extent of disease and underlying host immune function. White piedra can be treated with shaving of infected hair, followed by topical antifungal therapy, often ketoconazole shampoo daily for several months. Systemic *Trichosporon* infection is considerably more difficult to manage and portends a very poor prognosis, with reported mortality rates as high as 90% (Chagas-Neto *et al.*, 2009; Kontoyiannis *et al.*, 2004; Suzuki *et al.*, 2010).

In vitro susceptibility testing and case series reports suggest extended-spectrum azoles as therapies of choice. Testing of voriconazole and posaconazole against isolates reliably reproduce MIC values which suggest clinical efficacy (Araujo Ribeiro *et al.*, 2008; Chagas-Neto *et al.*, 2009). Fluconazole MICs range very widely, but fluconazole monotherapy was used successfully in a small series of Taiwanese patients with a 42% mortality (Ruan *et al.*, 2009). Documented MIC values for amphotericin B against various *Trichosporon* species are generally too high for achievable serum concentrations at conventional dosing (Chagas-Neto *et al.*, 2009; Rodriguez-Tudela *et al.*, 2005). Furthermore, clinical response rates in patients treated with amphotericin B hover around 25% (Girmenia *et al.*, 2005). Echinocandins have little to no activity against *Trichosporon*, should not be used as monotherapy for trichosporonosis, and breakthrough infections have been reported (Kimura *et al.*, 2018; Matsue *et al.*, 2006). However, *in vitro* data exist to suggest a synergistic effect of echinocandins with both amphotericin B and azoles, and several case reports document successful combination therapy (Li *et al.*, 2011b; Serena *et al.*, 2005). Based upon currently available data, most clinicians would utilize voriconazole for treatment of trichosporonosis (Arendrup *et al.*, 2014). While antifungal therapy is imperative for treatment of invasive *Trichosporon*, successful treatment appears to depend primarily upon resolution of neutropenia and reconstitution of host immunity.

Phaeohyphomycosis

Introduction

Phaeohyphomycosis, a term derived from the Greek *phaeo-* (dusky) and *hypho-* (hyphal), is a broad clinical term that refers to a variety of infections caused by a myriad of molds which are identified by brown-black coloration of their vegetative state (Ajello *et al.*, 1974). This feature is caused by deposition of melanin within the cell wall of these pathogens, which are also known as dematiaceous fungi. Over 100 different fungal species are included as dematiaceous fungi, and reported to cause disease in humans at varying frequencies. They range widely through the taxonomy of the Fungi Kingdom, and are found within the Ascomycota, Basidiomycota, and Zygomycota phyla. This section will touch upon the clinical presentations of the more commonly seen species which comprise the phaeohyphomycoses.

Clinical Manifestations

Dematiaceous fungi cause a wide spectrum of disease in human hosts, ranging from asymptomatic colonization to life-threatening and devastating disseminated infection. Profoundly immunocompromised individuals, such as those with hematologic malignancy, solid organ or bone marrow transplant recipients, are at greatest risk of infection with this group of fungi; however, cases of phaeohyphomycosis have also been reported in apparently immunocompetent individuals. These pathogens are ubiquitous within the environment, and infection most frequently occurs by direct inoculation of the fungus, through trauma or medical procedures, or from inhalation of spores into the sinuses and respiratory tracts. Pathogens are generally implicated in certain clinical syndromes and treatment may vary depending upon the specific fungus isolated.

Cutaneous phaeohyphomycosis

All dematiaceous molds are capable of causing cutaneous disease, such as dermatomycosis or onychomycosis. Treatment with debridement of involved tissue or short courses of antifungal agents is typically sufficient for eradication in all but the most immunosuppressed individuals.

Subcutaneous phaeohyphomycosis

Subcutaneous disease is a common manifestation of phaeohyphomycosis, frequently caused by *Curvularia*, *Exophiala*, and *Phialophora* species. Symptoms include development of subcutaneous cysts and/or ulcerations, with or without satellite lesions. In immunocompetent persons, lesions may persist unchanged for years without treatment.

Keratitis

Most often fungal keratitis is caused by a traumatic injury to the cornea, either from accidental harm or surgical injury. Among the dematiaceous molds, Curvularia, *Exophiala*, and *Exserohilum* are commonly implicated. The hyalohyphomycoses, such as *Aspergillus*, *Fusarium*, and *Purpureocillium* (*Paecilomyces*) frequently cause keratitis and will be discussed later in this chapter.

Fungal sinusitis

Fungal sinusitis manifests in three predominant forms – allergic fungal sinusitis, eumycetoma, and invasive sinusitis. The first two are localized diseases without tissue invasion that produce a local eosinophilic response, and are often managed surgically. Conversely, invasive fungal sinusitis is an aggressive disease characterized by bony destruction, which may progress intracranially if left unchecked. Of the dematiaceous molds, *Alternaria* and *Curvularia* species are often involved. Other fungi producing similar disease include *Aspergillus* and the mucormycoses, discussed elsewhere.

Disseminated phaeohyphomycosis

Disseminated infection implies systemic disease from an inoculating injury, such as inhalation through the respiratory tract or penetration through the skin. Patients are most commonly immunosuppressed, either following transplantation, or secondary to profound neutropenia from hematologic malignancy or chemotherapy. Several species are known for their neurotropism and propensity to cause brain abscesses, such as *Cladophialophora bantiana*, *Exophiala* (*Wangiella*) *dermatiditis*, *Ochroconis gallopava*, and *Rhinocladiella mackenziei*. Others, such as *Lomentospora prolificans* cause fungemia and/or pulmonary disease in immunocompromised hosts.

Common Species Causing Phaeohyphomycosis

Alternaria species

The genus *Alternaria* includes over 100 species, of which two – *A. infectoria* and *A. alternata* – form the near totality of human infection. The predominant syndromes caused by *Alternaria* species are cutaneous and subcutaneous phaeohyphomycosis (Ferrandiz-Pulido *et al.*, 2019), although fungal sinusitis (Pesic *et al.*, 2015), ocular keratitis, onychomycosis (Arrese *et al.*, 1996), and disseminated disease (Mirhendi *et al.*, 2013) also occur. The most common risk factors for developing disease are hematologic malignancy with neutropenia, and solid organ or hematologic transplantation. Cutaneous and ocular disease typically results following a traumatic or surgical inoculation, resulting in either localized soft tissue infection or keratitis, respectively.

Culture of *Alternaria* is most effectively realized on potato-carrot agar. Morphologically, *A. infectoria* and *A. alternata* form septate, melanized hyphae, and may be distinguishable by their conidia formations. *A. alternata* conidia demonstrate multiple, branching, cylindrical beaks, whereas *A. infectoria* displays scarce, tubular conidia with secondary long, multiseptated conidiophores (Pastor and Guarro, 2008). Reliable species identification is made through sequencing of the ITS region, with particular attention to the length of the ITS-4 amplicon (de Hoog and Horre, 2002; Leahy *et al.*, 2010). Experience with mass spectrometry for identification of *Alternaria* at the genus and species level is limited and not yet validated for clinical microbiology (Panda *et al.*, 2015).

In vitro susceptibility testing of *Alternaria* isolates is minimal. Data that exists suggests resistance to flucytosine and fluconazole, and a wide range of MICs to amphotericin B (Pujol *et al.*, 2000). Itraconazole, voriconazole, and posaconazole demonstrate excellent *in vitro* antifungal activity against *Alternaria* (Cuenca-Estrella *et al.*, 2006). Clinical experience with

itraconazole is greatest, but increasing reports of success with posaconazole (Lyskova *et al.*, 2017) will likely make it a first-line drug for treatment of this fungus.

In addition to antifungal therapy, surgical management and withdrawal of immunosuppression, if possible, is recommended for invasive disease. Localized cutaneous disease may be cured with surgical management alone. Outcomes for alternariosis appear to be rather good, with relatively low mortality, likely due to its predilection to cause localized cutaneous disease without dissemination.

Bipolaris species

Three fungi formerly belonging to the genus *Bipolaris* are known to cause human disease – *B. australiensis*, *B. hawaiiensis*, and *B. spicifera*. These molds have recently been reclassified under the genus *Curvularia* based upon sequencing of the internal transcribed spacer and GPDH rDNA (Manamgoda *et al.*, 2012).

Cladophialophora species

Cladophialophora bantiana (previously classified as *Cladosporium bantianum*, *Xylohypha bantiana*, or *Torula bantiana*) is the major pathogenic mold of this genus, and the leading cause of cerebral phaeohyphomycosis. It has also been reported to cause pulmonary phaeohyphomycosis, although pulmonary colonization has not been associated with increased risk of central nervous system disease. In a large review of clinical cases, many patients had no identifiable underlying risk factor; however, a large plurality of individuals had undergone solid organ transplantation or received prolonged courses of corticosteroids (Kantarcioglu *et al.*, 2017).

Cerebral phaeohyphomycosis should be suspected in patients with imaging suggestive of intracranial abscess, and indolent symptoms of increased intracranial pressure (headaches, altered mentation, seizures) in the absence of fever. There are no specific findings for cerebral phaeohyphomycosis, and the differential diagnosis will commonly include malignancy and other infectious etiologies. Meningitis may also occur with or without brain abscess. Diagnosis is made by isolation of *C. bantiana* in culture or detection of hyphae on histopathology.

In clinical culture *Cladophialophora* grows as a mold with long, branching, hydrophobic conidia seen microscopically. Identification is most accurately made be sequencing the rDNA of the ITS region (Abliz *et al.*, 2004; Caligiorne *et al.*, 2005). Mass spectrometry is not yet reliable for identification of *Cladophialophora* due to deficiencies in the existing commercial databases (Singh *et al.*, 2017).

Treatment of cerebral phaeohyphomycosis caused by *C. bantiana* necessitates surgical excision/debulking and antifungal therapy. In the absence of these interventions, infection is uniformly fatal. Unfortunately, mortality still hovers around 70%, even with surgery and antifungal medications, regardless of the immune status of the patient (Kantarcioglu *et al.*, 2017). *In vitro* susceptibility data suggest good antifungal activity of itraconazole and posaconazole (Badali *et al.*, 2010). While voriconazole MICs are higher, voriconazole demonstrates higher oral bioavailability and central nervous system penetration than either itraconazole or posaconazole. Conversely, while amphotericin B MICs are low, CNS penetration is poor, likely limiting efficacy (Al-Abdely *et al.*, 2005). In mouse models, combination therapy with flucytosine, micafungin and posaconazole prolonged survival time over controls, single and double combination therapies (Marine *et al.*, 2009).

Other species of *Cladiophialophora* are implicated in human disease. *C. carrionni* is a frequent cause of chromoblastomycosis with a similar drug susceptibility profile to *C. bantiana* (Deng *et al.*, 2013). Other infrequently reported causes of disseminated phaeohyphomycosis include *C. arxii* and *C. devriesii* (Mitchell *et al.*, 1990; Tintelnot *et al.*, 1995).

Curvularia species

There are over 100 species within the *Curvularia* genus, but only a few are known to cause disease in humans. The predominant pathogens are *C. aeria*, *C. lunata* and *C. geniculata*. As mentioned above, several clinically relevant *Bipolaris* species have recently been reclassified as *Curvularia* species: *C. australiensis*, *C. hawaiiensis*, and *C. spicifera* (Manamgoda *et al.*, 2012).

The most common clinical syndrome caused by *Curvularia* species is allergic fungal rhinosinusitis (Nishioka *et al.*, 1987), but invasive fungal sinusitis has also been documented (Ebright *et al.*, 1999). Brain abscess (Gadgil *et al.*, 2013; Gonzales Zamora and Varadarajalu, 2019; Skovrlj *et al.*, 2014), endocarditis (Kaufman, 1971), endophthalmitis (Berbel *et al.*, 2011; Pathengay *et al.*, 2012), keratitis (Dorey *et al.*, 1997), mycetoma (Gunathilake *et al.*, 2014; Janaki *et al.*, 1999), peritonitis (Subramanyam *et al.*, 2016; Ujhelyi *et al.*, 1990), and disseminated disease (Vasikasin *et al.*, 2019; Rohwedder *et al.*, 1979) have also been reported.

Colonies of *Curvularia* grow rapidly and are characterized by brown or black-brown pigment, like other dematiaceous fungi. Conidia appear curved or lunate, with rounded ends and usually three to five septa. *Curvularia* cannot be reliably distinguished from *Bipolaris* morphologically, and sequencing of the glyceraldehyde-3-phosphate dehydrogenase gene is needed to accurately determine isolates at the species level (Jeon *et al.*, 2015).

Treatment of allergic fungal sinusitis is primarily achieved by surgical resection and oral corticosteroid therapy. Antifungal therapy has not been shown to be effective (Schubert, 2009), although occasionally itraconazole or amphotericin B are administered. Invasive or systemic disease necessitates antifungal therapy. Amphotericin B seems to have the most *in vitro* activity (da Cunha *et al.*, 2013). While MICs for the triazoles are generally higher, posaconazole and itraconazole likely retain some efficacy. There is no standard therapy for invasive *Curvularia* infection, but amphotericin B and azoles are typically utilized to varying degrees of success (Gonzales Zamora and Varadarajalu, 2019; Kalawat *et al.*, 2012; Moody *et al.*, 2012).

Exophiala species

Exophiala (*Wangiella*) counts over forty species in the genus, with a handful causing disease in human hosts. The pathogenic species are *E. dermatiditis*, *E. xenobiotica*, *E. oligosperma*, *E. lecaniicorni*, *E. phaeomuriformis*, *E. jeanselmi*, *E. bergeri*, and *E. mesophila*, listed in order of clinically observed frequency (Zeng *et al.*, 2007). Ubiquitous in the nature, *Exophiala* species have been primarily isolated from warm, humid environments (Babic *et al.*, 2018).

The most commonly seen infections caused by *Exophiala* species are cutaneous and subcutaneous phaeohyphomycosis. However, *Exophiala* displays a significant neurotropism – as do other members of the order *Chaetothyriales* – and *E. dermatiditis* commonly causes cerebral phaeohyphomycosis, almost exclusively in Asian countries (Li *et al.*, 2011a). The reason for this geographical preference is not well understood, but speculation has been given to potential increased inoculum exposure. Infections occur in both immunocompromised and immunocompetent individuals, with dissemination more frequent in those with impaired immunity. Fungemia is a rare event, although an outbreak of *E. dermatiditis* fungemia was described in oncologic patients in New York who received treatment with a contaminated IV flush solution (Vasquez *et al.*, 2018). Other reported infections caused by *Exophiala* include cases of pneumonia (Cohen and Stead, 2015; Suzuki *et al.*, 2012), endocarditis (Berger *et al.*, 2017; Patel *et al.*, 2013), peritonitis (Agarwal *et al.*, 1993; Lau *et al.*, 2003; Pinheiro *et al.*, 2019) and endophthalmitis (Clamp *et al.*, 2014; Homa *et al.*, 2018). While not representing infection, *Exophiala* species commonly colonize the respiratory tracts of patients with cystic fibrosis (Lebecque *et al.*, 2010).

Members of the genus are slowly growing dematiaceous fungi, which are best cultivated on selective agars, such as erythritol-chloramphenicol agar. In culture, colonies appear restricted, waxy and smooth. Initial growth appears yeast-like, but with maturation, cells thicken and darken, developing mycelium with cylindrical annellides. Conidia appear pale brown and accumulate at the ends of annellides. Species identification is best performed by gene sequencing of the ITS region (Nagano *et al.*, 2008), although mass spectrometry can also reliably differentiate species of *Exophiala* (Borman *et al.*, 2017).

In cases of cutaneous phaeohyphomycosis with *Exophiala*, surgical intervention may be curative. More invasive disease often necessitates combined surgical resection, if possible, and antifungal therapeutics. Posaconazole, voriconazole, itraconazole and isavuconazole all demonstrate activity against *Exophiala* species in *in vitro* assays (Fothergill *et al.*, 2009; Gonzalez, 2009). Most strains also appear susceptible to amphotericin B; however, an experimental mouse model suggests increased efficacy of posaconazole over amphotericin B in disseminated disease due to *Exophiala dermatiditis* (Calvo *et al.*, 2010). Echinocandins have little-to-no activity against *Exophiala* and breakthrough infections in patients on echinocandin prophylaxis are documented (Watanabe *et al.*, 2018). Human clinical experience is greatest with amphotericin B, posaconazole and voriconazole, all of which have been used successfully in case reports (Nachman *et al.*, 1996; Negroni *et al.*, 2004; Saito *et al.*, 2018; Vasquez *et al.*, 2018). For cerebral phaeohyphomycosis, current guideline-directed therapy consists of complete excision, or at least substantial debulking, coupled with voriconazole or posaconazole, with combination therapy considered in cases where surgical management is not possible (Chowdhary *et al.*, 2014).

Exserohilum rostratum

While there are over 30 species of fungi belonging to the genus *Exserohilum*, only *E. rostratum* is known to cause disease in humans. *E. longirostratum* and *E. mcginnisii* are reported in older medical literature as pathogenic species, but have since been found to be identical to *E. rostratum* on the basis of molecular sequencing (da Cunha *et al.*, 2012). An environmental fungus, found commonly in soil, *E. rostratum* is a rare cause of allergic or invasive fungal sinusitis; however, a large nosocomial outbreak of *Exserohilum rostratum* CNS infections occurred in the United States in 2012 due to a contaminated methylprednisolone supply (Smith *et al.*, 2013).

Exserohilum are closely related to the genera *Bipolaris* and *Curvularia*. Therefore, morphologies seen among these species are quite similar. Isolates form dark brown colonies, with aerial mycelium which appear wooly. Conidia are ellipsoidal, straight to slightly bent, and septate, with protruding hilum (Sharma *et al.*, 2014). Accurate species level identification is made by sequencing of the ITS region (da Cunha *et al.*, 2012). Mass spectrometry identification has not been proven for *Exserohilum* species.

Amphotericin B, followed by ketoconazole, has most often been used for therapy of invasive respiratory disease. The treatment of choice during the fungal meningitis outbreak caused by *Exserohilum* was voriconazole, owing primarily to its pharmacologic property of high central nervous system penetration (Kainer *et al.*, 2012). In *in vitro* studies, posaconazole, itraconazole and voriconazole display low MIC values, while isavuconazole MICs are notably higher (Chowdhary *et al.*, 2015). Echinocandin MICs vary widely and breakthrough cases have been reported (Chou *et al.*, 2007). Current expert opinion suggests treatment with amphotericin B alone or in combination with an azole for systemic disease, and voriconazole monotherapy for central nervous system disease (Pappas *et al.*, 2013).

Lomentospora prolificans

Classically, this species was included in the hyalohyphomycoses with other *Scedosporium* species; however, *L. prolificans* often exhibits melanized hyphae, a defining trait of the phaeohyphomycoses. Furthermore, *Lomentospora prolificans* (formerly *Scedosporium prolificans* or *S. inflatum*) was reclassified into its own genus on the basis of tubulin molecular sequencing (Lackner *et al.*, 2014a). To further contrast with *Scedosporium apiospermum*, it is rarely isolated from environmental sources, and appears geographically restricted, with most cases reported in Australia, Spain and the United States (Rodriguez-Tudela *et al.*, 2009; Seidel *et al.*, 2019).

Clinically, disease from *L. prolificans* ranges from asymptomatic colonization to disseminated, devastating, and often fatal, disease. Pulmonary colonization is well described and occurs in individuals with underlying cavitary or bronchiectactic disease, such as those living with cystic fibrosis (Sedlacek *et al.*, 2015). In immunocompetent patients, the most common disease entity encountered is localized cutaneous or subcutaneous disease, resulting from traumatic inoculation (Steinbach *et al.*, 2003). Invasive infection is seen in patients with profound immunosuppression, most often in individuals with hematologic malignancy and prolonged neutropenia, hematopoietic stem cell transplant recipients, or in patients after solid organ transplantation receiving alemtuzumab or anti-thymocyte globulin induction. When disseminated disease does occur, the lungs, central nervous system, eye, and heart are often involved, and blood cultures are frequently positive (Seidel *et al.*, 2019).

Diagnosis of *Lomentospora* infection is made by isolation of the organism in culture or visualization on histopathology. Isolation in culture is more readily achieved preventing overgrowth by *Aspergillus* species by use of a selective media, SceSel^{+}, which contains dichloran and benomyl (Rainer *et al.*, 2008). Differentiation of *L. prolificans* from *Scedosporium* species can easily be made by observation of morphologic and phenotypic characteristics. *L. prolificans* forms darkly pigmented colonies, is susceptible to cycloheximide, and displays flask-shaped conidiogenous cells. As noted previously, molecular sequencing of the ITS region can identify species within the *Scedosporium/Lomentospora* complex, with further sequencing of the tubulin gene allowing for species differentiation (Lackner *et al.*, 2014a). Mass spectrometry has been shown to be as effective as molecular testing for identification of clinical isolates to a species level (Coulibaly *et al.*, 2011; Sitterle *et al.*, 2014; Sleiman *et al.*, 2016).

Treatment of *Lomentospora* is problematic, due to its resistance to nearly all available antifungal therapy. Clinical isolates are resistant to amphotericin B, echinocandins, fluconazole, isavuconazole, posaconazole and flucytosine, and demonstrate variably high MICs to voriconazole and itraconazole (Castanheira *et al.*, 2012; Cuenca-Estrella *et al.*, 1999; Guinea *et al.*, 2008). Some combination therapies, such as voriconazole and micafungin (Heyn *et al.*, 2005), or terbinafine and itraconazole (Meletiadis *et al.*, 2000) have shown *in vitro* synergism. A murine model has demonstrated prolonged survival time and decreased fungal burden in mice treated with the combination of micafungin and voriconazole or amphotericin B (Rodriguez *et al.*, 2009). More recently, several investigational drugs have shown promise in combating *Lomentospora* infection. APX001 (fosfomanogepix), a novel antifungal acting against Gwt1p, a protein critical for cell well integrity, displays low MIC values when tested against *L. prolificans* (Rivero-Menendez *et al.*, 2019). Similarly, F901318 (olorofim), a dihydroorotate dehydrogenase inhibitor, has shown promising *in vitro* activity against clinical isolates (Biswas *et al.*, 2018; Wiederhold *et al.*, 2017).

Voriconazole therapy is most commonly utilized clinically due to its relatively lower MIC values. A review of 56 cases of invasive *Lomentospora* infection found that mortality rates were diminished with voriconazole therapy, but that combination with terbinafine did not further improve survival (Seidel *et al.*, 2019). However, on the basis of previously published case reports, most recent current European guidelines modestly recommend combination voriconazole and terbinafine therapy (Tortorano *et al.*, 2014). Surgical debridement of involved tissue and restoration of immune function are critical to improving outcomes of *Lomentospora* infection. Unfortunately, mortality from *Lomentospora* infection remains extremely high, with around 70%–80% of patients ultimately succumbing to their disease.

Hyalohyphomycoses

The hyalohyphomycetes constitute a diverse group of molds which are characterized by their lack of melanin deposition within the fungal cell wall (Ajello, 1986). This finding primarily distinguishes them from the phaeohyphomycetes, or dematiaceous molds, which were addressed previously within this chapter. Clinically encountered hyaline molds include *Aspergillus* (discussed elsewhere in this book), and *Fusarium*, *Purpureocillium* (*Paecilomyces*), *Scedosporium*, and *Scopulariopsis*, which will be touched upon here.

Fusarium

Predominantly phytopathogens, over 300 species of *Fusarium* have been identified, with over 20 identified as agents of human infection (Balajee *et al.*, 2009). Behind *Aspergillus* species, *Fusarium* species are the second most common cause of hyalohyphomycosis in certain patient populations (Marr *et al.*, 2002). Phylogenetic analyzes have shown that many *Fusarium* species can be classified as a species complex (Summerell, 2019). In human disease, four complexes appear to predominate: *Fusarium solani* species complex (*F. solani* sensu stricto, *F. keratoplasticum*, *F. falciforme*, *F. petroliphilum*), *Fusarium oxysporum* species complex (*F. oxysporum*), *Fusarium fujikuroi* species complex (*F. verticillioides*, *F. proliferatum*, *F. acutatum*), and *Fusarium dimerum* species complex (*F. dimerum*, *F. delphinoides*) (Muraosa *et al.*, 2017). *F. solani* and *F. verticillioides* comprise a plurality to majority of infections due to *Fusarium* species (Tortorano *et al.*, 2008). Infections occur in immunocompetent and immunocompromised hosts, but hematopoietic stem cell transplant recipients suffer from significantly increased risk (Nucci *et al.*, 2004).

Clinical disease caused by *Fusarium* species runs the gamut from localized skin and soft tissue infection to disseminated disease with fungemia. *Fusarium solani* is the most common cause of fungal keratitis, often occurring in tropical climates after traumatic injury (Cheikhrouhou *et al.*, 2014). Ocular infection has also been associated with contact lens use, including a worldwide outbreak from 2005 to 2006 related to use of an ineffectively fungistatic cleaning solution (Chang *et al.*, 2006; Khor *et al.*, 2006). Cases can result in permanent visual impairment by contiguous spread into the posterior chamber, resulting in endophthalmitis, a particularly feared complication. Skin and soft tissue infections include onychomycosis,

eumycetoma and colonization and infection of burn injuries. Importantly, individuals with hematologic malignancy are at increased risk of invasive fusariosis if *Fusarium* species are cultured from preexisting skin lesions (Varon *et al.*, 2014). Invasive fusariosis is most frequently seen in patients with acute leukemia or hematopoietic stem cell transplant patients, particularly patients having undergone mismatched allogeneic transplantation (Atalla *et al.*, 2015; Nucci *et al.*, 2004). In these patients, infection most often manifests as sinopulmonary disease, localized deep-seated infection, disseminated fusariosis, or fever of unknown origin. Clinically, fusariosis is difficult to distinguish from invasive aspergillosis, but some key distinctions may be seen. In pulmonary disease, CT imaging cannot reliably distinguish between aspergillosis and fusariosis; however, absence of the halo sign or occluded vessel sign suggest a *Fusarium* etiology (Sassi *et al.*, 2017). Additionally, patients with disseminated fusariosis often have positive blood cultures and skin manifestations, which are uncommonly seen in invasive aspergillosis (Bodey *et al.*, 2002; Nucci *et al.*, 2018; Torres *et al.*, 2003). Notably, serum Aspergillus antigenemia is not reliable for distinguishing between *Aspergillus* and *Fusarium* as there is significant cross-reactivity between these genera (Fernandez-Cruz *et al.*, 2019; Horn *et al.*, 2014; Nucci *et al.*, 2018; Tortorano *et al.*, 2012). *Fusarium* infections have been reported in cases of septic arthritis (Stempel *et al.*, 2015), endocarditis (Guzman-Cottrill *et al.*, 2004; Jorens *et al.*, 1990; Kassar *et al.*, 2016; Nunes *et al.*, 2013), peritonitis (da Silva-Rocha *et al.*, 2015; Mayr *et al.*, 2017; Shah *et al.*, 2014b), brain abscess (Chen *et al.*, 2017).

Fusariosis may be diagnosed by histopathology or culture. On histopathology, acutely-angled, branching, septate hyphae are visualized, as is seen in invasive aspergillosis, without distinguishing features to discriminate between *Fusarium* and *Aspergillus*. Recently, a small, single center study showed promise in obtaining accurate species diagnosis through quantitative PCR of the ITS region in clinical samples in formalin-fixed, paraffin-embedded tissue (Salehi *et al.*, 2016). This method may be useful to aid in identification when culture results are not forthcoming.

As noted previously, blood cultures are frequently positive (70%–80%) in patients with disseminated fusariosis due to its ability to form adventitial, yeast-like forms in tissue (Liu *et al.*, 1998). *Fusarium* cultures develop rapidly and form white colonies with a pink to violet center and lighter periphery. Microscopically, macroconidia, microconidia and chlamydospores may be seen. The presence of macroconidia allows for identification of *Fusarium* at the genus level, as macroconidia form a characteristic canoe, or banana, shape. Otherwise, *Fusarium* species may be confused with other pathogenic fungi. Discrimination at the species level utilizing morphologic techniques is difficult, time-intensive, and unreliable. Instead, molecular sequencing of the RNA polymerase II RPB2 subunit and translation elongation factor 1-alpha allow for accurate identification to a species level (Thomas *et al.*, 2019; van Diepeningen *et al.*, 2015). Mass spectrometry, through MALDI-TOF methods, are also increasingly reliable in identifying *Fusarium* species correctly, especially as databases improve (De Carolis *et al.*, 2012; Sleiman *et al.*, 2016; Triest *et al.*, 2015).

Fusarium species are intrinsically resistant to most antifungal agents, including flucytosine, miconazole, ketoconazole, itraconazole, fluconazole, and the echinocandins (Arikan *et al.*, 2001; Espinel-Ingroff, 2003; Pujol *et al.*, 1997; Reuben *et al.*, 1989; Speeleveld *et al.*, 1996). Among species, *F. solani* displays more resistance than other non-*solani Fusarium* species, including *F. verticillioides* and *F. oxysporum*, which retain some activity to voriconazole and posaconazole (Alastruey-Izquierdo *et al.*, 2008). Of the newer extended spectrum azoles, voriconazole, and posaconazole have variable, fungistatitic activity against *Fusarium* species, whereas isavuconazole is ineffective in *in vitro* studies (Gonzalez, 2009; Guinea *et al.*, 2008). Amphotericin B and natamycin retain the most efficacy against clinical *Fusarium* isolates (Reuben *et al.*, 1989; Rotowa *et al.*, 1990). Due the intrinsically resistant nature of *Fusarium*, combination antifungal therapy has been extensively studied *in vitro* and in animal models. One small *in vitro* study demonstrated no synergism between amphotericin B and voriconazole in *F. solani* and *F. oxysporum* isolates, but showed synergism with the combination of terbinafine and voriconazole (Cordoba *et al.*, 2008). Another *in vitro* study has shown synergism against *Fusarium* isolates with the combination of natamycin and voriconazole (Al-Hatmi *et al.*, 2016), while a third documented synergistic combinations of amphotericin B and ravuconazole, terbinafine and ravuconazole, and terbinafine and voriconazole (Ortoneda *et al.*, 2004). In a murine model, the combination of amphotericin B and voriconazole improved survival in a small cohort of mice infected with *F. solani*¸ although fungal burden was not decreased (Ruiz-Cendoya *et al.*, 2008).

Fusarium keratitis is often treated with topical natamycin, confirmed by the results of the Mycotic Ulcer Treatment Trial, which documented superior clinical outcomes with topical natamycin compared to topical voriconazole (Prajna *et al.*, 2013). In secondary analysis from the same study, individuals receiving adjunctive oral voriconazole were at decreased risk of corneal perforation or need for therapeutic penetrating keratoplasty (Prajna *et al.*, 2017). Onychomycosis is most often treated with terbinafine and voriconazole (Al-Hatmi *et al.*, 2018).

While mortality from invasive *Fusarium* infection remains high, clinical evidence suggests that utilization of high-dose voriconazole monotherapy or combination therapy with amphotericin B and voriconazole for treatment of fusariosis is increasingly practiced by physicians with improving survival (Jenks *et al.*, 2018; Nucci *et al.*, 2014). Indeed, current guidelines recommend combination therapy with liposomal amphotericin B and voriconazole as first-line therapy for invasive fusariosis (Tortorano *et al.*, 2014), along with surgical debridement and reversal of immunosuppression, if possible. Posaconazole is recommended as salvage therapy in patients failing first line therapy on the basis of small, uncontrolled clinical observations (Alexander *et al.*, 2008; Chowdhary *et al.*, 2014; Raad *et al.*, 2006). Studies have shown consistently poor outcomes in patients with persistent neutropenia (Fernandez-Cruz *et al.*, 2019; Nucci *et al.*, 2014; Nucci and Anaissie, 2007). On this fact, several investigators have evaluated the role of granulocyte transfusion and granulocyte colony-stimulating factor therapy as an adjunctive therapy in patients with invasive fusariosis (Kadri *et al.*, 2015). Despite the promise of adjunctive therapies, these studies remain clouded by confounders and the role of immune supplementation remains unclear.

Scedosporium

The genus *Scedosporium* (previously also *Pseudallescheria*) has undergone a major taxonomic revision in the last decade on the basis of phylogenetic analysis and simplification of nomenclature. As a result, currently 10 species of *Scedosporium* are recognized – *S. apiospermum*, *S. angustum*, *S. boydii*, *S. ellipsoideum*, and *S. fusoideum*, comprising the *S. apiospermum* species complex; and *S. aurantiacum*, *S. cereisporum*, *S. dehoogii*, *S. desertorium*, and *S. minutisporum* forming the remainder (Gilgado *et al.*, 2005, 2008; Lackner *et al.*, 2014a). Of these, *S. apiospermum* and *S. aurantiacum* are the most relevant agents of human disease. *Lomentospora prolificans* (formerly *S. prolificans* or *S. inflatum*) is no longer categorized with *Scedosporium* species and is discussed previously within this chapter.

Scedosporium species are ubiquitous having been cultured from agricultural soil, animal droppings, environmental water sources, and sewage (Rougeron *et al.*, 2018). Geographical differences exist, with *S. aurantiacum* far more commonly isolated in Australia, and *S. dehoogi and S. apiospermum* more frequently encountered in Europe (Harun *et al.*, 2010; Kaltseis *et al.*, 2009). Their omnipresence likely results in their frequent isolation from the respiratory tracts of patients with cystic fibrosis, where they are the second most common mold isolated, only trailing *Aspergillus* species (Cimon *et al.*, 2000; Engel *et al.*, 2019).

Infections with *Scedosporium* species affect both immunocompetent and immunocompromised hosts. In patients with intact immunity, traumatic inoculation results in most clinical manifestations and *Scedosporium* is a predominant cause of eumycetoma and fungal arthritis (Bonifaz *et al.*, 2014; Estrada *et al.*, 2012; Sampaio *et al.*, 2017; Tirado-Miranda *et al.*, 2001). *S.* apiospermum, *S. aurantiacum* and *S. boydii* have also precipitated fatal intracranial abscesses in victims of near-drowning events (Buzina *et al.*, 2006; Gari *et al.*, 1985; Katragkou *et al.*, 2007; Kowacs *et al.*, 2004). Furthermore, there are reports of fatal disseminated scedosporiosis following organ transplantation from nearly-drowned donors (Kim *et al.*, 2015; Leek *et al.*, 2016). Cases of keratitis (Saracli *et al.*, 2003a), peritonitis (Severo *et al.*, 1999), sinusitis (Loh *et al.*, 2018), and endocarditis (O'Bryan *et al.*, 2002) have also been documented in immunocompetent individuals.

Profoundly immunosuppressed individuals, including those with solid organ or hematopoietic stem cell transplants, as well as persons with the Acquired Immunodeficiency Syndrome, are at particularly increased risk of invasive and disseminated scedosporiosis. (Cortez *et al.*, 2008; Neofytos *et al.*, 2010; Tammer *et al.*, 2011). Neutropenia and receipt of T-cell depleting agents are also significant risk factors (Johnson *et al.*, 2014; Lamaris *et al.*, 2006). Disseminated scedosporiosis typically develops from haematogenous spread secondary to underlying pneumonia, and may result in fungemia (Husain *et al.*, 2005). Other presentations in immunocompromised hosts include intracranial abscess, endophthalmitis (Castiglioni *et al.*, 2002), meningitis and endocarditis (Uno *et al.*, 2014), and osteomyelitis (Ochiai *et al.*, 2003).

Diagnosis of *Scedosporium* infection requires isolation of fungi from a sterile body site or visualization of the mold by microscopic or histopathologic evaluation. In histopathology, *Scedosporium* cannot be readily distinguished from other hyalohyphomycetes, including *Aspergillus* or *Fusarium* species, however presence of hyphae branching at right angles and intravascular conidia may suggest the diagnosis (Kimura *et al.*, 2010). In culture, selective media such as SceSel^{+}, which contains dichloran and benomyl, can help to recover *Scedosporium* from clinical samples (Rainer *et al.*, 2008). As mentioned previously, *Scedosporium* species can be distinguished from *Lomentospora prolificans* as the latter is susceptible to cycloheximide, and produces flask-shaped conidiogenous cells. To identify *Scedosporium* to a species level, molecular sequencing and mass spectrometry are clinically available and accurate (Coulibaly *et al.*, 2011; Sitterle *et al.*, 2014; Steinmann *et al.*, 2011; Lu *et al.*, 2011).

Treatment of *Scedosporium* infection is complicated by the lack of available therapeutic antifungal agents. Amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole all display limited *in vitro* activity against *S. apiospermum* (Cuenca-Estrella *et al.*, 1999). The extended-spectrum azoles voriconazole and posaconazole retain variable activity against *S. apiospermum* isolates, with voriconazole demonstrating consistently lower MIC values when compared with posaconazole (Carrillo and Guarro, 2001; Lackner *et al.*, 2012; Meletiadis *et al.*, 2002). However, both antifungals have shown retained efficacy *in vivo* by decreasing fungal burden and prolonging survival in murine and guinea pig animal models (Capilla *et al.*, 2003; Capilla and Guarro, 2004; Rodriguez *et al.*, 2010). The newest triazole, isavuconazole, appears to be ineffective against all species of *Scedosporium*, based upon available MIC data (Guinea *et al.*, 2008; Lackner *et al.*, 2012). Echinocandins demonstrate significantly variable activity against *Scedosporium* species, and have been evaluated for synergism in combination with azole therapy. *In vitro* evaluation suggested possible synergism between voriconazole and micafungin (Heyn *et al.*, 2005). However, no survival benefit was seen with micafungin/voriconazole or micafungin/posaconazole combination therapy in an *in vivo* murine model (Lackner *et al.*, 2014b). Currently, recommended first-line therapy consists of voriconazole monotherapy for *S. apiospermum* infection. However, some clinicians would advocate for azole/polyene or azole/echinocandin combination therapy on the basis of case series (McCarthy *et al.*, 2018). At this time, two novel antifungal agents, APX001 (fosfomanogepix), and F901318 (olorofim) show promise for the treatment of *Scedosporium* infection and remain in development (Biswas *et al.*, 2018; Rivero-Menendez *et al.*, 2019; Wiederhold *et al.*, 2017).

Paecilomyces

These species are saprophytic hyaline molds distributed globally throughout the world, and a cause of microbiology laboratory contamination. Several species are known to cause human disease, including *Paecilomyces variotti*, *P. marquandii*, *P. javanicus*, *P. fumosoroseus*, *P. viridis*, and *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) (Aguilar *et al.*, 1998). Recently, *P. lilacinum* was reclassified into the newly created genus *Purpureocillium* on the basis of phylogenetic analysis

which places *Paecilomyces* species within the family *Trichomaceae* (*Eurotiales*) and *Purpureocillium lilicanus* within the family *Ophiocordycipitaceae* (*Hypocreales*) (Luangsa-Ard *et al.*, 2011).

Foreign medical devices, including central venous catheters (Chan-Tack *et al.*, 1999; Shing *et al.*, 1996), prosthetic cardiac valves (Allevato *et al.*, 1984; Haldane *et al.*, 1974; Kalish *et al.*, 1982; McClellan *et al.*, 1976), contact lenses and corneal implants (Almeida Oliveira *et al.*, 2019; Chen *et al.*, 2019), and peritoneal dialysis catheters (Uzunoglu and Sahin, 2017), account for the majority of infections caused by *Purpureocillium lilacinum* and *Paecilomyces* species. In immunocompromised individuals, invasive disease appears quite similar to disseminated fusariosis, with manifestation of skin lesions and fungemia (Chamilos and Kontoyiannis, 2005).

As a common contaminant of the microbiology laboratory, diagnosis of clinical disease is made by positive culture in combination with clinical manifestations of infection. *Paecilomyces* species grow rapidly on Sabouraud agar, ranging in color from yellow, pink, brown, or white. Isolates may be confused with *Penicillium* species, based upon similarities of their conidia, however phialides tend to be widely spaced in contrast to *Penicillium* (Sciortino, 2017). Molecular sequencing is available to discriminate to the species level on the basis of the ITS region and beta-tubulin gene (Houbraken *et al.*, 2010). MALDI-TOF-MS identification is promising, but will depend upon improvement of databases before clinical use is reliable (Chen *et al.*, 2015).

The reclassification of *P. lilacinum* into a distinct genus from the *Paecilomyces* helps to explain differences in phenotypic resistance. *P. lilacinum* isolates demonstrate significant resistance to most antifungals, including amphotericin B, fluconazole, itraconazole, flucytosine, and the echinocandins (Castelli *et al.*, 2008; Drogari-Apiranthitou *et al.*, 2012). In contrast, *P. variotii* isolates demonstrate preserved *in vitro* susceptibility to amphotericin B, itraconazole and potentially echinocandins (Castelli *et al.*, 2008; Drogari-Apiranthitou *et al.*, 2012). Both species demonstrate low MIC values for posaconazole, whereas only *P. lilacinum* appears susceptible to voriconazole (Castelli *et al.*, 2008; Drogari-Apiranthitou *et al.*, 2012). No first-line antifungal therapy has been definitively established, but treatment with voriconazole with posaconazole as salvage therapy may be a reasonable option. There are documented cases of successful treatment with voriconazole therapy (Martin *et al.*, 2002; Van Schooneveld *et al.*, 2008), and cases of successful salvage therapy with posaconazole (Almeida Oliveira *et al.*, 2019; Feldman *et al.*, 2016; Kim and Williams, 2014). As with all other hyaline molds, surgical debridement and resolution of underlying immunosuppression improve the chance of cure.

Scopulariopsis

Scopulariopsis is a saprobic, hyaline fungus found ubiquitously in the environment which is rarely pathogenic belonging to the *Microascales* order (Sandoval-Denis *et al.*, 2016). Several species have been documented to cause human infections, with *S. brevicaulis* most commonly implicated. Other less frequently seen pathogenic species include *S. candida*, *S. brumptii* (*Microascus paisii*), and *S. alboflavescens* to name a few (Delliere *et al.*, 2019; Kurata *et al.*, 2018).

Severity of infection caused by *Scopulariopsis* species varies predominantly with host immune status. Onychomycosis, keratitis, and localized infection occur most often in immunocompetent patients, although systemic infection, such as endocarditis has been reported following traumatic inoculation (Migrino *et al.*, 1995). Conversely, immunocompromised persons are at higher risk of invasive or disseminated infection (Helander and Stark, 2017; Neglia *et al.*, 1987), including pneumonia (Kurata *et al.*, 2018), empyema and pericardial effusion (Wuyts *et al.*, 2005), and brain abscess (Patel *et al.*, 1994). Inhalation of *Scopulariopsis* may also result in hypersensitivity pneumonitis in certain hosts (Grieble *et al.*, 1975).

Diagnosis is made by isolation of *Scopulariopsis* in culture, or visualization of narrow, branching septate hyphae on histopathology from a clinical specimen. When cultures are positive, colonies grow quickly ranging in color from white to gray. Conidiogenous cells are annellidic, with short, cylindrical stalks with a swollen base. Conidia are globose or ovate and unicellular (Sandoval-Denis *et al.*, 2016).

These species are inherently resistant to most antifungal therapies, making treatment difficult. No optimal therapy has been definitively determined and no guidelines are currently available to aid treatment (Tortorano *et al.*, 2014). *In vitro* susceptibility testing shows high MIC values for amphotericin B, flucytosine, fluconazole, itraconazole, posaconazole and voriconazole (Aguilar *et al.*, 1999; Skora *et al.*, 2015). Echinocandins have no activity against these species (Sandoval-Denis *et al.*, 2013). While terbinafine appears to have fungicidal activity, it has not been effective in clinical treatment of onychomycosis (Steinbach *et al.*, 2004). Susceptibility testing to isavuconazole has not been reported. Combination therapies have been evaluated *in vitro* with triple therapy of posaconazole, terbinafine, and caspofungin (Yao *et al.*, 2015) or amphotericin B, voriconazole, and anidulafungin (Martin-Vicente *et al.*, 2017) showing promise for *S. brevicaulis* isolates. There are a few case reports which announce successful treatment with combination posaconazole and terbinafine in solid organ transplant recipients (Pate *et al.*, 2016; Rakita *et al.*, 2015); however, mortality from invasive *Scopulariopsis* infections remains high. In addition to antifungal therapy, surgical debridement and correction of the underlying immunodeficiency are vital in treating these infections.

Emergomyces

A group of novel dimorphic fungi, *Emergomyces* was first identified in 1994 as the causative agent of a disseminated mycosis in an Italian woman with advanced HIV (Gori *et al.*, 1998). At that time, the species was classified as *Emmonsia* due to morphologic

similarities with the existing genus, as well as genetic analyzes. Thirteen additional cases of disseminated disease were described in South Africa in 2013 among HIV-infected individuals (Kenyon *et al.*, 2013). Further genetic analysis of these cases led to taxonomic revision and five species were identified within the family Ajellomycetaceae: *Emergomyces africanus*, *E. canadensis*, *E. europaeus*, *E. orientalis*, and the type species, *E. pasteurianus* (Dukik *et al.*, 2017). With the exception of *E. pasteurianus* these fungi appear to be geographically restricted in accordance with their species names – that is cases of *E. africanus* have been documented in South Africa and Lesotho (Schwartz *et al.*, 2017), *E. canadensis* in Canada and the United States (Schwartz *et al.*, 2018), *E. europaeus* in Germany (Wellinghausen *et al.*, 2003), and *E. orientalis* in China (Wang *et al.*, 2017). Interestingly, the environment of *Emergomyces* species has not yet been identified, and infection of non-human animals has not been observed (Cronje *et al.*, 2018).

Of the reported cases of *Emergomyces*, a vast majority of patients are suffering from the Acquired Immunodeficiency Syndrome, although a small number of cases in apparently immunocompetent individuals exist. Disseminated emergomycosis appears to be the norm, presenting with cutaneous lesions and pulmonary involvement, which may take a variety of forms (Schwartz *et al.*, 2015).

Cultures are readily positive from skin biopsies, and form tannish colonies which become raised and furrowed with maturation. In their mold form, they are identified by right-angled, branching hyphae with the presence of "florets" – secondary conidiophores with a small, spherical conidia. Definitive species identification may be made by sequencing of the ITS region and β-tubulin genes (Dukik *et al.*, 2017). Importantly, *Emergomyces* species may cross-react with both *B. dermatitidis* DNA probing and *Histoplasma* urine antigen testing (Schwartz *et al.*, 2018; Schwartz *et al.*, 2015).

Very limited data exists regarding the treatment of *Emergomyces* infections. Currently susceptibility data suggest efficacy of amphotericin B, itraconazole, voriconazole, posaconazole, isavuconazole, anidulafungin, and micafungin. These fungi are resistant to fluconazole and flucytosine (Dukik *et al.*, 2018; Maphanga *et al.*, 2017). In general, patients are given induction therapy with amphotericin B, followed by prolonged courses of azole therapy, typically itraconazole, in a fashion similar to treatment for disseminated histoplasmosis. Infection is uniformly fatal without antifungal therapy, although mortality rates still approximate 50% with treatment (Schwartz *et al.*, 2015).

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Fungal Infections in Children

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Introduction

Children commonly encounter fungi in the environment, but infrequently develop disease. In healthy children, fungal infections most commonly present as mucocutaneous disease, while invasive fungal infections (IFD) occur primarily in immunocompromised and hospitalized children. In fact, *Candida spp.* are the third most common cause of nosocomial infections in children (Wisplinghoff *et al.*, 2003). Many of the known risk factors for IFD in adults (i.e., hematologic malignancies, ICU admission, and organ transplantation) confer a similar risk of disease in children. There are however important differences in the epidemiology, diagnostic and therapeutic approaches to fungal infections between children and adults, such as unique cohorts that exhibit increased susceptibility to fungal infections. This includes neonates (especially pre-term neonates) and children with primary immunodeficiencies for whom IFD has a very significant impact on morbidity and mortality. Recognition of this fact has led to the successful use of anti-fungal prophylaxis to prevent IFD in these children. Furthermore, the study of fungal disease in children, especially those with defined immune defects, has significantly advanced our understanding of fungal diseases and host response, including the importance of TH17 immunity in preventing mucocutaneous disease. The most common IFD in children are caused by *Candida* and *Aspergillus spp.* and are reviewed in this chapter with a special emphasis on these unique at-risk groups of children.

Candida Infections

Neonates

Invasive candidiasis

Epidemiology

Neonates are disproportionately at risk for invasive candidiasis (IC) with a reported incidence on the order of 11 per 100,000 live births per year (or 15 cases per 10,000 neonatal admissions) (Benedict *et al.*, 2018; Zaoutis *et al.*, 2007). IC is not only more common in neonates but associated with a worse prognosis when compared with the same disease in older children (Hsu *et al.*, 2018). The highest rates of IC occur in premature babies and the risk of IC can be readily stratified based on the extent of prematurity. IC has been reported to occur in 4%–8% of babies weighing less than 1000 g also known as extremely low birth weight infants (ELBW) (reviewed in Kelly *et al.*, 2015). Both the mortality and morbidity of IC are high with death or neurodevelopmental impairment being reported in as many as 73% of ELBW neonates (Benjamin *et al.*, 2006). A variety of medical practices have been identified as risk factors for IC in neonates, including antibiotic exposure (especially 3rd generation cephalosporins), the presence of indwelling catheters, parenteral nutrition, and H2-antagonists. The presence of necrotizing enterocolitis and other GI abnormalities have also been reported as risk factors for invasive candidiasis in neonates (Benjamin *et al.*, 2006). A decrease in the rates of neonatal IC has been linked to the institution of central line care protocols and anti-fungal prophylaxis regimens (reviewed in Benedict *et al.*, 2018). Other fungi like Aspergillus and Malassezia can occasionally cause disease in neonates, though much less frequently than candida.

Host

Skin and mucosal colonization of the neonate occur quickly after birth as a result of both horizontal and vertical transmission. Candidal colonization often precedes invasive disease and may occur in as many as 23%–60% of neonates, with the higher rates reported in premature neonates (Saiman *et al.*, 2001; Kicklighter *et al.*, 2001). Nonetheless, the minority of colonized neonates go on to develop invasive disease (Baley *et al.*, 1986). Intrauterine transmission of candida infection (via ascending infection or transplacental spread) resulting in congenital disease occurs but is rare despite the relatively common occurrence of candidal vaginitis during pregnancy. In general, the placenta appears to efficiently limit transmission of fungal disease from mothers to neonates as evidence by low rates of congenital fungal infection (Patel *et al.*, 2012). Neonates with congenital candidiasis usually present within the first day of life with a diffuse, erythematous rash that can be variable in appearance (i.e., macular, vesicular, pustular and desquamating). Blood stream infection with dissemination to other organs may be present at the time of the appearance of the rash or may occur with progression of disease, especially in the absence of prompt therapy (Kaufman *et al.*, 2017).

The specific immune deficiencies that lead to an increased risk of candidiasis in neonates are poorly understood. Animal model experimentation demonstrates that pups are more susceptible to IC when compared to adult animals, supporting the hypothesis that immunologic immaturity of the newborn contributes to an increased susceptibility to candida infection (Trofa *et al.*, 2011). The skin and vernix caseosa of full-term infants, which is lacking or underdeveloped in preterm neonates, produce anti-microbial peptides that have anti-candida activity and may help prevent candida colonization (Marchini *et al.*, 2002). In vitro studies suggest that reduced neutrophil function in neonates (including phagocytosis and oxidative burst) does not play a role as a risk factor for invasive disease in this cohort (Destin *et al.*, 2009), even though neutrophil defects are often a primary risk factor for IC.

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Nonetheless, the s-lectin, galectin, which is secreted by neutrophils and is an opsonin for candida is deficient in newborns (Linden *et al.*, 2013). A variety of other immune factors that are developmentally immature in the neonate have been hypothesized to contribute to an increased susceptibility candida infection, including decreased complement components, a decreased number of innate host cells, a decreased and TH2 skewed inflammatory response (reviewed in Michalski *et al.*, 2017).

Pathogen

The most common candida species to cause IC in neonates are *C. albicans* and *C. parapsilosis* (Trofa *et al.*, 2008). Other non-albicans species cause disease in neonates, but much less commonly, including *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, and *C. lusitaniae*. The emergence of fluconazole resistance and the appearance of non-albicans strains has been a concern due to the use of fluconazole prophylaxis in NICUS (Brion *et al.*, 2007). However, in a recent review of 137 ELBW infants with IC, fluconazole resistance was only present in isolates from 3% of infants (Autmizguine *et al.*, 2018). Nonetheless, NICU outbreaks of fluconazole resistant, non-albicans candida have been described (Juyal *et al.*, 2013; Khan *et al.*, 2007).

Candida species exhibit a variety of virulence traits, though their role in neonatal disease are unknown. This includes secreted enzymes, which contribute to invasion and host damage, like aspartyl proteases and lipases. In vitro features, including adhesion and cytotoxicity have been reported to correlate with increased virulence of Candida strains isolated from neonates with IC (Bliss *et al.*, 2012). In animal models of neonatal disease lipase deficient mutants of both *C. parapsilosis* and *C. albicans* show decreased virulence relative to wild type strains, highlighting the importance of this enzyme in causing disease (Trofa *et al.*, 2011). An agglutinin protein, which mediates cellular adhesion and invasion, has also been linked with virulence in animal pup experimentation (Liu and Filler, 2011).

The enhanced virulence of *C. albicans* relative to other species of candida may be in part related to its relative unique tendency to form hypha during infection. Biofilm formation also appears to be an important virulence trait for *C. albicans* in the neonatal intensive care unit (NICU) setting. Like most candida, *C. albicans* forms a biofilm that promotes both adhesion to foreign material and decreased susceptibility to anti-fungal therapy. The biofilm of *C. parapsilosis* is biochemically and structurally unique. It appears to confer an enhanced ability to bind to foreign materials, especially in environments with high glucose and lipid concentrations, which is often seen with NICU babies who are receiving parenteral nutrition (reviewed in Chow *et al.*, 2012).

Disease manifestations and outcome

IC in neonates typically presents after the second week of life with sepsis that is indistinguishable from bacterial causes of sepsis. While thrombocytopenia is common, it is not specific for IC and can also be seen with bacterial sepsis. In one study, approximately 26% of ELBW neonates with IC died within 14 days of diagnosis (Benjamin *et al.*, 2006). Persistent candidemia is commonly observed in infected neonates despite anti-fungal therapy (Benjamin *et al.*, 2006). Furthermore, delayed catheter removal is associated with delayed sterilization of the blood and increased mortality (Karlowicz *et al.*, 2000). Dissemination to multiple organ systems is not infrequent with documented central nervous system (CNS) involvement occurring in approximately 8% of patients (Benjamin *et al.*, 2006). Thus, all neonates with IC should be evaluated for metastatic candida disease. Even in the absence of CNS infection, candida sepsis has been associated with worse developmental outcomes when compared with non-infected neonates (Benjamin *et al.*, 2006; Friedman *et al.*, 2000). Other reported sequelae to occur more commonly in neonates with IC include chronic lung disease, retinopathy of prematurity and peri-ventricular leukomalacia (Friedman *et al.*, 2000).

Mucocutaneous candidiasis

Epidemiology

Oropharyngeal candidiasis (thrush) and candida diaper dermatitis are commonly encountered fungal infections in healthy neonates. Thrush appears as white plaques on the buccal mucosa, tongue or palate of the mouth and can cause discomfort during feeding. Erythema of the tongue, soft palate or buccal mucosa and cracking of the corners of the mouth can also occur. Candida dermatitis typically results from secondary infection of an irritant diaper dermatitis, resulting in a scaly erythematous rash in the diaper area with occasional satellite lesions. While usually self-limited in full-term infants, candidal diaper dermatitis can be a risk factor for the development of systemic candidal infection in premature infants.

Thrush is most commonly seen among babies younger than 1 month of age. It is estimated that approximately 2%–7% of healthy infants develop thrush (Krol and Keels, 2007). In older children, immunocompromising conditions such as HIV and diabetes can also lead to oral candidiasis. Inhaled corticosteroids, frequent or prolonged antibiotic courses, chemotherapy or immunosuppressive medications can also predispose to oral candidiasis. Persistent oral candidiasis or recurrences in children older than 6 months of age should prompt investigation for possible immunodeficiency (Krol and Keels, 2007).

Pathogen and host

C. albicans accounts for approximately 70%–80% of cases of oral candidiasis, with *C. glabrata* and *C. tropicalis* less commonly implicated (Krol and Keels, 2007). Similar to oral candidiasis, 80%–90% of candidal diaper dermatitis is due to *C. albicans*, with *C. tropicalis*, *C. parapsilosis*, and *C glabrata* found less commonly (Bonifaz *et al.*, 2016). While Candida is a common oral commensal, the presumed age-related factors that promote symptomatic disease in neonates is poorly understood. The pathogenesis of diaper dermatitis is multifactorial with friction from diapers, moisture, exposure to lipases and proteases from feces and urine, and changes in skin microflora, and increases in pH all contributing to maceration of the skin. The resultant impairment of the stratum corneum, promotes invasion by *C. albicans* in the stool (Bonifaz *et al.*, 2016). Premature infants are at particularly high

risk due to a thinner stratum corneum (Sikic Pogacar *et al.*, 2018) and a higher skin pH, that takes longer to normalize (Adam, 2008). A higher skin pH leads to greater activity of fecal enzymes including lipases and proteases which causes increased irritation to the skin (Bonifaz *et al.*, 2016).

Primary Immunodeficiency and Candida Infection

Invasive candidiasis

Primary immunodeficiencies consist of over 300 different disorders that result in impaired immunity. Depending on the underlying immune defect, children with primary immunodeficiencies (PIs) are at an increased risk for developing candida infection that can manifest as IC or chronic mucocutaneous candidiasis (CMC). Disorders of phagocytic function are in particular noted for an increased risk of IC. Children with chronic granulomatous disease (CGD), for example, have defective NADPH oxidase activity resulting in impaired oxygen free radical production by neutrophils. This results in an increased susceptibility to both invasive aspergillus and candida infections. While IC is less common than invasive aspergillosis for CGD patients, it is still an important cause of disease with a reported incidence ranging from 6% to 10% (Antachopoulos *et al.*, 2007; Winkelstein *et al.*, 2000; Henriet *et al.*, 2013). Lymphadenitis, meningitis, and bloodstream infection are the most common forms of disease in children with CGD, while mucocutaneous candidiasis is rarely encountered (Winkelstein *et al.*, 2000). Affected infants typically present with non-specific symptoms of irritability and fever, but may have localizing signs such as lymphadenopathy or hepatosplenomegaly. Myeloperoxidase deficiency, which results in decreased hypochlorous acid production by neutrophils and defective killing of *Candida spp.* in vitro, also confers an increased risk of IC. However, affected patients typically also have other predisposing factors for IC like diabetes (Antachopoulos *et al.*, 2007). Other primary immunodeficiencies resulting in defective neutrophil function have variably be reported to confer an increased risk of IC, including leukocyte adhesion defect and congenital neutropenias (Antachopoulos *et al.*, 2007).

Chronic mucocutaneous candidiasis

Several PIs have been associated with CMC, a condition characterized by chronic skin, nail, oral and genital infections with *Candida spp*. Affected patients develop plaques on the oral, esophageal or genital mucosa and thickened skin and nails. For some PIs, CMC is the primary manifestation of the underlying immune defect, while for others it is a part of a constellation of symptoms. The PIs associated with CMC generally have impaired TH17 immunity, though this may occur as a result of different mechanisms. This observation is consistent with animal experimentation and highlights the importance of IL-17 in the host response to mucosal candida infection (reviewed in Conti and Gaffen, 2015). Job's syndrome, also known as Hyper Immune IgE Syndrome (HIES), is a PI associated with multiple manifestations including CMC, for which the underlying immunodeficiency was only recently recognized. In these patients, mutations in genes that encode the signal transducer and activator of transcription 3 signaling protein (STAT3) result in defective Th17 cell differentiation (Okada *et al.*, 2016). Patients typically have extremely elevated serum IgE levels, decreased Th17 cells, characteristic facial features, eczema, and recurrent pulmonary and staphylococcal skin abscesses. A large cohort study of 60 patients with AD-HIES demonstrated that 85% developed CMC, including oral, esophageal, genital, cutaneous candidiasis as well as chronic onychomycosis (Okada *et al.*, 2016). Other PIs associated with CMC include severe combined immunodeficiency (SCID), STAT1 gain-of-function mutations, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), and mutations in IL-17, IL-12, and IL-23. For some of these conditions (i.e., SCID and IL-12 receptor deficiency), both CMC and IC can occur (reviewed in Okada *et al.*, 2016).

Children with mutilations in caspase recruitment domain-containing protein 9 (CARD9) are at risk for both CMC and IC. IC for these patients has a peculiar tendency to involve the central nervous system (CNS) although other organ systems including eyes and bone may also be involved (Corvilain *et al.*, 2018). CARD9 is expressed by neutrophils and monocytes and is part of the signaling pathway that is downstream of dectin-1 and dectin-2, which are C-type lectin receptors that are important for fungal recognition. Affected patients have both impaired neutrophil-mediated killing of *Candida spp.* in vitro and decreased levels of TH17 cells, which may explain their predisposition for both IC and CMC. Besides candida infections, these patients are also at increased risk for other fungal diseases, including non-pulmonary aspergillosis, extensive dermatomycosis, as well as subcutaneous and invasive phaeohyphomycosis.

Diagnosis

Culture

While blood culture is considered the gold standard for the diagnosis of IC, its sensitivity has been reported to vary between 20% and 70% (Clancy and Nguyen, 2013; Avni *et al.*, 2011). In some instances, this is due to the presence of end-organ disease in the absence of fungemia. In patients with fungemia, the number of circulating organisms is often low, making it difficult to isolate the organism. This is especially true in children and premature neonates, for whom the volume of blood that is available for testing is low, in some instances less than 1 ml. Of note, the concentration of circulating yeasts in the blood has been reported to vary based on the infecting species and age of the patient, with higher loads reported for neonates and with disease due to *C. parapsilosis* (Pfeiffer *et al.*, 2011). In immunocompromised children, the detection of Candida in the urine can be an indicator of systemic disease and should be investigated accordingly. This is especially true for premature neonates for whom the detection of Candia in the urine has been associated with an increased risk of death and impaired neurodevelopmental outcome., similar to that see in those with candidemia (Wynn *et al.*, 2012).

Fungal antigen and molecular testing

Given the limits of blood culture testing for IC, fungal antigen testing has been developed to enhance the diagnosis of IC. β-D-glucan (BDG) is a polysaccharide constituent of the candida cell wall. This polysaccharide is found in a variety of fungi, but notably not present in Mucormycetes or Cryptococcus. False positives can occur in a variety of clinical settings and complicate the interpretation of results. This includes bacteremia, intravenous immunoglobulin therapy, certain antibiotics (i.e., piperacillin tazobactam, meropenem), and hemodialysis with use cellulose filters (Sulahian *et al.*, 2014; Alexander *et al.*, 2010). Elevated BDG levels have also been reported in children with hematologic malignancies that were colonized with Candida (Wynn *et al.*, 2012). The BDG assay was developed by testing in adults and the appropriate cut-off values for children are not well characterized. Current pediatric data is limited by several factors, including small study size, retrospective nature and differences in methodology (reviewed in Cohen *et al.*, 2020; Lehrnbecher *et al.*, 2016). It is likely that the appropriate pediatric cut-off is not only different from the adult value but likely to vary between different pediatric cohorts. For example, several NICU studies suggest higher cut off values (105–125 pg/ml) are need to discriminate between IC and other serious illness (Goudjil *et al.*, 2013; Cornu *et al.*, 2018) Monitoring of BDG levels in the CSF may be helpful in monitoring response to antifungal therapy (Salvatore *et al.*, 2016). Mannan is another fungal polysaccharide, which has been used diagnostically for IC though minimal pediatric data exists. PCR based assays have been increasingly studied for the diagnosis of IC as they offer rapid turn around and high sensitivity. A PCR combined with a magnetic resonance detection system (T2Candida Panel) has been recently licensed by the FDA for the detection of Candia directly from blood. The assay detects 5 species of Candida, including *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. The sensitivity of this assay is reported to be 1 colony-forming unit/ml with a turn-around time of 3–4 h (Mylonakis *et al.*, 2015; Beyda *et al.*, 2013). Preliminary pediatric studies suggest good agreement of this assay with blood culture testing (Hamula *et al.*, 2016).

Aspergillus Infections

Among children with invasive mold infections, *Aspergillus spp.* are responsible for as many as 75% of cases (Wattier *et al.*, 2015). Many of the epidemiologic, clinical and diagnostic features of invasive aspergillosis (IA) in children are similar to those seen in adults, but there are important differences, which are reviewed in this section. Aspergillus also causes hypersensitivity lung disease in children with underlying respiratory disease, including cystic fibrosis and asthma. In these instances, colonization of the airway by *Aspergillus species* can result in an exacerbation of underlying respiratory disease (Vicencio *et al.*, 2014).

Invasive Aspergillosis

Epidemiology

Children with hematologic malignancies with or without stem cell transplantation make up the majority of IA cases. Children with primary immunodeficiency and those who have undergone solid-organ transplantation, especially heart and lung recipients are also at risk for IA. IA may also occur in children with lupus and preterm neonates (Silva *et al.*, 2015; Gallais *et al.*, 2017).

Among children with hematologic malignancies, most but not all studies suggest the highest rates of IA in AML patients who typically have more prolonged periods of neutropenia. Likewise, children with recurrent ALL also appear to have an increased risk of IA (Papachristou *et al.*, 2019). Affected children typically have one or more immunologic risk factors for the development of IA including neutropenia, corticosteroid therapy or other immunosuppressive therapy (i.e., calcineurin inhibitors). IA has been reported to cause 4.79% of culture-proven infections in HSCT recipients, though rates tend to vary among sites. Most infections occur in the first 100 days following transplant and often occur in the context of concurrent bacterial, viral or other fungal infections. Graft versus host disease and the immunosuppression used to treat this entity are a recognized risk factor for IA. When compared to HSCT, children undergoing solid-organ transplantation are significantly lower risk of IA with reported rates typically less than 1% (Zaoutis *et al.*, 2006). The exception to this is lung transplantation (especially in patients with cystic fibrosis) where aspergillosis may occur in as many as 5% of patients (Zaoutis *et al.*, 2006).

Among the primary immunodeficiencies (PIs), chronic granulomatous disease (CGD) is most closely linked to enhanced susceptibility to aspergillus infection. CGD occurs in 1/200,000 births and results from an impaired ability to generate an oxidative burst with a resulting decrease in superoxide free radical production secondary to defects in nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. IA is one of the most common causes of serious infections in children with CGD, affecting approximately 40% of patients (Marciano *et al.*, 2015; Blumental *et al.*, 2011). Differences in the incidence of IA among CGD patients appears to be attributable to differences in residual NADPH activity, and associated oxidative burst activity. The X-linked form of disease accounts for approximately 80% of all CGD. X-linked CGD is the most severe form of CGD and is associated with earlier presentation (most by age 2) and earlier onset of IA (Bortoletto *et al.*, 2015).

IA may also occur in other primary immunodeficiencies that result in either quantitative or qualitative defects in neutrophil function (i.e., leukocyte adhesion defect, congenital neutropenias, Wiskott–Aldrich Syndrome). In children with CARD9 deficiency, which is associated with chronic mucocutaneous candidiasis, defects in neutrophil migration may result in extra-pulmonary aspergillosis (Rieber *et al.*, 2016). T cell defects can also be associated with an increased risk of IA. In children with Job's syndrome

(also known as Hyper-IgE syndrome), aspergillus is known to superinfect pneumatoceles and invade blood vessels. Approximately 20% of patients with Hyper-IgE syndrome develop IA and 17% of these infections lead to death (Chandesris *et al.*, 2012). IA may also occur in newborns, though it is significantly much less common than invasive candidiasis. Primary cutaneous aspergillosis with and without secondary dissemination is well described in premature newborns, typically in association with nosocomial contamination of areas of the skin that are damaged (Woodruff and Hebert, 2002).

Organism

Similar to IA in adults, *A. fumigatus* is the most common species isolated from children with IA, with the most commonly involved site being the lungs (Burgos *et al.*, 2008). Other Aspergillus species that have been implicated in pediatric IA include *A. flavus*, *A. terreus*. *A. niger*, and *A. nidulans*. A unique characteristic of IA in CGD is the high frequency of *A. nidulans* infections, ranging from 38% to 47% of cases (reviewed in King *et al.*, 2016). The basis of increased frequency of *A. nidulans* in CGD patients is poorly understood and may not be related to ROS deficiency (Henriet *et al.*, 2012). There has been increasing recognition of infections due to less common species of Aspergillus in patients with primary immunodeficiencies. This includes the organisms, *A. pseudoviridinutans*, *A. tanneri*, *A. udagawae*, which may have unusual anti-fungal susceptibility patterns (reviewed in Seyedmousavi *et al.*, 2018).

Pathogenesis

While a complete review of the pathogenesis of IA is beyond the scope of this chapter the study of IA in children with CGD has led to a better understanding of the role of NADPH in the host response to infections and will be reviewed here. IA begins with the inhalation of conidia, which are able to bind to respiratory epithelium. Conidia that avoid macrophage killing germinate to form hyphae, which are targeted by infiltrating neutrophils. The host response to the conidial and hyphal forms are distinct, though both rely to varying degrees upon the generation of oxygen free radicals to limit organism growth. The NADPH defects associated with CGD appear to result in increased susceptibility to Aspergillus infections as a result of several different mechanisms including impaired intracellular killing activity of neutrophils and possibly by macrophages. Decreased ROS production also hinders the release of preformed proteases by neutrophils and the development of neutrophil extracellular traps (NETS). NETS consist of extracellular collections of neutrophil-derived chromatin and granules, which are essential in limiting the growth of the hyphae, which are too large to undergo phagocytosis. Gene therapy which allowed for the restoration of NET formations has been reported to result in a cure of refractory IA in a child with CGD (Bianchi *et al.*, 2009).

Decreased ROS levels have also been hypothesized to contribute to the exuberant inflammation, which is typical in children with CGD and IA. Histopathologic exam of affected tissues typically reveals extensive granuloma formation with infiltrating neutrophils containing fungal elements. Of note, angioinvasion by hyphal elements which is characteristic of IA in non-CGD patients is absent, suggesting that ROS is not necessary to prevent this aspect of disease. Several different anti-inflammatory mechanisms of ROS have been proposed including increased proinflammatory cytokine and chemokine production as well as impaired neutrophil apoptosis (reviewed in Segal *et al.*, 2012). In mice, decreased ROS production interferes with tryptophan metabolism which results in enhanced inflammation through augmented IL-17 activity, though the role of this process in children with CGD is unclear (Romani *et al.*, 2008).

Clinical

In children with IA, in association with an underlying malignancy, the lungs are the most common site of involvement though other organ systems, including the sinuses, bone, skin, and brain, can be involved by both direct and hematogenous extension of infection. More the one organ system is involved in as many as 35% of cases (Abbasi *et al.*, 1999). The presentation of IA depends on the underlying risk factor and the organ system involved. In children with hematologic malignancies, a diagnosis of IA is commonly first considered as a result of abnormal radiographic studies of the chest or sinuses, which were done for the evaluation of fever in the context of neutropenia (Abbasi *et al.*, 1999). However, patients may also present with symptoms indicative of the site of disease, including but not limited to skin lesions, facial swelling or increased nasal drainage, respiratory symptoms, and headache (Abbasi *et al.*, 1999). Despite therapy, the mortality of IA in this cohort is high, with rates ranging from 22% to 52.5%, with the highest mortality reported in stem cell recipients (Zaoutis *et al.*, 2006; Burgos *et al.*, 2008). Other reported risk factors for death include GVHD, steroid therapy, immunosuppression, and surgery following diagnosis (Burgos *et al.*, 2008).

In children with CGD, the lungs are also the primary site of disease in the majority of cases. Extension of infection into the bones, mostly in the adjacent thoracic cavity, has been reported to occur in 41%–74% of cases (Henriet *et al.*, 2013). Dissemination to the brain can also occur though this is much less common. Symptoms of IA may be absent in 1/3 of the cases or non-specific such as generalized malaise and failure to thrive. In a French study of CGD patients admitted with invasive mold infections, respiratory symptoms and fever were present in 55% and 38% of patients respectively (Blumental *et al.*, 2011). IA in children with CGD may also present as a chronic progressive infection and occasionally as an acute, severe pneumonitis due to exposure to a high inoculum of conidia (aka mulch pneumonitis) (Henriet *et al.*, 2013). Overall, signs and symptoms of IA tend to be more subtle in children with CGD compared to children with underlying hematologic malignancies. This is especially true for *A. nidulans* disease, even though this infection is particularly aggressive and more likely to be associated with local extension into the thoracic bones and death (Henriet *et al.*, 2012; Dotis and Roilides, 2004).

Diagnosis

Radiologic features

Plain X rays and CT scans are useful in the diagnosis of pulmonary IA in children. However, the most common radiologic features are non-specific and include: segmental and multi-lobar consolidation, perihilar infiltrates, multiple small nodules, peripheral nodular masses, and pleural effusions (Thomas *et al.*, 2003; Taccone *et al.*, 1993; Katragkou *et al.*, 2017). Air crescent and halo signs, which are commonly seen in adult cases of IA appear to be less commonly observed in children (Burgos *et al.*, 2008; Taccone *et al.*, 1993). This is especially true for children with CGD, for whom angio-invasion is not present. It is important to note that the progression of radiologic findings does not necessarily correlate with clinical response (reviewed in Steinbach, 2010).

Culture

Blood cultures in children with IA are typically negative and culture of affected tissue is considered the gold standard of IA diagnosis. Isolation of the organism in culture allows for speciation and susceptibility testing. This is particularly important for children with CGD and other PIs who receive chronic anti-fungal prophylaxis and who are at increased risk for infections due to non-fumigatus species with reduced anti-fungal drug susceptibility (Seyedmousavi *et al.*, 2018). For children with hematologic malignancy and HSCT, some but not all studies indicate a higher yield for an infectious diagnosis with lung biopsy compared to BAL, though this procedure is associated with higher complication rates (Qualter *et al.*, 2014; Chellapandian *et al.*, 2015). Lung biopsy has been reported to have a higher yield over BAL for the diagnosis of IA in CGD (including adults) (King *et al.*, 2016; Salvator *et al.*, 2015).

Fungal antigen and molecular studies

Due to the low incidence of fungemia and positive blood cultures in patients with IA, additional tools have been developed to assist in the diagnosis of this disease. Some of these assays are based upon the detection of fungal/Aspergillus polysaccharides, including galactomannan (GM) and β-D-glucan (BDG) in the serum or other biologic fluids. GM testing is more specific for IA than BDG testing, as BDG can be found in a large number of fungi, including *Candida spp*. and Pneumocystis. Both tests are commonly employed as screening tools in children with fever and neutropenia and in diagnostic scenarios, (i.e., in patients with suspected disease). These tests were originally developed based on adult studies and appear to have overall less diagnostic utility in children for unclear reasons. A relatively recent metanalysis highlights the high degree of variability testing results in pediatric studies (Lehrnbecher *et al.*, 2016). GM testing was found to have an overall sensitivity of 68% and 89% when used for screening and diagnosis, respectively. The positive and negative predictive values ranged from 0% to 100% and 70%–100%, respectively. Less data exists for the role of GM testing in bronchoalveolar fluid (BAL) of pediatric patients. Reported sensitivity and positive predictive values range from 72% to 82% and 58%–82%, respectively (Desai *et al.*, 2009; de Mol *et al.*, 2013) Aspergillus PCR assays have also been developed, though again pediatric data is lacking. In adults, PCR testing of blood appears to have limited utility, while testing of BAL and affected tissue has increased sensitivity (reviewed in Lehrnbecher *et al.*, 2018). PCR technology also offers the potential for both the identification of species and anti-microbial resistance, though additional refinements in the current assay are necessary. It is important to note that, the utility of serum fungal antigen and PCR testing is even more limited in children with CGD. The basis for false-negative results in this cohort is unclear but could be related to differences in pathogenesis (i.e., lack of angioinvasion in CGD patients) and the use of chronic anti-aspergillus prophylaxis.

Antifungal Treatment in Children With IFD

Anti-fungal prophylaxis is routinely used for children with a variety of conditions who are at high risk for IFD, including prematurity, CGD, AML, aplastic anemia, HSCT and solid-organ transplantation. For neonates, the decision to institute anti-fungal prophylaxis should be based on the local incidence of IFD along with the presence of risk factors, i.e., prematurity and antibiotic usage (Pappas *et al.*, 2016). Fluconazole is the most commonly used agent for prophylaxis in the NICU. There have been multiple studies documenting the safety and efficacy of fluconazole prophylaxis in preventing IC in preterm neonates (Cleminson *et al.*, 2015). For children with CGD, universal anti-fungal prophylaxis with an aspergillus active agent is the standard of care (Thomsen *et al.*, 2016). Itraconazole is generally used for this purpose and has been reported to decrease the incidence of IFD by 50% or more (Beaute *et al.*, 2011; Gallin *et al.*, 2003). Itraconazole, however, is poorly absorbed following oral administration, and absorption with the liquid formulation is superior to that observed with capsules. Dosing for younger children (i.e., less than 13 years) is done on a mg per kg basis, though currently recommended doses have recently been reported to result in subtherapeutic levels (Leong *et al.*, 2019). In all children receiving anti-fungal prophylaxis, the potential for breakthrough infection with resistant fungi is always a concern, and this has been described for both neonates and children with CGD (Sarvikivi *et al.*, 2005; Warris *et al.*, 2002).

Specific treatment recommendations for invasive fungal infections are beyond the scope of this chapter, but pediatric guidelines are available elsewhere (Pappas *et al.*, 2016; Ullmann *et al.*, 2018; Patterson *et al.*, 2016). In the absence of primary pediatric data, many treatment recommendations are based on findings from adult studies combined with PK data from pediatric studies, which may be small in size. In some instances, anti-fungal therapy in children is further limited by the lack of even PK data. For example, there is no current pediatric data isavuconazole dosing, though pediatric studies are underway.

There are currently four classes of antifungals (azoles, echinocandins, polyenes and pyrimidine analogs) that are used for the treatment of IFD in adults and children. For all four classes, there are important pediatric dosing considerations. Doses in children

are typically based on a mg/kg and have to take into account age-related differences in pharmacokinetics and pharmacodynamics. For example, the serum half-life and terminal elimination phase of amphotericin-B, a polyene antifungal, are both longer in neonates than in older children (Baley *et al.*, 1990). It also has better cerebrospinal fluid (CSF) penetration among pre-term neonates when compared to adults (Stockmann *et al.*, 2014). Furthermore, rates of nephrotoxicity associated with amphotericin-b deoxycholate are considerably less in infants and children when compared to adults. Current treatment guidelines, accordingly, still include amphotericin-B deoxycholate as first-line therapy for invasive candidiasis in neonates with lipid preparations of amphotericin, fluconazole and echinocandins listed as alternatives (Pappas *et al.*, 2016). In contrast, amphotericin B deoxycholate is now infrequently used in adults for treatment of IC because of concerns of renal toxicity. It should be noted that the recommendation for amphotericin-b deoxycholate for neonatal IC is based primarily on reported experience as opposed to comparison trials with other anti-fungal agents, for which there is little data (Driessen *et al.*, 1996). In a recent phase III study, which compared micafungin versus amphotericin B deoxycholate for the treatment of IC in the neonate, 30 subjects were enrolled. No differences in fungal free survival and tolerability between the two agents were noted (Benjamin *et al.*, 2018). The choice of which anti-fungal to use for the empiric treatment of IC in the NICU should take into account several variables, including organ system involvement, local resistance patterns, prior exposure to anti-fungal therapies (i.e., prophylaxis) and presence of renal insufficiency.

Voriconazole is another anti-fungal agent for which age-related differences in pharmacokinetic properties have important dosing and therapeutic implications for use in children with IFD. Due to its improved efficacy and decreased toxicity, voriconazole has replaced amphotericin as the primary drug of choice for the treatment of IA. However, attaining therapeutic levels can be problematic in younger children (i.e., <12 years of age), presumably due to a combination of factors including dose-related variability in kinetics and impaired oral absorption (Walsh *et al.*, 2004; Karlsson *et al.*, 2009). Racial differences in CYP2C19 alleles, which code for the main enzyme responsible for voriconazole metabolism, also contribute significantly to variation in voriconazole levels in children.

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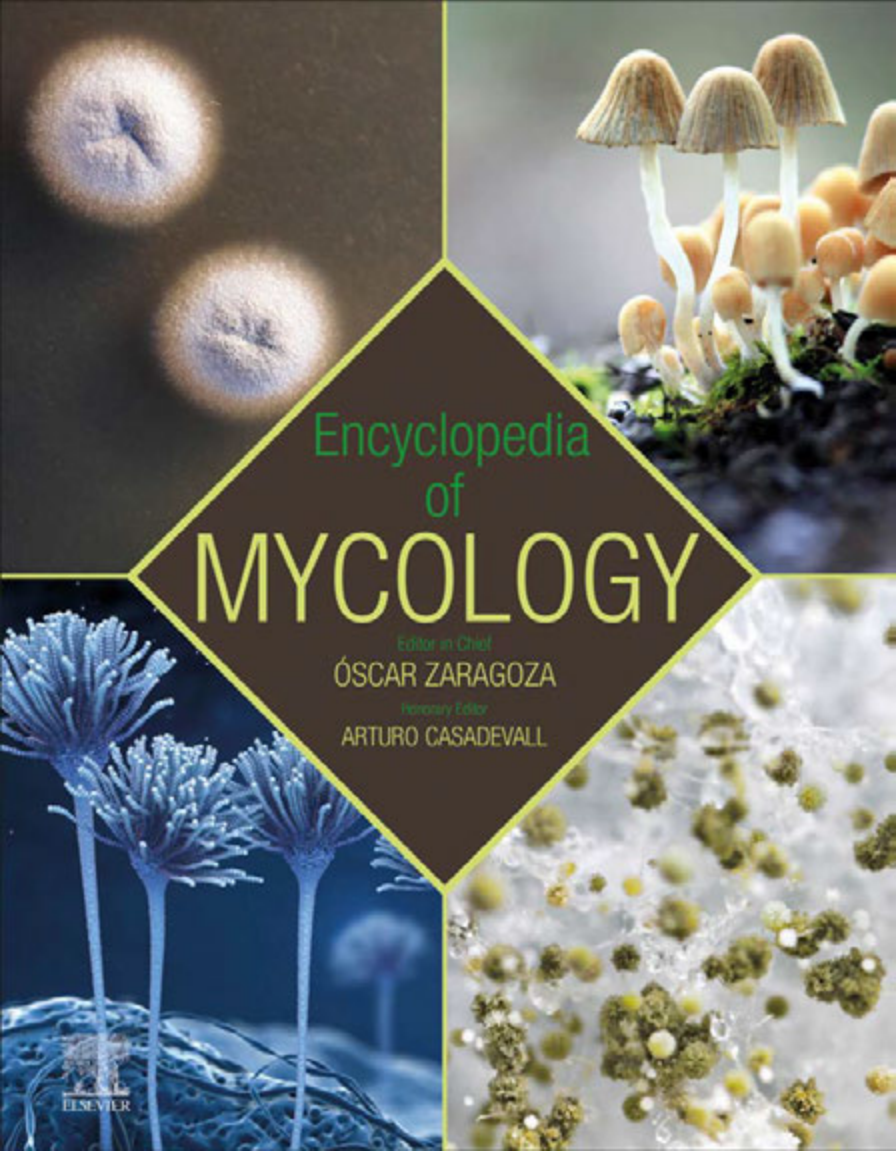

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Exposure to Fungi in Health Care Facilities

Raquel Sabino, Department of Infectious Diseases, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal

Epidemiology of Nosocomial Fungal Infections

Fungal diseases affect over a billion people across the globe (Bongomin *et al.*, 2017) and are an increasingly common cause of morbidity and mortality in hospitalized patients. There are multiple factors that predispose to the development of these infections such as neutropenia, stem cell transplantation, hypogammaglobulinemia, T-cell dysfunction, and/or mucosal damage. Gastrointestinal surgery or other extensive surgeries, use of invasive medical devices, high exposure to antibiotics, diabetes, corticosteroid therapy, prolonged hospitalization, and interinstitutional transfer of patients are other risk factors for the development of fungal infections (Fridkin and Jarvis, 1996; de La Rosa *et al.*, 2002; Sadfar and Maki, 2002; McCarty and Pappas, 2016). Healthcare-associated invasive fungal infection (IFI) outbreaks have been increasing in immunocompromised patients and are associated to suboptimal hospital environmental conditions, transmission via healthcare workers' hands, contaminated medical products, and transplantation of infected organs (Benedict *et al.*, 2017).

Significant statistical associations are often found between the degree of fungal contamination of air and hospital surfaces and the number of fungal infections in hospitalized immunocompromised patients (Kanamori *et al.*, 2015). When an imbalance occurs between the patient's immune system and the surrounding environment fungal infections are more likely to arise. In hospital units with immunocompromised patients, environmental quality (fungal burden and species) plays a key role in the development of infection.

In a "One Health" approach, which is aimed at designing and improving strategies, actions, legislation, and research that involves multiple sectors for achieving better public health outcomes, healthcare facilities have a great impact in health promotion among the several issues that affect the quality of population health (Gola *et al.*, 2019).

In fact, hospital environment plays a crucial role in the epidemiology of fungal infections. The majority of nosocomial fungal outbreaks occurred in patients exposed to building environments with inadequate air filtration, ongoing construction, water damage, and air pressure differentials (Benedict *et al.*, 2017).

Airborne infection is considered a major route of transmission in hospitals, and prevention of airborne pathogens is critically important to control hospital infections (Tong *et al.*, 2017). According to some studies (Srikanth *et al.*, 2008; Viegas *et al.*, 2016), air sampling is not sufficient to assess the risk of fungal infection in a hospital environment as airborne fungal particles eventually settle on surfaces and sampling is essential to identify potential risks and sources of contamination. The most frequent sources of fungal contamination within hospital environment are air-conditioning and ventilation systems, particles, ornamental plants, flowers, fresh fruits, food, water, construction work in and around hospital buildings, occupants or visitors, or even insects (Kordbacheh *et al.*, 2005; Pantoja *et al.*, 2009). Therapeutic swimming pools and nebulizers have also been pointed out as possible sources of nosocomial fungal infections (Angenent *et al.*, 2005; Peckham *et al.*, 2016). The degree of environmental fungal contamination can further increase dramatically in combination with other factors such as the presence of a favorable microclimate (humidity and temperature) and other geoclimatic factors (as the season of the year), the population density within a specific hospital/ward, and developed activities, among others. In the hospital setting, modes of transmission and portals of entry for the etiological agents responsible for nosocomial fungal infections vary according to the pathogen involved. Fungal infection may occur by direct or indirect contact with contaminated surfaces or objects, through the hands of health professionals, by ingestion and by inhalation of contaminated particles or bioaerosols (Leung and Chan, 2006; Sabino, 2015).

The epidemiology of IFIs in immunocompromised patients is rapidly changing due to several factors, namely the advances in medical care. Moreover, global warming (including climatic oscillations) may select for environmental species that have acquired thermotolerance, a key step toward pathogenesis to humans. In addition, important virulence factors have developed in environmental fungi, because they are essential for yeast survival in the environment. Thus, fungi traditionally regarded as non-pathogenic to humans have virulence factors similar to those of their pathogenic relatives (Richardson and Lass-Flörl, 2008; Kanamori *et al.*, 2015; de S Araújo *et al.*, 2017).

Aspergillus and *Candida* species are responsible for most nosocomial fungal infections in immunocompromised patients. However, other fungi have emerged as agents of nosocomial fungal infection, namely *Fusarium* species, *Pseudallescheria boydii*, species belonging to the subphylum Mucormicotina, and yeasts like *Malassezia* or *Saccharomyces*. **Table 1** shows the most frequent opportunistic fungal species, associated with nosocomial infections. Some of these emerging pathogens are, in some cases, resistant to the available antifungals, potentiating the threat of novel fungal diseases (de S Araújo *et al.*, 2017).

The most common potentially pathogenic fungi can survive and persist in bioaerossols and surfaces for many days or even months and can therefore be a constant source of transmission.

As nosocomial fungal infections progress rapidly and fungi are increasingly resistant to available antifungal agents, efforts are urgently needed for infection prevention, early diagnosis, and effective treatment. Preventive measures are of great importance in the control of nosocomial fungal infections, and should include environmental surveillance and strict application of cleaning procedures. Training health professionals and improving their knowledge of fungal infections is also of particular importance in preventing and controlling nosocomial fungal infections (Neely and Orloff, 2001).

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Table 1 Most frequent fungal species associated with nosocomial infections

Yeasts	*C. albicans* *Candida auris* *C. glabrata* (complex) *C. parapsilosis* (complex) *C. tropicalis* *Pichia kudriavzevii* *Merozyma guilliermondii* *Malassezia* spp. *Rhodotorula* spp. *Saccharomyces* spp.	Yeast-like fungi	*Trichosporon* spp. *Geotrichum* spp.
		Subphylum Mucormicotina	*Lichtheimia corymbifera* *Cunninghamella* spp. *Mucor* spp. *Rhizopus* spp. *Rhizomucor* spp.
Aspergillus spp.	*A. fumigatus* (section) *A. niger* (section) *A. terreus* (section) *A. flavus* (section)		
Other hyaline molds	*Sarocladium* spp. *Fusarium* spp. *Paecyllomyces* spp. *Purpureocillium lilacinus* *Scedosporium* spp. *Pseudolesheria boydii*	Dematiaceous molds	*Alternaria* spp. *Bipolaris* spp. *Curvularia* spp. *Exophiala* spp.

Note: Adapted from Richardson, M., Lass-Flörl, C., 2008. Changing epidemiology of systemic fungal infections. Clin. Microbiol. Infect. 14 (Suppl 4), 5–24. doi:10.1111/j.1469-0691.2008.01978.x. Alangaden, G.J., 2011. Nosocomial fungal infections: Epidemiology, infection control, and prevention. Infect. Dis. Clin. North Am. 25 (1), 201–225. doi:10.1016/j.idc.2010.11.003. Bougnoux, M.E., Brun, S., Zahar, J.-R., 2018. Healthcare-associated fungal outbreaks: New and uncommon species, New molecular tools for investigation and prevention. Antimicrob. Resist. Infect. Control 7, 45. doi:10.1186/s13756-018-0338-9.

About 20%–50% of hematological patients show evidence of IFI at autopsy (Traoré *et al.*, 2002). The mortality attributed to *Candida* systemic infections ranges from 8% to 53% in allogeneic bone marrow transplant recipients, while in the spread of filamentous fungi such as *Aspergillus*, *Fusarium*, and infections caused by fungi belonging the subphylum Mucormicotina this rate ranges from 56% to 95% (Martino *et al.*, 2002; Fukuda *et al.*, 2003; Roden *et al.*, 2005).

In the following pages, the most frequent fungal genera and species associated with nosocomial fungal infections will be discussed in more detail.

Candida

Infections caused by yeasts belonging to *Candida* genus are classified as candidiasis (includes candidemia). From 150 *Candida* species described, some are part of our normal microbiota and only 10% are known to be responsible for infections in humans, *C. albicans* being the most frequent etiological agent (Yapar, 2014). Nosocomial bloodstream infections caused by *Candida* species have increased in recent decades, mainly due to the multiple medical resources currently used, such as chemotherapy, antibiotics, catheters, and parenteral nutrition (Perlroth *et al.*, 2007; Safdar *et al.*, 2001; Pemán *et al.*, 2004; Bedini *et al.*, 2006; Pfaller and Diekema, 2007). In an European study (European Centre for Disease Prevention and Control, 2013a), *Candida* sp. was the 5th most common microorganism isolated from bloodstream infections (7.4% isolates).

Among invasive candidiasis, candidemia is estimated to account for 2–8 infections per 10,000 hospital discharges in recent studies from the United States and Europe (Alangaden, 2011). The true incidence of healthcare-associated candidemia is likely to be since approximately 50% of blood culture are negative (Alangaden, 2011).

Although *C. albicans* remains the most frequently isolated species, numerous studies have detected changes in the epidemiology of these infections, with a significant increase in the number of infections caused by non-*albicans* species, namely *C. tropicalis* and *C. parapsilosis* (complex), *C. glabrata* (complex), and *Pichia kudriavzevii* (former *C. krusei*) (Safdar *et al.*, 2001; Capoor *et al.*, 2005; Almirante *et al.*, 2005). Some studies mention that non-*albicans* species are the cause of two-thirds of candidemia cases (Posteraro *et al.*, 2015). One of the main reasons for this rising is the introduction of fluconazole prophylaxis/therapy, which led to the positive selection of azole-less susceptible/resistant species (Berkow and Lockhart, 2017). *Candida* sp. can be found temporarily or permanently in the gastrointestinal tract in about 40%–50% of the healthy adult population (Eggimann *et al.*, 2003) and in the genital tract of about 20% of healthy women (Sobel, 2005). Although *Candida* sp. does not constitute the normal flora of the skin, it may transiently colonize fingers or skin folds (Trofa *et al.*, 2008).

The longer length of hospital stay, the greater chance of *Candida* colonization, given the longer exposure to risk factors. A previous colonization by *Candida* spp. has been reported as an important risk factor for the development of systemic infection (Pittet *et al.*, 1994; Dalle *et al.*, 2008). Inanimate environmental surfaces and the colonized patients themselves may constitute a

reservoir from which other patients can acquire the fungus and, in some cases, develop candidemia (Hota, 2004; Stephan *et al.*, 2002). Systemic infection caused by *C. albicans* and *C. tropicalis* is usually preceded by colonization (endogenous transmission), while invasive disease caused by *C. parapsilosis* may occur without prior colonization and is often transmitted horizontally from contaminated external sources (exogenous transmission), such as infusions, parenteral feeding, catheters, prostheses, or hands of health professionals or visitors (Weems *et al.*, 1987; Raad and Bodey, 1992; Levin *et al.*, 1998; Lupetti *et al.*, 2002; Kan *et al.*, 2002; Deshpande, 2003; San-Miguel *et al.*, 2004; van Asbeck *et al.*, 2007; Trofa *et al.*, 2008).

Candida parapsilosisis is associated with approximately 25% of *Candida* infections in European hospitals, and it is now the second most commonly isolated Candida species from blood cultures in Europe, Canada, and Latin America (Trofa *et al.*, 2008; Sabino *et al.*, 2010a). This species is often described as responsible for nosocomial outbreaks and may be a frequent agent of candidemia. This species survives on surfaces for long periods of time (more than 14 days) and is able to form biofilms, allowing a high survival capacity in a hospital setting. Cell surface hydrophobicity has been associated with the initial adherence of *C. parapsilosis* to surfaces (Panagoda *et al.*, 2001), and the production of slime has been linked to the tendency of *C. parapsilosis* to adhere to the surfaces of intravascular plastic catheters and prosthetic devices (Raad and Bodey, 1992; Panagoda *et al.*, 2001). Adherence to tissues or medical devices precedes the biofilm formation, presumably resulting in a change in organism morphology and behavior (Trofa *et al.*, 2008). Biofilm-forming potential is cited as a reason that patients with *C. parapsilosis*–infected catheters should have the device removed (Barchiesi *et al.*, 2016) because the ability to grow as a biofilm is directly related to clinically significant disease.

For a better understanding of its epidemiology, several typing methods are used and microsatellite analysis has been reported as highly discriminant (Sabino *et al.*, 2010b; Vaz *et al.*, 2011; Reiss *et al.*, 2012; Diab-Elschahawi *et al.*, 2012; Romeo *et al.*, 2013; Barchiesi *et al.*, 2016). Analysis of *C. parapsilosis* isolates from hospital environment showed great genotypic diversity; however, the same or very similar genotypes were also found, suggesting that environment could be the source of some strains and that infections due to *C. parapsilosis* may be mainly related with exogenous transmission to the patient.

Recently, *C. auris* emerged as an etiological agent of candidemia. This species has been associated with hospital environments, being found in several different hospital surfaces, where it can survive for long periods. This species may be resistant to various classes of antifungals and is associated with high mortality in patients, given the limited treatment options (Chatterjee *et al.*, 2015). *C. auris* has a high propensity for patient-to-patient transmission, probably related to contamination of the hospital environment or transient colonization of medical devices, equipment, or people. *C. auris* can persist on surfaces in healthcare environments. *C. auris* has been cultured from multiple locations in patient rooms, including both high-touch surfaces, such as bedside tables and bedrails, and general environmental surfaces farther away from the patient, such as windowsills. *C. auris* has also been identified on mobile equipment that is shared between patients, such as glucometers, temperature probes, blood pressure cuffs, ultrasound machines, nursing carts, and crash carts (see "Relevant Websites section"). This multidrug-resistant species is rapidly spreading worldwide, with several described outbreaks. One of those was found to be linked to reusable axillary temperature probes (Eyre *et al.*, 2018). The role of healthcare workers in spreading *C. auris* is still unknown. However, during investigation of the outbreak in the United Kingdom, *C. auris* was isolated from the nares of a nurse who was providing care for a patient who was heavily colonized (Schelenz *et al.*, 2016). Moreover, an outbreak investigation in Colombia isolated *C. auris* from the hands of two healthcare workers and the groin of one out of six healthcare workers. Whole-genome sequencing (WGS) established that these were genetically identical to strains isolated from a patient and surround environment (Escandón *et al.*, 2019). It is a species of difficult identification since it can be confused with other yeasts species (*C. haemulonii*, *C. sake*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, or other non-*albicans Candida* species). Correct species identification is achieved through biochemical or mass spectrometry methods, whose devices already have their databases updated, that is, they already contain this species' profile. Confirmation of species identification should be done by sequencing specific DNA regions (as the D1/D2 region of ribosomal DNA) or specific polymerase chain reaction targeted to *C. auris*.

Difficulties with laboratory identification and lack of knowledge about this new species may result in potential dissemination of this microorganism and lack of awareness of outbreaks that may exist.

Given the highlighted questions raised with this new species, special recommendations regarding infection control measures in healthcare settings have been produced, such as (see "Relevant Websites section"):

- Adherence to hand hygiene.
- Appropriate use of transmission-based precautions based on setting.
- Application of all defined precautions for contact transmission.
- Considerations for single rooms and roommate pairings for people on contact precautions, which might be considered based on a single pathogen (as *C. auris*) without regard to cocolonizing organisms as a measure to control transmission during an acute outbreak.
- Cleaning and disinfecting the patient care environment (daily and terminal cleaning) and reusable equipment with recommended products and on a more frequent schedule.
- Follow all manufacturers' directions for use of surface disinfectants and applying the product for the correct contact time. Some products with fungicidal activity may not be effective against *C. auris*, and products solely dependent on quaternary ammonia compounds are not effective.
- Interfacility communication about patient's *C. auris* status when a patient is transferred to another healthcare facility, namely nursing homes.

- Screening contacts of newly identified case patients to identify *C. auris* colonization.
- Laboratory surveillance of clinical specimens to detect additional cases.
- Change protective equipment (if worn) and performing hand hygiene when moving between roommates.

Aspergillus

The filamentous fungi most frequently associated with hospital infections is *Aspergillus*, especially in patients from hematological and bone marrow transplant wards. *Aspergillus* conidia are very small and easily inhalable and can colonize the upper and lower respiratory tract (Walsh *et al.*, 2008).

Worldwide, it is estimated that over 30 million of patients are at risk of developing invasive aspergillosis due to the use of corticosteroids and/or other immunosuppressive therapies and that about ~250,000 persons will develop the disease annually. Diagnosis is difficult and often too late. The associated mortality is 50% if the infection is treated and >99% if not treated (see "Relevant Websites section"). The most frequently *Aspergillus* sections isolated from clinical settings are *Fumigati*, *Flavi*, *Nigri*, and *Nidulantes*, which are also the most frequent in the environment (Warris *et al.*, 2001). Invasive aspergillosis generally involves inhalation of conidia or hyphae and further growth in human internal milieu. Vonberg and Gastmeier (2006) reviewed all cases of invasive aspergillosis and concluded that *Aspergillus* is able to cause disease in environments with less than 1 colony-forming unit m^{-3} of air. They recommended that at-risk patients should not be exposed to *Aspergillus* and concluded that prevention from all routes is critical (see "Relevant Websites section").

A significant association was found between *Aspergillus* isolates of the patient's room and *Aspergillus* isolates colonizing the respiratory tract of lung transplant recipients (Bonnal *et al.*, 2015), emphasizing the environmental risk of contamination within hospital wards.

One key concern is the risk of aspergillosis associated with hospital construction. In a previous review, construction work accounted for about half (49%) of the causes of hospital-acquired aspergillosis outbreaks (Vonberg and Gastmeier, 2006). Accordingly, there have been many efforts to control environmental fungal contamination using appropriate ventilation systems, such as high-efficiency particulate air filters (Sehulster *et al.*, 2004; Benet *et al.*, 2007; Kanamori *et al.*, 2015). Routine air sampling is not recommended; however, where major works are to be undertaken it may be useful to establish baseline levels of *Aspergillus* in the air and continue to monitor during construction work to detect increased counts, which should prompt additional preventive measures (Sehulster *et al.*, 2004).

Infection control practitioners must consider some key factors when addressing land excavation and building demolition. To minimize the risk of fungal infection and contamination of the healthcare environment during the demolition, excavation, and construction phases of the build, infection control risk assessments must be undertaken prior to commencement and as needed during the build. Such risk assessments document the type of build, the risk category in relation to patients and departments, and the class of barriers required throughout each phase of the works. Major construction and/or renovation activities require the establishment of a multidisciplinary team including but not limited to the healthcare facility major projects manager, hospitals engineer, infection prevention and control, clinical and department managers, environmental services manager, and architects and contracted building supervisors (Harrington, 2016).

Traditionally, clinical microbiology laboratories have relied heavily on morphology-based identification methods to differentiate *Aspergillus* species. This allowed several genetically distinct species to be misidentified (Balajee *et al.*, 2007, 2009). In fact, many species share morphological features and can only be identified by sequencing specific genes, which has led to the clustering of species with overlapping morphologies into sections.

Thus, with the development of molecular tools, the number of *Aspergillus* species involved in human infections is continuously increasing.

Aspergillus Fumigati section, composed of *A. fumigatus* sensu stricto and its cryptic species, is the most frequent isolated *Aspergillus* section in clinical products and is also often isolated from environmental sources. Antifungal resistance in *A. fumigatus* is emerging (European Centre for Disease Prevention and Control, 2013b) and reduced susceptibility is associated with higher mortality (Verweij *et al.*, 2016). In *A. fumigatus*, *cyp51A* gene encodes lanosterol 14-α-sterol demethylase, which is required for the biosynthesis of ergosterol, an essential component of the fungal cell membrane and mutations in this gene and its promoter are in the basis of the triazole resistance. Numerous mutations in *cyp51A* have been reported that confer resistance to triazoles *in vitro*.

Reasons for the increased resistance include the use of prophylactic antifungals/treatment with azoles, and the use of agricultural azoles (Verweij *et al.*, 2016). The molecular basis of resistance to triazoles in *A. fumigatus* mainly involves the occurrence of non-synonymous mutations in the *cyp51A* gene that cause structural alterations due to amino acid substitutions and are sufficient to induce resistance to some or all triazole drugs. These resistant mutations often evolve during prolonged azole treatment in patients with chronic forms of aspergillosis (Hagiwara *et al.*, 2016). On the other hand, other mutations are linked to environmental transmission; the environmentally driven polymorphism TR34/L98H consists of a tandem repeat (TR) of 34 bases in the promoter of the *cyp51A* gene and a leucine-to-histidine change at codon 98 (Mellado *et al.*, 2007); this polymorphism is globally widespread in environmental and clinical isolates (Chowdhary *et al.*, 2015, 2017). Another *cyp51A* -mediated resistance alteration that leads to high-level voriconazole resistance, TR46/Y121F/T289A, has also been described in *A. fumigatus* (van der Linden *et al.*, 2013).

The inhalation of antifungal-resistant isolates of environmental origin is currently a major concern of the scientific community (Chowdhary *et al.*, 2015, 2017). Resistance to triazoles may severely limit treatment options and has been associated with poor

prognosis regarding the course of the infection (Snelders *et al.*, 2008; Verweij *et al.*, 2009). In an environmental screening performed by Godeau *et al.* (2019), 83 azole-resistant *A. fumigatus* isolates with a TR34/L98H mutation were obtained from the indoor air, soil, and dust samples of a French hospital. Some studies also suggest differences in virulence of environmental isolates, so appropriate environmental control practices are important for the prevention of nosocomial infections caused by *Aspergillus*. The European Centre for Disease Prevention and Control (2013b) recommended to improve epidemiological surveillance, to develop molecular methods to detect triazole resistance, and to investigate the environmental origin of *A. fumigatus* resistance through environmental and laboratory studies.

In a study performed by Sabino *et al.* (2014), during a one-year period, in four seasonal samplings, air and surfaces samples were collected from the Hematology, Oncology, and ICU wards of a Portuguese Central Hospital. *Aspergillus* was the most frequently recovered fungal genus, followed by *Cladosporium* and *Penicillium*. Antifungal-resistant isolates were also detected, especially cryptic species belonging to the genera *Aspergillus*. In comparison with *Aspergillus* isolates collected from other sources, a significant association was found between different environmental sources and the distribution of different sections, with hospital environment showing greater variability (Sabino *et al.*, 2014). Among the 75 *Aspergillus* isolates from hospital environment, 10 different sections were detected and 25 different species were identified by β-tubulin and calmodulin sequencing, including a high percentage of cryptic species (i.e., not sensu stricto) (59%) (Sabino *et al.*, 2016). Although aspergillosis caused by cryptic species remain less frequent than infections caused by "sensu stricto" isolates, the eventual high prevalence of cryptic species in hospital environment may change the "regular" antifungal susceptibility profile, increasing the number of resistant strains, which implies a potential danger, especially in wards with immunocompromised patients.

Because of the high mortality rate associated with invasive aspergillosis in vulnerable patients, it is essential to minimize the risks associated to environmental exposure and to understand the biodiversity and concentration of cryptic *Aspergillus* species in air and aerosols, such as those from hospital plumbing. Knowledge of the resistance patterns of *Aspergillus* isolates is important to assess changes in the epidemiology of the organisms studied and to evaluate new therapeutic options.

Mucorales

Mucormycosis is a life-threatening infection by fungi of the order Mucorales, which is associated with mortality rates that exceed 50% (Roden *et al.*, 2005). The agents of mucormycosis are ubiquitous in soil and decaying vegetation, and infection may be acquired by inhalation, ingestion, or contamination of wounds with sporangiospores from the environment (Gonzalez *et al.*, 2002). As with *Aspergillus* species, nosocomial spread of Mucorales may occur by way of air-conditioning systems, particularly during construction (Gonzalez *et al.*, 2002; Roden *et al.*, 2005; Spellberg *et al.*, 2005). According to a review performed by Walther *et al.* (2019), the most common source of outbreaks was contaminated medical devices that are responsible for 40.7% of the outbreaks followed by contaminated air (31.3%). The most prevalent species were *Rhizopus arrhizus* and *R. microsporus* causing 57% of the outbreaks. The genus *Rhizomucor* was dominating in outbreaks related to contaminated air while outbreaks of *Lichtheimia* species and *Mucor circinelloides* were transmitted by direct contact. Outbreaks with the involvement of several species were also reported.

Focal outbreaks of Mucorales have also been associated with the use of contaminated adhesive bandages or tape in surgical wound dressings, resulting in primary cutaneous mucormycosis (Roden *et al.*, 2005; Spellberg *et al.*, 2005). In addition to causing infection in immunocompromised patients, the Mucorales may also cause lethal infections in a broader and more heterogeneous population, such as patients with diabetes, patients receiving deferoxamine therapy, injection drug users, and patients with no apparent immune impairment (Gonzalez *et al.*, 2002). Invasive mucormycosis is clinically similar to aspergillosis and is marked by angioinvasion and tissue infarction (Spellberg *et al.*, 2005). Person-to-person transmission cannot be excluded because Mucorales can sporulate on wounds (Walther *et al.*, 2019).

Nosocomial infections caused by Mucorales have been reviewed (Roden *et al.*, 2005). Clusters of cutaneous infections have occurred in orthopedic and cardiothoracic patients, children with leukemia, and burn patients. These infections were associated with adhesive dressings possibly contaminated with *Rhizopus* and *Lichtheimia* spp. (Gonzalez *et al.*, 2002).

Fusarium

Nosocomial infections caused by members of the genus *Fusarium* have emerged over the past two decades (Pfaller and Diekema, 2010). In the immunocompromised patients, especially those with hematologic malignancies and recipients of allogeneic hematopoietic stem cell transplantation, *Fusarium* can disseminate in the organism causing a systemic and invasive infection (fusariosis) (Nucci and Anaissie, 2007; Pfaller and Diekema, 2010; Lass-Florl and Cuenca-Estrella, 2017). *Fusarium solani* (complex of species) is the most frequent species involved in fusariosis (50% of cases), followed by *Fusarium oxysporum* (complex of species) (20%), *Fusarium verticillioides* (complex of species), and *Fusarium moniliforme* (complex of species) (10% each). *Fusarium* spp. may show an antifungal susceptibility profile marked by high level of resistance; however, some isolates can be susceptible in vitro to amphotericin B, voriconazole (Alastruey-Izquierdo *et al.*, 2008; Al-Hatmi *et al.*, 2015), notwithstanding, the mortality rate of disseminated fusariosis may exceed 75%.

Most infections are believed to be caused by airborne transmission (Moretti *et al.*, 2018); however, contamination of the water system in the hospital has been reported to result in dispersal of airborne conidia (Anaissie *et al.*, 2001). Water distribution systems in hospitals were identified as potential reservoirs for species of Fusarium, where they are thought to be responsible for nosocomial infections (Anaissie *et al.*, 2001; Litvinov *et al.*, 2015). *Fusarium* conidia in water may be aerosolized into the air when the water passes through taps and showers and then inhaled by immunocompromised patients (Anaissie *et al.*, 2001). Since they may be aerosolized into air and inhaled by patients, it is essential to implement prophylactic measures in hospitals to control invasive fusariosis by using filters on taps and showers. DNA sequencing has demonstrated evidence of widespread distribution of clones that were similar to isolates recovered from nosocomial infections in patients (Moretti *et al.*, 2018).

Other Fungi Associated With Hospital Infections

Outbreaks caused by *Scedosporium* spp. have been reported in patients with leukemia undergoing chemotherapy (Alvarez *et al.*, 1995; Ruiz-Diez *et al.*, 1997; Guerrero *et al.*, 2001). Recently, after molecular distinction among species and taxonomic differentiation (Ramirez-Garcia *et al.*, 2018), it was concluded that almost half of the cases of *Scedosporium* spp. infections occurred in immunocompetent hosts, whereas 70% of *Lomentospora prolificans* infections occurred in patients with immunocompromising conditions, mostly malignancy or solid organ transplantation (Seidel *et al.*, 2019). *Lomentospora prolificans* isolates were pan-resistant to virtually all systemically active antifungals, including azoles, terbinafine, and amphotericin B (Seidel *et al.*, 2019).

Paecilomyces and *Purpureocillium* species are of clinical interest because of their pathogenicity and resistance to antifungal agents. *Paecilomyces variotii* is a commonly occurring species in air and food, and it is also associated with many types of human infections, such as fungemia, endocarditis, peritonitis, and osteomyelitis (Williamson *et al.*, 1992; Cohen-Abbo and Edwards, 1995; Chan *et al.*, 1996; Kovac *et al.*, 1998).

In a study performed by Olm *et al.* (2019), *Purpureocillium lilacinum* genomes from infant and neonatal intensive care unit environmental samples shared 99.999% average nucleotide identity, highlighting the potential of hospital-associated fungi to colonize hospitalized infants.

A study performed by Nucci *et al.* (2002), documents the occurrence of nosocomial *Exophiala jeanselmei* fungemia in high-risk patients and confirms the potential role of contaminated water in nosocomial fungal infection. Given the prevalence of *Exophiala* spp. in cystic fibrosis patients' lungs (Grenouillet *et al.*, 2018), the possible transmission of this genus from the hospital environment may be a risk, especially to these patients.

Novel Methods and Future Perspectives

To identify the causative airborne microorganisms, high-volume air samplers have been recently used for collection, and species identification is starting to be performed using a culture-based method but with DNA sequencing analysis as well with the Illumina MiSeq and HiSeq 2000 sequencing systems (WGS) (Tong *et al.*, 2017).

The study of a hospital's metagenome can allow physicians, researchers, and other medical staff a full knowledge of the microbial communities present inside medical wards. This is expected to give information on the presence of certain fungi in highly restricted areas where critical patients are admitted, and therefore to have a considerable impact on public health. Metagenomic strategies are not only an important tool for characterization of the genetic diversity in whole communities, but also represent an indispensable approach for detection of rare fungal taxa (by revealing new Operational taxonomic unit) (see "Relevant Websites section").

For WGS to become applicable for real-time investigations, time and cost of generating and analyzing WGS data need to be reduced. WGS methods are already widely used for investigating bacterial and viral outbreaks. However, most fungal genomes are at least 10 times larger than those of bacteria and viruses; therefore, considerably more time and resources are needed to generate and process fungal genomes. Development of a curated public database containing assembled genomes of major fungal pathogens will significantly accelerate analyses and implementation of WGS into public health by providing reference genomes and control strains for assessing genetic diversity in a population as well as facilitate data sharing among institutions (Litvintseva *et al.*, 2015).

Final Remarks

Fungal infections remain serious and underappreciated causes of illness and death. Much can be done to prevent the consequences of these infections, although environmental exposure to these agents may not be entirely avoidable in the community. Continued public health efforts toward defining, characterizing, and tracking the emergence of fungal infections can help to focus studies on priority infections and settings (Brandt and Park, 2013).

When we focus on fungal contamination in healthcare facilities, we have to think not only of the hospital environment but also of other healthcare facilities, such as nursing homes. In these cases, it is important to emphasize that prevention measures of

healthcare associated fungal infections should be implemented in the same as or similar conditions to in the hospital, to avoid microorganism transmission and dissemination.

Descriptive epidemiology (i.e., detailed assessment of patients' demographic characteristics, clinical histories, and the geographic and temporal distribution of cases) is essential for generating hypotheses about the potential source of infection. For example, cases occurring over several weeks to months and scattered geographically suggest a common source outbreak that involves a widely distributed product, especially when case patients have undergone similar medical treatments (Smith *et al.*, 2013; Mikosz *et al.*, 2014).

To reduce fungal infections in hospital settings, antimicrobial stewardship programs should promote the appropriate use of antimicrobials, leading to better patient outcomes and a reduction in the spread of infections caused by multidrug-resistant fungi. These programs can help educate medical professionals about best practices for continuity of care, following the patient from the inpatient to the outpatient setting. Prevention and infection control strategies are equally important. These would include participating in and leading efforts within the hospital setting to improve antifungal prescribing practices, ensuring that the right antifungal is given to the right patient at the right time. Fungal infections should be a more visible part of the larger national campaign to reduce hospital-acquired and drug-resistant infections. Inpatient infections continue to cause high percentages of avoidable deaths and contribute to waste and high healthcare costs.

(see "Relevant Websites section").

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Further Reading

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Elderly Exposure to Fungi: A Review Study

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Introduction

Indoor air quality is an increasing concern since people spend 90% of their time indoor. The majority of exposure to air pollution happens indoors rather than outdoors, despite the focus being on the latter. Indoor air quality in elderly care centers (ECC) is an emerging important issue arising in the last decade because of the increase of (1) life expectancy, (2) number of individuals residing in ECC, and (3) population aging, which is correlated with the inversion of the age pyramid (United Nations UN, 2012; GEP/MSSS, 2010). However, given the magnitude of the ECC population that is ageing, and the considerable amount of time spent by elders within ECC, information linking contamination of ECC by particles, micro-organisms and exposure of elders continues to be sparse (Almeida-Silva *et al.*, 2014a,b). Thus, societies must provide environments that guaranty the wellbeing of vulnerable groups like the elderly.

The older population is growing faster than the total population in practically all regions of the world – and the difference in growth rates is increasing (United Nations UN, 2012). Since 1996 until 2008 the number of adults aged >65 years increased 31% (from 380 million to 500 million). According to the United Nations UN (2013) the percentage of total population aged 60 years or over in the world was 11% for the year 2010 and is estimated to be 18% for 2050. Although indoor concentration and number of carcinogenic air pollutants has been decreasing since the 1950s (Weschler, 2009), all of the previously evidences, plus the changes on life-style and the fact that people spend a large part of their life inside indoor environments have promoted an increase on exposure to indoor air pollutants (Byčenkienė *et al.*, 2009; Zhao *et al.*, 2009; Dales *et al.*, 2008; Leech *et al.*, 2002; Klepeis *et al.*, 2001). Consequently, we are witnessing an intensification of studies developed by the scientific community concerning Indoor Air Quality (IAQ) and its effects upon health (Canha *et al.*, 2012a,b; Franck *et al.*, 2011; World Health Organization WHO, 2010; Saliba *et al.*, 2009; Fraga *et al.*, 2008; Fromme *et al.*, 2007; Kosonen and Tan, 2004; Lee *et al.*, 2002; Allen and Miguel, 1995). Indoor air pollution is caused by a combination of several factors: hazardous substances that are emitted from the outdoors, buildings, construction materials, furnishings, equipment, inadequate ventilation, indoor human activities, etc. (Almeida-Silva *et al.*, 2015; Canha *et al.*, 2013; Viegas *et al.*, 2010; Weschler, 2009). Physical factors such as air temperature, air velocity and relative humidity are usually used as indicators of thermal comfort, in IAQ studies. The main chemical and biological parameters used to characterize the IAQ are carbon monoxide (CO) and dioxide (CO_2), the volatile organic compounds (VOC), the formaldehyde (H_2CO), the ozone (O_3), the particulate matter (PM), fungi and bacteria. Several studies positively correlated indoor exposure to microorganisms and microbial components with adverse health effects including headache and respiratory symptoms (Douwes *et al.*, 2003). Considering specifically fungi, their spores are complex agents that may contain multiple hazardous components. Health hazards may differ across species because fungi may produce different allergens and mycotoxins. Moreover, some species also infect humans (Eduard and Halstensen, 2009). Most infections occur in immunocompromised hosts or as a secondary infection, following inhalation of fungal spores or the toxins produced by them (Srikanth *et al.*, 2008). It is known that some people are sensitive to molds and exhibit symptoms such as dry nose, wheezing, and red or itchy eyes or skin when exposed to molds. Severe reactions have also been observed among workers exposed to large amounts of molds in occupational settings including fever and shortness of breath. Exposure to mold or dampness may also lead to development of asthma in some individuals (Centers for Disease Control and Prevention CDC, 2017).

Methodology

In order to identify the scientific publications to be included in this review and analysis, three search engines were used: Web of Science (WoS), Scopus, and Online Knowledge Library (b-on). This strategy allowed to access to the largest number of existing publications under this topic. These search engines were selected due to its interdisciplinary nature, in order to cover several disciplines – from mycology to environmental science.

The search methodology was developed under three steps.

First Step

The follow keywords were selected and search in all selected search engines:

A. "elderly" + "indoor" + "fung*".
B. "old*" + "indoor" + "fung*".
C. "old*" + "indoor air" + "fung*".

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Second Step

After the first approach a list of inclusion and exclusion criteria was created, in order to do a deep and refined selection of the retrieved publications. Inclusive criteria: publications that studied indoor environments related to elderly people; publications in English and Portuguese. Exclusive criteria: publications before the year 2000; publications in duplicate; the follow keywords – "child*", "infant*" and "work". **Table 1** presents the number of publications under the study in first and second methodological steps.

Third Step

A database with all selected articles was elaborated. During this process all duplicate publications were erased, as well as publications that didn't present any quantitative or qualitative data regarding fungi concentrations in elderly homes. The studies included in this integrative review were selected based on their content. In terms of content, each publication selected for this literature review included results based on empirical data on the relation between fungi concentrations assessed indoors and elderly or old people. Considering the purpose of this study, the possible influence of sociodemographic variables was not considered in this chapter, since it was considered irrelevant.

At the end, 11 publications (**Table 2**) were compiled to be discussed in the next sub-article.

Results and Discussion

Table 2 summarizes the selected publications after the application of the three methodological steps described previously.

The temporal distribution of the 11 publications identified according to the stated criteria was not uniformed. A total of 9% covered the period between 2005 and 2010; 27% represented the period between 2010 and 2015; 64% were from the period between 2015 and 2019. The largest proportion of studies identified in recent years, or in other words since 2015, may be due to the fact that scientific studies about indoor air pollutants in elderly care centers only effectively began in the second half of the 20th century, with scientific production on this theme intensifying from this period onwards.

The selected publications are from Europe (73%) and Asia (27%). **Fig. 1** represents the general characterization of the selected publications according to the country in study. It is possible to observe that Portugal shows the highest percentage (55%) of developed works on the studied field, followed by Asiatic Continent (Iran, Korea and China). This shows a Portuguese great concern about this topic and goes in line with the European society' evolution: (1) in 2014 the Portuguese ageing rate was higher that the European average, with 21% of the population aged 65 or over (Eurostat, 2014); (2) according to Eurostat, Portugal will have the oldest European

Table 1 Number of publications identified according to each keywords' group, search engine and inclusion and exclusion criteria

Keywords	*A*		*B*		*C*	
Methodological Step	*1*	*2*	*1*	*2*	*1*	*2*
WoS	46	17	313[a]	–	212	19
Scopus	15	9	111	14	57	9
b-On	22	11	96	15	47	10

[a]Due to the big amount of generated data and the heterogeneity of the results this keywords' group for WoS was rejected.

Table 2 Selected publications after the three methodological steps

Title	*Year*	*Ref.*
Airborne microbiological characteristics in public buildings of Korea	2007	(Kim and Kim, 2007)
Biological air contamination in elderly care centers	2014	(Aguiar *et al.*, 2014)
Fungal Contamination Assessment in Portuguese Elderly Care Centers	2014	(Viegas *et al.*, 2014)
Indoor air quality and thermal comfort in elderly care centers	2015	(Mendes *et al.*, 2015)
Indoor and outdoor exposure to ultrafine, fine and microbiologically derived particulate matter related to cardiovascular and respiratory effects in a panel of elderly urban citizens	2015	(Karottki *et al.*, 2015)
"*Avaliação da qualidade do ar interior em lares de idosos, 2013–2014: projeto GERIA*" (Portuguese)	2016	(Cano *et al.*, 2016)
The impact of indoor air quality and contaminants on respiratory health of older people living in long-term care residences in Porto	2016	(Mendes *et al.*, 2016)
Airborne fungal diversity inside a nursing home in Edirne, Turkey	2017	(Yilmaz *et al.*, 2017)
Bioaerosol exposure and circulating biomarkers in a panel of elderly subjects and healthy young adults	2017	(Faridi *et al.*, 2017)
Indoor environmental conditions in urban and rural homes with older people during heating season: a case in cold region, China	2018	(Fan *et al.*, 2018)
"*Qualidade do ar interior em ambiente geriátrico no Nordeste de Portugal*" (Portuguese)	2018	(Madacussengua *et al.*, 2019)

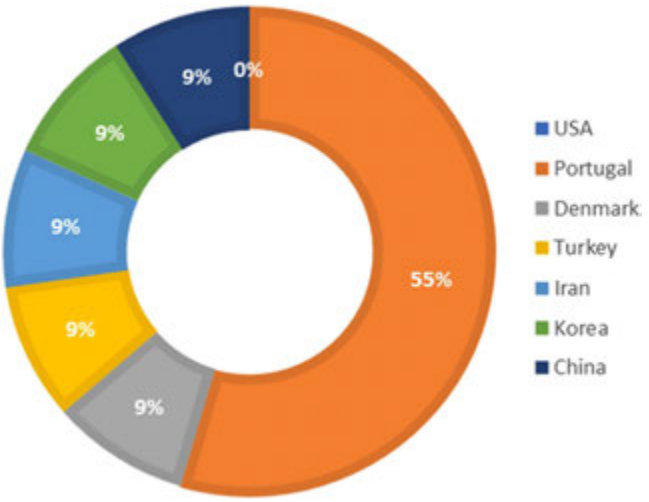

Fig. 1 Publications distributed according to its localization.

Table 3 Detailed information regarding the applied methodology

Ref.	*Indoor*	*Outdoor*	*Equipment*	*Agar*	*Incubation conditions (duration (days); temperature (°C))*
(Kim and Kim, 2007)	x	x	Six-stage cascade impactor (Andersen, Model 10–800)	MEA with chloramphenicol	3–5d; 20–25°C
(Aguiar *et al.*, 2014)	x	x	Merck Air Sampler MAS-100	MEA	3d; 25°C
(Viegas *et al.*, 2014)	x	x	Merck Air Sampler MAS-100	MEA with chloramphenicol	5–7d; 27°C ± 2°C
(Mendes *et al.*, 2015)	x	x	Merck Air Sampler MAS-100	MEA	25°C
(Karottki *et al.*, 2015)	x	–	Electrostatic Dust Cloths (for 15 days)	DG-18	3–7d; 25°C
(Cano *et al.*, 2016)	x	x	Merck Air Sampler MAS-100	MEA	4–5d; 25°C
(Mendes *et al.*, 2016)	x	x	n/d	n/d	n/d
(Yilmaz *et al.*, 2017)	x	x	Merck Millipore M Air T	RBCA	7d; 25°C
(Faridi *et al.*, 2017)	x	x	QuickTake® 30	SDA with chloramphenicol	3–7d; 20–28°C
(Fan *et al.*, 2018)	x	-	Adherent material applied in floor surfaces	DG-18	5d; 25°C
(Madacussengua *et al.*, 2019)	x	x	Surface Air System	RBCA	3–5d; 25°C

Note: n/d: Not described.

population in 2050, with almost 50% of the population with more than 55 years old. The justification is given by a historical drop in fertility rates, increased average life expectancy and, in some cases, migration patterns (Eurostat, 2019) (see Figure SI 1).

Regarding the methodology of each study it was possible to observe (**Table 3**) that 63% (n = 7) of the selected works used impaction as sampling method, either using the Merck Air Sampler MAS-100 (n = 4), the Six-stage cascade impactor (Andersen, Model 10–800) (n = 1), the Merck Millipore M Air T (n = 1), or the Surface Air System air sampler (n = 1) (**Table 2**). Of those, 5 used malt extract agar (MEA) (two with chloramphenicol added) and 2 Rose-Bengal Chloramphenicol Agar (RBCA). Faridi *et al.* (2017) used the QuickTake® 30 as air sampling device, with a spore trap cassette system, and Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol as culture medium. Other two studies used a different methodology, based on passive methods: adherent material applied in floor surfaces (Fan *et al.*, 2018); and Electrostatic Dust Cloths (Karottki *et al.*, 2015) using Dichloran Glycerol Agar (DG-18) as culture medium. The applied methodologies regarding temperature and incubation time are not equal through the selected works. For the impaction-based sampling studies, three (Madacussengua *et al.*, 2019; Kim and Kim, 2007; Aguiar *et al.*, 2014) describe similar conditions, with incubation periods from 3 to 5 days in a temperature between 20–25°C. A slight difference could be observed in Cano *et al.* (2016) and Fan *et al.* (2018) with a duration and temperature of incubation around 4–5 days and 25°C, respectively. The other studies, Yilmaz *et al.* (2017), Faridi *et al.* (2017), Karottki *et al.* (2015) and Viegas *et al.* (2014), applied more incubation time for its samples: 5–7 days, and incubation temperatures between 25°C and 29°C. Mendes *et al.* (2015) only presented the temperature of incubation, 25°C.

Although the number of publications is scarce, it is interesting to notice the small divergence of reported results across Europe and Asia: median concentrations (considering all studies) were 350 CFU m^{-3} and 406 CFU m^{-3} respectively, and the most frequently found genera were *Cladosporium* sp., *Penicillium* sp. and *Aspergillus* sp. regardless the region. Knowing that weather

conditions and geography-related vegetation (Jędryczka, 2014; Núñez *et al.*, 2016) are the most accountable factors contributing to airborne fungi diversity, a wider heterogeneity in the results was expected.

Overall, the main findings reported in these publications were:

(1) Fungi species found in indoor air were similar to the ones found outdoor;
(2) *Penicillium sp.*, *Aspergillus sp.* and *Cladosporium sp.* were the dominant genera of fungi both indoor and outdoor;
(3) Levels of fungi indoor were weakly but significantly correlated with the levels of PM2.5;
(4) Median concentration levels of indoor fungi exceed the median outdoor levels during winter season;
(5) Fungi median indoor concentrations were slightly above references in winter season, and peak values in both seasons (winter and summer) were concerning;
(6) Significant positive correlations were found between temperature and *Aspergillus sp.*, *Cladosporium sp.* and *Alternaria sp.* levels;
(7) Correlation between circulating biomarkers, especially IL-6 and white blood cells counts, and fungal aerosols exposure among elderly subjects was found;
(8) Fungi levels in rural houses were higher than in urban houses.

No Health Authority or equivalent organization has already set specific limits for indoor air pollutants' levels regarding environments harboring particular groups of individuals, as children, the elderly, patients, and other immunologically vulnerable individuals. In addition, for fungi, the only existing regulation for indoor levels is specifically set for indoor environments as a measure of good indoor air quality, yet the values vary greatly between countries/entities. For instance: the European Confederation Commission recommends levels of fungi under 2000 CFU m^{-3}; the World Health Organization (World Health Organization WHO, 2009), under 500 CFU m^{-3}; the American Industrial Hygiene Association (AIHA) under 1000 CFU m^{-3} (EL-Morsy, 2006); the Turkish Standards Institute under 1000 CFU m^{-3} [TS 12281/Nisan 1997] (EL-Morsy, 2006); the Portuguese government sets the limit of indoor air fungi level at the level of concomitant outdoor air fungi level (Ordinance no 353-A/2013).

The levels of fungi found for elderly care centers varied between 5 CFU m^{-3} and 1343 CFU m^{-3} among the studies in review. Depending on the location, these levels could or not be acceptable. If the indoor concentrations of airborne fungi are higher that the outdoor concentration, the presence of indoor generative sources may be suspected. Nevertheless, from a health perspective the risk that fungi exposure poses is much more related to the susceptibility of the individuals rather than to general limits. As observed in one of the publications a correlation between circulating biomarkers of inflammation – IL-6 and white blood cells count – and fungi exposure could be established for a population of older people. Among older adults, asthma-related hospitalizations have already been associated with the presence of mold in the home environment. (Hsu *et al.*, 2018). These data are suggestive that much more studies are needed to assess the influence of fungi in indoor air and older-people's health.

Conclusion

The growing number of aged populations is a consequence, among others, of the better health conditions that medical progress has enabled. These populations, despite their longevity, may present new challenges has their immune system, resistance, past diseases, and overall frailty may predispose them differently to otherwise non-threatening substances and contaminants. This still growing new reality coupled with changes in lifestyles and environment makes it a priority to fully assess the risk that indoor air contaminants exposure poses to these populations. Establishing safe exposure limits specifically considering these populations needs would result in improved health and quality of life, and a significant reduction in costs with healthcare.

Appendix A Supplementary Material

Supplementary data associated with this chapter can be found in the online version at doi:10.1016/B978-0-12-809633-8.21036-4.

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Integrating Fungi in the Drinking Water Regulation and in Guidelines for Materials in Contact With Drinking Water. Is there Room for Change?

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Introduction

Water is one of the essential natural resources supporting life on Earth. In times of rapid changes in the atmosphere, and increasing global warming, humanity is facing the ongoing environmental crisis. This can be seen by melting glaciers, long periods of drought and on the other side severe weather events, like hurricanes and floods (Handmer *et al.*, 2012). Meanwhile, the urban environment is expanding in many areas, and affecting surface and underground water sources due to the anthropogenic factors, such as construction work, water flow remediation and pollution (Cheremisinoff, 1997). Care for safe drinking water becomes now more than ever, one of the humanity's main present and future goals (Novak Babič *et al.*, 2017). Free access to safe drinking water must be ensured for everyone, as stated by the United Nations Human Rights Council in the Resolution 64/292 (UN, 2010). Yet, due to the above listed natural and anthropogenic factors, not every fresh water is safe for use in terms of being free of chemicals and microorganisms affecting health (WHO, 2011). Daily delivery of fresh and safe drinking water is still a challenge in many countries worldwide. World Health Organization (WHO) regularly updates its guidelines with recommendations on methods for water cleaning, disinfection and transport, together with the most problematic chemical- and microbiological agents affecting human health (WHO, 2011). The final decision on the hazardous agents, included in directives, and the application of water cleaning methods is responsibility of each Member-state and national team, who possess the on-the-field situation knowledge, regarding the water chemical and microbiological suitability (EEC, 1998). Although many microorganisms, particularly the ones associated with gastro-intestinal illnesses, are already part of the regular monitoring, fungi remain excluded. However, their presence in water is known for a long time. It is frequently associated to "moldy" odor, and to changes in taste and color of water (Department for Environment, Food & Rural Affairs, 2011; Novak Babič *et al.*, 2017, 2018). Also fungi as part of microbial biofilms have been recognized in different lines of industry using tap water for their operations, often causing clogging of small-diameter pipes, filters and grids (Douterelo *et al.*, 2016; Liu *et al.*, 2017). Species of the genera *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Stachybotrys* and *Trichoderma* were commonly related to biodegradation of materials used for water transport, either metal or concrete (Andersen *et al.*, 2011; Hurtado-McCormick *et al.*, 2016). Yet, the choice of used material in water producing companies has no unified legislative approach in terms of microbiological parameters and depends on in-country authorities and industrial financial budget (4MS, 2011). However, building materials in contact with drinking water have a significant effect on colonization and later proliferation of fungi, establishment of biofilms and production of fungal secondary metabolites (Department for Environment, Food & Rural Affairs, 2011; Novak Babič *et al.*, 2017). Fungal particles and their metabolites may affect human health when released with drinking water via direct contact or aerosolisation. Health implications due to fungal presence and possible proliferation within drinking water distribution systems already raised concerns in the past (Novak Babič *et al.*, 2017). In times of prolonged human life span and raising occurrence of antimicrobial resistance, fungi became studied as potential infectious agents with occasional, but expanding anti-mycotic resistance (Chiller, 2016). The latter is particularly worrisome for the genera *Aspergillus*, *Candida* and *Fusarium*, due to their opportunistic nature, speed of resistance emergence and the ubiquitous presence in nature (Rivero-Menendez *et al.*, 2016; De Hoog *et al.*, 2019).

In terms of broadening the knowledge on fungi in drinking water the present article enlightens the current legislative background and the main future concerns regarding the inclusion of fungal parameters in water directives. Additionally, we present the European on-the-field situation on fungi in drinking water, pointing out the most common genera with the major effect on materials in drinking water transport network and effect on human health.

Why are Fungi in Drinking Water a Present and a Future Legislation Problem?

Regulation and Legislation

Drinking water is a necessity and through history humanity learned that poor water quality significantly affects health and may be even deadly (Gray, 2014). To date, the World Health Organization (WHO) recommended monitoring of many different species of protozoa, bacteria and viruses (WHO, 2011). Protozoa include *Acanthamoeba* spp., *Cryptosporidium hominis*, *C. parvum*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia intestinalis*, and *Naegleria fowleri*. Drinking water should be monitored for bacterial species *Burkholderia pseudomallei*, *Campylobacter jejuni*, *C. coli*, *Escherichia coli* and enterohaemorrhagic *E. coli*, *Francisella tularensis*, *Legionella* spp., *Leptospira* spp., *Mycobacteria* (nontuberculous), *Salmonella typhi* and other salmonellae, *Shigella* spp., *Vibrio cholerae*, and cyanobacteria. Also, Coliphages, Adenoviruses, Astroviruses, Enteroviruses, Hepatitis A, Hepatitis E, Noroviruses, Rotaviruses,

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and Sapoviruses are in the WHO recommendation list. Many more are on the "suggested" list (WHO, 2011). Microbial species selected for monitoring by each country may vary due to the geographical positions, related climate factors (e.g., temperature and relative humidity) and the potential for the presence of specific taxa of microorganisms in drinking water (Percival *et al.*, 2014). However, there is a void in the WHO recommendation for algae and fungi. Recommendations only state that any algae causing bad taste or odor, and toxins producers should be absent, while "*Fungi causing bad taste and odor should be minimized*" (WHO, 2011). Therefore, European Member-states and other countries do not list any specific fungal taxa among parameters for water monitoring (EEC, 1998; US EPA, 2016). There is nonetheless a modest fungal contribution within the heterotrophies plate count, which indicates changes in microbial concentration, like ingress or regrowth in water distribution systems (WHO, 2003), but with no specific regulatory value since the compliance is defined as "*no abnormal change*" (Novak Babič *et al.*, 2017). Despite this framework of regulatory void for fungi in drinking water, a few countries decided, nonetheless, to include fungi as a parameter for drinking water quality. Within the European Union (EU) the Czech Republic requires microscopic observation of all microorganisms in concentrated drinking water samples taken at consumers' taps with a parametric value of 50 individuals per milliliter, including filaments and spores (Ministry of Health, 2004). Hungary uses similar approach, with the difference of parametric values being set separately for each group of organisms. The value for fungi at end-point users is 0 individuals per 1 l (Ministry of Health, 2001). The only legislation on fungi in drinking water to date requiring the direct detection of fungi by culture dependent method is the Swedish legislation. Their parametric value for "microfungi" (including molds and yeasts) is set to 100 colony forming units CFU per 100 mL (NFA, 2001). Many reasons contribute to the global legislative void of fungal parameters in drinking water, the main ones being the lack of knowledge on fungal groups in water and the lack of uniform approach for their detection and identification.

The Lack of Knowledge on the "Core Fungal Species" Connected to the General Water Quality and/or Human Diseases

The first reason could be the lack of knowledge on a potential common group of fungal taxa (i.e., "core species") connected to either general water quality and/or human diseases. Conceptually speaking, fungal water biota is highly dependent on natural features, like the use of main raw water sources, pollution and climate, thus non-core fungal contaminants can drastically vary among different countries (Novak Babič *et al.*, 2017, 2018). For these reasons, common fungi that may represent an equivalent criterion for global water quality, including effects on taste, odor, material degradation and mycotoxin production may be quite difficult (Novak Babič *et al.*, 2017).

Similarly was observed for the determination of water-borne fungi causing human diseases. Contrary to bacteria and viruses, water-borne fungi more likely pose a long-term health hazard via the production of secondary metabolites than the onset of an immediate disease (Department for Environment, Food & Rural Affairs, 2011; Novak Babič *et al.*, 2017). In cases of disease development, fungal infections usually have a long incubation period and the primary source of infection can become problematic to trace (De Hoog *et al.*, 2019). Also, more than one fungal genus may cause infections, depending mainly on the state of the host's immune system. The vast majority of patients suffering from an invasive fungal infection frequently suffer from an underlying disorder, such as diabetes mellitus, cystic fibrosis, cancer, AIDS or they suffered severe burns or underwent an organ transplantation (De Hoog *et al.*, 2019). Infections caused by fungi are thus primarily limited to certain groups of immunocompromised people and are usually not highly contagious among humans as in the case of some bacteria and viruses (De Hoog *et al.*, 2019). Drinking water was thus to date rarely designated as a direct source of fungal infections in regular circumstances.

Setting Equivalent Methods for Enumeration or Cultivation of Fungi

The second reason may occur when setting the equivalent methods for enumeration or cultivation of fungi. The main problem is high diversity of fungal growth forms (Novak Babič *et al.*, 2017). Proliferation does not occur exclusively by unicellular particles displacement, but instead fungi may germinate from different kind of spores, pieces of hyphae, yeasts or meristematic clumps (De Hoog *et al.*, 2019). Due to these differences microscopic enumeration often shows discrepancies in comparison to the growth on solid media (Novak Babič *et al.*, 2017). Also, not all fungi related to water quality or diseases are able to grow on only one selected medium (usually Sabouraud or potato-dextrose agar). In future more cultivation media should be used to broaden the knowledge on water-borne fungi, which may raise costs and prolong identification (Gonçalves *et al.*, 2006; Novak Babič *et al.*, 2017) and more common approaches should be employed.

Correct and Uniform Identification of Fungi

Another problem is fungal identification to the species level. Microscopic observations are rarely correct (De Hoog *et al.*, 2019). Even after visible growth on a common cultivation media, not all fungi form distinctive macro- and micro-morphological features. Some genera may thus not be identified correctly in laboratories equipped with only the basic equipment (Novak Babič *et al.*, 2017). The molecular approach is a good alternative to microscope identification, but sometimes even the internal transcribed spacer (ITS) used as a general fungal barcode fails to identify fungi to the genus or species level, prolonging identification time and increasing costs (Irinyi *et al.*, 2015). Lastly, correct and uniform identification of fungi may represent an issue for laboratories lacking people trained in mycology, since fungal taxonomy is currently facing a huge restructuring, known

under the term "one fungus – one name", frequently resulting in old names being recently replaced or combined based on recognition of both sexual and asexual forms (Taylor, 2011).

Exploring Fungi in Water Distribution Systems

Fungi may not be the usual parameter related to water quality monitoring, but they were nevertheless studied by many European research groups during the last 30 years (Novak Babič *et al.*, 2017). For this chapter we included and analyzed reports on water-related fungi from 19 European countries: Austria, Belgium, Finland, France, Germany, Greece, Hungary, Italy, Norway, Poland, Portugal, Serbia, Slovakia, Slovenia, Spain, Sweden, The Netherlands, Ukraine and United Kingdom. These studies included raw water sources (either groundwater or surface water) used for preparation of potable water, as well as finished drinking water at consumers' endpoint (Novak Babič *et al.*, 2017). Although the factors influencing fungal presence vary amongst countries, we looked for the fungi commonly occurring in water, regardless of geographically bound disparities: the "core-species".

"Core Species" and Diversity of Fungal Communities Depend on the Main Raw Water Source

Geographic location and the climate of countries have an important influence on the fungal diversity. The main factors influencing the occurrence of fungi in raw water are temperature, humidity, rainfall rate, presence of different plants (Wurzbacher *et al.*, 2011), and geological features determining the position of the aquifers. Geological features may not vary only between the countries, but also within a country. In some cases, countries have only one type of water source, whilst others may have access to both surface water and groundwater, or even to mixtures of both, as regularly seen in the karst systems (Novak Babič *et al.*, 2017). One of the important factors, observed particularly in large valleys and basins with a high population density (urban environment), is water pollution with organic or inorganic substances (Gray, 2014). Plastics, crude oil and other complex hydrocarbons can be degraded by fungi in the presence of water. These compounds thus represent the ideal scaffold for fungal attachment and proliferation (Steffen *et al.*, 2002). This phenomenon usually affects local areas but plays an important role in cases of natural disasters (e.g., floods, earthquakes, drought, fire), or disasters caused by humans (e.g., oil spills) (Novak Babič *et al.*, 2018). During such disasters, waters are often displaced, and can carry fungal propagules to longer distances. As a consequence, a shift in fungal biota may occur (Novak Babič *et al.*, 2018).

Considering the above-mentioned facts, fungal diversity in the drinking water of European countries often shows locally highly abundant genera or species related to the specifics of the water or country (Novak Babič *et al.*, 2017). Presence of certain locally isolated fungal genera should not be excluded just because they do not fit into the general European picture. Instead, they should be carefully evaluated for each country, especially if the most abundant fungi increase after unexpected events and belong to the opportunistic pathogens.

Despite different geographical locations, geological features, techniques applied on the raw water sources for their cleaning, as well as different fungal cultivation and identification techniques used by different laboratories, some conclusions on fungal water biota of European countries can be summarized. It seems that the location of the raw water source has the most important effect on the presence of fungi in final drinking water (Novak Babič *et al.*, 2017, 2018). Countries using surface water as the main water source more frequently reported the presence of soil- and plant-degrading fungi in final drinking water, such as *Penicillium*, *Aspergillus*, *Botrytis*, *Geotrichum*, *Mucor*, *Rhodotorula*, *Scopulariopsis* and *Trichoderma* (Pereira *et al.*, 2010; Novak Babič *et al.*, 2017). These genera were reported from 20% to 26% countries, with *Penicillium* being most frequently detected (in 26% of countries).

Conversely, countries with groundwater related tap water frequently isolated the genera *Alternaria*, *Aspergillus*, *Phialophora*, *Cyphellophora*, *Exophiala* and *Paecilomyces* (Göttlich *et al.*, 2002; Heinrichs *et al.*, 2013; Biedunkiewicz *et al.*, 2014). The percentage of countries reporting their presence ranged from 26% to 47%, with *Alternaria* and *Aspergillus* being the most abundant (47%) (Novak Babič *et al.*, 2017). Melanised, black fungi with thick cell walls and fungi with small diameter of spores were generally more likely present in groundwater (Novak Babič *et al.*, 2017). The reason remains unknown, but it could be hypothesized as related to the low porosity of rocks, presence of certain ions, like calcium and magnesium, and low organic material, promoting growth of oligotrophic species (Novak Babič *et al.*, 2017).

The above listed fungi add to differences in quality of captured raw water and may also significantly affect the final drinking water quality.

The Most Common Fungi Isolated From European End-Point Drinking Water

Raw water sources with diverse fungal biota are used for production of drinking water. Depending on the general quality of the raw water, complying with the regulating Directive, several mechanic-chemical steps may be applied on the captured raw water (EEC, 1998; WHO, 2011). Since there is no legislation requiring monitoring of fungi in natural water, consequently there is little data about the influence of certain water cleaning steps on the removal of fungi from natural water sources (Novak Babič *et al.*, 2017). However, in vitro studies conducted with single fungal strains have reported removal between 8% and 90% of fungi using mechanical treatments (e.g., sand filtration, coagulation, flocculation), and inactivation of 97%–99% of fungi with free chlorine

concentration reaching 2 ppm (Department for Environment, Food & Rural Affairs, 2011; Pereira *et al.*, 2013). Despite water cleaning procedures and general good quality of drinking water complying with the Directive's parameters, fungi often remain present and reach end-point consumers. Species reported as a cross-section of fungi from surface water, groundwater and drinking water from European countries are *Acremonium* spp., *Acrostalagmus luteoalbus*, *Alternaria alternata*, *Aspergillus clavatus*, *A. flavus*, *A. fumigatus*, *A. niger*, *A. terreus*, *A. ustus*, *A. versicolor*, *Aureobasidium* spp., *Beauveria bassiana*, *Cadophora malorum*, *Cephalosporium* spp., *Cladosporium cladosporioides*, *C. halotolerans*, *C. herbarum*, *Coniochaeta hoffmannii*, *Didymella molleriana*, *Epicoccum nigrum*, *Fusarium oxysporum*, *F. solani*, *Geotrichum* spp., *Gliocladium* spp., *Isaria farinosa*, *Lecanicillium lecanii*, *Leptosphaeria* spp., *Paecilomyces variotii*, *Penicillium brevicompactum*, *P. chrysogenum*, *P. corylophilum*, *P. glabrum*, *P. spinulosum*, *Phialophora fastigiata*, *Phoma herbarum*, *Ph. leveillei*, *Pilidium concavum*, *Purpureocillium lilacinum*, *Sarocladium strictum*, *Stachybotrys chartarum*, *Trichoderma harzianum*, *T. viride*, *Rhodotorula* spp., and *Mucor* spp. (Department for Environment, Food & Rural Affairs, 2011; Novak Babič *et al.*, 2017). While most of the listed fungi occurred in a low number of countries, 25%–50% of EU countries regularly reported presence of the genera *Rhodotorula* (25%), *Alternaria*, *Mucor* (both 32%), *Paecilomyces* (37%), *Phoma*, and *Aureobasidium* (both 42%) in all three water types (Novak Babič *et al.*, 2017). The most commonly detected or isolated fungi belong to the genera *Trichoderma*, *Fusarium*, *Acremonium* (all 63% countries), *Aspergillus* (68%), *Penicillium* (74%) and *Cladosporium* (84%) (Novak Babič *et al.*, 2017). Presence of other yeasts beside *Rhodotorula* was not determined in detail, either because they were not included in the studies or they were overlooked due to used cultivation techniques, usually promoting growth of filamentous fungi (Novak Babič *et al.*, 2017).

Fungal genera reported from drinking water from more than 50% of European countries, despite of the origin of raw water source, may be considered as a part of the core fungal biota. As widespread, they very likely influence also the biofilm formation and biodegradation of materials in contact with drinking water. Additionally, they should not be overlooked when discussing the possible effect on health among European population.

Fungal Effect on Used Building Materials in Water Distribution Networks

After cleaning procedures freshly produced drinking water is usually retained in reservoirs before it is distributed to consumers. The networks of pipes and reservoirs are built of various materials and alloys (Nlzoh and Nijz, 2016). The most commonly used materials for water transport are concrete, mortar, gypsum, ceramic tiles, plastic materials (e.g., polyethylene (PE) and its derivates – PEX, GFRP, and rubber), and different metal alloys (e.g., copper and its alloys; Cu-Zn, Cu-Zn-As, Cu-Zn-Pb, Cu-Zn-Pb-As, etc.) (4MS, 2011). Each of the used materials reacts differently with water and thus their long-life stability depends greatly on the chemical features of water, and disinfectants used, particularly chlorine, and its by-products (e.g., chloramines) (Gray, 2014). For now, the European Union (EU) does not have a uniform approach for quality and adequacy for materials and their products in contact with drinking water. However, four member states (4MS; France, Germany, The Netherlands and the United Kingdom) issued standardized procedures for the approval of building materials and final products for water networks (4MS, 2011; Nlzoh and Nijz, 2016). The document sets also standard procedures in order to determine possible corrosion and growth promotion of total coliforms at 37°C and total microbial count at 22°C, but does not directly include fungi (Nlzoh and Nijz, 2016). However, studies conducted during the last two decades frequently linked the occurrence of fungi in water networks to the choice of building materials. Materials more susceptible to biodegradation may result in elevated growth of biofilms, affecting the stability of residual disinfection, slowing water flow and weakening shear forces (Department for Environment, Food & Rural Affairs, 2011).

Colonization and Biodegradation of Materials in Contact With Drinking Water Induced by Water-Related Fungi

Most widely used building materials in water industry are concrete, metal and plastic materials. Plastic in particular constitutes almost 90% of materials used to build pipes in new constructions (Andrady and Neal, 2009; Bertron, 2014). The number of microorganisms in water significantly increases in certain parts of the supply systems which promote biofilm formation (**Fig. 1**).

Although biofilm formation occurs independently of the hydrophobicity or hydrophilicity of the material, the available reports show higher presence of fungi and bacteria on rough surfaces and those made of iron or steel in comparison to plastic materials (Doggett, 2000; Grabińska-Łoniewska *et al.*, 2007). The main reason is probably the reaction of metals with residual chlorine in water, lowering its harmful effect on microbial biofilms (Le Chevallier, 1999).

Surface characteristics and material composition exerts a strong selective pressure on fungal biodiversity. The most used building material for large water retention centers, storage tanks, and main pipes for water transport is rough concrete (Bertron, 2014). After prolonged time of being in direct contact with water, its relative humidity raises above 90%, representing ideal conditions for growth of water-borne, hydrophilic fungi (WHO, 2009). Concrete is often colonised by the filamentous genera *Bjerkandera*, *Sporothrix*, *Chaetomium*, and *Penicillium*, while yeast biota on concrete is constituted mainly of *Candida*, *Debaryomyces*, *Kluyveromyces*, *Meyerozyma* and *Rhodotorula* species (Andersen *et al.*, 2011; Hurtado-McCormick *et al.*, 2016). In addition, species *Aspergillus fumigatus*, *A. melleus*, *A. niger*, *A. versicolor*, *A. ochraceus*, *Mucor racemosus* and *M. spinosus* colonize concrete combined with different adhesives (Andersen *et al.*, 2011). Finishing materials, such as plaster, gypsum or fiberglass are usually colonised by *Acremonium*, *Cladosporium*, *Penicillium*, *Stachybotrys*, *Ulocladium*, and *Phoma* (Andersen *et al.*, 2011; **Fig. 2**). Depending on the core species, fungal growth and metabolism may affect concrete in two ways: (i) local scaling due to the production of organic acids, as

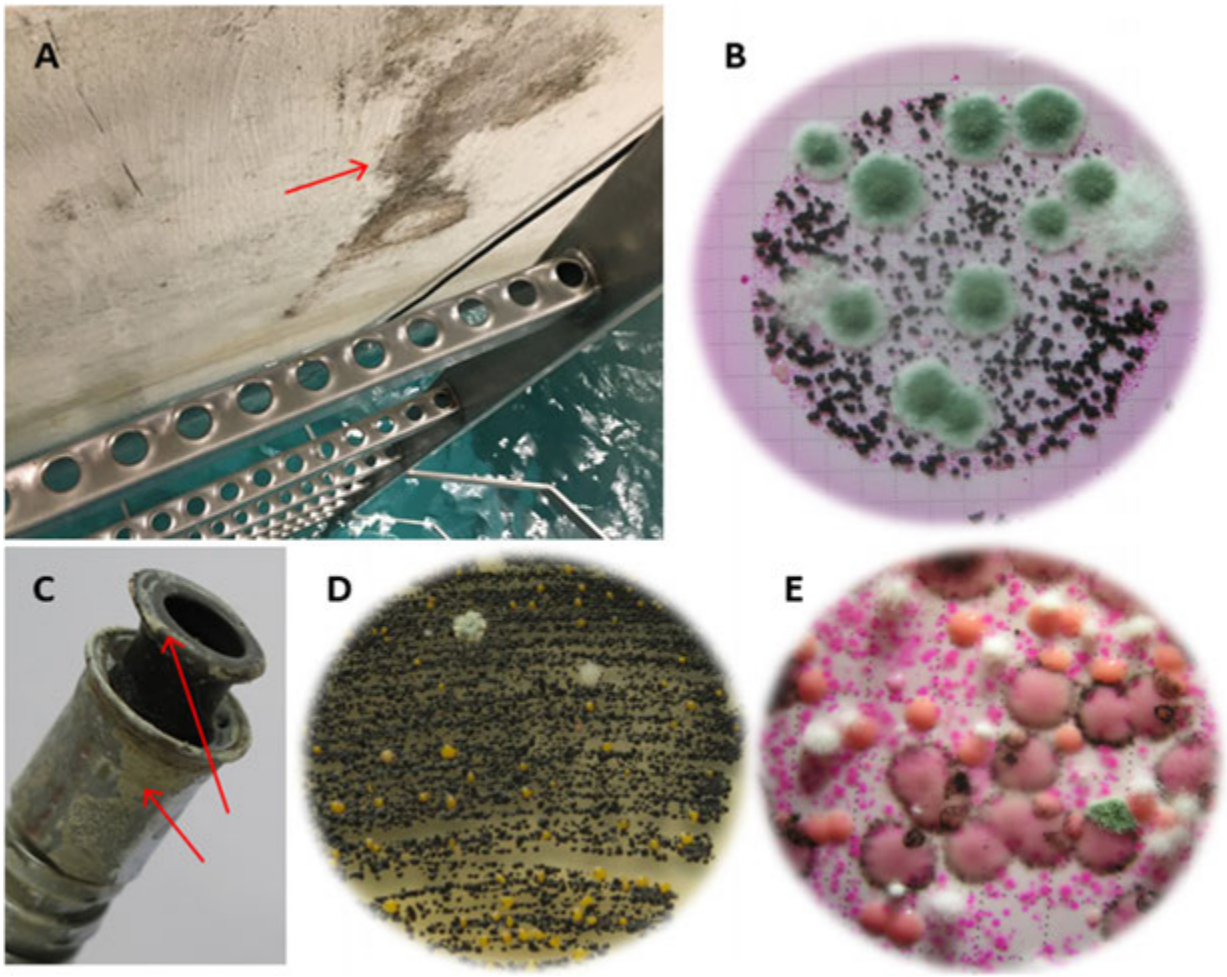

Fig. 1 The presence of fungi on various materials in contact with water, in untreated water and in drinking water. A: Fungal colonies on the surface of the concrete coating in water storage facility (indicated by the red arrow); B: *Aureobasidium melanogenum* (black colonies) and *Aspergillus* sp. (green colonies) isolated from 1 L of untreated water on DRBC agar; C: Visible biofilm on the metal and rubber parts of the pipe (marked with red arrows); D: Sample taken from the inside of the rubber tube after incubation on the SABG agar with the addition of penicillin and streptomycin, visible bacteria *Sphingomonas* sp. (yellow colonies) and black yeast *Exophiala* sp.; E: DRBC agar with visible *A. melanogenum* (large, pink colonies), *Rhodotorula mucilaginosa* (red yeast), *Bisifusarium dimerum* (white colonies), and *Aspergillus fumigatus* (green colony) isolated from 1 L of chlorine-treated drinking water sampled from the pipe at end-point user.

shown for *Aspergillus*, *Penicillium* and *Stachybotrys* (Andersen *et al.*, 2011; Luo *et al.*, 2018); (ii) dissolution of $Ca(OH)_2$, and precipitation of calcite on the surface of fungal hyphae as confirmed for *Trichoderma reesei* and *Candida tropicalis*. These changes are well known under the term "self-healing concrete" (Luo *et al.*, 2018; Menon *et al.*, 2019).

While concrete covers large areas due to its use for water retention, metal alloys (e.g., different types of brass and stainless steel) represent smaller percentage of materials in contact with water. They are usually used as a part of ductile pipes (ductile iron), for water pipes with small diameter, and for couplings and counters (Hurtado-McCormick *et al.*, 2016). Water-associated fungi damage metal materials locally, causing extensive separation into layers, pitting and intercrystallite corrosion (Semenov *et al.*, 2006). Ductile iron and iron have been colonised and degraded by *Penicillium ochrochloron*, *P. glabrum* and *P. janthinellum* and ubiquitous yeast *Debaryomyces hansenii* (Hurtado-McCormick *et al.*, 2016). Destruction of aluminum and magnesium alloys has been observed for *Aspergillus niger* (Semenov *et al.*, 2006). Also, aluminum and steel wires are commonly colonised by *A. niger*, as well as *A. amstelodami*, *Chaetomium globosum*, *Stachybotrys atra* and various fungi of the genera *Aureobasidium*, *Alternaria* and *Stemphylium* (Semenov *et al.*, 2006). On the contrary, copper wires have been reported as the most resistant to fungal colonization, with genus *Penicillium* being the only one detected (Semenov *et al.*, 2006). *Penicillium* spp., *Aspergillus fumigatus* and *Sarocladium kiliense* are often involved in biocorrosion of multiphase and mild steels (Rovetta *et al.*, 2014; Imo *et al.*, 2018). Additionally, metal parts, like grids at the end of the indoor faucets harbor yeasts like *Candida parapsilosis* and black yeasts *Exophiala* spp. (Zupančič *et al.*, 2016). Although zinc has been known as rarely colonised by fungi, several zinc parts of shower also harbored *Penicillium chrysogenum*, *P. purpurogenum* and *P. lilacinus*, together with yeasts *Debaryomyces hansenii*, *Rhodotorula mucilaginosa*, and *Cryptococcus diffluens* (Hurtado-McCormick *et al.*, 2016).

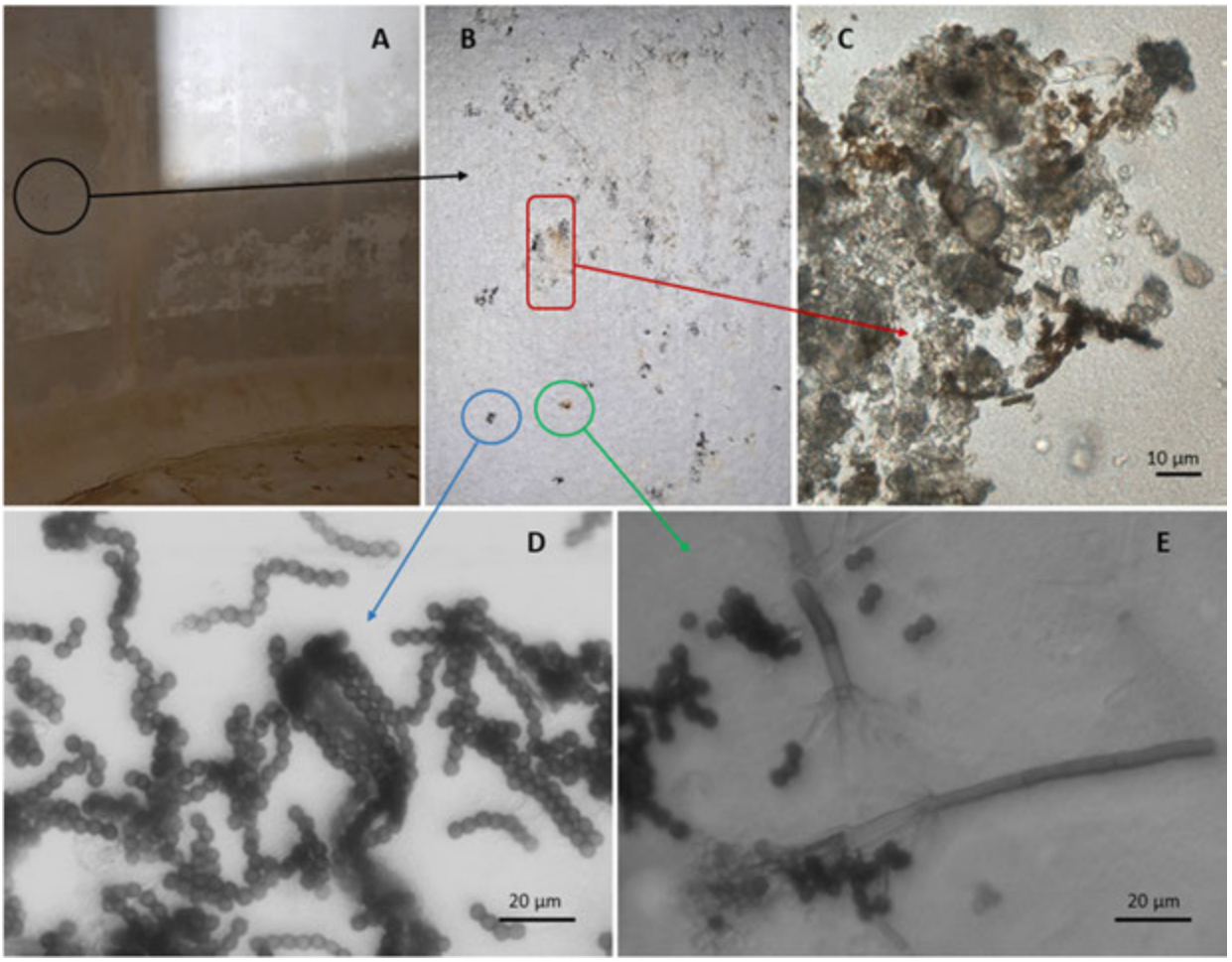

Fig. 2 Biodegradation of mortar and concrete inside water storage tank occurring under usual water level. A: Cementious ceiling of water storage tank, affected by biodegradation with visible brown and black colonies (indicated by black circle); B: closer photo of visible fungal colonies with mixed genera on the ceiling. Tape samples of colonies, indicated with red, green and blue colors, were taken for further microscopical analysis; C: biodegradation with several fungi seen as in-deep growth of hyphae and meristematic clumps, mechanically degrading mortar; D: monoculture forming large chains of spores appearing directly from the mortar surface; E: mixed fungal culture with visible chains of dark spores and centrally pale brown conidiophores with 3–6 phialides and slimy heads full of transparent conidia. The latter was identified as *Musicillium theobromae*.

During last decades, plastic materials, such as polyvinyl chloride (PVC) and high or medium density polyethylene (HDPE/MDPE), became widely used in water transportation, particularly in household water networks due to easier cleaning, slower biofilm formation, and consequently lower biodegradation potential in comparison to metal (Semenov *et al.*, 2006; Fish *et al.*, 2016). Petroleum-based plastics, such as polyethylene (PE), poly(ethylene terephthalate) (PET), polystyrenes (PS), polyurethanes (PUs), and polyvinyl chloride (PVC), are highly resistant to microbial biodegradation (Hyde *et al.*, 2019). Plastic pipes also do not react with residual chlorine, as is often the case with metal alloys, and thus provide long-distance quality of transported water (Department for Environment, Food & Rural Affairs, 2011). So called "soft-plastics" like neoprene, nylon, silicones and rubber seals, mainly used for sealing couplings and the end-point pipes, are more prone to fungal colonization (Naismith *et al.*, 2017). Due to roughness they represent a good scaffold for yeasts *Candida* spp., *Cystobasidium sloofiae*, *Meyerozyma guilliermondii*, *Naganishia* spp., *Rhodotorula mucilaginosa* (many of which act as primary colonizers) and black yeast-like fungi *Aureobasidium melanogenum*, *Cladophialophora* spp., *Exophiala* spp., *Phialophora* spp., *Cyphellophora* spp. and *Rhinocladiella similis* (Zupančič *et al.*, 2016; Novak Babič *et al.*, 2017). Additionally, molds of the genera *Phoma*, *Scedosporium*, *Cladosporium* and *Fusarium* can be commonly detected (Hamada and Abe, 2009; Zupančič *et al.*, 2016). Filamentous fungi of the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cephalosporium*, *Fusarium*, *Penicillium*, *Rhizopus*, and *Trichoderma* have been recognized as the main agents of microbiological damaging of the PVCs (Semenov *et al.*, 2006). Compact plastic materials, as plasticized polyvinyl chloride (pPVC) are usually colonised and degraded by filamentous fungi *Alternaria alternata*, *Aspergillus* spp., *Aureobasidium pullulans*, *Bipolaris* spp., *Epicoccum nigrum*, *Paecilomyces lilacinus*, *Penicillium* spp. (Webb *et al.*, 2000; Hyde *et al.*, 2019). Other fungi like *Aspergillus flavus*, *A. niger*, and *Fusarium* spp. degrade polycaprolactone (PCL), *Talaromyces* and *Penicillium* species degrade biopolymeric polyhydroxybutyrate (PHB), and *Aspergillus terreus*, *A. tubingensis*, *Chaetomium globosum*,

Curvularia senegalensis, *Pestalotiopsis microspora*, and *Trichoderma* spp. were reported for degradation of polyurethane (PU), particularly polymers with ether bond, more susceptible to fungal degradation than polyurethanes with ester bonds (Hyde *et al.*, 2019; Semenov *et al.*, 2006). It has been shown that also the use of certain metals like iron and magnesium oxide as additives to plastics can promote the attachment and growth of certain fungi (Semenov *et al.*, 2006), while zinc oxide and titanium oxide diminish it (Semenov *et al.*, 2006). Fungal colonization of plastic materials becomes more problematic with time, due to the release of potentially toxic substances from degraded plastic into drinking water (Semenov *et al.*, 2006).

Fungi from drinking water represent important components of biofilm, containing up to 8.9 CFU cm^{-2} of yeasts and 4.0–25.2 CFU cm^{-2} of filamentous fungi (Doggett, 2000; Grabińska-Łoniewska *et al.*, 2007). They usually enter the biofilm symbiosis as secondary colonizers, actively producing extracellular polymeric substances, particularly humic acids and aliphatic constituents (Claus *et al.*, 1999). Active growth in hyphal form also enables better bacterial attachment and contributes to biofilm stability (Harding *et al.*, 2009). Fungal biofilms can be fully formed within 48–72 h in experimental systems mimicking real water network conditions (Fish *et al.*, 2016), as shown with yeasts *Candida*, *Saccharomyces*, *Naganishia* (former *Cryptococcus*) and *Aureobasidium*, and molds *Acremonium*, *Aspergillus*, *Penicillium*, *Coriolus*, *Neocosmospora*, and *Trichoderma* (Harding *et al.*, 2009; Kauffmann-Lacroix *et al.*, 2016; Douterelo *et al.*, 2018). Fungal biofilms in water pipes frequently raise concerns in hospital environments due to the possible health effect and in industrial water networks, due to slowing work processes and clogging (Fish *et al.*, 2016). Such investigations most frequently revealed the presence of opportunistic species of the genera *Aspergillus*, *Candida*, *Exophiala*, *Fusarium*, *Malassezia*, *Ochroconis*, *Penicillium*, *Phialophora*, *Phoma* and *Rhinocladiella* (Novak Babič *et al.*, 2017).

Biodeterioration and biodegradation of material occurs with the maturation of formed biofilms starting by chemical and later followed by mechanical degradation. Microorganisms can either mobilize (solubilize) or immobilize (accumulate, precipitate) metals (Dighton and White, 2017). Mobilization occurs via redox processes, hydrolysis, chelation, or methylation. Immobilization includes redox processes, complexation, biosorption and biomineralization (Dighton and White, 2017). Complexation of materials happens due to the local production of high concentrations of chemically aggressive metabolites, such as organic and mineral acids, bases, CO_2, alcohols, and sulfur compounds (Dighton and White, 2017; Hyde *et al.*, 2019). Acid's production is well known for the species *Aspergillus niger*, *A. brasilensis*, *A. flavus*, *A. terreus* and *A. itaconicus*, commonly producing high concentrations of succinic, itaconic, and L-malic acid (Dighton and White, 2017). Second most frequent producers of acids are species of the genus *Penicillium*, producing succinic, gluconic and (–)-trans-2,3-epoxysuccinic acid (Dighton and White, 2017). Other acid producers, frequently isolated from water, belong to the genera *Fusarium* (succinic acid), *Paecilomyces*, *Talaromyces* (both produce (−)-trans-2,3-epoxysuccinic acid), and zygomycetous *Actinomucor*, *Mucor*, *Cunninghamella*, *Circinella* and *Rhizopus*, producing fumaric acid (Hyde *et al.*, 2019; Dighton and White, 2017). Basidiomycetes isolated from water (e.g., *Stereum hirsutum*) rarely produce acids, but they may produce ethanol as one of the main precursors in the metabolic acid production (Hyde *et al.*, 2019). Effects of chemical dissolution of materials can be noticed as "swampy" odor and taste of drinking water (Department for Environment, Food & Rural Affairs, 2011).

Once materials become weak due to the chemical dissolution, fungi may additionally degrade them by mechanical disruption caused by in-depth growth of hyphae (Bertron, 2014), as was shown for water-related genera *Alternaria*, *Aspergillus*, *Cephalosporium*, *Cladosporium*, *Mucor*, *Penicillium*, *Rhizopus*, and *Trichoderma* (Shinkafi and Haruna, 2013; Luo *et al.*, 2018). Effects of mechanically degraded material (usually predominant on concrete and metals) are visible in a range from a slight change of color to pieces of corroded metal present in water. These effects might not be toxic, but they can be unpleasant for most consumers and should be avoided (WHO, 2011; Department for Environment, Food & Rural Affairs, 2011).

How Big is the Health Risk Due to Fungal Presence in Tap Water for End-Point Users?

Once established, biofilms are hard to remove from water-retention reservoirs and pipe systems (Fish *et al.*, 2016). On the long-term, the phenomenon leads to altered color, taste and odor of water and, additionally, to the production of allergenic and irritating compounds (MVOCs) (Department for Environment, Food & Rural Affairs, 2011; Novak Babič *et al.*, 2017). In cases of people with immune deficiency fungi may also initiate an infection (Novak Babič *et al.*, 2018; De Hoog *et al.*, 2019).

Development of Fungal Disease due to Contaminated Water

Besides drinking, consumers use potable water also for daily activities such as cooking, washing and personal hygiene, where they come in contact with fungi directly via the skin and mucosae while washing, digestive system while drinking, and through the respiratory system by inhaling aqueous aerosols (Novak Babič *et al.*, 2017, 2018). Shower hoses can be colonised by *Aspergillus glaucus*, *Cladosporium* spp., *Exophiala mesophila*, *Fusarium fujikuroi* species complex, *Malassezia restricta*, *Penicillium* spp., and *Schizophyllum commune* (Moat *et al.*, 2016). Some studies linked elevated numbers of fungi in the bathrooms' air and on surfaces with the use of showers (Anaissie *et al.*, 2003). Molds were recovered in 70% of samples and included fungi often responsible for asthma symptoms, hypersensitivity pneumonitis and skin irritation (i.e. *Alternaria*, *Acremonium*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Paecilomyces*, and *Penicillium*) (De Hoog *et al.*, 2019). Water-borne fungi, such as *Acremonium*, *Aspergillus*, *Candida*, *Cladosporium*, *Cryptococcus*, *Exophiala*, *Fusarium*, *Penicillum*, and *Rhodotorula* are often reported from dental unit waterlines, especially after prolonged chemical treatment with chlorine and elimination of dominant bacteria (Porteous *et al.*, 2003; Göksay *et al.*, 2013;

Lisboa *et al.*, 2014; Damasceno *et al.*, 2017). Special attention should be given to dialysis water in hemodialysis units, where melanised water-borne fungi of the genera *Cadophora*, *Cladosporium*, *Cladophialophora*, *Exophiala*, *Phialemonium*, and *Rhinocladiella* can colonize silicone-based materials for water transport (Figel *et al.*, 2013; Kadaifciler *et al.*, 2013; Mesquita-Rocha *et al.*, 2013).

In general, water-borne fungi of the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida*, *Cladosporium*, *Fusarium*, *Malassezia*, *Mucor*, *Penicillium*, *Rhodotorula* and *Trichoderma* are regularly present on skin, oral cavity and gastrointestinal tract mycobioms (Underhill and Iliev, 2014; Damasceno *et al.*, 2017). Moreover, species of genera *Aspergillus*, *Cladosporium* and *Scedosporium* can be found transiently in the respiratory tract of healthy individuals, causing transient immune responses like coughing and irritation (Underhill and Iliev, 2014). The above listed fungal genera are opportunistic pathogens, often reported as causative agents of skin, hair and nails infections, and respiratory diseases, such as allergies, asthma and sinusitis (De Hoog *et al.*, 2019). Serious and life-threatening systemic fungal infections may develop in individuals suffering from autoimmune disorders, cystic fibrosis, long-term hospitalization and impaired immune system due to previous viral or bacterial infection, diabetes, cancer or transplantation (Anaissie *et al.*, 2003; De Hoog *et al.*, 2019). Globally, these groups of patients are mainly at risk to get infected with water-related fungi of the genera *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium*, *Mucor*, and causative agents of chromoblastomycoses (e.g., *Cladophialophora*, *Exophiala* and *Fonsecaea*) (Bongomin *et al.*, 2017).

While most fungal infections are treated with azole compounds, a sudden increase of azole-resistant strains in recent years causes great concern. In particular, species of the genus *Aspergillus* are the predominant fungi developing resistance to azoles, which is believed to have been triggered by the mass use of azole-containing fungicides in agriculture (Verweij *et al.*, 2009). Crops' protection products are not present only in soil but can be detected also in water, leading to the selective pressure, resulting in propagation of azole-resistant strains (Hollomon, 2017). Such occurrences are higher in surface water, due to direct contact with sewage water collected from crops, as shown for *Candida* spp. (Monapathi *et al.*, 2017). In Netherlands they already confirmed drinking water as the main vector for azole resistant *Aspergillus fumigatus* in infected patients (Warris *et al.*, 2003). These incidences put tap water under scrutiny when suspecting infections with azole-resistant fungi (Monapathi *et al.*, 2017).

Indirect Health Effects Due to the Secretion of Fungal Secondary Metabolites

Another way for fungi to affect health is the production of VOCs and mycotoxins. Fungi, in particular the presence of the genera *Acremonium*, *Aspergillus*, *Penicillium* and *Phialophora*, affect the odor and taste of drinking water due to the production of geosmine (Department for Environment, Food & Rural Affairs, 2011). Other VOCs that can be frequently detected in tap water systems are phenols, alkanes, alkenes, and monoterpenes, some of which are genotoxic (Hurtado-McCormick *et al.*, 2016) or they may cause intoxications (Kauffmann-Lacroix *et al.*, 2016). Particularly macrocyclic trichothecenes, alkaloids, fumonisin B, and ochratoxin A are neurotoxic upon ingestion or inhalation of aerosols (French *et al.*, 2019). Recent research of data from 923 Counties of the United States of America (USA) found a significant correlation between mortality due to motor neurone diseases (MND) and the prevalence of well water use. For the first time there was speculation about prolonged exposure (e.g., via drinking, inhaling) to mycotoxigenic fungi from well water as the possible cause of sporadic amyotrophic lateral sclerosis (ALS) as the most common MND disease in adults (French *et al.*, 2019). Similarly, studies on ALS conducted in Spain (Alonso *et al.*, 2015, 2017) and Italy suspected drinking water and fungi to be the possible cause of sporadic ALS (Vinceti *et al.*, 2010). The mycotoxigenic hazard is presumably lower in chlorinated water in comparison to unchlorinated water (Mata *et al.*, 2015), due to the complete degradation of aflatoxin B1, occurring 30 min after exposure to chlorine (McKenzie *et al.*, 1997). However, the same effect was not recorded for aflatoxins B2 and G2, even after several days of water chlorination (McKenzie *et al.*, 1997; Mata *et al.*, 2015). Their study on the presence of mycotoxins in bottled water and tap water confirmed the instability of mycotoxins in tap water treated with chlorine, while in bottled water aflatoxin B2 was the most frequently detected mycotoxin, followed by aflatoxin B1, aflatoxin G1 and ochratoxin A. In all cases levels were below the European maximum limits for mycotoxins in food products (Mata *et al.*, 2015). Ubiquitous fungi *Cladosporium*, *Penicillium* and *Fusarium* were isolated in most of those samples, while other mycotoxin producing genera, *Aspergillus*, *Rhizopus*, *Trichoderma*, *Acremonium*, *Beauveria*, *Mucor*, *Paraconiothyrium*, *Cadophora*, and *Cunninghamella*, were isolated only sporadically (Mata *et al.*, 2015). Although mycotoxins were not frequently present in chlorinated drinking water, low- or even unchlorinated water should be tested for fungi described as mycotoxin producers, particularly when drawn from lakes, rivers and wells, or from karst systems.

Conclusions

Due to the currently unresolved issues, such as lack of a uniform approach for fungal count and correct identification of isolates, future inclusion of fungi in states' or national legislations for drinking water quality remain unclear, but nevertheless necessary. Fungal presence in water should be carefully considered in construction of water networks, and in health-care institutions, particularly hospitals and intensive care units, where water-transmitted fungi can pose a serious health risk. European countries should be aware of the most common and frequently isolated opportunistic fungi. In cases where surface water is used as the main water source for the production of drinking water these are the genera *Penicillium*, *Aspergillus*, *Botrytis*, *Geotrichum*, *Mucor*, *Rhodotorula*, *Scopulariopsis* and *Trichoderma*. Groundwater derived tap water can be the source of fungi of the genera *Alternaria*, *Aspergillus*, *Phialophora*, *Cyphellophora*, *Exophiala* and *Paecilomyces*. The most commonly detected fungi at consumers' endpoints, unregarding

the raw water source are *Rhodotorula*, *Alternaria*, *Mucor*, *Paecilomyces*, *Phoma*, *Aureobasidium*, *Trichoderma*, *Fusarium*, *Acremonium*, *Aspergillus*, *Penicillium*, and *Cladosporium*. These genera represent the core fungal biota of European tap waters. They can have a significant effect on mixed biofilm formations, material degradation and consumers' health. However, any other fungal genera observed in excess numbers in a particular geographic area should be taken into consideration for quality assessment of drinking water. The following points are recommendations to lower fungal contaminants in distribution networks and downstream at the end-user's access points.

Lowering the Effect of Water-Borne Fungi on Construction of Water Networks

Although the 4MS initiative (4MS, 2011) is not a requirement, future materials should be tested for microbial colonization (including fungi) before being integrated in water transport networks. Materials showing high affinity for fungal colonization and degradation could be replaced with more stable ones. This would contribute to prolonged stability of residual chlorination effect, lower biofilm formation and slowing of material degradation. Summing up, such preventive measurements could lower the economic costs due to later construction repairs.

Lowering Effect of Water-Borne Fungi on Human Health

Fungal implications for health could be lowered with the use of end-point filtration systems in order to minimize aerosolisation of fungal particles in drinking water. Such systems are particularly highly recommended for hospitals and healthcare facilities where people with impaired immune systems stay for prolonged times. Concentrations of mycotoxins in drinking water are presumably low, but should be monitored in cases of natural emergencies, such as prolonged floods or droughts that can promote fungal growth in water systems. Additional chlorination, in particular of surface water used as raw water source may nullify the toxic effect of certain mycotoxins and is thus recommended in cases of natural disasters, but also for small water treatment plants operating with surface water or water of mixed origin.

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Mycological Studies in Cultural Heritage

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Biodeterioration, Fungi and Cultural Heritage

Several definitions have been suggested for biodeterioration, generally considered as "any undesirable change in a material brought about by the vital activities of organisms" (Sterflinger and Piñar, 2013). This can translate into physical and/or chemical damage and, as the title of this article states, the affected materials or objects may be deemed with historic, artistic and economic relevance and classify as Cultural Heritage (Griffin *et al.*, 1991). Fungi (as bacteria, archaea and lichens) assume an important role here and are virtually everywhere and colonize virtually any material, be it ancient or modern, private or public, organic or not (Sterflinger and Piñar, 2013). Because they are ubiquitous, the study on the influence fungi have on works of art or cultural heritage involves analyzing the dynamics established between them and a vast set of artistic supports: e.g., canvas, paper, parchment, wood, textiles, murals, or stone. It would be impossible to address all of these in this chapter but several reviews can be consulted for further reading (Choi, 2007; Coutinho *et al.*, 2015; Gaylarde *et al.*, 2003; Gu, 2003; McNamara and Mitchell, 2005; Pinheiro, *et al.*, 2019; Sequeira *et al.*, 2019; Sterflinger and Piñar, 2013; Warscheid and Braams, 2000; Zyska, 1997). Instead, the next lines will be dedicated to explaining the relationship between fungi and examples of organic and inorganic cultural heritage supports. After these examples, the issues of treatment and prevention are also addressed.

Fungal Biodeterioration Mechanisms and Impact on Cultural Heritage

Organic Supports: Paper Based Objects

Great part of the History of mankind is registered in the form of documents or works of art on paper support. Due to its hygroscopicity and organic content (mainly cellulose), paper is particularly susceptible to mycological deterioration. The source of raw material, additives and manufacture processes have greatly changed throughout papermaking history and these additions have also influenced its bioreceptivity., i.e., the proneness to colonization by living organisms (Guillitte, 1995). For instance, in order to reduce ink spreading and increase the bonding between fibers, paper was usually sized with polysaccharides, proteins or resins, like starch flour, gelatin or rosin, and ultimately alkaline synthetic compounds (Barrett, 1989; Hubbe and Bowden, 2009). Fillers, like clay or chalk were also added to the paper pulp to increase paper opacity (Cappitelli and Sorlini, 2005). These elements may constitute an additional and easier to absorb food source for fungi, and therefore strongly influence the intensity and results of fungal action on paper (Pinzari *et al.*, 2006).

Filamentous microfungi are considered the most important biodeteriogens of paper based collections due to their capacity to grow at average relative humidity (RH) levels (above 65%), to produce an array of deteriorating enzymes and metabolites (Sterflinger and Pinzari, 2012), and also due to the fact that fungal contamination is very easy to occur. Bacteria can also deteriorate paper materials, but since fungi require less moisture to develop, the environmental conditions normally found in libraries, archives or museums are more prone to the growth of fungi than that of bacteria.

When paper is colonized by fungi, besides the hyphae penetrating in the paper matrix causing physical alterations, several excreted metabolic products also accumulate in the material. **Fig. 1** shows the cycle of fungal development and how it affects paper based cultural heritage assets but the same scheme can be applied to other substrates.

The decomposition of cellulose by cellulolytic fungi is mainly due to the production and secretion of a complex array of extracellular cellulases, which hydrolyze the cellulose macromolecule into small water soluble sugars that can then be processed by the fungi (Bergadi *et al.*, 2014). Other enzymes, like amylases or proteases decompose paper additives, causing loss of mechanical resistance. Non-enzymatic metabolites, like acids or metal ions also cause accelerated depolymerization of cellulose (Hastrup *et al.*, 2011). Furthermore, the biogenic formation of calcium oxalate crystals promoted by fungi can change the calcium carbonate alkaline reserve in paper and be a source of chemical and mechanical damage (Pinzari *et al.*, 2010).

The deterioration caused by all these fungal compounds will cause a gradual loss of mechanical strength in paper (Sequeira *et al.*, 2017). On an advanced stage of deterioration by fungi, paper acquires a felted consistency, with little or no mechanical resistance and its manipulation may result in losses of material and information (**Fig. 2**).

Besides chemical and physical damage, fungi often cause aesthetic alterations on paper through the production of colored pigments (Melo *et al.*, 2019), which interfere with the readability and aesthetic value of the object (**Fig. 3**).

The most commonly identified fungi from discolored paper/book materials (identified in more than five studies) are presented in **Table 1**.

Most species in **Table 1** belong to the *Aspergillus* or *Penicillium* genus, both airborne conidia producers, with a high potential for dissemination and contamination. Almost all of these commonly identified fungi are great cellulase producers. It is also relevant to

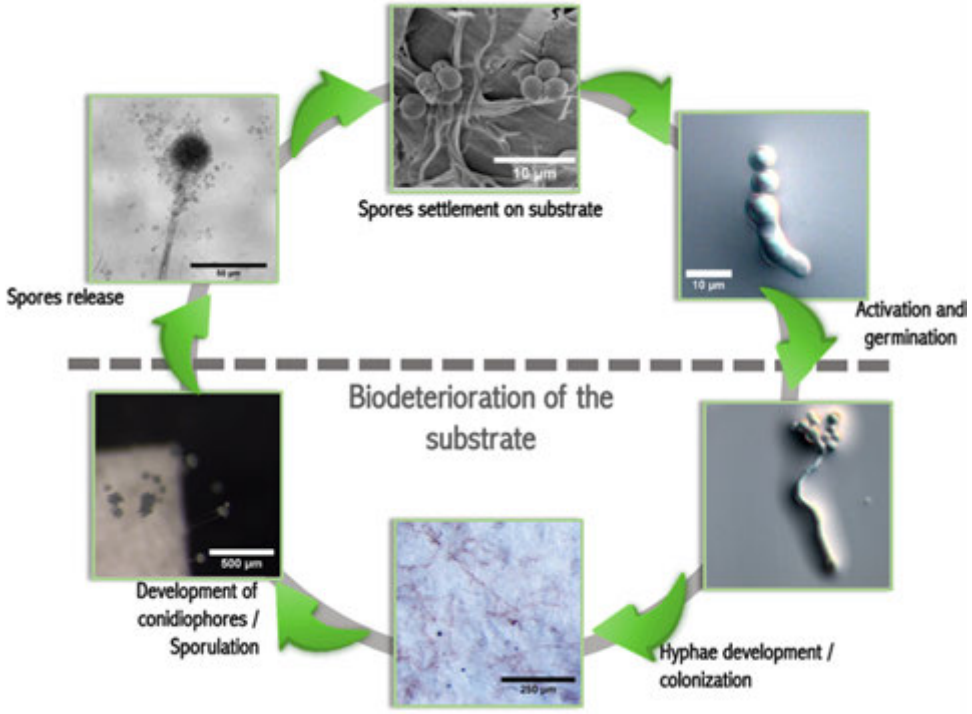

Fig. 1 Life cycle of fungi and how they interact with paper-based substrates. The development of hyphae and formation of conidiophores/sporulation phases are visible to the naked eye and may affect readability and aesthetic value.

Fig. 2 Manuscript song book, 16th century, private collection. The felted consistency and colored fungal metabolites may be observed in greater detail in the right magnified rectangle.

highlight the presence in this table of important mycotoxin producers, such as *Stachybotrys chartarum* and *Chaetomium globosum*, which reinforces the importance of skin and respiratory tract protection gear when dealing with colonized paper collections.

Organic Supports: Photographic Collections

Photographic items are multi-layered materials composed of a wide assortment of constituents, generally having a common structure: a support layer (substrate) and a binder layer (emulsion) containing the microscopic image forming particles. Several photographic substrates have been used since the invention of photography in the early 19th century, such as metal plates, glass plates, cellulose nitrate, cellulose acetate, polyethylene terephthalate, or polyethylene naphthalene films, or paper (Roldão, 2018).

Fig. 3 Example of colored fungal stains on an etching (private collection).

The emulsion could be composed of albumen, collodion or gelatin (Puškárová *et al.*, 2018). Silver, pigments or dyes were the most common picture forming particles used along history. Besides all these variations, photographic items can be delivered in several different formats, such as prints, negatives, slides, microfilm rolls or microfiches (Florian *et al.*, 1994). All these variables will contribute to the level of fungal bioreceptivity of photographic items, once favorable temperature and humidity growth conditions are met. Synthetic and semi-synthetic polymers, such as polyethylene and cellulose nitrate and acetate (substrates), respectively, are less biodegradable than natural polymers such as cellulose (paper substrate) or proteins (gelatin, albumin emulsion), and a closer similarity to natural polymers will result in a higher bioreceptivity for synthetic polymers (Gu, 2003). When comparing black and white (b&w) and color negatives and slides, a tendency for higher fungal susceptibility on color chromogenic materials was observed (Lourenço and Sampaio, 2009). Fungal growth was affected by the reduced silver particles in the b&w images, while the dyes in colored images had no significant impact on the fungi (Lourenço and Sampaio, 2009).

Fungi will degrade the photographic materials due to hyphae penetration in the photographic layers, by the excretion of specific enzymes and organic acids, decomposing the polymeric substrates and emulsions into smaller molecules, or by etching glass and metal plates supports to extract minerals or metallic ions. Fungi have also shown to alter the distribution of silver crystals in the gelatin-silver emulsions, reducing the silver ions and forming silver nanoparticles on the fungal cell wall (Sclocchi *et al.*, 2013).

Gelatin emulsions, for instance, are easily degraded by proteolytic fungi, leading to the solubilization and disintegration of the photographic image (Puškárová *et al.*, 2018). As the emulsion or substrate are consumed by fungi, image material will be lost, causing significant disturbance or total loss of information in photographic objects. This is particularly relevant in objects that are meant for projection, like slides, in which a small fungal blemish will represent a large defacement on the image, when amplified and projected (**Fig. 4**).

In the last decades, a few studies have been published covering the fungal contamination of photographic collections, including gelatin-silver prints, albumen prints, film-based and glass-based negatives, slides, tintypes and ambrotypes (**Table 2**).

Most species belong to the common genera *Aspergillus*, *Cladosporium*, and *Penicillium* (**Table 2**). Only recently, culture independent fungal identification has been performed to identify the fungal species dwelling on photographic materials (Bucková *et al.*, 2014; Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017), bringing to light new fungal species, identified for the first time in these materials, such as species from the genera *Malassezia* or *Hyphodontia*. Nevertheless, mycological research on photographic collections is still scarce, and the biodeterioration mechanisms of such complex multi-layered materials are yet to be fully unveiled, along with proper conservation treatments to stabilize or reverse them.

Inorganic Supports: Stone Artefacts and Monuments

Deterioration of stone occurs through physical, chemical and biological processes, in isolation or in combination (Wakefield and Jones, 1998) and is intimately related to stone bioreceptivity and exposure to climatic conditions (Pinheiro *et al.*, 2018). So, against our primary impression that only organic based artefacts suffer from the action of microorganisms, stone, one of the materials used by mankind to express itself, is also susceptible to their action. In fact, microbial action can account for 20%–30% of stone deterioration (Wakefield and Jones, 1998).

Table 1 The most commonly identified fungal species in discolored paper/book materials – identification methods (CD – culture dependent; CI – culture independent; CDMB – culture dependent molecular biology), enzymes and color production

Fungal species	*Identification methods*			*Colors*	*Enzymes*
	CD	*CI*	*CDMB*		
Aspergillus flavus Link	(Nol *et al.*, 2001; Zotti *et al.*, 2008; Bankole, 2010; Borrego *et al.*, 2012; Castillo *et al.*, 2016)		(Oetari *et al.*, 2016; Okpalanozie *et al.*, 2018; Teixeira *et al.*, 2018)	yellow, green, orange	Cellulases (–/+++) Amylases (++/+++) Gelatinases (+) Proteases (+) (Das *et al.*, 1997; Gopinath *et al.*, 2005; Janda *et al.*, 2009; Pitt and Hocking, 2009; Samson, *et al.*, 2000; Saleem and Ebrahim, 2014; Anaya *et al.*, 2016; Oetari *et al.*, 2016; Okpalanozie *et al.*, 2018)
Aspergillus fumigatus Fresen.	(Das *et al.*, 1997; Nol *et al.*, 2001; Lourenço and Sampaio, 2005; Bankole, 2010)	(Di Bonaventura *et al.*, 2003; Krakova *et al.*, 2012)	(Mesquita *et al.*, 2009; Castillo *et al.*, 2016; Oetari *et al.*, 2016)	Turquoise-grey, dark green	Cellulases (+/+++) Amylases (+++) Gelatinases (+) Lipases (++) Proteases (Pasanen *et al.*, 1991; Das *et al.*, 1997; Tepsic *et al.*, 1997; Samson *et al.*, 2000; Chang *et al.*, 2004; Gopinath *et al.*, 2005; Janda *et al.*, 2009; Pitt and Hocking, 2009; Oyeleke *et al.*, 2010; Low *et al.*, 2011; Saleem and Ebrahim, 2014)
Aspergillus niger Tiegh	(Das *et al.*, 1997; Nol *et al.*, 2001; Lourenço and Sampaio, 2005; da Silva *et al.*, 2006; Michaelsen *et al.*, 2009; Bankole, 2010; Borrego *et al.*, 2012)		(Bergadi *et al.*, 2014; Oetari *et al.*, 2016; Pietrzak *et al.*, 2017; Coronado-Ruiz *et al.*, 2018; Krakova *et al.*, 2018; Okpalanozie *et al.*, 2018; Teixeira *et al.*, 2018)	Black/dark brown (melanin and hexahydroxyl pentacyclic quinoid colorants)	Cellulases (–/+++) Amylases (–/+++) Gelatinases (++) Proteases (+) (Fogarty and Kelly, 1979; Das *et al.*, 1997; Samson *et al.*, 2000; Parra and Magan, 2004; Gopinath *et al.*, 2005; Janda *et al.*, 2009; Jorgensen *et al.*, 2011; Borrego *et al.*, 2012; Bergadi *et al.*, 2014; Saleem and Ebrahim, 2014; Anaya *et al.*, 2016; Oetari *et al.*, 2016; Okpalanozie *et al.*, 2018)
Aspergillus terreus Thom	(Fabbri *et al.*, 1997; Ricelli *et al.*, 1999; Nol *et al.*, 2001; Zerek, 2003)	(Michaelsen *et al.*, 2010; Pinheiro, 2014)	(Pinheiro, 2014)	Yellow-brown	Cellulases (+++) Amylases (–/++) Gelatinases (+) (Samson *et al.*, 2000; Pitt and Hocking, 2009; Shahriarinour *et al.*, 2011; Saleem and Ebrahim, 2014)
Aspergillus versicolor (Vuill.) Tiraboschi	(Hyvarinen *et al.*, 2002; Zerek, 2003; Lourenço and Sampaio, 2005; da Silva *et al.*, 2006)	(Michaelsen *et al.*, 2009, 2010; Pinheiro, 2014; Okpalanozie *et al.*, 2018)	(Mesquita *et al.*, 2009; Sato *et al.*, 2014; Oetari *et al.*, 2016; Coronado-Ruiz *et al.*, 2018; Krakova *et al.*, 2018; Teixeira *et al.*, 2018)	White, yellow, orange, green, flesh, pink, greyish green	Cellulases (+/++) Amylases (+/+++) Gelatinases (+++) Lipases (+) (Reiss, 1978; Samson *et al.*, 2000; Pitt and Hocking, 2009; Adan and Samson, 2011; Borrego *et al.*, 2012; Saleem and Ebrahim, 2014; Oetari *et al.*, 2016)

Chaetomium globosum Kunze	(Das *et al.*, 1997; Szczepanowska and Cavaliere, 2000; Corte *et al.*, 2003; Lourenço and Sampaio, 2005)	(Rakotonirainy *et al.*, 2007)	(Mesquita *et al.*, 2009; Pietrzak *et al.*, 2017; Coronado-Ruiz *et al.*, 2018; Sequeira *et al.*, 2019)	Dark brown, chrome, slight yellowish or purplish tinge	Cellulases (+/+++) Amylases (++) Proteases (Florenzano, 1949; Lakshmikant, 1990; Das *et al.*, 1997; Sharma and Shukla, 2008; Pitt and Hocking, 2009; Abdel-Azeem *et al.*, 2016; Saleem and Ebrahim, 2014; Andersen *et al.*, 2016)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	(da Silva *et al.*, 2006; Zotti *et al.*, 2008)		(Mesquita *et al.*, 2009; Michaelsen *et al.*, 2009; Coronado-Ruiz *et al.*, 2018)	Olive, bluish grey or black	Cellulases (-/+++) Amylases (-/++) Lipases (-/+) Proteases (-/+) (Sautour *et al.*, 2002; Gopinath *et al.*, 2005; Pitt and Hocking, 2009; Borrego *et al.*, 2012; Saleem and Ebrahim, 2014; Anaya *et al.*, 2016; Coronado-Ruiz *et al.*, 2018)
Epicoccum nigrum Link	(Corte *et al.*, 2003)	(Michaelsen *et al.*, 2009; Pinheiro, 2014)	(Karakasidou *et al.*, 2018; Teixeira *et al.*, 2018)	Orange, brown, red, pink, yellow, green, black	Cellulases (+/++) Amylases (+/+++) Proteases (-) (Samson *et al.*, 2000; Pitt and Hocking, 2009; Favaro *et al.*, 2011)
Penicillium chrysogenum Thom	(Fabbri *et al.*, 1997; Ricelli *et al.*, 1999; Corte *et al.*, 2003; Michaelsen *et al.*, 2009; Borrego *et al.*, 2012)	(Di Bonaventura *et al.*, 2003; Michaelsen *et al.*, 2010; Karakasidou *et al.*, 2018)	(Mesquita *et al.*, 2009; Michaelsen *et al.*, 2009; Bergadi *et al.*, 2014; Sato *et al.*, 2014; Sterflinger and Engel, 2014; Pietrzak *et al.*, 2017; Coronado-Ruiz *et al.*, 2018; Krakova *et al.*, 2018; Teixeira *et al.*, 2018; Sequeira *et al.*, 2019)	Dull blue, greyish turquoise, dull green blue-green, yellow Cellulases (+/+++)	Amylases (-/+++) Proteases (-/+) (Samson *et al.*, 2000; Sautour *et al.*, 2002; Pitt and Hocking, 2009; Chinedu *et al.*, 2011; Bergadi *et al.*, 2014; Saleem and Ebrahim, 2014; Anaya *et al.*, 2016; Andersen *et al.*, 2016)
Penicillium citrinum Thom	(Corte *et al.*, 2003; Lourenço and Sampaio, 2005; da Silva *et al.*, 2006; Borrego *et al.*, 2012)	(Rakotonirainy *et al.*, 2007)	(Oetari *et al.*, 2016; Karakasidou *et al.*, 2018; Okpalanozie *et al.*, 2018; Sequeira *et al.*, 2019)	Greyish Orange, greyish turquoise; bright yellow	Cellulases (-/+++) Amylases (+/+++) Proteases (+) Lipases (+) (Samson *et al.*, 2000; Gopinath *et al.*, 2005; Pitt and Hocking, 2009; Borrego *et al.*, 2012; Saleem and Ebrahim, 2014; Okpalanozie *et al.*, 2018)
Penicillium commune Thom	(Michaelsen *et al.*, 2009; Borrego *et al.*, 2012)		(Bergadi *et al.*, 2014; Sato *et al.*, 2014; Krakova *et al.*, 2018; Sequeira *et al.*, 2019)	Greyish turquoise to dull green	Cellulases (+++) Amylases (+) Proteases (+) (Samson *et al.*, 2000; Pitt and Hocking, 2009; Borrego *et al.*, 2012; Bergadi *et al.*, 2014)
Penicillium spinulosum Thom	(Zerek, 2003; Lourenço and Sampaio, 2005; Zotti *et al.*, 2007, 2008)		(Pietrzak *et al.*, 2017; Krakova *et al.*, 2018)	Mycelium white; pinkish or pale brown; orange brown or red brown	Cellulases (+) (Ruegger and Tauk-Tornisielo, 2004; Pitt and Hocking, 2009)
Stachybotrys chartarum (Ehrenb.) S. Hughes	(Das *et al.*, 1997; Fabbri *et al.*, 1997; Ricelli *et al.*, 1999; Castillo *et al.*, 2016)		(Sato *et al.*, 2014; Sequeira *et al.*, 2019)	Black, grey, brown, olivaceous brown	Cellulases (-/+++) Amylases (+) (Samson *et al.*, 2000; Flannigan *et al.*, 2011; Saleem and Ebrahim, 2014; Sato *et al.*, 2014; Andersen *et al.*, 2016)

Note: Enzymatic potential is assigned with (+ + +) high; (+ +) medium; (+) low; or (−) null levels.

Adapted from Pinheiro, A.C., Sequeira, S.O., Dinis, M.F.M., 2019. Fungi in archives, libraries and museums: A review on paper conservation and human health. Crit. Rev. Microbio. doi:10.1080/1040841X.2019.1690420.

Stone bioreceptivity is higher on stones with higher values of open porosity and water absorption by capillarity rise (Miller *et al.*, 2006, 2010). Adhesion and settlement of organisms are also crucial aspects and these translate into surface roughness (Miller *et al.*, 2012).

Among the most frequent agents of biodeterioration of stone are algae, cyanobacteria, bacteria, lichens and, of course, fungi. Each of these microorganisms displays biochemical features that allow them to damage stone substrate, something called biochemical deterioration (Griffin *et al.*, 1991). And chemically, fungi produce a vast array of acids and these allow fungi to weaken and dissolve the stone matrix. This effect may, however, vary between fungal species and different mineral substrates (Gadd, 2007). Many of such acids, such as lactic, oxalic or gluconic can etch the stone and function as chelating agents, solubilizing and dragging cations out of the stone matrix in a process known as leaching (Griffin *et al.*, 1991). Oxalic acid, one of the most common organic acids excreted by fungi and calcium oxalate (as whewellite and weddellite, both responsible for the appearance of a yellow-brown film on stone), have been observed in close proximity to fungal hyphae in situ (Pinna, 1993; Wakefield and Jones, 1998). Being (mostly and in normal conditions) aerobic organisms, fungi also produce carbon dioxide which becomes carbonic acid when in contact with water or moisture and this contributes to dissolution of the stone and soluble salt formation (Griffin *et al.*, 1991).

Fungi can also act mechanically upon stone. Their hyphae can infiltrate more or less deeply into the stone substrate (Griffin *et al.*, 1991) and cycles of swelling and shrinkage ease water seepage which increases the inflicted damage when it freezes and thaws inside the stone (McNamara and Mitchell, 2005). Hyphae also make way for the transportation of organic compounds and nutrients making it an enticing environment for bacteria to thrive (McNamara and Mitchell, 2005). The forces exerted by hyphal progression can lead to substantial disruption of the stone matrix, lifting and flaking stone surface applications such as paint, pigment and consolidants (Wakefield and Jones, 1998).

Several reviews have been elaborated on the relationship between microorganisms and stone monuments (Gaylarde *et al.*, 2003; Griffin *et al.*, 1991; McNamara and Mitchell, 2005; Warscheid and Braams, 2000). Many studies separate the members of stone microbial communities since each displays specific traits, but studying them as community is also insightful since synergies appear to take place early on in order to assure the communities survival in the sometimes harsh stone environment (Wakefield and Jones, 1998).

The microbiological communities that normally act on stone develop in a succession. Fungi are heterotrophic and require the presence of organic material prior to their development (Griffin *et al.*, 1991). So, the microbial colonization of stones commonly starts with phototrophic organisms (such as algae, cyanobacteria, mosses and higher plants) which build up a visible protective biomass film enriched with carbohydrates, growth factors and antibiotics on an otherwise almost nutrient-depleted stone surface (Warscheid and Braams, 2000).

The chemolitoautotrophic (bacteria) and chemoorganotrophic (bacteria and fungi) microorganisms follow these first colonizers. While the first group is especially relevant for the transformation of inorganic compounds into acids (such as nitric, sulfuric and nitrous, which they later release into the stone) the chemoorganotrophic group uses organic substrates as energy source and release their also very corrosive organic acids (Warscheid and Braams, 2000). In general, fungi use a slightly narrower range of carbon-based substrates than bacteria and present a larger cell mass which enables them to accommodate considerable enzymatic machinery and produce a wide variety of potential stone deteriogens (Wakefield and Jones, 1998). These chemoorganotrophic fungi are able to penetrate into the rock material by hyphal growth and are usually found in stone crusts. They exert their biochemical activity due to the excretion of organic acids or by oxidation of mineral-forming cations, preferably iron and manganese (Burford *et al.*, 2003).

The type of fungus present in stone is related to the surrounding climate. The common airborne fungi – *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Aureobasidium*, *Phoma* and *Ulocladium* – are usually present in humid and moderate climates (Pinheiro *et al.*, 2018). *Exophiala*, a black yeast, has also been identified in stone (Warscheid and Braams, 2000) and when water and nutrient availability is reduced, lichens, black yeasts and microcolonial fungi – such as *Capnobotryella* – start to appear (Scheerer, 2008; Sterflinger and Piñar, 2013). Dematiaceous fungi (black fungi) are frequent in many stone types where they resemble carbonaceous deposits, which hampers their correct identification. By excreting melanins, they are responsible for the discoloration of stone surfaces and darkening of gypsum crusts (Wakefield and Jones, 1998; Warscheid and Braams, 2000). These melanins are persistent and function as a protection against UV radiation, predation, desiccation and increase cell resistance to pressure (Wakefield and Jones, 1998).

The aesthetic impact alone of the variations in color and texture of stone colonizers is an impetus for stone treatments such as cleaning or biocide application (Wakefield and Jones, 1998). At this point it is relevant to point out the ability exhibited by fungi to attack polymeric substances, including the ones conservators use for protective reasons: by-products of natural or synthetic substances applied during restoration treatments can act as a carbon source (Crispim and Gaylarde, 2005; Pinna, 1993; Scheerer *et al.*, 2009; Skipper and Skipper, 2014; Wakefield and Jones, 1998). This adds to the importance fungal contamination assessment has for stone cultural heritage (Warscheid and Braams, 2000) since the establishment of an heterotrophic community becomes possible even without the participation of phototrophs. After the community has been identified measures to reduce their impact and prevent further damage include reducing water exposure and nutrient obtainability. Biocides may be applied after compatibility tests are performed and care must be taken as artificial selection can clear the way for one species to take over (Warscheid and Braams, 2000) (see Section "Treatment and Preventive Measures for Fungal Contamination in Cultural Heritage" for more details).

Inorganic Supports: Ceramics and Glazed Tiles

Traditional ceramics are clay-based and, putting it in simple terms, are made of clay mixed with water and shaped during the forming process. After this is achieved the object is left to dry in order to become harder and submitted to fire for permanent

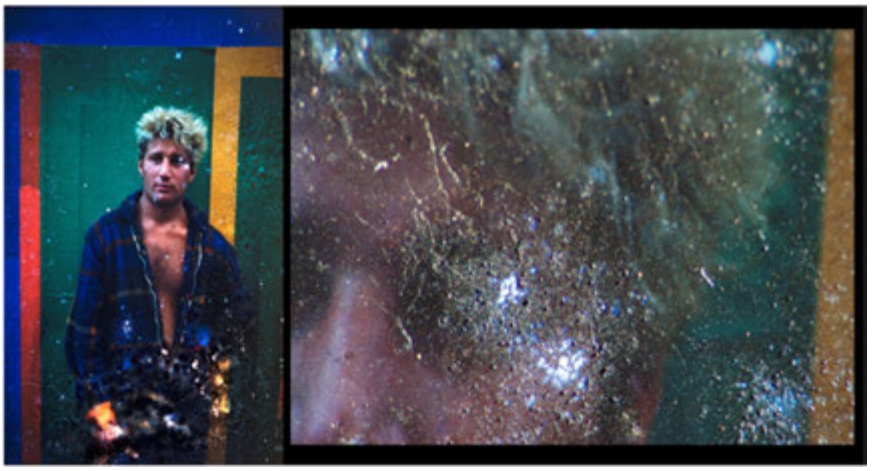

Fig. 4 Color slide colonized by filamentous fungi – left: full image; right: detail, where image loss is already observed. © Nederlands Fotomuseum/Ed van der Elsken, Photo credits: Katrin Pietsch.

hardening. Both type of raw materials and temperature used to harden the ceramic body are extremely relevant in the final composition, texture and porosity. Sometimes, the final product receives a finishing coating: engobe (colored coating of fine-grained clay that is applied over the ceramic body to reduce surface roughness) or glaze (vitreous coating used to enhance mechanical strength). Glaze finishes are more common on floor and wall tiles. One of the most beautiful elements used in built Cultural Heritage is the glazed wall tile which, by Near East influence, gained great importance in Portugal, Spain and later in Brazil (Coutinho *et al.*, 2015; Oliveira *et al.*, 2001; Pereira *et al.*, 2009).

Biological deterioration and its extent in glazed wall tiles is, as happens in stone, dependent on a series of preexisting conditions. Rainwater, sun heat, air currents and pollution affect outdoor glazed ceramics while inside microclimatic conditions such as liquid water and vapor ascending through the porous system of the ceramic body may also give rise to pathologies. A network of fissures is formed, soluble salts may arise on the surface and the glaze fracture is followed by subsequent detachment (Silva *et al.*, 2013a,b). Characteristics of the substrate are also worth mentioning. As also happened with stone, porosity and surface roughness play key roles in determining primary bioreceptivity in glazed wall tiles but because these are coated with a crystalline phase embedded in a glassy matrix this impermeable coating is very smooth. Pristine glazed materials tend to display a higher resistance to microbial colonization (Coutinho *et al.*, 2015). Of course, this changes once the substrates show the effects of environmental conditions and they become liable to the development of microorganisms. The microorganisms encountered in architectural ceramics, glazed tiles included, are similar to the ones mentioned for stone. A dark tarnishing detected on 19th century outdoor polychrome tiles in Brazil was attributed to Cyanophyte and Bacillariophyte algae (Oliveira *et al.*, 2001; Silva *et al.*, 2013a,b), colored alterations noticed in 20th century polychrome tiles covering the facade of an "Art Nouveau" building in Italy were attributed to cryptoendolithic cyanobacteria (Silva *et al.*, 2013a) and algae/fungi may give rise to green stains, particularly in blue-and-white glazes from the 16th to the 18th century (Silva *et al.*, 2013b).

Unlike stone and other substrates and despite being described as a common malaise very few studies have sought to identify the colonizing microorganisms on outdoor ceramic materials along with their mechanisms and effects (Coutinho *et al.*, 2015; Sand, 1997) on outdoor ceramic materials. The less studied ceramics typology are the glazed wall tiles (13%) but because these are classified as cultural heritage the relevance of these studies gains importance and they are predicted to increase in future times. Also, being classified as cultural heritage means such assessments on glazed wall tiles can trace an eventual association between a given community and the tile's composition, since this is known and crucial before any conservation intervention. Eight studies are presented in the review by Coutinho *et al.* (2015) and include bacteria, cyanobacteria, algae and fungi. Nonetheless, lichens and bryophytes can also be found. Regarding fungi, **Table 3** presents the species or genera identified in glazed tiles, the method used to identify them and the reference study.

Most are Ascomycota (90%) and only a minor part is represented by Basidiomycota. The fungi *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium* and *Phoma* were also detected in bricks (not shown). Based on the fact they are widespread airborne fungi with a large capacity to survive in different environments, their presence in all substrates is not surprising. Fungi display the wider diversity when compared with other ceramic architectural feature, such as bricks and roofing tiles (Coutinho *et al.*, 2015).

Lichens are also worth mentioning when discussing glazed tiles (and stone, for that matter). They consist of algal and fungal cells in close association, forming a large thallus which is visually detected, hence its importance in cultural heritage. Lichens are associated with the formation of a calcium oxalate patina (McNamara and Mitchell, 2005; Sabbioni and Zappia, 1991) resulting from the reaction of oxalic acid with the matrix. Although the release of corrosive acids is seen as damage inducing in this case this patina can help prevent the colonization by black fungi (Ascaso *et al.*, 1998; Pinna, 2014) (**Fig. 5**).

As happens for other materials used to create Cultural Heritage and on which biodeterioration occurs, the use of classic culturing methods coupled with molecular methods delivers a more trustworthy scenario (Coutinho *et al.*, 2015, 2013;

Table 2 Fungal species identified from photographic materials. Identification methods: CD – culture dependent; CI – culture independent; CDMB – culture dependent molecular biology

Fungal species	*Identification methods*	*Photographic material*	*References*
Alternaria sp.	CI	Prints, albumen prints	(Bucková *et al.*, 2014; Puškárová *et al.*, 2016)
Aspergillus nidulans	CD	Glass negatives	(Zyska *et al.*, 1988)
Aspergillus niger	CD	Prints	(Guiamet *et al.*, 2011)
Aspergillus penicilioides	CI	Albumen prints	(Puškárová *et al.*, 2016)
Aspergillus reptans	CD	Glass negatives	(Zyska *et al.*, 1988)
Aspergillus sp.	CD; CI	Prints; film negatives; slides; tintype; ambrotype, albumen print	(Borrego *et al.*, 2015, 2010; Lavin *et al.*, 2014; Lourenço and Sampaio, 2005; Puškárová *et al.*, 2016)
Aspergillus versicolor	CD; CDMB	Glass negatives; albumen prints	(Puškárová *et al.*, 2016; Zyska *et al.*, 1988)
Basipetospora sp.	CD	Prints; film negatives	(Lourenço and Sampaio, 2005)
Beauveria brongniartii	CI	Negative films	(Bucková *et al.*, 2014)
Bjerkandera adusta	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Ceriporiopsis gilvescens	CI	Prints	(Bucková *et al.*, 2014)
Cordyceps sp.	CI	Negative films	(Bucková *et al.*, 2014)
Chaetomium elatum	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Chaetomium globosum	CI	Albumen prints	(Puškárová *et al.*, 2016)
Cladosporium chladosporioides	CD, CI	Glass negatives; prints; film negatives; slides	(Lourenço and Sampaio, 2005; Sclocchi *et al.*, 2017; Zyska *et al.*, 1988)
Cladosporium macrocarpum	CI	Prints	(Sclocchi *et al.*, 2017)
Cladosporium ramotenellum	CI	Albumen prints, prints	(Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017)
Cladosporium sp.	CI; CD	Prints; negative films; albumen prints	(Borrego *et al.*, 2015; Bucková *et al.*, 2014; Puškárová *et al.*, 2016)
Coprinellus bisporus	CI	Prints	(Bucková *et al.*, 2014)
Corynespora sp.	CI	Albumen prints	(Puškárová *et al.*, 2016)
Eurotium halophilicum	CI	Albumen prints, prints	(Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017)
Eurotium sp.	CD	Tintype, albumen print	(Borrego *et al.*, 2015)
Fusarium sp.	CD	Prints	(Grbić *et al.*, 2013)
Galactomyces geotrichum	CI	Prints, negative films	(Bucková *et al.*, 2014)
Geotrichum sp.	CI	Albumen prints, prints	(Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017)
Gnomonia setacea	CI	Albumen prints	(Puškárová *et al.*, 2016)
Gymnopus erythropus	CI	Negative films	(Bucková *et al.*, 2014)
Humicola sp.	CD	Prints	(Grbić *et al.*, 2013)
Hyphodontia nespori	CI	Prints	(Bucková *et al.*, 2014)
Hyphodontia radula	CI	Prints	(Sclocchi *et al.*, 2017)
Malassezia sp.	CI	Albumen prints	(Puškárová *et al.*, 2016)
Malassezia globosa	CI	Prints	(Sclocchi *et al.*, 2017)
Malassezia restricta	CI	Prints	(Sclocchi *et al.*, 2017)
Mucor plumbeus	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Nectria sp.	CI	Albumen prints	(Puškárová *et al.*, 2016)
Nectria haematococca	CI	Prints	(Sclocchi *et al.*, 2017)
Paecilomyces lilacinus	CD	Glass negatives	(Zyska *et al.*, 1988)
Paecilomyces variotti	CD	Glass negatives; prints	(Grbić *et al.*, 2013; Zyska *et al.*, 1988)
Penicillium canescens	CD	Glass negatives	(Zyska *et al.*, 1988)
Penicillium chrysogenum	CDMB	Albumen prints; prints	(Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017)
Penicillium cyaneum	CD	Glass negatives	(Zyska *et al.*, 1988)
Penicillium cyclopium	CD	Glass negatives	(Zyska *et al.*, 1988)
Penicillium expansum	CD	Glass negatives	(Zyska *et al.*, 1988)
Penicillium simplicissimum	CD	Glass negatives	(Zyska *et al.*, 1988)
Penicillium sp.	CD, CDMB	Prints; film negatives; slides; glass slides; tintype; ambrotype	(Borrego *et al.*, 2015, 2010; Guiamet *et al.*, 2011; Lavin *et al.*, 2014; Lourenço and Sampaio, 2005; Puškárová *et al.*, 2016; Sclocchi *et al.*, 2017)
Penicillium thomii	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Phlebia sp.	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Pleosporales sp.	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Pleurotus pulmonarius	CDMB	Albumen prints	(Puškárová *et al.*, 2016)
Rhodotorula sp	CD	Glass negatives	(Zyska *et al.*, 1988)
Rhizopus microsporus	CI	Negative films	(Bucková *et al.*, 2014)
Toxicocladosporium strelitziae	CI	Prints	(Bucková *et al.*, 2014)
Trebouxia impressa	CI	Albumen prints	(Puškárová *et al.*, 2016)
Trichoderma viride	CD	Prints	(Grbić *et al.*, 2013)
Trichosporum aquatile	CI	Albumen prints	(Puškárová *et al.*, 2016)
Ulocladium sp.	CD	Prints	(Grbić *et al.*, 2013)

Table 3 Fungal species identified from ceramic glazed tiles. Identification methods: CD – culture dependent; MB – molecular biology

Fungi	*Method used*	*References*
Alternaria sp.	CD	(Coutinho *et al.*, 2011)
Aspergillus (flavus; sydowii; versicolor)	CD	(Pedi *et al.*, 2009)
Arthrinium sp.	CD	(Coutinho *et al.*, 2011)
Aureobasidium pullulans	MB	(Giacomucci *et al.*, 2011)
Capnobotryella sp.	MB	(Coutinho *et al.*, 2013)
Capronia sp.	MB	(Coutinho *et al.*, 2013)
Chrysosporium sp.	CD	(Coutinho *et al.*, 2013)
Cladosporium sp.	CD	(Coutinho *et al.*, 2013)
Devriesia imbrexigena	CD	(Crous *et al.*, 2012)
Epicoccum sp.	CD	(Coutinho *et al.*, 2011)
Fellomyces sichuanensis	MB	(Coutinho *et al.*, 2013)
Fusarium sp. (solani)	CD	(Pedi *et al.*, 2009)
Geotrichum candidum	CD	(Pedi *et al.*, 2009)
Gliocladium sp.	CD	(Coutinho *et al.*, 2011)
Hortaea thailandica	MB	(Coutinho *et al.*, 2013)
Kockovaella schimae	MB	(Coutinho *et al.*, 2013)
Monodictys levis	CD	(Pedi *et al.*, 2009)
Neofusicoccum sp.	CD	(Coutinho *et al.*, 2011)
Penicillium sp. (citrinum, fellutanum, waksmanii)	CD	(Pedi *et al.*, 2009)
Pestalotiopsis sp.	CD	(Coutinho *et al.*, 2011)
Phoma herbarum	MB	(Giacomucci *et al.*, 2011)
Saccharata sp.	MB	(Coutinho *et al.*, 2013)
Sporobolomyces coprosmae	MB	(Giacomucci *et al.*, 2011)
Stigmina sp.	MB	(Coutinho *et al.*, 2013)
Trichoderma sp.	CD	(Coutinho *et al.*, 2011)
Non-identified fungi	Scanning Electron Microscope (SEM)	(Costa *et al.*, 2013)
Non-identified yeasts	CD	(Coutinho *et al.*, 2011)

Fig. 5 (A) Glazed ceramic street name sign, Travessa das Peras, in Évora, Portugal; (B) detail, where lichens and surface detachment is visible. Photo credits: Catarina Pinheiro.

McNamara and Mitchell, 2005; Sequeira *et al.*, 2019). Classic culturing methods give us insight on the viability of the fungal community as well as allowing the metabolic study of the identified species. However, because not all collected samples are composed of viable specimens and/or we may not be giving these specimens the right media to thrive, molecular biology protocols are programmed to, ideally, deliver us all the viable, unviable, dead and alive elements of the fungal population. Technique limitations such as DNA extraction, Polymerase chain reaction inhibition or preference for one species over the other or even overwhelming amount of data per sample (as is the case when using NGS to identify entire communities) are some of the reasons why a polyphasic approach, such as the one performed by Coutinho *et al.* (2013) and mentioned in **Table 1**, is preferable. SEM imaging is perfect to detect if a fungal element is present in a damaged or corroded area of the artefact but is very hard to achieve an identification by its sole use.

Treatment and Preventive Measures for Fungal Contamination in Cultural Heritage

It is now an accepted fact that fungi cause severe chemical and physical damage on virtually all supports used to create cultural heritage. Some of them were described above. The defacing and eventual loss of reading urges those responsible to prevent fungal contamination altogether, diminish its impact or recover the damaged artefacts.

The costs increase substantially once there are "infected" artefacts or spaces and there is a need to advance to treatment options (Sterflinger and Piñar, 2013). The chemical methods available to perform this are restricted by two rules: (1) they must be

compatible with historic materials and (2) they must comply with health and environment regulations. Few fall within this scope (Sequeira *et al.*, 2012). One of the most famous biocides used to treat fungi contaminated documents was ethylene oxide, very effective but also very damaging and toxic (Sequeira *et al.*, 2012). Its effects can still be noted today when fumigated books are opened for reading or conservation purposes.

A biocide treatment also brings an additional concern. Very often, as shown in Section "Fungal Biodeterioration Mechanisms and Impact on Cultural Heritage", there is a mixed community of microorganisms and their susceptibility to the biocide applied varies. It is fairly easily to understand the selective pressure this puts on the microbial community, and the Lascaux Cave is a perfect example of the effects successive biocide treatments can have: the over development of white fungal stains by *Fusarium solani*, *Pseudomonas fluorescens* (a bacteria), and black fungal species such as *Ochroconis lascauxensis*, *O. anomala* and *Exophiala castellanii* (Sterflinger and Piñar, 2013).

As far as physical methods are concerned, gamma radiation is very effective against fungi and their spores (Silva *et al.*, 2013a). However, the necessary dose to achieve the desired results must be in excess of 10–20 kGy and so this method may also affect many historic materials. So, for the time being gamma radiation is restricted to specific cases and must be closely monitored not just for its safety but also to measure its effectiveness. And so is the case for anoxia and heat/cold or modification of light treatments (Sequeira *et al.*, 2012; Sterflinger and Piñar, 2013).

Prevention-wise, performing periodic biological contamination assessments and monitoring the cultural heritage assets is pivotal (de Carvalho *et al.*, 2018; Silva *et al.*, 2013a). To effectively prevent fungal damage it is also necessary to assure climate control and frequent cleaning (Sterflinger and Piñar, 2013). Measuring RH and temperature on the environment surrounding a material can provide a generic indication of the risk of microbial biodeterioration, but care must be taken since these measurements are usually performed in a determined area of collections repositories, not reflecting the conditions in every niche and corner. Local microclimates with very high water activity can be generated by ineffective ventilation and surface temperature inhomogeneity in a room with an otherwise low RH (Sterflinger and Pinzari, 2012). Also, in already colonized materials, the humidity preserving biofilm will help fungi survive in lower environmental HR (Camuffo, 1998). By keeping the water activity of cultural heritage materials under 0.60, and temperature less than 20°C, with a good air circulation, the development of fungi can be avoided (Allsopp *et al.*, 2004; Arai, 2000; Florian, 2002; Raschle, 2001; Valentin, 2010). Nevertheless, the technology and/or building characteristics needed for an efficient climate control are not available to all the heritage repository institutions in the world.

UNESCO (2007) considers climate change as one of the most serious threats impacting on the conservation of cultural heritage. With climate change, the intensity, frequency and seasonality of extreme events, such as intense rainfall or floods, are projected to increase, together with a rise in global mean temperatures. This will lead to an increased risk of biodeterioration, even in altitudes and latitudes that may not have been previously concerned by such threats (UNESCO, 2007). This increased risk intensifies the need for an urgent development of control and mitigation strategies to protect our cultural heritage from biodeterioration.

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How to Asses Fungal Contamination in School Environments

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Introduction

Indoor air quality (IAQ) includes biological and non-biological components that can affect the health and wellbeing of humans (Awad *et al.*, 2018). The biological components of the air in a building (such as the mycobiota) can cause a number of health problems, including immunologic reactions, toxicity, infection, and irritation (Awad *et al.*, 2018; Seltzer and Fedoruk, 2007). As such, the air quality in indoor buildings is of the upmost importance (Aydogdu *et al.*, 2005).

The microorganisms in indoor air have different sources, mostly outdoor air or anthropogenic sources, but, and because fungi can use all organic materials, including building materials, they thrive in an indoor environment, especially in humid conditions (Salonen *et al.*, 2015; Awad *et al.*, 2018).

The IAQ becomes even more important when we focus on buildings like schools, due to the commonality of respiratory illnesses in children, especially asthma (Aydogdu *et al.*, 2005; Su *et al.*, 2001). Several factors can be connected with childhood asthma, including environmental factors; also, it has been shown that children who live in damp and moldy buildings have more respiratory symptoms than children who live in less damp places (Su *et al.*, 2001). Because children have been shown to spend about 85% of their time in either school or at home, it is very important to study the air quality in both these dwellings (Su *et al.*, 2001).

Additionally, the association between moisture damage in school buildings, microbial presence due to excess moisture and the adverse health effects has been described previously (Aydogdu *et al.*, 2005; Hussin *et al.*, 2011; Meklin *et al.*, 2002). Still, there are no health-based guidelines for indoor dampness or microbial contamination (Karvala, 2012; WHO, 2009).

Because young children are more vulnerable to exposure to their environment than adults, and because fungal contamination can be used to assess the quality of indoor air, studying the fungal contamination in a building, in this case school buildings, is one way to assess the IAQ in schools (Cabral, 2010; Awad *et al.*, 2018).

This study aims to perform a scope review regarding the fungal contamination assessments performed on school's indoor environment and to determine a protocol for fungal contamination assessment to be used by the competent authorities and researchers.

Materials and Methods

This study reported the search of available data published between January 1st, 2000 and December 31st, 2018. The initial search terms were developed to identify research in the IAQ assessment in scholastic environments, focusing on the fungi contamination assessment. The databases chosen were PubMed, Scopus and Web of Science (WoS). Searches were carried out in English. The search terms via PubMed were "fungal contamination in school environments". This search strategy led to 3573 papers in all databases. Articles that did not meet the inclusion criteria and duplicates were excluded from further analysis (**Table 1**).

The diagram describes the different phases of the selection of papers (**Fig. 1**).

Results

A total of 21 articles were analyzed. Air sampling was performed with different techniques in the different studies, most commonly by active air sampling through impaction method (9 out of 21 studies) (Dacarro *et al.*, 2003; Viegas *et al.*, 2010; Hussin *et al.*, 2011; Roda *et al.*, 2011; Chen *et al.*, 2012; Rojas and Aira, 2012; Madureira *et al.*, 2015; Salonen *et al.*, 2015; Hoseinzadeh *et al.*, 2017), or two or more complementing techniques (in 6 out of the 21 studies) (Santilli and Rockwell, 2003; Wilson *et al.*, 2004; Godwin and Batterman, 2007; Cai *et al.*, 2009; Norbäck *et al.*, 2016; Awad *et al.*, 2018). Other techniques used for air fungal contamination assessment included air filtration (Santilli and Rockwell, 2003; Godwin and Batterman, 2007), settled dust (Santilli and Rockwell, 2003; Cai *et al.*, 2009; Norbäck *et al.*, 2014, 2017; Awad *et al.*, 2018), electrostatic dust collector (Vesper *et al.*, 2015), spore traps (Wilson *et al.*, 2004), surface swabs (Wilson *et al.*, 2004; Cai *et al.*, 2009; Norbäck *et al.*, 2016), tape lift method (Wilson *et al.*, 2004), and open plate method (Aydogdu *et al.*, 2005; Aydogdu and Asan, 2008; Norbäck *et al.*, 2016; Reddy and Srinivas, 2017).

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Table 1 Inclusion and exclusion criteria in the articles selected

Inclusion criteria	*Exclusion Criteria*
Articles in English language	Articles in other languages (for articles)
Articles published from January 1st, 2000 to December 31st, 2018	Articles published prior to 2000
Articles published anywhere	
Articles related to fungi assessment in school environments	Articles related to biologic samples from students (exclusive)
Articles related to school environments up to high school (including kindergarten)	Articles containing information from university environments
Scientific original articles on the topic	Abstract of congress, Reviews/State of the art, reports: used for discussion
Identification of fungi to the genera level (at least).	

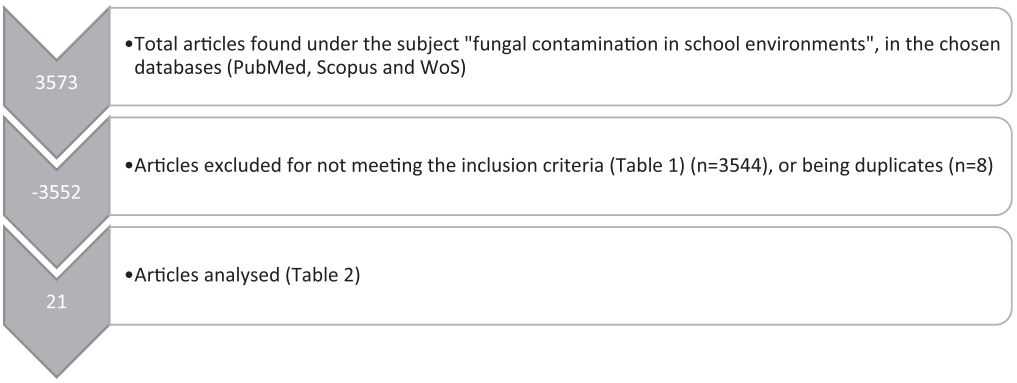

Fig. 1 Phases of the selection of articles.

Fungal identification was performed by either morphological identification (in 14 out of the 21 studies) (Dacarro *et al.*, 2003; Wilson *et al.*, 2004; Aydogdu *et al.*, 2005; Aydogdu and Asan, 2008; Viegas *et al.*, 2010; Hussin *et al.*, 2011; Roda *et al.*, 2011; Chen *et al.*, 2012; Rojas and Aira, 2012; Salonen *et al.*, 2015; Hoseinzadeh *et al.*, 2017; Reddy and Srinivas, 2017; Awad *et al.*, 2018) or by fungal DNA detection (in 5 out of the 21 studies) (Cai *et al.*, 2009; Norbäck *et al.*, 2014, 2016, 2017; Vesper *et al.*, 2015), but never with both techniques. Also, in 2 of the studies, the fungal identification techniques were not sufficiently described (Santilli and Rockwell, 2003; Godwin and Batterman, 2007).

For all studies the most common fungal genera found were described, but some studies complemented this information with seasonal variations (4 out of 21 articles) (Aydogdu and Asan, 2008; Roda *et al.*, 2011; Reddy and Srinivas, 2017; Awad *et al.*, 2018), sampling site variations (2 out of 21 articles) (Godwin and Batterman, 2007; Roda *et al.*, 2011), and indoor/outdoor air contamination comparisons (10 out of 21 articles) (Dacarro *et al.*, 2003; Santilli and Rockwell, 2003; Wilson *et al.*, 2004; Godwin and Batterman, 2007; Aydogdu and Asan, 2008; Viegas *et al.*, 2010; Hussin *et al.*, 2011; Roda *et al.*, 2011; Salonen *et al.*, 2015; Awad *et al.*, 2018).

The most commonly found fungi genera were either *Aspergillus*/*Penicillium* (in 5 out of the 21 articles) (Godwin and Batterman, 2007; Cai *et al.*, 2009; Norbäck *et al.*, 2014, 2016, 2017), *Cladosporium* (in 5 out of the 21 studies) (Wilson *et al.*, 2004; Aydogdu and Asan, 2008; Viegas *et al.*, 2010; Chen *et al.*, 2012; Madureira *et al.*, 2015), *Penicillium* (also in 5 studies) (Aydogdu *et al.*, 2005; Roda *et al.*, 2011; Rojas and Aira, 2012; Salonen *et al.*, 2015; Hoseinzadeh *et al.*, 2017), *Aspergillus* (in 4 studies) (Santilli and Rockwell, 2003; Hussin *et al.*, 2011; Reddy and Srinivas, 2017; Awad *et al.*, 2018), or *Alternaria* (in 1 study) (Dacarro *et al.*, 2003).

From the ten studies that highlight the indoor/outdoor air contamination differences, three articles study only fungal species differences (Aydogdu and Asan, 2008; Viegas *et al.*, 2010; Hussin *et al.*, 2011), while two of them show higher fungal contamination in indoor environments (Dacarro *et al.*, 2003; Santilli and Rockwell, 2003), and five higher fungal counts outdoors (Wilson *et al.*, 2004; Godwin and Batterman, 2007; Roda *et al.*, 2011; Salonen *et al.*, 2015; Awad *et al.*, 2018) (**Table 2**).

Discussion

The present study focuses on the fungal contamination assessments performed in school environments made in the past twenty years. *Aspergillus*, *Penicillium*, *Cladosporium*, and *Alternaria* were the fungal genera most often described as dominant in the indoor air from all the selected papers. *Aspergillus* spp. was the most often connected with respiratory infections, as well as Mucorales order (Chowdhary *et al.*, 2016). Among *Aspergillus* genera, one of the most relevant concerns is their toxigenic potential (Viegas *et al.*,

Table 2 Data obtained from the chosen articles

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Fungal identification*	*Conclusions*	*References*
PubMed	"Determination of aerial microbiological contamination in scholastic sports environments"	Italy	11 high school and college gyms in the province of Pavia, Italy	Air impaction	Morphological identification	Most common fungi during heated periods: *Alternaria*, *Cladosporium*, and *Penicillium*. Most common fungi during non-heated periods: *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum nigrum*, *Fusarium*, *Penicillium,* and *Phoma*. *Aspergillus* and *Epicoccum nigrum* were always present. Indoor contamination was always superior to outdoor contamination.	Dacarro *et al.* (2003)
	Fungal contamination of elementary schools: a new environmental hazard	USA	Two public schools in southern Connecticut.	Air impaction, filter cassette air sampling and settled dust		For school 1: In 70% of the indoor samples the fungal counts exceeded the outdoor values; *Alternaria*, *Botrytis*, *Curvularia*, *Epicoccum*, and *Stachybotrys* were detected only in indoor samples. *Aspergillus*, *Cladosporium*, and *Penicillium* were found both indoors and outdoors, but the levels of *Aspergillus* and *Penicillium* were higher in some indoor sites. For school 2: total mold spore counts ranging from 2600 to 9000 spores. m^{-3}. The most affected rooms were the Special Education room and gymnasium.	Santilli and Rockwell (2003)
	Indoor air quality in Michigan schools	USA	Four elementary schools and five middle schools in southeast Michigan. Within each school, at least one art room, one miscellaneous use room, two classrooms, two science rooms, and two offices were selected.	Filter cassette air sampling	Morphological identification	Five mold genera were detected, with *Aspergillus*/ *Penicillium* as the most common, followed by *Cladosporium*. For most species and schools, outdoor levels exceeded indoor levels, except for *Aspergillus*/ *Penicillium*. Portable classrooms had higher bioaerosol concentrations than conventional classrooms.	Godwin and Batterman (2007)
	"Airborne fungi and bacteria in child day care centers and the effectiveness of weak acid hypochlorous water on controlling microbes"	Taiwan	Child day care centers	Air impaction	Morphological identification	Most commonly found fungi: *Cladosporium*, Yeast, *Nonsporium*, and *Penicillium*. Toxigenic *Aspergillus versicolor* and *A. flavus* detected.	Chen *et al.* (2012)

	Endotoxin, Ergosterol, Fungal DNA and Allergens in Dust from Schools in Johor Bahru, Malaysia-Associations with Asthma and Respiratory Infections in Pupils	Malaysia	Eight junior high schools in Johor Bahru, Malaysia.	Settled dust	Fungal DNA detection	Total fungal DNA and Asp/Pen DNA were found in all dust samples. *A. versicolor* DNA, *S. chartarum* DNA, and *Streptomyces* DNA were detected in 87%, 31%, and 31% of the classrooms, respectively. *S. chartarum* DNA was common in low levels.	Norbäck *et al.* (2014)
	Mold contamination in schools with either high or low prevalence of asthma	USA	A water-damaged school and a comparison school.	EDC sampling	Fungal DNA detection	Ten Group 1 molds, including *Aspergillus ochraceus* and *A. unguis*, were found in greater concentrations in the water-damaged school samples. Two Group 2 molds, *Aspergillus ustus* and *Cladosporium cladosporioides,* were significantly more abundant in the water-damaged school, while *Cladosporium herbarum* was more abundant in the comparison school.	Vesper *et al.* (2015)
	Ocular symptoms and tear film break up time (BUT) among junior high school students in Penang, Malaysia – Associations with fungal DNA in school dust	Malaysia	Eight junior high schools in Penang, Malaysia.	Settled dust	Fungal DNA detection	Fungal DNA and Asp/Pen DNA were found in all vacuumed dust samples. *A. versicolor* DNA was detected in 67% of the classrooms and *Streptomyces* DNA in 94%. *S. chartarum* DNA was found in one classroom (low levels).	Norbäck *et al.* (2017)
Scopus	Identification, Remediation, and Monitoring Processes Used in a Mold-Contaminated High School	USA	3-year-old high school	Air impaction, spore traps, surface swabs and tape lift sampling	Morphological identification	Fungal concentrations inside were lower than outside. *Cladosporium* sp. was the dominant fungi in the outside air. Inside counts reflected the outside counts.	Wilson *et al.* (2004)
	Airborne culturable fungi in naturally ventilated primary school environments in a subtropical climate	Australia	25 randomly selected primary schools in the Brisbane Metropolitan Area, Australia.	Air impaction	Morphological identification	Indoor fungi concentrations were lower than the outdoor concentrations. *Penicillium* most common fungi indoor, followed by *Cladosporium* and *Aspergillus*.	Salonen *et al.* (2015)
	Mold Allergens in Indoor Play School Environment	USA	Two play schools.	Open Petri-plate method	Morphological identification	Most common fungi: *Aspergillus*, *Cladosporium*, *Penicillium, Alternaria, Fusarium*, and *Curvularia*. Monsoon season showed less concentration of mold spores compared to winter season.	Reddy and Srinivas (2017)
Web of Science	Monitoring of Fungi and Bacteria in the Indoor Air of Primary Schools in Edirne City, Turkey	Turkey	Classroom, corridor, and canteen of five primary schools	Petri Plate Gravitational Settling Method	Morphological identification	*Penicillium, Cladosporium*, and *Alternaria* were the most common fungi.	Aydogdu *et al.* (2005)

(*Continued*)

Table 2 Continued

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Fungal identification*	*Conclusions*	*References*
Airborne fungi in child day care centers in Edirne City, Turkey	Turkey		Classroom, sleeping room, and eating room of four child day care centers (CDCC)	Petri Plate Gravitational Settling Method	Morphological identification	The highest fungal concentration was found in June, and the lowest in January and February. *Cladosporium, Penicillium, Alternaria, Aspergillus, Epicoccum*, and *Stemphylium* were the most common genera, both indoor and outdoor, with differences in sequence.	Aydogdu and Asan (2008)
Quantitative PCR analysis of fungal DNA in Swedish day care centers and comparison with building characteristics and allergen levels	Sweden	Eleven	allergen-avoidance day care centers (AADCs) and eleven ordinary day care centers (ODCs)	Surface swabs and settled dust	Fungal DNA detection	Total fungal DNA was detected in 91%, Asp/Pen DNA in 34% and *S. chartarum* DNA in 6% of all rooms.	Cai *et al.* (2009)
Air fungal contamination in two elementary schools in Lisbon, Portugal	Portugal	Canteen,	library, and classrooms of two elementary schools in Lisbon, Portugal	Air impaction	Morphological identification	The two most commonly isolated fungi were *Cladosporium* sp. and *Penicillium* sp. The youngest school presented 8 different genera and the oldest presented 11 different genera. All the indoor spaces from both schools presented fungal species different from outdoor ones.	Viegas *et al.* (2010)
Characterization of Bacteria and Fungi Bioaerosol in the Indoor Air of Selected Primary Schools in Malaysia	Malaysia	Five	primary schools in Hulu Langat, Malaysia	Air impaction	Morphological identification	The most common indoor airborne fungi were *Aspergillus, Penicillium, Fusarium, Rhizopus, and Zygomycetes*.	Hussin *et al.* (2011)
Assessment of indoor environment in Paris child day care centers	France		Playroom and bedrooms of 28 Parisian child day care centers.	Air impaction	Morphological identification	Most common fungi were *Penicillium* and Cladosporium, followed by *Aspergillus*. Whatever the season, indoor total airborne fungal levels were higher in playrooms than in bedrooms, and outdoor total fungi levels were significantly higher than indoor levels. Levels of predominant genera were higher in hot season.	Roda *et al.* (2011)

Fungal biodiversity in indoor environments in Havana, Cuba	Cuba	One school in	Havana, Cuba	Air impaction	Morphological identification	*Penicillium* sp. was the most common species, followed by *Chrysonilia* sp.	Rojas and Aira (2012)
Assessment and determinants of airborne bacterial and fungal concentrations in different indoor environments: Homes, child day care centers, primary schools and elderly care centers	Portugal	50	classrooms in 9 child day care centers; 73 classrooms in 20 primary schools	Air impaction	Morphological identification	Most common fungi in child day care centers: *Cladosporium* sp., *Penicillium* sp., *Rhodotorula* sp., and *Aspergillus* sp. Most common fungi in primary schools: *Cladosporium* sp., *Penicillium* sp., *Rhodotorula* sp., and *Alternaria* sp.	Madureira *et al.* (2015)
Rhinitis, Ocular, Throat and Dermal Symptoms, Headache and Tiredness among Students in Schools from Johor Bahru, Malaysia: Associations with Fungal DNA and Mycotoxins in Classroom Dust	Malaysia	Eight	secondary schools in Johor Bahru, Malaysia.	Surface swab and open Petri dish sampling	Fungal DNA identification	Total fungal DNA and Asp/Pen DNA detected in all classrooms in both sampling methods. *A. versicolor* DNA detected in 70% of the Petri dish samples, while *S. chartarum* was found in 13% and *Streptomyces* DNA in 87% of the samples. *A. versicolor* DNA was detected in 56% of the swab samples, while *S. chartarum* DNA was detected in 3% and *Streptomyces* DNA in 28% of the samples.	Norbäck *et al.* (2016)
Indoor air fungus bioaerosols and comfort index in day care child centers	Iran	Four	different day care centers (DCC) all located at urban sites.	Air impaction	Morphological identification	The most common fungi were *Penicillium* spp. and *Cladosporium* spp.	Hoseinzadeh *et al.* (2017)
Air microbial quality in certain public buildings, Egypt: A comparative study	Egypt	Two	schools and two child day care centers.	Air impaction and settled dust	Morphological identification	Higher fungal concentrations were found in the winter indoors. *Aspergillus, Penicillium, Cladosporium* and *Alternaria* were the dominant fungi. Fungal concentrations were always higher in the outdoor environment, except in one of the primary schools.	Awad *et al.* (2018)

2019a). *Aspergillus flavus* is the main producer of the carcinogenic aflatoxin B1, and the cause for the most aflatoxin contaminations in food and feed; ochratoxin A, another mycotoxin with carcinogenic and neurotoxic potential, is also produced by several *Aspergillus* strains (Noonim *et al.*, 2008; Okoth *et al.*, 2018). Therefore, besides their infectious potential, *Aspergillus* species are also dangerous because of their toxigenic potential, hence the danger of their presence in school environments (Viegas *et al.*, 2019a).

Additionally, in the last couple a years, due to the improved knowledge on mycological infections, the number of species associated with health conditions has increased, including now *Fusarium*, *Penicillium*, and others; even though *Aspergillus* spp. are still considered to be the major cause of asthma with fungal sensitization, other fungi, such as *Alternaria* and *Cladosporium* spp., have been discovered to also be involved (Chowdhary *et al.*, 2016; Baxi *et al.*, 2016). Considering that these fungi were the most common found in schools, scholastic environments should be consider as areas with high risk of exposure to allergens and harmful fungal species.

Microbial air sampling falls under two categories: active air sampling (impaction, impinger or filtration) and passive air sampling (including surface sampling, dust analysis by settled dust collection (Santilli and Rockwell, 2003; Cai *et al.*, 2009; Norbäck *et al.*, 2014, 2016, 2017; Awad *et al.*, 2018) by EDC sampling (Vesper *et al.*, 2015), by open dish plate method (Aydogdu *et al.*, 2005; Aydogdu and Asan, 2008; Reddy and Srinivas, 2017; Norbäck *et al.*, 2016), by surface swabs (Wilson *et al.*, 2004; Cai *et al.*, 2009; Norbäck *et al.*, 2016), and other methods) (Haig *et al.*, 2016). Both categories have their advantages and disadvantages. Passive air sampling is usually more easily available, relinquishing in most cases the need for specific equipment, usually more economic and less obtrusive (Haig *et al.*, 2016). But these methods are not easily standardized, leading to difficulties when trying to compare the results obtained (Haig *et al.*, 2016). Active air sampling on the other hand is very easily standardized, because the equipment used can be programmed to draw a specific amount of air, leading to an easy report of the results (Méheust *et al.*, 2014). However, active air sampling is usually more expensive, since it requires specific equipment, and it can't describe the fungal contamination in an indoor environment for a long period of time, giving only an idea of the load in a specific moment (Viegas *et al.*, 2019b). Several different active and passive air sampling methods were applied in the different studies, but they were only applied jointly in some cases (Santilli and Rockwell, 2003; Wilson *et al.*, 2004; Godwin and Batterman, 2007; Cai *et al.*, 2009; Norbäck *et al.*, 2016; Awad *et al.*, 2018). A recent study in healthcare facilities in Portugal highlighted the need for the use of both sampling methods, because some toxigenic fungal species were identified only in specific sampling methods (Viegas *et al.*, 2019b). Therefore, when selecting a sampling strategy for schools environments, more than one sampling method should be applied to obtain a more accurate risk characterization (Viegas *et al.*, 2019a,b,c). Active sampling must be used in concurrence with the passive methods, with surface sampling to identify moldy spots on the walls and to assess the effectiveness of the cleaning, dust analysis (by either settle plate, EDC or settled dust vacuuming) to evaluate the fungal contamination/accumulation over time, and even the sampling of the HVAC filters in buildings that use artificial ventilation, in order to study the impact of the ventilation in the building's users (Méheust *et al.*, 2014). Thus, the analysis of HVAC filters can provide an idea of the fungal contamination in the air of buildings that use mostly artificial ventilation (Viegas *et al.*, 2019a).

When analyzing the samples obtained from either active or passive sampling, we can obtain different information, such as the fungal contamination or the presence of specific fungal compounds, such as mycotoxins (Méheust *et al.*, 2014; Viegas *et al.*, 2019c). The choice of what to analyze depends on the goal of the research, but when describing the contamination in a specific building, and because the same sample can be used for different analysis, it is important to obtain the most complete picture as possible, with information on all possible sources of pathogenicity for the occupants (Méheust *et al.*, 2014). This includes the analysis of the fungal contamination and the presence of the metabolites. Fungal detection can be performed by a vast array of methods, including the culture-based methods and microscopical identification, molecular detection, and biochemical testing (Méheust *et al.*, 2014). Culture-based methods and microscopic identification has been the gold standard for years, and is highly studied and applied, in not only academia but also in medicine (Kozel and Wickes, 2014). This method is easily applied in a high variety of samples, can be used for susceptibility testing, and it is relatively cheap (Kozel and Wickes, 2014; Méheust *et al.*, 2014). However, the microscopic identification requires a very well-trained user, and this method cannot detect the non-viable fungal load in the air (Kozel and Wickes, 2014; Méheust *et al.*, 2014). Molecular detection by PCR is a fast method and allows the identification of both the viable and non-viable fungal load in samples (Méheust *et al.*, 2014). However, this method requires a previous DNA extraction of the sample, is prone to contamination during the process, and the detection in environmental samples can be difficult, due to the contamination of the sample (Méheust *et al.*, 2014; Viegas *et al.*, 2019a). In all 21 studies, only one identification method was used, either morphological identification or molecular detection. Therefore, different analysis methods should be used when studying the indoor fungal contamination; indeed, the detection of specific fungal components, such as allergens or mycotoxins, is also important when describing the potential health effects on occupants (Viegas *et al.*, 2019b).

Out of the 21 studies, in only eight of them was the air sampling performed more than once (Awad *et al.*, 2018; Aydogdu *et al.*, 2005; Aydogdu and Asan, 2008; Chen *et al.*, 2012; Dacarro *et al.*, 2003; Hussin *et al.*, 2011; Reddy and Srinivas, 2017; Roda *et al.*, 2011). Several studies have shown that the fungal concentration in the indoor air of a building is influenced by meteorological factors (Reddy and Srinivas, 2017). Therefore, when establishing a protocol for fungal contamination assessment indoors (such as school environments), it is important to consider sampling in different months/seasons, depending on the climate of the region (Reddy and Srinivas, 2017).

Recently, a multi-approach sampling protocol using active and passive air sampling methods was applied in public health centers in Portugal (Lisbon) (Viegas *et al.*, 2019a,b). This study showed that, when using several passive air sampling techniques together, the qualitative assessment of the fungal contamination in the different centers was highly improved, with the identification of possibly pathogenic species in only one specific sampling method and in a specific media (Viegas *et al.*, 2019a,b). Considering that school

environments can be somewhat compared with health centers in the sense that they are highly frequented, and mostly by individuals with decreased immune systems (children in the case of schools, patients in the case of the healthcare centers), the use of different sampling techniques, different sampling media and even different fungal identification tools (culture-based and molecular) in concurrence is of the upmost importance when drawing a protocol for any public building.

The information drawn from the studies analyzed in this chapter, and others performed in different indoor environments (Viegas *et al.*, 2019a,b) allow the authors to suggest a protocol for sampling and analyses that should be considered when assessing the fungal contamination in a school environment:

(1) Apply a checklist beforehand, taking note of the complaints of the users of the building relating to possible effects of fungal contamination (allergy/asthma symptoms, including coughing, wheezing, irritation in the eyes and nose area, etc.), the overall state of the building materials (cracks on the walls, visible signs of water damage, mold on the walls and ceiling, etc.), and the seasonal ventilation of the building (number of windows open, natural/ artificial ventilation, etc.) (Viegas *et al.*, 2019a).
(2) Perform several sample collections throughout the year, considering the weather variations of the area. For example, consider hot and cold seasons, and even dry and wet seasons, to paint the most complex picture of the fungal environment of the building, and the weather-related variations of the contamination (Reddy and Srinivas, 2017).
(3) Using a multi-approach sampling method that includes both active air sampling (impaction and impinger) and passive sampling, with surface sampling, settled dust, EDC, and even HVAC filters in buildings that use artificial ventilation (Viegas *et al.*, 2019a).
(4) Analyze the samples by more than one method, to provide a more accurate characterization of the contamination, and to obtain information that goes beyond the fungal contamination, including information on the presence of allergens (very important in school environments), mycotoxins, and others.

In conclusion, the disparity between sampling and analysis methods between the studies of fungal contamination in school environments found highlights the need for a more comprehensive protocol when assessing fungal contamination in susceptible environments such as this one. The authors propose a multi-approach method that can yield the most thorough results out of the sampling in school environments, and that can be applied in other types of buildings.

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Airborne Fungi in Workplace Atmospheres: Overview of Active Sampling and Offline Analysis Methods Used in 2009–2019

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Introduction

The fungi are ubiquitous and are found in many different environments including workplaces where they can be use deliberately (e.g., food industry) or not. Occupational fungal aerosols are associated with various immunoallergic or toxinic respiratory disorders among workers (Eduard, 2009; Knutsen *et al.*, 2012) like asthma, rhinitis, hypersensitivity pneumonitis, organic dust toxic syndrome, and chronic bronchitis. A fungal aerosol is a complex matrix that depends on the investigated environments and that often presents a huge variety in particles sizes, in composition and in concentration. It may include different types of fungal particles such as spores, strands or fragments of mycelium that can be isolated or agglomerated. Such aerosols may be composed of culturable, viable, dormant or dead fungal cells and associated microbial compounds or metabolites such as (1,3)-β-D-glucans, mycotoxins, and allergens that can all be causative agents of workers diseases. Occupational environments may present a wide fungal biodiversity, composed of many different species. Fungal aerosols found in workplace atmospheres are even more complex because fungi are often mixed with other contaminants such as bacteria, dust or gas for example. Like airborne chemical pollutants, fungal concentrations are known to be highly variable over time including for a single working day, and in space within a company or even in a workshop. Measurements of airborne fungal concentrations in workplace atmospheres contributes to estimate the risks associated with workers' exposures, helps to better understand the complexity of occupational bioaerosols or may be used to check the effectiveness of control and prevention measures.

Usually, measurement methods consist of two main stages. The sampling aims to separate the airborne fungal particles from the air stream and to collect the pollutants on or inside a medium. The analysis of the sample aims to determine the quantity and/or the composition of collected fungal particles and associated compounds.

A lot of technical and scientific information is already available. Previous published articles and reviews well described the principles and mechanisms of collection involved in devices used for bioaerosol sampling (Verreault *et al.*, 2008; Grinshpun, 2010; Reponen *et al.*, 2011; Ghosh *et al.*, 2015; Haig *et al.*, 2016; Lindsley *et al.*, 2017; Reponen, 2017; Amato *et al.*, 2018; Mainelis, 2020) or aimed at presenting available methods for quantification and characterization of bioaerosols (Grinshpun, 2010; Ghosh *et al.*, 2015; Lindsley *et al.*, 2017; Mensah-Attipoe and Täubel, 2017; Duquenne, 2018; Fröhlich-Nowoisky *et al.*, 2018; King *et al.*, 2020).

The objective of this chapter is to provide an overview of the sampling and analysis methods actually used for measurement of fungi in workplace atmospheres. This inventory describes in a factual way the practices really implemented in more than 200 research works published between 2009 and 2019. This original and unusual approach aims to complement and to enrich the information already given in previous published reviews.

General Presentation of the Reviewed Articles

This work is not a systematic review but selected articles had to fulfill all of the following inclusion and exclusion criteria.

- The study was peer reviewed and published in English from 2009 to 2019.
- Measurements targeted, at least, one fungal aerosol parameter. Articles only focused on bacteria or viruses for example were not considered.
- Measurements were performed in, at least, one workplace atmosphere or occupational location. Thus, many environments were not considered, such as home, hotel, school, church, subway, railway station, neighborhood of an industrial site, outdoor urban or rural area (e.g., city, forest, garden, landscape), large-scale region (e.g., desert, Alaska, Antarctic, costal area, troposphere, rain event), etc. Laboratory-based results (generation and characterization of experimental bioaerosols in a controlled chamber) were also excluded.
- Measurements of airborne fungi were performed using an active sampling device coupled with at least one offline method of quantification and characterization. Passive air sampling techniques (sedimentation settling plate, dust fall collector, passive electrostatic sampler, etc.) and surface sampling methods were not considered. Likewise, real-time detection techniques using biological particles fluorescence properties were also excluded.

The overview synthesizes data from 208 articles (the detailed list is available in online supplementary information SI). This corresponds to between 14 and 24 articles per year, coming from nearly 75 different peer-reviewed international journals.

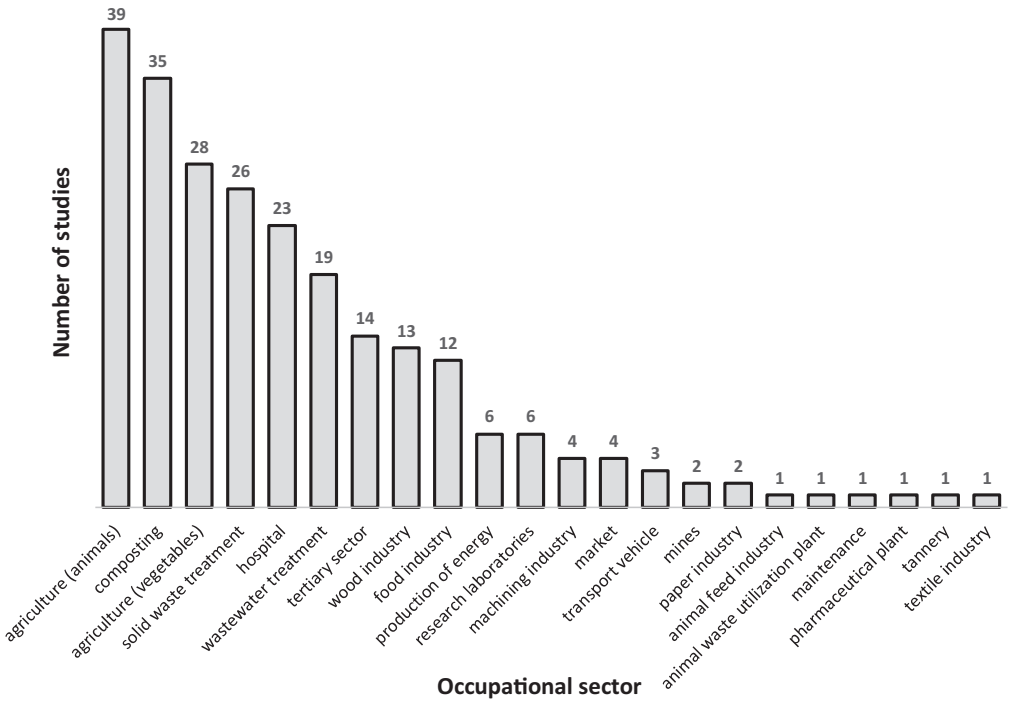

Fig. 1 Number of articles vs. investigated occupational sector for the 2009–2019 selected articles (one study can provide either measurements of airborne fungal aerosols in only one workplace atmosphere, nor several).

Although not fully exhaustive, the reviewed articles represent however, a large part of the scientific published field studies on occupational fungal aerosols during the 2009–2019 decade.

These studies focused on numerous work environments grouped into 22 occupational sectors (**Fig. 1**). The most investigated types of workplaces belonged to the following main sectors: agriculture (poultry or swine facilities, horse stables, grain handling, greenhouses, slaughterhouses, dairy farms, etc.), composting (sewage sludge, green waste, food waste, etc.), solid waste treatment (waste collection, sorting facilities, municipal landfills, etc.), hospital and wastewater treatment. There is a strong inhomogeneity between those environments that led to several publications each year and other workplaces for which less than five articles were published in 10 years. If research studies and use of measurement methods improve knowledge about fungal aerosols and the resulting workers' exposures in some workplaces, **Fig. 1** also clearly shows that others occupational sectors were poorly documented during this decade. Otherwise, it can be noted that six of the nine most documented sectors (i.e., appearing in >10 articles) are considered in the scientific community as highly contaminated (Eduard and Heederik, 1998; Oppliger and Duquenne, 2016) and show occupational situations with high exposures potential for workers: agriculture (animals and vegetables), composting, solid waste treatment, wood and food industry.

Overview of Active Samplers Used for Airborne Fungi Characterization in the Workplace Atmospheres

Through the 208 investigated articles, ~ 50 different bioaerosols samplers were used to perform airborne fungi field measurements. Over the 2009–2019 decade, we observed that little effort was made in scientific published works to standardize the usual practices of each team worldwide, making difficult the comparison of the results of different studies with each other. However, the complexity and variability of occupational fungal aerosols (particles sizes, composition, concentration, etc.) and the need to use suitable measurement strategies make it difficult to consider only one sampler as a reference method (Mainelis, 2020). If 72% of the articles used a single device to perform sampling of airborne fungi, 21% and 5% of them used two or three different samplers, respectively. Only three articles used four different fungal samplers in their sampling strategy.

These ~ 50 different samplers were classified into three large families (**Fig. 2**): the inertial impactors, which represent $\sim 48\%$ of the actually used sampling methods, the filtration samplers ($\sim 40\%$) and the liquid samplers/impingers ($\sim 12\%$). The principles and mechanisms of collection involved in these fungal samplers will not be described in detail as this information can be found elsewhere (some examples of published articles and reviews have been proposed in the introduction).

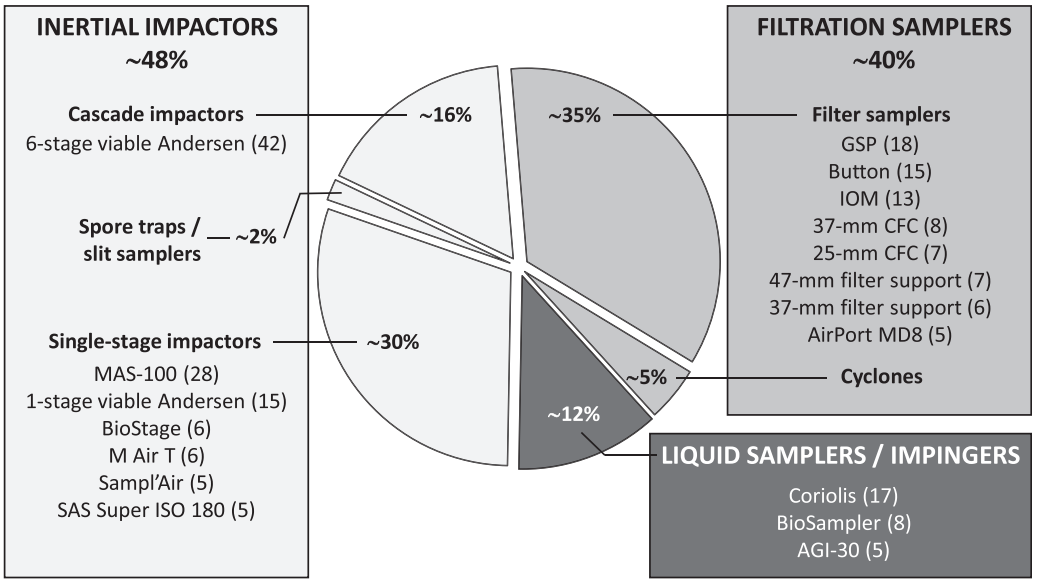

Fig. 2 Distribution of the ~50 different samplers used in the 2009–2019 investigated articles according to their principles of fungal particles selection/collection. The 18 cited samplers were used in ≥5 articles (e.g., GSP has been encountered in 18 different articles). The 35 others samplers that are not mentioned have each been used in only 1–4 articles throughout the 208 investigated published works.

Inertial Impactors

In impaction methods, fungal particles are separated from the air stream by their inertia: larger ones are collected on an impaction surface to be analyzed and smaller ones, that do not impact, follow the air stream towards the exit of the impactor or another downstream impaction stage.

~80 articles used 15 different models of single-stage culture-based impactors (the detailed list is available in Table S1 in SI). Many of them have been characterized for their physical collection and biological efficiencies or compared in term of performances. Such portable microbial samplers mainly differ in number, dimension or shape of inlet nozzles and jet-to-plate distance. The Reuter Centrifugal Sampler (RCS®) is specific because it is based on both centrifugal and inertial impaction forces for the biological collection on a manufacturer-supplied agar strip. The encountered air flowrates are also significantly different depending on the device: e.g., 10 L min^{-1} for the Burkard sampler for agar plates; 28.3 L min^{-1} for the 1-stage Andersen impactor/BioStage®; 100 L min^{-1} for the AirIdeal® 3P™, MAS-100®, RCS®, Sampl'Air™ or SAS Super 100; up to 140 and 180 L min^{-1} for the M Air T® and SAS Super 180/Duo 360, respectively. Except for RCS, single-stage impactors are usually loaded with one standard 90-mm Petri dishes containing agar. Malt extract agar (MEA) and Sabouraud dextrose agar (SDA) were the collecting medium the most used in single-stage impactors. To a lesser extent, many different other types of agar were also used in a few articles: e.g., dichloran-glycerol agar (DG18), Rose Bengal agar (RBA), potato dextrose agar (PDA), Czapek-Dox agar, Wort agar, or Waskman agar. In ~50% of cases, the agar was supplemented with antibiotics such as chloramphenicol, streptomycin, penicillin and gentamicin as antibacterial agents to promote the growth of fungal species only. Agar's desiccation may be associated with single-stage impaction as moisture is removed by high air stream passing over the collecting medium plate. Consequently, the encountered sampling durations were most often ranged from 30 s to 3 min, and almost exclusively less than or equal to 10 min. Only five articles used durations greater than or equal to 15 min. However, the use of such short sampling periods remains of limited interest to extrapolate the average concentration levels over longer periods of several hours that could be more representative of full working time and thus of the real worker's exposure. Although the use of such single-stage impactors in highly contaminated sites can lead to overloaded culture plates and difficulties in enumeration due to overlap of colonies, we observed that they were used as much in non-industrial environments (hospital, office, laboratory, etc.) as in industrial sectors (composting, metalworking, animal farming, etc.).

A few articles used slit samplers (the shape of the impactor nozzle is rectangular) or spore traps (the detailed list is available in Table S2 in SI). Despite Hirst-type spore trap are mainly dedicated for collection of fungal spores and pollen grains, such impactors were not widely encountered in the investigated occupational field studies (**Fig. 2**). The air flowrates were between 5 and 15 L min^{-1} depending on the device. The impaction stage consisted of an adhesive tape or a coated microscope slide for direct microscopical investigations. Despite the Allergenco MK-III and the Burkard glass slide samplers were previously studied (Portnoy *et al.*, 2000) and are frequently mentioned in bioaerosols reviews, these two inertial impactors have only been encountered once in the selected published articles for airborne fungi characterization in workplace atmospheres (see Table S2 in SI).

The 6-stage viable Andersen cascade impactor, one of the oldest bioaerosol samplers (Andersen, 1958), was also still the most used with 42 articles out of 208 (**Fig. 2** and Table S3 in SI). Since a long time, this model of cascade impactor which has collecting stages with 50% cutoff diameter between ~0.65 μm (lowest stage) and ~7 μm (top stage) is widely used for determining the size distribution of

culturable microorganisms. Two other versions have been encountered in a few articles (see Table S3 in SI): a 2-stage and a 3-stage Andersen impactors. The expected air flow rate of Andersen cascade impactors (28.3 L min^{-1}) was correct in almost all the articles. Still to avoid drying of the agar or overloading of culture plates, the encountered sampling time was limited to 0.5–5 min for ~80% of the articles, while ~20% of them show sampling duration superior to 10 min. Highest sampling time of 30 min were only chosen in low concentration environments (office, laboratory and hospital). MEA was the collecting medium the most used in Andersen cascade impactors (~63%). Four other types of agar were also used in ~15 articles: RBA, SDA, PDA, and DG18. In a third of the works using the Andersen cascade impactor, the agar was supplemented with antibiotics to promote the growth of fungal species only. Only ~60% of the investigated articles clearly mention that they adjust the number of colonies counted on an agar plate using the positive-hole correction factor in order to take into account the fact that multiple fungi may deposit through an impactor hole (Macher, 1989). In the 208 investigated articles, only one work used another cascade impactor to measure the size distribution of fungal particles (Simon and Duquenne, 2014). It was a Marple that consists of eight impaction stages (34-mm Mylar® circular disks with six radial slots, 2 L min^{-1}) and presents cut-off aerodynamic particles diameters between 0.52 and 21.3 μm, depending on the impaction stage.

Filtration Samplers

In filtration methods, fungal particles are collected by passing air stream through porous membrane filters, based on the five following mechanisms: inertial impaction, interception, gravitational settling, diffusion, and electrostatic attraction.

Because filtration may achieve high physical collection efficiency and remains an easy-to-use method to sample robust microorganisms such as fungi, the filter samplers are widely employed in the workplace atmospheres (**Fig. 2**). More than 15 different models were encountered in ~85 articles (the detailed list is available in Table S4 in SI). Some of them have long been known to industrial hygienists and are, despite some limitations (Görner *et al.*, 2010), representative of the inhalable fraction: GSP (3.5 L min^{-1}), Button (4 L min^{-1}), IOM (2 L min^{-1}), 25-mm or 37-mm closed-face cassette (2 L min^{-1}), PAS-6 (2 L min^{-1}) and 7-hole (2 L min^{-1}). Only one team, that considers that the thoracic fraction is the most suitable for describing the exposure of dairy farmers to organic dust, used thoracic filter samplers (see Table S4 in SI): CATHIA-T (7 L min^{-1}) and PPI-T (2 L min^{-1}). Only one work (Haatainen *et al.*, 2009) investigated the use of a size-selective polyurethane foam contained within a standard 2 L min^{-1} IOM to achieve the respirable fraction. Through the 208 selected articles, few of them sampled the fungal aerosols according to an environmental convention, using the following devices (Table S4 in SI): Personal Environmental Monitor (PM2.5 at 10 L min^{-1}) or PM10 static high-volume samplers (500–3500 L min^{-1}).

In ~20% of the articles with filter samplers, it was not clearly specified which filter holder was used: e.g., "filtration system", "filtration support", "in-line filter holder", "filter capsules" or sometimes just referred with the commercial name of the sampling pump or with a trade name for which no information seems available. We then created the following categories to list these unclear sampling methods (**Fig. 2** and Table S4 in SI): 25-mm, 37-mm, or 47-mm filter support and just "filter support" when the filter diameter was not even specified. In such cases, the inlet or selection characteristics of fungal particles are unknown; no one can say if the measurement was done according to environmental conventions (PM10 or PM2.5), occupational conventions (inhalable, thoracic, respirable) or none.

Finally, the 2.5 kg portable AirPort MD8 sampler (30–50 L min^{-1}) was used in a few articles (Table S4 in SI). The encountered sampling times were below ~30 min to try to avoid complete drying of the 3.0 μm pore size 80-mm gelatin membrane filter.

~5% of the encountered sampling methods were cyclones (**Fig. 2** and Table S5 in SI): e.g., GK 2.69 (1.6 L min^{-1}) for thoracic fraction, SCC1.062 Triplex (3.5 L min^{-1}) for PM1 fraction or different models of microcentrifuge-tube cyclones (1-stage BC112, 2-stage BC221, and BC251, between 2 and 4 L min^{-1}).

Filter samplers allow longer sampling times than agar plates' impactors or liquid samplers, for example. For nearly 85% of the selected articles using a filtration method, the sampling time was between 15 min and 9 h; in ~50% of cases, the encountered sampling times were greater than 180 min. Such long period of sampling may lead to desiccation and loss of culturability of sensitive microorganisms (e.g., vegetative bacterial cells) but the biological efficiency is much better for resistant ones (e.g., fungi and others spore forming species). The sampling durations equal to or lower than 10 min were rare and exclusively associated with the use of a gelatin membranes. Only three studies use extremely long sampling times (>20 h).

Polycarbonate was the collecting membrane the most used in filter samplers (~50%), followed by gelatin filters (~20%) and polytetrafluoroethylene (Teflon, PTFE – ~12%). In ~10% of the articles which used filtration sampling, glass fiber filters were used, mostly to perform (1,3)-β-D-glucans analysis. Some other filter types were also used in a few articles: e.g., polyvinyl chloride (PVC), mixed cellulose ester, nitrocellulose and quartz microfiber.

Liquid Samplers/Impingers

Liquid samplers and impingers collect fungi directly into a liquid. Maybe due to several issues (liquid evaporation, possible need of periodic replenishments, re-aerosolization or internal losses of already collected material), liquid-based samplers were only used in ~12% of the investigated works to collect occupational fungal particles, known to be hydrophobic (**Fig. 2**).

~30 articles used 4 different liquid samplers (the detailed list is available in Table S6 in SI).

Coriolis family of samplers (mainly the Coriolis μ model) was used in 17 articles out of 208 (**Fig. 2**). Coriolis is a wetted-wall cyclone, which utilizes centrifugal impaction and swirling cyclonic mechanism to collect fungi into a liquid. In the investigated articles, it was frequently operated at flow rate of 300 L min^{-1} (200 L min^{-1} in only two works) and with 10 or 15 mL of collecting

liquid. Encountered in 8 articles, the BioSampler (12.5 L min^{-1}) is an all-glass swirling aerosol collector that utilizes centrifugal forces and tangential impingement into 20 mL liquid vessel as sampling principles (Willeke *et al.*, 1998). The All Glass Impinger (AGI-30, 12.5 L min^{-1}, 20 mL of liquid) (Han and Mainelis, 2012) and the CIP 10-M, that does not require an external pump for active sampling (10 L min^{-1}, 2 mL of liquid in a rotating cup) (Simon *et al.*, 2016), were found in ~5 articles each among the 208 selected articles.

About half of the encountered liquid-based samplers were filled with sterile distilled water and the other half with phosphate buffer saline (PBS) solution. In ~50% of these works, water and PBS were complemented with Tween-20 and Triton X-100, respectively. Other surfactant (Tween-80) and antifoaming agent were used in one and two articles, respectively. Despite viscous collection fluid could minimize evaporation and increase the sampling duration, only one investigated article used diluted mineral oil; this may be explained by the fact that the microorganism extraction is then often considered somewhat challenging.

For nearly 85% of the selected articles using a liquid-based collection, the sampling time was between 1 and 30 min; few others were included between 40 and 120 min. Such a majority of short sampling durations is mainly guided by the rapid evaporation of the water-based liquid rather than the objective of the study and the associated sampling strategy.

Static Versus Personal Sampling

Regarding the sampling strategies used in the 208 investigated articles or the large size of certain devices (e.g., single-stage or 6-stage Andersen impactors), static sampling (>80%) were more frequently encountered than personal sampling (<20%). For most static sampling points, samplers were positioned at around 1.5–1.7 m from the ground, the approximate height of the human respiratory tract. The personal samplers that were carried out in the workers' breathing zone (region within 30 cm of the worker's mouth and nose) were exclusively the following: Button, CFC, CIP 10-M, cyclones (Dorr-Oliver, GK 2.69, BC221, etc.), GSP, IOM, PAS-6, PPI-T. All these well-known aerosol samplers are not dedicated to fungi but are also commonly used for many years for sampling other particulate pollutants (wood dust, metals, dust not otherwise specified, etc.). Except for CIP 10-M which has a built-in pump and is used at 10 L min^{-1}, the others encountered personal samplers have air flowrates less than or equal to 4 L min^{-1} and need to be used with an additional personal sampling pump.

Overview of Downstream Analyses Used for Airborne Fungi Characterization in the Workplace Atmospheres

This bibliographic work provides a comprehensive view of the downstream analyses that are actually performed in scientific works to quantify/identify fungi and fungal components in the collected air samples from occupational atmospheres. The measurement principles of these analyses will not be described in detail as this information can be found elsewhere (some examples of published articles and reviews have been proposed in the introduction). ~20 different analysis methods were encountered and were classified into two large families (**Fig. 3** and Tables S7 and S8 in SI): (1) culture-based methods comprising colonies' enumeration and identifications from culturable fungi and (2) culture-independent methods comprising microscopic analysis, qPCR and PCR, biodiversity and metabolites analyses. In the majority of the investigated articles (~52%), two different analyses methods were used. Then, 35% and 11% of the selected articles used one or three methods, respectively. Four different types of analysis methods used in the same work are few with only five articles concerned (~2%).

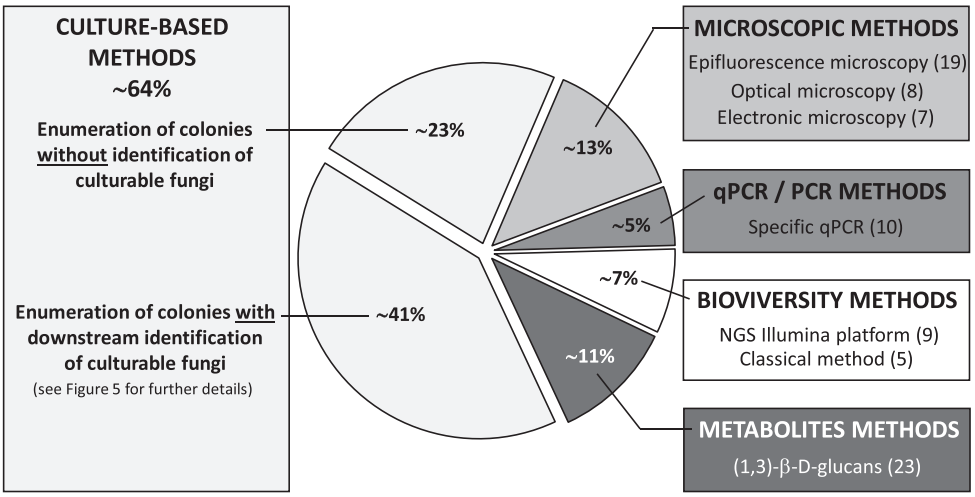

Fig. 3 Distribution of the ~20 different analyses used in the 2009–2019 investigated articles according to their principles of fungal particles detection. The 7 cited analyses were used in ≥5 articles (e.g., epifluorescence microscopy has been encountered in 19 different articles).

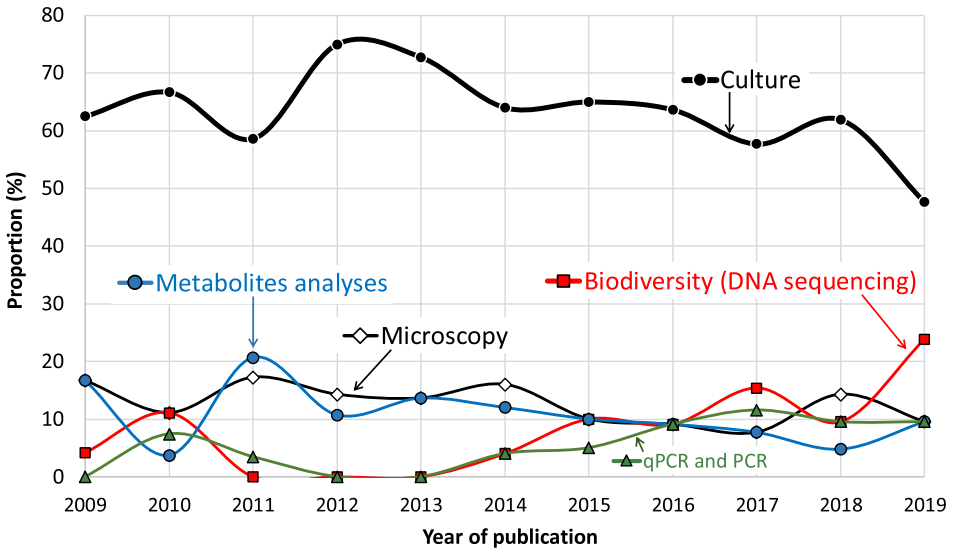

Fig. 4 Evolution of the proportion of the use of culture-based and culture-independent methods in the 208 selected articles during the 2009–2019 decade (one study can either provide measurements of airborne fungal aerosols using only one analysis, nor several).

Culture-Based Methods/Overview

In the 2009–2019 decade, culture was still the most used method to characterize fungi in workplaces (**Figs. 3** and **4**) and was used in ~80% of the 208 investigated articles. This analysis method enumerates the culturable fungi that are able to form colonies on agar medium in laboratory growth conditions (Mensah-Attipoe and Täubel, 2017). **Fig. 4** seems to show a slowdown in the use of culture in recent years, with a decrease of ~30% between 2013 and 2019 throughout the considered articles.

Culture-Based Methods/Enumeration of Culturable Fungi

Analysis by culture is first associated with quantification of airborne fungal concentrations (expressed in number of colony forming units per cubic meter of sampled air, $CFU \cdot m^{-3}$). It can be done either directly by impaction on solid agar, the most used sampling method in combination with culture (68% of the articles in which culture is performed), nor indirectly using filtration or liquid samplers for which a volume of the eluate or the sampling liquid, or serial dilutions of it, is spread on the surface of agar medium. For a long time and despite known limits (Duquenne, 2018), such a quantification of the culturable fraction of the fungal community is considered as a first approach of the evaluation of the fungal contamination in the workplace atmospheres and/or of the workers' exposure.

For enumeration, different broad agar media, which support the growth of a wide number of fungi, were encountered. MEA was the most used with ~47% of the selected articles, followed by three other media observed in a similar proportion (~15%): RBA, SDA and DG18. To a lesser extent, some others media such as PDA or Blood agar were used in less than 10 articles. In ~40% of cases, these broad growth media were supplemented with at least one antibiotic, mainly chloramphenicol.

Culture-Based Methods/Identification of Culturable Fungi

In ~65% of the articles in which culture was done, a subsequent identification of the genera or species of the grown colonies was performed. Among the different methods that were used to identify culturable fungal isolates, description of macroscopic and microscopic phenotypes was the most frequently encountered (**Fig. 5**). Biomolecular, biochemical, and chemical identification methods were also used in ~30 articles. 34 articles used a combination of two or three of these different culture-based methods to perform the identification of fungi.

~53% of the identification methods performed were based on macroscopic and/or microscopic morphological criteria, most often after subculturing. Standard taxonomic keys, well described in previous reviews (Lindsley *et al.*, 2017; Fröhlich-Nowoisky *et al.*, 2018), were usually applied to perform the identification of culturable fungi: e.g., colonies morphology, ability to grow on selective media or specific temperatures to discriminate mesophilic and thermotolerant fungi. Microscopic observation also often helped to reveal which types of fungi are concerned. Stains (e.g., lactophenol cotton blue) were sometimes cited to examine fungal element and help to resolve these colony structures using microscopic-based approaches.

Identification of the cultivated fungi using their genetic material represented ~9% of the articles associated to culture (**Fig. 5**). Briefly, the principle of this technic is to extract DNA from pure cultures before amplifying the region of interest by polymerase chain reaction (PCR). The PCR products are then purified and sequenced. The sequence obtained are finally compared with existing sequences in databases using alignment software (Duquenne, 2018; Fröhlich-Nowoisky *et al.*, 2018). Within these DNA-based methods, the

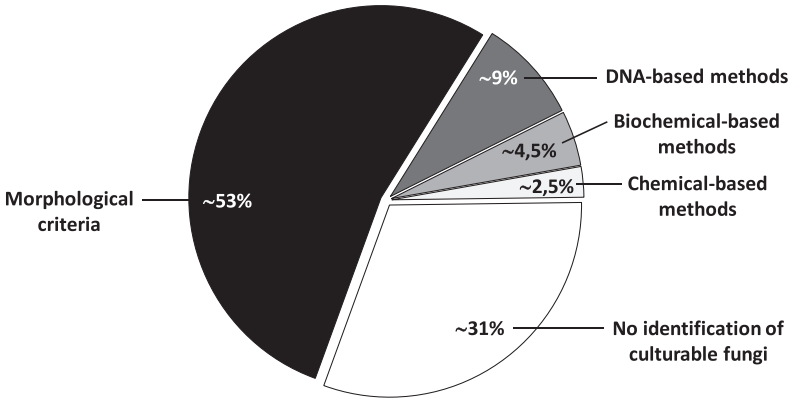

Fig. 5 Proportions of the different culture-based identification methods encountered in the investigated articles in which culturable fungi were measured (one study can either provide identification of culturable fungi using only one method, nor several).

nuclear ribosomal internal transcribed spacer (ITS) gene sequence of fungi was frequently used. The gene coding for β-tubulin to identify *Aspergillus* and/or *Penicillium* was found in a few articles (Roussel *et al.*, 2012; Plewa-Tutaj and Lonc, 2014). The D1–D2 region of 25S RNA and the HKGF2 gene coding for β-tubulin gene was analyzed in only one article (Simon and Duquenne, 2014). Specific PCR followed by gel electrophoresis were done in two articles to identify species (Hansen *et al.*, 2010; Donát *et al.*, 2012).

Identification based on biochemical methods were almost exclusively performed with API™ miniature test. These tests are specifically used for yeast identifications (API 20C) and none are existing for filamentous molds. One article reported the use of Biolog™ with Microstation ID (Paba *et al.*, 2014). These miniatures systems consists of a wide range of phenotypic and metabolic tests to be performed providing metabolic profiles that are compared to a database containing profiles of known microbial isolates (Duquenne, 2018).

Identification based on chemical methods were used in only five of the investigated articles ($\sim$2.5%, **Fig. 5** and Table S7 in SI). Four were using Maldi-TOF mass spectrometry method. The Maldi-TOF method compare the proteomic profiles obtain from pure culture to a database in order to identify the microorganism (Ghosh *et al.*, 2015; Duquenne, 2018). Gaz chromatography method for studying fatty acid composition of the fungi was investigated in one article (Park *et al.*, 2011).

Culture-Independent Methods/Generality

Culture-based methods only target the fraction of airborne occupational fungi that remain culturable after sampling and laboratory analysis. Cultivation is known to dramatically underestimate the total population and the real diversity of environmental samples (Ghosh *et al.*, 2015). Therefore, a large part of the fungi as well as their components or metabolites cannot be detected or quantified with culture-based analyses. Not all the numerous different methods (e.g., microscopy, molecular techniques, flow cytometry, ATP assays, etc.) which are usually listed in classic reviews, were found in the present factual inventory work. $\sim$100 of the 208 selected articles used one or several culture-independent analyses, alone or in addition to culture-based analyses.

Culture-Independent Methods/Microscopic Analysis

Various microscopic methods were used to perform enumeration of viable and non-viable fungi in occupational samples. Epifluorescence microscopy was the most used method ($\sim$56% of the articles using microscopy) and was often coupled with acridine orange for DNA staining. Other fluorochromes were used in a few articles: e.g., DAPI and Calcofluor-White. Widely used to count the total fungal spores expressed in spores $\cdot$ m^{-3}, it was also sometimes associated to a total fungal count of both spores and hyphal fragments or a total airborne microorganisms count including bacteria and fungi.

Bright field microscopy was encountered in $\sim$25% of the articles using microscopy and was often associated with spores traps/slit samplers (Hirst-type and Burkard glass slide sampler). Indeed, such samplers allows collecting fungal particles directly on a microscope slide to facilitate counting and observation. A further identification step was sometimes associated either with a germination step or by morphological criteria. In a few articles, eluate was spread directly on a slide, dried and stained using glycerin gelly to count fungal spores and identified them morphologically. In only one article (Cho *et al.*, 2011), the eluate was filtrated through a MCE filter that was placed on a glass slide, then made transparent and stained before counting.

Total fungal spores can also be count and characterize (e.g., size and morphology) using scanning electronic microscopy (see Table S8 in SI). More recently, fungal spores and/or submicronic and larger fungal fragments were observed and differentiated using field emission scanning electronic microscopy, with prior immunolabeling.

Culture-Independent Methods/Molecular Methods

The principle of these techniques are based on the extraction and purification of DNA from air sample without prior cultural step, and then on the amplification of the region of interest by PCR.

qPCR was used in 14 articles, mainly to quantify fungal aerosol from air sample. Specific qPCR for targeted species (as *A. fumigatus*) or genera (e.g., *Aspergillus*, *Penicillium* or *Alternaria*) were used in six articles (see Table S8 in SI). In three articles, quantification was performed without identification because using universal 18S rRNA or ITS primers for "total" fungi. In five other articles, qPCR was not performed to quantify but only to detect and identify some specific taxa (e.g., *A. flavus*, *A. fumigatus* and *A. ochraceus* in poultries). Specific PCR followed by gel electrophoresis was performed in one article to identify specific strain of *Trichoderma harzianum*. In many of the above-mentioned articles using qPCR and PCR, the sampling was performed using the Coriolis μ and its 300 L min^{-1} flow rate.

DNA sequencing methods including Sanger, 454 pyrosequencing and Illumina sequencing platforms provided new insight into the characterization of fungal aerosol. In the 2009–2019 selected articles, such methods of biodiversity analysis allowed characterizing the fungal communities in various occupational sectors: e.g., composting, agriculture, coalmines, solid waste treatment, and hospital. **Fig. 4** shows that, since 2014, the percentage of articles using these techniques is gradually increasing.

Pre-amplification PCR of the extracted and purified DNA contained in the air sample consists in the first step. Then, for diversity analysis, genes coding for ribosomal subunits 18S rRNA or ITS are often used as targets for PCR amplification. The fragments can be sequenced by classical techniques after PCR products being cloning or directly sequenced after TTGE or D-HPLC separation of PCR products (see Table S8 in SI). Since 2014, sequencing was mainly performed with high throughput sequencing (also called next generation sequencing) with 454 pyrosequencing or Illumina platforms (see Table S8 in SI). These methods permits in theory to detect all fungal taxa present in a sample independently of their culturability or viability. NGS results are then often presented as relative abundance of a given fungal taxon (Mensah-Attipoe and Täubel, 2017).

Based on sequencing with Illumina platforms, two articles used a metagenomics approach (whole genome sequencing) to study the total airborne microbial communities (see Table S8 in SI). It consists in the sequencing of all nucleic acid sequences of a complex sample by whole genome shotgun approach. Sequences are then reconstructed and identified by comparison with databases. One interesting work investigate the comparison of the diversity measured by ITS sequencing with Illumina with that obtained by a high number of fungal qPCR (Unterwurzacher *et al.*, 2018).

Culture-Independent Methods/Metabolites Analysis

Fungal metabolites or other constituents are also frequently investigated because they can be measured as "molecular signatures" of the biological nature in the occupational atmosphere (Fröhlich-Nowoisky *et al.*, 2018). 1,3-β-D-glucan, a polysaccharide found in the fungal cell wall under a triple helical structure and associated to potential health effects by inhalation (Grinshpun, 2010; Ghosh *et al.*, 2015; Mensah-Attipoe and Täubel, 2017), was measured in 23 selected articles (see Table S8 in SI). Glucans were mainly quantified ($\sim$80%) by enzymatic assay with some commercial kits like Glucatell or Fungitic g test, while a few studies used method based on immunoassay.

Ergosterol is the major sterol found in membrane of fungal spores and hyphae. As it is found almost exclusively in fungi, ergosterol is thus considered as a good indicator to evaluate total fungal biomass (Mensah-Attipoe and Täubel, 2017). This structural component was studied in three articles by gaz chromatography to measure fungal contamination in occupational workplaces (see Table S8).

Mycotoxins, a secondary metabolite of fungi, is considered potentially harmful by inhalation. Moreover, identification of specific mycotoxins can help identify the presence of certain fungal species. In the selected articles, multi-mycotoxins (e.g., aflatoxin B1, ochratoxin A. deoxynivalenol, gliotoxin, zearalenone) were investigated by liquid chromatography to assess farmers (Lanier *et al.*, 2010) and grain workers (Niculita-Hirzel *et al.*, 2016) exposures.

ELISA method was used in one article (see Tables S8) to perform quantification of antigens from specific fungal species (*A.fumigatus*, *A. versicolor*, *P. chrysogenum*, *Cladosporium* spp.). Similarly, a new metaproteomic analysis of proteins present in bioaerosol from composting, wastewater treatment and agriculture (vegetables) environment was performed using nano-HPLC Tandem Mass Spectrometry in one article. It allowed the identification of proteins from different fungal species (e.g., *A. flavus*, *A. clavatus*, *C. albicans*).

Conclusion

Several previous published reviews aimed at exhaustively describing the existing measurement methods to characterize bioaerosols. Using an unusual approach that factually inventory the sampling and analysis methods actually used for measurement of fungi in workplace atmospheres, this work provides an overview of practices really implemented in numerous research articles published between 2009 and 2019.

The highlights of this complementary overview are:

- $\sim$50 different active samplers and $\sim$20 different offline analysis methods were encountered to measure fungal aerosols in work environments.

- The airflow rate for a given sampler, the collecting medium (filter, liquid, agar, etc.) and the sample preparation steps and analysis (agar, operating conditions, elution, reagent, processing time, etc.) can even be different from one study to another.
- Filter samplers (GSP, Button, IOM, closed-face cassette, etc.), single-stage impactors or 6-stage viable Andersen impactor were the most used sampling methods.
- Filter samplers were almost the only ones to be used in order to carry out workers' personal sampling and/or long-term sampling (>180 min).
- A large number of selected articles did not clearly specify which fractions of the aerosol was targeted (inhalable, thoracic, respirable, PM10, PM2.5). Some articles also used imprecisely described sampler or unusual samplers' airflow rates leading to the sampling of an unknown fungal aerosol fraction.
- Despite known drawbacks, culture-based methods were still, by far, the most widely used analysis. Enumeration of fungal colonies was often associated with identification of species, most of the time using morphological criteria, leading to a first approach of the species present in workplace atmospheres.
- Characterization of fungal diversity, using NGS methods for example, was gradually increasing since 2014.

Therefore, as already stated by previous authors and due to diversity in the sampling and analysis procedures but also in the reporting protocols, the data comparison between published studies remains challenging and is sometimes impossible. Each single scientific article has its own sampling objectives and strategy; it can only provide incomplete results and a partial picture regarding presence, quantification, complexity and workers' health impact of occupational fungal aerosols. To date, it seems preferable to consider the contribution of several articles carried out in a given occupational sector as complementary without trying to compare them. It is of course desirable that, during the decade 2020–2030, the scientific community work together to develop better harmonized fungal aerosol measurement methods and reporting guidelines.

Appendix A Supplementary Information

Supplementary data associated with this chapter can be found in the online version at doi:10.1016/B978-0-12-819990-9.00009-3.

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Fungal Contamination of Sawmills

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Introduction

Fungi as Occupational Health Risks at Sawmills

Sawmills manufacturing sawn boards, timber frames, glulam, decking, flooring, joinery, fencing, and several other wood products exist across the world. Several thousand people are employed in the sawmill industry, including several European countries (Austria, Belgium, England, Scotland, Northern Ireland, Croatia, Denmark, Finland, France, Germany, Latvia, Norway, Romania, Sweden, Switzerland), Canada, USA, New Zealand, Australia, India, and several African countries (**Fig. 1**, See "Relevant Websites section"). Only in the European Union, there are approximately 35,000 sawmills that employ about 250,000 people (See "Relevant Websites section"). Sawmill workers are exposed to wood dust and multiple wood-associated chemicals and microbiota, including fungi (Straumfors *et al.*, 2018; Demers *et al.*, 2000; Douwes *et al.*, 2000; Duchaine *et al.*, 2000a; Eriksson *et al.*, 1996; Rosenberg *et al.*, 2002). Although several components in the sawmill aerosol can cause health effects, the exposure to fungal spores has been of particular interest, and is associated with respiratory symptoms and hypersensitivity pneumonitis (HP) among sawmill workers (Wimander and Belin, 1980; Eduard *et al.*, 1992a, 1993; Halpin *et al.*, 1994b). The severity of health effects resulting from fungal exposure and the number of workers this potentially includes, have received attention for decades and still rises concerns in the sawmill industry. Continuous exposure reduction and control will have great impact on both workers' health and the society's health economy.

The possible inhalable exposure to fungi along the process line of sawmills is dependent on the growth and occurrence of fungi, but also on the degree of fungal aerosolization, that is dependent on the work process, task, and technological installation. This review intends to discuss these factors and potential health outcomes linked to fungal exposure in sawmills.

Fungal Exposure in Sawmills

Many types of fungi accompany trees during natural life and decay, i.e., biotrophic, necrotrophic, saprophytic and endophytic fungi. Once the three is cut, wood logs are stored in piles sorted by dimension on dedicated yards outdoors before debarking and cutting at the sawmills. During this time, the timber is kept humid so to stabilize the wood and prevent cracking. As a result, logs are often colonized by endogenous fungi, fungi from the local environment (Dickinson and Levy, 1990; Strong *et al.*, 2005) and bacteria (Rossell *et al.*, 1973). A large part of the potential fungal contamination inside sawmills is avoided by debarking the logs before they enter the sawmill. Logs are cut into planks inside the saw department, and the green timber is sorted according to their dimension. After sawing, the green timber will be stored for a variable time length before it is further processed. In this period, fungi may continue to grow on the timber if the water content is high, as is often the case before kiln drying and during the first few hours in the drier. Dried timber with surface colonization is handled in the sorting of dry timber department, where the timber is sorted by quality and dimension. Although fungal particles, including spores and fragments, are released into the air and subsequently inhaled by workers at relatively high levels during handling of the timber at any stage in the sawmill process line, the transition between dryer and sorting of dry timber has been associated with the highest exposure. Dried and sorted timber are further processed at the planer department (see **Fig. 2** for an overview of the process line at sawmills). Maintenance and transport workers engaged in many stages of the process line.

A proper exposure assessment is necessary to evaluate health effects of work place exposure. Personal sampling is recommended for the quantitative determination of suspected agents. The methods for fungal aerosol sampling, detection, and quantification is decisive for the levels and details of reported fungal exposure in occupational settings. This will have impact on how we can evaluate health risk related to fungal exposure. In the following sections, the results of fungal exposure in sawmills is presented with some emphasis on the different methodology used in order to highlight this aspect.

Exposure Measurements and Identification by Cultivation of Aerosol Samples From Sawmills

A review of the literature shows that the average levels of fungal exposure in sawmills range between 0.8×10^3 and 50×10^3 CFU per m^3 when assessed by cultivation on artificial media followed by counting of colony forming units (cfu) (**Table 1**). Cultivation of fungal particles is the most common method for measuring fungal exposure in sawmills as this method is relatively cheap and enables both quantification (cfu counts) and taxonomical characterization based on of the macro morphological appearance of colonies, their characteristics in culture and light microscopic observation of the micromorphology (Eduard and Heederik, 1998). The cultivation of sawmill aerosols have shown that the composition of fungal species includes *Cladosporium* spp., *Alternaria* spp., *Aspergillus spp*., *Penicillium* spp., *Trichoderma* spp., *Paecilomyces* sp., *Rhizopus* spp. and *Mucor* spp. in sawmills in Canada (Duchaine and Meriaux, 2000; Cormier *et al.*, 2000), Croatia (Klaric *et al.*, 2012), Poland (Dutkiewicz *et al.*, 2001a), Italy (Gioffre *et al.*, 2012), UK (Halpin *et al.*, 1994a), and Norway (Eduard *et al.*, 1988). The study from Croatia revealed the presence of *Chrysonilia sitophilia*

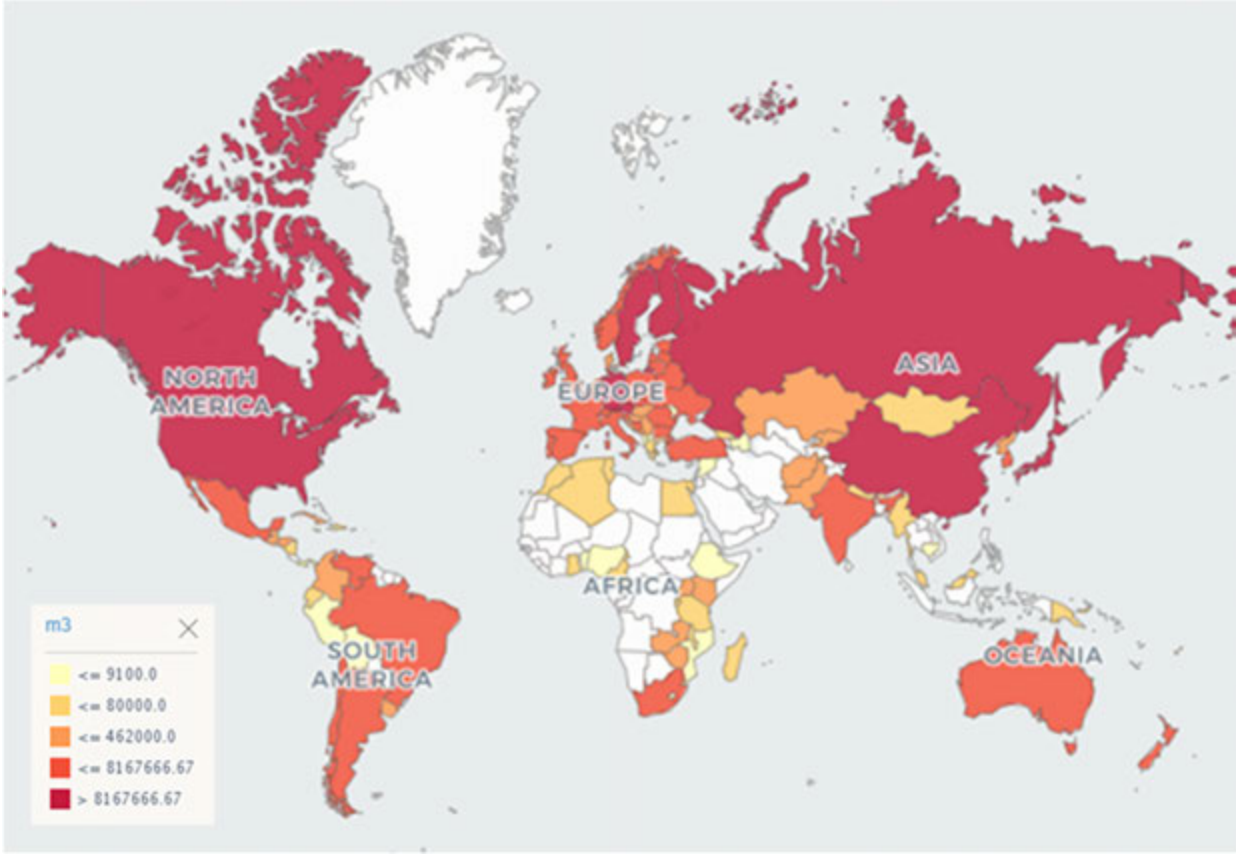

Fig. 1 Sawnwood, coniferous production quantity by country. Average 2016–2018. (FAOSTAT 02.20).

in addition to *Penicillium sp* and *Paecilomyces sp* (Klaric *et al.*, 2012). Although cultivation is commonly used to assess fungal exposure, it is important to have in mind that results may be biased towards rapidly growing fungi that outcompete and inhibit slowly growing species (Wu *et al.*, 2000; Macneil *et al.*, 1995). Furthermore, since aggregates of many propagules will be counted as one colony and since many species are no longer cultivable after aerosolization, colony counting will underestimate the total number of fungal propagules in the air.

Exposure Assessment of Both Live and Dead Fungi by Microscopy

The use of fluorescence or scanning electron microscopy (SEM) to assess fungal exposure has the advantage of including non-viable and non-cultivable fungal particles. Fluorescence and confocal microscopy can be used for identification and quantification of fungal aerosols after staining with dyes (Green *et al.* 2005a,b) or fluorochromes (Palmgren *et al.*, 1986; Nayak *et al.*, 2011). To our knowledge, only Roponen *et al.* have used epifluorescence microscopy with the CAMNEA method to estimate fungal exposure in sawmills and reported personal exposure levels ranging between 2.15×10^5 and 14.6×10^5 spores per m^3 in a Finish sawmill (Roponen *et al.*, 2002; **Table 1**). The mayor limitation of this method is that the natural dense pigmentation of many spores may mask the fluorescence of the dyes employed from staining and lead to underestimations of the exposure (Burge, 1995).

The high resolution and field depth obtained by SEM enables the visualization of detailed characteristics of fungal particle surface morphology, shape and size. A rough estimation indicate that spore count with SEM gives averagely ten times higher counts as cfu counts (Eduard, 2009). Hence, quantitatively SEM may give more precise spore exposure estimates than cultivation. Since both viable/cultivable and non-viable/dead fungal particles have antigens, allergens, toxins and immune-modulating components such as β-1,3-glucans, SEM represents a generally broader health relevant estimate. SEM have been used to measure the exposure levels of fungal spore in several sawmill studies (Eduard *et al.*, 1993; Faerden *et al.*, 2014; Straumfors *et al.*, 2018; Dahlqvist and Ulfvarson, 1994). In a longitudinal study in the 1980s, Eduard *et al.* reported that wood trimmers were exposed to up to 10^7 spores/m^3, AM 4×10^6 spores/m^3(Eduard *et al.*, 1993; **Table 1**). Micro morphological recognition of spores from *Rhizopus microsporous* and *Paecilomyces variotii* in the samples were enabled by SEM comparison of spores from pure cultures. As such, wood trimmers' exposure was estimated to 1.3×10^4 *R.microsporous* spores/m^3 and 130×10^3 *P.variotii* spores/m^3 (Eduard *et al.*, 1993).

Over a period of nine years, Færden *et al.* observed a reduction in sawmill workers spore exposure, from AM 9.8×10^6 spores /m^3 in 2001 to AM 0.04×10^6 spores/m^3 GM (GSD) 0.02×10^6 spores/m^3 (3.3) in 2009 (Faerden *et al.*, 2014). This was believed to be

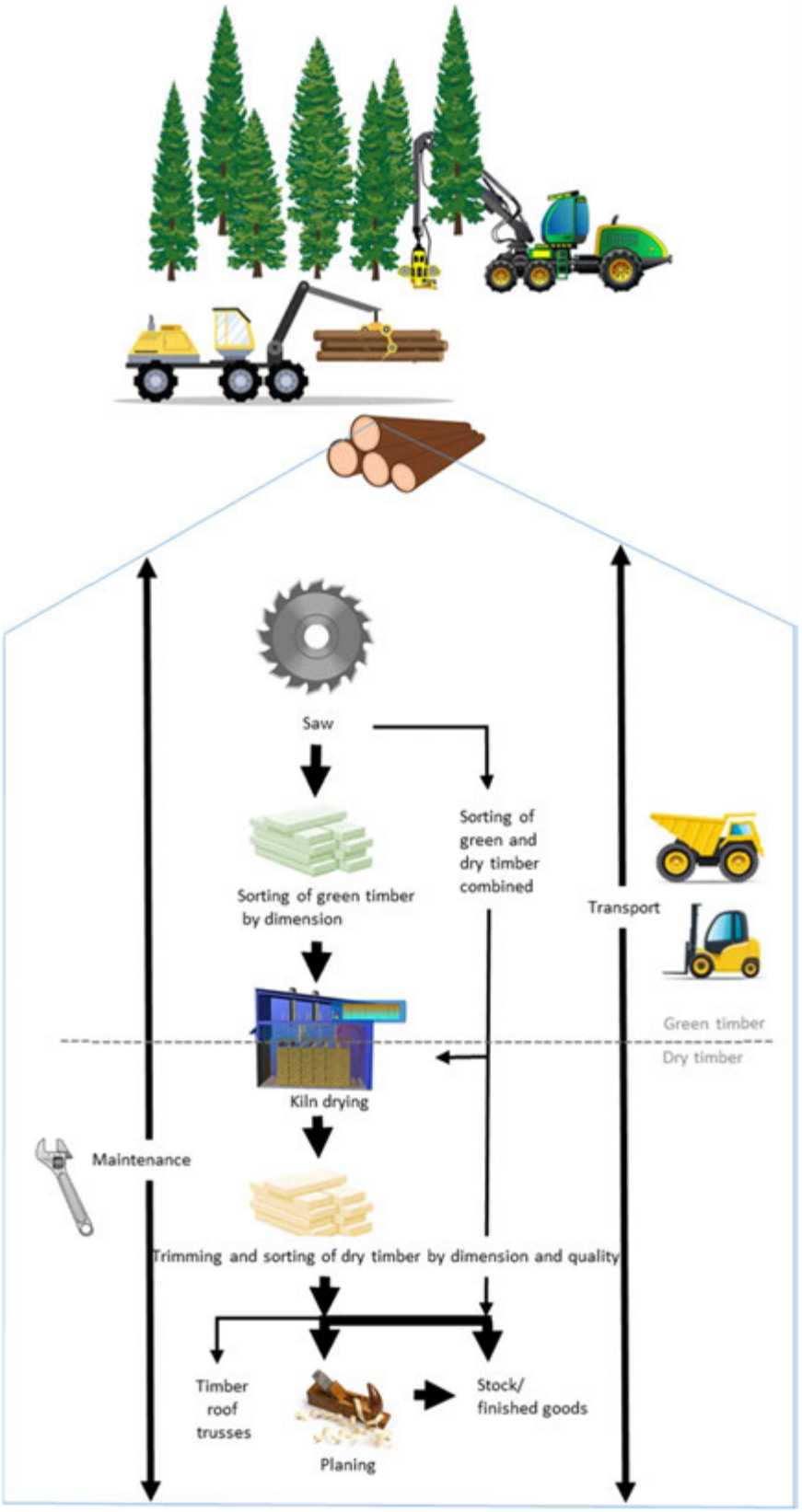

Fig. 2 Process overview from forestry to timber production at sawmills. The review of fungi related to sawmills is defined to the work processes at sawmills. Arrows show the process direction and arrows with a greater thickness indicated the parts that are most common. Adapted from Straumfors, A., Foss, O.A.H., Fuss, J., *et al.*, 2019. The inhalable mycobiome of sawmill workers: Exposure characterization and diversity. Applied and Environmental Microbiology 85.

Table 1 Review of studies on fungal exposure in sawmills

Fungal components	*Unit*	*N*	*GM*	*GSD*	*Median*	*Min*	*Max*	*AM*	*Fungal species*	*Country*	*Wood type*	*Method*	*Reference*
Fungal community composition	OTU	87							Whole mycobiome	Norway	Spruce & pine	ITS2 meta-barcoding	Straumfors *et al.* (2019)
Fungal spores	$\times 10^5/m^3$	86	0.4	3.8						Norway	Spruce & pine	FESEM	Straumfors *et al.* (2018)
Fungal spores	$\times 10^5/m^3$	476	0.4	4.2						Norway	Spruce & pine	FESEM	Straumfors *et al.* (2018)
Fungal fragments	$\times 10^6/m^3$	69	2	3.2						Norway	Spruce & pine	IG-FESEM	Straumfors *et al.* (2018)
Submicronic fungal fragments	$\times 10^6/m^3$	69			0.1	<dl	6.2	0.6		Norway	Spruce & pine	IG-FESEM	Afanou *et al.* (2018)
Large fungal fragments	$\times 10^6/m^3$	69			1.9	0.09	21.8	3		Norway	Spruce & pine	IG-FESEM	Afanou *et al.* (2018)
Fungal spores	$\times 10^5/m^3$	79	0.2–98	2.7–8.9				0.4–98		Norway	Spruce	SEM	Faerden *et al.* (2014)
Asp f1 allergen	ng/g	15			Sawing: 65.5 Parquetry: 40.7	<lod	120			Croatia	Beech/oak/fir/ash	ELISA	Prester and Macan (2014)
Asp f1 allergen	ng/g	20			Sawing: 39.2 Parquetry: 30.3	15.1	77.2			Croatia	Beech/oak/fir/ash	ELISA	Prester and Macan (2014)
Fungal colonies	$\times 10^3$cfu/m^3	30			8.62	0.8	14.4			Croatia	Oak/beech/ ash/fir/ spruce	Culture	Ljubičić *et al.* (2013)
Fungal colonies	$\times 10^3$cfu/m^3	30			3.41	0.86	8.78			Croatia	Oak/beech/ash	Culture	Ljubičić *et al.* (2013)
Fungal colonies	$\times 10^3$cfu/m^3	150						1.6–7.3	Viable characterized	Croatia	Beech/oak/fir	Culture	Klaric *et al.* (2012)
Fungal colonies	$\times 10^3$cfu/m^3	36	2.25			0.10	30.9			South Korea		Culture	Park *et al.* (2010)
Fungal colonies	%	36				9.2	21.3	16.3		South Korea		Culture	Park *et al.* (2010)
Fungal colonies	%	36				78.7	90.8	83.7		South Korea		Culture	Park *et al.* (2010)
Alt a1 allergen	ng/g	15			<LOD					Croatia	Oak/beech/ash	ELISA	Prester *et al.* (2010)
Fungal colonies	$\times 10^3$cfu/m^3	111						14.78		Switzer-land	90% coniferous (spruce/fir)/10% broadleaf trees	Culture	Rusca *et al.* (2007)
Fungal colonies	$\times 10^3$cfu/m^3	47				4.3	35.1	14.78	Viable characterized	Switzer-land	90%spruce, 10% larch and/or fir	Culture	Oppliger *et al.* (2005)
β-1,3-glucan	μg/m^3	52	0.84	3.32		<lod	77.2	3.84		Canada	Western Hemlock/ Douglas fir	EIA	Ronald *et al.* (2003)
β-1,3-glucan	μg/m^3	57	1.02	3.86		<lod	53.6	3.54		Canada	Western Hemlock/ Douglas fir	EIA	Ronald *et al.* (2003)
β-1,3-glucan	μg/m^3	61	2.52	5.16		<lod	121	10.41		Canada	Engelmann spruce/ white spruce/ Lodgepole pine/ Balsam fir	EIA	Ronald *et al.* (2003)
β-1,3-glucan	μg/m^3	53	4	5.96		<lod	226	18.94		Canada	Engelmann spruce/ white spruce/ Lodgepole pine/ Balsam fir	EIA	Ronald *et al.* (2003)
Fungal spores	$\times 10^5/m^3$	11				2.15	14.6	6.68	Viable characterized	Finland		CAMNEA	Roponen *et al.* (2002)

Fungal colonies	$\times 10^3$cfu/m^3	7						4.2	Viable characterized	Poland	Pine & silver fir	Culture	Dutkiewicz *et al.* (2001a,b)
Fungal colonies	% of total	7						50.4		Poland	Pine & silver fir	Culture	Dutkiewicz *et al.* (2001a,b)
Fungal colonies	$\times 10^4$cfu/m^3	12						0.13–1.1	Viable characterized	Canada	Spruce/fir	Culture	Duchaine *et al.* (2000a,b)
Fungal colonies	$\times 10^4$cfu/m^3	1						3.4		Canada	Pine	Culture	Duchaine *et al.* (2000a,b)
β-1,3-glucan	μg/m^3	37				$<$lod	92.5			New Zealand			Douwes *et al.* (2000)
Fungal colonies	$\times 10^4$/m^3	50	2.97	2.77		0.23	9.51	4.03		Australia	Eucaluptus	Culture	Mandryck *et al.* (1999)
β-1,3-glucan	ng/m^3	54	1.37	4.42		0.16	11.7	3.28		Australia	Eucaluptus	LAL	Mandryck *et al.* (1999)
β-1,3-glucan	ng/m^3	28	0.26	2.53		0.1	1.56	0.4		Australia	Eucaluptus	LAL	Mandryck *et al.* (1999)
Fungal colonies	$\times 10^3$cfu/m^3	63		2.77				3–50	Viable characterized	Australia	Eucalyptus	Culture	Alwis *et al.* (1999)
β-1,3-glucan	ng/m^3	54	1.37	4.42				3.28		Australia	Eucalyptus	LAL	Alwis *et al.* (1999)
β-1,3-glucan	ng/m^3	28	0.26	2.53				0.4		Australia	Eucalyptus	LAL	Alwis *et al.* (1999)
Fungal colonies	$\times 10^3$cfu/m^3	15					1000	50		Sweden		Culture	Dahlqvist and Ulfvarson (1994)
Fungal colonies	$\times 10^3$cfu/m^3	26					75			Sweden		Culture	Dahlqvist and Ulfvarson (1994)
Fungal spores	$\times 10^3$/m^3	72	1300	6.8				4400	Viable characterized	Norway	Spruce/pine	SEM	Eduard *et al.* (1993)
Fungal spores	$\times 10^3$/m^3	85	920	5.4				3100	Viable characterized	Norway	Spruce/pine	SEM	Eduard *et al.* (1993)
Fungal colonies	$\times 10^3$cfu/m^3	36			2.95	2.1 (IQR)	4 (IQR)		Viable characterized	Sweden		Culture	Dahlqvist *et al.* (1992)
Fungal spores	$\times 10^3$/m^3	36			120	68 (IQR)	280 (IQR)			Sweden		SEM	Dahlqvist *et al.* (1992)
Fungal colonies	$\times 10^3$cfu/m^3	15			3.7	2.1 (IQR)	4.9 (IQR)		Viable characterized	Sweden		Cultivation	Johard *et al.* (1992)
Fungal spores	$\times 10^5$/m^3	15			1.3	0.71 (IQR)	2.6 (IQR)		Viable characterized	Sweden		light microscopy	Johard *et al.* (1992)

Abbreviation: AM, arithmetic mean; EIA, enzyme immunosorbent assay; GM, Geometric mean; GSD, Geometric standard deviation; IG-FESEM, Immunogold Field emission scanning electron microscope (SEM); (IQR), Inter quartile range; LAL, Lumulus Amebocyte lysate assay; OUT, operational taxonomic unit; SEM, Scanning electron microscopy.

due to modernization and major improvements at different stages of the wood processing line to prevent fungal exposure among sawmill workers since the 1980. Straumfors and colleagues recently showed that both inhalable and thoracic fungal exposure among sawmill workers at 11 Norwegian sawmill today were GM (GSD) 0.4×10^5 spores/m^3 (3.8–4.4) (Straumfors *et al.*, 2018), i.e., at the same level as Færden *et al.* reported in 2009. This indicates that the declining trend have stagnated after about 200 × reduction over 30 years.

Fungal Fragments – A Large Part of Fungal Aerosol in Sawmills

In spite of the reduced spore exposure over the years, the health risk associated with fungal exposure at sawmills is still of great concern. The use of a novel immunogold labeling method for FESEM where fungal fragments were gold-labeled and counted in a complex environmental sample matrix (Afanou *et al.*, 2015a) recently revealed that the fungal aerosols consists of fragments of various size and shape in addition to spores (Afanou *et al.*, 2015b). The fungal aerosol in sawmills was dominated by large fungal fragments (Afanou *et al.*, 2018). Fragments in the sub-micrometer size were also detected (AM: 0.6×10^5 per m^3) in addition to larger fragments (AM: 3×10^5 per m^3) and spores (AM: 1.4×10^5 per m^3) (**Table 1**; **Fig. 3**). This indicates that the fungal aerosol exposure is underestimated by spore counts, but may be included in cultivation if the fragments are viable. The quantification of fragments has not been included in fungal exposure measurements in sawmills previously, and hence not their specific association with health effects. The contribution of fragments in the development of occupational diseases related to fungal exposure is therefore still not known, and need to be clarified in the future.

Revelation of the Sawmill Mycobiome by Metabarcoding

Although SEM can reveal detailed characteristics of surface morphology, shape and size, the identification of airborne fungal particle to species level is difficult. In contrast, DNA-based methods are more robust in targeting all fungal particle types having DNA; viable or dead spores, hyphae and fragments. Molecular methods based on DNA provide a comprehensive approach for fungal identification with chosen level of specificity. Although DNA based assessment of fungal exposure in indoor and various workplaces has been reported (Viegas *et al.*, 2016; Vesper, 2011; Adams *et al.*, 2013), this approach has not been used to the best of our knowledge, in fungal exposure assessment in sawmills until recently. High throughput sequencing (HTS)-based DNA metabarcoding-technology are gaining wide acceptance to capture the complete composition of microbial communities in different environments, including air samples with different size fractions (Cao *et al.*, 2014; Adams *et al.*, 2015; Bartram *et al.*, 2011; Lindahl *et al.*, 2013). DNA metabarcoding of the fungal ITS2 region recently revealed the complete spectrum of inhalable fungi in sawmills, showing a much broader and complex fungal profile than previously known (Straumfors *et al.*, 2019). The most common orders detected among the ascomycetes were the Capnodiales (3.3% of the OTUs and 13.2% of the reads), Eurotiales (1.1% of the OTUs and 9.3% of the reads), Saccharomycetales (1.7% OTUs and 6.8% reads) and Microascales (0.5% OTUs and 6% reads), whereas Tremellales (3.4% OTUs and 6.9% reads), Sporidiobolales (1.2% OTUs and 3.6% reads), Polyporales (4.9% OTUs and 3.4% reads) and Cystofilobasidiales (1% OTUs and 2.8% reads) were most common among basidiomycetes. Among the most abundant species regardless of phyla were the human pathogens *Candida* spp. (Ascomycete) and *Cryptococcus* spp (Basidiomycete). The second most abundant order, Eurotiales, includes species such as *Aspergillus fumigatus* and *Paecilomyces variotii* that previously were detected as dominating species by cultivation, but these species were detected in very low abundances by metabarcoding. Logs were debarked in an automated station before entering the sawmills, and according to Cormier *et al.*, may *Paecilomyces* and *Trichoderma* primarily occur in the bark (Cormier *et al.*, 2000), which may explain the low prevalence. However, relatively high prevalence of *Rhizopus microsporous* and *Mucor* spp. was detected, which is in line with some of the previous studies detecting these species by SEM and cultivation (Dutkiewicz *et al.*, 2001a; Eduard *et al.*, 1992b). Although the most common order among the Zygomycetes was Mucorales, where these species belong, the abundance of Zygomycetes was in general very low compared to ascomycetes and basidiomycetes. The prevalence and relative abundance of the allergenic *Alternaria alternata* was moderate. Overall, the fungal composition differed significantly across sawmills, departments, wood types and seasons.

Bioactive Fungal Components in Sawmill Aerosol

Fungal exposure measurements includes also the detection of fungal antigens, allergens, toxins and β-1,3-glucans. These bioactive fungal components may all elicit or modulate a response upon inhalation of fungal aerosols, and some of them have been used as markers of fungi in many settings (Madsen *et al.*, 2009; Halstensen *et al.*, 2007). In sawmills Alwis *et al.* measured β-1,3-glucan in respirable (AM: 0.4 ng/m^3) and inhalable (AM: 3.28 ng/m^3) dust, and found an association between this fungal derived β-1,3-glucan exposure and airways symptoms reported by workers in sawmills processing eucalyptus in Australia (Alwis *et al.*, 1999). Similarly, β-1,3-glucan levels ranging between 0.40 and 92.5 μg/m^3 were reported in the inhalable fraction of dust collected in sawmills in New Zealand, and although no direct cause-and-effect relationship were established, the exposure levels of endotoxin and beta-glucans were sufficient to potentially contribute to the observed symptoms (Douwes *et al.*, 2000). From settled dust collected in sawmills processing hardwood in Croatia, Prester and Macan reported the presence of Asp f 1 allergen (range: <LOD (3.6 ng/g dust) – 120 ng/g dusts) (Prester and Macan, 2014) but not the Alt a1 allergen (<LOD(120 ng/g dust)) (Prester *et al.*, 2010).

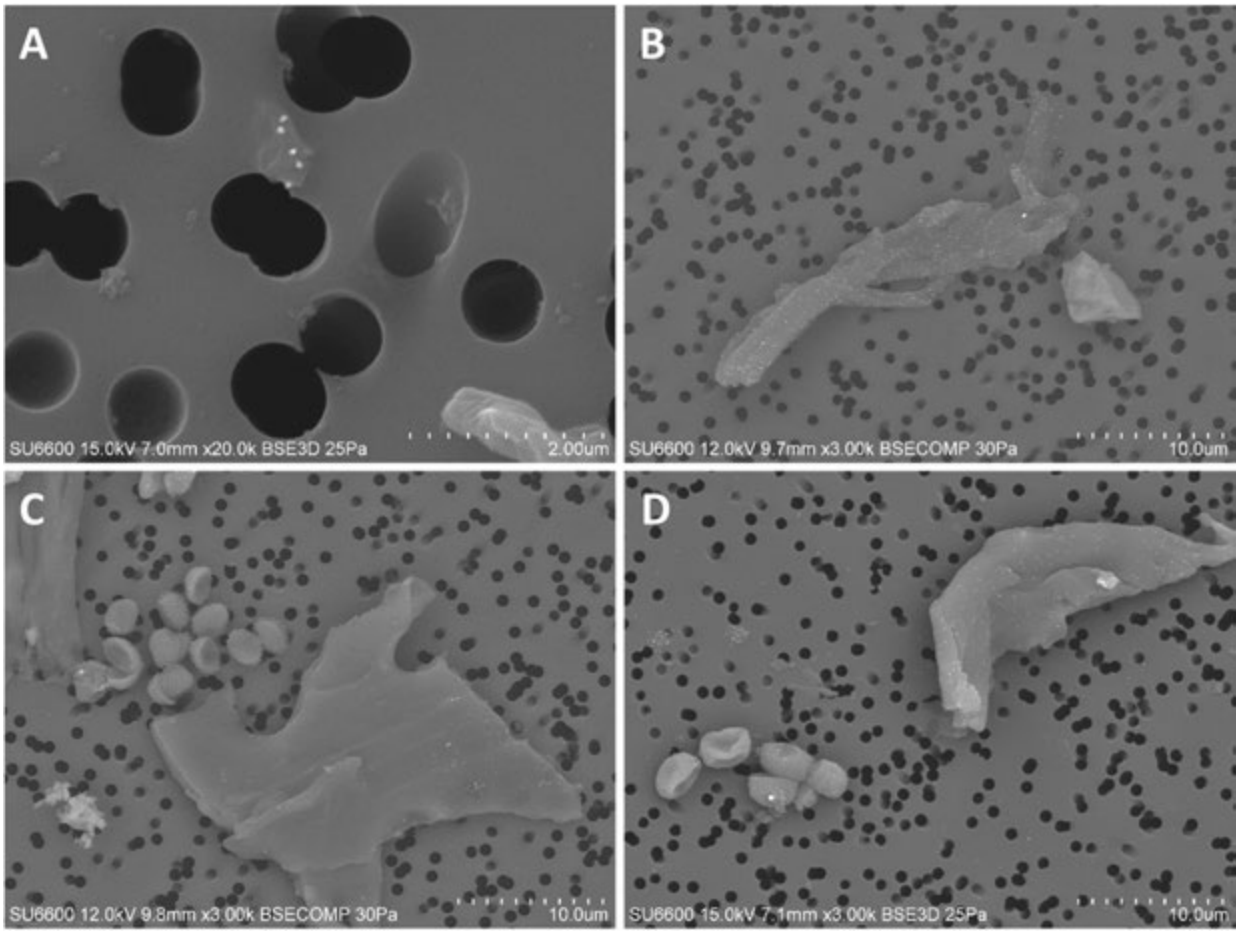

Fig. 3 Micrographs of fungal spores and immunolabelled fragments from sawmill samples. (A) Immunolabelled fragment in the submicrometer size; (B) Immunolabelled larger fungal fragment; (C) Partially labeled fragment and unlabeled fragment and (D) Labeled larger fragment and unlabeled spores. Photo: Anani Afanou, STAMI.

Mycotoxins, as being secondary metabolites of fungi, are inherently associated with fungi, and may exert a wide range of toxic effects in mammals. Mycotoxins have been recognized as part of the occupational exposure in moldy work environments such as grain industry and other agricultural industries (Straumfors *et al.*, 2015; Viegas *et al.*, 2018). In spite of the importance of fungi recognized in sawmills, only two reports of mycotoxins in sawmills have, to our knowledge, been published (Land *et al.*, 1987; Land *et al.*, 1993). Isolates of A. fumigatus collected from kiln dryers at Swedish sawmills were shown to be producers of verruculogen and fumitremorgin C, that exerted mild to strong tremorgenic effect in rats (Land *et al.*, 1987). Thirty-two percent of A. fumigatus strains isolated from 21 sawmills were tremorgen-producers, and the toxins were detected in variable concentrations in the conidia of 7 strains. However, no mycotoxin was detected isolates from different kinds of wood substrates, such as pine, spruce, birch, dried versus non-dried wood, bark, leached wood and wood after various sterilization methods (Land *et al.*, 1993). Although the presence of fungi capable of producing mycotoxins under particular conditions were detected in sawmills suggests that sawmill workers may inhale mycotoxins, the possible role of mycotoxins in HP or other health effects of sawmill workers have not been further studied.

Overall, independent of the method used, large variances between fungal exposure measurements have been observed. An occupational exposure limit (OEL) of 1×10^5 spores m^{-3} has been proposed based on experimental and epidemiological evidence of the lowest observed effect level (LOEL) for exposure to general fungal spores (Eduard, 2009). Although one cannot expect to remove all fungal exposure in sawmills, the industry need to continue improvements of technology and practices that contributes to keep the exposure as low as possible.

Sawmills as Potential Reservoirs of Azole Resistant Fungi

Emerging azole-resistant fungi caused by environmental selection pressure related to the use of azole fungicides in agriculture and floriculture have been described recently (Vermeulen *et al.*, 2013; Verweij *et al.*, 2013; Schoustra *et al.*, 2019). Sawmills also use azole-containing fungicides for impregnation and other wood preservatives, and may as such represent a potential source of pressure for the emerging azole-resistant fungi. Azole-resistant *Aspergillus fumigatus* and *Candida* spp. are of particular concern as

they can cause a wide spectrum of health effects, including invasive aspergillosis (IA) and bloodstream infection, respectively, with high mortality rate (Verweij *et al.*, 2009, 2013; CDC, 2019). Treatment of these opportunistic human pathogens is dependent on azole-containing anti-fungal clinical treatment. Occupational environments may therefore represent a reservoir of potential azole-resistant fungi contributing to the challenges related to the treatment of diseases caused by *A. fumigatus* and *Candida spp*. Azole-resistant A. fumigatus with the TR_{34}/L98H mutation has indeed been detected in sawmills (Jeanvoine *et al.*, 2017), whereas resistant *Candida* spp. is, to our knowledge, not reported in this setting.

Determinants of Fungal Exposure in Sawmills

Knowledge of the life cycle of fungi and the conditions at the work place that are favorable for fungal growth and dispersion is of great importance when trying to understand the variability of fungal exposure in both intensity and composition at any work place. Factors related to the work, such as job type, department, raw materials, job organization, and technical installations may influence the personal fungal exposure. The collection of such information during sampling is valuable for identifying whether or not such factors are determinants of exposure. Finding determinants of exposure is valuable for learning about the exposure variations in different occupations, and for identifying where the largest risks are and where potential interventions can be necessary in order to reduce exposure and improve the workers' health.

Wood Type

The most commonly used wood types that are cut and processed at sawmills are spruce (*Picea abies*) pine (*Pinus sylvestris*) and fir (Douglas fir, *Pseudotsuga menziesii*), but in Australia and some African countries, also Eucalyptus are being processed. Theses wood types are often used in large building constructions, whereas beech, oak and ash are use more in house interiors. Several types of trees, including spruce and pine, have natural resistance against microorganisms (Pearce, 1996; Straumfors *et al.*, 2019). Nevertheless, the wood type may be a determinant of fungal exposure, as reported in Polish and Norwegian sawmills, where the fungal exposure was highest when working with spruce compared with pine (Straumfors *et al.*, 2020; Dutkiewicz *et al.*, 2001a). It was speculated that spruce may be more prone to fungal infection in warm and humid conditions than pine, but different ways of handling and processing various wood types based on their characteristics, may also contribute to differences in fungal exposure. A study of the inhalable mycobiome of sawmill workers revealed that the workers were exposed to significantly different proportions of several fungal genera depending on the wood type they were handling (Straumfors *et al.*, 2019; **Fig. 4**). The overall fungal composition of the exposure was, however, only slightly affected by the wood type processed in the sawmills. Higher fungal richness was also observed during work with spruce compared with pine, but the average OTU-richness per sample was not significantly different between the two wood types, suggesting that the difference may be due to factors in the sawmills that are not related to the wood types. Whereas the exposure to fungi in the phyla Ascomycota versus Basidiomycota were similar when working with spruce or pine, the relative proportion of Basidiomycota was higher when working with both wood types, suggesting either alternative work organization or different competition in dispersal or growth among fungi when mixed wood types are handled at the sawmills. The differences in diversity (Shannon, not richness), evenness and top 10 OTU abundance between sawmills, suggest that other sawmill specific conditions than different wood types indeed play a role (Straumfors *et al.*, 2019).

Departments

Although the process line in all sawmills are relatively similarly organized, with a green side before drying of the timber and a dry side after drying of the timber, the place of highest exposure has deviated somewhat between studies. Work in the saw and with sorting of green timber have been shown to represent the highest fungal exposure in Norwegian sawmills (Straumfors *et al.*, 2018). Work with sorting of green timber also resulted in significantly higher levels of fungal fragments, compared with the other departments (Afanou *et al.*, 2018). The saw department and the sorting of dry timber departments were by mixed effect modeling shown to give the highest fungal spore exposure intensity. More detailed task-based models showed furthermore that controlling the saw in the planing department and cleaning in the sorting of dry timber department were associated with higher spore exposure estimates than the other tasks (Straumfors *et al.*, 2020). In a Swiss study, sorting tasks were shown to represent significantly higher exposure to fungi compared to planing, debarking and saw tasks grouped together, despite the small number of samples per site (Oppliger *et al.*, 2005). Debarking has previously been identified as an activity with the highest microbial exposure (Duchaine *et al.*, 2000b), but this work is nowadays fully automated in some sawmills, and although there may be high levels of airborne fungi during this process, it will not represent any exposure risk at normal process operations, as no operator will be present. DNA metabarcoding of the ITS2 region of inhalable mycobiome in sawmills has revealed distinct fungal compositional profiles between departments across companies (Straumfors *et al.*, 2019), which is in agreement with a previous study that used cultivation as fungal detection method (Dutkiewicz *et al.*, 2001a). This indicates that workers in different job groups not only are exposed to different levels of airborne fungi, but also to different fungal compositional profiles depending on the department and the sawmill (**Fig. 4**). A systematic turnover of fungal diversity was observed throughout the process line from dominating orders of *Saccharomycetales* in the saw, *Saccharomycetales* and *Eurotiales* in the sorting of green timber department, *Eurotiales*, *Tremellales*, and *Capnodiales* in the planing department, and *Eurotiales* and *Tremellales* in the

sorting of dry timber department. The fungal composition in the sorting of green timber department were almost completely overlapping with the saw, both being "green" departments where the timber has not been dried yet. Logs entering the saw are newly debarked, and thus are expected to bring along and release microorganisms related to the bark and the external natural environment. While some of this seemed to carry through to the sorting of green timber, other species also were detected. Although it has not been shown that the community composition in heart wood and sapwood of *Picea abies* and *Pinus sylvestris* is different (Leonhardt *et al.*, 2019), fungi growing inside the wood may be released at later stages of the process line. Indeed, the exposure profile of dominating species observed in the sorting of dry timber department and the planer department, respectively, were completely different with no overlap with other departments (**Fig. 4**). The fungi found here may also have occurred on green timber before being kiln dried, and during storage of dried timber, and thereafter transported on the surface of the timber planks to the sorting department where they were aerosolized.

Seasons

An effect of season on the fungal exposure level may be related to variations in temperatures and relative humidity that will influence the growth conditions for fungi. There could also be conditions at the work place that differs by season, and that will affect the exposure. Considerably higher levels of airborne fungi have been recorded in April-May and July compared with the rest of the year (Klaric *et al.*, 2012). The fungal exposure have been shown to be 2.2 times higher for spores and 2.5 times higher for fragments in summer season compared with winter season (Straumfors *et al.*, 2020). The fungal spore exposure level in a trimming department with relapsing episodes of HP was also higher in winter (4.8×10^6) than in summer (5.3×10^5) (Faerden *et al.*, 2014). Also a higher fungal richness has been observed in summer than in winter, and workers were exposed to different species by season (Straumfors *et al.*, 2019). This is most likely due to more favorable fungal growth conditions and higher rate of spore dispersal in the warm and humid summer season, and in concordance with earlier studies where seasonal differences in fungal communities associated with plant leaves (Davey *et al.*, 2012), roots (Mundra *et al.*, 2015) and soil (Santalahti *et al.*, 2016) were observed. Interestingly, there were systematic seasonal differences in taxonomic composition, with more ascomycetes during winter and more basidiomycetes during summer. This fits into the rough classification of ascomycetes being more tolerant to environmental stress, in this case the harsh winter conditions. Especially many yeast, which are mainly decomposers, were relatively more abundant during winter. This corresponds well with the findings from Santalahti *et al.* showing that saprotrophic fungi, which are metabolically active when photosynthesis conditions are less favorable (Baldrian *et al.*, 2012), dominate during winter compared with the growth season in boreal Scots pine forest (Santalahti *et al.*, 2016). The polypore *Fomitopsis pinicola*, a widespread brown-rot fungus in the boreal forest landscape, was relatively more abundant during summer, when it is expected to be vegetatively more active. However, the abundance of the 10 most common OTU was higher in winter than in summer. This relationship might be related to the evenness of the samples, i.e., if there are fewer species during winter, these will appear as more abundant relatively. Hence, the summer season most likely represents the most diverse fungal exposure. This was also supported by ordination analyzes showing broader ordination configuration of samples collected in summer compared with winter (**Fig. 4**). Overall, these results highlight that the seasonal shift in fungal communities related to the sawmills may impose variation in occupational exposure.

Other Factors That Impact Fungal Exposures at Sawmills

The type and amount of fungi that colonize the logs in the storage yard will depend on temperature and humidity and the storage time at the particular conditions. i.e., the fungal profile that workers are exposed to is partly be dependent on the fungi that enters the sawmill together with the logs, which again is a result of time and conditions for storage of the logs. Production volume, how much timber is processed per year, may have impact on the fungal presence and the workers exposure. The more timber processed, the higher may the occurrence of natural fungal contamination from the fresh logs become. The persistence of a generally higher fungal level or the growth of particular species at any point along the timber production line, will be dependent on how the sawmill is dimensioned for its production volume and the flow-through time of the timber, i.e., the duration from logs are entering the saw until the cut timber is collected by the costumers. Factors of importance here includes drying and storage capacity, good routines for cleaning sawdust, and exposure reducing measures, such as dust extractors and ventilation. Færden *et al.* hypothesized that the reduced occurrence and intensity of HP symptoms in trimmers was due to reduction of the outdoor storage time of sawn timber before drying that resulted in less fungal growth on the wood and improved drying process with new kiln (Faerden *et al.*, 2014).

It should be noted that many other non-fungal components of the aerosol at sawmills may cause health effect individually and in combination, and the exposure determinants of the individual components may differ between each exposure component (Straumfors *et al.*, 2020, 2018).

Reducing the Exposure to Fungi

Any action to reduce fungal growth and to reduce the dispersion of fungal particles into the work place aerosol will reduce fungal exposure. Dust extractors and functional ventilation are important exposure reducing measures. However, because each company have their own particular solution of the technological installations, is hard to compare the technological factors between companies in order to identify what is most efficient in exposure reduction. By changing operator position during the shift, workers will even out between them any potential high exposures due to particular tasks. In this respect may job rotation

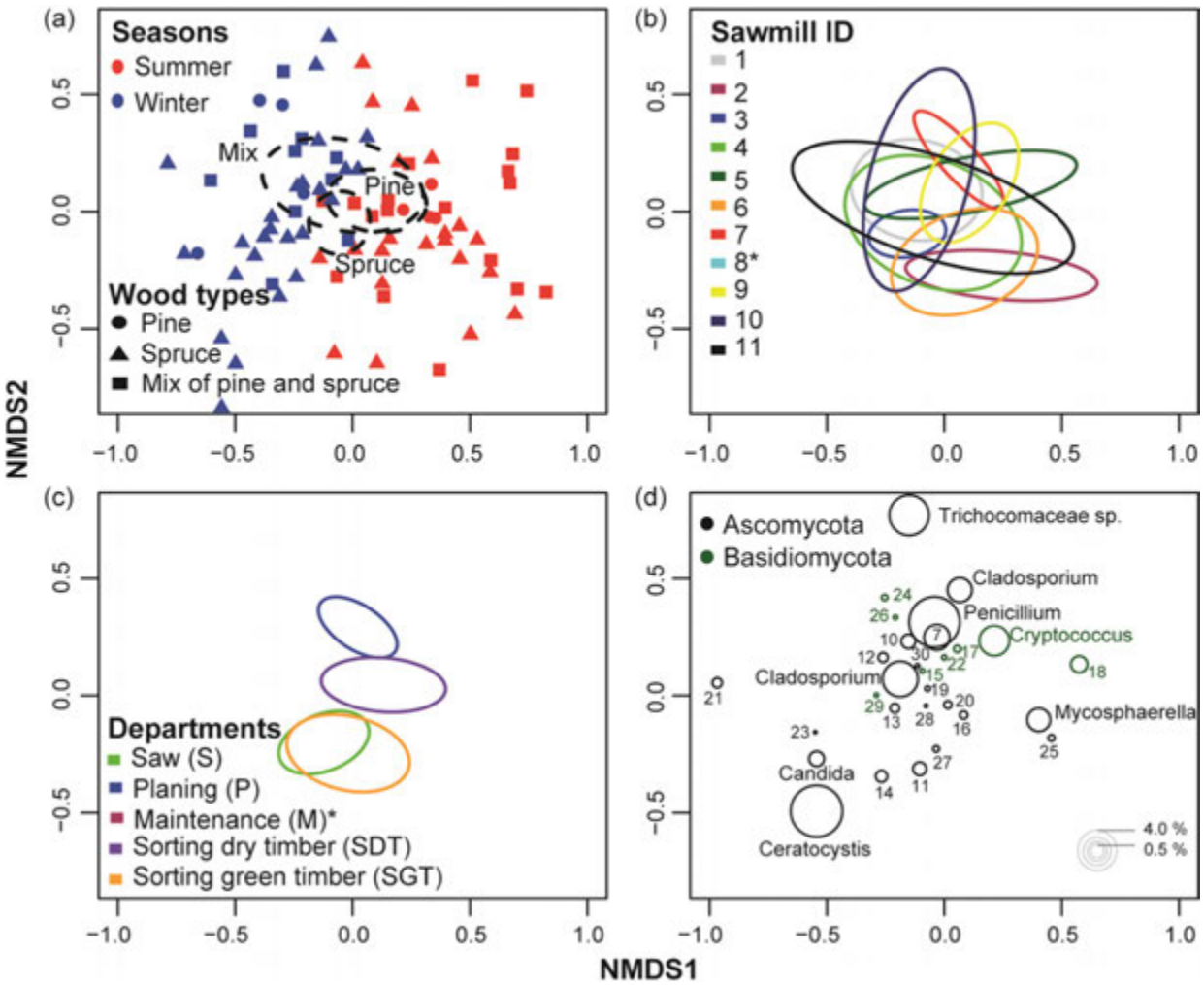

Fig. 4 Variation in fungal community composition by wood type, season, sawmill and departments. Nonmetric multidimensional scaling (NMDS) ordination analysis of fungal communities in personal air samples from Norwegian sawmills. (a) The plot represents 84 different personal air samples coded by color for the sampling season (summer and winter) and coded by shape for the different wood species (pine, spruce, or a mix of the two wood species) processed on the sampling day. (b) Differently colored ellipses represent the 95% confidence interval around the centroids of individual companies. (c) Differently colored ellipses represent the 95% confidence interval around the centroid for different departments at all sawmills. (d) NMDS ordination analysis showing the fungal operational taxonomic unit (OTU; species) composition from all sawmills. The ordination plot is based on all fungal OTUs present, but only the 30 most common OTUs are shown here. Each of these OTUs represented, on average, at least 0.5% of the total reads. The top 30 OTUs accounted for 49% of the total reads. All factors (season, wood type, sawmill, and department) had significant effects ($P \leq 0.05$) on the ordination configuration. An asterisk denotes a sawmill or department with too few samples for inclusion in the ordination analysis. Rendered from Straumfors, A., Foss, O.A.H., Fuss, J., *et al.*, 2019. The inhalable mycobiome of sawmill workers: Exposure characterization and diversity. Applied and Environmental Microbiology 85; Copyright AEM 2019.

contribute to reduce the average exposure during a workday. The use of personal respiratory protection need to be evaluated in particular high-exposure situations and when sufficient exposure reduction is not possible.

Health Effects of Fungal Aerosol Inhalation in Sawmills

Exposure to fungal spores has been associated with HP and other respiratory symptoms among sawmill workers (Wimander and Belin, 1980; Eduard *et al.*, 1992b, 1993; Halpin *et al.*, 1994b; Douwes *et al.*, 2001).

Hypersensitivity Pneumonitis (HP)

HP has, in particular, been reported among sawmill workers handling wood and wood products. Symptoms of and clinical diagnosed cases of HP were previously frequently reported among workers employed in trimming departments of sawmills. A

non-allergic immunological response to the inhalation of *R. microsporous* spores was considered as being the cause. This was confirmed by the presence of serum IgG antibodies against *R. microsporous*, positive precipitating antibodies to the antigens from these species, and recurrent episodes of characteristic symptoms, such as cough, chills fever, 4–8 h after exposure (Faerden *et al.*, 2014). HP has been associated with antigens from *Rhizopus microsporous* (spruce/pine), *Alternaria*, *Aspergillus*, *Trichoderma koningii* (Halpin *et al.*, 1994a), *Thermo actinomycetes vulgaris* (moldy wood chip), but also *Cryptostroma corticale* (maple bark) and *Aureobasidium pullulans* (redwood) (Enarson and Chan-Yeung, 1990). Wood trimmers had higher levels of serum IgG towards *Rhizopus microsporous* and *Paeocilomyces variotii* than sawmill workers with other tasks with assumed lower fungal exposure (Eduard *et al.*, 1992b). Although the occupational fungal exposure were reduced and the frequency of HP symptoms declined over 9 years, workers still reported HP symptoms several times in a year (Faerden *et al.*, 2014), specifically related to handling of moldy timber.

The validity of exposure estimates in relation to health effects depend on our ability to differentiate between species. This is particularly important when examining specific responses such as allergic asthma, allergic rhinitis and HP. Several of identified species may provoke other immunological and allergic responses, also shown by the presence of specific antibodies in the blood of workers (Cormier *et al.*, 2000; Dutkiewicz *et al.*, 2001b; Eduard *et al.*, 1993).

Many fungal species commonly found in sawmills are sources of allergens, and inducers of IgE-mediated sensitization and causes of atopic respiratory diseases such as rhinitis and asthma. However, IgE mediated reactions to inhaled fungi does not seem to be common in sawmills, but rather a non-atopic, neutrophilic inflammation. In a Croatian study, only one of 96 sawmill workers had positive skin prick reaction to *Rhizopus nigricans*, whereas the reaction towards *Cladosporium herbarum*, *Alternaria alternate*, *Aspergillus niger*, and *Penicillium notatum* were negative for all (Klaric *et al.*, 2012). Rydjord *et al.* detected increased level of *R. microsporous* specific serum IgG in sawmill workers, particularly trimmers, and a decreased level of IgE compared with controls (Rydjord *et al.*, 2007). In a study of sawmill workers reaction to work-related airborne allergens, Dutkiewicz *et al.* observed an association with work-related symptoms and positive immunological response only in for a limited number of allergens tested, including fungi (Dutkiewicz *et al.*, 2001b). Atopy is present among wood workers in similar proportions to the general population (Klaric *et al.*, 2012).

Cedar sawmill workers have higher reported prevalence of asthma compared to a reference population, but fungi is not believed to be the main causal agent for this. On the contrary, Western red cedar (*Thuja plicata*) is regarded mold-resistant, and no cases of HP has been reported after handling this wood type (Enarson and Chan-Yeung, 1990).

Other Inflammatory Airway Responses Among Sawmill Workers

Airway inflammation is commonly induces by constituents of microorganisms. Although microbial species may have different toxicity, the importance of species identification is less obvious for "non-specific" responses such as inflammation of the airways, non-allergic asthma, bronchitis and organic dust toxic syndrome (ODTS), also called inhalation fever. Organic dust toxic syndrome (ODTS) is caused by high dose exposures to fungal spores in moldy materials in several occupations, including sawmills (Sorenson *et al.*, 1998; Rask-Andersen *et al.*, 1994; Enarson and Chan-Yeung, 1990), but can also be induced by bacteria, endotoxins and mycotoxins.

Workers exposed to wood dust showed evidence of chronic airway obstruction, but no unspecific bronchial hyperreactivity when compared with controls (Enarson and Chan-Yeung, 1990). The causal agent for this is not clarified, but seemed to vary with the dust content. Dahlqvist and colleagues reported a mild obstructive lung function deterioration in Swedish wood in trimmers exposure to up to 10^6 cfu/m^3 compared with other sawmill workers exposed to several times less fungi (Dahlqvist *et al.*, 1992; Dahlqvist and Ulfvarson, 1994).

Chronic bronchitis is shown to be more prevalent in sawmill workers than among grain handlers, with an additive effect of tobacco smoking, but reversible upon cessation of exposure (Enarson and Chan-Yeung, 1990). Although signs of low intensity alveolar inflammation were observed in wood trimmers exposed to low concentrations of dust, fungi and bacteria (Johard *et al.*, 1992), significant differences has not always been observed between exposed sawmill workers and controls (Cormier *et al.*, 2000). Cormier and colleagues showed that overall, sawmill workers with low exposure levels had little or no evidence of respiratory health problems related to their work environment.

Other symptoms, such as irritation of the eyes (burning, itchiness, and lacrimation) or nose (sneezing, itchiness, and rhinorrhea) is usual among sawmill workers, but can be related to nuisance dust, several components of the dust and not necessarily only related to fungal exposure (Demers *et al.*, 1997). As dust contains multiple components potentially affecting the health, it is important to take into account health effects of all components. Very few studies have been designed to evaluate individual and combined effects of the mixed exposure at sawmills.

β-1,3-glucans are glucose polymers with variable molecular weight and degree of branching (Williams, 1997) that are cell wall components of most fungal families, including human pathogens such as *Candida* and *Aspergillus*, but not Zygomycetes. β-1, 3-glucans are also produced by algae (laminarin), bacteria (curdlan), and plants (callose), and elicit potent biological responses across several kingdoms, including plants, insects, and animals (Jacobs *et al.*, 2003; Brown *et al.*, 2003). In humans, β-1,3-glucans can induce inflammation or modify concomitant biological responses to endotoxins or allergens by either enhancing or inhibiting their effects (Young and Castranova, 2005). Working in New Zealand pine sawmills has been associated with an increased prevalence of cough symptoms, and eye and nose irritation (Douwes *et al.*, 2001). The causal agent was not clear, as shown by a poor dose-response relationship to dust exposure, but exposure levels of endotoxin and beta-glucans were sufficient to potentially contribute to the observed symptoms (Douwes *et al.*, 2000). The authors stressed that other causal agents such as resin acids, terpenes and other microbial components could contribute, and ought to be included in exposure assessments.

Immune Modulating or Positive Effects of Fungal Diversity

Protective effects associated with fungal exposure have also been reported (Ege *et al.*, 2011; Stein *et al.*, 2016), and it is increasingly recognized that it is the fungal compositional distribution in the exposure that is important to health (von Mutius, 2007; Jatzlauk *et al.*, 2017; Ege *et al.*, 2011; Stein *et al.*, 2016; Haahtela *et al.*, 2015; von Hertzen *et al.*, 2011). The idea is that a rich diversity will modulate all potential effects and reflect a balanced mycobiome, rather than a particular species composition that is beneficial. An imbalance between natural species may cause dominance of few particular species including pathogens that may result in negative health effects. The immune modulating effect of β-1,3-glucans may be part of this balance.

Conclusions and Future Perspectives

There will always be a potential for exposure to aerosols containing fungal particles at sawmills, since wood is natural substrate for many fungi. Sawmill workers were exposed to up to 10^7 spores/m^3 in the 80s, but this have been reduced over 200 times over the past 30 years due to improved drying technology and other exposure reducing measures in the sawmill industry. Concomitantly has the frequency and severity of reported HP been reduced, but is still reported several times a year for some workers, and ODTS and other inflammatory and respiratory impairments are frequently observed. Recent studies have shown that the fungal exposure at sawmills have a broad species diversity, and consists primarily of fungal fragments. Additionally is the fungal exposure level and composition shown to differ between seasons, wood types, continents and departments. The use of exposure determinants may be useful for evaluating the potential health effects and exposure reducing measures. The presence of resin acids, bacteria and terpenes that individually or in combination may exert health effects complicates the exposure picture further, also because exposure determinants differ between individual components. The evaluation of health effects from this complex fungal exposure challenges the previous perception of causal agents, revealing the need for continuing investigations of how sawmill workers health is affected by fungal exposure. Particularly should the contribution of fungal fragments to health effects be addressed. Further studies are needed to determine factors that shapes the fungal diversity in sawmills, and to elucidate how the differences in the complete fungal spectra relate to health effects, in order to become able to apply the new knowledge for improving workers health.

Appendix A Supplementary Material

Supplementary data associated with this chapter can be found in the online version at doi:10.1016/B978-0-12-809633-8.21044-3.

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Next-Generation Sequencing in Environmental Mycology. A Useful Tool?

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Introduction

Since the rapid development of next-generation sequencing, more commonly referred to as high-throughput sequencing (HTS) methods in the recent years, fungi have been the underdogs of the microbial world, especially in bioaerosol studies. Particularly, studies describing fungal exposure in different occupational environments have been limited by traditional culture methods that underestimate the broad spectrum of fungi present in the air (Peccia and Hernandez, 2006). There are potential risks in the human inhalation of fungal spores in an occupational scenario where the quantity and diversity of fungi is high (Shams-Ghahfarokhi *et al.*, 2014; Ekhaise and Ighosewe, 2008; Douglas *et al.*, 2018). Although some health problems are already known to be associated with fungal exposure in certain work environments, the risk may be underestimated due to the methods used (Fabian *et al.*, 2005; Peccia and Hernandez, 2006). The presence of previously undocumented fungi in aerosol samples represents one of the limitations in the understanding of the impacts of fungal exposure on respiratory health (Bush *et al.*, 2006; Tischer and Heinrich, 2013). The harmfulness of fungal aerosol mixes could be dependent upon the composition of elements, synergy among the elements, and factors attributable to source and growth conditions (Fröhlich-Nowoisky *et al.*, 2016; Aleksic *et al.*, 2017). The absence of a universal method to explore fungal exposure in occupational settings is not surprising due to the limitations of the actual methods. To date, many questions are still unanswered in the use of molecular methods to characterize the fungal diversity of aerosols. The hypothesis is that, although essential in the study of biodiversity, traditional approaches based on the cultivation of fungi induce a bias in the representation of fungi in aerosols (Fabian *et al.*, 2005; Peccia and Hernandez, 2006). Thus, a method based on HTS with the appropriate barcode could be optimal to reveal the diversity of fungal aerosols and to provide exposure data with a maximally complete image of the presence of fungi (Tedersoo *et al.*, 2015, 2018). For example, applying HTS in soil samples has helped the explanation of the fungal role in many other ecosystems (Nilsson *et al.*, 2019a,b). Nonetheless, the literature is still not decisive in terms of the genomic region to use as target for the enrichment and sequencing of fungi (Tedersoo *et al.*, 2015; Wang *et al.*, 2014). The motivation behind writing this chapter is the desire to introduce an overview of new approaches to study fungal aerosols. The reason behind this motivation was to compensate the gaps caused by the traditional approaches used in the study of fungal exposure in occupational environments. The traditional methods consist of cultivating fungal species collected from air samples to study their concentration and diversity. The gap in the literature caused by the application of cultivation methods solely is linked to the specific representation of the cultivable and viable portion of fungal aerosols. The underrepresented portion of the fungal community might have a significant role in the adverse health effects related to exposure. Thus, it is necessary to consider not only the tip of the iceberg, but also look at the bigger picture of fungal aerosols. The willingness to fill the gap of the occupational studies with the non-viable or the uncultivable portion of fungal aerosols took us to consider the application of molecular tools. These tools, amongst others, consist of HTS of the genomic DNA extracted from aerosol samples taken from environmental settings, as streamlined by targeting various fungal universal barcodes. In addition, culturomics, which consists of the application of multiple culture conditions, combined with amplification and sequencing for the identification of previously unidentified colonies, is also mentioned for its ability to offer complementary images of fungal diversity identified by molecular tools (Amrane *et al.*, 2018). Also, with HTS becoming more popular, more bioinformatic tools are available, and choosing the right tool is a prerequisite to a valid diversity analysis. The author was also motivated by the desire to present an overview of the bioinformatic tools available that can offer the readers the freedom to control every step and parameter in the process of sequence treatment and diversity analyses.

Environmental Exposure to Fungi

From an occupational health perspective, enhancing our understanding of the risks related to exposure requires attention. The relevance of bioaerosol data is needed for risk assessment and risk management (Mbareche *et al.*, 2019a,b,c). Industrial and agricultural environments are the major considerable concerns in occupational health issues due to the presence of potential fungal sources (e.g., raw organic materials), and the occurrence of operations releasing harmful bioaerosols (e.g., mechanical operations such as wood planning, straw chopping, animal bedding, hay handling, and compost pile turning). The generation of large amounts of bioaerosols in confined spaces is what make this type of exposure of importance in occupational health. Bio-waste facilities are characterized by notable concentrations of fungal aerosols due to the fungal activity responsible of the waste degradation and the daily activities related to composting (Wéry, 2014; Bonifait *et al.*, 2017; Dubuis *et al.*, 2017; Mbareche *et al.*, 2017). Intensive animal farming practices in confined buildings containing many animals (e.g., cows, pigs, poultry, cattle) are also associated with extreme exposure to airborne microbes. The variety of possible sources (e.g., animals, feces, feed, litter) present at farms leads to the emission of complex mixtures of biological particles (Gilbert and Duchaine, 2009; Lanier *et al.*, 2010; Létourneau *et al.*, 2010; Tsapko *et al.*, 2011; Douglas *et al.*, 2018). Moreover, the constantly changing nature of the microbial composition over time and space makes health risk evaluation complicated for farm workers and nearby residents (Iversen *et al.*,

2000; Schiffman *et al.*, 2005). Environmental scientists, public health practitioners, and epidemiologists continue to insist that insufficient exposure assessments are one of the main reasons for the absence of bioaerosol exposure limits and strategies to mitigate risk (Douglas *et al.*, 2018; Walser *et al.*, 2015; Mubareka *et al.*, 2019).

Studies in occupational health are pointing toward adverse health effect, especially respiratory symptoms, among workers exposed to high levels of fungal aerosols (Gilbert and Duchaine, 2009; Douglas *et al.*, 2018). Health-related exposure limits are crucially needed for implementing procedures to decrease workers' exposure to an acceptable level. This field of bioaerosols could make great progress by using suitable measurable parameters, developing standardized detection methods for exposure markers, combining different detection methods to compensate the limitations of different approaches, and applying new analysis methods to identify the potential hazard incurred by workers in different occupational settings. As stated by Mubareka *et al.* (2019), the field of applied bioaerosol research can benefit from having an open network research (collaboration of different specialists); a shared technical protocol and training programs; engaging knowledge users; enhancing capacity-building for response measures (development of best practices). Particularly for fungi, culture methods have been the golden standard for all the training programs and the build-up knowledge on fungal concentrations, and taxonomy in different environments.

Cultivating Fungi

Although HTS methods are increasing the possibility for fungal studies, data production from cultivating fungi cannot be underestimated. Isolation of fungal species is essential to understanding and studying phenotypic and genotypic characteristics of single isolates and to better assess biodiversity. Antifungal resistance is a current trend that represents a major clinical challenge for treating invasive fungal infections (Wiederhold, 2017). Like for bacteria, clinicians are facing the limited stock of available antifungal agents. Antifungal resistance has been found in species of *Candida albicans*, *Aspergillus fumigatus*, *Scedosporium*, and *Fusarium* worldwide (Wiederhold, 2017). Even if using molecular methods allow the identification of resistance genes, cultivating fungi carrying the gene of interest is key for the determination of antifungal resistance phenotypes. Furthermore, culture methods provide the link between antifungal resistance measured in the environment, and antifungal resistance detected in human clinical specimens. In addition, cultivation of fungi inform on the antifungal concentrations range, which is an important factor in invasive fungal infections treatments. In sum, both molecular and cultivation methods are needed to get the full spectrum of resistomes. Another example of the advantages of cultivating fungi is the intracellular parasites, microbes that are capable of growing and reproducing inside a host cell. Some fungal intracellular parasites include *Histoplasma capsulatum* and *Cryptococcus neoformans* (Sebghati *et al.*, 2000; Feldmesser *et al.*, 2000). Efforts in associating optimized culture conditions, and detection methods allowed a better understanding of the mechanisms used by intracellular pathogens (Orfila, 1996). In general, subjects with a T cell deficiency are particularly affected by intracellular pathogens (Carneiro-Sampaio and Coutinho, 2007). One common example, more related to occupational exposure, of the importance of cultivating fungi is the production and characterization of antibodies to environmentally important fungal species. It's a technique that has been used to describe exposure and workers' susceptibility to fungi in different workplaces (Eduard, 2009). Finally, even some molecular techniques require the cultivation of fungi. Reverse Sample Genome Probing (RSGP) is a method used to analyze microbial composition of the most dominant culturable fungal species by using genome microarrays. One of the steps of the RSGP is the isolation of genomic DNA from pure cultures (Voordouw *et al.*, 1991).

Now that the conviction is undeniable that a pure fungal culture remains essential for the reasons cited above, cultivating fungi in diversity studies is problematic. Indeed, most fungal species are difficult to isolate using common culture methods. Growth media and conditions are not known for most fungi. The ones that are used commonly (Sabouraud and Rose-Dextrose Agar; Malt Extract Agar) may advantage some species and be less fit for others. The bias could create an imbalance in the fungal species represented in bioaerosols. In particular, considering that the fungal kingdom is one of the most diverse one (Blackwell, 2011). Viability is not the only condition fungi can cause harm once inhaled. Cultivating airborne fungi for concentration measures and diversity analyses is still current amongst bioaerosol researchers. Distinctly, exposure to bioaerosols in waste work environments has received a particular attention for the use of culture methods. In a 2016 study, Madsen and collaborators have identified 23 fungal species, and a total concentration of fungi up to 4.6×10^4 fungi m^{-3} inside the truck cap during waste collection (Madsen *et al.*, 2016). A more recent study of 2018 has set to evaluate the risk of occupational exposure to viable fungi in five waste-sorting industries, identifying a total of 13 different species with a mean count of 1.6×10^4 total fungi m^{-3} (Santos *et al.*, 2018). In beginning of 2019, Madsen and collaborators identified 21 different fungal species with a personal exposure of 6.5×10^4 CFU m^{-3} in cardboard waste sorting facilities. Outdoor environments are also still characterized using culture methods. In 2016, a fungal exposure analyses in various outdoor settings identified 18 different fungal species, with *Cladosporium*, *Penicillium*, *Aspergillus*, *Alternaria*, and *Rhizopus* being the most abundant (Ghiasian *et al.*, 2017). Also in 2018, a study monitoring airborne fungi in different outdoor spaces has identified *Aspergillus*, *Penicillium*, and *Cladosporium* as the dominant genera (Ziaee *et al.*, 2018). In addition, a thesis from the University of Iowa, published in 2018 as well, described cultivable airborne fungal concentrations and diversity in 100 different indoor and outdoor locations registering different climates of the United States. The study identified close to 40 fungal species (Messer, 2018). Indoor studies are also familiar with the use of culture methods for the assessment of cultivable airborne fungi (Liu *et al.*, 2018). A recent review of common indoor fungi has identified *Aspergillus*, *Penicillium*, *Cladosporium*, *Alternaria*, and *Fusarium* as the major fungi in hospital interior environments (Marques do Nascimento *et al.*, 2019). A survey of 71 classrooms has also used cultivation methods to address schoolchildren indoor exposure to fungi (Madureira *et al.*, 2018).

A rookie in the -omics team (metagenomics, metatranscriptomics, metabolomics, etc.), which is a part of the culture methods family, appeared in recent years as a promising new technique in microbiology. As indicated in the name, culturomics consists of the application of high-throughput culture conditions combined to 16S rRNA amplification and sequencing or matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) for the identification of previously unidentified colonies. Although the first mention goes back to 2012, culturomics gained popularity in recent years (Lagier *et al.*, 2012, 2015, 2016; Amrane *et al.*, 2018; Kambouris *et al.*, 2018; Hosny *et al.*, 2019). Most of the studies using culturomics were done to explore the gut microbiota, with a focus on bacterial communities. Future efforts in culturomics could include fungi, as the actual knowledge on high-throughput culture conditions of fungi is very limited compared to bacteria. For fungi, studies combining culturomics and HTS in human gut have arrived to the same conclusions about the complementarity of both approaches in describing fungal diversity of the gut (Hamad *et al.*, 2017). Fungal bioaerosol studies can benefit from the complementarity of these approaches to better understand exposure and its impact on health.

In consideration of the complexity and the diversity of fungi in different ecosystems, one would expect such diversity to be mirrored in bioaerosols too. Yet, occupational, indoor and outdoor bioaerosol studies characterizing fungal exposure around the world refers to the same major key players in the fungal kingdom, as demonstrated above. Could it be that the underdogs of the fungal kingdom are awaiting to be put on the spotlight by applying the right methods? Shade and his collaborators have demonstrated brilliantly the complementarity of culture-dependent and culture-independent approaches to studying bacterial diversity in soils. The premise of their study is that culture-dependent methods reveal bacteria from the rare biosphere and provide supplemental information to that obtained using a HTS approach. The latter method would be beneficial for the most abundant taxa. This brings the hypothesis that all these years microbiologist have been studying the rare biosphere (Shade *et al.*, 2012). Thus, combining culture-based methods with HTS can provide a richer portrait of microbial diversity than does either approach alone. Airborne fungi in different environments and conditions deserve the same attention to better understand, not only the ecological importance of fungi in the air, but also to validate the members contributing to the adverse health effects of being exposed to them.

To sum up, cultivating fungi alone underrepresent the extent of airborne fungal diversity and their exposure in occupational settings. As mentioned previously in this chapter, the application of HTS methods will provide us with necessary information in order to complete the portrait of fungal diversity.

High-Throughput Sequencing

The use of molecular methods in microbial studies is based on the detection of genetic material of organisms present in a given sample. Applying these methods to different environments permitted the identification of the microorganisms present, and a better comprehension of the environmental impacts and ecological roles of microbial communities (Venter *et al.*, 2004; Delmont *et al.*, 2011; Walter and Ley, 2011; Philippot *et al.*, 2013). The countervailing approach of molecular biology to culture methods can be appreciated in the representation of the non-viable or the uncultivable (unknown culture conditions) portion of microbes, extending the measurement to a total biomass (uncultured + cultured). In addition, it became a common knowledge that only a few percentage (less than 1%) of microbes can be recovered by enrichment culture. Although it was first demonstrated for bacteria from soil and aquatic environments (Amann *et al.*, 1995; Pace, 1997), other studies, measuring both total and culturable microbes, showed the same tendency for bacteria and fungi in bioaerosols (Peccia and Hernandez, 2006). For example, in 2005, Fabian and collaborators showed that the percentage of culturability in outdoor air was 0.1% for bacteria (trypticase soy agar), and 0.001% for fungi (malt extract agar; Fabian *et al.*, 2005). Without a doubt, the reliance on culturing alone is a limitation in bioaerosol studies.

Molecular methods do not assess organisms viability or integrity. However, some negative health impacts, like allergies, do not rely exclusively on the viability of microbes. The enormous potential of molecular biology methods for describing bioaerosols, expanding the diversity of microbes that can be detected, identified, and quantified counterweight the critics. Other major challenges are associated with the detection of nucleic acid to identify and quantify microbes, and they can be summarized as follows: (1) concentrating and retrieving the biological material from aerosol samples prior to analyses; (2) choosing the right genomic regions to amplify and/or to sequence in order to obtain the most accurate information about the microbial content of the environment sampled. Specific genes from different organisms can be detected using quantitative real-time amplification (qPCR), which lead to a concentration of specific microbes in air. Virulence factors genes, metabolic operons or antibiotic resistance genes can also be quantified from environmental samples (Chizhikov *et al.*, 2001).

David Stahl developed the concept of molecular microbial ecology based on the sequencing of the 16S rRNA gene region and comparing it with databases (Amann *et al.*, 1990). This concept has been used for the last decades to study microbial diversity in environmental samples. Detection and identification of bacteria can be achieved using the conserved 16S rRNA prokaryotic gene, after centrifugation of liquid or filtered and re-suspended air samples. For fungi, two major challenges are confronted: they are not efficiently recovered from liquid air samples using centrifugation protocols used for bacteria and archaea (Mbareche *et al.*, 2019a,b,c) and the choice of the genomic region to be used that will lead to a representative image of bioaerosols content is difficult. The eukaryotic conserved regions, 18S rRNA gene and ITS regions, can be used depending on the context of the study. Even though those regions have highly conserved functions in their respective organisms, there are some distinct differences between their variability in eukaryotes compared to prokaryotes. For example, while the V6 region in 16S rRNA gene has been considered as variable and well-suited for assessing bacterial diversity (Huse *et al.*, 2008), its equivalent in the 18S rRNA is more conserved in eukaryotes and it is often avoided for that reason (Sogin, 1991; Hadziavdic *et al.*, 2014). The relevance of the ITS region to describe fungal diversity in aerosols will be covered later in the next sub-section.

Although they might not give a direct information on dissemination and transmission of diseases, using molecular approaches to detect DNA sequences from an environment give crucial information. Indeed, they enable us to link aerosol content directly to the source and have a better evaluation of the air contamination. For example, detecting high concentrations of *Aspergillus fumigatus* in a composting environment using qPCR is a good indicator that workers might be at great risk of developing health problems, regardless of the viability of *Aspergillus fumigatus* in our samples. Thus, using HTS approach can give an indication about the most abundant microorganisms in an environment. Then, qPCR can be applied to have a concentration per cubic meter of a specific genus. This will add a biomass indicator to the diversity portrait. Finally, the type of exposure can be investigated in regard to the transmission and the dissemination of microbial agents.

The HTS method was applied to bio-waste and agricultural environments with successful outcomes in the description of a large specter of fungal diversity. The results obtained from composting facilities showed that the fungal composition was dependent on the type of waste treated. The differences between the two types of compost were statistically significant, and were driven by the class of Eurotiomycetes, which were more abundant in carcass and placenta composting, while Sordariomycetes were dominant in domestic composting. Very diverse fungal profiles were obtained for the two types of composting environments, in the compost pile and air samples. Although, the taxonomic profiles showed a clear link between the source and bioaerosols, some taxa of interest were detected in higher proportions in air samples compared to the composting piles. This suggests a higher risk of inhalation by daily exposed workers by these particular fungi (Mbareche *et al.*, 2017). For Biomethanization, the results obtained from the diversity analyses confirmed the relation between the fungal composition and the type of waste treated, previously observed in the compost study, as the taxonomic profiles were different for both facilities. Like the compost environment, a large diversity profile was observed in bioaerosols from both facilities showing the presence of pathogenic fungi. This observation served as a proof of the validity of the approach applied because, it was able to catch the difference in fungal composition of bioaerosols released from both facilities. Plus, quantifying the fugal biomass with qPCR allowed the addition of an absolute quantification, rather than just a relative abundance. The combination of both approaches offers a more complete idea on the type of fungal exposure (Mbareche *et al.*, 2018). Similar methods were applied to air collected from five dairy farms. Concentrations of *Penicillium* and *Aspergillus* reached a maximum value of 10^6 gene m^{-3} of air. The taxonomic profiles obtained from the five dairy farms were different, proving that the method is able to capture important players in the fungal community, and that fungal composition may vary depending on different conditions of each farm. Many opportunistic fungi were identified in air samples of dairy farms, suggesting a potential harm on daily exposed individuals. The intake of culture methods on fungal diversity showed a complementarity of both approaches in the most abundant taxa. In other words, when the most abundant fungi were considered HTS and culture gave complementary taxonomic profiles. However, when all the taxa were considered, HTS undoubtedly outperformed the culture approach in identifying fungi in aerosols from dairy farms.

This paper serves as an endorsement of the methodology applied in terms of fungal analyses in aerosol samples, from the sample treatment to the diversity analyses (Mbareche *et al.*, 2019a,b,c).

High-Throughput Sequencing Platforms

Many commercial HTS platforms have got involve in the sequencing business since the completion of the first draft of the human genome by the traditional Sanger sequencing. As a consequence, the cost of genome sequencing had dropped drastically (Schloss, 2008). The specific paradigm applied by each platform includes sample preparation, amplification (when applied), followed by a massive cycle of parallel sequencing. These steps influence the quality, quantity, and biases of the output sequences, which control the platforms usefulness for specific applications. Since the HTS outbreak, some sequencers have stopped offering services, therefore, they will not be covered in this chapter. Other platforms, although important, are also not mentioned because of their infrequent use in microbial ecology studies. Examples of these cases include, 454 pyrosequencing by 454 Life Sciences, part of Roche Diagnostics; Qiagen-intelligent bio-systems sequencing -by-synthesis by IBS, acquired by Qiagen; Polony sequencing by Polonator; Sequencing-by-ligation by ABI SOLiD; and DNA nanoball sequencing by Complete Genomics, acquired by the Beijing Genomics Institute. Detailed information about the chemistry and sequencing technologies used by these platforms can be found in reviews like the one conducted by Morey *et al.* (2013).

Currently, Illumina platforms dominate the HTS market. The sequencing by synthesis is performed on bridge amplicon obtained by PCR amplification. This type of sequencing consists of the polymerase-catalyzed addition of reverse terminator fluorescent labeled bases. The bases are added simultaneously to the reaction and compete to link with oligo primed cluster fragments. Only one base can be attached in a cycle, preventing other bases of subsequent attach. A coupled-charge device (CCD) camera scans the flow cell capture the image. After each imaging, 3′ blocking is chemically removed, and the process is repeated. Illumina have a set of sequencers: MiSeq, NextSeq, and HiSeq optimized for various applications. All Illumina models have an overall error rates below 0.1%, with the most common one being substitutions (Dohm, *et al.*, 2008). The MiSeq is set as a fast, personal benchtop sequencer, with a 4-h run time and designed for amplicon-based sequencing, and also suited for sequencing of small genomes. The HiSeq series were created for application where a deeper sequencing is needed. For example, the HiSeq 2500 gives an output of 1 Tb in 6 days, while the HiSeq X Ten is capable of giving 1.8 Tb in 3 days. Recently, Illumina released the HiSeq 3000/4000 platforms with a run time and an output between the HiSeq 2500 and the HiSeq X Ten (Reuter *et al.*, 2015).

Ion torrent sequencing is another popular platform amongst microbial ecology researchers. Amplification is obtained my emulsion-PCR, similar to 454 pyrosequencing platform. The sequencing is based on a semiconductor where hydrogen ions are

generated and detected after dNTP insertion, rather than by emission of light like Illumina. The detection is performed by a semiconductor chip that measures pH changes induced by the release of hydrogen ions during DNA extension. Ion Torrent Systems wanted to improve sequencing times by omitting the time consumption of imaging steps, which reduces the overall cost. The original benchtop sequencer is Ion PGM with an output capability up to 1 Gb and a speed of runs of 2 h. A second sequencer was released in 2012, the Ion Proton, 10 times more outputs (10 Gb). However, the read length is 200 bp compared to 400 bp for Ion PGM. Ion Proton is more suitable for whole-transcriptome analyses, whereas Ion PGM is suited for targeted sequencing. Recently, a third platform was released under the name of Ion GeneStudio S5, which increased the maximum read length to 600 bp and an output of 1.5–4.5 Gb with a run time of 22 h. Insertions and deletions are the most common error type in Ion Torrent chips, with an error rate of 1%.

There are two other sequencing technologies, although less popular than Illumina and Ion Torrent, that are worth the mention because of their potential overtake over current sequencing technologies in the near future, in the case of particular applications. Pacific Biosciences commercializes the single-molecule real time (SMRT) designed by Nanofluidics. Briefly, a capped DNA template is produced by ligation of a single-stranded hairpin adapter onto the ends of the digested DNA, and by using a strand displacing polymerase, the DNA molecule is sequenced. While polymerization is occurring continuously, the DNA sequence is read in real time with a video recording the fluorescent signals emitted. Each SMRT cell produces 50 k reads (up to 1 Gb) in 4 h, and an average read length of more than 14 kb. But, as with most SMRT, error rate are high (11%), and consist primarily on insertions and deletions. Oxford Nanopore Technologies developed a nanopore based single molecule sequencing platform. In short, DNA is fragmented and adapters are ligated: the first adapter is linked to a motor enzyme and the second is a hairpin oligonucleotide that is linked to a motor protein. The nanopore flow cell contains hundreds of microwells, each comprises a biological nanopore. A molecular motor protein measure the changes of current characteristics induced by the bases passing through the pores. The output is >90 Mbp for an 18 h run time, and a maximum length >60 kb. SMRT sequencing is usually suited for small bacterial and viral whole genome sequencing.

HTS methods were since adapted to study microbial diversity in bulk environmental samples. One of the most used techniques is the targeted sequencing approach, where a specific gene marker is amplified and sequenced with the defining goal of species identification. This method is also referred to as metabarcoding, environmental barcoding, or amplicon sequencing. Basically, this approach changed the field of microbial molecular ecology by targeting whole communities at the same time, instead of isolating individual members. In the particular case of the marker genes, amplicons produced are suitable for phylogenetic analyses, which is important for phylogenetic diversity given the rapid pace of new sequences generated in various environments. The accuracy of the results obtained from the amplicon-based HTS approach is in the gene marker selection. These markers are selected based on their presence across the organisms of interest and having sequence variations between the species. The 16S rRNA gene is the most uses gene marker for prokaryotes. However, eukaryotes have many markers options depending on the organisms studied. For example, animals are most studied by targeting the mitochondrial gene COI (Hebert *et al.*, 2003). For plants, the rbcl and matK has been used as the gene markers in diversity studies (Hollingsworth *et al.*, 2009). For fungi, the ITS region within the rDNA is considered to be the universal fungal gene marker (Roe *et al.*, 2010; Dentinger *et al.*, 2011; Schoch *et al.*, 2012). One particularity of the ITS region is that the sequence length vary between fungal taxa, unlike the length of the 16S rRNA gene used for bacteria, which is similar. For example, in Ascomycota and Basidiomycota the sequence lengths range between 600 and 900 bp (Toju *et al.*, 2012). Amplicon-based HTS approaches involve the amplification of the target gene marker before its sequencing. The most commonly used high-throughput sequencers have a maximum read length limitation. For Illumina MiSeq and HiSeq, maximum sequence length are 2×300 bp, and 2×125 bp, respectively. For In torrent Ion PGM, the maximum sequence length is 400 bp. This restriction forces the choice between ITS1 or ITS2 when applying the amplicon-based HTS approach, as the 5.8S gene does not contain a sufficient number of informative sites that can be used to design primers that can be informative for the differentiation between species. The ITS region is composed of three sub-regions: ITS1, 5.8S, and ITS2, and it is an inserted region separating the small ribosomal subunit 18S and the large ribosomal subunit 28S. To the best of our knowledge, there are no relevant studies that explored the potential of both sub-regions in the characterization of fungal communities in bioaerosols. A strategy that is actually being considered for publication is using both subregions, coupled with shotgun metagenomics, to demonstrate their potential and relevancy for a global fungal characterization by coming up with a standardized methodology for the assessment of fungal exposure in occupational settings. The results obtained are unequivocal toward ITS1 outperformance to ITS2 in terms of richness, and taxonomic coverage. The differential abundance analysis did demonstrate that some taxa were exclusively detected only by ITS2, and vice versa for ITS1. However, the shotgun metagenomic approach showed a taxonomic profile more resembling to ITS1 than ITS2. Based on these results, neither of the barcodes evaluated is perfect in terms of distinguishing all species. Using both barcodes offers a broader view of the fungal aerosol population. However, with the actual knowledge, we strongly recommend using ITS1 as a universal fungal barcode for quick general analyses of diversity and when limited financial resources are available, primarily due its ability to capture taxonomic profiles similar to those obtained using the shotgun metagenomic approach (Mbareche *et al.*, 2020).

Another molecular ecology method used to profile microbes is metagenomics. This HTS method involves sequencing of all the genomic material in a sample without a specific target enrichment. Thus, it allows the analyses of mixed communities in environmental samples. Other names for metagenomics are environmental genomics or shotgun sequencing, to differentiate it from amplicon sequencing. An advantage of this approach is the absence of an amplification step prior to DNA sequencing, avoiding in PCR amplification biases or selective primer biases. Because no particular gene is targeted, the functional potential of a sample can be explored, in addition to taxonomic analyses. Also, the metabolic potential of organisms from different environmental samples under different conditions can be compared. Depending on the sequencing depth and the genome size, reconstruction of individual

microbial genomes can be achieved by bioinformatics' *de novo* assembly programs. A recent study published in Nature Microbiology assembled 8000 prokaryote genomes 1500 metagenome sequences available in public databases (Parks *et al.*, 2017). However, fungi do not benefit yet from this achievement as the size of eukaryotic genomes and their complexity make it difficult. Improving sequencing platforms and machine learning assisted bioinformatics may make this possible in the future. In addition, taxonomic assignment may be biased toward microbes with the whole genomes available in the database used.

Due to the fact that not only gene markers are sequenced, other sequenced genes of the genomes may be hard to identify to the species level with confidence. Also, the sequencing depth needed to cover the diversity of an environmental sample is technically higher if the shotgun sequencing is used versus amplicon sequencing. As mentioned above, with HTS and SMRT sequencing technologies advances, the output increases, and the costs decreases. Combined to the attractive advantage of PCR-free, and the continually growing databases of annotated genomes, metagenomics popularity will continue to grow amongst bioaerosol researchers.

Although the sequencing-based approaches described in this chapter are popular and their use is widespread amongst microbial ecology investigators in general, and only recently for bioaerosol specialists, they present key limitations. Certainly, bioaerosol studies applying modern microbial molecular ecology methods should move from descriptive to bringing answers and solutions to decision makers, to ultimately reduce exposure and improve occupational health. This goal can be achieved by continuing the development of standardized laboratory methods, improving bioinformatics algorithms and their usage by scientists, curating more complete and better qualified databases, and combining data from different analyses. A perfect example of data integration is the combination of HTS outputs and culturomics to improve the understanding of microbial communities. Reference databases could use the help of professional taxonomist, whose expertize on isolates identification can enhance the identification of new species. As a consequence, the relative abundance of unidentified taxa will decrease. As amplicon-based HTS approaches depends on database for taxonomic identification, bioaerosol exposure studies applying these approaches could identify a larger number of airborne microbes, and thus, have a closer look to the real diversity of bioaerosols.

In addition to error rates and types associated to each sequencing platform, the high accuracy of homopolymers, and the GC-rich regions amplification problems, short reads lengths produced by most current platforms limit the ability to characterize large regions, with insertions and deletions, and structural variation (Ross *et al.*, 2013). Improvements in the long reads sequencing technologies can help overcome this limitation. Long HTS reads from technologies such as PacBio (see "Relavant Websites section") and Oxford Nanopore (see "Relavant Websites section") are gradually becoming available. To put it in context, one downfall of the actually most used sequencing platforms is the short sequencing reads. For example, we often choose a partial sub-region among the nine variable sub-regions (V1 to V9) of the full 16S rRNA gene so that the chosen region is fully sequenced by Illumina MiSeq (2×300 bp), HiSeq 2500 Illumina (2×125 bp) or Ion PGM (400 pb). With the increasing demand of more accurate methods to analyze microbial communities from different environments, sequencing longer amplicons (e.g., the full-length 16S rRNA gene) is appealing. Primarily, because of its potential to better identify bacteria at the species level. Indeed, the high similarity of the 16S rRNA amplicon sequences usually used with Illumina or Ion Torrent make the accurate species level identification difficult. To overcome this situation, recent microbiome studies have used the nanopore sequencing platform to sequence the full-length 16S rRNA amplicon from the mouse gut microbiota. A comparison of the nanopore (full-length 16 rRNA gene) and short-read (V3 and V4) Illumina sequencing data showed that there were no significant differences in major taxonomic units, with 89% of similarity between the two approaches. Moreover, both sequencing data were highly similar at all taxonomic resolutions except the species level. At the species level, nanopore sequencing allowed identification of more species than short-read sequencing, facilitating the accurate classification of the bacterial community composition (Shin *et al.*, 2016). With these results in mind, applying the same approach to sequence the full-length ITS region represent a compelling avenue for fungal amplicon-based HTS methods, without having to choose between ITS1 or ITS2. However, a proof of concept should be conducted to fine-tune library preparation, including the best set of primers to amplify the ITS region. In addition, UNITE has already started to include long PacBio reads comprising the full ITS region, including few hundreds bases of the LSU gene, to the database (Nilsson *et al.*, 2019a,b).

Like for other environments, fungi in aerosols could benefit from the myriad of methods available to answer questions related to their occupational impact. Amplicon-HTS, shotgun metagenomics, metatranscriptomics, culturomics, and qPCR can all bring insightful information depending on the questions asked. What is the source of fungal bioaerosols? What are the environmental factors favorizing aerosolization of fungi? Who is there? How are they interacting? Is this interaction susceptible to amplify the harm? What is the metabolic potential? Are there any differential expression of genes involved directly or indirectly to fungal pathogenesis? The answers to these questions could take us to answer other types of occupational questions. Is it possible to eliminate the source? How can we reduce the exposure? Can we control the factors favorizing aerosolization? Could the measures proposed change the quality and quantity of fungal exposure?

The success of the combination of multiple methods to study fungi in aerosols reside in the creativity applied to analyze the data. Machine learning is a subset of artificial intelligence based of algorithms and statistical models that computers use to perform specific tasks without a definite instruction for each task (Bishop, 2006). Concretely, the algorithms build mathematical models from a set of training data to predict the next decision without being programmed to do so. In the context of molecular ecology, data mining is the machine learning field of study closely related to the analyses needed for HTS data. Data mining focuses on discovering patterns in large datasets with the use of machine learning, statistics and databases. It allows the exploration of data analyses through unsupervised learning. Supervised machine learning include support-vector machines and it is possible when more data information are available.

Recent work on HTS environmental DNA (eDNA) have showed that machine learning could overcome many limitations of taxonomy-based eDNA bioassessment (Cordier *et al.*, 2018). The algorithms used could predict accurately biotic indices values from

HTS data, regardless of the taxonomic identification of the sequences. In the Cordier study, prokaryotic and eukaryotic ribosomal markers yielded the same accuracy of the predictive models to assess the environmental impact of marine aquaculture. This suggest that the use of predictive models with training datasets from HTS on relevant molecular markers that comprises different potential bioindicator taxa have the potential to overcome some limitations of the traditional methods. Thus, the combination of microbial HTS with machine learning algorithms can be highly effective in filling important gaps of microbial ecology studies. For example, machine learning models were previously used to predict effluent concentration in a wastewater treatment plant (Guo *et al.*, 2015). In addition, microbial community compositions helped the establishment of predictable effluent parameters, like temperature, suspended solids, biochemical oxygen demand, using machine learning's vector regression models (Liu *et al.*, 2016). The application of machine learning to bioaerosols could include scaling-up both spatial and temporal resolution for larger and more ambitious monitoring of exposure. It could be used to predict microbial source tracking, and provide a clearer vision of the environmental impact of bioaerosols.

The challenges related to sequencing technologies can be managed from a bioinformatics point of view. Indeed, bioinformatics analyses can have an impact on diversity analyses such as the clustering algorithms, the percent identity threshold and taxonomy assignment tools (BLASTn vs. Naïve Bayesian Classifier). In fact, the bioinformatics part of HTS studies have become an unavoidable field of science to integrate to microbial molecular ecology studies, and bioaerosol are not an exception.

Bioinformatics

The avalanche of big data generated by HTS platforms at an extraordinary rate has made bioinformatics indispensable to modern molecular ecology. Actually, there is a certain reluctance toward bioinformatics by some scientists due to the command line tools and programming skills needed for sequence processing and diversity analyses. Therefore, bioinformatics should be made accessible to a vast majority of potential daring users. Knowing which analyses to conduct and which tools to apply remains confusing for bioaerosol scientists, as a litany of tools and data resources are now available for characterizing microbial communities. Currently, microbial molecular data from bioaerosol studies are obtained predominantly using 16S rRNA gene sequencing surveys that provide a characterization of bacterial and archaeal diversity. In studies using 16S rRNA gene sequencing, the choice of primer set depends on a number of factors, including compatibility with previous studies and the specificities of the primers (Soergel *et al.*, 2012). 16S rRNA gene sequence data from different microbial environments present bioinformatical, statistical, and computational challenges. The most widely used bioinformatics tools are QIIME and mothur (Schloss *et al.*, 2009; Caporaso *et al.*, 2010). Both packages are open source and have online tutorials and forums that takes the users to a step-by-step analysis of the 16S rRNA gene sequences. Although the bioinformatics pipeline may seem to be the same for eukaryotic marker genes as for bacterial marker genes, there are no standardized bioinformatics protocols for ITS rRNA gene sequences as for 16S rRNA gene analyses. The lack of a standard marker gene and a reference database may be the reason behind that. The bioinformatics workflow used for the ITS sequences differs greatly from the one for the 16S rRNA gene in the alignment algorithm as the ITS region is of different lengths compared to the 16S rRNA gene. For this reason, amongst others, OTU clustering, reference databases, assigning taxonomy, diversity metrics, data visualization, and statistical modeling should be specific to ITS sequences analyses for the characterization of fungal communities. The UNITE database is often used for ITS sequence-based analyses of fungal sequences (Abarenkov *et al.*, 2010). On the other hand, the 18S rRNA gene can, generally, be used to analyze eukaryotic communities in the same manner as 16S rRNA genes are used. The SILVA database contains many eukaryotic regions and can be used for such analyses. However, one should confirm that the region of the 18S gene amplified discriminates between the taxa studied and should be aware that the 18S rRNA gene is not sufficient to characterize the fungal diversity and will likely end up to be of questionable utility.

When it comes to HTS, PCR errors are common, and are sometimes difficult to detect. Chimeras, which are caused by incomplete extension of DNA strand during amplification that make up a recombination between two sequences, can cause biases in diversity result, particularly the alpha metrics (Ley *et al.*, 2008; Haas *et al.*, 2011). Multiple chimera checking software are available and they often have different filtering methods. One should look at the one that fits adequately with the project (Ley *et al.*, 2008; Edgar *et al.*, 2011; Quince *et al.*, 2011; Wright *et al.*, 2012).

Data filtering is often made at the same stage as demultiplexing where each sequence is assigned to a sample based on the barcode information. Quality threshold includes quality scores, read length and the presence of ambiguous base calls. After quality filtering, one way to identify the microbial groups is through OTU analyses where sequences are clustered together based on sequence identity. As a matter of fact, there is different OTU clustering algorithms and the use of any one affects greatly the findings and interpretations of data. In *de novo* OTU clustering, sequences are clustered into OTUs by comparing them to the whole dataset, without the use of a reference (Westcott and Schloss, 2015). In contrast, closed OTU clustering uses a reference sequence database, whereby the sequences that don't match the reference sequence database are discarded. Open-reference OTU clustering is a two-step process combining both of the last algorithm described. First, closed-reference OTU clustering is done followed by *de novo* clustering of sequences that fail to match to the reference database. Each of the clustering methods have pros and cons and should be chosen with a particular attention. For example, if one must combine datasets that have sequences from different regions of the 16S rRNA gene or ITS rRNA gene, the closed reference algorithm should be used, as sequences from different regions of the same rRNA gene would cluster into different *de novo* OTUs. In contrast, using the closed reference algorithm in samples coming from an undiscovered environment may lead to a big percentage of the sequences being discarded due to a failure to match a reference database.

Labeling the OTUs is the next step using taxonomy assignment algorithm with a reference database. For the 16S rRNA gene, the three main databases are: Greengenes, Ribosomal Database Project (RDP), and SILVA (Cole *et al.*, 2009; McDonald *et al.*, 2012;

Quast *et al.*, 2013). For fungi, the UNITE database is the most used one. The microbial diversity analysis is typically described within samples (alpha diversity) and between samples (beta diversity). Multiple scripts for data visualization are available through QIIME, mothur and R. A particular attention should be given when choosing the classification and clustering methods depending on the metadata information available about the samples. For example, classification methods can be used to determine which taxa differ between predefined groups of samples. On the other hand, clustering methods does not make use of any prior knowledge about the samples. Both methods use in-between sample distance metrics and the specificities of each method used can affect the outcome and the interpretation of the analyses. For this reason, it is mandatory to use several different ways of classification and clustering to ensure that the existence of clusters is not dependent on just one set of parameters.

Diversity analyses include alpha and beta measures, statistical significance of sample groupings, differential abundance of taxa in different groups of samples, taxonomic analyses, and correlations between abundances (relative or absolute) and numerical metadata (e.g., days, temperature, relative humidity, etc.). Different taxonomic assignment method exist (not specific to amplicon-based HTS): similarity-based (BLAST, BOLD, METAPHYLER, MG-RAST); phylogeny-based (NJ, SAP, PPLACER, EPA); composition-based (KNN Classy method by mothur, OTU-picking by QIIME, RDP Classifier, UCLUST); hybrid methods (FUZZYD2, MEGAN); Bayesian treeless methods (Coalescent Assigner, Segregating Sites Assigner); Machine learning (BPSI, SVM classifier). At last, statistical modeling can be used to explain the variations observed between the different samples. As many covariates can be included and that many parameters can be statistically controlled, we strongly recommend the consultation of a statistician or a biostatistics expert during the experimental design and the analysis stages.

Recently, a new trendy method don't impose a dissimilarity threshold to analyze HTS gene markers nor a reference database. Amplicon Sequence Variants (ASVs) prone a better amplicon resolution by distinguishing sequence variants differing by one nucleotide (Eren *et al.*, 2015; Tikhonov *et al.*, 2015; Callahan *et al.*, 2016; Edgar, 2016; Amir *et al.*, 2017; Callahan *et al.*, 2017). ASVs most prominent advantage, compared to OTU-based methods, is the combination of the benefits from overcoming limitations inherent to closed-reference and *de novo* methods. For instance, closed-reference OTUs cannot document biological variations outside of the reference database used for their construction. On the other hand, the validity of *de novo* OTUs outside of the dataset in which they were defined is also questionable, which make cross-studies comparison invalid. While ASVs capture all biological variations present in a dataset, and ASVs inferred from a given dataset can be reproduced in future datasets and validly compared (Callahan *et al.*, 2017). However, ASVs method also comes with its share of limitations. Allowing 100% sequence similarity may lead to a wrong differentiation between the SNPs of the same species. In addition, the zero percent difference may give an extremely high number of ASVs in a sample, which, in return, causes the missing of the core microbiome information (unpublished data). Above all, the same genome can contain multiple ASVs if there is multiple copies of the targeted gene. For this matter, ASVs can be validly compared between studies, only when the same primers were used on the targeted gene. Furthermore, the high variability of the ITS region makes us reconsider the automatic replacement of the traditional OTUs by ASVs. To sum up, ASVs and *de novo* OTUs are more precise in describing diverse biological sequences in a less represented environment in reference databases like bioaerosols, compared to closed-reference OTUs. Most importantly, no matter the methodology used, downstream analyses should consider the methodological differences, accordingly.

The diverse biological sequences are identified by comparison to a database. As mentioned throughout this chapter, UNITE is the database used for the molecular identification of fungi. Without a doubt, it is a better choice when working with the fungal ITS region, as other databases, like NCBI or SILVA are not specifically curated for ITS identification. The UNITE database is regularly updated and annotated; thus, it continues to show tremendous improvements in the sequence management of fungal ITS (Nilsson *et al.*, 2019a,b). Although some databases contain an enormous number of sequences due to the fact that sequence deposition is easy an open to anybody, the risk of erroneous identification is also multiplied. In the contrary, UNITE is subject to quality control by ITSx and UCHIME in an attempt to reject non-ITS and chimeric sequences. When working with a less characterized environment like air (in comparison to soil and water), the quality control of sequences in a database is greatly appreciated. However, the representative sequences of ITS1 and ITS2 in UNITE is not known. According to the results obtained in Chapter 2, more unidentified fungi and plant identification were associated to ITS2 in datasets obtained from air samples in compost, biomethanization, and dairy farms. Earlier this year, UNITE curators stated that they are working on adding more ITS2-derived HTS studies to UNITE (Nilsson *et al.*, 2019a,b). It would be valuable to address the current state of ITS2 sequences in the UNITE databases. In addition, annotated UNITE sequences covers not only taxonomic names, but also country of collection, and substrate of collection. Currently, UNITE uses the default NCBI Taxonomy classification complemented by Index Fungorum and MycoBank. Sequences with a confusing taxonomic annotation are flagged by experienced users for manual correction by UNITE curators. In the long term, UNITE is re-annotating, and re-identifying conflicting taxonomic information. The original names are kept for reference tracking accuracy.

In molecular microbial ecology studies, phylogeny allows the study of the evolutionary relation between species based on mathematical algorithms that compares DNA sequences. Particularly, phylogeny analyses are useful when comparing microbial community members between different samples, and by incorporating information on the relative relatedness between the microbes. Thus, the use of a reliable phylogenetic tree is necessary when analyzing diversity with a phylogenetic measure. A phylogenetic tree is a diagram with branches showing the evolutionary relationships between species. Because the 16S rRNA gene sequence contains both highly conserved regions for primer design (PCR-amplification) and hypervariable regions to identify phylogenetic characteristics of microbes, it became the most widely used marker gene for profiling bacterial communities (Lane *et al.*, 1985; Tringe and Hugenholtz, 2008; Yang *et al.*, 2016). As ITS sequences are subject to intraspecific variability the construction of a phylogenetic tree is not recommended (Nilsson *et al.*, 2008). Actually, different tree construction methods

(clustalw, and different programs within the PHYLIP package) led to conflicting phylogenetic results using the same dataset (unpublished data). This issue is known amongst the scientific community studying fungal diversity. Therefore, when analyzing fungal diversity with ITS datasets, measures including phylogenetic information are not recommended. Hopefully, many alternatives are available to compare microbial composition between samples without the use of phylogeny. Another popular alternative amongst phylogeny aficionados is the use of the 18S rRNA gene sequences as a phylogenetic marker for the fungal kingdom. Although the 18S rRNA have promising characteristics to build a phylogenetic framework for fungi (Yarza *et al.*, 2017), it shows a poor potential in describing the full extent of fungal diversity (Liu *et al.*, 2015).

Although the bioinformatics workflows described above were developed for different environments many of the same issues apply to microbial communities of different habitats. Thus, they can be applied to bioaerosol samples as well. In general, the different projects presented in this chapter encourage researchers entering the field to use bioinformatics tools to analyze the microbial molecular ecology results obtained in different bioaerosol environments. This active research area needs more standardized protocols that will help build a fungal bioaerosol public database that will allow cross-comparative studies around the world and give this field of study a great dash.

Conclusion

This chapter presented a comprehensive framework for the utility of HTS in the study of fungi in aerosols. The concern came mainly from the significant gap in fungal diversity of aerosols in occupational environments. Certainly, having a wider portrait of fungi that could represent a risk for exposed individuals in concerned environments represent an important piece of the puzzle for occupational exposure studies. Plus, combining the description of the fungal profiles of bioaerosols to other components like quantifying the fungal biomass, the association with traditional culture methods, the association of aerosols with the source, and the environmental variables explaining the fungal community composition served as a methodology approval and has brought new answers to fill the gap of fungi exposure in occupational environments. Looking ahead, key research directions which could follow up on the methodological contributions of this chapter could include the HTS of long reads using the PacBio or Nanopore technology covering the whole ITS region. The outcome could be compared to the approach targeting ITS1 or ITS2 to characterize fungal diversity in aerosols. In terms of occupational exposure, future works could include the measuring of health problems amongst the workers of the sampled environment. This way, fungal composition and concentration can be linked to specific health conditions, especially if the number of visits in the sampling campaign is very high. For this matter, it is important to shift attention to new ways of designing bioaerosol exposure studies in order to prevent the replication of previous flaws (e.g., different study designs, methods and analyses which makes it impossible to compare the results). Eventually, the goal is to set exposure guidelines referring to health-related exposure limits.

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Relevant Websites

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Fungal Contamination of Swimming Pools and Fitness Centers

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Introduction

Studies already reported that swimming pool facilities have contributed to the dissemination of fungal infections (Seebacher *et al.*, 2008). Most of the studies performed focused on dermatophytes and health effects (tinea pedis and onychomycosis) (Ali-Shtayeh *et al.*, 2003; Gudnadóttir *et al.*, 1999). However, besides dermatophytes, other clinically relevant fungi such as Fusarium spp., Aspergillus spp., and Candida spp., were showed on different environmental matrices from swimming pools facilities, such as surfaces, water and in indoor air (Buot *et al.*, 2010; Jankowski *et al.*, 2017; Viegas *et al.*, 2011).

Tinea pedis and onychomycosis are prevalent among swimming pool visitors (Kamihama *et al.*, 1997; Shemer *et al.*, 2016) and workers (Viegas *et al.*, 2011). Indeed, surfaces from the facilities may contain skin fragments from infected persons leading to the infection spread when infected skin fragments adhere to the feet of uninfected individuals while walking barefoot on the contaminated surfaces (English and Gibson, 1959).

To assess mycological contamination, it's important to consider the fungal spore's dispersion on air and surfaces. Type of sporulation and spore characteristics must be considered (size, density, colony structure and rugosity) as well as additional environmental characteristics, such as surface vibrations, smoothness and role as substrate (Roussel *et al.*, 2008). As such, culturing air samples as a stand-alone sampling approach to assess indoor fungal contamination is not enough. Indeed, surface analysis complements the fugal contamination characterization and can be used in order to identify contamination sources. It may also be apply to allow the evaluation of the efficacy of surface cleaning and disinfection procedures (Stetzenbach *et al.*, 2004).

Besides the sampling approach also the assays employed can influence the data obtained to perform the exposure assessment. Indeed, several constraints are being reported regarding the use of culture based-methods (Degois *et al.*, 2017; Viegas *et al.*, 2019a) and suggesting the use of molecular tools in parallel (Viegas *et al.*, 2019a).

This study aims to perform a scope review regarding the fungal contamination assessments performed on swimming pools and fitness centers aiming the identification of further research needed on these specific facilities.

Materials and methods

The available data from January 1st, 2000 to December 31st, 2019 was reviewed. The terms used were to identify studies were the fungal contamination in public swimming pools and fitness centers was assessed. PubMed, Scopus and Web of Science (WoS) were the databases chosen, and the search terms were "fungal contamination in swimming pools" and "fungal contamination in fitness centers". From this search, a total of 2907 papers were recovered from all databases. Articles that did not meet the inclusion criteria and duplicates were excluded from further analysis (**Table 1**). After eliminating the duplicates and articles that did not meet the criteria, a total of fifteen articles were further analyzed.

Results

The initial research resulted in a total of fifteen articles. From those articles, nine referred to indoor swimming pools alone (Brandi *et al.*, 2007; Ekowati *et al.*, 2018, 2017; Fadaei and Amiri, 2014; Itah and Ekpombok, 2004; Jankowski *et al.*, 2017; Karimi *et al.*, 2019; Rafiei and Amirrajab, 2010; Rasti *et al.*, 2012); one referred to both indoor and outdoor pools (Papadopoulou *et al.*, 2008), which studied indoor and outdoor public swimming pools, one hospital hydrotherapy pool and one private outdoor pool; one studied an hospital physical therapy pool (Buot *et al.*, 2010); three articles studied gymnasiums/sports centers with swimming pools (Małecka-Adamowicz *et al.*, 2019; Viegas *et al.*, 2011, 2010); and one article studied gymnasiums (Ramos *et al.*, 2016).

From all the studies considered, four of them used only water sampling (Fadaei and Amiri, 2014; Itah and Ekpombok, 2004; Papadopoulou *et al.*, 2008; Rasti *et al.*, 2012); three used only air sampling (Małecka-Adamowicz *et al.*, 2019; Ramos *et al.*, 2016; Viegas *et al.*, 2010); and two of them used only surface sampling (Buot *et al.*, 2010; Jankowski *et al.*, 2017). The remaining six studies combined either two or three of these matrixes: Rafiei and Amirrajab (2010), Ekowati *et al.* (2017) and Karimi *et al.* (2019)

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Table 1 Criteria for inclusion and exclusion of the articles used

Inclusion criteria	*Exclusion criteria*
Articles in English language;	Articles in other languages (for articles)
Articles published from January 1st, 2000 to December 31st, 2019	Articles published prior to 2000
Articles published anywhere	
Articles related to fungi assessment in swimming pools and fitness centers	Articles related to biologic samples from gym/pool users (exclusive)
Articles related to swimming pools and fitness centers	Articles containing information from natural water sources
Scientific original articles on the topic	Abstract of congress, Reviews/State of the art, reports: used for discussion
Identification of fungi to the genera level (at least)	

combined water and surface sampling; Viegas *et al.* (2011) combined air and surface sampling; and Brandi *et al.* (2007) and Ekowati *et al.* (2018) used all three matrixes to assess fungal contamination (Brandi *et al.*, 2007; Ekowati *et al.*, 2018, 2017; Karimi *et al.*, 2019; Rafiei and Amirrajab, 2010; Viegas *et al.*, 2011).

From the nine articles that used water sampling (Brandi *et al.*, 2007; Ekowati *et al.*, 2018, 2017; Fadaei and Amiri, 2014; Itah and Ekpombok, 2004; Karimi *et al.*, 2019; Papadopoulou *et al.*, 2008; Rafiei and Amirrajab, 2010; Rasti *et al.*, 2012), all but one (Fadaei and Amiri, 2014), which used pour plate technique used the membrane filtering technique to analyze this matrix. All six articles that sampled air (Brandi *et al.*, 2007; Ekowati *et al.*, 2018; Małecka-Adamowicz *et al.*, 2019; Ramos *et al.*, 2016; Viegas *et al.*, 2011, 2010) did so using air impaction technique. Six studies performed surface sampling: the majority of them (five studies) did so with contact plates (Brandi *et al.*, 2007; Buot *et al.*, 2010; Ekowati *et al.*, 2018, 2017; Jankowski *et al.*, 2017), two of them performed sampling using sterile carpet (Karimi *et al.*, 2019; Rafiei and Amirrajab, 2010), and two of them used surface swabs (Karimi *et al.*, 2019; Viegas *et al.*, 2011).

All but three of the analyzed studies used morphological identification exclusively: two of them used both morphological and molecular identification (Brandi *et al.*, 2007; Ekowati *et al.*, 2017), and one used molecular identification alone (Ekowati *et al.*, 2018).

Sampling was, in some cases, performed twice in the same day, once in the morning, before activities, and in the evening (Itah and Ekpombok, 2004; Ramos *et al.*, 2016). Sampling was performed in different seasons (Brandi *et al.*, 2007; Buot *et al.*, 2010; Fadaei and Amiri, 2014; Jankowski *et al.*, 2017; Papadopoulou *et al.*, 2008; Rafiei and Amirrajab, 2010; Rasti *et al.*, 2012), whereas in some studies was only performed once (Ekowati *et al.*, 2018, 2017; Karimi *et al.*, 2019; Małecka-Adamowicz *et al.*, 2019; Viegas *et al.*, 2011, 2010).

Penicillium sp. was the most commonly found fungal species in air samples from some of the analyzed studies (Brandi *et al.*, 2007; Itah and Ekpombok, 2004). *Fusarium* sp. was the most reported in the surface samples (Brandi *et al.*, 2007; Buot *et al.*, 2010; Viegas *et al.*, 2011), while in the water samples the most common was *Alternaria* sp. (Buot *et al.*, 2010).

In the samples from Rafiei and Amirrajab (2010), Rasti *et al.* (2012), Fadaei and Amiri (2014), and Karimi *et al.* (2019), the most prevalent fungus was *Aspergillus* sp., and *Candida* sp. was the most prevalent in Jankowski *et al.* (2017). The most prevalent species in Ekowati *et al.* (2017, 2018) was *Phialophora oxyspora*.

Cladosporium sp. was the main source of contamination in several studies on air samples (Viegas *et al.*, 2010, 2011) and in the afternoon samples (Małecka-Adamowicz *et al.*, 2019; Ramos *et al.*, 2016), whereas in the morning samples of Ramos *et al.* (2016) the most significant contamination was from *Chrysosporium* sp. (**Table 2**).

Discussion

Due to this scope review it was possible to conclude that there is a wide spectrum of environments that include a swimming pool in their facilities. Indeed, it was possible to observe that although the majority was to develop only the activity of swimming in the facilities (Brandi *et al.*, 2007; Ekowati *et al.*, 2018, 2017; Fadaei and Amiri, 2014; Itah and Ekpombok, 2004; Jankowski *et al.*, 2017; Karimi *et al.*, 2019; Papadopoulou *et al.*, 2008; Rafiei and Amirrajab, 2010; Rasti *et al.*, 2012), others include different services available (Buot *et al.*, 2010; Małecka-Adamowicz *et al.*, 2019; Ramos *et al.*, 2016; Viegas *et al.*, 2011, 2010). As such, different variables favoring the fungal dissemination should be considered due to the different activities performed indoors (Roussel *et al.*, 2008).

As in other indoor environments studied (Santilli and Rockwell, 2003; Viegas *et al.*, 2019a, 2014; Wilson *et al.*, 2004) we should consider for assessing this specific indoor environment different sampling approaches. Active sampling such air impaction applied by several studies analyzed (Brandi *et al.*, 2007; Ekowati *et al.*, 2018; Małecka-Adamowicz *et al.*, 2019; Ramos *et al.*, 2016; Viegas *et al.*, 2011, 2010) combined with passive methods, namely surface swabs, contact plates or carpet samples (Brandi *et al.*, 2007; Ekowati *et al.*, 2018, 2017; Karimi *et al.*, 2019; Rafiei and Amirrajab, 2010; Viegas *et al.*, 2010) can give a more accurate characterization of fungal contamination. Different passive sampling methods are being used in other indoor environments and can be an added value also in this indoor environment. Indeed, settled dust and electrostatic dust collectors have already being reported as retrieving a more diversified fungal contamination data comparing with other passive methods (Viegas *et al.*, 2019b, 2018).

Table 2 Articles used in this review

Database	*Country*	*Type of environment analyzed*	*Type of sample obtained*	*Sampling method*	*Fungal identification*	*Results*	*Reference*
PubMed	Nigeria	Ten swimming pools	Water samples	Water samples	Morphological identification	The most common fungal groups identified were *Penicillium* sp., *Rhizopus* sp., *Aspergillus versicolor*, *Fusarium* sp., *Trichophyton mentagrophytes*, *Mucor* sp., *Candida albicans*, *Aspergillus niger*, and *Absidia* sp	(Itah and Ekpombok, 2004)
	Italy	10 Italian swimming pools	Water and surface samples (swimming pool edge, shower pans and locker room floors)	Settle plates, air impaction, water samples	Morphological and molecular identification	*Penicillium* spp., *Aspergillus* spp., *Cladosporium* spp. and *Alternaria* sp., were present in all air and surface samples from the swimming area. *Fusarium* spp. was the most common fungi isolated from surface samples	(Brandi *et al.*, 2007)
	Greece	Three indoor swimming pools (a teaching pool, a competition public pool, a hydrotherapy pool) and two outdoor swimming pools (a hotel semi-public and a residential private pool)	Water samples	Water samples	Morphological identification	The swimming pool with the highest prevalence of isolates and multi-resistant isolates was the hydrotherapy pool. *Candida albicans*, *Aspergillus* spp., *Mucor* spp., *Alternaria* spp., *Rhizopus* spp., *Trichophyton* spp., and *Penicillium* spp. were all isolated	(Papadopoulou *et al.*, 2008)
	Switzerland	Hospital physical therapy swimming pool	Surface samples from three showers, three synthetic carpets and two tiled locations	Contact plates	Morphological identification	All samples were positive for fungi. *Candida famata* was the most common yeast in shower areas. *Fusarium* spp. was present in all studied areas	(Buot *et al.*, 2010)
	Iran	Ten indoor public swimming pools	Water samples from the swimming pools and foot-washing sink. Environmental samples from shower bath area, margin of pool walls, dressing rooms, and slippers	Sampling using a piece of sterile carpet	Morphological identification	The most common dermatophytes were *Trichophyton mentagrophytes*, *T. rubrum*, *T. verrucosum* and *Epidermophyton floccosum*. *Aspergillus* sp., *Penicillium* sp. and *Mucor* sp. were the most common saprophytic fungi	(Rafiei and Amirrajab, 2010)
	Iran	Two indoor swimming pools	Water samples and surface samples (showers, dressing rooms and bottom of the swimming pools)	Not described	Morphological identification	The most prevalent fungi were *Aspergillus* sp., *Penicillium* sp., *Nocardia* sp., *Cladosporium* sp., *Rhizopus* sp., *Scopulariopsis* sp., *Fusarium* sp., and *Mucor* sp. The most contaminated sites were the showers	(Fadaei and Amiri, 2014)
	Netherlands	Six pools in a swimming pool facility	Water and surface samples	Contact plates and surface swabs, water samples	Morphological and molecular identification	Highest fungal concentrations in water and on surfaces were found in the recreational pool and on a flexibeam, respectively. *Phialophora oxyspora* and *Phoma* spp. were the most frequently identified groups	(Ekowati *et al.*, 2017)

	Poland	Eight indoor swimming pools	Surface samples from shower sinks and women's dressing room floors	Contact plates	Morphological identification	*Candida* spp., *Rhodotorula rubra*, *Aspergillus* spp. and *Trichophyton mentagrophytes* were the most common fungi	(Jankowski *et al.*, 2017)
	Netherlands	Seven swimming pool facilities	Water and floor surface samples, one air sample, and surface samples from one of the flexibeams. When possible, samples were taken from the cleaning tools used to clean the floors	Water samples, contact plates and air impaction	Molecular identification	Maximum fungal concentrations were found on surfaces. *Phialophora oxyspora* and *Trichosporon dohaense* were the most frequently isolated species, but only found in surface samples. *Penicillium* spp. and *Aspergillus* spp. were the dominant fungi in water and air	(Ekowati *et al.*, 2018)
	Iran	Two indoor swimming pools	Water samples, surface samples and carpet samples	Water samples, membrane filtration technique and carpet samples	Morphological identification	The pools were contaminated with dermatophytes (*Trichophyton mentagrophytes* and *Epidermophyton flucosomes*), yeasts, and opportunistic saprophytic fungi	(Karimi *et al.*, 2019)
WoS	Portugal	Ten gyms with swimming pools	Air samples	Air impaction	Morphological identification	Twenty-five different species of fungi were identified, with *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp., *Mucor* sp., *Phoma* sp. and *Chrysonilia* sp. as the most common	(Viegas *et al.*, 2010)
	Iran	Four indoor public swimming pools	Water samples	Water samples	Morphological identification	Twelve species of fungi were isolated, with *Aspergillus* sp., *Penicillium* sp., *Rhizopus* sp. and *Fusarium* sp. as the most common. The highest fungal contamination was observed in summer	(Rasti *et al.*, 2012)
	Portugal	Three gymnasiums in the city of Lisbon	Air samples	Air impaction	Morphological identification	In the morning period, the most common fungal species were *Chrysosporium* sp., *Chrysonilia* sp., *Neoscytalidium hialinum*, *Sepedonium* sp. and *Penicillium* sp.; *Cladosporium* sp., *Penicillium* sp., *Chrysosporium* sp., *Acremonium* sp. and *Chrysonilia* sp. were more prevalent at night	(Ramos *et al.*, 2016)

(*Continued*)

Table 2 Continued

Database	*Country*	*Type of environment analyzed*	*Type of sample obtained*	*Sampling method*	*Fungal identification*	*Results*	*Reference*
	Poland	Several sports facilities in Poland	Air samples	Air impaction	Morphological identification	At the sports field, *Cladosporium* sp. is the most common fungal group, as well as in the swimming pool and martial arts room. At the remaining sites their contribution was considerably lower. Several humidity indicator species, such as *Aspergillus fumigatus*, *A. versicolor*, *Trichoderma* sp., *Penicillium* sp., *Phialophora* sp., *Fusarium* sp. and *Ulocladium* sp. were recorded at indoor swimming pools. The air at the gym and sports hall contained molds of *Fusarium* sp. and *Acremonium* sp., as well as *Cladosporium* sp. The fitness room was contaminated with *Penicillium* sp	(Małecka-Adamowicz *et al.*, 2019)
Scopus	Portugal	Ten indoor gyms with swimming pools	Air and surface sampling (in the floor surrounding the swimming pool; the floor surrounding the Jacuzzi; the stair's access to the swimming pool area; the floor of the training studios where most of the barefoot activities are performed and the changing/ locker rooms from each gender)	Air impaction and surface swabs	Morphological identification	In air samples: 25 species of molds were identified, with *Cladosporium* sp., *Penicillium* sp., *Aspergillus* sp., *Mucor* sp., *Phoma* sp. and *Chrysonilia* sp. as the most common. In surface samples: 37 different species of molds were isolated and the most frequent genera before and after cleaning and disinfection were *Fusarium* sp., *Penicillium* sp. and *Scytalidium* sp	(Viegas *et al.*, 2011)

As regards the assays to be performed culture-based methods are crucial for assessing the exposure to microbial contamination, since the viable component of the fungal contamination, shows an higher impact regarding health effects (Cooley, 2000; Croston *et al.*, 2016; Huttunen *et al.*, 2003; Viegas *et al.*, 2019c). However, they should be combined with real-time PCR assay for targeting the indicators of harmful fungal contamination specific for each indoor environment, to better assess fungal contamination (Degois *et al.*, 2017; Viegas *et al.*, 2017). Of note, culture-based methods can reveal less abundant fungi in some indoor environments, when compared with more refined molecular tools. However, though in less counts some *Aspergillus* sections (opportunistic pathogens) found as the most prevalent in some studies (Fadaei and Amiri, 2014; Karimi *et al.*, 2019; Rafiei and Amirrajab, 2010; Rasti *et al.*, 2012) may represent a health risk for visitors and also workers who are exposed on a daily basis (Mbareche *et al.*, 2019).

Conclusions

Future endeavors should be performed to achieve an exposure assessment protocol specific for the indoor environments that comprise swimming pools, since they can work as additional fungal sources. The decisions regarding the sampling methods and the laboratory tools to apply to accomplish an accurate fungal contamination exposure assessment should be supported by scientific guidance to allow the risk characterization of users and workers.

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Occupational Fungal Exposure and Assessment on Animal Production

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Introduction

In buildings where intense animal breeding occurs, the air is usually contaminated with a high prevalence of microorganisms (Dutkiewicz *et al.*, 1994; Seedorf *et al.*, 1998; Radon *et al.*, 2002; Jo and Kang, 2005; Lee *et al.*, 2006; Sowiak *et al.*, 2011; Sowiak *et al.*, 2012). However, the sector of animal production where the highest prevalence of microorganisms was reported was poultry breeding (Seedorf *et al.*, 1998; Radon *et al.*, 2002; Sowiak *et al.*, 2012).

It is well known that fungi in indoor farm environments emerge and sporulate easily in low quality bedding or polluted feedstuffs. Pollutants accumulated in confined animal buildings can present a serious health hazard for workers (Karwowska, 2005; Chmielowiec-Korzeniowska *et al.*, 2018). Insufficient ventilation and the presence of particulate matter raise the risk of exposure to bioaerosols (Viegas *et al.*, 2019b). Bioaerosols consist of airborne bacteria, fungi, viruses and their by-products, endotoxins and mycotoxins (Oppliger *et al.*, 2008; Just *et al.*, 2009; Viegas *et al.*, 2012a) and also non-viable particles, formed by items such as feces, litter, feed and feathers (Viegas *et al.*, 2012a).

Airway inflammation caused by a non-allergic mechanism has been well established as the main respiratory health problem for those working in animal production buildings with heavy dust exposure, especially in swine and poultry farms (Olenchock *et al.*, 1982; Mutel and Donham, 1983; Rylander, 1986; Melbostad *et al.*, 1997; Iversen *et al.*, 2000; Kirkhorn and Garry, 2000; Viegas *et al.*, 2012b). Among fungi the genera *Aspergillus, Penicillium, Cladosporium, Alternaria, Rhizopus, Scopulariopsis* and *Trichophyton* are commonly found inside breeding buildings (Gemeinhardt and Wallenstein, 1985; Lugauskas *et al.*, 2004; Jo and Kang, 2005; Sowiak *et al.*, 2012). Thus, the inhalation of fungal spores has been linked to hypersensitive lungs (Mutel and Donham, 1983; Viegas *et al.*, 2012b). *Aspergillus* species have been closely related among the filamentous fungi to asthma exacerbations and other allergic respiratory diseases (Viegas *et al.*, 2019a). Particles with an average aerodynamic diameter of 1–4 μm, like *A. fumigatus* conidia, are found in the lower respiratory tract since they are small enough to pass through the terminal airways and enter the pulmonary alveoli (Viegas *et al.*, 2019a).

Occupational exposure can occur by inhalation of the dust produced during the processing and handling of contaminated crops and feed. This contamination path creates harmful health effects for workers (Chang *et al.*, 2001; Cormier *et al.*, 2000; Reboux *et al.*, 2006; Viegas *et al.*, 2019a,b) and for animals, especially for avian species (Lair-Fulleringer *et al.*, 2006).

Respiratory problems are well-recognized by these workers, resulting in elevated morbidity and mortality from certain respiratory disorders. Several studies have shown that the prevalence of work-related respiratory problems such as wheeze, cough and dyspnea is extremely high (23–50 per cent) (Rylander *et al.*, 1990; Skórska *et al.*, 1998; Radon *et al.*, 1999; Viegas *et al.*, 2019a,b). The main respiratory symptoms due to fungi exposure include allergic bronchopulmonary mycoses (ABPM), pneumonitis with hypersensitivity, fungal sinusitis, allergic rhinitis, and extreme fungal sensitized asthma (SAFS) (Simon-Nobbe *et al.*, 2008; Viegas *et al.*, 2019a,b). Additionally, under Hazardous Biological Agents Regulation, enforced by Directive 2000/54/EC of the European Council, farmers are classified into the groups of occupationally exposed to biological agents at their workplace. It is also important to highlight that most of the work on airborne fungi in various agricultural environments used only culture-based sampling methods (Predicala *et al.*, 2002; Lubiński *et al.*, 2005; Chmielowiec-Korzeniowska *et al.*, 2018). Therefore, these studies neglected non-cultural airborne fungi, which can be as allergic or harmful as culturable fungi (Dutkiewicz *et al.*, 2011; Chmielowiec-Korzeniowska *et al.*, 2018) and, enhance health risks to animal production workers.

As such, this study aims to gather and highlight information regarding animal production environments, its dominant species, the most commonly used sampling and analytical methods and of which show more reliable and accurate results when assessing or monitoring fungal burden in these kinds of occupational environments.

Materials and methods

For this study was performed a well-structured search of published data and information in the public domain between 1st of January 2000 and 31st of June 2020. Baseline parameters were selected following the PRISMA methodology to identify and select studies referring to animal production occupational environments that assessed fungi, selecting only English and original

Table 1 Inclusion and exclusion criteria in the articles selected

Inclusion criteria	*Exclusion criteria*
Articles published in the English language;	Articles published in other languages
Articles published from 1st January 2000 to 31st June 2020	Articles published prior to 2000
Articles published in any country	
Articles related to fungi assessment in animal production settings	Articles related exclusively to biological samples or without mention of fungi quantification.
Original scientific articles on the topic	Abstracts of congress, reports, reviews or state of the art articles

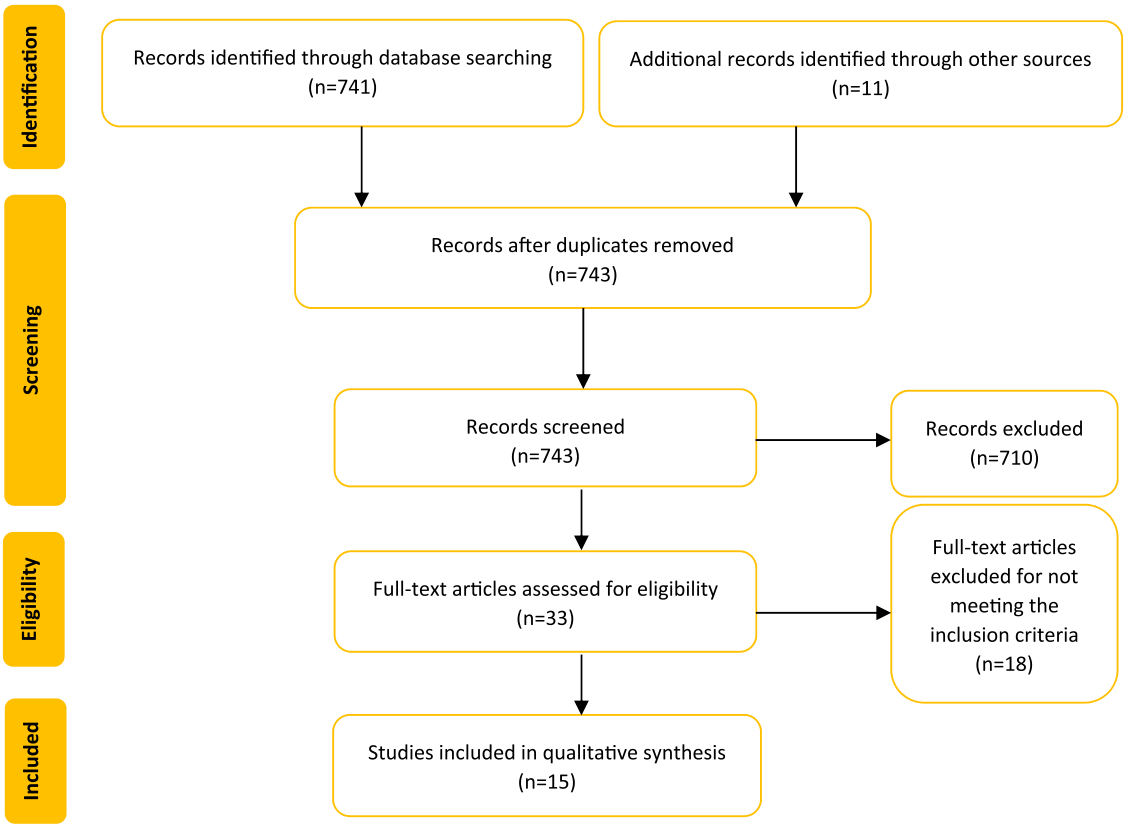

Fig. 1 PRISMA methodology of selection of papers.

papers. Appropriate search terms were employed in the selected databases (PubMed, Scopus and Web of Science), mainly focusing in occupational settings and fungi assessment. However, since the number of papers selected in the databases were lower than the expected, search terms were also employed in other sources, so that more robust results were achieved. The final result of this search resulted in 15 original papers and all the articles that do not meet the selected inclusion criteria were excluded (**Table 1**). The main findings of the studies were highlighted and compared for consistency, resulting in a final selection of papers (**Fig. 1**).

Results

As a result of the structured literature review, a total of 15 articles were selected since the inclusion criteria were verified (**Table 2**). Different occupational environments regarding animal production (poultry houses, swine farms and other facilities) were considered. The sampling and analytical methods used, as well as the main findings found in each study were analyzed.

The main setting found among the selected studies were poultry (Kim *et al.*, 2008; Sabino *et al.*, 2012; Sowiak *et al.*, 2012; Viegas *et al.*, 2012a,b; Lawniczek-Walczyk *et al.*, 2013; O'Brien *et al.*, 2016) and swine farms (Adhikari *et al.*, 2004; Kim *et al.*, 2008; Sabino *et al.*, 2012; Viegas *et al.*, 2013; Costa *et al.*, 2014; Chmielowiec-Korzeniowska *et al.*, 2018; Viegas *et al.*, 2018b) making a

Table 2 Papers included in the review

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Analytical methods*	*Results*	*Main findings*	*References*
PubMed	Occupational exposure to airborne microorganisms, endotoxins and β-glucans in poultry houses at different stages of the production cycle	Poland	3 Poultry houses	Personal air filtration and impaction air sampling	Culture-based methods	*Aspergillus* spp. *Penicillium* spp. *Scopulariopsis* spp.	Professional activities in poultry farms are associated with constant exposure to large quantities of airborne microorganisms. Filter method showed incomplete information regarding qualitative results, however it stands as a good reference for total microbial counts.	(Lawniczek-Walczyk *et al.*, 2013)
	Occupational exposure level of pig facility workers to chemical and biological pollutants	Poland	1 Swine farm	Stationary air filtration	Culture-based methods	*Aspergillus* spp. *Cladosporium* spp. *Fusarium* spp. *Penicillium* spp. *Rhizopus* spp. *Ulocladium* spp. *Trichoderma* spp.	VOC concentration exceeded Polish recommended values regardless of the season, however ventilation plays a major role on the microbiological contamination.	(Chmielowiec-Korzeniowska *et al.*, 2018)
Scopus	Effects of disinfectant fogging procedure on dust, ammonia concentration, aerobic bacteria and fungal spores in a farrowing-weaning room	Italy	1 Swine farm	Impaction air sampler	Culture-based methods	Fungal spore range: 0 CFU m^{-3}–1,84 $\times$ 10^2 CFU m^{-3}	The reduction of fungal spores and the smaller fractions of particles were associated with the oxidizing effect of the disinfection procedure.	(Costa *et al.*, 2014)
	Pulmonary illness as a consequence of occupational exposure to shrimp shell powder	Canada and Norway	3 Shrimp processing units	Stationary air filtration	Culture-based methods, MALDI-TOF	*Aspergillus fumigatus, Penicillium chrysogenum, Penicillium rugulosum*	This study showed that exposure to shrimp powder aerosols have a negative impact on the workers health, due to the high fraction of inhalable dust and microorganisms present.	(Bertelsen *et al.*, 2016)
	High throughput genomic sequencing of bioaerosols in broiler chicken production facilities	USA	Poultry farms	Personal air filtration	Illumina genome sequencing and analysis	Ascomycota phylum prevalence: 1.4%	The reduction of fungal spores and the smaller fractions of particles were associated with the oxidizing effect of the disinfection procedure.	(O'Brien *et al.*, 2016)
Web of Science	Airborne contaminants in conventional laboratory rabbit rooms	Finland	2 Rabbit rooms	Personal and stationary air filtration	Culture-based methods	*Aspergillus versicolor, Aspergillus* sp. *Cladosporium* sp. *Penicillium* sp.	Good ventilation and hygiene may reduce airborne contaminants concentration, however pathogenic species were found that may pose a health hazard to the workers.	(Kaliste *et al.*, 2002)

(*Continued*)

Table 2 Continued

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Analytical methods*	*Results*	*Main findings*	*References*
	Evaluation of environmental circumstance within swine and chicken Houses in South Korea for the production of safe and hygienic animal food products	South Korea	6 Swine houses and 7 Chicken houses	Impaction air sampling	Culture-based methods	Swine houses fungal range: 1.2×10^1 CFU m^{-3}–2.5×10^4 CFU m^{-3} Chicken houses fungal range: 6.8×10^2 CFU m^{-3}–1.0×10^5 CFU m^{-3}	The values found in this study suggests that these facilities may not represent a health hazard to the workers, however its reported that the environments of swine and poultry facilities contribute to the development of respiratory tract diseases and other symptoms.	(Kim *et al.*, 2008)
	Air contaminants in animal production: the poultry case	France	7 Poultry farms	Impaction air sampling	Culture-based methods	*Aspergillus* sp. *Penicillium* sp. *Rhizopus* sp. *Scopulariopsis brevicaulis*	Exposure level assessment to bioaerosols in these occupational settings are needed due to the already reported cases of hypersensitivity pneumonitis and other respiratory symptoms. Environmental factors may influence fungal load.	(Viegas *et al.*, 2012a)
	Production of recycled manure solids for use as bedding in Canadian dairy farms: II. Composting methods	Canada	12 Experimental dairy rooms	Impinger air sampling	Culture-based methods	Culturable fungi range: 3.0×10^2 CFU m^{-3}–5.6×10^4 CFU m^{-3}	Composting materials may have effects on the concentration of spore-forming organisms in milk. In humid climate the temperature should be kept at thermophilic temperatures to avoid the increase of the microbial load.	(Fournel *et al.*, 2019)
Other Sources	Assessment of human exposure to airborne fungi in agricultural confinements: Personal inhalable sampling versus stationary sampling	USA	1 Swine farm and 1 Dairy farm	Personal and stationary air filtration	Culture-based methods	Stationary air sampling: *Aspergillus*/ *Penicillium* sp. *Cladosporium* sp. Personal air sampling: *Aspergillus*/ *Penicillium* sp. *Cladosporium* sp.	No major difference was detected between the two sampling methods. Button samplers seem to be a reliable method do assess the workers exposure to inhalable fungi in agricultural environments.	(Adhikari *et al.*, 2004)
	Occupational Exposure to *Aspergillus* by Swine and	Portugal	7 Poultry farms and 7 Swine farms	Impaction air sampling,	Culture-based methods	*Aspergillus* genus prevalence: 22% *Aspergillus*	High fungal load was verified in these kinds of facilities, mainly of *Aspergillus* and *Penicillium*	(Sabino *et al.*, 2012)

Poultry Farm Workers in Portugal			surface sampling by swabbing		*versicolor, Aspergillus flavus, Aspergillus fumigatus*	species. Total fungal load surpassed the recommended values for poultry houses in all the air samples.	
Fungal aerosol in the process of poultry breeding-quantitative and qualitative analysis	Poland	5 Poultry farms	Stationary air filtration	Culture-based methods	Acremonium sp. *Acremonium strictum, Aspergillus* sp. *Aspergillus fumigatus,* Candida sp. *Candida tropicalis,* Geotrichum sp. Penicillium sp. *Penicillium aurantiogriseum,* Scopulariopsis sp.	Fungi concentrations found often exceeded reference limit values. 88% of the fungal microflora were composed by molds and also pathogenic species were found.	(Sowiak *et al.*, 2012)
Comparison of indoor and outdoor fungi and particles in poultry units	Portugal	7 Poultry farms	Impaction air sampling	Culture-based methods	*Aspergillus flavus*, *Aspergillus* sp. *Penicillium* sp. *Rhizopus* sp. *Scopulariopsis brevicaulis*	Poultry farms are a reservoir of fungi and particles that may be re-aerosolized into the air resulting in emission of potential pathogenic fungi into the air, thus increasing the health hazards present in the work environment.	(Viegas *et al.*, 2012b)
Fungal Contamination in Swine: A Potential Occupational Health Threat	Portugal	7 Swine farms	Impaction air sampling, surface sampling by swabbing, bulk sampling of floor coverage	Culture-based methods	*Aspergillus versicolor*, *Cladosporium* sp. *Chrysosporium* sp. *Geotrichum* sp. *Mucor* sp. *Scopulariopsis brevicaulis* *Trichoderma* sp.	*Stachybotris* sp. should not be neglected when assessing swine barns due to its slow growth and to its toxin production potential. Pig houses have many possible sources of contamination that may also affect outdoor air quality and pose a threat to human health.	(Viegas *et al.*, 2013)
A Novel Multi-Approach Protocol for the Characterization of Occupational Exposure to Organic Dust—Swine Production Case Study	Portugal	5 Swine farms	Impaction and Impinger air sampling, surface sampling by swabbing	Culture-based methods, RT-PCR	Air samples: *Aspergillus* section *Circumdati*, *Aspergillus* section *Versicolores*, *Cladosporium* sp. Feed samples: *Cladosporium* sp. Surface samples: *Cladosporium* sp. *Scopulariopsis brevicaulis* Floor samples: *Penicillium* sp. *Alternaria* sp.	The use of different culture media, passive methods and molecular tools helps to achieve a more accurate mycobiota exposure assessment. Swine farm workers were exposed to a combination of several risk factors.	(Viegas *et al.*, 2018b)

total of 12 out of 15 articles. Two dairy farms (Adhikari *et al.*, 2004; Fournel *et al.*, 2019), a rabbit room (Kaliste *et al.*, 2002) and a shrimp processing plant (Bertelsen *et al.*, 2016) were also analyzed.

The air impaction method was used in 8 of the 15 studies (Kim *et al.*, 2008; Sabino *et al.*, 2012; Viegas *et al.*, 2012a,b, 2013; Lawniczek-Walczyk *et al.*, 2013; Costa *et al.*, 2014; Viegas *et al.*, 2018b), making it the most common method overall followed by the air filtration method used in 7 of the 15 studies (Kaliste *et al.*, 2002; Adhikari *et al.*, 2004; Sowiak *et al.*, 2012; Lawniczek-Walczyk *et al.*, 2013; Bertelsen *et al.*, 2016; O'Brien *et al.*, 2016; Chmielowiec-Korzeniowska *et al.*, 2018). Other methods were also employed, such as surface swabs (Sabino *et al.*, 2012; Viegas *et al.*, 2013, 2018b), impinger method (Fournel *et al.*, 2019) and bulk sample collection (Viegas *et al.*, 2013). The exclusive use of active methods were the most used approach reported in 12 out of the 15 studies (Kaliste *et al.*, 2002; Adhikari *et al.*, 2004; Kim *et al.*, 2008; Sowiak *et al.*, 2012; Viegas *et al.*, 2012a,b; Lawniczek-Walczyk *et al.*, 2013; Costa *et al.*, 2014; Bertelsen *et al.*, 2016; O'Brien *et al.*, 2016; Chmielowiec-Korzeniowska *et al.*, 2018; Fournel *et al.*, 2019) and the simultaneous use of active and passive methods (Sabino *et al.*, 2012; Viegas *et al.*, 2013, 2018b) was observed in 3 of the 15 studies. However, it was not observed in any study the use of passive sampling methods as a stand-alone method.

Regarding analytical methods culture-based methods was the most common approach across all studies, being performed in 14 out of the 15 studies (Kaliste *et al.*, 2002; Adhikari *et al.*, 2004; Kim *et al.*, 2008; Sabino *et al.*, 2012; Sowiak *et al.*, 2012; Viegas *et al.*, 2012a,b; Viegas *et al.*, 2013; Lawniczek-Walczyk *et al.*, 2013; Costa *et al.*, 2014; Bertelsen *et al.*, 2016; Chmielowiec-Korzeniowska *et al.*, 2018; Viegas *et al.*, 2018b; Fournel *et al.*, 2019) while the use of molecular tools for analytical assessment were observed only in 3 out of the 15 studies (Bertelsen *et al.*, 2016; O'Brien *et al.*, 2016; Viegas *et al.*, 2018b). *Aspergillus* section *Nidulantes* (formerly *Aspergillus* section *Versicolores*) (Kaliste *et al.*, 2002; Sabino *et al.*, 2012; Viegas *et al.*, 2013, 2018b) and *Scopulariopsis brevicaulis* (Viegas *et al.*, 2012a,b, 2013, 2018b) were both found in 4 out of 15 studies making both species the most identified across all studies.

Aspergillus was the genera most common among all the selected studies, found in 10 out of 15 studies (Kaliste *et al.*, 2002; Adhikari *et al.*, 2004; Sabino *et al.*, 2012; Sowiak *et al.*, 2012; Viegas *et al.*, 2012a,b; Lawniczek-Walczyk *et al.*, 2013; Viegas *et al.*, 2013; Bertelsen *et al.*, 2016; Chmielowiec-Korzeniowska *et al.*, 2018; Viegas *et al.*, 2018b), in which were reported species belonging to *Aspergillus* section *Fumigati* (*Aspergillus fumigatus*) (Sabino *et al.*, 2012; Sowiak *et al.*, 2012; Bertelsen *et al.*, 2016) and *Aspergillus* section *Flavi* (*Aspergillus flavus*) (Sabino *et al.*, 2012; Viegas *et al.*, 2012b). *Penicillium* was reported in 9 out of 15 studies (Adhikari *et al.*, 2004; Sowiak *et al.*, 2012; Viegas *et al.*, 2012a,b; Lawniczek-Walczyk *et al.*, 2013; Bertelsen *et al.*, 2016; Chmielowiec-Korzeniowska *et al.*, 2018; Viegas *et al.*, 2018b), *Scopulariopsis* sp. in 6 out of 15 studies and *Cladosporium* sp. in 5 out of 15 studies.

Discussion

The use of passive methods for sampling occupational environments allows a better quantification of the number of fungal species present and a sampling that is representative of longer periods of time (Viegas *et al.*, 2015a,b, 2017). The collection and analysis of feed and floor coverage samples during exposure assessment in animal production environments is an important tool as these matrices function as passive methods that are critical for bioburden assessment (Hayleeyesus and Manaye, 2014; Viegas *et al.*, 2020b). This approach was employed in 3 out of the 15 studies regarding swine and poultry farms (Sabino *et al.*, 2012; Viegas *et al.*, 2013, 2018b), considering these type of environmental matrices ensures that the microbiological characterization of the environment is carried out in a more comprehensive and complete way (Klánová and Hollerová, 2003; Stetzenbach *et al.*, 2004; Viegas *et al.*, 2016a,b, 2019b).

Regarding the analytical methods, in most papers (12 out of 15) culture-based methods were the only one used to perform fungal identification. However, this method has some disadvantages that should be considered such as the growth speed of some fungal species that can influence others when we´re considering mixed cultures. As an example, the overgrowth of a specific species in a mixed culture, may cause a chemical competition and consequently lead to the inhibition of the growth of other species (Viegas *et al.*, 2015a,b; Dias and Viegas, 2020). That evidence supports the need to combine culture-based methods with molecular tools as we can see in 3 out of the 15 papers analyzed (Bertelsen *et al.*, 2016; Viegas *et al.*, 2018b). Nonetheless, it is important to highlight that all three papers used different molecular tools, and that these methods detain some specific characteristics that makes them more efficient such as its speed, extreme analytical sensitivity of detection, accuracy and the possibility to detect and identify dead or dormant microorganisms, as well as toxigenic strains from specific fungal species (Amann *et al.*, 1995; MacNeil *et al.*, 1995; Viegas *et al.*, 2015a,b; Dias and Viegas, 2020). Considering all the above, the use of molecular tools as an ally to culture-based methods should always be considered. Regarding the species prevalence, most studies (11 out of 15) presented *Aspergillus* spp. as one of the species with the highest prevalence. It has been reported that some sections of *Aspergillus* genera are opportunistic and increase the appearance of infections in immunocompromised individuals, and also of antifungal resistance, both in the clinical and in the environment (Fairlamb *et al.*, 2016; Nature Microbiology, 2017; Viegas *et al.*, 2020a,c; Dias and Viegas, 2020). There is also some other factors in *Aspergillus* spp. that gives them a higher occupational risk such as the pathogenic and toxigenic potential of some *Aspergillus* sections (*Flavi*, *Nidulantes*, *Circumdati*, *Nigri*, *Terrei*, *Fumigati*,….) (Varga *et al.*, 2015, 2018a, 2020a,c) that may cause several health complications such as chronic pulmonary aspergillosis, allergic bronchopulmonary aspergillosis and invasive aspergillosis (Lamoth, 2016). Therefore, the assessment of this genus should always be recommended. In animal production, the One Health approach should be considered, since these occupational environments require a dynamic relationship between the environment, animals and humans. When dedicated to animal production, it considers the impact caused by the products that come directly from the animals (e.g., Milk, Eggs, etc) in public health while guarantees food safety by establishing controlling measures (Predicala *et al.*, 2002; Chmielowiec-Korzeniowska *et al.*, 2018). Therefore, it is important to insure that high-quality water, feed, and equipment are used and precautions are placed in place to avoid the occurrence of

environmental contamination and disease transmission within the herd and animal handlers, safeguarding all animal products (Predicala *et al.*, 2002; Chmielowiec-Korzeniowska *et al.*, 2018).

Conclusions

Animal production is an occupational environment with scientific evidence about occupational exposure to microbial agents, and more specifically to fungi. The available research is scarce, nevertheless all the information gathered with this review highlights the importance of monitor this type of environment and implement protocols to assess fungal burden, specially *Aspergillus* sections, since they can cause adverse health problems due to their pathogenic and toxigenic potential.

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Further Reading

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Fungal Prevalence on Waste Industry – Literature Review

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Introduction

Bioaerosols are microbial fragments, constituents of cells and airborne biological particles that can consist of fungi, bacteria, pollen, fragments, constituents, particulate matter (PM_{10}), and by-products of cells, that may be viable or nonviable (Dowd and Maier, 1999; Douwes *et al.*, 2003; Viegas and Gomes, 2014; Pearson *et al.*, 2015). Exposure to bioaerosols can lead to a number of pathologies, such as: non-allergic irritative reactions (upper and lower respiratory tracts, asthma, contact dermatitis), inflammatory (chronic bronchitis), allergic or immunoallergic reactions (rhinitis, sinusitis, allergic asthma, hypersensitive pneumonitis, and organic dust toxic syndrome), and infections like invasive aspergillosis in immunosuppressed individuals (Bünger *et al.*, 2000; Douwes *et al.*, 2003; Viegas *et al.*, 2015).

Microbiological exposures associated with waste can occur indoors, where waste is stored, or outdoors during its collection and it may be influenced by the process of sorting, transfer or cleaning (Wouters *et al.*, 2000; Lavoie and Dunkerley, 2002; Abou-Elwafa and El-Bestar, 2014).

In this type of highly contaminated environment, one of the most prevalent genera is *Aspergillus* spp. (Viegas *et al.*, 2017b). However, there are a few more genera/species that are also considerably prevalent such as *Aureobasidium pullulans, Epicoccum nigrum, Fusarium sambucinum, Beauveria bassiana, Rhizopus stolonifer, Phoma, Absidia, Mucor, Rhizopus, Penicillium*, and yeasts (Grisoli *et al.*, 2009).

In addition, it is important to highlight that workers may be exposed to the same potential hazards as the general population, although exposure and danger may be different due to waste disposal by workers and, as a result, proximity to pollution sources and a more contaminated indoor environment (Rushton, 2003).

It has been shown that waste management releases airborne microorganisms and bioaerosols into the atmosphere around waste facilities (Viegas and Gomes, 2014) and that temperature, relative humidity, and wind speed affect concentrations and viability of airborne microorganisms (Jones and Harrison, 2004). Consequently, this type of industry and facilities are considered critical in terms of occupational exposure to fungal contamination (Heinonen-Tanski *et al.*, 2009; Hameed and El Gendy, 2013) as they provide optimal conditions for fungal growth (Thirumala *et al.*, 2012).

The installation of a central ceiling diffuser to provide downstream fresh air, as well as to keep workers ' breathing zones free of pollutants, and cleaning procedures are preventive measures that are usually applied in sorting cabinets (Marchand *et al.*, 1995). However, it's important to consider that waste-sorting it's crucial to maximize recycling (Viegas *et al.*, 2020) and that additional monitoring should be performed to evaluate the workers exposure (Marchand *et al.*, 1995) and to solve remaining contamination and, consequently, exposure problems.

Materials and Methods

This study is based on a research of the available information/data on fungal occupational exposure in waste industry, published between 1st of January 2010 and 31st of December 2019, following the PRISMA methodology. The databases chosen were b-on, PubMed and WOS and the keywords used were "fungi", "occupational exposure", and "waste industry". Searches were carried out in English. However, since the last two databases led to a small number of results (<20 papers), and that the papers were the same in b-on results, those databases were excluded, and only b-on results were considered to this study. This search led to nearly 1010 papers in b-on database. Articles that did not meet the inclusion criteria were excluded from further analysis (but some of them were used for introduction and discussion) (**Table 1**) (**Fig. 1**).

Results

Among the 20 papers included in this study (**Table 2**), analysis was performed regarding the occupational environment studied, which country belongs the industry, sampling methods applied, the environmental matrix which assays were applied, and the most prevalent fungal species found.

Table 1 Inclusion and exclusion criteria

Inclusion Criteria	*Exclusion Criteria*
Articles published in the English language;	Articles published in other languages
Articles published from 1st of January 2010 to 31st of December 2019	Articles published prior to 1st of January 2010
Articles related to fungi assessment in waste industry	Articles related exclusively to biologic samples from patients
Original scientific articles on the topic	Abstracts of congress, reports, reviews/state of the art articles
Articles published in any country	

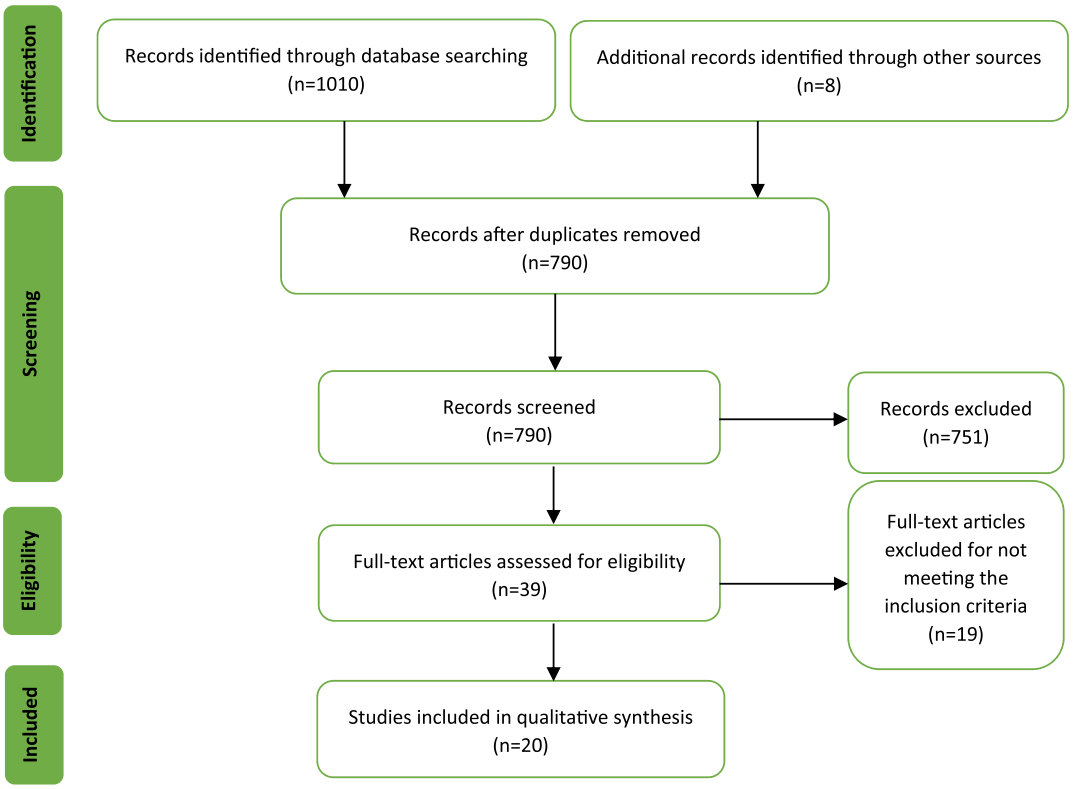

Fig. 1 PRISMA methodology of selection of papers.

Regarding the environmental matrix, most studies (14 out of 20) presented air as the only type of sample (Issever *et al.*, 2011a,b; Burkowska *et al.*, 2014; Taylor *et al.*, 2015b; Hameed *et al.*, 2015; Černá *et al.*, 2017; Madsen *et al.*, 2016; Bonifait *et al.*, 2017; Gladding and Gwyther, 2017; Wolany and Płaza, 2017; Afsharnia *et al.*, 2018; Mbareche *et al.*, 2018; Madsen *et al.*, 2019; Line *et al.*, 2019), the remaining studies presented air samples in parallel with samples collected by passive sampling methods (Viegas and Gomes, 2014; Taylor *et al.*, 2015a; Viegas *et al.*, 2015, 2016, 2017b).

In most studies (7 out of 20), the air samples were only collected by impaction methods (Issever *et al.*, 2011a,b; Burkowska *et al.*, 2014; Taylor *et al.*, 2015a,b; Wolany and Płaza, 2017; Afsharnia *et al.*, 2018), some other (3 out of 20) used only impingement methods (Hameed *et al.*, 2015; Gladding and Gwyther, 2017; Mbareche *et al.*, 2018) and a few others (4 out of 20) used only filtration methods (Viegas *et al.*, 2017a; Černá *et al.*, 2017; Madsen *et al.*, 2016; Line *et al.*, 2019). The remaining studies combined either two active sampling methods (2 out of 20) (Bonifait *et al.*, 2017; Madsen *et al.*, 2019), two active sampling methods and one passive sampling method (1 out of 20) (Viegas *et al.*, 2017a,b) or one active sampling method and one passive sampling method (3 out of 20) (Viegas and Gomes, 2014; Viegas *et al.*, 2015, 2016). The passive sampling used in those six studies was swabbing (Viegas and Gomes, 2014; Taylor *et al.*, 2015a,b; Viegas *et al.*, 2015, 2016, 2017b), collected compost (Taylor *et al.*, 2015a,b), and filters from the air conditioning system of forklifters (Viegas *et al.*, 2017b).

Concerning the methods of fungal identification, most studies (12 out of 20) used only culture-based methods (Issever *et al.*, 2011a,b; Burkowska *et al.*, 2014; Taylor *et al.*, 2015a,b; Hameed *et al.*, 2015; Černá *et al.*, 2017; Madsen *et al.*, 2016; Gladding and Gwyther, 2017; Wolany and Płaza, 2017; Afsharnia *et al.*, 2018; Madsen *et al.*, 2019). Additionally, some studies (6 out of 20) combined culture-based methods with molecular tools (Viegas and Gomes, 2014; Viegas *et al.*, 2015, 2016; Bonifait *et al.*, 2017;

Table 2 Papers included in the review

Setting	*Environmental Matrix*	*Sampling*	*Assays Applied*	*Results (Most prevalent fungi found – Genera/species)*	*Country*	*Reference*
Occupational exposure -Respiratory functions of the people working in solid waste storage centers	Air	Impaction	Culture-based methods	*Culture-based methods:* Aspergillius sp and Cladosporium sp	Turkey	Issever *et al.* (2011a)
Fungal Flora at Solid Waste Storage Centers and Their Potential Allergenic Effects on the Workers	Air	Impaction	Culture-based methods	*Culture-based methods:* *Aspergillus* sp. and *Cladosporium* sp.	Turkey	Paper *et al.* (2011)
Exposure of Workers of Municipal Landfill Site to Bacterial and Fungal Aerosol	Air	Impaction	Culture-based methods	*Culture-based methods:* *Penicillium spp., Cladosporium spp. Alternaria* spp. *and Aspergillus spp.*	Poland	Burkowska *et al.* (2014)
Fungal contamination in two plants handling solid waste management – one waste sorting plant and one incineration plant	Air Surface (swabs)	Impaction and swabbing	Culture-based methods Molecular tools Particulate matter assessment	- *Waste sorting plant* *Culture-based methods:* *Air and surface samples:* Aspergillus *sections* Nigri, Fumigati, *and* Flavi *Molecular Tools (qPCR):* No species amplified - *Incineration plant* *Culture-based methods:* Air samples - *Penicillium* sp. and *Aspergillus* sections *Fumigati* and *Flavi* Surface samples - *Penicillium* sp. and *Aspergillus* sections *Fumigati* and *Nigri* *Molecular Tools (qPCR):* No species amplified	Portugal	Viegas and Gomes (2014)
Microbial contamination within working environments of different types of composting plants	Air Surface (swabs) Compost	Impaction	Culture-based methods	Culture-based methods: *Aspergillus fumigatus, Cladosporium (C. cladosporioides, C. herbarum, C. macrocarpum), and Rhizopus oryzae*	Poland	Taylor *et al.* (2015a)
Occupational risk of exposure to biological agents in a waste packaging glass sorting plant	Air	Impaction	Culture-based methods	*Culture-based methods:* *Penicillium* spp.	Portugal	Taylor *et al.* (2015b)

(*Continued*)

Table 2 Continued

Setting	*Environmental Matrix*	*Sampling*	*Assays Applied*	*Results (Most prevalent fungi found – Genera/species)*	*Country*	*Reference*
Fungal burden in waste industry: an occupational risk to be solved	Air Surface (swabs)	Impaction and swabbing	Culture-based methods Molecular Tools	*Before cleaning* Culture-based methods: Air (indoor): *Penicillium* sp., *A. fumigatus, A. niger,* Surface: *A. niger, E. herbarium* Air (outdoor): *Penicillium* sp., *A. fumigatus, A. niger* *Molecular Tools (qPCR):* *A. fumigatus* *After cleaning* *Culture-based methods:* Air (indoor): *A. niger complex and Penicillium* sp. *Surface: A. niger complex and Ulocladium sp.* *Air (outdoor): A. fumigatus* complex *Molecular Tools (qPCR):* *A. fumigatus*	Portugal	Viegas *et al.* (2015)
Airborne fungal pollution at waste application facilities	Air	Impingement	Culture-based methods	*Culture-based methods:* *Aspergillus* sections *Fumigatus and Flavi, Penicillium spp. and Cladosporium* spp.	Egypt	Hameed *et al.* (2015)
Analysis of surfaces for characterization of fungal burden waste management plants and wastewater treatment plants	Air Surface (swabs)	Impaction and swabbing	Culture-based methods Molecular tools	*Culture-based methods:* *Cladosporium* sp., *Penicillium* sp., *A. fumigatus* complex, *A. versicolor* complex and *A. flavus* complex. *Molecular Tools (qPCR):* *A. flavus complex (toxigenic strains),* *A. fumigatus complex and Stachybotrys chartarum*	Portugal	Viegas *et al.* (2016)
Exposure to airborne fungi during sorting of recyclable plastics in waste treatment facilities	Air	Filtration	Culture-based methods	*Culture-based methods:* *Penicillium spp. and Aspergillus* spp.	Czech Republic	Černá *et al.* (2017)
Waste Workers' Exposure to Airborne Fungal and Bacterial Species in the Truck Cab and During Waste Collection	Air	Filtration	Culture-based methods	*Culture-based methods:* *Penicillium* spp.	Denmark	Madsen *et al.* (2016)

Workers' exposure to bioaerosols from three different types of composting facilities - domestic, manure, carcass	Air	Impingement and impaction	Culture-based methods Molecular Tools	*Culture-based methods:* *Thermophilic actinomycetes* and *A. fumigatus* *Molecular Tools (qPCR):* *Penicillium* and *A. fumigatus*	Canada	Bonifait *et al.* (2017)
Occupational exposure to airborne fungal contamination of forklift drivers in waste sorting facilities	Filters from the air conditioning system of forklifters	Filtration	Culture-based methods Molecular tools Mycotoxins analysis Cytotoxicity assay	*Culture-based methods: Aspergillus* sections *Circumdati* and *Nigri.* *Molecular Tools (qPCR):* *Aspergillus* section *Fumigati*	Portugal	Viegas *et al.* (2017a)
Aspergillus spp. prevalence in different Portuguese occupational environments - waste management plants and wastewater treatment plants	Air Surface (swabs)	Impingement, impaction and swabbing	Culture-based methods Molecular Tools	*Culture-based methods:* *Aspergillus* sections *Nigri*, *Fumigati* and *Flavi* *Molecular Tools (qPCR):* *Aspergillus* section *Fumigati*	Portugal	Viegas *et al.* (2017b)
Potential release of bioaerosols from containers as a result of reduced frequency residual waste collections	Air	Impingement	Culture-based methods	*Culture-based methods:* *A. fumigatus* and Thermotolerant fungi (species unidentified)	UK	Gladding and Gwyther (2017)
Characteristics of airborne fungi in some Polish wastewater treatment plants	Air	Impaction	Culture-based methods	*Culture-based methods:* *C. Cladosporioides*	Poland	Wolany and Płaza (2017)
Identifying and Determining Dispersion Boundary Bio-aerosols of Bacterial and Fungal Pathogens from Municipal Waste Collection Containers	Air	Impaction	Culture-based methods	*Culture-based methods:* *Aspergillus* spp., Yeast and *Alternaria* spp.	Iran	Afsharnia *et al.* (2018)
Fungal bioaerosols in biomethanization facilities	Air	Impingement	Molecular Tools (qPCR and NGS)	*Molecular Tools (qPCR):* *Penicillium spp., Aspergillus* spp. *and Aspergillus fumigatus*	Canada	Mbareche *et al.* (2018)
Expanded cardboard waste sorting and occupational exposure to microbial species	Air	Filtration and impaction	Culture-based methods	*Culture-based methods:* *Penicillium commune, P. italicum and P. brevicompactum*	Denmark	Madsen *et al.* (2019)
Occupational exposure during treatment of offshore drilling waste and characterization of microbiological diversity	Air	Filtration	Culture-based methods	*Quantitative Results:* 20 CFU/mL	Norway	Line *et al.* (2019)

Viegas *et al.*, 2017a,b), and the remaining two studies used either only molecular tools (Mbareche *et al.*, 2018), or culture based methods achieving only quantification of fungal contamination (Line *et al.*, 2019).

Through culture-based methods, *Aspergillus* spp. and *Penicillium* spp. were the most commonly found fungi (18 out of 20) (Issever *et al.*, 2011a; Burkowska *et al.*, 2014; Viegas and Gomes, 2014; Taylor *et al.*, 2015a,b; Viegas *et al.*, 2015; Hameed *et al.*, 2015; Černá *et al.*, 2017; Madsen *et al.*, 2016; Viegas *et al.*, 2016; Bonifait *et al.*, 2017; Viegas *et al.*, 2017a,b; Gladding and Gwyther, 2017; Afsharnia *et al.*, 2018; Mbareche *et al.*, 2018; Madsen *et al.*, 2019). However, some studies presented different fungi with a considerable prevalence like *Cladosporium* spp. (Issever *et al.*, 2011a,b; Burkowska *et al.*, 2014; Taylor *et al.*, 2015a; Hameed *et al.*, 2015; Viegas *et al.*, 2016; Wolany and Płaza, 2017), *Rhizopus* spp. (Taylor *et al.*, 2015a), and *Alternaria* spp. (Burkowska *et al.*, 2014; Afsharnia *et al.*, 2018).

When applying molecular tools the most prevalent genera detected (5 out of 20) were *Aspergillus* spp. (Viegas *et al.*, 2015, 2016; Bonifait *et al.*, 2017; Viegas *et al.*, 2017a,b), and two studies had another fungal species such as *Stachybotrys chartarum* (Viegas *et al.*, 2016) and *Penicillium* spp. (Bonifait *et al.*, 2017).

A few studies (10 out of 20), identified *Aspergillus* spp. to the section level (Viegas and Gomes, 2014; Taylor *et al.*, 2015a; Viegas *et al.*, 2015; Hameed *et al.*, 2015; Viegas *et al.*, 2016; Bonifait *et al.*, 2017; Viegas *et al.*, 2017a,b; Gladding and Gwyther, 2017; Mbareche *et al.*, 2018). The most prevalent *Aspergillus* sections were *Fumigati* (Viegas and Gomes, 2014; Viegas *et al.*, 2015; Hameed *et al.*, 2015; Viegas *et al.*, 2016; Bonifait *et al.*, 2017; Viegas *et al.*, 2017a,b; Gladding and Gwyther, 2017), followed by *Nigri* (Viegas and Gomes, 2014; Viegas *et al.*, 2015, 2017a,b) and *Flavi* (Viegas and Gomes, 2014; Abdel Hameed *et al.*, 2015; Viegas *et al.*, 2016, 2017b).

Discussion

It is well known that waste management has an enormous influence as a release agent of a wide range of different microbiological agents (Pillai and Ricke, 2002; Viegas and Gomes, 2014). Fungi can affect human health in several ways that result in health outcomes such as infections, allergic reactions, irritation and toxic reactions (Fischer and Dott, 2003; McGinnis, 2004; Viegas *et al.*, 2016). Studies to perform exposure assessment to fungi in waste handling are increasing in several countries (Nielsen *et al.*, 1995; Poulsen *et al.*, 1995; Wouters *et al.*, 2006; Lavoie *et al.*, 2006; Park *et al.*, 2011; Madsen *et al.*, 2016), and the 20 papers used in this review are following this trend.

Regarding the analyzed papers, as previously reported, most of them (19 out of 20) used air as an environmental matrix, being impaction the most common sampling method (thanking into account studies with one and more sampling methods). Since this method relies only on culture based-methods, this can be a feature from this sampling approach since the viability of micro-organisms can affect their inflammatory and/or cytotoxic potential (Croston *et al.*, 2016), and thus it is possible to conclude about the inflammatory potential that varies according to the microbial composition of the bioaerosol (Timm *et al.*, 2009).

Impaction samplers collect particles from air with a specific flow rate and place them on a collecting surface such as Petri dishes containing agar medium (Institute APT, 1980). However, it is important to highlight that this sampling method has some disadvantages, since only assesses organisms that are culturable, that represent only one or two percent of the total air spore. Another disadvantage are the fact that in indoor environments the air is not uniform in space or time, it is always affected by the type of activity (Stellman, 1998) and its intensity, and that the high velocity of the air flow may result in the loss of some particles (Institute APT, 1980).

Taking all that into account, it is important to consider the use of passive sampling methods as a complement to active sampling methods. As previously reported, some studies (5 out of 20) combined an active sampling method (impaction) with a passive sampling method (surface samples (swabs)). Those two methods combined, increase the sensitivity of the assessment and eliminate the described disadvantages of the impaction samplers (Viegas *et al.*, 2016).

In this type of industry, there are passive sampling methods other than swabbing that can be useful to provide a more accurate exposure assessment to fungal contamination. Protection devices used by workers, such as Filtering Respiratory Protective Devices (FRPD), are used as an individual protection for workers and they can replicate the real scenario of exposure by inhalation since the FRPD is covering the workers airways (Viegas *et al.*, 2020). Therefore, the results obtained by the FRPD are similar to the results obtained in studies that combined active (air sampling) and passive (surface sampling) sampling methods (Viegas *et al.*, 2015, 2017a, 2020). Furthermore, FRPD can be suggested and desirable as a sampling method, since it has the ability to collect the sample of a wider period of time, just like any other passive sampling method, and also because it allows the combination of different approaches such as culture-based methods and molecular tools, to complement the study and the results (Viegas *et al.*, 2020).

Most papers (12 out of 20) used only culture-based methods to preform fungal identification, however, it is important to notice that this method has its own disadvantages such as how the growth speed and requirements of different fungal species affect other species in mixed cultures. For instance, the fast growth of a specific species may cause its overgrowth and consequently a chemical competition that can inhibit the growth of other species (Viegas *et al.*, 2015) and subsequently lead to lack of accuracy in the study results. Those reasons support the use and combination of culture-based methods and molecular tools, as seen in some papers (6 out of 20) of this study. Molecular tools detain some characteristics that allows them to be more efficient than other methods such as its accuracy, speed, it's extreme analytical sensitivity of detection and the fact that allows the detection and identification of dead or dormant microorganisms, as well as toxigenic strains from specific fungal species (Amann *et al.*, 1995; MacNeil *et al.*, 1995; Viegas *et al.*, 2015). Therefore, the complement between culture-base methods and molecular tools should always be recommended.

In both methods, as previously mentioned, *Aspergillus* spp. appeared with the highest prevalence and that is a big concern in terms of occupational health since it has been reported an increase of infections caused by opportunistic *Aspergillus* in immunocompromised patients, and also an increase of antifungal resistance, both in the clinical and in the environment (Fairlamb *et al.*, 2016; Nature Microbiology, 2017; Viegas *et al.*, 2020). Another fact that represents a high occupational risk is that several *Aspergillus* species have a pathogenic potential (Varga *et al.*, 2015; Viegas *et al.*, 2018, 2020) such as *A. flavus, A. niger, A. terreus, A. versicolor, A. calidoustus, and A. nidulans* that can cause several health conditions like allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis and invasive aspergillosis (Lamoth, 2016). Consequently, measures should be taken to implement and standardize the proper assessment of the prevalence of this genera, as recommended in Viegas *et al.* (2017b).

Conclusions

Although waste industry is an occupational environment with an increasing scientific evidence about occupational exposure to microbial agents, the information gathered with this review highlights the importance of monitor and implement protocols to assess fungal burden, focusing on *Aspergillus* species.

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Aspergillus in Indoor Environments

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Sources of Mold and Mold-Derived Fragments or Metabolites and Elevated Mold Exposure

Fungi normally present in indoor environments represent a mixture of species (reviewed in Adams *et al.*, 2013; Du *et al.*, 2021; Haleem Khan and Mohan Karuppayil, 2012). Under normal circumstances where obvious indoor fungal contamination is absent, the fungal propagules that predominate in indoor environments arise from three primary reservoirs: (1) the phylloplane/phyllosphere (the ecology of the surface of leaves considered to be a habitat, especially for yeasts and filamentous fungi), (2) the pedosphere (the outermost layer of the earth that is composed of soil and subject to environmental processes), and (3) the dermatophane (skin surface) (Adams *et al.*, 2013).

Phylloplane fungi comprise an ecologically similar grouping of unrelated, dry-spored fungal taxa that inhabit plant leaf surfaces (Levetin and Dorsey, 2006). In northern regions, phylloplane taxa predominate in the aerospora during the growing season. Owing to their abundance in the outdoor air, aerosols derived from these fungi, including spores, hyphal fragments and cellular debris, filter into indoor environments through normal activity and openings in the building envelop, primarily windows and ventilation systems. Characteristic taxa in this category include certain species of *Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, rust and smuts, agarics, and brackets (Basidiomycetes).

Fungi originating from soil reservoirs also commonly are found in indoor environments (Frac *et al.*, 2018). These mostly wet-spored fungi may enter buildings on footwear, particularly common where footwear is not removed upon entry into a building. Alternatively, soil containing fungal material may become aerosolized during building works or heavy excavation activities and these dust aerosols may be transported indoors on air currents or via human movements or actively through the actions of mechanical air handling systems. Taxa in this category include species of *Aspergillus*, *Acremonium*, *Fusarium*, *Trichoderma* and *Penicillium*. Demolition, gardening, sweeping and strong winds will also contribute to the indoor environment mycoflora. The release of fungal spores is most often seasonal. Molds (filamentous fungi) growing on plant surfaces show peak airborne concentrations seasonally in accordance with the life cycles of the plants. In northern, temperate climates, airborne levels of these molds decrease substantially during the winter.

Virtually all buildings contain molds, but some are moldier than others due to a variety of factors including water ingress, damp, wet cells and the annual hygrothermal performance of buildings (reviewed in Du *et al.*, 2021). Indoor molds can be remarkably tolerant of dry conditions but none can live without moisture. Excessively moldy buildings generally have a source of moisture leading to unusually heavy mold growth. The source of the moisture may be a damp or flooded basement, a dripping pipe, a roof in need of repair or some other fairly obvious cause. In most cases the mold can be seen growing on walls or other materials in contact with the moisture. Sometimes the moisture can occur inside walls and not be apparent (termed interstitial mold). A common but not obvious cause of moisture in cold climates is condensation. Severely moldy buildings may have a musty smell, but not necessarily. Sometimes the only sign of a problem is persistent poor health of the occupants, such as headaches, nausea, respiratory symptoms, for example, exacerbation of asthma.

Like other *Aspergillus* species, *A. fumigatus* has a worldwide distribution (Ashu *et al.*, 2017; Sewell *et al.*, 2019). This probably results from the production of numerous airborne conidia, which easily disperse by air movements and possibly by insects. The composition of the atmosphere has a great impact on mold growth, with humidity being the most important variable. *A. fumigatus* grows optimally with water activity (a_w) between 0.86 and 0.96. The optimum temperature for *A. fumigatus* to grow is 37°C, but fungal growth can be observed at temperatures ranging from 12°C to 48°C.

This review will focus primarily on where *Aspergillus* is found in built environments. It is present in air, and in dust found in infrequently-cleaned places, particularly attics and cellars, on potted ornamental plants and flowers, on shutters, carpets and in vacuum cleaners (reviewed in Mousavi *et al.*, 2016). This organism has also been identified in many food types, especially spices, tea, biscuits and fruits. Some of these reservoirs are not limited to community indoor environments and are also found in hospitals and workplaces. Important sources of *Aspergillus* in outdoor environments include soil, plant debris, rotten vegetation, manure, sawdust litter, bagasse litter, animal feed, bark chippings, on animals. Water may be an alternative source of *Aspergillus* conidia or where *Aspergillus* biofilms are formed in water distribution systems (reviewed in Richardson and Rautemaa-Richardson, 2019).

What is Aspergillosis?

Aspergillosis is seen worldwide (see "Relevant Websites section"). Manifestations include allergic aspergillosis (rhinosinusitis and bronchopulmonary, often complicating asthma and cystic fibrosis) (estimated number of cases >10 million), chronic pulmonary aspergillosis (~ 3 million), invasive aspergillosis (incidence $>300{,}000$ annually), and superficial disease (notably keratitis, otomycosis, and trauma or burn wound infections) (Kosmidis and Denning, 2015).

The genus *Aspergillus* includes over 300 species, divided into 22 sections (Gautier *et al.*, 2016). Around 20 species have so far been reported as causative agents of opportunistic infections in humans, although many cryptic species are now implicated, so the term complex is added to species names, unless definitive identification has been conducted. Among these, *Aspergillus fumigatus* is the most commonly isolated species, followed by *A. flavus* and *A. niger*. *A. nidulans*, *A. terreus*, *A. calidoustus*, and *A. versicolor* are among the other species less commonly isolated. In respiratory samples from patients with chronic and allergic forms of pulmonary aspergillosis, fifty-six (2.5%) isolates were identified as *Aspergillus* cryptic species by sequencing of ITS, BenA and CalM gene loci (Bongomin *et al.*, 2018). Common cryptic species were *Aspergillus montevidensis*, *A. sydowii* and *A. calidoustus* It is important to identify these species because of the high frequency of antifungal drug-resistance.

Is Aspergillosis Community Acquired or a Hospital-Associated Infection?

Differentiation between nosocomial and community aspergillosis is important for investigation, exposure pathways and prevention, and to understand whether the indoor environment is a source of exposure (reviewed in Nicolle *et al.*, 2011). Nosocomial (hospital-acquired) infections require appropriate infection control measures to avert additional cases. Nosocomial invasive aspergillosis occurs during hospitalization or is caused by *Aspergillus* acquired during hospital stay. Such infections should not be evident when patients are admitted to the hospital. Furthermore, the definition of nosocomial aspergillosis is based on epidemiological criteria, such as the time lapse between admission and onset, or microbiological criteria. This definition might be difficult to apply to invasive aspergillosis which often afflicts patients with severe immunosuppression or who have undergone solid organ transplantation. Identification of the source may be difficult which could either be outside or inside the hospital. Another significant issue is the lack of valid and reproducible data on the incubation period. The incubation duration of IA is influenced by different individual, environmental and mycological determinants, including the severity of immunosuppression (for example, neutropenia), the aggressiveness of the *Aspergillus* strain and air quality. A better understanding of early events related to invasive aspergillosis onset will help to prevent this disease for which the prognosis still remains very poor.

Investigation of Indoor Biotic Fungal Communities

The investigation of indoor microbial ecology has been based primarily on culture, but more recently it has been facilitated by nucleic acid sequence-based methods, (Adams *et al.*, 2013; Vesper *et al.*, 2007). Together, these approaches have identified fungi that grow indoors when moisture is available. From recent studies that have relied on nucleic acid amplification techniques to assess total fungal assemblages it is clear that fungi in settled dust are diverse, that variation between buildings can be large and fluctuate with the outdoor air, and that, as a result, there may not be a typical profile of indoor fungi that distinguishes different types of buildings (reviewed in Rintala *et al.*, 2012; Du *et al.*, 2021).

Ongoing research in Norway has used next generation sequencing to decipher the mycobiome of indoor environments (See Relevant Websites Section). The project has combined air sampling with high-throughput DNA sequencing (HTS) and qPCR. HTS is particularly useful in characterizing the microbial communities present in environmental samples, for example, dust and air from buildings. It was found that regional climate factors significantly affected both outdoor and indoor mycobiomes, owing to an ingress of spores from outdoor sources. Furthermore, features of the buildings studied and their occupants also influenced the indoor mycobiomes, in particular, the detection of *Penicillium*, *Aspergillus* and *Botrytis* found colonizing building materials. In contrast, outdoor mycobiomes were distinctly enriched in rock-inhabiting fungi, which include lichen-forming fungi (Lecanorales) and black fungi (Chaetothyriales and Capnodiales). The investigators anticipate that the project will have a relevant impact on related scientific areas (microbiology of the built environment, fungal ecology and human exposure), as well as on commercial activities related to building microbiology and material biodeterioration.

An extensive survey by Visagie and colleagues highlighted that xerophilic *Aspergillus* species (those species that thrive in dry environments and have a low water requirement) are one of the dominant genera of the indoor environment (Visagie *et al.*, 2017). A survey of xerophilic fungi isolated from Canadian and Hawaiian house dust resulted in the isolation of 1039 strains; 296 strains belonged to *Aspergillus* and represented 37 species. *Aspergillus* sect. *Aspergillus* (formerly called *Eurotium*) was one of the most predominant groups from house dust with nine species identified. Among all strains, two new species were found: *A. mallochii* and *A. megasporus*.

General Considerations of *Aspergillus* Growth and Proliferation

To understand whether the indoor environment is a conducive place for *Aspergillus* to grow it is important to review the growth requirements and ecological niches where *Aspergillus* has been found to understand exposure pathways.

Nutrients

Aspergillus assimilates preformed organic matter; carbohydrates being the preferred carbon source (Rhodes, 2006). *Aspergillus* can readily absorb and metabolize a variety of soluble carbohydrates, such as glucose, xylose, sucrose, and fructose. It is characteristically well suited to using insoluble carbohydrates such as starches, cellulose, and hemicelluloses, as well as very complex hydrocarbons such as lignin. Saprophytic fungi, such as *Aspergillus* obtain their food from dead organic material; parasitic fungi do so by feeding on living organisms (usually plants), thus causing disease.

Water

Most fungi require very high water availability and rapidly dry out, or senesce, under dry conditions (Andersen *et al.*, 2011). However, fungi are also able to tolerate much lower water availability than other organisms. The most xerophilic organism, *Xeromyces bisporus*, can grow at a water activity (a_w) of 0.62, while many other species such as *Eurotium*, *Aspergillus*, *Wallemia*, and *Penicillium* commonly contaminate low water activity environments (a_w 0.75–0.90). Xerophilic molds are also extremely common in indoor environments but are often neglected and not found if the detection method uses high water activity isolation media. Some species of *Aspergillus*, for example, *A. penicilloides*, is a halophyte (Nazareth and Gonsalves, 2014). This species requires a high salt-content culture medium to support its growth.

pH

For most fungi, a pH range of 5.5–6.5 seems to be suitable for their maximum growth and sporulation, but the hydrogen environment of fungi is difficult to study because they change the pH of their environment as they grow. A typical example is *A. niger*, which produces citric and other organic acids and thus lowers the pH of the substrate. Generally speaking, fungi can tolerate a wide range of pH.

Oxygen

Aspergillus has the capacity to function facultatively under aerobic and anaerobic conditions. Oxygen is used for oxidative metabolism, to generate energy. However, it is also essential for the biosynthesis of sterols, unsaturated fatty acids, and some vitamins.

Temperature

Aspergillus species can normally tolerate the range of temperature of the environment where they are found growing. However, active growth will usually be associated with a limited range of temperatures. There are different definitions of temperature requirements, but those fungi that grow between 15°C and 35°C are usually called mesophilic, and those growing above this range are termed thermophilic. Many *Aspergillus* species are thermotolerant. Temperature affects specific growth rate, and sporulation. High or low temperatures may cause the *Aspergillus* to enter dormancy.

Light

Light does not play a major part in the metabolism and growth of fungi. For the cultivation and sporulation of common species, light seems not to be a limiting factor. Most fungi, including *Aspergillus* species develop well in the dark in outdoor and indoor environments. UV radiation can reduce the viability of conidia, especially in air. It has been found that 600 mWs/cm^2 of UV at 260 nm could potentially be used for the inactivation of *Aspergillus niger*, *Penicillium citrinum*, and *Cladosporium cladosporioides* (Levetin *et al.*, 2001). The study demonstrated the effectiveness of UV is where levels of fungi growing on insulation within air-handling units (AHUs) in an office building and levels of airborne fungi within AHUs were measured before the use of germicidal UV light and again after 4 months of operation. The fungal levels following UV operation were significantly lower than the levels in control AHUs.

Where Does *Aspergillus* Proliferate?

Decaying vegetation is the primary ecological niche of *Aspergillus fumigatus* (Rhodes, 2006). The organism also lives in outdoor soil, and rural areas are a major source of contamination. Other reservoirs are compost heaps, hay barns and bird droppings and a variety of indoor biotic environments. Some of these ecological niches are examined further.

Soil

Aspergillus species appears to spend most of its life growing as a saprophyte in the soil, where it plays an important role as a nutrient recycler, supported by plant and animal debris. The ability of *Aspergillus* to survive in harsh conditions allows it to easily

out-compete other organisms for substrates in the soil or on plants. The fungus overwinters either as mycelium or as resistant structures known as sclerotia, for example, *A. flavus*. The sclerotia either germinate to produce additional hyphae or they produce conidia (asexual spores), which can be further dispersed in the soil and air.

Water

Fungi in drinking water may alter the taste and odors of the water. Health problems are possible, including mycotoxin exposure, direct infection and allergy. More studies are needed on this subject. Surveys of fungi in drinking water have recovered many different taxa, including *Aspergillus* (reviewed in Richardson and Rautemaa-Richardson, 2019). Contamination tends to arise from surface reservoirs and not from deep ground wells. This variation is often attributed to factors such as raw water source (surface versus well), water temperature patterns, treatment patterns and maintenance of distribution systems. Additionally, it was reported that fungi can pass through treatment processes by means of leaks in the system, or from air in contact with water stored in distribution system reservoirs, and can even survive water disinfection with chlorine.

Water-Damaged Building Materials

Andersen and colleagues estimated the qualitative and quantitative diversity of fungi growing on damp or water-damaged building materials (Andersen *et al.*, 2011). An additional aim of the study was to determine if associations existed between the most commonly found fungal species and different types of building fabric. More than 5300 surface samples were taken by means of V8 contact plates from materials with visible fungal growth. Fungal identifications and information on building material components were analyzed using multivariate statistic methods to determine associations between fungi and material components. The results confirmed that *Penicillium chrysogenum* and *Aspergillus versicolor* were the most common fungal species in water-damaged buildings. The results also showed *Chaetomium* spp., *Acremonium* spp., and *Ulocladium* spp. to be very common on damp building materials. Analyzes show that associated mycobiotas exist on different building materials. Interestingly, associations were found *between Aspergillus fumigatus, A. melleus, A. niger, A. ochraceus, Chaetomium* spp., *Mucor racemosus, Mucor spinosus*, and floor-related materials.

Colonization of Buildings and Building Fabric

Many manufactured products are susceptible to mold attack. Painted walls, particularly in humid places such as showers, can become overgrown by certain fungi, notably species of *Phoma* and *Exophiala*. Wallpapers also serve as a source of nutrition for some molds. Commonly cited are *Scopulariopsis* species which have been reported to grow on wallpapers containing arsenic pigments and to release very poisonous gases. With modern wallpaper pigments, which contain no arsenic, this cannot happen.

Modern heating systems are designed to remove humidity from buildings, creating opportunities for xerophiles to dominate indoor fungal communities. Due to their xerophilic physiology species of section *Aspergillus* makes them significant for the built environment. Here, these species are among the primary colonizers of building materials (Visagie *et al.*, 2017). Also of concern is the growth of these fungi in museums or libraries on historic artefacts such as books, carpets or paintings (reviewed in Visagie *et al.*, 2017). They also commonly grow on/in leather, dust, softwood, a variety of textiles and even dried specimens in herbaria.

Haleem Khan and colleagues answered this question within the context of the spectrum of molds found colonizing a wide range of damp building materials (reviewed in Haleem Khan and Mohan Karuppayil, 2012). A key feature of many of the studies is an understanding that fungi are able to grow on almost all natural and synthetic materials, especially if they are hygroscopic or wet. Inorganic materials get frequently colonized as they absorb dust and serve as good growth substrates for *Aspergillus fumigatus* and *Aspergillus versicolor*. Wood is highly vulnerable to fungal attack. *Cladosporium* and *Penicillium* (*P. brevicompactum* and *P. expansum*) colonize wooden building materials. Kiln dried wood surfaces are more susceptible to fungi. Acylated wooden furniture, wood polyethylene composites, plywood and modified wood products are susceptible to colonization by *Aspergillus*, *Trichoderma* and *Penicillium*.

Inner wall materials used in buildings, such as prefabricated gypsum board, highly favors the growth of *Stachybotrys chartarum* especially when it becomes damp due to it be hygroscopic but interestingly, *Aspergillus fumigatus* does not appear to be a primary colonizer of gypsum board. Paper and glue used in indoor surfaces are very good growth substrates for most of the indoor fungi. Fiber glass insulation and ceiling tiles support the growth of a number of fungi, including *A. versicolor*. *Aspergillus* and *Penicillium* grow superficially on painted surfaces.

Is Dust a Biotic Environment That Supports the Survival of *Aspergillus?*

House dust is a complex mixture of inorganic and organic material that supports a diverse population of fungal species (reviewed in Rintala *et al.*, 2012). Filamentous molds are able to grow and proliferate in dust only if there is enough moisture present, which in turn is dependent on the relative humidity of the indoor environment. Most of the fungal population originates from sources other than the dust itself, these being outdoor air and other outdoor material, such as vegetation or dirt brought into buildings, occupants of the buildings themselves, including pets and fungal growth on moist building materials. Based on numerous culture

and nonculture based studies, *Penicillium*, *Aspergillus*, *Cladosporium* are the most commonly found in dust depending on the dust type, detection method, type of the indoor environment and season, among other factors. Fungal assemblages in different house dust types usually share the same dominant species; however, alterations in the composition are caused by differing sources of microbes for different dust types. For example, mattress or pillow dust is dominated by species originating from the user of the mattress (Woodcock *et al.*, 2006). Further studies have explored the occurrence of fungi in furniture stuffings and upholstery as well as house dust in relation to asthmatic reactions and allergic sensitization (reviewed in Rintala *et al.*, 2012). The house dust components of fungal origin may include intact fungal conidia, spores, and spore clumps, as well as fragments of spores and hyphae. The size and shape of intact fungal spores varies from tiny, round to ovoid 2–5 μm conidia of *Aspergillus* and *Penicillium*, to large, oblong 50 μm conidia of *Alternaria* and *Helminthosporium*. Fungal fragments vary in size from submicrometer (<1 μm) particles to larger hyphal fragments. Fungal species capable of proliferating in house in the absence of moisture include extreme xerophiles: for example, *Eurotium repens*, *Aspergillus penicilloides* and mixed *Penicillium* species (reviewed in Rintala *et al.*, 2012). Of importance in mechanically ventilated indoor environments is that air filters and ventilation ducts are also colonized by fungi (Noris *et al.*, 2011).

Aspergillus Spore Dispersal in Indoor Air

What are the processes that influence the dispersal and selection regimes of molds in the indoor space? First, it is clear that the majority of indoor air fungi are derived from outdoor air. Second, the variation in fungal communities between houses, for example, is large and partly attributable to selection pressures imposed by resident behavior. A third finding is that airborne fungal communities vary among rooms within a building based on room use. Furthermore, it is expected that cleaning and cooking patterns and exposure to the outdoors will have a predictable effect on the composition of the indoor fungal communities and that the air in rooms with water sources (bathrooms and kitchens) would have a higher burden of resident fungi.

Numerous studies have determined the pattern of fungal diversity and composition in indoor air in specific in indoor environments in order to identify processes supporting the array of species. A number of surveys have looked at airborne fungal profiles over seasons of the year seasons, both indoors and outdoors, within and between houses. Fungal communities indoors were diverse and strongly determined by dispersal from outdoors, and no fungal species were found as indicators of indoor air. There was a seasonal effect on the fungi found in both indoor and outdoor air, and quantitatively more fungal biomass was detected outdoors than indoors. Interestingly, room and occupant behavior had no detectable effect on the fungi found in indoor air. These surveys confirm previous reports that outdoor air fungi dominate the spectrum of indoor air. A study reported by the author and colleagues demonstrated that there were only marginal differences in the profile of indoor molds in British homes (Vesper *et al.*, 2005). Analysis of 26 mold indicator species revealed that the homes fell into two clusters, tentatively identified as "atypical" and "typical" mold conditions.

Starting from the premise that the indoor mycobiome is thought to comprise of spore dispersal from the outdoor mycobiome and the occupants' mycobiome combined with selective pressures imposed by the occupants' behaviors and the building itself Adams *et al.* determined the pattern of fungal diversity and composition in indoor air within 1-month time periods during two seasons in a university housing facility (Adams *et al.*, 2013). Fungal assemblages indoors were diverse and strongly determined by dispersal from outdoors. No fungal taxa were found as indicators of indoor air and indoor environments. There was a seasonal effect on the fungi found in both indoor and outdoor air, and quantitatively more fungal biomass was detected outdoors than indoors. A strong signal of isolation by distance (IBD, differences between populations at opposite ends of a more or less continuous group of populations) existed in both outdoor and indoor airborne fungal assemblages, despite the small geographic scale in which this study was undertaken (approximately 500 m). Moreover, room and occupant behavior had no detectable effect on the fungi found in indoor air. These results show that at the local level, outdoor air fungi dominate the patterning of indoor air. *Aspergillus* spp. were found in 48% of indoor air samples and in 65% of outdoor samples.

Numbers of Inhaled Conidia

Several assumptions have to be made when discussing the inhalation of *Aspergillus* conidia in indoor environments (reviewed in McCluskey, 2020). Firstly, airborne concentrations of *A. fumigatus* are greater outdoors than they are indoors, provided that the building fabric does not support the growth of the fungus and the environmental conditions are not conducive to spores dispersal. Secondly, the typical concentration of viable *A. fumigatus* spores encountered are outdoors has been estimated to be <10 CFU/m^3. Thirdly, the concentrations of *A. fumigatus* spores may vary significantly indoors based on the characteristics and maintenance of the occupied building, including the level of settled dust. Fourthly, the average person is a healthy individual, breathing comfortably with a tidal volume of 500 ml. Lastly, *A. fumigatus* spores are evenly distributed throughout the air and remain so in the lungs during breathing. Tidal volume is the term used to describe the normal volume of air a person inhales and exhales in a single breath with no forced effort. Therefore, it is being presumed that the with each breath, 500 ml of air is entering the lungs. Further, the normal range for breaths per minute in a healthy adult is considered between 12 and 20 respirations per minute. Thus, a healthy person may take 720–1200 respirations per hour or 17,280–28,800 per day. This translates into a healthy person inhaling and out 8.64–14.4 m3 of air per day. With a conservative assumption that the concentration of *A. fumigatus* spores in the air is nine

CFU/m^3, a healthy person might inhale up to 78–130 viable *A. fumigatus* spores per 24-h period. Naturally, there are a number of limitations of attempting to calculate such a number. For instance, some of the assumptions made ignore known characteristics or *A. fumigatus* spores, such as the day to day variability, and possibly even the variability between different areas of the same geographic location. Additionally, there is a lack of data to what is considered normal, or even generally safe, airborne viable spore counts. Further, there is evidence to suggest discordance between *A. fumigatus* spore levels and outbreaks of aspergillosis (reviewed in McCluskey, 2020). Finally, while the calculation here suggests a relatively low number of 78–130 viable *A. fumigatus* spores are inhaled per day, others have estimated that this number could be as high as 200 (reviewed in Dagenais and Keller, 2009). Notably though, these estimates are suggested in countries were typical concentrations of airborne *A. fumigatus* are greater than <10 CFU/m^3.

In continental temperate climates *A. fumigatus* spore counts in outdoor environments are generally <10 CFU/m^3 with little evidence of seasonal variation, for example in northern Europe (Alshareef and Robson, 2014). In other parts of the world, seasonal variation may explain reports suggesting that *A. fumigatus* spore counts are higher in summer months. Some surveys have suggested that higher concentrations of *A. fumigatus* spores are found in winter (reviewed in McCluskey, 2020). Over a two-year period including a 14-month period of renovation work in a Tunisian hospital, *Aspergillus* spp. were isolated in 59.7% of 494 air samples and in 52.8% of 1579 surface samples taken in various patient rooms (Haleem Khan and Mohan Karuppayil, 2012). *Aspergillus* section *Nigri* (72.7%) was the most frequent. *Aspergillus* contamination peaked in autumn and winter on surface and in summer and autumn in air samples and was higher during the renovation work period. In Tunisia, *Aspergillus* section *Nigri* and *Flavi*, but not *Fumigati*, are chiefly involved in invasive aspergillosis.

Diversity of Molds in Social Housing

A study in the US used molecular diagnostic tools to evaluate fungal communities on surfaces and in airborne dust in multiple units of a social housing complex (Sylvain *et al.*, 2019). The investigators surveyed 21 flats and characterized the apartments as either a unit with visible mold or no visible mold. They sampled airborne fungi from dust settled over a month-long time period from the outdoors, in units with no visible mold, and units with visible mold. In the units with visible mold the investigators additionally sampled visible fungal colonies from bathrooms, kitchens, bedrooms, and living rooms. They found that fungal biomass in settled dust was greater outdoors compared to indoors, but there was no significant difference of fungal biomass in units with visible mold and no visible mold. Interestingly, they found that fungal diversity was reduced in units with visible mold compared to units with no visible mold and the outdoors. Units with visible mold harbored fungal communities distinct from units with no visible mold and the outdoors. Colonies of fungi collected from units with visible mold were dominated by two allergenic species, *Cladosporium sphaerospermum* and *Cladosporium halotolerans*. *Aspergillus fumigatus* was not found in any of the indoor samples.

Does the Presence of *Aspergillus* in Home Environments Present a Risk Factor for Invasive Aspergillosis?

In contrast to what is known about exposure to *Aspergillus* in hospitals, little is known about the impact of indoor fungal exposure of hematology patients in their home environment. Rocchi and colleagues investigated the mold exposure of hematology patients both at home and at hospital to assess their risk of acquiring invasive aspergillosis (Rocchi *et al.*, 2014). Fungal exposure was assessed by quantifying opportunistic molds at hospital during hospitalization and in homes of 53 hematology patients. Invasive aspergillosis was diagnosed in 13 of 53 patients and invasive fungal infection in one patient. In hospital, no opportunistic species, or low levels of opportunistic species, were found in 98% of weekly samples. Only 2% of hematology intensive care unit samples showed a high level of *Aspergillus fumigatus* spores in corridor air. Five patients with invasive aspergillosis were hospitalized during these periods. Seven of the 53 homes (5/14 homes of patients with invasive aspergillosis or invasive fungal infection and 2/39 homes of patients without invasive aspergillosis) had a percentage of *A. fumigatus* and *A. flavus* to total mold as high as 15%. The study reinforces the dogma that minimizing exposure to *Aspergillus* in hospital is essential, and that continuous surveillance in the patients home could help to further reduce the risk of acquiring invasive aspergillosis. Also, advice should be given such as avoiding activity in highly contaminated rooms (for example, a cellar, attic, garage), cleaning rooms where there is visible mold, installing air purifiers, investigating areas of dampness, delaying discharge from hospital, or introducing health monitoring before patients return home if a high risk of fungal infection is suspected.

In marked contrast a survey carried out by Schweer and colleagues determined that there was no correlation between domestic ambient-air spore counts and invasive aspergillosis (Schweer *et al.*, 2016). By air sampling the authors found no correlation between airborne *Aspergillus* spores in patients' homes and development of probable or proven invasive aspergillosis. The authors emphasize that attempts to show a correlation between the presence or level of *Aspergillus* spores in both home and hospital environments and the incidence of invasive aspergillosis have failed repetitively in different study set-ups (reviewed in Schweer *et al.*, 2016). Correlation between spore counts and invasive aspergillosis in the domestic setting is generally more difficult to interpret since the precise time spent in the surroundings by a patient is significantly more variable than in the hospital. Further inference is that the infectious inoculum size and exposure time-frame for developing invasive aspergillosis are unknown.

Aspergillus Mycotoxins in Indoor Environments

Mycotoxin-producing *Aspergilli* (*Circumdati*, *Flavi*, and *Nigri*) may cause respiratory disorders. To explore this further Jakšić *et al.* analyzed air and dust samples from flooded indoor environments (Jakšić *et al.*, 2020). Eleven different species were identified based on their calmodulin marker: *A. ochraceus*, *A. ostianus*, *A. pallidofulvus*, *A. sclerotiorum*, and *A. westerdijkiae* (*Circumdati*); *A. flavus* (*Flavi*); and *A. tubingensis*, *A. welwitschiae*, *A. niger*, *A. piperis*, and *A. uvarum* (*Nigri*). *A. flavus* dominated among airborne isolates (25%) while 56% of the dust borne Aspergilli were identified as *A. tubingensis* and *A. welwitschiae*. The ability of identified isolates to produce mycotoxins aflatoxin B1 (AFB1), fumonisin B2 (FB2), and ochratoxin A were assessed by LC-MS analysis. All ochratoxin A (OTA)-producing *Circumdati* belonged to *A. westerdijkiae*. In the section, *Flavi A. flavus* produced AFB1 while *A. welwitschiae* and *A. niger* (section *Nigri*) produced FB2. The major conclusion from this study was that water damage substrates supported the growth of aflatoxigenic *A. flavus* in indoor environments. However, the causal relationship of exposure to the mycotoxins produced by these Aspergilli with adverse health effects is yet to be determined.

Aspergillus and Hospital Environments

This review has focused on domestic indoor environments. However it is deemed useful to summarize where *Aspergillus* is found in hospital and office environments. In addition to those environmental niches discussed above additional reservoirs of *Aspergillus* growth have been identified in ceiling voids, roller-blind casings, fire proofing material, ventilation ducting and disfunctional air handling units (Gheith *et al.*, 2015). Active growth in these spaces and on these materials will result in spore formation and dispersal. In hospitals *Aspergillus* specifically has been found contaminating such adhesive tapes, arm bands, plaster. Shower heads within which *Aspergillus* forms biofilms are recognized a risk factor for vulnerable patients in hospitals.

Is the Presence of *Aspergillus Fumigatus* in Indoor Environments Reflected in the Mycobiome of the Respiratory Tract?

Colonization of indoor environments by *Aspergillus* may become significant, and eventually result in *Aspergillus*-related lung disease when the microbiological composition of the normal flora in the respiratory tract is disturbed, such as in the case of an underlying disease, an altered immunological status of the host, or a decrease in ciliary activity of the mucous pulmonary epithelium. The underlying diseases that predispose to aspergillosis could thus provide significant insights into which alterations in the respiratory tract microbiome might predispose to aspergillosis. The fungi that reside in the human lungs represent an understudied but medically relevant community. This thesis has been reviewed by Richardson *et al.* (2019). From the few studies about the lung mycobiome, it is clear that there are fungi in both the healthy and diseased respiratory tract, that these fungi vary widely between individuals, and that there is a trend toward lower fungal diversity among individuals with chronic or progressive respiratory disease. Another dimension to the above discussion is the persistence or clearance of fungal conidia and hyphal fragments in healthy or debilitated individuals from the various levels of the respiratory tract. The primary route of human infection is via the inhalation of these airborne conidia, followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, conidia that are not removed by mucociliary clearance encounter epithelial cells or alveolar macrophages, the primary resident phagocytes of the lung. Alveolar macrophages are primarily responsible for the phagocytosis and killing of *Aspergillus* conidia as well as the initiation of a proinflammatory response that recruits neutrophils to the site of infection. Conidia that evade macrophage killing and germinate become the target of infiltrating neutrophils that are able to destroy hyphae. The risk of developing invasive aspergillosis results primarily from a dysfunction in these host defenses in combination with virulence attributes that permit *A. fumigatus* survival and growth in this pulmonary environment. It is patently clear from the surveys reviewed that healthy individuals and patients are exposed to a diverse collection of fungal taxa and species in outdoor and indoor environments. As we have seen, *A. fumigatus* is able to grow and sporulate on a variety of organic substrates in outdoor indoor niches and release conidia into the air. Mycobiome studies of air and dust demonstrate this very clearly. The appearance of *Aspergillus* in indoor locations is most likely as a result of migration of conidia from outside considering that there are few substrates and microenvironments to support the growth of *Aspergillus* in well-maintained homes. The interesting question is whether the lung mycobiome reflects the diversity of fungi in outdoor and indoor environments. Richardson *et al.* suggest that based on their review of the lung mycobiome, that the answer is no (Richardson *et al.*, 2019). *Aspergillus* species are under-represented in many respiratory tract mycobiome analyzes.

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Fungal Exposure in Agricultural Environments – A Review

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Introduction

In agricultural environments it's possible to find fungi in many stages of their development. The presence of these microorganisms poses as a threat to the final quality of the products, to its workers, and most of the times causing significant losses on the annual crops harvest (Korbas, 2004; Weber, 2007; Suchorzyńska and Misiewicz, 2009). Fungi can be easily found in this kind of environments due to the good growth conditions that are provided by the raw materials present in this environment and for its storage temperatures (Soroka *et al.*, 2008).

The fungal variety present in agricultural matrices are influenced by several environmental factors, such as the type of crop and cultivation, the local climate and the application of fertilizers and pesticides (Girsch and Weinhappel, 2004; Szwajkowska-Michalek *et al.*, 2010).

Although the normal presence of fungi in the environment, the exposure is mostly through dust particles released from cereals and other agricultural products during handling and storage operations (Mbareche *et al.*, 2019). This kind of particles represents a real hazard regarding the pathogenesis of respiratory tract diseases in farmers (Nardell and Macher *et al.*, 1999). Fungi are among the particle's components (Jenerowicz *et al.*, 2012), and due to the daily activities, their suspension in the air is frequent, promoting workers´s exposure. Particles can be responsible for numerous health problems due to its ability to be airborne, thus classifying itself as a vector for infectious diseases (WHO, 1999; Roy and Milton, 2004; Yu *et al.*, 2004; Li *et al.*, 2007; Eames *et al.*, 2009).

The level of the impact on the workers' health from their exposure to bioaerosols will depend of 4 main factors: Size of the particles, chemical compositions, microbiological composition and its concentration on the environment (Żukiewicz-Sobczak *et al.*, 2012). Indeed, studies report that workers in agriculture environments are exposed to high levels of microbial components (Żukiewicz-Sobczak *et al.*, 2012; Menga *et al.*, 2016), being already reported symptoms related to cough, inflammation of the eyes and nose, asthma and others (Melbostad and Eduard, 2001; Eduard *et al.*, 2004; van der Walt *et al.*, 2010; Chowdary *et al.*, 2011; Schlünssen *et al.*, 2011; Żukiewicz-Sobczak, 2013a). In the specific case of exposure to fungi it was already reported as being related with several symptoms in the eyes and nose and to cough (Melbostad and Eduard, 2001) as well as to asthma and work-related respiratory symptoms (Eduard *et al.*, 2004; Schlünssen *et al.*, 2011).

Although the major concern when exposed to an environment is the fungal contamination, mycotoxins, produced by some fungal species, are also of concern, since can origin chronic and acute effects. Some of these mycotoxins also have carcinogenic and mutagenic characteristics, causing damage in internal organs, lowering the organism natural defences and accelerating the development of tumors (Maestrelli *et al.*, 2009; Frac *et al.*, 2015).

The more frequently reported fungi in agricultural settings are species from the genera *Aspergillus, Alternaria, Fusarium, Penicillium,* and *Cladosporium*. The *Alternaria alternata is* also a quite common species in agricultural environments (Saur *et al.*, 1984; Krysińska-Traczyk *et al.*, 2003; Maestrelli *et al.*, 2009; Żukiewicz-Sobczak *et al.*, 2012; Frac *et al.*, 2015; Kozak *et al.*, 2016).

The climate conditions are one of the most selective factors for the prevalence of species in this kind of environments. Indeed, species of the genera *Fusarium* prefer high levels of persisting humidity and temperatures above 20°C (Brennan *et al.*, 2003, 2005), while *Aspergillus* prefers higher temperatures in between 30°C and 35°C for optimal growth (Kozakiewicz and Smith, 1994; Lasram *et al.*, 2010; Shehu and Bello, 2011).

This systematic review aims to provide a broad overview of the state of art in the developed subject, indicating the most critical scenarios, among agriculture environments has the highest prevalence of fungal species and which methods were applied to assess the exposure to fungi on agricultural environments.

Materials and Methods

This study was done based on a systematic search of available information and data published in the public domain between the period of 1st January 2000 and 31st November 2019.

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Baseline search parameters were established to identify and select studies in agricultural environments accessing fungi and mycotoxins. Appropriate search terms were employed to the selected databases (PubMed, B-on, and Web of Science). All the searches on the databases were done in English, the strategy of research mainly focused on agricultural settings, on fungi and mycotoxins, selecting only English and original papers.

The final result of this search strategy resulted in 18 PubMed, B-on, and Web of Science papers, all the articles that did not meet the selected inclusion criteria were excluded (**Table 1**). The findings were compared for consistency and discrepancies were highlighted; a final selection of papers was fixed.

The diagram describes the different phases of the selection of papers (**Fig. 1**).

Table 1 Inclusion and exclusion criteria in the articles selected

Inclusion Criteria	*Exclusion Criteria*
Articles published in the English language;	Articles published in other languages
Articles published from 1st January 2000 to 31st November 2019	Articles published prior to 2000
Articles published in any country	
Articles related to fungi assessment in agricultural settings	Articles related exclusively to biologic samples from patients or without mention of fungi quantification.
Original scientific articles on the topic	Abstracts of congress, reports, reviews/state of the art articles

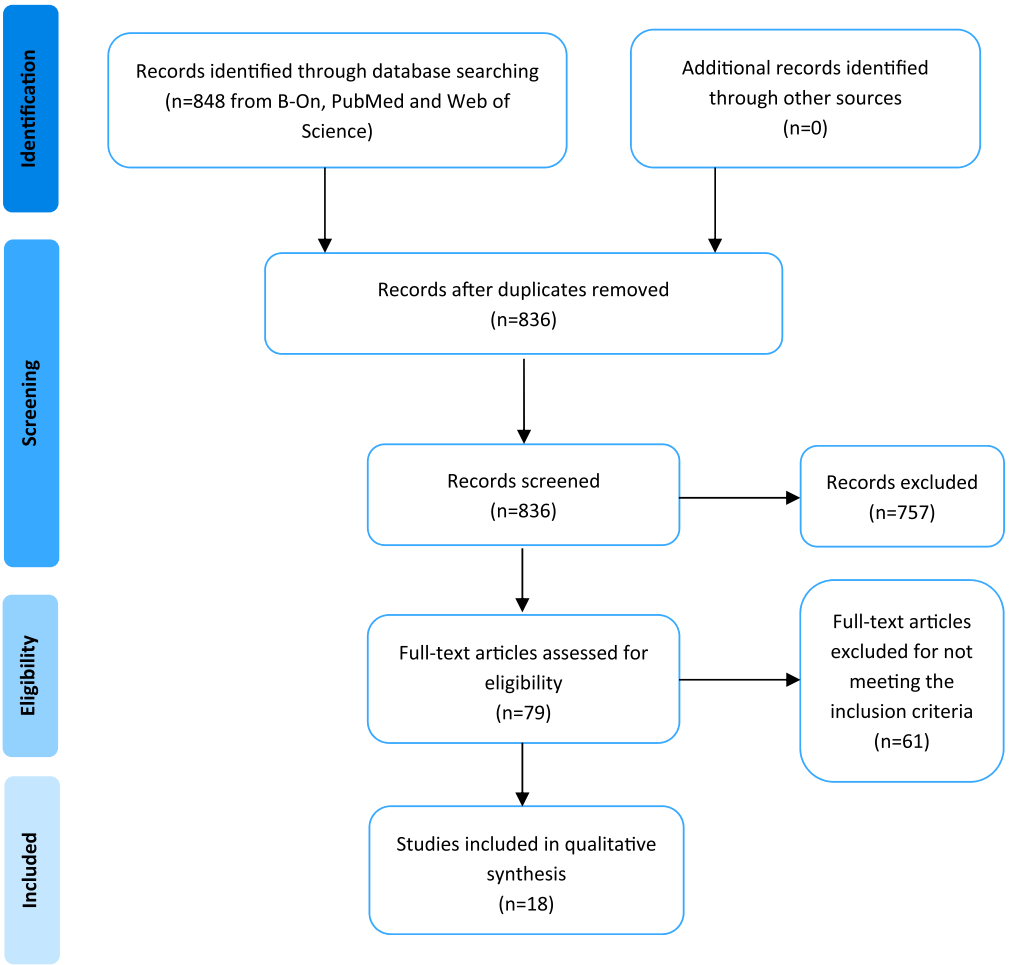

Fig. 1 PRISMA based selection of articles.

Results

A total of 18 articles were analyzed regarding agricultural environments (Crop handling, processing plants, and other agricultural activities), the sampling methods used, the analytical methods, how the fungal identification was performed and the main findings of each of the selected studies.

The majority of the studies analyzed were set on farms (10 out of 18), the others were developed on agricultural processing plants (5 out of 18) and in agricultural handling sites (3 out of 18). Three studies used organic and conventional farms for comparison purposes (Żukiewicz-Sobczak *et al.*, 2012, 2013b,c).

The most frequent sampling method used across all studies was air filtration methods, used in 8 of the 18 studies (Adhikari *et al.*, 2004; Góra *et al.*, 2004; Skórska *et al.*, 2005a,b; Lee *et al.*, 2006; Madsen, 2006; Halstensen *et al.*, 2007; Madsen *et al.*, 2012). The exclusive use of active sampling was the most common approach in the selected studies (10 out of 18 studies) followed by the use of passive methods (5 out of 18), while the combination of the two types of sampling was only stated in 3 studies (Krysinska-Traczyk *et al.*, 2004; Shenoi *et al.*, 2005; Madsen *et al.*, 2012). Among the passive methods used in the studies the most common was the direct sampling of grain or other agricultural products (Reboux *et al.*, 2002; Krysińska-Traczyk *et al.*, 2007; Madsen *et al.*, 2012; Żukiewicz-Sobczak *et al.*, 2012, 2013b) but was also used as passive methods the collection of settle dust (Góra *et al.*, 2004; Krysińska-Traczyk *et al.*, 2007; Żukiewicz-Sobczak *et al.*, 2012) and the collection of soil samples (Shenoi *et al.*, 2005; Żukiewicz-Sobczak *et al.*, 2013b).

Across all studies the only method of fungal identification used was the morphological identification obtained by culture-based methods. *Alternaria alternata* was the most identified species across all studies, found in 9 out of 18 studies (Dutkiewicz *et al.*, 2002; Góra *et al.*, 2004; Krysinska-Traczyk *et al.*, 2004; Skórska *et al.*, 2005a,b; Krysińska-Traczyk *et al.*, 2007; Żukiewicz-Sobczak *et al.*, 2013b,c; Zawiślak *et al.*, 2019), although the genera more commonly found was the *Aspergillus* genera (15 out of 18 studies) in which species like *Aspergillus fumigatus* (Dutkiewicz *et al.*, 2002; Krysinska-Traczyk *et al.*, 2004; Skórska *et al.*, 2005a,b; Madsen, 2006; Madsen *et al.*, 2012; Zawiślak *et al.*, 2019), *Aspergillus flavus* (Żukiewicz-Sobczak *et al.*, 2012), *Aspergillus versicolor* (Żukiewicz-Sobczak *et al.*, 2013b), *and Aspergillus ochraceus* (Krysińska-Traczyk *et al.*, 2007) were also found.

Less prevalent than the *Alternaria* and *Aspergillus* genera, was also found *Mucor* sp. (7 out of 18 studies), *Fusarium* sp. (6 out of 18 studies) and *Candida* sp. (4 out of 18 studies) (**Table 2**).

Discussion

Alternaria, *Aspergillus*, *Mucor*, and *Fusarium* were the most common genera found across all the papers. Among these some genera raise a higher concern than others, such as the *Aspergillus* and *Mucor* genera. It was already reported high prevalence of both genera in indoor and occupational environments (Viegas *et al.*, 2017; Caetano *et al.*, 2019) leading to health hazards and the rise of cases of *Aspergillus* and *Mucor* infections (Kontoyiannis *et al.*, 2005; Bitar *et al.*, 2009; Auberger *et al.*, 2012).

Aspergillus is a very common filamentous fungal genus that can be found in many different environments, usually they are classified as saprophytes, but in some cases, they can also be pathogenic and cause harm to humans (Heitman, 2011; Seyedmousavi *et al.*, 2015). This genus is classified as an opportunistic pathogen that may cause a wide range of respiratory disorders (Hope *et al.*, 2005). The conidia are very persistent in the environment and can stay viable for several months. Additionally they are well suited for airborne dispersion (Pringle *et al.* 2005). Furthermore, the presence of *Aspergillus* should not be neglected due to some of its sections being able of producing toxic compounds (Varga *et al.*, 2015; Viegas *et al.*, 2018b) and also due to the increasing occurrence of antifungal resistance in clinic and environmental settings (Fairlamb *et al.*, 2016; Nature Microbiology, 2017).

Mucorales order is known to be prevalent in indoor and occupational environments (Caetano *et al.*, 2017, 2018a; Viegas *et al.*, 2018a), and it includes a variety of saprophyte species that may cause serious infections, as the mucormycosis (Caetano *et al.*, 2018b), or other serious health problems (Lavergne *et al.*, 2017). As mentioned Mucorales cases are on the rise and the increase of azole resistant strains. Their cross-resistance characteristics show a more critical scenario regarding the therapeutics and management of fungal infections (Leathers and Sypherd, 1985; Cuenca-Estrella, 2014). This problem is perhaps result of the drawback of exposure to antifungal drugs or other related chemicals that may lead to a review of the current therapy for these cases due to potential treatment failures (Nature Microbiology, 2017). Moreover, *Mucor* genera in Europe is one of the most identified fungi (Kwon-Chung, 2012; Lanternier *et al.*, 2012).

The level of exposure to fungi may be increased when present in certain settings where the levels of organic dust generated is high. The particles act as carriers of the microorganisms leading to the dispersion of the fungi being carried (Caetano *et al.*, 2018a; Viegas *et al.*, 2020). Scenarios such as this, where exposure to organic dust is high, may be found in agricultural environments (Nardell and Macher *et al.*, 1999; Jenerowicz *et al.*, 2012; Mbareche *et al.*, 2019).

Among the sampling methods employed in all the studies observed, the exclusive use of active methods were the most common approach (10 out of 18 studies) and with 5 out of 18 studies using exclusively passive methods, while the combined use of both methods were only stated in 3 out of 18 studies. The usage of passive and active sampling has some advantages and disadvantages. The passive methods are considered to be a very versatile technique to detect the microbial contamination in various settings, allowing a better understanding of the contamination present and an improved characterization of indoor environments (Viegas *et al.*, 2014, 2016a; Caetano *et al.*, 2017). Active sampling can provide information about the load in air at

Table 2 Data obtained from the chosen articles

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Analytical Methods*	*Results*	*Main findings*	*References*
PubMed	Exposure to airborne microorganisms, dust and endotoxin during flax scutching on farms	Poland	Five flax farms	Custom particle-sizing slit impaction sampler	Morphological identification	*Alternaria alternata, Aspergillus fumigatus, Candida* spp. *Fusarium* spp. *Mucor* spp. *Penicillium* spp.	Flax scutching represent high exposure concentrations to airborne organisms, adding to this the presence of allergenic and immunotoxic species were stated.	Krysinska-Traczyk *et al.* (2004)
	Exposure of hop growers to bioaerosols	Poland	Nineteen Hop farms	Air filtration, settle dust collection	Morphological identification	*Alternaria alternata, Alternaria humicola, Aspergilus candidus, Cladosporium elegantum, Cladosporium epiphyllum, Mucor mucedo, Mucor Racmosus, Penicillium citrinum, Penicillium* spp. *Trichoderma viride*	The exposure levels of hop growers are much lower than in other crops, although allergen species present still represent a potential hazard.	Góra *et al.* (2004)
	Exposure to Airborne Microorganisms, Dust and Endotoxin During Processing of Peppermint and Chamomile Herbs on Farms	Poland	Thirteen Herb Farms (peppermint and chamomile)	Personal and stationary air filtration	Morphological identification	*Alternaria alternata, Aspergillus fumigatus, Candida* spp. *Cladosporium cladosporioides, Cladosporium herbarum, Fusarium* spp. *Geotrichum cardinum, Oidiodendron* spp. *Penicillium* spp. *Rhodotorula rubra*	These farmers are exposed to large concentration of airborne microorganisms. At 50% of the sites sampled fungi were the dominant airborne microorganisms, where allergenic species were commonly found.	Skórska *et al.* (2005a)
	Size-selective assessment of agricultural workers' personal exposure to airborne fungi and fungal fragments	Taiwan	One corn farm and one mushroom farm	Personal Cyclone air sampler (impinger)	Morphological identification	*Aspergillus* spp. *Penicillium* spp. *Alternaria* spp. *Botrytis* spp. *Cercospora* spp. *Cladosporium* spp.	*Aspergillus, Penicillium and Cladosporium* represented 15%–99% of fungal concentration. *Alternaria* and *Botrytis* produces small metabolites (<1 μm) representing risk to human health.	Lee *et al.* (2011)
	Rye grains and the soil derived from under the organic and conventional rye crops as a potential source of biological agents causing respiratory diseases in farmers	Poland	Two organic farms and two conventional farms	Collection of rye grain and soil samples	Morphological identification	Organic Farms: *Alternaria alternata, Aureobasidium pullulans, Paecilomyces variotii, Penicillium citreo-viride, Penicillium glabrum, Penicillium lilacinum* Conventional farms: *Alternaria alternata, Aureobasidium pullulans, Cladosporium herbarium, Cladosporium oxysporum, Aspergillus versicolor, Penicillium citreo-viride, Penicillium funiculosum, Penicillium glabrum, Penicillium lilacium*	Significant exposure was verified due to hazardous agents present in grain and soil. Allergenic and toxigenic molds were also found.	Żukiewicz-Sobczak *et al.* (2013b)
Web of Science	Exposure to airborne microorganisms and endotoxins in a potato processing plant	Poland	Potato processing plant	Custom particle-sizing slit impaction sampler	Morphological identification	*Alternaria alternata, Aspergillus fumigatus, Aspergillus niger, Candida* spp. *Geotrichum candidum, Mucor* spp. *Rhizopus nigricans*	Potato processing plants represent high exposure concentrations to airborne microorganisms, mainly when handling dried potatoes.	Dutkiewicz *et al.* (2002)

(*Continued*)

Table 2 Continued

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Analytical Methods*	*Results*	*Main findings*	*References*
	Assessment of human exposure to airborne fungi in agricultural confinements: Personal inhalable sampling *Versus* Stationary sampling	USA	Grain handling site	Personal and stationary air filtration	Morphological identification	Stationary Samplers: *Aspergillus* spp. *Penicillium* spp. *Cladosporium* spp. *Alternaria* spp. Personal Samplers: *Aspergillus* spp. *Penicillium* spp. *Cladosporium* spp. *Alternaria* spp.	Potential hazardous fungi were found, but low variety of flora were verified. The results in the two methods were almost similar revealing that the sensitivity of button sampling is low in confined environments.	Adhikari *et al.* (2004)
	Exposure to Airborne Microorganisms, Dust and Endotoxin during Processing of Valerian Roots on Farms	Poland	Fifteen Herb farms (valerian root)	Personal and stationary air filtration	Morphological identification	*Alternaria alternata, Aspergillus candidus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus niger, Aspergillus terreus, Candida* spp. *Cylindrocarpon* spp., *Fusarium* spp. *Humicola* spp., *Monilia* spp. *Mucor mucedo, Mucor* spp. *Oidiodendron flavum, Penicillium* spp. *Rhizopus nigricans, Rhodotorula rubra, Trichoderma album, Trichothecium roseum*	Only after the drying of roots is verified the prevalence of *Aspergillus fumigatus*, this being one of the main hazards found due to the health risks posed to the respiratory tract.	Skórska *et al.* (2005b)
	Exposure to airborne microbial components in autumn and spring during work at Danish biofuel plants	Denmark	Five biofuel plants	Personal air filtration	Morphological identification	Total fungi (range from 1.1–130 $\times$ 10^4 cfu/m^3) Mesophilic fungi (range from 0.043–20 $\times$ 10^4 cfu/m^3) *Aspergillus fumigatus* (range from 0–2.48 $\times$ 10^4 cfu/m^3)	High exposure capable of causing health disorders were found. *A. fumigatus* were most found in wood chips.	Madsen (2006)
	Levels of fungi and mycotoxins in the samples of grain and grain dust collected from five various cereal crops in Eastern Poland	Poland	Twenty-four private farms	Collection of grain and grain dust samples	Morphological identification	*Alternaria alternata, Aspergillus ochraceus,* *Aspergillus niger, Cladosporium elegantulum, Cladosporium herbarum, Cladosporium lignicola, Geotrichum candidum,* *Monilia sitophila, Monosporium silvaticum, Mucor mucedo, Oidiodendron griseum, Oidiodendron fl avum,* *Penicillium* spp. *Prophytroma tubularis, Rhizopus artocarpi,* *Rhizopus nigricans, Rhodotorula rubra, Trichoderma Álbum*	Although the concentrations found were low, the prevalence of these fungi were almost constant (90%), leading to high bioavailability of mycotoxins through inhalation, causing possible chronic effects.	Krysińska-Traczyk *et al.* (2007)
	Determinants of microbial exposure in grain farming	Norway	Eleven grain farms	Personal and stationary air filtration	Morphological identification	Fungal spores (10^6/m^3) range:5200 Actinomycetes (10^6/m^3) range:3000 Hyphae (10^6 AU/m^3) range:200	Despite farmers practices to prevent fungal growth, were verified high exposure levels. Visible fungal damage were the main determinants of exposure to inhalable dust and its microbial components.	Halstensen *et al.* (2007)

	Organic dust toxic syndrome at a grass seed plant caused by exposure to high concentrations of bioaerosols	Denmark	Grass seed plant	Collection of grass seed samples, personal air filtration	Morphological identification	Mesophilic fungi (range from 6.9×10^5–7.95×10^6 cfu/m^3) *Aspergillus fumigatus* (range from 3.20×10^5–6.79×10^6 cfu/m^3)	It was found *A. fumigatus* to be released in very high quantities from the problematic seeds. All direct handling of the problematic seeds seemed to imply a serious exposure even after cleaning.	Madsen *et al.* (2012)
	Pathogenic fungi in the work environment of organic and conventional farms	Poland	Organic and conventional farms	Collection of wheat and wheat dust samples	Morphological identification	Organic Farms: *Aspergillus flavus, Cladosporium* spp. *Cladosporium macrocarpum, Fusarium* spp. Conventional Farms: *Cladosporium* spp. *Fusarium* spp., Gonatobotrys spp. *Ulocladium* spp.	Concentrations found in ecological farms were lower. The isolates show a direct relation between metabolites and health effects reported in farmers.	Żukiewicz-Sobczak *et al.* (2012)
B-On	Influence of buffered propionic acid on the development of micro-organisms in hay	France	Seventeen hay farms	Probe sampling of hay	Morphological identification	*Eurotium amstelodami, Alternaria* spp. *Mucorales* spp.	The use of BPA reduced greatly (40%–65%) the prevalence of fungal species present in hay.	Reboux *et al.* (2002)
	Dermatoses among paddy field workers – A descriptive, cross-sectional pilot study	India	Paddy field workers	Air and water sampling (culture collection), soil sampling	Morphological identification	*Aspergillus* spp. *Penicillium* spp. *Fusarium* spp. *Curvularia* spp. *Mucor* spp. *Synphelastrum* spp.	Working in paddy fields leads can traumatize the skin leading to lacerations, were microorganisms present in the grains can cause dermatitis.	Shenoi *et al.* (2005)
	Personal exposure to airborne dust and microorganisms in agricultural environments	USA	Three grain farms	Personal air filtration	Morphological identification	*Aspergillus* spp. *Penicillium* spp. *Cladosporium* spp. *Alternaria* spp, *Epicoccum* spp.	The exposure in the farms were mainly due to large particles with high fraction of fungal spores.	Lee *et al.* (2006)
	Grain dust originating from organic and conventional farming as a potential source of biological agents causing respiratory diseases in farmers	Poland	Organic and conventional farms	Settle dust collection	Morphological identification	Organic Farms: *Alternaria alternata, Aureobasidium pullulans, Cladosporium macrocarpum, Fusarium poae, Fusarium oxysporum* Conventional Farms: *Alternaria alternata, Cladosporium macrocarpum, Fusarium poae, Fusarium tricinctum*	Components of grain dust show potential risks containing toxigenic and allergenic molds. Most of Polish occupational diseases reported by farmers are caused by pathogens in bioaerosols.	Żukiewicz-Sobczak *et al.* (2013c)
	Microbiological analysis and concentration of organic dust in an herb processing plant	Poland	Herb processing plant	Impaction air sampler	Morphological identification	*Aspergillus fumigatus, Penicillium citriunum, Alternaria alternata*	The type of raw material processed at a processing line is an important factor. *A. fumigatus and P. citriunum* constituted the dominant flora.	Zawiślak *et al.* (2019)

the moment of sampling, since it only collects a portion of the air of the sampled site (Napoli *et al.*, 2012). Thus, the application of active methods coupled with passive methods is recommended, as this way it is possible to obtain more reliable data from long-term periods (Viegas *et al.*, 2017; Leppänen *et al.*, 2018). The integration of different kinds of methods allows the collection of a more refined and useful data without having to rely in a single method to represent a given scenario (Viegas *et al.*, 2017; Leppänen *et al.*, 2018).

Across all the studies that were analyzed it was stated a single analytical approach: morphological identification based on culture-based methods. This method it is considered a well-known and validated method for microbiological studies allowing to assess living and culturable microorganisms. It is easily recognizable and quantifiable the viable cells in a sample and has a high sensitivity when paired with the appropriate media (Figdor and Gulabivala, 2011). However, it has several drawbacks, such as the risk of external contamination, its time and resource intensive, it requires a certain level of skill to achieve optimal results and relies on the phenotypic biochemical characterization that may lead to favoring only some of the viable microorganisms (Jannasch and Jones, 1959; Amann *et al.*, 1995; Schmit and Lodge, 2005; Liu, 2011; Alsohaili and Bani-Hassan, 2018; Viegas *et al.*, 2019). The under-estimation is the major limitation of this method, since can dramatically underestimate the numbers and composition in the samples (Figdor and Gulabivala, 2011; Al-Awadhi *et al.*, 2013). Molecular tools can overcome some of the limitations, since provides a faster and more accurate description of the microbial communities present in each sample, compared to the culture-based methods (Alsohaili and Bani-Hassan, 2018). However, as the culture based-methods, molecular tools have also some restrains related to the preferential sequencing of some species, the use of inadequate primers or the result being the production of single bands (Polz and Cavanaugh, 1998; Sipos *et al.*, 2007). Thus, it is advised to use in parallel both methods. Indeed, it was reported that the single use of culture-based methods or molecular tools may lead to entirely different results (Al-Awadhi *et al.*, 2013), adding up the importance to apply both.

Conclusions

This study raises concern to occupational exposure to fungi on agriculture environments due to high concentrations of organic dust present and highlights the importance to use more than one sampling and assay methods to have an accurate exposure assessment.

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Fungal Contamination of Beaches

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Introduction

Mentioning beaches will evocate in most of us an image of golden sands, palm trees and an exotic cocktail in a coconut. Zooming in on the sand and water the picture is much less idyllic. A myriad of microorganisms, including viruses, bacteria, fungi, and protozoa, thrive in these ecosystems in complex patterns. Since beaches have an enormous potential for revenue, linking them to human infectious diseases outbreaks, could have significant economic repercussions (Sabino *et al.*, 2014a). Thus, the study of various aspects of potential pathogens and ways to minimize the risk of their involvement in human disease is of importance.

Although less studied than bacterial contaminants, mostly linked to fecal contamination, this review will focus on potential fungal pathogens. It will summarize the major findings as represented in scientific literature regarding the fungal contamination of sand and water on beaches, primarily in context of association to human health and wellbeing. It will not cover, specifically, the data of original research of the authors in this area, but rather mention it briefly.

Surveyed Beaches

Surveys of salt and sweet water beaches for potential pathogens were conducted in many countries and regions. Many of these surveys dealt only with fecal contamination indicator (FCI) bacteria.

Surveys on fungal contamination were carried out in various geographic areas, including a variety of water bodies. Surveys on beaches around the Pacific Ocean were reported from Hawaii (Kishimoto and Baker, 1969), California (Dabrowa *et al.*, 1964), Guadeloupe (Boiron *et al.*, 1983), Brazil (Gomes *et al.*, 2008; Vieira *et al.*, 2001; Menezes and Trindade, 1996, cited from Vieira *et al.*, 2001; Sanchez *et al.*, 1986; de Moura Sarquis and de Oliveira, 1996; Pinto *et al.*, 1992; Sato *et al.*, 2005; Zuza-Alves *et al.*, 2019), and Barbados Bridgetown, Puerto Rico and St. Maarten (Lourdes, 2019).

The second large saltwater body – the Atlantic Ocean was also surveyed, including beaches in Florida (Bergen and Wagner-Merner, 1977; Vogel *et al.*, 2007; Shah *et al.*, 2011), South Carolina (Stevens *et al.*, 2012), and Portugal (Romão *et al.*, 2017; Sabino *et al.*, 2011; Sabino *et al.*, 2014b, Abreu *et al.*, 2016; Pereira *et al.*, 2013; Mendes *et al.*, 1998; Brito *et al.*, 2019).

The Mediterranean is another saltwater body around which surveys were conducted. These included the coasts of Israel (Sheinman *et al.*, 2000; Ghinsberg *et al.*, 1994; Frenkel *et al.*, 2020), Morocco (Soussi Abdallaoui *et al.*, 2007), Spain (Borrego *et al.*, 1991; Izquiredo, Piera, Aledany and Lucena,1986, cited from Matavulj *et al.*, 2005; Roses Codinachs *et al.*, 1988; Larrondo and Calvo, 1989), Greece (Efstratiou and Velegraki, 2010; Papadakis *et al.*, 1997), Egypt (Sharaf, 2005), Italy (Mancini *et al.*, 2005), and France (Bernard *et al.*, 1988, cited from Matavulj *et al.*, 2005).

The Israeli beaches around the very high salt concentration water body – the Dead Sea – were surveyed as well (Buchalo *et al.*, 1998).

Results of surveys conducted on beaches of freshwater bodies were reported from Canada (Sherry *et al.*, 1979) and France (Chabasse *et al.*, 1986).

The Fungal Flora in Beaches

Unsurprisingly, considering the extreme variations in the conditions of the different beaches screened, the variety of isolated fungi comprises hundreds of genera and species. Three species of extreme halophile fungi were found even in the Dead Sea (Buchalo *et al.*, 1998). Moreover, the application of non-cultural screening methods has further incremented the gamut of fungal varieties found on beaches (Romão *et al.*, 2017).

Potentially pathogenic or allergenic fungi are often found on beaches. The most common are *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and the *Mucorales* (Matavulj *et al.*, 2005; Mancini *et al.*, 2005; Sabino *et al.*, 2014b). To the best of our knowledge, the question whether these environmental fungi are more frequent on the beaches than in other ecosystems has not been addressed. Other, potentially pathogenic, fungi isolated from beaches include *Scedosporium apiospermum* and *Sporothrix schencki* (Dabrowa *et al.*, 1964).

An important group of fungi that infect immunocompetent humans and animals are the dermatophytes. These fungi may be shed in the environment by hair or scales of infected humans and animals. It has been suggested (Szepetiuk *et al.*, 2010) that the abrasive action of the sand, especially if wet, may promote the shedding of these scales and thus increase the risk of infecting new hosts. In fact, Soussi Abdallaoui *et al.* (2007) isolated dermatophytes exclusively from wet sand. This is further facilitated by the skin's maceration due to its exposure to sea water and/or sand moisture.

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Paradoxically, dermatophytes were isolated relatively rarely from beaches (Kishimoto and Baker, 1969; Sousa, 1990; Abreu *et al.*, 2016; Szepetiuk *et al.*, 2010; Pereira *et al.*, 2013; Soussi Abdallaoui *et al.*, 2007; Sabino *et al.*, 2011; Larrondo and Calvo, 1989). It was postulated (Pereira *et al.*, 2013) that their survival under the conditions present on beaches is limited, which would explain the relatively rare person to person transmission (Mancini *et al.*, 2005). In fact, several authors noted that their prevalence is related to the influx of bathers to the beaches (Abreu *et al.*, 2016; Szepetiuk *et al.*, 2010). Sabino *et al.* (2011) found the presence of dermatophytes correlated with FCI organisms. Among the dermatophytes found most frequently were *Trichophyton rubrum* and those belonging to the *T. mentagrophytes* complex, probably of human origin and *Microporum canis*, most likely from animals. It is of interest to add that a recent study of the authors (unpublished data) in several beaches of the Mediterranean Israeli coast, did not detect dermatophytes in sand.

Among yeasts, *Candida* species are the most prevalent, especially *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C, guillermondii* (Sheinman *et al.*, 2000). Other frequently isolated yeasts belong to the *Rhodotorula* genus. Yeasts, especially *Rhodotorula* spp., were more abundant in the sand than in the water (Shah *et al.*, 2011). It is noteworthy that *C. albicans* and *Rhodotorula* spp. may be carried in the human gastrointestinal tract and thus their isolation may be an indicator of fecal contamination. In fact, several authors (Borrego *et al.*, 1991; Sabino *et al.*, 2011; Sherry *et al.*, 1979) found a correlation between the presence of these yeasts and bacterial fecal contamination indicators such as *Escherichia coli*, enterococci or *Clostridium perfringens* (Shah *et al.*, 2011). Zuza-Alves *et al.* (2019) found *C. tropicalis* isolates from one beach to be heterogeneous, relatively stable during one season and dispersed over long tracts of the beach, possibly through anthropogenic activities. More rarely, *Cryptococcus neoformans*, possibly from bird droppings was isolated (Kishimoto and Baker, 1969). Another Cryptococcus species, *Cr. uzbekistanensis*, with potential ability to infect humans (Powel *et al.*, 2012) was found in sand (Segal, unpublished data).

The Ecosystem

The seashore is an extremely variable environment (for a review see Whitman *et al.*, 2014). The exposure of the sand to water is an important source of this variability: it may be submerged (infra-tidal zone), bathed by the waves (swash zone), by the tides (inter-tidal zone) or dry (supra-tidal zone). Moreover, sea spray, influenced by wind, may have an impact on the sand's moisture.

Climatic variations such as intensity and duration of exposure to sun radiation, wind causing movement of the sand, rain, and temperature may influence the beach microbiome.

The chemical composition of the sand and its physical characteristics may influence the quantity, composition, and tendency to form biofilms of the microbiome. Parameters of significance are the content in organic material, acidity, and salinity. Grain size may influence the impact of winds on the diffusion of point contaminations and the channels in the sand through which microorganisms can diffuse.

The composition of the microbiome may vary in time due to differences in the multiplication ability of the various micro-organisms and the synergistic or antagonistic interaction between them.

Sources of microbial contamination may be anthropogenic activities originating from the extent of people frequenting the area, effluents running through it or contamination of nearshore seawater. An additional factor to be considered are animal excrements such as bird droppings or pet feces.

All these variables and their interaction have a significant impact on the composition of the microflora in general and the mycoflora in particular. Their study is important for geographical comparisons, as indicators of contamination and the possible presence of pathogenic fungi.

Sand seems to be a more suitable environment as yeasts are more abundant in it than in water. Moreover, dry sand seems to be more richly populated by yeasts than wet sand (Vieira *et al.*, 2001; Papadakis *et al.*, 1997; Sato *et al.*, 2005). This may be due to sand filtering and amassing yeast cells brought in by high tides and providing a nutrient rich, protected environment (i.e., crannies) (Vogel *et al.*, 2007; Ghinsberg *et al.*, 1994; Mancini *et al.*, 2005). Interestingly, FCI bacteria seem to prefer the moist areas (Whitman *et al.*, 2014). Moreover, the adsorbing surface of the sand grains, their mineral composition and oxygenation may influence the extent of fungal contamination of the sand (Stevens *et al.*, 2012). Abreu *et al.* (2016), found, however, no impact of sand grain size on the level of microbial contamination. *In-vitro* experiments showed that dry-wet cycles and higher temperatures had a negative impact on the survival of some, but not all, fungi associated with beaches. Salinity had no influence on fungal survival in these experiments (Anderson, 1979).

Several authors (De Moura Sarquis and De Oliveira, 1996; Mendes *et al.*, 1998; Chabasse *et al.*, 1986) found seasonality of fungal beach contamination that varied with the fungal species involved. Abreu *et al.* (2016) found no such phenomenon.

Human and Animal Impact on Beach Contamination

Anthropogenic activities may influence the littoral mycoflora. Such is the case for fungi belonging to the *Scedosporium/Pseudallesceria/Lomentospora* complex, often associated with sewers (Al-Yasiri *et al.*, 2017; Alm *et al.*, 2018).

While examining several beaches, Abreu *et al.* (2016) found a heavier burden of fungal contamination on artificial beaches. They attributed this finding to the disruptions of natural water currents and a higher popularity.

Interestingly, pollution seems to cause a decrease of potentially pathogenic microorganisms (Gomes *et al.*, 2008). The interaction between microorganisms may induce self-purification (Chabasse *et al.*, 1986). Such interaction between *Streptomyces* spp. and *Aspergillus* spp., isolated from a Queensland (Australia) beach, were demonstrated in-vitro by Khalil *et al.* (2019).

Beaches may be contaminated by animals that are frequenting it. Gulls and other sea bird droppings are a potential source for such important pathogens as *Cryptococcus neoformans* (Kishimoto and Baker, 1969). In case these birds feed on landfills they may act as vectors transferring microorganisms to the beaches they frequent (Al-Yasiri *et al.*, 2017).

The Relevance of Beach Contamination on Human Health

Human exposure to microorganisms that contaminate beaches and adjacent water may be by ingestion, inhalation and cutaneous exposure causing, respectively, gastrointestinal, respiratory/allergic and skin reactions. Recreational activities in these areas increase the risk due to swallowing of sand and sea waste, especially in children (Abreu *et al.*, 2016), exposing large skin areas for prolonged periods to the sand (i.e., getting buried in it) and inhalation resulting from the heads' relative proximity of the sand (DeFlorio-Barker *et al.*, 2018).

The most widespread and consequently most studied and monitored danger to public health stems from fecal contamination. Among these studies, those focusing on bacterial fecal contamination indicators such as coliforms, enterococci and clostridia are the large majority, whereas fungal microorganisms have received significantly less attention. It was repeatedly noted that levels of fungal contamination of beaches coincided with areas and periods of more intensive human presence (Kishimoto and Baker, 1969; Sherry *et al.*, 1979; Bergen and Wagner-Merner, 1977; Gomes *et al.*, 2008; Vogel *et al.*, 2007; Papadakis *et al.*, 1997). Stevens *et al.* (2012) found, however, that there was no accumulative effect between bathing seasons and non-bathing seasons.

The extent to which beach fungal contamination can be connected to human disease has been rarely assessed (Sabino *et al.*, 2014a), possibly due to the difficulty of gathering adequate data. Among surveys dealing with this topic only a minority includes fungi. In a review, Prüss (1998) noted that there is a dose related association between FCI bacteria and exposure to contaminated recreational waters. Fungal contamination was not included in the review. Heaney *et al.* (2009) and Heaney *et al.* (2012) found that gastrointestinal symptoms but not non-enteric illness was associated with digging in sand and/or getting buried in it and were related to levels of FCI in the sand. (for a more detailed review of bacterial beach contaminants and human disease see Whitman *et al.* (2014)). Although not specifically examined, other authors (Borrego *et al.*, 1991; Sabino *et al.*, 2011; Sherry *et al.* (1979)) have found that yeasts of the genera *Candida* and *Rhodotorula* are associated with such contamination, as well. Fleisher *et al.* (2010) found that a significantly higher risk exists for bathing beach-frequenters to develop gastrointestinal symptoms, acute febrile respiratory illness and skin illness relative to non-bathers. The latter was correlated to levels of contamination with enterococci, the only microorganism tested. No such connection was found for gastrointestinal or respiratory symptoms, caused by other microbial agents. Arnold *et al.* (2013) found that bathers had a higher risk of enteric symptoms, ear inflammation or rash but could not correlate these findings with levels of FCI bacteria. Marino *et al.* (1995) found skin infections to be associated with FCI microorganisms, including *C. albicans* but not dermatophytes. On the other hand, other authors (Chabasse *et al.*, 1986; Colford *et al.*, 2007) found no association between FCI and health risk. Thus, there is currently no unequivocal evidence that FCI microbial counts can be predictive of public health risks for beachgoers (Whitman *et al.*, 2014). The latter observation has undergone currently a revision and restrictive measures are undertaken or proposed by current guidelines for beach contamination (note next section), at this stage only for bacteria, with perspective to add such measures also for fungi.

The gamut of studies on microbial contamination of beaches has been greatly expanded in the last decades due to the states of immunosuppression becoming more common, due to pathological conditions or of iatrogenic origin. Unfortunately, some of the more susceptible populations, such as children are a major component of the populations exposed to these fungi. Increased awareness to these risks has generated a vast number of scientific publications dealing with this topic, which is based on the advancement of methodology, such as metagenomics and chromatography.

Guidelines/Recommendations

Several guidelines and recommendations focused on sampling and criteria for safe use of beaches have been published, such as the United Nations Environment Programme and the World Health Organization documents (for a review see Kamizoulis and Saliba, 2004). For a partial list of relevant websites see Sabino *et al.* (2014a) recommended screening beach sand for human pathogens regularly and the adoption of universal sampling and analysis methods to make inter-laboratory comparison of results meaningful.

Considering the extreme variability of the conditions that may influence the quantity and quality of microorganisms on the beach, repeatability of results may be extremely difficult. Moreover, the timespan during which the results have repercussions on the safety of the beaches for human use is relatively short due to the relatively fast temporal changes in some of these conditions and the possibility of different survival time and reproduction rate of the various microorganisms (Sabino *et al.*, 2011). This complicates attempts to associate human pathology with a specific beach microbial profile since both must be tested in parallel.

Summary

(1) This chapter summarized the literature on fungal contamination of beaches over the globe.
(2) The chapter is based on literature reports from different geographic, climatic and demographic sites.
(3) It included both salt and sweet water bodies.
(4) The surveyed literature revealed a great variety of fungal species, both yeasts and molds, which could be found in sand and/or in water.
(5) A number of studies indicated possible associations between fungal contamination and deleterious effects on human health and well-being
(6) In view of lack of regulatory measures regarding fungal contamination of beaches, the collection of data on the subject, might possibly serve as a database for initiation of regulations for public health.

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Fungal Exposure and Relevant Recreational Settings

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Fungi and Recreational Water Environments

Recreational water environments are often where we wear less protective clothing against environmental factors, like when we are at the beach. Possible activities that can foster exposure to fungi in recreational waters' sites and settings include the following:

(1) Bathing in recreational and medicinal waters (public swimming pools, beaches, saunas, spas, etc.)
(2) Sunbathing
(3) Water sports (rowboat, kayaking, surfing, fishing, diving, etc.)

Exposure

Swimming Pools

Fungal infection can be a substantial health concern at public swimming pools, due to the prevalence of fungi on hard surfaces, mainly those partly submerged in water, as published by Ekowati *et al.* (2018). This author postulated that dermatophytes tend to follow the trajectory of the users, from the pool, to the dressing and shower areas, aided by cleaning instruments. These types of fungal contamination are present mainly on solid surfaces in swimming-pool environments. The recognition of public swimming pools as a high risk for *tinea pedis* and onychomycosis is not a new subject. The first time dermatophytes were successfully isolated from shower floors was in 1947 but they still remain an issue today. This is not surprising, though, if we consider that worldwide, 25% of the population suffers from a superficial fungal infection of some kind. Besides the fungi listed above, the yeasts *Candida* and *Rhodotorula* are frequently encountered in water in swimming pools (Brandi *et al.*, 2007; Jankowski *et al.*, 2017).

Water-borne fungi often thrive in recreational facilities, such as pools and especially high humidity saunas. These can include black yeasts and yeast-like *Aureobasidium*, *Cladophialophora*, *Cyphellophora*, *Exophiala*, *Phialophora*, *Phoma*, and *Ochroconis* (Matos *et al.*, 2002; Hamada and Abe, 2009; Novak Babič *et al.*, 2018). The damp, warm environment and necessary nutrients for the growth of black fungi make them a probable nuisance for facility management, but above all a potential problem for the health of the visitors and users. Some of the black fungi are considered opportunistic pathogens, causing superficial infections and in some cases of trauma, invasive infections, mainly in form of mycetoma, but occasionally as a disseminated (systemic) infection. Although the most commonly reported health issues related to leisure activities in swimming facilities are on average caused by dermatophytes, other fungi present in high concentrations may affect well-being by triggering an allergic response in susceptible individuals. Simon-Nobbe *et al.* (2008) describe *Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia*, and *Penicillium* as triggers of rhinitis; *Alternaria*, *Aspergillus*, *Cladosporium*, *Helminthosporium*, *Epicoccum*, *Aureobasidium*, and *Penicillium* as triggers of allergic asthma; and *Malassezia*, *Sccharomyces cerevisiae*, *Alternaria*, *Candida*, and *Trichophyton* as triggers of atopic dermatitis.

Other health implications, such as intoxication due to mycotoxins, sinusitis, asthma, and infections of the respiratory tract, are not likely to occur in occasional visitors but should be taken into consideration for workers in such facilities. Amongst the most problematic molds on wet concrete and finishing materials on ceilings indoors is the presence of *Stachybotrys* spp. Prolonged exposure to toxic metabolites by these fungi can lead to respiratory infections, irritation, and allergies (Novak Babič *et al.*, 2018). Fungal presence in water and on surfaces is nonetheless difficult to control due to fungal resistance to most agents that can be used for their removal, namely bleach and UV radiation (DEFRA, 2011; Novak Babič *et al.*, 2018).

While dermatophytes, yeasts and yeast-like fungi dominate lower parts of wet surfaces where exposure happens through skin, upper ceilings often promote growth of filamentous fungi transferred by air with possible effects on our respiratory system. Viegas *et al.* (2011) found no association between air and surface contaminants, either in quality (species), or quantity (number of colony-forming units (CFU) per sample volume or area, respectively). In this study, the majority of the air contaminants in 200 L of air samples were *Cladosporia* (36.6%), *Penicillia* (19.0%), and *Aspergilli* (10.2%), whereas in surfaces, *Fusaria* showed the strongest presence, with 19.1% of positive samples. *Fusaria* is in fact a species complex that tends to prefer warmer but not dry climates, soil, and plants, as reported by many authors, but also shows up in aquatic habitats (Lamprecht *et al.*, 2011). Dominant species of this genus may vary between fresh water and seawater (Palmero *et al.*, 2009). The relevance in aquatic habitats of *Fusarium* species is their association with keratitis in patients with lesions after exposure to water. Contact lenses are also a risk factor for this pathology (Ahearn *et al.*, 2008). *Fusarium* is, however, not the only fungus able to cause keratitis. Khater *et al.* (2014) studied 66,303 cases of microbial keratitis and determined that 43.3% of the cases were of fungal origin (mostly *Aspergillus*), although in this study, the patients were mainly adults aged between 40 and 60 years, who had close contact with soil and outdoor sewage.

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Sunbathing

Sunbathing is an activity that does not necessarily imply direct contact with recreational waters. By the coast, inevitably, people tend to get their daily dose of vitamin D production and energy at the beach. Considering that most beaches at the coast are composed of sand, which is where recreational beach users tend to spend most of their visiting time, one must wonder why has the regulatory community only worried about water as a potential source of disease. In fact, sand can harbor all kinds of pathogens and even other health threats (e.g. glass fragments and hematophagic flies, some of which are vectors of zoonoses).

Currently, the bathing water regulation is based on the World Health Organization's "Guidelines for safe recreational water environments", published in 2003 (WHO – World Health Organization, 2003). These guidelines recommend a limit of 200 enterococci to accept a 5% risk of developing an ailment associated with human fecal pollution during bathing. While fungi are not mentioned *per se* in the guidelines, they may be included in the equation to determine 5% risk of ailment. On the other hand, management of sand contamination is not included in the guidelines or subsequent regulations, even implicitly. The concept of sand contaminants is pointed out in chapter 6 of the guidelines (where even fungi are addressed) but without any actual conclusions or recommendations of parameters and respective levels. There has been a considerable amount of work published with results and discussions on the raison d'être of looking at contaminants in sand. In September 2014, a meeting in Lisbon, Portugal gathered professionals of several areas of expertise to discuss the most sensible approach for sand contaminant analyses, amongst other microbiological public health issues. Up for discussion were parameters, their limits, analytical methods and sampling representativeness as they relate to monitoring sand contamination. The group included bacteriologists, virologists, medical mycologists, and parasitologists both as scientists and technical staff, public health professionals, and beach managers. The outcome of that meeting was a white paper authored by a smaller group of participants who published the meeting's observations and recommendations (Solo-Gabriele *et al.*, 2016). **Table 1** lists those.

The group discussion focused on water environments but many of the recommendations are also valid for other sand environments, like sandboxes and recreational parks (see Section "Sandboxes"). The proposed structure for fungal analysis is a simple test based on undifferentiated colony count that can be performed in any water microbiology lab, unless there is reason to specifically identify or detect a species, like an overwhelming presence or outbreak. This requires a reference laboratory or at least an accredited procedure because the recent taxonomic revolution that is taking place in medical mycology, together with emerging anti-fungal resistance, makes the correct identification of fungi a detail of importance, especially during an outbreak.

Table 1 Observations and recommendations of the white paper addressing sand microbial contaminants

Observations

Beaches are a significant contributor to the pathogen load to which beach users are exposed.
At beaches not impacted by sewage, the source of pathogens originates from the local site and includes human visitors, animals, local runoff and the release of microbes from sand.
Studies have identified the presence of pathogens in sand and factors other than point source pollution that contribute to their presence (e.g. moisture, wrack, wildlife, domestic animals, beach morphology, currents).
Epidemiological studies have shown that children who play in sand are subject to higher rates of illness than those not playing in sand.
Measurements of fungi have not been included in beach epidemiological studies.
Considerable evidence exists that sand can serve as a reservoir of enteric microorganisms and fungi, which can be vehicles of disease transmission.
Highlight: Contaminated sands present health and economic costs that can and should be known by decision makers, communities and by individuals.

Recommendations

Identification of recreational beach areas.
Identification of disease-causing agent(s) (viruses, bacteria, fungi and protozoa).
Sampling strategy, control and monitoring programmes.
Methods that estimate risks from pathogens in the sand.
Assessment of exposure to microbes in the sand by contact, ingestion and inhalation.
Tools for identification of sources of microbes.
Evaluation of alternative fecal indicator organisms, to determine the presence of sewage and human waste.
Detection and quantification of microbe levels for specific pathogens with culture-based methods, q-PCR and beach metagenomes.
Determination of whether FIOs are indicative of fecal pollution that carries pathogens or if they have separated from their original source through survival and regrowth.
Development of regulatory standards that reflect microbial sources, pathogens they contain, and associated health risks.
Development of reliable sand collection methods since pathogen contamination at sandy beaches tends to be patchy.
Determination of beach sand quality at freshwater vs. marine beaches.
Assessment of beach sand quality based on contamination by land and air.
Creation of standardized methods to recover and disinfect pathogens from different types of sands.

Highlight: Available evidence should be evaluated by both scientists and regulators with a view to filling the data gaps, which should be followed by sound policy development for safeguarding public health.

Note: Adapted from Romão, D., Sabino, R., Veríssimo, C., *et al.*, 2015. Children and sand play: Screening of potential harmful microorganisms in sandboxes, parks, and beaches. Current Fungal Infection Reports 9, 155–163.

The relevance of sand as an exposure setting in beach and public health was confirmed by Heaney *et al.* (2012) with a controlled epidemiological study of 4,999 beach users and 144 wet sand samples tested for F+ coliphage, *Enterococcus*, *Bacteroidales*, fecal *Bacteroides*, and *Clostridium* spp. The study confirmed an increase in gastrointestinal illness associated with playing in sand on a beach. Although raising awareness to the confirmation of sand as an exposure vector at a beach, the study did not include other microbes or dry sand. Although fungi are known to be plated also directly from water, it is in the dry sand that most of the fungal contaminants can be found, despite the extreme temperatures and solar radiation to which they may be subjected. In fact, some of these fungi, as mentioned above, thrive on radiation. Finding black molds in beach sand seems to be a common situation (Romão *et al.*, 2015). Like other fungal groups, melanised fungi are brought to sand via different carriers – plants, animals, through anthropogenic influences including pollution, and water. However, they do differentiate from others in high concentrations of melanin in their cell walls and unique growth in thick meristematic clumps. Melanin has a protective role against irradiation, but also uses radiation to produce energy and thus helps fixate the micronutrients necessary for fungal development (Dadachova *et al.*, 2007; Dadachova and Casadevall, 2008). Thus, melanised fungi like *Trimmatostroma*, *Exophiala*, *Coniosporium*, *Phaeotheca*, and *Lichenothelia*, are often found on dry solid rocks, covered with salts and exposed to UV radiation (Sterflinger, 1998).

Extremophiles, such as the genera *Hortaea* and *Wallemia* have been isolated from salterns and are frequently present in ponds with high concentrations of salt. Prolonged exposure to those fungi was described as *tinea nigra*, where *Hortaea werneckii* grew on palms of salt harvesting workers. Occasional exposure can be related to spa treatments where mud from salterns is used. While *Hortaea* is widely present in seawater and sand, other black yeasts, such as *Cladophialophora*, *Aureobasidium*, and *Exophiala* likely occur more locally in association with the presence of long-chained hydrocarbons, namely oils and their derivates (Prenafeta-Boldú *et al.*, 2006; Isola *et al.*, 2013). Also, *Aureobasidium* and *Exophiala* at beaches in Greece have been associated with geochemical properties of sand (Efstratiou and Velegraki, 2009).

How relevant are fungi at the beach, in terms of exposure? Nobody knows. The most common dermatophytes and the most commonly found yeast in human infections, *Candida albicans*, do not seem to survive the heat of the sand at a sunny beach. They sometimes can be found in highly populated beaches, suggesting a recent contamination of anthropogenic origin, rather than resilience. This does not exclude the chance of a transfer between individuals before inactivation, nor does it exclude less sunny beaches that may foster longer survival times. More work is needed in order to assess the actual risk of fungal ailments of recreational beach users.

Also, if we want to consider looking into a possible outbreak, how do we know that the number of cases isolated from recreational beach users did not contaminate the sand, rather than being contaminated by the sand? Fungal outbreaks are usually slow and scattered, with low numbers of cases. They are not explosive like those associated with viruses and certain bacteria. Many patients may have been infected during their holidays and then go home before the onset of the symptoms, rendering the connection between a beach visit and infection unlikely to be found. Residents can be screened during a bathing season, but the local area includes many other possible indirect contamination surfaces/media besides beach sand. It is thus extremely difficult to find an outbreak, unless obvious in a geographically confined yet relevant number of cases. But there may be an alternative to detection, based on modeling of expectations and Quantitative Microbial Risk Assessment (QMRA), as described later in this chapter.

Water Sports

Water related sports may represent excellent relaxation activities, but can also be dangerous. They are often associated with superficial injuries, and in more serious cases, deep trauma, or even drowning; which can represent risk factors for several fungal infections. Fungi posing risks to people involved in water sports are species of *Cylindrocarpon*, *Microsporum*, *Phialemonium*, *Rhinocladiella*, and zygomycetous genera *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, *Rhizomucor*, and *Saksenaea* (Novak Babič *et al.*, 2018). These fungi have been associated with a variety of infections from localized to disseminated, depending on the host's immune system. Fungi clearly connected to water-transmitted disease are those of *Scedosporium apiospermum* complex causing near-drowning syndrome, with characteristic cerebral abscesses or pulmonary infection developed after weeks of exposure.

In some areas of the globe, endemic fungi may be a problem for water sports. Sipsas and Kontoyiannis (2008) ran a meta-study to review cases of invasive fungal infections (IFI), looking into the lifestyle and contexts of the patients and concluded in terms of water sports in endemic areas for *Blastomyces* spp., that there is a certain amount of risk associated with the activity.

Cave Exploration

In 2018, a group of 12 members of a junior football team and their assistant-coach went cave exploring in Thailand and heavy rains caused a landslide trapping them in the cave for 17 days. Once rescued, they were all quarantined in an isolation room because of any potential infections myriad pathogens that can be transmitted in caves: Marburg hemorrhagic fever, leptospirosis (Weil's disease), rabies, borreliosis (cave fever), melioidosis (Whitmore's disease), and histoplasmosis (Gorvett, 2018).

Bats inhabit caves and their excreta, named guano, is a breeding ground for many micro- and macroorganisms. Bat guano inside caves in endemic areas for *Histoplasma capsulatum* pose an infection threat by this fungus. The fungus causes histoplasmosis, also known as "cave disease", and is well known in North and Central America as a cause of pneumonia. This histoplasmosis is often misdiagnosed, which leads to an inappropriate therapeutic approach with antibiotics. Immunocompetent individuals have

about a 5% chance of developing the disease when exposed to environmental levels of the fungus. These odds change to between 50% and 100% chance of getting the infection when exposed to higher levels of the fungus, like in caves.

Histoplamosis has a second, disseminated, stage of the disease, which can happen many years after the primary infection. *H. capsulatum* survives intracellularly in the reticuloendothelial system and may be reactivated mainly (not exclusively) with old age or immune-suppression (Ashbee *et al.*, 2008). Management of the disseminated histoplasmosis, if successful, is achieved by administration of systemic anti-fungal therapy.

In the United States of America, *H. capsulatum* inhabits the soil in the eastern and central parts of the country, especially in the Ohio and Mississippi River Valleys. It is however not exclusive to North and Central America. It has been found in South America, Africa, India, and Southeast Asia (Ashbee *et al.*, 2008).

Gardening and Camping

Gardening is a wonderful opportunity to commune with nature but some of the plants that we deal with have structures that may pierce our skin. *Sporothrix*, the infectious agent causing sporotrichosis, or rose gardener's disease as it is commonly known, is an IFI. *Sporothrix* is one of the most famous agents associated with gardening but also in terms of occupational exposure; it seems to affect people working with tree nursing, garden centers, forestry and hay bale handling (CDC, 2019). Sporotrichosis has a higher prevalence in tropical and temperate climates despite being present in all the world. Japan, India, Mexico, Brazil, Uruguay, Peru, and United States (associated with pine seedlings and moss manipulation (Barros *et al.*, 2011)) are endemic areas for this fungus. *Scopulariopsis brevicaulis*, *Saksenaea vasiformis*, *Scedosporium prolificans*, and *Exophiala jeanselmei* have also been found as minority gardening IFI agents (Sipsas and Kontoyiannis, 2008).

Different species of plants and trees seem to be connected also with symptoms known under the term "farmer's lung disease". The causative agents of the disease are highly irritant spores and mycotoxins wallimidione, walleminone and walleminol (walleminol A) produced by *Wallemia* spp. These fungi are xerophilic, osmophilic, halophilic, and chaophilic and can thrive on pollen, dry plant material, like hay or leaves, or in the presence of highly concentrated salts or sugars. Spores disperse in large quantities via air during hot and dry weather periods (Zajc and Gunde-Cimerman, 2018).

Lastly, *Cryptococcus gattii sensu lato* is making its way around the world, including Europe, despite being previously considered a tropical and subtropical climate fungus (Hagen *et al.*, 2012). The number of cases is increasing; there are several genotypes known and from those it has been concluded that some of those cases are allochthonous but a high percentage (40%) are autochthonous, many of which are in the Mediterranean region. This yeast can be found in decaying vegetable matter primarily, but it has also been isolated from animals and soil. It can stay dormant in its hosts and manifest itself much later. Unlike its close relative, the opportunistic *Cryptococcus neoformans*, *Cryptococcus gattii sensu lato* is a pathogen. *C. gattii* causes a pneumonia-like infection, with persistent cough, shortness of breath, chest pain and fever and if it spreads to the brain, patients get a cryptococcal meningoencephalitis, experiencing headache, fever, neck pain, nausea, and vomiting, sensitivity to light, confusion or changes in behavior (CDC, 2015). Susceptibility tests *in vitro* suggest that different genotypes react differently to various antifungals. Therapy may involve a combination of Amphotericine B with Fluconazol (Chen *et al.*, 2014) and management may require corticosteroids and draining in order to relieve the intracranial pressure (Franco-Paredes *et al.*, 2015). A more recent approach recommends a triplet of antifungals, lipid Amphotericin B formulation and 5-Flucytosine for 2–6 weeks followed consolidation/maintenance therapy with fluconazole for 12 months or longer.

As mentioned above, *Fusarium* spp. is a common fungus in the ground and care should be taken with rubbing eyes in order to prevent fungal keratitis, by this but also by other fungi. Soil is full of microbes, many of which are fungal opportunists and allergenic taxa, as mentioned above relative to swimming pools (*Alternaria*, *Aspergillus*, *Bipolaris*, *Cladosporium*, *Curvularia*, *Helminthosporium*, *Epicoccum*, *Aureobasidium*, *Penicillium*, *Malassezia*, *Saccharomyces cerevisiae*, and *Trichophyton*). Susceptible campers must take care and avoid camping sites with moist ground in order to avoid turning a camping event into a trip to hell.

Sandboxes

Undoubtedly, where there is sand and there are children, there will be a confluence of both. Children love to build structures on sand, like castles and dams (depending on water availability); they like to dig and even to bury themselves and others, often ending up eating some of the sand. 190 mg/day was the estimate of sand ingested by children without pica (extraordinary tendency to eat sand – van Wijnen *et al.*, 1990) at the beach. There is usually concomitant animal life on sand, especially outside of beach visitation hours. Considering that children get sand on their skin, eyes, ears, hair, nails and mucosae, and that prowling animals, rodents and birds shed whatever they have on their skin besides leaving excreta behind, it is easy to envision why sand should be monitored for pathogens.

Municipalities and public health protection offices have occasionally expressed concerns about microbial contaminants in sandboxes of public parks and beaches. A good example is a publication by European researchers with the result of such worries, in and around Lisbon, on sandboxes from three elementary schools and two kindergartens. The results were unsettling, but not necessarily surprising: *"Potentially pathogenic fungi were isolated from all samples besides one. Fusarium dimerum (32.4%) was found to be the dominant species in one park and Chrysonilia spp. in the other (46.6%). Fourteen different species and genera were detected, and no*

dermatophytes were found. Of a total of 14 species and genera, the fungi most isolated from the samples of the elementary schools were Penicillium spp. (74%), Cladophialophora spp. (38%) and Cladosporium spp. (90%). Five dominant species and genera were isolated from the kindergartens. Penicillium spp. was the only genus isolated in one, though with remarkably high counts (32,500 colony-forming units per gram). In the other kindergarten Penicillium spp. *were also the most abundant species, occupying 69% of all the fungi found."* (*Fusarium dimerum* has changed to *Bisifusarium dimerum* (Lombard *et al.*, 2015; Viegas *et al.*, 2016). The authors therefore recommend that sand be cleaned and replaced regularly.

Beach sand was addressed in another heading in this chapter (See Section "Sunbathing"). The different matrices pose different concerns, since the microorganisms at the beach tend to be less concentrated than in sandboxes.

Climate Change – The Game Changer

The days of geographically circumscribed endemic fungal infections are over. Until not many years ago, if you were a physician practicing somewhere in Europe and you got a patient with pneumonia, you would probably not suspect an endemic fungal infection like blastomycosis or histoplasmosis, unless your patient traveled recently to North America and mentioned it. Generally, endemic fungi remain endemic for now, but maintain the potential for changes in range associated with climatic alterations. The problem is that known pathogens and opportunists may experience new niches, displacement or range expansion due to extreme weather events associated with climate change and in concert with globalization (McIntyre *et al.*, 2017). This may include endemic fungi, as already may have happened in the Pacific Northwest region of North America, with a Vancouver Island outbreak caused by *Cryptococcus deuterogattii* which has historically been endemic to tropical and subtropical climates (Kidd *et al.*, 2004). There is still some controversy over the origin of this pathogen, which some authors believe to have originated in a mutant lineage originating somewhere else in the West-coast of North America (Ma *et al.*, 2009). More recently, however, a new taxonomy for this *C. gatii* complex was proposed by Hagen *et al.* (2015).

There is a known movement of air carrying fungal particles from Africa to America (Kellogg and Griffin, 2003), no doubt associated with the jet streams that flow westbound and converge at the equator. This may have been the cause of propagation of the plant pathogen *Hemileia vastatrix* (Bowden *et al.*, 1971) responsible for coffee leaf rust, a fungal infection devastating entire crops of coffee in South America since 1970. McIntyre *et al.* (2017) may have focused on the general alterations of climate because of the expansion of high temperatures beyond their usual latitudes. However, together with globalization, extreme weather events are generating the biggest concerns and the strangest clinical cases. The following three examples illustrate the worries about climate change and extreme weather events:

(1) Central Europe has reported bacterial *Vibrio cholerae* necrotizing fasciitis in bathers of inland water catchments (Hirk *et al.*, 2016);
(2) There are cases of mucormycosis associated with wood fragments flying at high speeds during hurricane-like winds (Neblett Fanfair *et al.*, 2012);
(3) A case of mucormycosis associated with catastrophic floods with a near-drowning situation, in Mandra, Attica in Greece, in November 2017 (Sympardi *et al.*, 2019).

Cases like these suggest that public health protection agencies need to advise the public on susceptibility factors more actively during extreme weather events and for climate change preparedness. For that, knowledge gathering is crucial. Benedict and Park (2014) prepared a review on published cases of natural disasters and fungal infections. In this review, coccidioidomycoses were the leading infections with over 100 cases, both in a 1977 dust storm and the 1994 Northridge earthquake in the USA. Many of these kinds of situations, however, take place not only due to natural disasters but frequently in recreational settings (Sipsas and Kontoyiannis, 2008).

Quantitative Microbial Risk Assessment (QMRA)

In a public health context, and especially in the face of climate change and globalization that can lead to changes in fungal contaminant ranges, it will be important to look toward understanding human health outcomes resulting from exposure to fungi. Quantitative Microbial Risk Assessment (QMRA) can be a powerful tool for understanding how contaminant concentrations are related to risks to human health in areas like gardens, beaches, swimming pools, and caves.

QMRA relies upon contaminant concentration data, combined with an expected exposure pathway from the environment to the human host (**Fig. 1**), to determine the concentration (exposure dose) of contaminants that humans are likely to be exposed to during their activities. From there, QMRA applies that calculated exposure dose to an existing dose-response model. This model then predicts the probability of a health response (e.g., infection, illness, death, etc.), given the calculated exposure dose (**Fig. 2**, Haas *et al.*, 2014). This probability of a health response is also known as the "risk" in quantitative microbial risk assessment and can be used to inform management and policy guidance for controlling contamination in the local environment.

Recent research has indicated that bacterial and viral health risks at beaches can be substantial, but that they are also highly dependent upon both the concentration of the bacteria or viruses and the exposure pathway. For instance, it has been found that

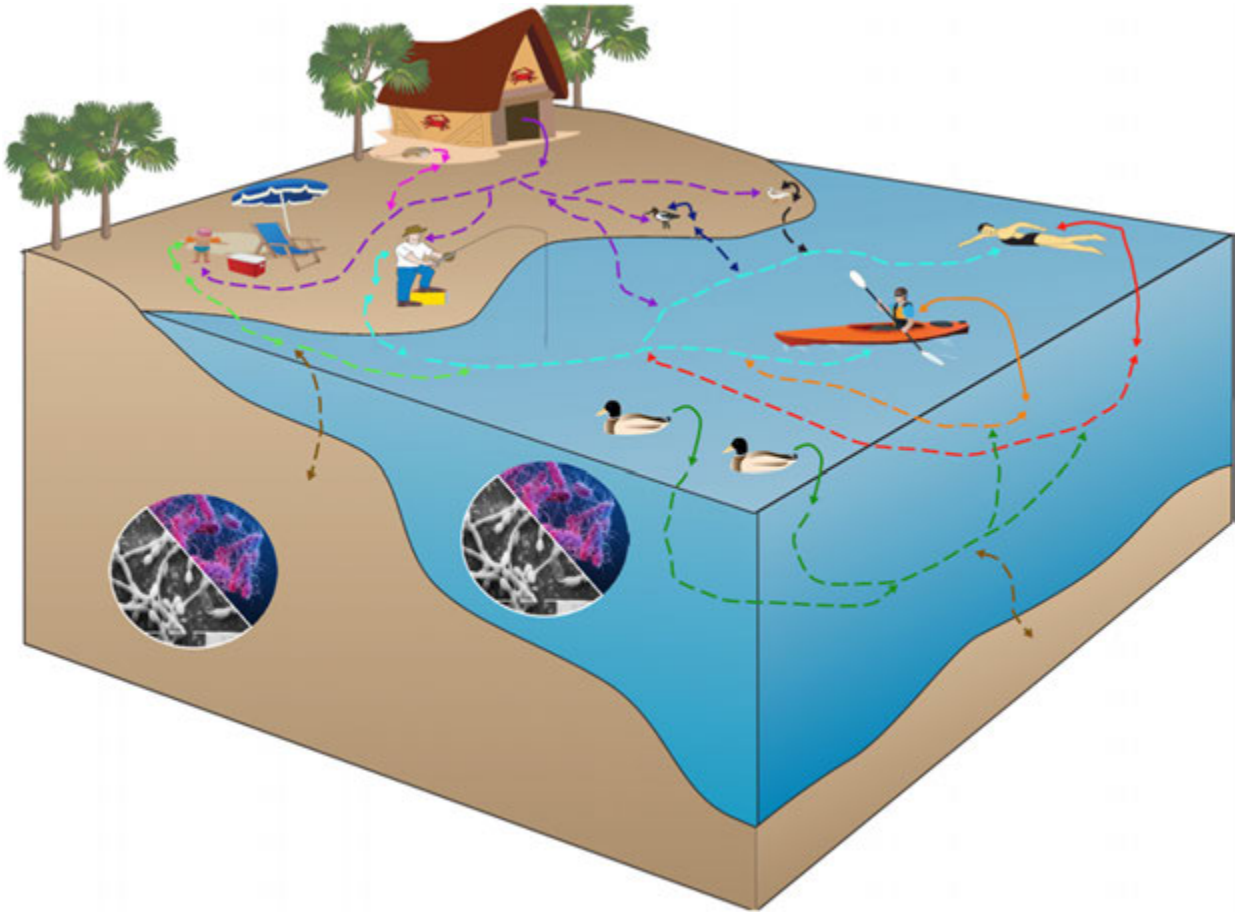

Fig. 1 Potential exposure pathways that human and animal hosts may be subject to at the beach. Different colors represent distinct pathways. Dashed lines represent transport of contaminants through the water or sand media; solid lines denote transfer of contaminants between the environment and human or animal hosts. Image created using vector graphics from Tracey Saxby, Kim Kraeer, Lucy Essen-Fishman, Jane Thomas, and Kate Moore, Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/) and images of microorganisms from the US Centers for Disease Control and Prevention. Reproduced from Jabra-Rizk, M.A., Falkler, W.A., Meiller, T.F., 2004. Fungal biofilms and drug resistance. Emerging and Infectious Diseases 10 (1), 14–19. doi:10.3201/eid1001.030119.

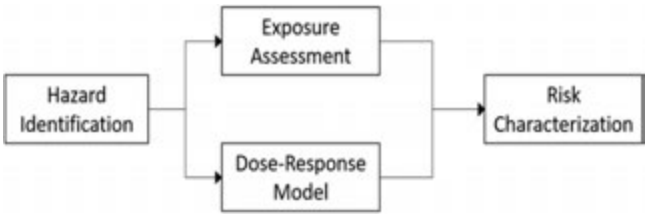

Fig. 2 General flow of development for a QMRA. Contaminant concentration observations are used in hazard identification and exposure assessment, while exposure pathways from the environment to human hosts are used in the exposure assessment. Epidemiological or feeding study observations are used with exposure assessment results in the dose-response modeling process, to calculate overall risk.

contaminant concentrations can be much higher in sand than in recreational water (Heaney *et al.*, 2012), due to the accumulation of contaminants in biofilms (Piggot *et al.*, 2012). However, the overall health risks associated with sand contamination may be comparable to or less than those associated with water contact because of the more complex exposure pathways that sand contaminants must take to infect humans.

A variety of QMRAs have been developed for recreational water in beach settings (Dickinson *et al.*, 2013; Eregno *et al.*, 2016; Juntunen *et al.*, 2017; Shibata and Solo-Gabriele, 2012; and others) and swimming pools (Peters *et al.*, 2017). These risk assessments, however, are exclusively in the context of contamination by bacteria or viruses; there is very little risk assessment work that is focused on fungal contamination in recreational areas. QMRAs developed for mycotoxin in agricultural environments

(Wu, 2004; Han *et al.*, 2013) may provide examples to draw from in developing fungal QMRAs for recreational settings. However, their applicability is limited because of the sensitivity of human health outcomes and risks to differences in both the microbial taxon and the setting/exposure pathway. It will therefore be crucial for ongoing research to focus on characterizing exposure pathways and developing epidemiologically-based dose-response models for fungal contaminants in areas like beaches, swimming pools, spas, gardens, and caves.

Conclusion

Currently, there is a rising awareness of the global burden of fungal infections. Bongomin *et al.* (2017) estimated nearly 2 billion people are suffering from a hair, skin or nail infection and tens of millions of from mucosal candidosis. They also describe the main fungal agents responsible for these infections at a global scale: *"Although the epidemiology of fungal diseases has greatly changed over the past few decades, Aspergillus, Candida, Cryptococcus species, Pneumocystis jirovecii, endemic dimorphic fungi such as Histoplasma capsulatum and Mucormycetes remain the main fungal pathogens responsible for the majority cases of serious fungal disease. Candida albicans is the main agent responsible for mucosal disease, Aspergillus fumigatus for most allergic fungal disease and Trichophyton* spp., *especially T . rubrum, for skin infections."* According to the same study, 150 million people suffer from serious fungal infection. Many of which are in less healthy economies but serious fungal infections have one common denominator throughout the whole world: the survival rate of the patient starts, *grosso modo*, at 50% and ends in 0% (Hagen, 2018). Mortality is currently over 1.6 million, which is similar to tuberculosis and three times that of malaria. Despite these numbers, there is no policy to fight fungal infections by health authorities anywhere but the WHO recently included two pathologies in the tropical neglected diseases list: Chromoblastomycosis and Mycetoma (WHO – World Health Organization, 2016).

One of the weak points in public policy management of fungal infections is neglecting their treatment rate and not considering fungi as a group of diverse pathologies and infection agents. This posture would make sense considering that all fungal infections are handled with a handful of antimicrobial agents and approaches. The biochemistry of our cells, of protozoan parasites and of fungi is very similar due to small evolutionary distances, so there are not many options for selectively treating an infection, leaving the patient unharmed.

The best policy in public health protection from fungal infections is by preventing them instead of managing or treating them, given the low treatment efficacies. However, it has been called a neglected epidemic (Armstrong-James *et al.*, 2014) in the particular context of HIV/AIDS patients but likely for others as well.

This chapter focused on exposure in leisure contexts, and the main goal of the authors is to raise awareness for the possible fungal ailments associated with leisure activities in the most common practices and contexts, because awareness leads to prevention and management.

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Assessment of *Aspergillus* Section *Fumigati* in Occupational Environments – A Bibliographic Review

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Introduction

High levels of fungal particles can be found in various occupational settings making workers to be exposed to these microorganisms and their components, such as viable and non-viable spores or conidia and hyphal fragments for considerably long periods of time, thus increasing the risk for occupational respiratory diseases (Eduard, 2008).

Aspergillus can be found in almost every environment since they are ubiquitous in the environment and easily disseminated in the air (Sabino *et al.*, 2019). The *Aspergillus* genus is characterized by its filamentous fungi with septate hyphae and with specific reproductive structures of this genus that are the conidia (Park and Mehrad, 2009). Among *Aspergillus* genus, the designations actually represent sections (or complexes) of closely related species (also referred to as cryptic species) that cannot be clearly distinguished morphologically (Lamoth, 2016).

Among *Aspergillus* genus, *Aspergillus* section *Fumigati*, is one of the main responsible for invasive aspergillosis and other considerable risk factors (Baddley, 2011). This fungus has bluish-green colonies and has a high growth rate (Xavier *et al.*, 2006). *Aspergillus* section *Fumigati* is a thermophilic species with growth rates that occur even at high temperatures (55°C) and can guarantee its survival at temperatures around 70°C (Fritzler *et al.*, 2007; Albrech *et al.*, 2010; Pohl *et al.*, 2011). In most cases this species can be isolated from a wide variety of substrates and at temperatures in between 12°C and 70°C. However, it is at a temperature of 37°C and at a pH of 3.7–7.6 that it has an optimal growth rate (Kozakiewicz and Smith, 1994).

Aspergillus section *Fumigati* is a natural recycler, as this fungus has a very versatile metabolism that allows it to meet all its nutritional requirements under the most diverse environmental conditions (Gibbons *et al.*, 2012). It has enzymes in its constitution that allows to obtain nitrogen and other compounds through the degradation of plant cells (Tekaia and Latgé, 2005; Abad *et al.*, 2010). This ability allows this fungus to respond to nutritional restrictions, but also to a physiological versatility for the use of different substrates to obtain nutrients. Thus, *Aspergillus* section *Fumigati* can compete with other fungi and bacteria in the environment (Anastasi *et al.*, 2005). Given these characteristics, it is quite common to find this type of fungi in places that contain a lot of organic matter, such as gardens, greenhouses or even in composting areas, in these cases the percentage of this species present in the environment can be around 35%–75% (Anastasi *et al.*, 2005). In addition to these saprophytic environments, this fungus can also express other virulence factors that allow it to adhere and invade host cells, survive in environments of hypoxia and nutritional scarcity, such as the lung environment (Panepinto *et al.*, 2003; Schrettl *et al.*, 2004; Moreno *et al.*, 2007; Willger *et al.*, 2008; Liu *et al.*, 2010; Gravelat *et al.*, 2013; Bertuzzi *et al.*, 2014; Pongpom *et al.*, 2015; Liu *et al.*, 2016).

Some specific characteristics make this fungus an omnipresent pathogen, such as: its high capacity for survival and proliferation in a wide variety of environments, great efficiency of dispersion mainly in the air, because its conidia have physical characteristics that provide the ability to reach the distal airways (Brakhage and Liebmann, 2005; Rhodes, 2006) and the ability to adapt to their host's environment (Latgé, 2001; Anastasi *et al.*, 2005; Tekaia and Latgé, 2005; Hohl and Feldmesser, 2007; Dagenais and Keller, 2009; Abad *et al.*, 2010). These characteristics make *Aspergillus* section *Fumigati* a very competitive fungus, where the temperature, pH and nutrients are not ideal for a wide spectrum of microorganisms. It is in these environments that *Aspergillus* section *Fumigati* thrives due to the lack of competition in the environment, making this fungus an excellent opportunistic pathogen (Latgé, 2001).

This species is considered the major cause of invasive aspergillosis that represents a danger to human life, as this is a saprophytic fungus that is present in most environments, and in this way causing human exposure to *Aspergillus* section *Fumigati* almost daily. This type of infections is initiated by inhalation of conidia, which in a normal situation are quickly eliminated from the host organism, however in the case of immunocompromised individuals it can be the cause of invasive mycosis (Rhodes, 2006; Kwon-Chung and Sugui, 2013).

To access its presence in the environment active methods can be applied, usually by the use of devices that sample air through impaction, impingement or filtration methods (Mandal and Brandl, 2011; Viegas *et al.*, 2015). Passive sampling methods can be also applied and provide an assessment of the harmful part of the airborne population that accumulates on a surface (French *et al.*, 1980; Pasquarella *et al.*, 2000).

Azoles are currently considered the first-line drugs to combat the fungal presence, as they act directly on the production of ergosterol, which is an essential constituent of the fungal cell membrane and its consistency (Diaz-Guerra *et al.*, 2003). In the last

15 years, this type of drugs has maintained a prominent position regarding the treatment and prevention of diseases originating in the genus *Aspergillus* (Patterson *et al.*, 2016). It is scientifically reported that resistance to this type of drugs may be an intrinsic phenotype of the *Aspergillus* genus, more specifically in the case of *Aspergillus* section *Fumigati*. The acquisition of this capacity may be due to exposure to fungicides in agricultural areas or in a clinical environment (Denning *et al.*, 1997; Verweij *et al.*, 2007; Van der Linden *et al.*, 2011; Meis *et al.*, 2016). Currently, resistance to azoles is already reported in environmental isolates from *A. fumigatus* (Snelders *et al.*, 2008; Jeanvoine *et al.*, 2017; Viegas *et al.*, 2020).

Additionally, *Aspergillus* section *Fumigati* produces a variety of secondary metabolites that can alter the function of the host cells and thus contribute to their virulence. The genome of this fungus has approximately 34 gene groups that can express these secondary metabolites, of these few have been extensively studied (Inglis *et al.*, 2013). Among all of them, the metabolite of *A. fumigatus* best documented is gliotoxin, this toxin has multiple effects on the host, from damage to the respiratory epithelium, production of reactive oxygen species, inhibition of phagocytosis, or even the apoptosis of the immune system cells (Mullbacher *et al.*, 1985; Amitani *et al.*, 1995; Pahl *et al.*, 1996; Stanzani *et al.*, 2005; Orciuolo *et al.*, 2007; Liu *et al.*, 2018).

It is reported in several studies that the inhalation of particles contaminated with fungi or bio-components produced by them can lead to the contraction of allergic reactions and the development of respiratory diseases (Macher *et al.*, 1999). In the literature, it is also reported that these components present in aerosolized particles can cause asthma, allergic alveolitis, toxic organic dust syndrome, byssinosis, chronic bronchitis, allergic rhinitis, mucosal irritation, sick building syndrome, chronic fatigue syndrome, aspergillosis, other infectious diseases and even cancer (Dutkiewicz and Górny, 2002; Krysińska-Traczyk *et al.*, 2003; Jahnz-Rozyk *et al.*, 2011).

This issue is of great importance, especially for occupational and indoor environments, that were already reported to have high levels of azole-resistant *Aspergillus* isolates that may result in an increased occupational risk and in difficult clinical management (Chowdhary *et al.*, 2013; Snelders *et al.*, 2008; Verweij *et al.*, 2013).

Thus, a correct intervention to assess occupational exposure to *Aspergillus* section *Fumigati* is of upmost importance for the risk characterization and management. This study intends to perform a systematic search review, to verify which methods for sampling and analyses are currently used by the scientific community and to discuss which of them present more accurate results.

Materials and methods

This study was based on a systematic search for information and data that have been published in free access sources during the period of 1st January 2000 to 31st December 2020. In order to comply with the rigor of demand, specific parameters were established to assist in the search for information in order to identify and select articles related to occupational environments where the search for *Aspergillus* section *Fumigati* is carried out. The search terms were carried out in the selected database (PubMed), in English and giving preference to articles that focused on *Aspergillus* section *Fumigati* assessment in occupational environments. This research resulted in 25 articles from PubMed, all articles that were not accepted by the inclusion criteria were excluded (**Table 1**).

The selection of articles was elaborated in a structured way with different phases throughout the process. All the steps are described in the following diagram (**Fig. 1**).

Results

A total of 25 articles related to occupational environments were included and analyzed (Hospital environments, farms, animal production and composting were the most common environments). Sampling methods assays, species identification and the main findings were analyzed. Most of the studies analyzed were carried out on farms (10 out of 25). The remaining were carried out in hospital environment (7 out of 25), composting stations (4 out of 25), archives (2 out of 25), a biodiesel plant (1 out of 25) and in flower fields (1 out of 25).

Table 1 Inclusion and exclusion criteria for the selected articles

Inclusion criteria	*Exclusion criteria*
Articles published in the English language;	Articles published in other languages
Articles published from 1st January 2000 to 31st December 2020	Articles published prior to 2000
Articles published in any country	
Articles on *Aspergillus fumigatus* in occupational environments	Articles related exclusively to biological samples from workers
Identification of *Aspergillus* section *Fumigati*	*Aspergillus fumigatus* not identified in the study
Original scientific articles on the topic	Abstracts of congress, reports, reviews/state of the art articles

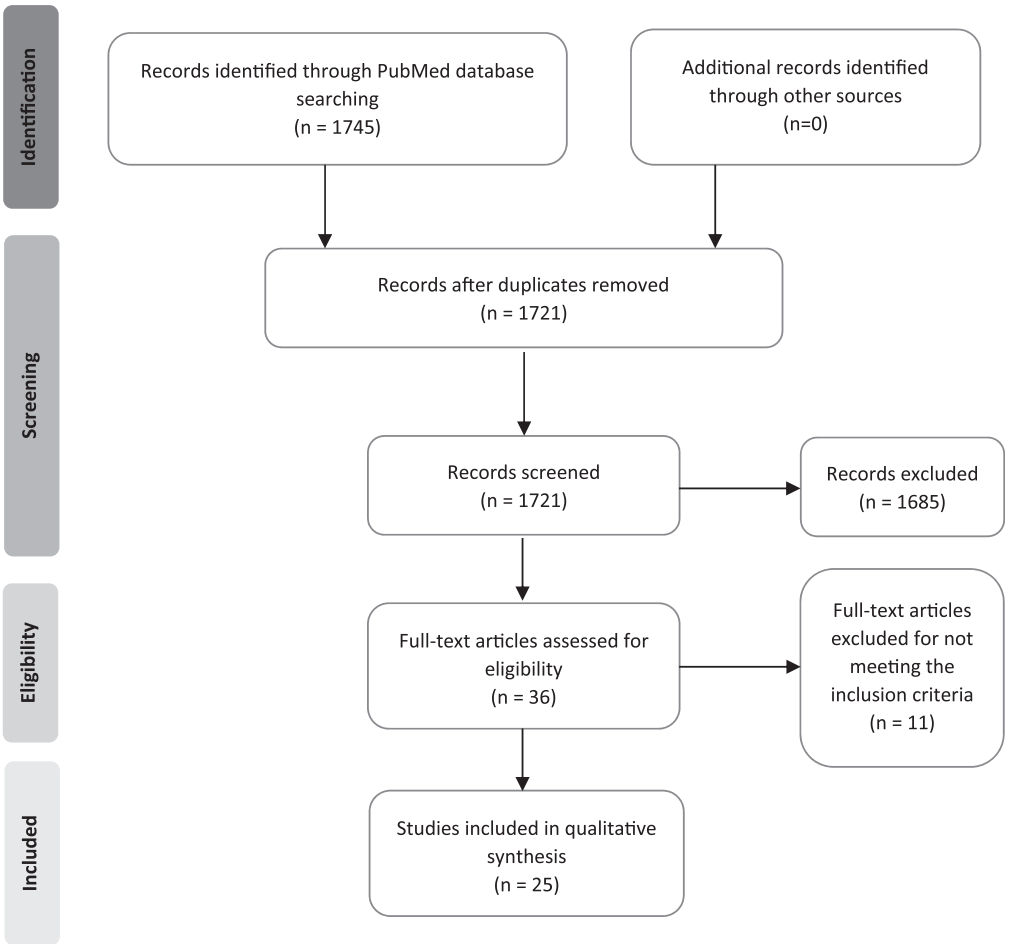

Fig. 1 Article selection based on the PRISMA method.

The most frequent sampling strategy among all the studies analyzed was the exclusive use of active sampling methods (11 of 25). However, although the use of passive methods was lower, their use was verified with some frequency (7 of 25). The combined use of the two types of sampling was also found in 7 studies (Zielinska-Jankiewicz *et al.*, 2008; Madsen *et al.*, 2012; Caggiano *et al.*, 2014; Madsen *et al.*, 2015; Viegas *et al.*, 2016a; Demuyser *et al.*, 2019; Madsen *et al.*, 2020). The most used active sampling method was air sampling through impaction method (Augustowska and Dutkiewicz, 2006; Zielinska-Jankiewicz *et al.*, 2008; Gniadek *et al.*, 2011; Ross *et al.*, 2011; Chang *et al.*, 2013; Caggiano *et al.*, 2014; Martínez-Herrera *et al.*, 2016; Rudramurthy *et al.*, 2016; Viegas *et al.*, 2016a; Demuyser *et al.*, 2019), but the method of sampling air by filtration was also widely used (Skórska *et al.*, 2005; Mackiewicz and Szponar, 2009; Timm *et al.*, 2009; Madsen *et al.*, 2012; Chang *et al.*, 2013; Madsen *et al.*, 2015; Priyamvada *et al.*, 2017; Madsen *et al.*, 2020). Regarding passive methods, the collection of bulk samples was the most observed approach (Storm *et al.*, 2010; Madsen *et al.*, 2012; Queiroz *et al.*, 2013; Carvalho *et al.*, 2016; Alvarez-Moreno *et al.*, 2017; Trovato *et al.*, 2018; Schoustra *et al.*, 2019) but surface sampling (Zielinska-Jankiewicz *et al.*, 2008; Caggiano *et al.*, 2014; Demuyser *et al.*, 2019; Madsen *et al.*, 2020) and settled dust sampling (Madsen *et al.*, 2015; Skóra *et al.*, 2016) were also used.

As for the analytical procedures, the exclusive use of culture-based methods was observed in 8 of the 25 studies (Skórska *et al.*, 2005; Augustowska and Dutkiewicz, 2006; Zielinska-Jankiewicz *et al.*, 2008; Gniadek *et al.*, 2011; Ross *et al.*, 2011; Caggiano *et al.*, 2014; Martínez-Herrera *et al.*, 2016; Rudramurthy *et al.*, 2016), while the exclusive use of molecular tools was not verified. The combined use of culture-based methods and molecular tools was the most common scenario (16 out of 25) (Prigione *et al.*, 2004; Mackiewicz and Szponar, 2009; Timm *et al.*, 2009; Storm *et al.*, 2010; Madsen *et al.*, 2012; Chang *et al.*, 2013; Queiroz *et al.*, 2013; Carvalho *et al.*, 2016; Skóra *et al.*, 2016; Viegas *et al.*, 2016a; Alvarez-Moreno *et al.*, 2017; Priyamvada *et al.*, 2017; Trovato *et al.*, 2018; Demuyser *et al.*, 2019; Schoustra *et al.*, 2019; Madsen *et al.*, 2020). Overall, culture-based methods were the most frequent approach verified in all of the 25 studies (Prigione *et al.*, 2004; Skórska *et al.*, 2005; Augustowska and Dutkiewicz, 2006; Zielinska-Jankiewicz *et al.*, 2008; Mackiewicz and Szponar, 2009; Timm *et al.*, 2009; Storm *et al.*, 2010; Gniadek *et al.*, 2011; Ross *et al.*, 2011; Madsen *et al.*, 2012; Chang *et al.*, 2013; Queiroz *et al.*, 2013; Caggiano *et al.*, 2014; Madsen *et al.*, 2015; Carvalho *et al.*, 2016; Martínez-Herrera *et al.*, 2016; Rudramurthy *et al.*, 2016; Skóra *et al.*, 2016; Viegas *et al.*, 2016a; Alvarez-Moreno *et al.*, 2017; Priyamvada *et al.*, 2017; Trovato *et al.*, 2018; Demuyser *et al.*, 2019; Schoustra *et al.*, 2019; Madsen *et al.*, 2020), followed by the use

Table 2 Data obtained from the chosen articles

Title	*Country*	*Environmental description*	*Objective*	*Sampling methods*	*Analytic methods*	*Range/Prevalence*	*Main findings*	*References*
Development and Use of Flow Cytometry for Detection of Airborne Fungi	Italy	1 Composting plant, 1 forest, 1 damp dwelling	Develop an analysis method based on FCM for rapid quantification of fungal load	Air sampling by impinger	Culture-based methods, FCM	Microscopy: 3.46×10^4 CFU m^{-3}–7.70×10^5 CFU m^{-3} FCM: 3.80×10^4 CFU m^{-3}–5.30×10^5 CFU m^{-3} *Aspergillus* section *Fumigati* gave R^2 values of 0.97 and 0.99 in the first and second simulated tests.	The degree of correlation between the FCM and microscopy counts was quite high. The use of flow cytometry proved to be feasible and more accurate than microscopy, however it was only used for the total load in the environment and requires further investigation.	Prigione *et al.* (2004)
Exposure to airborne microorganisms, dust and endotoxin during processing of valerian roots on farms	Poland	15 Valerian root farms	Assess the level of microorganisms present in this type of farms	Air sampling by personal and stationary filtration	Culture-based methods	Total fungi: 0.15 CFU m^{-3}–1.35×10^5 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 57.5%	The prevalence of *Aspergillus* section *Fumigati* is only verified after the roots are dried, this being one of the main dangers encountered due to the risks it poses to the respiratory system.	Skórska *et al.* (2005)
Variability of airborne microflora in a hospital ward within a period of one year	Poland	1 Pulmonology ward	Study the variability of the microflora present in the air of a pulmonology ward for 1 year	Impaction air sampling	Culture-based methods	Total fungi: 9.9 CFU m^{-3}–96.1 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 77%	*Aspergillus* section *Fumigati* proved to be the most prevalent fungus among all observed fungi The study showed that exposures to these fungi add to respiratory complications and contribute to sick building syndrome.	Augustowska and Dutkiewicz (2006)
Microbiological contamination with molds in work environment in libraries and archive storage facilities	Poland	3 Archives e 1 Library	Evaluate microbiological contamination in libraries and archives	Impaction air sampling, surface sampling with contact plates	Culture-based methods	Total fungi: 20 CFU m^{-3}–2.9×10^3 CFU m^{-3} *Aspergillus* section *Fumigati*: 80 CFU m^{-3}–5.0×10^2 CFU m^{-3}	Although visible signs of contamination were not verified, the study revealed the presence of allergenic and toxigenic species, such as *Aspergillus* section *Fumigati*, and some of these species exceeded reference values for occupational environments.	Zielinska-Jankiewicz *et al.* (2008)
Respiratory disorders in two workers of customs depositories occupationally exposed to moldy tobacco	Poland	2 Tobacco warehouses	Develop a microbiological assessment of potential health hazards in the workplace	Air sampling by filtration	Culture-based methods, GC-MSMS	Total fungi: 110 CFU m^{-3}–310 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 41.9%–70%	Professionals in this area are exposed to fungi, especially those of the genus *Aspergillus* and *Penicillium*, which due to the poor ventilation in the environment and presence of nutrients proliferate and easily aerosolize.	Mackiewicz and Szponar (2009)
Assessment of the Total Inflammatory Potential of Bioaerosols by Using a Granulocyte Assay	Denmark	22 Biodiesel plants	Develop a fast and low-cost method for microbiological analysis of bioaerosols in occupational environments	Air sampling by filtration	Culture-based methods, NAGase analysis	*Aspergillus* section *Fumigati*: BD–4.4×10^4 CFU m^{-3}	The analysis of reactive oxygen species gives reliable information on the inflammatory potential of bioaerosols present in a given environment.	Timm *et al.* (2009)

Dynamics in the microbiology of maize silage during whole-season storage	Denmark	20 Dairy farms	Monitoring of microbiological variations in different seasons	Collection of corn silage	Culture-based methods	Total fungi: $\pm 1 - \pm 2.5$ log CFU g^{-1} *Aspergillus* section *Fumigati*: 11%–60%	There is a great variation of the fungi present between periods. The storage of corn for long periods is the main cause of changes in the mycobiota.	Storm *et al.* (2010)
Cytotoxicity of Aspergillus Fungi Isolated from Hospital Environment	Poland	Hospital rooms	To evaluate the cytotoxicity of fungi of the genus *Aspergillus* isolated from hospital environments	Impaction air sampling	Culture-based methods	Total fungi: 5 CFU m^{-3}–2.37×10^3 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 37%	Most of the *Aspergillus* tested were cytotoxic. Ambient temperatures are favorable for the survival of these species and the production of metabolites.	Gniadek *et al.* (2011)
Refurbishment works in a hospital during normal operation	Germany	2 Surgical wards and 1 Intensive care area	Monitor the fungal load and particulate matter after infiltrations	Impaction air sampling	Culture-based methods	Total fungi: <10 CFU m^{-3}–3.6×10^2 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 0%	Although there is an increase in the number of particles in the restoration period, the number of viable fungi did not show to be influenced by the works, but by the type of affluence that the rooms have in terms of staff, patients or family members.	Ross *et al.* (2011)
Organic dust toxic syndrome at a grass seed plant caused by exposure to high concentrations of bioaerosols	Denmark	2 Grass seed factories	Assess the relationship between ODTS and the management of grass seeds	Collection of seed samples, air sampling by filtration	Culture-based methods	*Aspergillus* section *Fumigati*: 9.0×10^3 CFU kg^{-1}–4.50×10^9 CFU kg^{-1}	Microorganisms were aerosolized 10^7 times more in problematic seeds than in reference seeds Respiratory protective equipment has proven to be efficient and should be used at all times.	Madsen *et al.* (2012)
Bioaerosols from a Food Waste Composting Plant Affect Human Airway Epithelial Cell Remodeling Genes	Taiwan	1 Composting station	Evaluate the distribution of microorganisms in a composting station	Air sampling by filtration and impaction	Culture-based methods, PCR	*Aspergillus* section *Fumigati*: 1×10^2 CFU m^{-3}–1×10^6 CFU m^{-3}	The levels of *Aspergillus* section *Fumigati* found are not an effective indicator for monitoring workers 'health, underestimating the impact on these workers' health.	Chang *et al.* (2013)
Fungal contamination and determination of fumonisins and aflatoxins in commercial feeds intended for ornamental birds in Rio de Janeiro, Brazil	Brazil	Poultry feed industries	Determine mycobiota distribution and quantify mycotoxins in poultry feed	Collection of feed samples	Culture-based methods	Total fungi: 100 CFU g^{-1}–2.9×10^6 CFU g^{-1} *Aspergillus* section *Fumigati* isolated: 36%	Several toxigenic species have been identified, with *Aspergillus* section *Fumigati* being among the most prevalent species.	Queiroz *et al.* (2013)
Mold contamination in a controlled hospital environment: a 3-year surveillance in southern Italy	Italy	5 Intensive care units	Analyze the presence of molds in the air and on the surfaces of operating theaters and controlled environments	Impaction air sampling, surface sampling with contact plates	Culture-based methods	Surfaces Total Fungi: 0 CFU cm^{-2}–0.35 CFU cm^{-2} *Aspergillus* section *Fumigati* isolated: 20% Air sampling Total Fungi: 80 CFU m^{-3}–187 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 28.6%–76.2%	The most contaminated location in these intensive care units was the oncology pediatric area, largely due to the presence of visitors, animators or family members Areas that have undergone construction or are under reconstruction show higher concentrations of *Aspergillus* in the air, due to the resuspension of particles.	Caggiano *et al.* (2014)
Microbial diversity in bioaerosol samples causing ODTS compared to reference bioaerosol samples as measured using	Denmark	1 Grass seed factory	Check the microbiological communities present in problematic and	Air sampling by personal filtration, collection of dust samples	Culture-based methods, MALDI-TOF, PCR, NGS	*Aspergillus* section *Fumigati* on workers: 1.48×10^6 CFU m^{-3}–2.10×10^6 CFU m^{-3} *Aspergillus* section *Fumigati* on seeds:	Determination of microbiological profiles can be performed when using MALDI-TOF, 98% of the species identified in culture-based methods could be identified through MALDI-TOF.	Madsen *et al.* (2015)

(*Continued*)

Table 2 Continued

Title	Country	Environmental description	Objective	Sampling methods	Analytic methods	Range/Prevalence	Main findings	References
Illumina sequencing and MALDI-TOF			non-problematic seeds			7.50×10^5 CFU m^{-3}–9.00×10^6 CFU m^{-3}		
Occurrence of mycotoxins and yeasts and molds identification in corn silages in tropical climate	Brazil	36 Corn silos	Identify fungi and detect mycotoxins in corn silos	Collection of corn samples	Culture-based methods, Rep-PCR	Total fungi: 2.60 log CFU g^{-1}–3.84 log CFU g^{-1} *Aspergillus* section *Fumigati* isolated: 41.7%	All samples in which *Aspergillus* section *Fumigati* fungal growth was found can be isolated.	Carvalho *et al.* (2016)
Fungal diversity and Aspergillus species in hospital environments	Mexico	2 Hospitals	Assess the fungal diversity present in 2 hospitals	Impaction air sampling	Culture-based methods	Total fungi: 8.53 CFU m^{-3}–85.98 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: $\pm$5%–$\pm$10%	The most abundant species in both hospitals are also clinically relevant The thermal tolerance phenotypic characteristic of species belonging to the *Fumigati* section was confirmed	Martínez-Herrera *et al.* (2016)
High fungal spore burden with predominance of Aspergillus in hospital air of a tertiary care hospital in Chandigarh.	India	1 Tertiary care hospital	Study the mycoflora of areas with A/C and without A/C	Impaction air sampling	Culture-based methods	*Aspergillus* section *Fumigati* spores: 0–63.6 CFU mm^{-3}	The existence of an A/C system did not show any influence on the levels of fungal contaminants A uniform distribution of fungal species was verified throughout the seasons due to the tropical climate of India.	Rudramurthy *et al.* (2016)
Evaluation of Microbiological and Chemical Contaminants in Poultry Farms	Polonia	13 Poultry farms	Assess the chemical and microbiological contamination present in poultry settle dust	Settle dust sampling, gravimetry	Culture-based methods	Total fungi: 1.1×10^5 CFU g^{-1}–3.2×10^6 CFU g^{-1} *Aspergillus* section *Fumigati* isolated: 5.3%	Analyzes of dust, microorganisms and other metabolites suggest that this work environment poses a risk of respiratory complications for its workers It is advisable to monitor the effectiveness of these workers' respiratory protective equipment.	Skóra *et al.* (2016)
Occupational exposure to fungi and particles in animal feed industry	Portugal	1 Feed industry	Characterize fungal exposure in different jobs in the fattening industry	Surface swab, Impaction and impinger air sampling	Culture-based methods, qPCR	Surfaces Total Fungi: 0 CFU m^{-2}–1.8×10^5 CFU m^{-2} *Aspergillus* section *Fumigati* isolated: 6.3% Air sampling Total Fungi: 0 CFU m^{-3}–144 CFU m^{-3} *Aspergillus* section *Fumigati* isolated: 46.6%	Task-focused exposure assessment provides a better exposure assessment, rather than the personal 8-h continuous assessment The particles found are able to reach the distal respiratory system, and these are problematic because they can act as a transport and source of nutrients for pathogenic or toxigenic species,	Viegas *et al.* (2016a)
Azole-resistant Aspergillus fumigatus harboring TR34/L98H, TR46/Y121F/T289A and TR53 mutations related to flower fields in Colombia	Colombia	32 Flower fields	Assess the environmental presence of drug-resistant species	Collection of soil samples	Culture-based methods, analysis of mutations, azole-resistance test	*Aspergillus* section *Fumigati* isolated: 20%[a]	Of the eight triazoles registered for floriculture in Columbia 50% of them had no effect on *Aspergillus* section *Fumigati* The study demonstrates the phenotypic resistance present in this species.	Alvarez-Moreno *et al.* (2017)
Seasonal variation of the dominant allergenic fungal aerosols – One year study from southern Indian region	India	1 University	Investigate the prevalence of 3 fungal species for an entire year	Air sampling by filtration	Culture-based methods, RT-PCR	*Aspergillus* section *Fumigati* (copies of DNA m^{-3}): 18–80	The highest concentrations *of Aspergillus* section *Fumigati* could be seen in seasons with less relative humidity and with stronger winds.	Priyamvada *et al.* (2017)

Environmental Isolates of Multi-Azole-Resistant Aspergillus spp. in Southern Italy	Italy	8 Farms	Increase awareness of *Aspergillus* environmental mutations	Collection of soil, fruit, vegetable and cereal samples	Culture-based methods, PCR, azole-resistance test	*Aspergillus* section *Fumigati*: 0–2.36% *A. fumigatus* sensu stricto: 0%–2.36%	There was a high prevalence of phenotypic resistance in *Aspergillus* not belonging to the *Fumigati* section, indicating that other species considered common may be present in the environment with assigned resistance.	Trovato *et al.* (2018)
Airborne Aspergillus fumigatus contamination in an intensive care unit: Detection, management and control	Belgic	1 Secondary care hospital	Description of an outbreak of *Aspergillus fumigatus*	Surface swab, impaction air sampling	Culture-based methods, MALDI-TOF	Surface swab: 1.0×10^1 CFU cm^{-2}–1.0×10^1 CFU cm^{-2} *Aspergillus* section *Fumigati*: 0 CFU cm^{-2} Air sampling: 1 CFU cm^{-3}–6.4×10^2 CFU cm^{-3} *Aspergillus* section *Fumigati:* $>1 \times 10^1$ CFU m^{-3}	There is a correlation between the number of conidia in the air and the number of cases of invasive aspergillosis. *A. fumigatus* was cultivated from different air samples. In this perspective, dust containing *Aspergillus* spores is difficult to avoid in an indoor setting, therefore air sampling must be done regularly in order to control this risk factor.	Demuyser *et al.* (2019)
Environmental Hotspots for Azole Resistance Selection of Aspergillus fumigatus, the Netherlands	Holland	5 Contamination Hotspots	Find possible sources of multi-resistant *A. fumigatus*	Collection of household and agricultural waste	Culture-based methods, PCR	*Aspergillus* section *Fumigati:* 1.5×10^3 CFU m^{-3}–2.3×10^5 CFU m^{-3}	The risk of developing resistance is considered low in composting environments, even with the presence of azoles. The risk of developing resistance varies according to local conditions, so it is important to maintain a conscious use of fungicides.	Schoustra *et al.* (2019)
Towards a risk evaluation of workers' exposure to handborne and airborne microbial species as exemplified with waste collection workers	Denmark	8 Trucks and 16 Waste collecting workers	Assess and support the possibility to perform a risk assessment based on bioaerosol exposure	Surface swab, air sampling by filtration	Culture-based methods, MALDI-TOF	Air sampling: 1.2×10^3 CFU m^3–1.8×10^5 CFU m^3 *Aspergillus* section *Fumigati*: 6.7×10^4 CFU m^3	Waste collection workers are exposed to a vast number of microbial species. 37 species of fungi were identified, making the risk evaluation hard based on so many species, although it is possible to some degree to evaluate the workers' exposure based on species identification. A. *Fumigatus, A. niger anf P. italicum* shown to be highly inflammogenic species.	Madsen *et al.* (2020)

[a]Article that only assess prevalence.

of PCR for detection in 7 of the 24 studies (Chang *et al.*, 2013; Madsen *et al.*, 2015; Carvalho *et al.*, 2016; Viegas *et al.*, 2016a; Priyamvada *et al.*, 2017; Trovato *et al.*, 2018; Schoustra *et al.*, 2019).

The range of results varied from Below Detection to 9×10^6 CFU m^{-3} for air sampling (Timm *et al.*, 2009; Madsen *et al.*, 2015), 100 to 4.5×10^6 CFU g^{-1} for bulk sampling (Madsen *et al.*, 2012; Queiroz *et al.*, 2013) and Below Detection to 1.8×10^5 CFU cm^{-2} for surface sampling (Viegas *et al.*, 2016a) (**Table 2**).

Discussion

As mentioned, in order to perform a microbiological assessment, two kinds of sampling methods can be employed, the active and passive. The use of these methods has certain advantages and disadvantages. Regarding active methods they can obtain direct estimates of the number of culturable microorganisms in a given sample (Zollinger *et al.*, 2006; Viegas *et al.*, 2015). The volumes and times of sampling can be established and thus provide a more accurate estimate (Zollinger *et al.*, 2006; Viegas *et al.*, 2015). Some active sampling methods also allow for sample dilution which can be an asset in more contaminated environments (Thorne and Heederik, 1999; Viegas *et al.*, 2015). However, if the fungi present are in small numbers they may be misrepresented (Macher, 2001), due to risk of dehydration of microorganisms with the use of some of the active methods (De Nuntiis *et al.*, 2003).

Passive methods are considered necessary to ensure the bioburden assessment (Hayleeyesus and Manaye, 2014; Viegas *et al.*, 2020), to complement the microbiological characterization of the environment disclosing the identification of contamination sources and the assessment of the efficacy of cleaning procedures (Klánová and Hollerová, 2003; Stetzenbach *et al.*, 2004; Viegas *et al.*, 2016b, 2019). When compared with active methods, the passive methods show an advantage in the number of fungal species detected and counts, since they can collect contamination for longer periods (Viegas *et al.*, 2015, 2017), making this kind of sampling procedures an important complementary method to the active approach (Viegas *et al.*, 2016c, 2019). Thus, the usage of passive and active methods coupled together allow a better understanding of the contamination present and an improved characterization of indoor environments (Viegas *et al.*, 2014, 2016b; Caetano *et al.*, 2017; Viegas *et al.*, 2019, 2020). Indeed, active methods provide information about load and the passive methods allow to obtain a detailed information about contamination increasing the accuracy of the exposure assessment and consequently the risk characterization (Viegas *et al.*, 2017).

Regarding the assays to be applied, molecular tools show more reliable results, not only because of their limit of quantification allowing to detect lower values of conidia in the environment (McDevitt *et al.*, 2004), but also, in cases where there are species with very high prevalence's in the environment. Indeed, this situation can lead to the misidentification of species or even the non-identification of less prevalent species (Elliott *et al.*, 2000; Levine and Manders, 2004; Stoodley *et al.*, 2011; Jacovides *et al.*, 2012; Rudkjøbing *et al.*, 2016). The use of these methods, such as PCR provide accurate results achieving sensitivities of 79% and specificities of 94% (Tuon, 2007; Sun *et al.*, 2011; Avni *et al.*, 2012). Additionally, also provide a mean to analyze environmental air samples, allowing to correlate exposure to airborne fungi and the possible adverse health effects (McDevitt *et al.*, 2004). Although these methods present reliable results, they also have some limitations, such as, the requirement of expensive equipment, the possibility of the occurrence of false negatives due to the presence of inhibitors that may be present in the chemical composition of microorganisms or false positives due to the presence of DNA in the environment or in the laboratory (Lauri and Mariani, 2009). An important drawback in occupational exposure assessments is the fact that the molecular tools make no distinction between viable and non-viable microorganisms (Lauri and Mariani, 2009; Pritt, 2015; Madsen *et al.*, 2020).

Culture-based methods, on the other hand are reported to be less sensitive and require a higher time to achieve results than molecular methods (Hope *et al.*, 2005), although they present lower costs and allow the identification and analysis of resistances present in a given sample, making it a good tool for resistance surveillance of *Aspergillus fumigatus* (Montesinos *et al.*, 2017). Culture based-methods are also a reliable tool that allows to assess viable microorganisms in a given environment (Madsen *et al.*, 2020), this being an important factor to take in consideration in an occupational environment due to the higher ability to grow or reproduce that may represent an infectious potential for humans (Altug *et al.*, 2009). Indeed, the cell being viable causes production of gliotoxin, thus enhancing its cytotoxicity (Nouri *et al.*, 2015).

Conclusion

This study brings evidence that in order to have an accurate and reliable *Aspergillus* section *Fumigati* risk characterization, the combined use of active and passive sampling and the analysis of viable and non-viable components are needed.

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Screening of Fungal Azole Resistance in Different Environmental Samples

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Introduction

Fungi are responsible for skin diseases, infections of lung and other organs and bloodstream infections due to its presence in several environments (CDC, 2020b). These environments, where fungi can be present, include clinical sites, agriculture fields, food and feed industries, among others, as well as household environments (Hicks *et al.*, 2005; Park *et al.*, 2010; Cabo Verde *et al.*, 2015).

The fungi most commonly reported by the Centers for Disease Control and Prevention (CDC) are *Aspergillus* sp., *Blastomyces* sp., *Candida* sp., *Coccidioides* sp., *Cryptococcus* sp., *Histoplasma* sp., *Paracoccidioides* spp., *Pneumocystis jirovecii*, *Taloromyces* sp., *Sporothrix* sp. (CDC, 2020b). These fungi are responsible for diseases such as Aspergillosis, Blastomycosis, Candidiasis, Coccidioidomycosis, Cryptococcosis, Histoplasmosis, Pneumocystis and other diseases (NIAID, 2018). Some of these fungi, namely, *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps*, among others, can produce mycotoxins, representing an additional concern for public health (Viegas *et al.*, 2018c).

Although potentially all populations are exposed to fungi, since they are ubiquitous (i.e., present in all environments), fungi can easily cause infections on specific population groups such as elderly, immunocompromised patients and children (Silva, 2010; Jain *et al.*, 2010; Aguiar *et al.*, 2014).

A more recent concern for the public health is the development of fungal resistance to drugs. Such ability is related to the capacity of adaptation of fungi when exposed to drugs (Jensen, 2016). Antimicrobial resistance has been described as product of healthcare practices and their overuse (WHO, 2020). However, antifungals have also been overused in agricultural environments due to the growing need for quality and productivity improvements in food production (Brauer *et al.*, 2019). The use of fungicides in agriculture is also related to climate change and its influence on the distribution of fungi (Viegas *et al.*, 2016). A vast group of drugs used to inhibit fungal growth are azoles. Azoles are molecules that present an azole ring that interferes with biosynthesis of ergosterol (a component of fungal cell membrane), causing its death (Sheehan *et al.*, 1999).

Fungal resistance to azoles has been described as a result of mutations in the genome. Most of these genomic changes are related to inhibition of lanosterol 14α-demethylase enzyme, conferring fungal resistance to drugs that act at the level of ergosterol synthesis of the fungal cell membrane (Marichal *et al.*, 1999; Marie and White, 2009). These mutations in the fungal genome represent a major concern for the management of fungal infections in patients, which can affect various parts of the body. Failure on the treatment of these patients due to fungal resistance can lead to chronic health problems, and even death (CDC, 2020a).

The aim of this review article is to assess methodologies for fungi sampling and screening of azole resistance in environmental samples, as well as to describe the most reported resistant fungi in these environments. This work is important to evaluate the next steps on azole screening, for the development and future application of effective control measures.

Materials and Methods

This study reports the search of available data published between January 1, 2000 and January 31, 2020. The initial search terms were developed to identify studies on the fungal resistance to azoles in different environmental samples. The databases chosen were PubMed, Scopus, and Web of Science (WoS). Searches were carried out in English. The Search terms via PubMed were "fungal resistance in environmental samples" This search strategy led to 71 papers in all databases. Articles that did not meet the inclusion criteria and duplicates were excluded from further analysis (**Table 1**).

The diagram describes the different phases of the selection of papers (**Fig. 1**).

Results

Soil sampling was the most described (14 out of 18 studies) sampling method for the screening of azole resistance in environmental samples (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012; Yang *et al.*, 2012; Astvad *et al.*, 2014; Sharma *et al.*, 2015; Bader

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Table 1 Inclusion and exclusion criteria in the articles selected

Inclusion criteria	*Exclusion Criteria*
Articles in English language	Articles in other languages
Articles published from January 1, 2000 to January 31, 2020	Articles published prior to 2000
Articles related to azole resistance	Articles related to biological samples
Articles related to environmental samples	Articles related to clinical and hospital samples
Scientific original articles on the topic/Journal Articles	Congress abstracts, Reviews, Reports
Identification of fungi to the genera level (at least).	
Articles related to Humans	Articles related to other species

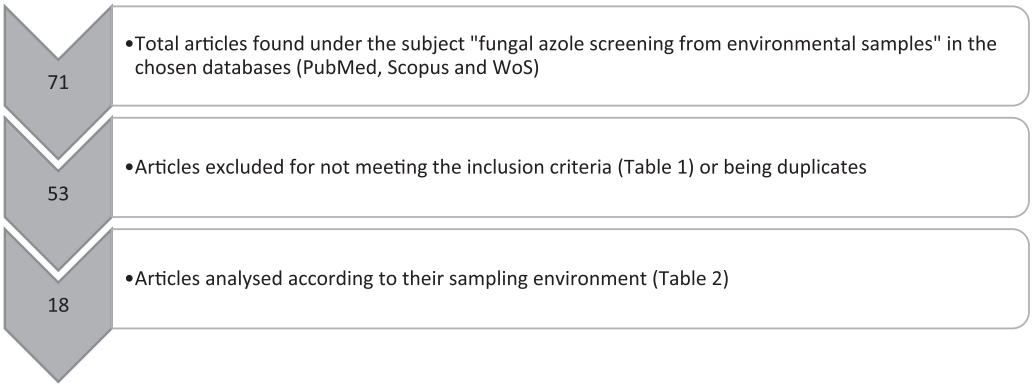

Fig. 1 Phases of the selection of articles.

et al., 2015; Toyotome *et al.*, 2016; Chowdhary *et al.*, 2016; Tangwattanachuleeporn *et al.*, 2017; Santoro *et al.*, 2017; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020). Air sampling was performed with two technique in three out of the 18 analyzed studies (**Table 2**): two by active air sampling with impaction method (Bromley *et al.*, 2014; Wang *et al.*, 2018) and one by collecting air conditioning filters (Viegas *et al.*, 2018a,b,c). In the studies of Bromley and Wang, soil samples were also collected as complementary method (Bromley *et al.*, 2014; Wang *et al.*, 2018). Water sampling was mentioned in one article only (Milanezi *et al.*, 2019).

The screening of azole resistance was performed using four methods, as follows: CLSI M38-A2 broth microdilution in 7 studies (Chowdhary *et al.*, 2012; Yang *et al.*, 2012; Toyotome *et al.*, 2016; Chowdhary *et al.*, 2014a,b; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Milanezi *et al.*, 2019); EUCAST microdilution method in 9 studies (Mortensen *et al.*, 2010; Astvad *et al.*, 2014; Bromley *et al.*, 2014; Bader *et al.*, 2015; Sharma *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Wang *et al.*, 2018; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020); DMI sensitivity test in one study (Santoro *et al.*, 2017); and screening azole on supplemented media in another study (Viegas *et al.*, 2018a,b,c). CLSI M38-A2 broth and EUCAST microdilution reference methods were the most described (16 out of 18 studies).

Among the analyzed studies there were different fungal species tested for azole resistance: *Aspergillus* sp. (2 out of 18) (Bromley *et al.*, 2014; Viegas *et al.*, 2018a,b,c), *Aspergillus fumigatus* (13 out of 18) (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012, 2014a,b; Astvad *et al.*, 2014; Sharma *et al.*, 2015; Bader *et al.*, 2015; Toyotome *et al.*, 2016; Tangwattanachuleeporn *et al.*, 2017; Santoro *et al.*, 2017; Vaezi *et al.*, 2018; Wang *et al.*, 2018; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020), *Aspergillus* section *Nigri and Aspergillus* section *Candidi* (1 out of 18) (Viegas *et al.*, 2018a,b,c), *Candida* spp. (1 out of 18) (Milanezi *et al.*, 2019), *Candida tropicalis* (1 out of 18) (Yang *et al.*, 2012) and *Fusarium solani* (1 out of 18) (Homa *et al.*, 2018).

Only in two studies, assessing soil samples, azole resistance was not detected (Astvad *et al.*, 2014; Toyotome *et al.*, 2016). Azole resistance was detected in most studies (16 out of 18) (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012; Yang *et al.*, 2012; Chowdhary *et al.*, 2014a,b; Bromley *et al.*, 2014; Sharma *et al.*, 2015; Bader *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Santoro *et al.*, 2017; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Wang *et al.*, 2018; Viegas *et al.*, 2018a,b,c; Milanezi *et al.*, 2019; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020). Among these, it was explored in two studies that azole resistance was led by mutations (Chowdhary *et al.*, 2012; Chen *et al.*, 2020).

Fungal resistance to itraconazole was described in 13 studies (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012, 2014a,b; Bromley *et al.*, 2014; Sharma *et al.*, 2015; Bader *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Viegas *et al.*, 2018a,b,c; Vaezi *et al.*, 2018; Wang *et al.*, 2018; Homa *et al.*, 2018; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020). Fungal resistance to voriconazole was described in 13 studies (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012, 2014a,b; Bromley *et al.*, 2014; Sharma *et al.*, 2015; Bader *et al.*, 2015; Tangwattanachuleeporn *et al.*, 2017; Santoro *et al.*, 2017; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Wang *et al.*, 2018; Viegas

Table 2 Data obtained from the chosen articles

Database	*Title*	*Country*	*Environment/samples description*	*Sampling methods*	*Azole resistance analyses methods*	*Main findings*	*References*
PubMed	*Azole susceptibility in clinical and environmental isolates of Aspergillus fumigatus from Eastern Hokkaido, Japan*	Japan	Soil samples from Tokachi area of Eastern Hokkaido	Soil samples	CLSI M38-A2 broth microdilution	From the 91 isolates were susceptible to medical azole. It suggests that TR34/L98H- or TR46/Y121F/T289A-type are not prevalent in the samples area.	Toyotome *et al.* (2016)
	Multi-azole-resistant Aspergillus fumigatus in the environment in Tanzania	Tanzania	Soil and woody debris samples from the surroundings of Kilimanjaro Christian Medical Center, Tanzania	Soil samples	CLSI M38-A2 broth microdilution	Out of the 108 isolates, 4 were found voriconazole resistance and 11 voriconazole and itraconazole resistant. All isolates were also resistant to the 10 azole fungicides tested.	Chowdhary *et al.* (2014a,b)
	First Detection of TR46/Y121F/T289A and TR34/L98H Alterations in Aspergillus fumigatus Isolates from Azole-Naive Patients in Denmark despite Negative Findings in the Environment	Denmark	Soil samples were collected from indoor flowerpots of a university hospital in Copenhagen, from an amusement park in the center of Copenhagen, conventionally grown fields and organic grown fields.	Soil samples	EUCAST method	The azole resistance was not detected in any of the soil samples.	Astvad *et al.* (2014)
	Aspergillus prevalence in air conditioning filters from vehicles: Taxis for patient transportation, forklifts, and personal vehicles	Portugal	Air conditioning filters from vehicles: 19 samples from taxis, 17 samples from forklifts and 37 samples from personal vehicles	Air conditioning filters sampling	Screening in azole-supplemented media	In forklifts filters, *Aspergillus* section *Nigri* was the most prevalent species grown in azole-supplemented media being 76% in 4 mg/L of itraconazole, 43% in 1 mg/L of voriconazole and 0,33% in 0,05 mg/L of posaconazole; In taxis filters, it was only found *Aspergillus* section *Candidi* identified in 4 mg/L of itraconazole. In filters from personal vehicles no *Aspergillus* species were detected.	Viegas *et al.* (2018a,b,c)
Scopus	*Comparison of Human and Soil Candida tropicalis Isolates with Reduced Susceptibility to Fluconazole*	China	Soil samples from agriculture fields; petroleum contained soil; sludge soil and agricultural fields	Soil samples	CLSI M38-A2 broth microdilution	From 11 soil C. tropicalis isolates, 6 were less susceptibility to fluconazole. Soil could be a source of *C. tropicalis* with reduced susceptibility to azole type of antifungal drugs.	Yang *et al.* (2012)

	Occurrence of azole-resistant species of Aspergillus in the UK environment	United Kingdom	Air sampling and soil sampling	Air sampling collected by impaction method; Soil samples	EUCAST method	Azole resistance was only detected in field isolates. These results support the hypothesis that the use of azoles in agriculture may lead to environmental fungi resistance of clinical importance.	Bromley *et al.* (2014)
	Antifungal susceptibility of yeasts isolated from anthropogenic watershed	Brazil	Water samples collected along Guaíba lake course.	Water samples	CLSI M38-A2 broth microdilution	From the 95 isolates, 16.8% were resistant to Amphotericin B. It was possible to identify among the isolates, *Candida* spp. and *Debaryomyces* spp.	Milanezi *et al.* (2019)
	High Azole Resistance in Aspergillus fumigatus Isolates from Strawberry Fields, China, 2018	China	Soil samples from agricultural farms or greenhouses located in 8 cities of China.	Soil core samples	EUCAST method	From 63 soil samples, of 206 *A. fumigatus* isolates, 21 (10.2%) were triazole resistance. Nineteen out of these 21 isolates had mutation on cyp51A gene.	Chen *et al.* (2020)
	Triazole-resistant Aspergillus fumigatus harboring G54 mutation: Is it de novo or environmentally acquired?	India, Romania, Tanzania	Soil and woody debris samples from flowerbeds, parks of hospitals, agricultural fields from Moshi, Tanzania.	Soil and woody debris samples	EUCAST method	From a total of 81 samples, was detected triazole-resistant *A. fumigatus* in 25% of the samples (20/81). In 16 out of these this 20 samples, a resistance mechanism was detected, associated with a mutation.	Sharma *et al.* (2015)
Web of Science	*Clonal Expansion and Emergence of Environmental Multiple-Triazole-Resistant Aspergillus fumigatus Strains Carrying the TR34/L98H Mutations in the cyp51A Gene in India*	India	Soil samples collected from flowerbeds of nurseries, hospital parks, cotton trees, tea gardens, soil containing bird excreta, decayed wood of tree trunks from different regions of India.	Soil samples	CLSI M38-A2 broth microdilution	The results suggest that exposure to *A. Fumigatus* to azole fungicides in the environment causes cross-resistance to medical triazole.	Chowdhary *et al.* (2012)
	Fungicide-driven alterations in azole-resistant Aspergillus fumigatus are related to vegetable crops in Colombia, South America	Colombia	Soil samples from several crops (potatoes, maize, carrots, strawberries, peas, and soil ready to be planted).	Soil samples	EUCAST method	15 samples of *A. fumigatus* grew on agar supplemented with ITZ and VOR. The study evidence the dissemination of azole-resistant *A. Fumigatus*, with high genetic diversity in vegetable crops in Colombia	Alvarez-Moreno *et al.* (2019)

(*Continued*)

Table 2 Continued

Database	Title	Country	Environment/samples description	Sampling methods	Azole resistance analyses methods	Main findings	References
	South Indian Isolates of the Fusarium solani Species Complex from Clinical and Environmental Samples: Identification, Antifungal Susceptibilities, and Virulence	India	Soil and plant parts samples from gardens, parks, yards and agricultural fields in the surroundings of Coimbatore.	Soil samples	CLSI M38-A2 broth microdilution	The first generation triazoles, fluconazole and itraconazole, as expected, were not effective against all isolates tested. The results also lead to the conclusion that the intensive agriculture use of azole compounds, the fusaria has not developed resistance against the imidazole class of antifungals.	Homa *et al.* (2018)
	Pesticide behavior in paddy fields and development of azole-resistant Aspergillus fumigatus: Should we be concerned?	Iran	Soil samples from rice farms around Mazandaran	Soil samples	CLSI M38-A2 broth microdilution	From the results, 31 of the 108 isolates were identified as *A. fumigatus* and 4 were recognized as azole-resistant for itraconazole and voriconazole. The study recognized the pesticides behavior, as tricyclazole in the rice paddy fields, having an effective role in the development of azole-resistance that requires detailed information.	Vaezi *et al.* (2018)
	Prevalence, mechanisms and genetic relatedness of the human pathogenic fungus Aspergillus fumigatus exhibiting resistance to medical azoles in the environment of Taiwan	Taiwan	Air samples and soil samples from hospitals, communities, and farmlands	Air samples collected by impaction method; Soil samples	EUCAST method	From 29 samples, 34 isolates displayed resistance to itraconazole, voriconazole and posaconazole. The results emphasize the need of surveillance where azole fungicides are applied.	Wang *et al.* (2018)
	Environmental Study of Azole-Resistant Aspergillus fumigatus and Other Aspergilli in Austria, Denmark, and Spain	Austria, Denmark, and Spain	Soil samples from park in the center of Copenhagen, compost bags from Denmark, Austria and Spain	Soil samples	EUCAST method	From the Danish soil samples, 4 *A. fumigatus* isolates displayed high azole resistance. One *A. Lentulus* isolate with voriconazole resistance was detected in Spain.	Mortensen *et al.* (2010)
	Prevalence of azole-resistant Aspergillus fumigatus in the environment of Thailand	Thailand	Soil samples from urban and agriculture sites across Thailand	Soil samples	EUCAST method	All the isolates were resistant to itraconazole, 2 showed additional resistance to posaconazole and one other to voriconazole.	Tangwattanachuleeporn *et al.* (2017)

Environmental Isolates of Azole-Resistant Aspergillus fumigatus in Germany	Germany	Soil samples from environment	Soil samples	EUCAST method	All the isolates showed resistance to at least one of the antifungals. The resentence results agree with applied molecular tools.	Bader *et al.* (2015)
Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of Aspergillus fumigatus isolates from organic substrates with special emphasis on compost	Italy, Spain, and Hungary	56 soil samples from meadow/forest	Soil samples	DMI sensitivity testing	It was used 4 DMI fungicides such as: epoxiconazole, difenoconazole, imazilil and posaconazole being imazilil the one with the most antifungal activity followed by posaconazole; Difenoconazole was about 5–7 times less active and epoxiconazole 15–24 times; 3 resistant isolates were tested also for voriconazole e itraconazole being 100-fold less active compared to posaconazole	Santoro *et al.* (2017)

et al., 2018a,b,c; Alvarez-Moreno *et al.*, 2019; Chen *et al.*, 2020). Fungal resistance to posaconazole was described in 11 studies (Mortensen *et al.*, 2010; Chowdhary *et al.*, 2012; Bromley *et al.*, 2014; Bader *et al.*, 2015; Sharma *et al.*, 2015; Santoro *et al.*, 2017; Tangwattanachuleeporn *et al.*, 2017; Homa *et al.*, 2018; Wang *et al.*, 2018; Viegas *et al.*, 2018a,b,c; Chen *et al.*, 2020). Eight studies also described resistance to other antifungal drugs, non-azoles, such as Amphotericin B (Mortensen *et al.*, 2010; Yang *et al.*, 2012; Chowdhary *et al.*, 2014a,b; Bader *et al.*, 2015; Santoro *et al.*, 2017; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Milanezi *et al.*, 2019).

Discussion

This review focus on azole resistance screening in environmental samples. Regarding sampling methodology, the collection of air, water, and soil samples was described in the analyzed studies. Air sampling was performed using the impactor method, which was proven to be the most efficient for fungal assessment in the environment, including in clinical environments (Nesa *et al.*, 2001). The soil sampling appears to be commonly used as a single method for environmental sampling (Toyotome *et al.*, 2016; Chowdhary *et al.*, 2014a,b; Astvad *et al.*, 2014; Yang *et al.*, 2012; Chen *et al.*, 2020; Sharma *et al.*, 2015; Chowdhary *et al.*, 2012; Alvarez-Moreno *et al.*, 2019; Homa *et al.*, 2018; Vaezi *et al.*, 2018; Mortensen *et al.*, 2010; Tangwattanachuleeporn *et al.*, 2017; Bader *et al.*, 2015; Santoro *et al.*, 2017).

In fact, in outdoor environments the fungal load present it the air can be very high (Uddin and Chakraverty, 1994; Shelton *et al.*, 2020). This might explain why some studies only used soil sampling: using the impactor method would request the lowering of the air liters pumped into the plates so they would not be overgrowth. By doing so, the samples collected could not be representative of the microbial load present in these outdoor environments (Yao and Mainelis, 2007). It is important, however, to assess the fungi present in the air in outdoor environments. Impinger methods, due to the use of liquid samples, allow obtaining dilutions to avoid countless growth in media plates, while allowing the use of molecular tools for both fungal molecular detection and further genome sequencing (Viegas *et al.*, 2015, 2018a,b,c).

Regarding the analyzed environments, most of them are related to outdoor environments such as agricultural fields or surrounding parks of hospitals, including flowerbeds and forest/meadow. The similarities between azoles used in the clinical practice and agricultural fungicides present in these environments show a link between fungal resistance and the development of resistance of human fungal pathogens caused by azole pressure in the environment and in clinical practice, increasing public health concerns (Hollomon, 2017).

Studies have shown that vehicle filters also represent a source of fungal contamination (Aquino *et al.*, 2018; Simmons *et al.*, 1997). Fungal contamination of air conditioning filters, either in vehicles or in buildings and resultant health effects represent a matter of concern for both public health and occupational health. While microbial identification (besides bacterial and fungal counts) is important for a more accurate risk characterization, additional assessment of fungal resistance to azoles is important for a better characterization of exposure and health risks in these environments (Viegas *et al.*, 2018a,b,c). For this sampling, the analysis of air conditioning filters turn out to be very efficient, since it is possible to dilute the samples and use molecular methods for fungal quantification and identification, complementary to culture-based methods (Viegas *et al.*, 2015, 2018a,b,c).

An important aspect for fungal proliferation is climate change and anthropogenic actions, which contribute to events such as floods (NCA, 2020). These occurrences help in the spread and transmission of fungi and can be present in wells, puddles or even lakes, also representing a very important environment to be analyzed (OSHA, 2020; Milanezi *et al.*, 2019).

Most of the studies search for azole resistance in fungi with some clinical significance for human health, such as *Aspergillus* spp., *Aspergillus fumigatus*, *Aspergillus* section *Nidulantes*, *Aspergillus* section *Nigri*, *Aspergillus* section *Candidi*, *Candida tropicalis*, *Fusarium solani*, *Penicillium* sp.

Aspergillus spp. was described in many environments and reported worldwide (Gaffi, 2020). The consequences of aspergillosis are very well described not only as allergenic reactions but also other chronic diseases in lungs (Richardson and Rautemaa-Richardson, 2019). The health concern about these genera is exacerbated by the toxigenic species such *Aspergillus flavus* and *Aspergillus fumigatus* (Viegas *et al.*, 2020). *Aspergillus fumigatus* is the most studied *Aspergillus* species for its resistance against the three most used azoles in patient's treatment: itraconazole, voriconazole and posaconazole, specially, in patients with lung diseases (Hamprecht *et al.*, 2018; Morio *et al.*, 2017). Fungal resistance to azoles was also described for *Aspergillus fumigatus* in agricultural samples (Berger *et al.*, 2017). *Aspergillus* spp. and consequent aspergillosis happens to be the larger cause of dead by fungal disease (Latgé, 1999).

Candida spp. appears to also be a very well reported genera for multiple health problems such as bloodstream infections. The *Candida* resistance against fluconazole represents a public health concern (Ben-Ami *et al.*, 2012). All the other studied genera (*Fusarium solani*, *Penicillium* sp.) are related with respiratory tract infections with high reported resistance to azoles (Chowdhary *et al.*, 2016).

According to the sampling methods used to test the susceptibility of fungi to azoles, the methods commonly used are EUCAST (Tudela *et al.*, 2008; Arendrup *et al.*, 2012) and CLSI M38–A2 broth microdilution (Treviño-Rangel and González, 2015; Espinel-Ingroff and Canton, 2008). In these methods, the azoles frequently used to test fungi susceptibility are itraconazole, voriconazole and posaconazole (Chowdhary *et al.*, 2014a,b; Viegas *et al.*, 2018a,b,c) being these azoles used as the first-line treatments in aspergillosis therapy (Mroczyńska *et al.*, 2020; Rudramurthy *et al.*, 2016) and molecularly similar to the azole fungicides used in agricultural practices, for instance, in crop protection (Bowyer and Denning, 2013; Santoro *et al.*, 2017).

Some studies describe a correlation between the resistance of *Aspergillus* spp. to azoles and a mutation in the *cyp51A* gene, which increases the number of aspergillosis infections (Rajendran *et al.*, 2016).

In conclusion, the biggest challenge is to ensure a global surveillance of azole resistance in all the environments, in order to establish an accurate scenario of exposure risk both in the clinic and in the environment. Therefore, more studies in different environments, especially in occupational environments, on sampling and azole resistance testing, are necessary to achieve this goal. Besides broadening sampling methodologies, it is also recommended to use complementary methods for fungal characterization so that it can better describe what exists in these environments. In relation to the studies performed, especially in agricultural environments, it is important to evaluate fungal resistance in more environments to better characterize the fungi present and their resistance to azoles.

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Further Readings

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Assessment of Azole Resistance in Healthcare Facilities

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Introduction

Fungal infections are emerging as an important cause of human disease around the globe (Bongomin *et al.*, 2017). Fungal infections include superficial mycoses, such as pityriasis versicolor, and systemic mycoses, such as aspergillosis. Systemic invasive fungal infections are commonly related to situations where patients are immunocompromised, such as cancer and AIDS patients, during corticosteroid therapies and others (Bongomin *et al.*, 2017; Enoch *et al.*, 2016).

Among the most commonly used antifungal drugs are amphotericin B, 5-flucytosine and azoles (Willey *et al.*, 2008). Azoles are a group of antifungal agents with broad spectrum of activity. They are divided in two main groups, according to the base molecule: imidazole based azoles, such as clotrimazole, ketoconazole, miconazole, and triazole derivatives, such as itraconazole, voriconazole, and posaconazole. Their mechanism of action is focused on the inhibition of the fungal cytochrome P450 3A enzyme, lanosine 14-α-demethylase, blocking the conversion of lanosterol into ergosterol, resulting in a depletion of this compound, which alters the fluidity of the fungal membrane (Rang *et al.*, 2016).

During recent years there has been an increase of resistance to the available antifungal agents, becoming a significant public health concern especially among individuals at higher risk of opportunistic fungal infections such as immunocompromised individuals (Barker and Rogers, 2006; Wiederhold, 2017). Azole resistance may develop in consequence of fungal exposure to azole compounds, which may occur during a patient's treatment with these antifungal drugs or through the use of azole fungicides similar to clinical azole drugs, such as in agriculture (Snelders *et al.*, 2008). Among acquired antifungal resistance, the azole resistance has been noticeable increasing, being found in isolates of *Candida* and *Aspergillus*, mostly *A.* section *Fumigati* (Wiederhold, 2017).

Healthcare facilities must provide a controlled environment in order to guarantee the security of patients and avoid the dissemination of potentially pathogenic airborne contaminants. Thus, indoor air quality must be regularly monitored and controlled in healthcare facilities, and must also include a rigorous biological assessment, in order to be able to characterize the risk of contamination by pathogenic microorganisms for patients, staff, and visitors (Qudiesat *et al.*, 2009).

The present study aims to perform a scope review of the assessments of fungal resistance to azole drugs performed on healthcare facilities' indoor environments in the last decade and to suggest a methodological approach for the screening of fungal resistance in those environments.

Materials and methods

In this study, available data on azole resistance in healthcare facilities' indoor environments, including hospitals and primary healthcare centers, published between January 1, 2000 and October 31, 2019 was searched, following the PRISMA methodology. The initial search terms were chosen to identify studies on the assessment of azole resistance in healthcare facilities' environments, focusing on fungal sampling and screening of azole resistance. The databases chosen were PubMed, Scopus, and Google Scholar. Searches were carried out in English. The Search terms were "Azole Resistance" and "Hospital Indoor Environments". This search strategy led to nearly 125 papers in all databases. Articles that did not meet the inclusion criteria and duplicates were excluded from further analysis (**Table 1**).

The diagram describes the different phases of the selection of papers (**Fig. 1**).

Results

A total of 12 articles were analyzed regarding the environmental setting (hospitals or other healthcare facilities), sampling methods, fungal identification methods, type of azole resistance identified (and the used methods, when possible), and the main conclusions of each study.

Most studies described the assessment of azole resistance in hospital environments (11 out of 12), the exception being one study performed in primary health care centers (Caetano *et al.*, 2019). Six studies mentioned indoor and outdoor sampling for comparison purposes (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019), and three studies compared the resistance phenotypes in environmental and clinical samples (Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019).

Air sampling was the most frequent sampling method (10 out of 12 studies) (Panagopoulou *et al.*, 2007; Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; San Juan *et al.*, 2017; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019;

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Table 1 Inclusion and exclusion criteria in the articles selected

Inclusion Criteria	*Exclusion Criteria*
Articles published in the English language;	Articles published in other languages
Articles published from 2000 to present	Articles published prior to 2000
Articles published in any country	
Articles related to fungi assessment in healthcare institutions environments	Articles related exclusively to biologic samples from patients
Articles related to hospital environments including other healthcare facilities	Articles containing information exclusively from non-health related environments
Original scientific articles on the topic	Abstracts of congress, reports, reviews/ state of the art articles
Identification of fungi to the genera level (at least)	

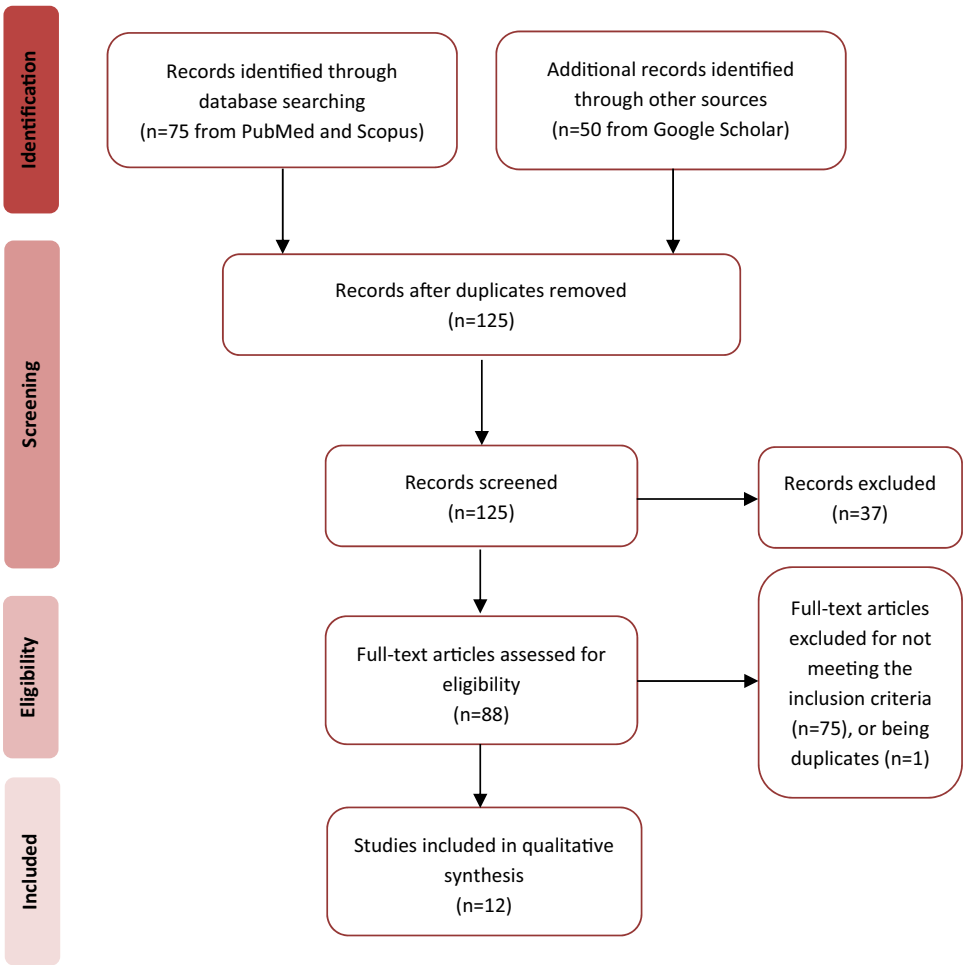

Fig. 1 PRISMA based selection of articles.

Monteiro *et al.*, 2019; Viegas *et al.*, 2019a). Air sampling was the only sampling method used in five studies (San Juan *et al.*, 2017; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019), and used in parallel with passive sampling methods in other five studies (Panagopoulou *et al.*, 2007; Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Viegas *et al.*, 2019a).

Passive sampling methods used for the assessment of fungal contamination in the analyzed studies were water sampling (Panagopoulou *et al.*, 2007; Snelders *et al.*, 2009; Ahmad *et al.*, 2014), surface swabs (Panagopoulou *et al.*, 2007; Savastano *et al.*, 2016; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Viegas *et al.*, 2019a), soil sampling (Snelders *et al.*, 2009), settled dust (Caetano *et al.*, 2019; Viegas *et al.*, 2019a), electrostatic dust collector (EDC) (Caetano *et al.*, 2019; Viegas *et al.*, 2019a), HVAC filter sampling (Caetano *et al.*, 2019), and staff hands and lab coats (Savastano *et al.*, 2016).

Regarding methods of fungal identification, all studies performed morphological identification. One study on *Candida* sp. performed physiological tests additional to morphological identification (Savastano *et al.*, 2016). Eight studies used molecular detection of fungal DNA in parallel with morphological identification (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Loeffert *et al.*, 2018; Cho *et al.*, 2019; Chen *et al.*, 2019; Monteiro *et al.*, 2019; Viegas *et al.*, 2019a).

Most of the analyzed scientific articles (10 out of 12) explicitly included terminology related to "resistance" or "azole-resistance" (Snelders *et al.*, 2009; Caetano *et al.*, 2019; Ahmad *et al.*, 2014; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Monteiro *et al.*, 2019) or "antifungal susceptibility" (Panagopoulou *et al.*, 2007; Mohammadi *et al.*, 2016; San Juan *et al.*, 2017; Cho *et al.*, 2019; Monteiro *et al.*, 2019) in the title, of which five specified *Aspergillus fumigatus* (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Loeffert *et al.*, 2018; Monteiro *et al.*, 2019) and three *Aspergillus* sp. (San Juan *et al.*, 2017; Chen *et al.*, 2019; Cho *et al.*, 2019).

Seven studies referred to CLSI protocols to determine azole-resistance (Panagopoulou *et al.*, 2007; Snelders *et al.*, 2009; Savastano *et al.*, 2016; Ahmad *et al.*, 2014; San Juan *et al.*, 2017; Chen *et al.*, 2019; Cho *et al.*, 2019), and five studies used the use of EUCAST guidelines (Caetano *et al.*, 2019; Mohammadi *et al.*, 2016; Loeffert *et al.*, 2018; Monteiro *et al.*, 2019; Viegas *et al.*, 2019a).

Aspergillus fumigatus was the most commonly described fungal species (7 out of 12 studies) (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019), with phenotype of azole resistance confirmed in five studies (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019), and one study reporting all *Aspergillus fumigatus* isolates, although not azole-resistant, susceptible to voriconazole and itraconazole at very high MIC's (Loeffert *et al.*, 2018). Other *Aspergillus* sp., reported as azole-resistant or with reduced susceptibility to azoles at tested concentrations, were *A. niger* (San Juan *et al.*, 2017; Cho *et al.*, 2019), *A. flavus* (Cho *et al.*, 2019), *A. versicolor* (Viegas *et al.*, 2019a), and *Aspergillus* cryptic species (Monteiro *et al.*, 2019). Regarding the evaluation of azole resistance of other fungal species, two studies described the occurrence of filamentous fungi able to grow in triazoles in Primary Healthcare Centers (Caetano *et al.*, 2019) and in one central hospital (Viegas *et al.*, 2019a), and another study the occurrence of azole resistance among several non-*albicans Candida* species (Savastano *et al.*, 2016).

Itraconazole-resistance was the most common type of azole resistance (6 out of 12 studies) referent to *A.* section *Fumigati* (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Loeffert *et al.*, 2018; Monteiro *et al.*, 2019), *A.* section *Nigri* (San Juan *et al*, 2017) and non-*Aspergillus* species (Caetano *et al.*, 2019), followed by multiazole-resistance (5 out of 12 studies) referent to *A.* section *Fumigati* (Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019) and non-*Aspergillus* species (Savastano *et al.*, 2016; Viegas *et al.*, 2019a). Reduced susceptibility to voriconazole was mentioned in three studies referent to *A.* section *Fumigati* (Loeffert *et al.*, 2018), *A.* section *Versicolores* (Viegas *et al.*, 2019a) and non-*Aspergillus* species (Caetano *et al.*, 2019), whereas reduced susceptibility to posaconazole or other azole drugs were mentioned in four studies referent to *A.* section *Fumigati* (Monteiro *et al.*, 2019) and non-*Aspergillus* species (Caetano *et al.*, 2019; Savastano *et al.*, 2016; Viegas *et al.*, 2019a).

Of the six studies that used molecular methods, five targeted fungal DNA mutations related to azole-resistance, namely, mutations among the TR_{34}/L98H codon (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Chen *et al.*, 2019; Monteiro *et al.*, 2019). In four of these studies mutations associated with azole resistance were effectively detected and related either with itraconazole-resistance or with multiazole-resistance (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Cho *et al.*, 2019; Chen *et al.*, 2019). Among all articles that described azole resistance, only one study described azole resistance non-related to any CYP51A gene mutation (Monteiro *et al.*, 2019). The occurrence of mutations with no phenotype of azole-resistance was not reported in the analyzed studies (**Table 2**).

Discussion

Hospital-acquired infections represent an important issue of public health and are repeatedly the cause of morbidity and mortality for patients admitted to hospitals. The bioburden in the hospital environment is unsurprisingly vast and includes opportunistic pathogens that originate not only indoor from patients, relatives or healthcare practitioners, but also from outdoor environmental sources being disseminated indoor (Viegas *et al.*, 2019a). In particular, exposure to mycobiota in indoor environments has been related to several adverse human health effects (Douwes *et al.*, 2003; Alberti *et al.*, 2001). *Aspergillus* sp. and *Candida* sp. have been the most frequently isolated agents in clinical samples corresponding to a high burden of the hospital-acquired fungal infections with high mortality (Nicolle *et al.*, 2011; Snelders *et al.*, 2009; Cho *et al.*, 2019; Savastano *et al.*, 2016). Increasing research on mycology and fungal infections in past years has clarified the role of a growing number of fungal species in different health conditions such as *Fusarium* and *Penicillium* spp., or *Alternaria* and *Cladosporium* spp. (Chowdhary *et al.*, 2016), which are fungal species commonly found in indoor environments, namely, hospitals and healthcare facilities (Caetano *et al.*, 2019; Viegas *et al.*, 2019a).

The transmission occurs in different ways according to the microorganism involved. *Aspergillus* sp. are ubiquitous saprophytic fungi and their conidia easily inhaled, while person-to-person transmission is unlikely. *Candida* spp. infections are primarily of an endogenous origin, but transmission may also occur through hands of staff or visitors, or through hospital devices (Savastano *et al*, 2016). Furthermore, the emergence of antifungal resistance is an important concern related to almost all main groups of pathogenic microorganisms, mostly described for *Aspergillus* section *Fumigati* (Snelders *et al.*, 2011; Verweij *et al.*, 2016; Fisher *et al.*, 2018) and non-*albicans Candida* species (Mar *et al.*, 2000; Savastano *et al.*, 2016). Azole resistance can develop by means of fungal exposure to azole compounds, which may occur in azole-treated patients or through the use of azole compounds in the

Table 2 Data obtained from the chosen articles

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Fungal identification*	*Azole resistance*	*Results*	*Conclusions*	*References*
Google Scholar	Filamentous Fungi in a Tertiary Care Hospital Environmental Surveillance and Susceptibility to Antifungal Drugs	Greece	One hospital, indoor	Air sampling, water sampling, surface swabs	Morphological identification	*CLSI reference method*	All 54 *Aspergillus* air isolates exhibited relatively low minimum inhibitory or effective concentrations for amphotericin B, itraconazole, voriconazole, posaconazole, micafungin, and anidulafungin.	Air and surface fungal loads vary in different departments. Environmental *Aspergillus* isolates lacked resistance to clinically important antifungal agents.	Panagopoulou *et al.* (2007)
	Possible Environmental Origin of Resistance of *Aspergillus fumigatus* to Medical Triazoles	Netherlands and USA	One hospital, indoor and outdoor	Air and water sampling (culture collection), soil sampling	Morphological and molecular identification	*CLSI reference method* *Microsatellite genotyping*	Five out of 248 (2%) indoor and six out of 49 (12%) outdoor *A. fumigatus* isolates grew on itraconazole. TR/L98H mutation related to itraconazole resistance (86%) with environmental and clinical azole-resistant isolates clustering together.	Invasive aspergillosis due to azole-resistant *A. fumigatus* might be caused by the acquisition of the resistant fungi, present in the hospital environment.	Snelders *et al.* (2009)
	Assessment of Azole Resistance In Clinical Settings by Passive Sampling	Portugal	Ten Primary Healthcare Centers, indoor	EDC sampling, HVAC filter sampling, settled dust	Morphological identification	*EUCAST reference method (adapted)*	No *Aspergillus* sp. grew on azole-media. Other filamentous fungal species grew on itraconazole (n = 9), voriconazole (n = 6), and posaconazole (n = 3) considering all sampling methods.	Passive sampling allows quantitative and qualitative characterization of fungal burden. EDC sampling provided the best characterization of fungal species diversity and should be included in the assessment of fungal resistance.	Caetano *et al.* (2019)
	Candida glabrata among *Candida* spp. from environmental health practitioners of a Brazilian Hospital	Brazil	One hospital, indoor	Surface swabs, hands and lab coats	Morphological identification and physiological tests	*CLSI reference method*	Resistant isolates of *C. albicans* were not found. All isolates of non-*albicans Candida* exhibited decreased susceptibility to miconazole and itraconazole, but susceptible to nistatin.	Surveillance programs are important to evaluate the trends of *Candida* species and their resistance profiles to antifungal drugs commonly used in medical practice.	Savastano *et al.* (2016)
PubMed	Occurrence of triazole-resistant *Aspergillus fumigatus* with TR34/L98H mutations in outdoor and hospital environment in Kuwait	Kuwait	One hospital, indoor and outdoor	Air and water sampling, surface swabs	Morphological and molecular identification	*CLSI reference method*	7% *A. fumigatus* isolates from indoor samples were resistant to itraconazole. TR_{34}/L98H mutation detected in all itraconazole-resistant isolates.	Triazole-resistant *A. fumigatus* with TR_{34}/L98H mutations in *cyp51A* is prevalent in Kuwait. The presence of triazole-resistant *A. fumigatus* in the hospital environment is a risk for susceptible patients.	Ahmad *et al.* (2014)

Determination of antifungal susceptibility patterns among the environmental isolates of *Aspergillus fumigatus* in Iran	Iran	Three hospitals, indoor	Air sampling, surface swabs	Morphological and molecular identification	*EUCAST reference method*	No azole-resistant isolates found among environmental isolates of *A. fumigatus.*	There were no azole-resistant fungi among environmental isolates obtained from these hospitals.	Mohammadi *et al.* (2016)
In vitro susceptibility of Cuban *Aspergillus* spp. strains of clinical and environmental origin	Cuba	Hospital, indoor	Air sampling	Morphological identification	*CLSI reference method, Etest*™	*A. niger* (5% of all isolates) was itraconazole-resistant.	Resistance to azoles presented low-frequency.	San Juan *et al.* (2017)
Prospective survey of azole drug resistance among environmental and clinical isolates of *Aspergillus fumigatus* in a French University hospital during major demolition works	France	One hospital – indoor and outdoor, and surveillance of invasive aspergillosis cases	Air sampling	Morphological and molecular identification	*EUCAST reference method (adapted*)	No *A. fumigatus* isolates were azole-resistant, although all exhibited susceptibility to voriconazole and itraconazole at very high MIC's.	Although no azole-resistant isolates were found, the presence of isolates susceptible to azoles at high MIC's alerts to the potential role of contaminated environments as sources of azole-resistant invasive aspergillosis	Loeffert *et al.* (2018)
Azole resistance in *Aspergillus* species in Southern Taiwan: an epidemiological surveillance study	Taiwan	One hospital, indoor and outdoor, *Aspergillus* clinical isolates, proximate farmlands	Air sampling	Morphological and molecular identification	*CLSI reference method*, Vipcheck™	10,2% of *A. fumigatus* isolates multiazole-resistant. 81% azole-resistant isolates contained the TR34/L98H mutation.	The presence of R34/L98H and TR46/Y121F/T289A isolates in hospital wards suggests that azole-resistant *A fumigatus* transmission could have a nosocomial origin.	Chen *et al.* (2019)
Epidemiology and antifungal susceptibility profile of *Aspergillus* species: comparison between environmental and clinical isolates from patients with hematologic malignancies	South Korea	One hospital, indoor/ outdoor, and *Aspergillus* clinical isolates	Air sampling	Morphological and molecular identification	*CLSI reference method*	Resistance rates to any azole: *Aspergillus fumigatus* 7%, *A. niger* 4%, and *A. flavus* 5%. Different TR34/L98H mutations were found in *A. fumigatus* azole-resistant isolates.	The azole resistance rate was not different between clinical and environmental isolates.	Cho *et al.* (2019)

(*Continued*)

Table 2 Continued

Database	*Title*	*Country*	*Environment description*	*Sampling methods*	*Fungal identification*	*Azole resistance*	*Results*	*Conclusions*	*References*
	Aspergillus species collected from environmental air samples in Portugal-molecular identification, antifungal susceptibility and sequencing of cyp51A gene on *A. fumigatus sensu stricto* itraconazole-resistant	Portugal	Two hospitals, indoor/ outdoor,and one waste water plant	Air sampling	Morphological and molecular identification	*EUCAST reference method*	*A. fumigatus sensu stricto* isolates*:* 48 isavuconazole-, 12 itraconazole- and 21 posaconazole-resistant. *Aspergillus* cryptic species: almost all were resistant to at least three azoles. None of the environmental isolates showed mutations on the *cyp51A* gene.	Most environmental isolates were *A. fumigatus sensu stricto*, and some were azole-resistant. The environment presence of cryptic species can be a risk factor for outpatients, increasing the probability of fungal infection while they are in ambulatory treatment.	Monteiro *et al.* (2019)
	Exposure assessment in one central hospital: A multi-approach protocol to achieve an accurate risk characterization	Portugal	One hospital, indoor	Air sampling, EDC sampling, settled dust, surface swabs	Morphological and molecular identification	*EUCAST reference method (adapted)*	*A. versicolor* and two other filamentous fungal species grew on voriconazole. *Penicillium* sp. grew on itraconazole and posaconazole, and *Chrysosporium* sp. on posaconazole.	Fungal contamination of indoor environments of hospitals and consequent exposure to less susceptible or resistant isolates may lead to complications in the management of fungal infections	Viegas *et al.* (2019a)

environment. If azole-resistant fungi are present in hospital environment, patients can potentially inhale resistant spores and consequently acquire an azole-resistant infection (Snelders *et al.*, 2009). This is particularly important in immunocompromised patients, more susceptible to developing invasive fungal infections (Snelders *et al.*, 2009; Mohammadi *et al.*, 2016; Cho *et al.*, 2019; Monteiro *et al.*, 2019; Savastano *et al.*, 2016). Therefore, the surveillance of azole-resistance in the hospital environment is of upmost importance so that healthcare facilities are prepared to control dissemination sources and provide a secure environment for their occupants (Caetano *et al.*, 2019).

In this review it was verified that the assessment of azole-resistance in the hospital environment is still incipient, as perceived by the limited number of studies (n = 12) published in the last decade, in spite of this environment being commonly related to infection acquisition (Wilson, 2018). In addition, the evaluation of azole resistance in the hospital environment in genera other than *Aspergillus* is not common, which makes it difficult to understand the extent of the azole resistance problem in this environment. In order to assess the risk of acquiring azole-resistant fungal infections in healthcare facilities, evaluations of the hospital indoor environment are crucial (Caetano *et al.*, 2019) and further studies on surveillance of azole resistance both in clinical isolates and in the environment are recommended (Annon, 2017; Talbot *et al.*, 2018; Chen *et al.*, 2019).

The most common microbial sampling strategy used in indoor environments is air sampling, either by active methods such as impaction or impinger, or passive air sampling by surface swabbing, dust collection, settled plates or electrostatic dust collectors. In the 12 analyzed studies different sampling strategies were used. Active air sampling, the gold standard to evaluate indoor air as it is easily standardized and comparable, was the strategy used in the majority of the analyzed studies (10 out of 12), of which five used it exclusively (San Juan *et al.*, 2017; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019). The parallel use of active and passive air sampling methods was described in five studies (Panagopoulou *et al.*, 2007; Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Mohammadi *et al.*, 2016; Viegas *et al.*, 2019a), and two studies resorted to passive sampling only (Savastano *et al.*, 2016; Caetano *et al.*, 2019). Since active air sampling methods allow the collection of contamination load only over a short period of time (*e.g.*, minutes), and passive air sampling methods collect contamination load over a long period of time (from days to several months), the combination of both can provide a more precise and in-depth assessment of the environment and be used for a more accurate evaluation of exposure risks (Viegas *et al.*, 2019b). Moreover, passive air sampling is more economic and less intrusive, the drawback being the difficulty in comparing results obtained by different means (Méheust *et al.*, 2014). Considering the hospital environment, the different activities performed there and limited access to specific areas, such as intensive care units or transplantation areas, it is easily understandable that less intrusive sampling methods are advantageous, as they do not require the presence of an external individual during long-term sampling campaigns. Noteworthy, passive air sampling may also enable the assessment of a broader range of mycobiota (Viegas *et al.*, 2019a) or evaluate microbial contamination in critical sources involved in infection transmission such as hands (in *Candida* sp. transmission) (Savastano *et al.*, 2016) or HVAC filters (in reaerosolization of fungal spores) (Caetano *et al.*, 2019) and, thus, better predict exposure risks. Therefore, a sampling strategy for hospital environments should consider more than one sampling method (Caetano *et al.*, 2019; Viegas *et al.*, 2019a).

Culture-based methods are of upmost importance for the measurement of viable microbial contamination and prediction of clinical significance being crucial in exposure assessment studies in health care facilities (Järvi *et al.* 2018). Interestingly, a significant number of studies resorted to molecular methods for fungal identification complementary to conventional culture-based methods (n = 8). Considering the growing role of molecular methods in species recognition, the "*Aspergillus* Systematics in the Genomic Era" working group proposed specific recommendations that include the use of ITS regions for inter section level identification and the β-tubulin locus for identification of individual species in the various *Aspergillus* sections (Balajee *et al.*, 2007). Fungal identification should, thus, rely both on culture-based and on molecular methods as closely related species with similar morphology (also referred to as cryptic species) are only distinguishable at the molecular level, while presenting variable antifungal susceptibility profiles (Lamoth, 2016; Cho *et al.*, 2019). For example, while resistance to triazoles is rare among *A. fumigatus sensu stricto*, decreased susceptibility to azoles and other antifungal agents is often observed for cryptic species (Alastruey-Izquierdo *et al.*, 2014). Moreover, in environmental samples with composite microbial burden, the competition for nutrients and the advantage of some species over others regarding media composition and incubation conditions (*e.g.*, moisture, temperature) may hinder the visible growth of important fungi with toxigenic or pathogenic potential, thus, representing an increased risk in health care facilities (Caetano *et al.*, 2019).

Since it was postulated that azole resistance has an environmental origin, as azole-resistant aspergillosis was reported in azole-naïve patients (Verweij *et al.*, 2002; Dannaoui *et al.*, 1999; Snelders *et al.*, 2009), the search for azole-resistance in the environment increased. In this review, six studies investigated the presence of azole resistance indoor and outdoor the hospital environment (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Cho *et al.*, 2019; Monteiro *et al.*, 2019). Noteworthy, five of them concluded that the presence of isolates from the *Fumigati* section of *Aspergillus* sp. that were triazole-resistant or susceptible to azoles at high MIC's in the hospital environment or it surroundings was a risk for susceptible patients (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Loeffert *et al.*, 2018; Chen *et al.*, 2019; Monteiro *et al.*, 2019). It appears that the development of resistant fungal isolates can be related to the widespread of fungicides in agricultural practices for plant protection and in material preservation such as paint, paper and wood, while it is also common in mattresses. Isolates that develop resistance to fungicides might be cross-resistant to medical triazoles (Verweij *et al.*, 2016). Azole resistance can also be either specific to the microorganism (*e.g.*, innate resistance of Mucorales order to voriconazole Caramalho *et al.*, 2017) or acquired by previously susceptible microorganisms in the course of therapy. Antifungal resistance can be microbiologic, clinical or both. Microbiologic resistance occurs when the fungal growth is inhibited by a much higher concentration of an antifungal compound in comparison to the range seen with wild-type strains, whereas clinical antifungal resistance is defined as when the concentration of an antifungal

agent is associated with a higher risk of therapeutic failure. When it is both clinical and microbiologic, the resistance is present when the isolates are not inhibited by the usual concentrations of an antifungal compound, with regular dosage schedules and/or when they demonstrate MIC's (minimum inhibitory concentrations) that fall within the range where it is likely to be present a specific microbial resistance, and the clinical efficacy against the isolate is not reliably shown in studies (Pfaller, 2012).

Present treatment options of fungal infections include polyenes, echinocandins, and triazoles. Azole resistance in clinical isolates of *A.* section *Fumigati* with *in vitro* resistance to itraconazole and voriconazole is increasing over the recent years and is a concern in the management of invasive aspergillosis (Snelders *et al.*, 2011). Azoles inhibit the ergosterol biosynthesis pathway through the inhibition demethylation of sterol 14-a-demethylase (CYP51). Azole resistance is commonly due to mutations in cyp51A gene target for azole antifungals (Snelders *et al.*, 2008). This resistance mechanism was recovered both in clinical and environmental samples (soil, compost, seeds, air, water, surface swabs) in four analyzed studies (Snelders *et al.*, 2009; Ahmad *et al.*, 2014; Chen *et al.*, 2019; Cho *et al.*, 2019), with one study identifying multi-azole-resistant environmental isolates from two hospitals and one waste water plant with no mutation on the cyp51A gene (Monteiro *et al.*, 2019). The mutations related to azole resistance found in these studies, located at the TR34/L98H and the TR46 codons in the CYP51A gene, have also been reported as some of the most commonly found mutations in the environment (Güngör *et al.*, 2018; Wiederhold *et al.*, 2016). This might indicate that the mutations found in the fungal species in hospital environments may come from the outdoor environment.

Standard antifungal susceptibility tests have been described for the detection of azole resistance in a reduced number of fungal species, including *Aspergillus* and *Candida* spp., with the Clinical and Laboratory Standards Institute (CLSI) (used in 7 studies) and the European Committee on Antimicrobial Susceptibility Testing (antifungal susceptibility testing [AFST]-EUCAST) (used in 5 studies). These guidelines for *in vitro* triazole testing in standardized conditions are mainly applied for the analysis of clinical isolates (EUCAST, 2019). Since *in vitro* triazole screening is not routinely performed in environmental isolates comprehensive studies on the antifungal susceptibility patterns are lacking. Moreover, these guidelines do not allow extensive analysis of complex and multi-contaminated samples, such as environmental samples.

The majority of the studies assessed the azole resistance among *Aspergillus* species, mainly from the *Fumigati* section. Further assessments of azole-resistance in clinical environments in other genera will provide important information to understand the extension of azole resistance in this type of environment. Comparing the articles, from 2007 to 2019, there has been an increase in the fungal species identified inside healthcare facilities' indoor environments that also displayed azole resistance or susceptibility to azoles only at high MIC's. The sophistication of microbiological and molecular methods of identification allied to a wide diversity of sampling methods will unveil a better characterization of the environmental bioburden as well as the contamination risk assessment in healthcare institutions. A better understanding of both factors is crucial for healthcare institutions to implement more effective practices and adequate cleaning and hygienization procedures to ensure the quality of their environment as well as the reduction of the risk of drug resistant infections.

Overall, the presence of azole-resistant fungi in the clinical environment and its relation with hospital-acquired azole-resistant infections in susceptible patients has been demonstrated and alert for the need of more research and environmental sampling, in addition to international surveillance studies of clinical isolates, in order to determine and track the spread of azole resistance in the environment.

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Climate Change and Aflatoxins Contamination in the Iberian Peninsula

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Introduction

It has been generally recognized by researchers and regulators that the expected climate change constitutes an important driver that will affect food sector, representing a public health issue that deserves particular attention. In one hand, climate change threatens our ability to ensure global food security, eradicate poverty and achieve sustainable development (Berbegal *et al.*, 2019). Climate change is associated with increasing temperatures and more extreme rainfall; it alters relationships among crops, pests, pathogens, and weeds; and it exacerbates several trends including declines in pollinating insects, increasing water scarcity, increasing ground-level ozone concentrations, and fishery declines (Myers *et al.*, 2017). Agricultural productivity is affected by changing rainfall patterns, drought, flooding and the geographical redistribution of pests and diseases (Anderson *et al.*, 2020), with consequent implications in the food availability, a key requirement for food security (**Fig. 1**).

On the other hand, despite less debated, climate change could also affect food safety (Miraglia *et al.*, 2009) (**Fig. 1**), namely through the occurrence of food safety hazards at various stages of food chain, from "farm to fork". The tendency to increase the use of agrochemicals to balance the effects of more frequent extreme weather events and water scarcity in some regions could become more frequent. In addition to pesticide residues, both chemical and microbiological risks are expected to impair food and feed safety as a consequence of climate change: in particular mycotoxins, marine biotoxins (phycotoxins), trace metals, among others (Miraglia *et al.*, 2009; Tirado *et al.*, 2010; Assunção *et al.*, 2018b; FAO, 2020).

Among the issues in food safety, the rise of crop diseases due to toxigenic fungi and the contamination of crops by toxic secondary metabolites – mycotoxins – constitute aspects of major importance (Moretti *et al.*, 2019). Mycotoxins are produced by different types of fungus belonging primarily to genera *Aspergillus*, *Penicillium*, and *Fusarium*. Although these low molecular weight toxic compounds occur worldwide and represent a global public and animal health threat, their prevalence and concentrations in food and feed may vary due to geographic and climatic differences, posing a serious risk to human and animal health worldwide (Ksenija, 2018; Moretti *et al.*, 2019). The mycotoxins of greatest concern to food and feed safety include aflatoxins, fumonisins, ochratoxins, trichothecenes, and zearalenone.

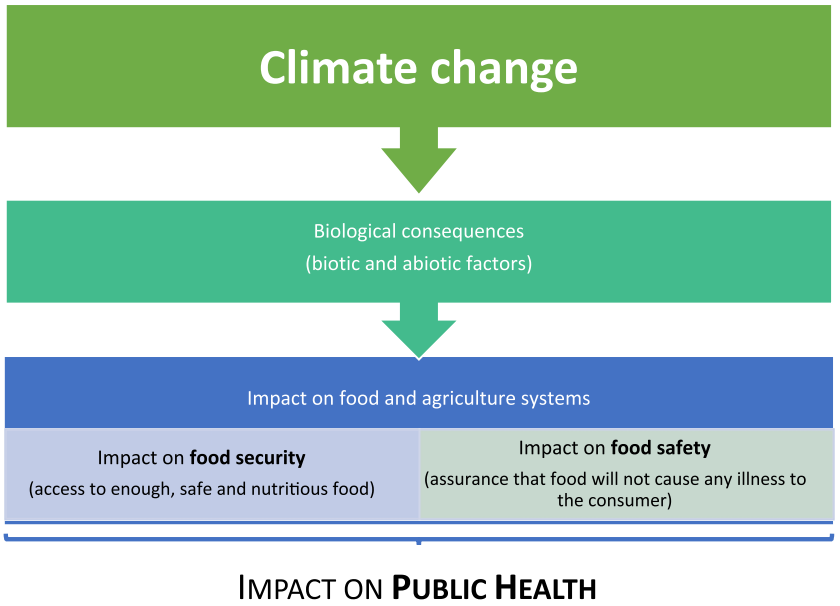

Fig. 1 Impact of climate change on food and agriculture systems.

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Table 1 Maximum permitted levels of mycotoxins in food and feed in the EU

	Foodstuffs	*Maximum levels (μg/kg)*[a]	*Animal feed*	*Maximum levels (mg/kg)*[b,c]
Aflatoxin B1	Cereals, cereal-based products, nuts/dried fruits, spices	2.0–12.0	Feed materials, complementary and complete feed	0.005–0.02
Sum of Aflatoxins B1, B2, G1, and G2		4.0–15.0		
Aflatoxin M1	Milk and milk products	0.050		
Aflatoxin B1	Foods intended for infants and young children (e.g., cereal-based foods)	0.10		
Aflatoxin M1		0.025		

[a]Regulation (EC) 1881/2006;
[b]Directive 2002/32/EC;
[c]Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12%.
Note: Adapted from European Commission, 2002. DIRECTIVE 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Official Journal of the European Union L140, 10–21. Available at: http://eur-lex.europa.eu/resource.html??uri=cellar:aca28b8c-bf9d-444f-b470-268f71df28fb.0004.02/DOC_1-&format=PDF. European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. Available at: http://eur-lex.europa.eu/legal-content/en/ALL/??uri=CELEX:32006R1881 (accessed 10.04.20).

Aflatoxins, the most toxic of mycotoxins, are genotoxic, carcinogenic and immunosuppressive substances, and cause both acute and chronic toxicity (Wu *et al.*, 2014). The four major aflatoxins known are B_1, B_2, G_1, and G_2. Aflatoxin B_1 (AFB_1) is the most potent naturally occurring chemical liver carcinogen known, being considered a significant risk factor for hepatocellular carcinoma (HCC) and classified as Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2012). Additionally, when animals that are intended for dairy production consume aflatoxin-contaminated feed, a metabolite, aflatoxin M1, is excreted in the milk (Strosnider *et al.*, 2006).

Cereals are often the most severely affected crops, and because they are a staple food for a large portion of humanity, mycotoxins are considered the most prevalent food-related health risk in field crops (Moretti *et al.*, 2019). Regarding aflatoxins, they are reported in several agricultural crops, mainly maize, peanuts, pistachio nuts, and cottonseeds (CAST, 2003). At the European level, regulations were established to set maximum levels of aflatoxins in food and feed (**Table 1**), contributing to reduce the human and animal exposure, and consequently the associated risk (European Commission, 2002, 2006).

The possible change in patterns of aflatoxin occurrence in food and feed crops due to climate change is a matter of concern that may require anticipatory actions (Battilani *et al.*, 2016; Van der Fels-Klerx *et al.*, 2016). During the last years, some studies were performed concerning the potential impact of climate change in the aflatoxins food and feed contamination (Van der Fels-Klerx *et al.*, 2016). Based on the results obtained, the authors were unanimous in considering different areas of Europe as Eastern Europe, Balkan Peninsula, and the Mediterranean regions as the most concerned areas, including the Iberian Peninsula. The authors also referred that aflatoxin contamination above the legal limit are expected to become more frequent in the future (Battilani *et al.*, 2016; Van der Fels-Klerx *et al.*, 2016).

Consequently, the establishment of the environmental conditions that determine the fungi growth, survival, and/or interactions with plants is essential to better understand the variation in the population structures of mycotoxigenic fungi and their ability to produce mycotoxins (Medina *et al.*, 2017), and to anticipate the potential impact of climate change.

Building on what was referred, through an overview of the current situation of aflatoxins contamination in food and feed in Portugal and Spain (Iberian Peninsula), the present article aims to answer two main questions: Are aflatoxins a current concern in the Iberian Peninsula? How the climate change could impact aflatoxins contamination and the risk of human exposure in Iberian Peninsula?

Are Aflatoxins a Current Concern in the Iberian Peninsula?

The occurrence of aflatoxins in cereal-based products intended for animal and human consumption has been reported in Portugal and Spain. Occurrence of aflatoxins in cereals and cereal-based products (food and feed) reported in 2007–2019 are summarized in **Tables 2** and **3** for Portugal and Spain, respectively.

The pattern of occurrence of aflatoxins in Portugal is what is generally verified in high-income countries, i.e., higher frequencies with usually low levels or levels below the regulation of contamination. Despite in Portugal a monitoring system is evaluating food and feed according to the EU regulations, these data are not publically available. Consequently, no published data are available for cereal grains and scarce data are available regarding the occurrence of aflatoxins in feed.

The occurrence of aflatoxins in Spain follows a similar pattern to the one presented for Portugal, i.e., higher frequencies with usually low levels or levels below the regulation of contamination. In Spain there are several studies reporting the occurrence of aflatoxins in cereals (maize, barley, wheat, malt, rice) and in cereal-based products (breakfast cereals, baby food, pasta, flours, and bread), and a few studies in feed.

Table 2 Occurrence of aflatoxins in cereal-based products (food and feed) in Portugal. The presented studies were published between 2007 and 2019

Mycotoxin	*Food product*	*LOD*[a] *($\mu g\ Kg^{-1}$; $\mu g\ L^{-1}$)*	*Positive Samples*[b] *n/n (%)*	*Range of Concentration ($\mu g\ Kg^{-1}$; $\mu g\ L^{-1}$)*	*Reference*
Food					
AFM_1	Infant cereals	0.001–0.004	4/19 (21.0%)	< LOD–0.018	Alvito *et al.* (2010)
AFB_1			6/19 (32.0%)	< LOD–0.009	
AFB_1	Breakfast cereals	0.001–0.011	18/26 (69%)	< LOD–0.130	Martins *et al.* (2018)
AFB_2			7/26 (27%)	< LOD–0.011	
AFG_1			1/26 (4%)	< LOD – 0.013	
AFG_2			0/26 (0%)	NA	
AFM_1			3/26 (12%)	< LOD–0.024	
AFB_1	Breakfast cereals	0.001–0.011	19/52 (37%)	< LOD	Assunção *et al.* (2018a)
AFB_2			13/52 (25%)	< LOD	
AFG_1	Infant cereals		3/52 (6%)	< LOD	
AFG_2	Biscuits		0/52 (0%)	< LOD	
AFM_1			11/52 (21%)	< LOD	
AFB_1	Wheat and rye flours	0.4–1.2	0/7 (0%)	< LOD	Cardoso *et al.* (2019)
AFB_2				< LOD	
AFG_1				< LOD	
AFG_2				< LOD	
Feed					
AFB_1	Cattle feed	1.0	374/1001 (37.4%)	1.0 – 74.0	Martins *et al.* (2007)

[a]Range of LODs for aflatoxins;
[b]number of positive samples/total number of samples (percentage of positive samples).
LOD – Limit of detection; AFB_1 – Aflatoxin B_1; AFB_2 – Aflatoxin B_2; AFG_1 – Aflatoxin G_1; AFG_2 – Aflatoxin G_2; AFM_1 – Aflatoxin M_1.
Note: Adapted from Assunção, R., Martins, C., Viegas, S., *et al.*, 2018. Climate change and the health impact of aflatoxins exposure in Portugal – An overview. Food Additives and Contaminants – Part A Chemistry, Analysis, Control, Exposure and Risk Assessment 35 (8), 1610–1621. Available at: https://doi.org/10.1080/19440049.2018.1447691.

To summarize, aflatoxins are occurring in food and feed products available in Portugal and Spain, generally in levels that not exceed established maximum limits.

Climate Change and the Impact on Aflatoxins Contamination and the Risk of Human Exposure

Fungal growth and mycotoxins' biosynthesis are influenced by several environmental factors such as the temperature, relative humidity, rainfall and water activity. Despite fungi can grow in a wide range of water activity and temperature, the optimal conditions for mycotoxins' biosynthesis are more restrict (Battilani *et al.*, 2020; Tai *et al.*, 2020). Data on ecological needs are available for *A. flavus*, with a suitable range of temperatures between 25–35°C and 28–30°C for fungal growth and aflatoxins production, respectively, and a suitable water activity ranging from 0.95 to 1.0 (Battilani *et al.*, 2020). As such, mycotoxins are highly dependent on climate, plant and storage-associated problems, but also influenced by non-infectivity factors (e.g., bioavailability of nutrients, insect damage, and other pest attack), that are also driven by climatic conditions (Medina *et al.*, 2014; Assunção *et al.*, 2018b).

Regarding how the climate has evolved in the last years, in Spain, climatic data have been registered officially since the 19th century (year 1869) (Brunet *et al.*, 2006). According to the Spanish State Meteorology Agency (AEMET) and after analyzing the annual mean temperature in mainland and Balearic Islands between 1900 and 2010, a general increasing trend in mean temperatures could be observed specially between the 70´s until 2000. For this period and in terms of yearly accumulated rainfalls, a high variability was observed at different time scales (Agencia Estatal de Meteorologia, 2020). Similar results were obtained in a paper presenting daily adjusted dataset composed of 22 Spanish daily temperatures records (the longest available at the time of the analysis) over 1850 and 2003 (Brunet *et al.*, 2006). This analysis showed that over mainland Spain highly significant rates of temperature increases occurred for maximum and minimum temperatures (0.12°C/decade and 0.10°C/decade, respectively) over 1850–2003. Likewise, the climatic trends in Portugal follow a similar pattern. From 1970 to nowadays, annual mean temperatures are increasing and there is a trend showing the increase of temperatures and a reduction of rainfall periods (Nunes *et al.*, 2020; Portal do Clima, 2020).

Considering the influence of environmental factors on fungal growth and mycotoxins' production, an impact of climate change in the occurrence of mycotoxins in cereals crops is also expected. There are several predictions relating climate change and crop production. For Southern Europe, it is foreseen an increase of 4–5°C with longer drought periods, that may result in increasing desertification and decreasing crop yields (Medina *et al.*, 2017). The impact of climate change in crops contamination by aflatoxins was predicted through a modeling approach that considered three scenarios: the present, +2°C and +5°C. The enlargement of

Table 3 Occurrence of aflatoxins in cereals and cereal-based (food and feed) products in Spain. The presented studies were published between 2009 and 2019

Mycotoxin	*Food product*	*LOD*[a] *($\mu g\ kg^{-1}$; $\mu g\ L^{-1}$)*	*Positive samples*[b] *n/n (%)*	*Range of concentration ($\mu g\ kg^{-1}$; $\mu g\ L^{-1}$)*	*Reference*
Food					
AFB_1	Barley	0.0005 – 0.015	123/123 (100.0%)	<LOD-0.34	Ibáñez-Vea *et al.* (2012)
AFB_2			21/123 (17.0%)	<LOD-0.04	
AFG_1			6/123 (5.0 %)	<LOD-0.61	
AFG_2			22/123 (18.0 %)	<LOD-0.10	
AFs			123/123 (100.0%)	<LOD-0.75	
AFB_1	Barley	0.01 – 0.015	13/105 (12.4%)	<LOD-0.061	Mateo *et al.* (2011)
AFB_2			3/105 (2.9%)	<LOD-0.06	
AFG_1			5/105 (4.8%)	<LOD-0.26	
AFG_2			3/105 (2.9%)	<LOD-0.05	
AFB_1	Wheat (durum semolina)	0.1	0/17 (0%)	<LOD	Herrera *et al.* (2009)
AFB_1, AFB_2	Malt	0.3 – 0.4	0/10 (0%)	Below the EU regulation	Rubert *et al.* (2010)
AFG_1, AFG_2			4/10 (40.0%)		
AFB_1	Rice	0.4 – 0.6	18/27 (66.7%)	<LOD-91.7	Suárez-Bonnet *et al.* (2013)
AFB_2			6/27 (22.2%)	<LOD-12.1	
AFG_1			25/27 (92.6%)	<LOD-78.7	
AFG_2			25/27 (92.6%)	<LOD-31.0	
AFs			27/27 (100.0%)	1.6-138.6	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Rice	0.01 – 0.13	0/10 (0%)	<LOD	Beltrán *et al.* (2013)
	Bran samples	0.05 – 0.3	0/67 (0%) (37 wheat bran and 30 oat bran)	<LOD	Vidal *et al.* (2013)
	Cereals	0.25 – 1.50	1/95 (1.05%)	n.d	Serrano *et al.* (2012)
AFB_1	Cereals	3.9 – 5.7 (LOQ)	2/18 (11.1%)	10-26	Romero-González *et al.* (2011)
AFB_2			0/18 (0%)	<LOQ	
AFG_1			1/18 (5.6%)	7.4	
AFG_2			0/18 (0%)	<LOD	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Maize	0.1 – 0.7	0/5 (0%)	<LOD	Beltrán *et al.* (2009)
AFB_1	Maize	n.d.	1/3 (33.3%)	2.7	Garrido Frenich *et al.* (2009)
AFB_2			1/3 (33.3%)	2.2	
AFG_1			1/3 (33.3%)	3.3	
AFG_2			1/3 (33.3%)	3.4	
AFB_1	Cereals for infants	0.002 – 0.003	42/91 (46.2%)	<LOD-3.11	Hernández-Martínez and Navarro-Blasco (2010)
AFB_2			36/91 (39.6%)	<LOD-0.41	
AFG_1			31/91 (34.1%)	<LOD-0.42	
AFG_2			10/91 (11.0%)	<LOD-0.07	
AFs			60/91 (65.9%)	<LOD-4.02	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Baby food	0.008 – 0.033	0/72 (0%)	<LOD	Cano-Sancho *et al.* (2013)
		0.1 – 0.7	0/7 (0%)	<LOD	Beltrán *et al.* (2009)
AFG2		0.05 – 0.45	1/35 (2.9%)	1.2	Rubert *et al.* (2012)
AFB1, AFB2, AFG1			0/35 (0%)	<LOD	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Bread	0.01 – 0.13	0/30 (0%)	<LOD	Beltrán *et al.* (2013)
AFB_1		0.08 – 0.3	4/80 (5.0%)	<LOD-7.1	Saladino *et al.* (2017)
AFB_2			13/80 (16.2%)	<LOD-5.3	
AFG_1			2/80(2.5%)	<LOD-2.9	
AFG_2			0/80 (0%)	<LOD	
AFB_1	Gofio (flour made from toasted grain)	0.008	21/94 (22.3%)	<LOD-0.17	Luzardo *et al.* (2016)
AFB_2			20/94 (21.3%)	<LOD-0.07	
AFG_1			8/94 (8.5%)	<LOD-0.12	
AFG_2			7/94 (7.4%)	<LOD-0.06	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Biscuits	0.01 – 0.13	0/10 (0%)	<LOD	Beltrán *et al.* (2013)
		n.d.	0/5 (0%)	<LOD	Garrido Frenich *et al.* (2009)

(*Continued*)

Table 3 Continued

Mycotoxin	*Food product*	*LOD*[a] *(μg kg⁻¹; μg L⁻¹)*	*Positive samples*[b] *n/n (%)*	*Range of concentration (μg kg⁻¹; μg L⁻¹)*	*Reference*
AFs	Popcorn kernels	0.01 – 0.04	2/30 (6.7%)	<LOD-3.72 AFB1 <LOD-3.1 AFs	Alborch *et al.* (2012)
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Instant cereal-breakfast beverages	0.3 – 0.4	0/5 (0%)	<LOD	Rubert *et al.* (2010)
AFs	Breakfast cereals	0.008 – 0.033	4/215 (1.9%)	<LOD-1	Cano-Sancho *et al.* (2013)
AFs (AFB_1, AFB_2, AFG_1, AFG_2)		0.01 – 0.13	0/10 (0%)	<LOD	Beltrán *et al.* (2013)
AFB_1		0.013 – 0.051	4/46 (8.7%)	<LOD-0.13	Ibáñez-Vea *et al.* (2011)
AFB_2, AFG_1, AFG_2			0/46 (0%)	<LOD	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)		n.d.	0/5 (0%)	<LOD	Garrido Frenich *et al.* (2009)
AFs	Flour	0.01 – 0.04	14/30 (46.7%)	<LOD-4.4	Alborch *et al.* (2012)
AFB_1		0.1 – 1	0/50 (0%)	<LOD	Rubert *et al.* (2011)
AFB_2			2/50 (4.0%)	<LOD-2	
AFG_1			2/50 (4.0%)	<LOD-0.72	
AFG_2			3/50 (6.0%)	<LOQ-1.2	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Pasta	0.01 – 0.13	0/10 (0%)	<LOD	Beltrán *et al.* (2013)
		0.1 – 0.7	0/6 (0%)	<LOD	Beltrán *et al.* (2009)
AFB_1	Pizza dough	0.08 – 0.3	14/60 (23.3%)	<LOD-9.50	Quiles *et al.* (2016)
AFB_2			19/60 (31.7%)	<LOD-0.67	
AFG_1			6/60 (10.0%)	<LOD-1.05	
AFG_2			0/30 (0%)	<LOD	
AFs			30/60 (50.0%)	<LOD-10.02	
AFG_2	Ready-to-eat food	0.15 – 0.3	1/8 (12.5%)	2.84	Carballo *et al.* (2018b)
AFB_1, AFB_2, AFG_1		0/8 (0%)	<LOD		
AFG_2		n.d	4/88 (4.5%)	0.17 (Mean value)	Carballo *et al.* (2018a)
AFB_1, AFB_2, AFG_1			0/88 (0%)	<LOD	
AFs (AFB_1, AFB_2, AFG_1, AFG_2)	Gluten free (bread, pasta and pastries)	0.008 – 0.033	0/18 (0%)	<LOD	Cano-Sancho *et al.* (2012)
	Ethnic foods (Mexican tortillas; corn and wheat flours; cuscus)	0.008 – 0.033	3/35 (8.6%)	<LOD-14.3	
Feed					
AFB1	Pig Feed	1 (LOQ)	7/228 (3.07%)	0.29-2.91	Arroyo-Manzanares *et al.*, (2019)
AFB2			3/228 (1.32%)	0.28-1.06	
AFG1			2/228 (0.88%)	0.22-0.44	
AFG2			0/228 (0%)	<LOD	
AF	Feed	0.3 (LOQ)	0/26 (0%)	<LOD	Griessler *et al.*, (2010)
AFB1	Feed (dairy cow feedstuffs)	0.003	58/78 (74.0%)	0.005-0.099	Hernández-Martínez and Navarro-Blasco, (2015)
AFB2		0.002	25/78 (32.0%)	<LOD-0.013	
AFG1		0.002	45/78 (58.0%)	<LOD-0.044	
AFG2		0.002	17/78 (22.0%)	<LOD-0.003	
AF			70/78 (89.7%)	0.003-0.211	
AFB1	Feed (swine, sheep, poultry, cattle, equine, rabbit breeding, aquaculture, feed material)	1	8/62 (12.9%)	n.a	Romera *et al.*, (2018)
AFB2		2	0/62 (0%)	<LOD	
AFG1		2	0/62 (0%)	<LOD	
AFG2		2	0/62 (0%)	<LOD	

[a]Range of LODs for aflatoxins;
[b]number of positive samples/total number of samples (percentage of positive samples); n.d. = Not determined.
LOD = Limit of detection; AFs – Sum of Aflatoxins; AFB_1 – Aflatoxin B_1; AFB_2 – Aflatoxin B_2; AFG_1 – Aflatoxin G_1; AFG_2 – Aflatoxin G_2.
Note: Adapted from Echarri, E., Vettorazzi, A., Lizarraga, E., *et al.*, 2019. Review of the analytical methodologies and occurrence data of aflatoxins in cereals and cereal-based foods in Spain. In: Kintzios, S., Mavrikou, S. (Eds.), Aflatoxins: Biochemistry, Toxicology, Public Health, Policies and Modern Methods of Analysis. New York: Nova Science Publishers, pp. 207–243.

aflatoxin risk zones could increase human and animal population chronic exposures to these mycotoxins. AFB_1 was predicted to become a food safety issue in maize in Europe, especially in the $+2°C$ scenario, the scenario of climate change considered as most probable in the coming years (Battilani *et al.*, 2016).

Under this context, considering the recently published EFSA's risk assessment of aflatoxins (EFSA Panel on Contaminants in the Food Chain CONTAM, 2020), the intake of total aflatoxins ranged in Portugal between 0.36 ng kg^{-1} bw day^{-1} (in infants) and

Table 4 Exposure to total aflatoxins (ng kg^{-1} bw day^{-1}) and risk characterization reported for Portugal and Spain, from 2012 to 2016 years

Country	*Survey*	*Age Group*	*Number of participants*	*Mean AFs – LB ng kg^{-1} bw day^{-1}*	*Mean AFs – UB ng kg^{-1} bw day^{-1}*	*Mean AFB_1 – LB ng kg^{-1} bw day^{-1}*	*Mean AFB_1 – UB ng kg^{-1} bw day^{-1}*	*MoE - LB*[a]	*MoE - UB*[a]
Portugal	IAN-AF 2015-2016	Infants	234	0.36	1.78	0.17	1.09	2,411	368
		Toddlers	571	1.01	5.03	0.49	3.64	820	110
		Other children	521	1.00	5.67	0.59	3.64	677	110
		Adolescents	633	0.62	3.84	0.38	2.44	1,065	164
		Adults	3102	0.44	3.06	0.22	1.35	1,785	296
		Elderly	509	0.34	2.58	0.29	1.32	1,373	303
		Very elderly	241	0.31	2.48	0.28	1.43	1,432	279
Spain	ENALIA 2012-2015	Infants	289	0.42	1.83	0.14	0.94	2,857	424
		Toddlers	326	0.97	5.34	0.51	3.15	787	127
		Other children	556	0.95	5.68	0.54	3.46	746	116
		Adolescents	609	0.60	3.85	0.37	2.37	1,070	169
	ENALIA2 2014-2015	Adults	669	0.36	2.19	0.29	1.71	1,400	234
		Elderly	264	0.32	2.00	0.26	1.44	1,542	277

[a]MoE determined for exposure to AFB_1 considering a $BMDL_{10}$ of 0.4 μg kg^{-1} bw day^{-1} for the induction of HCC by AFB_1 in male rats.

IAN-AF = National Food, Nutrition, and Physical Activity Survey of the Portuguese General Population; ENALIA = Spanish National Food Survey on children and adolescents; ENALIA2 = Spanish National Food Survey on adults, the elderly and pregnant women; AFs = Aflatoxins (Sum of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1); AFB_1 = Aflatoxin B_1; LB = Lower-bound approach; UB = Upper-bound approach; MoE = Margin of Exposure.

Note: Adapted from EFSA Panel on Contaminants in the Food Chain (CONTAM), 2020. Risk assessment of aflatoxins in food. EFSA Journal 18 (3). Available at: https://doi.org/10.2903/j.efsa.2020.6040.

5.67 ng kg^{-1} bw day^{-1} (in other children, aged between 36 months and 10 years) ng kg^{-1} bw day^{-1}, and in Spain between 0.42 ng kg^{-1} bw day^{-1} (in infants) and 5.68 ng kg^{-1} bw/day (in other children, aged between 36 months and 10 years) (**Table 4**). In a very tentative exercise considering the exposure of Portuguese and Spanish population reported in the EFSA's risk assessment, a prediction of the associated risk was estimated through the calculation of the Margin of Exposure (MoE), as presented in **Table 4**. These results also reinforced that children aged between 36 months and 10 years (identified in the table as other children) is the age group under higher risk in the both countries, presenting the lowest MoE. Consequently, careful attention should be dedicated to aflatoxins exposure in the Iberian Peninsula, taking into consideration the already identified risk and the potential of increasing in a climate change scenario.

Considering the carcinogenic effects associated with human exposure to aflatoxins, namely hepatocellular carcinoma, and the fact that exposure to aflatoxins in Iberian Peninsula is nowadays a reality (**Tables 2** and **3**), the impact on health was previously estimated and reported, quantifying the Disability-Adjusted Life Years (DALY). In 2015, the WHO estimated the burden associated with exposure to aflatoxins for the Europe region, where Portugal and Spain are included as 0.3 (0.1–0.7) DALY/year/100k (WHO, 2015). Assunção *et al.* (2018a), estimated a similar number of DALY/100,000 for Portugal, 0.08–0.30, based on previous exposure reports (Assunção *et al.*, 2018b). Recently, and based on data obtained under a human biomonitoring study, Martins *et al.* (2020) estimated that exposure to aflatoxins in Portugal corresponds to 0.13 annual extra-cases of HCC and 1.7 DALY/100,000 (Martins *et al.*, 2020).

European countries are already capacitated to anticipate and mitigate the effects of occurrence of mycotoxins in cereal crops. The effort of European countries has mainly an economic burden, i.e., the costs involved with sampling and analysis to meet regulatory and contract requirements, and hidden costs of destroyed or returned shipments and the time and expense of sourcing and purchasing replacement items (Logrieco *et al.*, 2018; Ndemera *et al.*, 2020). The scenarios that point to an increase of food safety issues will imply a continuous effort and a tighten surveillance of occurrence of mycotoxins. It is also possible that the impact of climate change will not be homogeneous within each country. As an example, Navarra, a region in the north of Spain, could be divided into four climatic areas and seven zones. Ibáñez-Vea *et al.* (2012) evaluated the occurrence of aflatoxins in 123 barley samples and compared samples from the central and southern region of Navarra (a region of Spain). In this study, the differences were minimal, except for AFG_1 that showed higher levels in one zone of the southern region. However, the samples were collected in two different years (2007 and 2008) and these results could also be biased by the different climatic conditions of each year. Indeed, some statistical difference were observed between years. Unfortunately, the study was not designed to collect the climatic records which refrain to have a clearer scenario.

Altogether, these results highlight the need for a continuous surveillance of levels of aflatoxins on feed and food consumed in Europe. The development of human biomonitoring studies is also needed so that the health effects associated with exposure to aflatoxins can be anticipated.

Conclusions and Future Perspectives

The occurrence of aflatoxins in foods marketed in Iberian Peninsula is proved to be a reality and a concern from a public health point of view. Additionally, and considering the quality control strategies implemented from farm to fork in Europe, the economic burden related with the occurrence of these mycotoxins should not be discarded. The environmental factors are a major influence on the levels of occurrence and this European region is foreseen to become affected in a near future, if climatic factors follow the current trends. New and more effective strategies to prevent the occurrence of aflatoxins in foods and consequently the associated health effects are needed in a near future. The development of monitoring strategies, not only for occurrence in foods and feed but also for monitoring the exposure of population, is an area requiring further efforts.

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The Usefulness of Human Biomonitoring in the Case of Mycotoxins Exposure Assessment

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Human Biomonitoring – What it is?

Exposure to contaminants can happen through different routes, such as inhalation, ingestion, and dermal absorption. The exposure routes are mainly defined by the context where exposure to a specific contaminant can take place (e.g., through food consumption or in the workplace) and by the contaminant physical and chemical properties (e.g., if it is volatile, exposure through inhalation can be facilitated). The amount of contaminant uptake is often termed as the "absorbed dose." The body burden of a specific contaminant is determined by factors, such as the contaminant's concentration in a specific environmental medium, its physical and chemical properties, and timing of exposure, as well as individual factors, such as uptake, metabolism, and excretion rates (Calafat, 2016; Ganzleben *et al.*, 2017). Human biomonitoring (HBM) considers all these important aspects by measuring the concentrations of a chemical or its metabolites in biological samples (WHO, 2015). HBM can be defined as "the method for assessing human exposure to chemicals or their effects by measuring these chemicals, their metabolites or reaction products in human specimens" (WHO, 2015).

Biomonitoring data directly reflect the total body burden or biological effect resulting from all routes of exposure, inter-individual variability in exposure levels and metabolism and excretion rates (WHO, 2015). Therefore, biomonitoring involves measurements of biomarkers in several biological samples, such as blood, urine, saliva, breast milk, sweat, feces, hair, teeth, and nails. The choice of biological sample is mainly dependent of the chemical toxicokinetics, metabolism, bioavailability, and the collection procedure that should be the less invasive as possible (WHO, 2015; Calafat, 2016). In this context, is easy to understand how important is the choice of biomarkers for the quality and pertinence of data to be obtained within a HBM study (IPCS/WHO, 1993, 2001). Only with the right biomarker is possible to formulate the correct assumptions about exposure (Calafat, 2016; Louro *et al.*, 2019). Additionally, there are some important aspects to consider for a successful HBM study such as the adequate selection of the study population, procurement and type of biological samples, and the choice of analytical methods (Calafat, 2016).

To sum up, HBM provides an integrated measure of exposure to chemicals from all sources and routes and when properly used, biomonitoring data can be reliably used to estimate internal doses in environmental epidemiology studies (Calafat, 2016).

Biomonitoring as an Exposure and Risk Assessment Tool

For a specific chemical, HBM can identify spatial and time trends, lifestyle contributing factors, and specific groups with higher risk. For knowing this implies considering chemicals proprieties and adapt sampling strategy to guarantee the representativeness of the results and their adequate interpretation. For instance, for chemicals that are excreted rapidly, cross-sectional biomonitoring data reflect recent exposure, while characterization of long-term exposure patterns at the individual level requires repetitive sampling (Calafat, 2016). All these considerations allow determining the exposure at individual level and consequently this is of high relevance for the risk assessment process (Martins *et al.*, 2019).

Recently, Louro *et al.* (2019) described the state of the art of HBM use in existing risk assessment schemes, presented examples of the use of HBM for risk assessment and the results of a survey made to regulatory risk assessors on the use of HBM in risk assessment. It was possible to conclude that although the HBM data in chemicals risk assessment can be found in almost all regulatory areas, the guidance on such use is generally either limited or missing. To surpass this some recommendations were made such as create an EU level guidance applicable to different regulatory schemes on the use of HBM in risk assessment to better support the integration of HBM in regulatory risk assessment. Additionally, scientifically sound health-based biological limit/guidance values (such as derived via the biological equivalents approach), preferably with at least some regulatory recognition should be established at EU and/or global (e.g., WHO, FAO) level. The same paper also recommended future research actions intending to the development of approaches to integrate existing HBM data, data using non-animal testing methods (in vitro for instance) in combination with computational modeling (PBTK) to generate more reliable information on toxicokinetic and to provide connections between adverse outcome pathways and human internal

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exposure levels. HBM data should also be used to validate integrated and aggregated external and internal exposure models (Louro *et al.*, 2019).

Biomonitoring Data to Support Risk Management Measures and Regulatory Actions

HBM is also an important tool to support environment and health policy-making because it can provide useful quantitative information regarding the actual exposure of a specific population to contaminants including emerging ones, as well as data regarding the resulting health effects and/or population susceptibility to these xenobiotic compounds. Therefore, HBM allows direct and more precise assessment of the distribution of risk in the population (WHO, 2015).

All this information can be used to prioritize actions and measures for policy making, to evaluate the effectiveness of the policy measures already in place and, consequently, to reduce exposure to hazardous substances (Ganzleben *et al.*, 2017).

In order to identify the most important exposure pathways as a basis for selection of the most relevant risk management measures and controlling emissions at source, HBM data on internal exposure should be combined with information on individual behaviors, diet, workplace environment, lifestyle, and monitoring data about environmental and/or food contamination. This contextual information combined with the development of exposure modeling can facilitate the identification of the most relevant exposure sources and pathways and support a review of the efficacy of legislation focused on specific chemical products or legislation dedicated to exposure pathways, such as air, drinking water or food or even the occupational environment (Ganzleben *et al.*, 2017; Viegas *et al.*, 2019).

In a more specific way, HBM can also support the implementation of new risk management measures and to evaluate the efficacy of the ones already in place in the workplaces. Therefore, HBM should be considered as an exposure assessment tool giving valuable information to the risk assessment and risk management process (Viegas, 2019). Unfortunately, HBM is seen frequently as only a health surveillance tool and not as an exposure assessment tool and this bring difficulties regarding the use of the information for risk management purposes (Santonen *et al.*, 2019; Viegas *et al.*, 2019).

What is a Mycotoxin and How We Can be Exposed?

Mycotoxins are fungi metabolites produced by specific fungal genera, mainly by *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps* (Bennett and Klich, 2003; Marín *et al.*, 2013; Viegas *et al.*, 2018). These natural toxins are small and stable molecules, with a low molecular mass. Until now, more than 300 mycotoxins have been identified and probably more will be discovered in the future. However, only 30 of these mycotoxins have been the focus of research work aiming to understand their possible toxic proprieties (Surai *et al.*, 2008). Mycotoxins can resist to adverse environmental factors such as high or low temperatures and can persist long after the death and disintegration of the fungal species responsible for their production (Halstensen, 2008). Therefore, this explains why measuring only fungi do not allow to conclude about exposure to mycotoxins. They are also difficult to eliminate or inactivate from the source even after being exposed to high temperatures such as boiling or roasting processes (Peraica *et al.*, 1999).

The most common and reported exposure source is the diet, either by direct exposure (through the consumption of contaminated agricultural food products) or by indirect exposure (through the consumption of food derived from animals fed with contaminated feedstuffs, e.g., milk or meat) (Marín *et al.*, 2013, 2018).

From a public health and economic perspectives, the more important mycotoxins are aflatoxins, trichothecenes, zearalenone (ZEA), patulin, ochratoxin A (OTA), and fumonisins (Schatzmayr and Streit, 2013; Wu, 2014; Abrunhosa *et al.*, 2016; Lee and Ryu, 2017). At European level, the occurrence of mycotoxins is regulated by the European Commission aiming to protect consumers from its health effects, by the Regulation 1881/2006 and its amendments (European Commission, 2006).

In 2004, it was put in place a survey program, monitoring the presence of five mycotoxins/mycotoxin groups (aflatoxins, ZEA, deoxynivalenol (DON), fumonisins, and OTA) in feed and feed raw materials worldwide. The samples comprised essentially raw materials also used to make feed, such as wheat and wheat bran (9%), barley (8%), silage (8%), soybean meal (4%), distillers dried grain with solubles (DDGS; 2%), corn gluten meal (1%), rice and rice bran (1%), straw (1%), and other feed ingredients (e.g., cotton seed, sorghum, cassava, peanut, copra, etc., 12%). The samples were originated from different places in the world, namely Asia (45%) and Europe (37%), with 15% from Americas, 2% in Africa and 1% in the Middle East (Schatzmayr and Streit, 2013). From the results, it was possible to conclude that mycotoxin occurrence and contamination is strongly dependent on regional climatic conditions (frequently linked to extreme weather events or at least uncommon weather conditions) and that mycotoxins are ubiquitously present in feed and co-occurrence is a common feature. The data also allowed to conclude that the prevalence of mycotoxins in food is comparable to the one found in feed, even though at a lower level (Schatzmayr and Streit, 2013).

Aflatoxin B1 (AFB_1) is probably the most hazardous mycotoxin found in agricultural products since it is recognized as an hepatocarcinogen, inducing DNA adducts leading to genetic changes in target liver cells (Chen *et al.*, 1997; Vineis and Xun, 2009). It is commonly found on several food commodities worldwide being the most reported the grains and peanuts (Rocha *et al.*, 2014). *Aspergillus flavus* and *Aspergillus parasiticus* are the most common species associated with aflatoxin contamination (Varga *et al.*, 2015; Lamoth, 2016). There are also the emerging mycotoxins such as fusaproliferin, moniliformin, beauvericin, and

enniatins produced mainly by *Fusarium* species; ergot alkaloids produced by *Claviceps*; and altenuene, alternariol, alternariol methyl ether, altertoxin, and tenuazonic acid produced by *Alternaria* species (Bottalico and Logrieco, 1998; Barkai-Golan and Paster, 2008; Marín *et al.*, 2013). These mycotoxins deserve particular attention since, currently, they are unregulated and were shown to occur frequently in agricultural products. The evidence of their incidence is rapidly increasing and gaps in toxicological knowledge have been identified for several compounds not allowing a proper risk assessment (Gruber-Dorninger *et al.*, 2017).

In addition to exposure through diet, humans, and animals can also be exposed to mycotoxins by inhalation of contaminated dusts in indoor and occupational settings where respirable particles below 1.0 μm may be present as particulate material that can act as carriers for mycotoxins (Straumfors *et al.*, 2014; De Santis *et al.*, 2019). In occupational settings, exposure can happen when organic dust containing mycotoxins is released during tasks such as storage work, loading, handling, or milling contaminated materials (grain, waste, and feed), and others such as caring for animals in animal husbandry settings (Viegas *et al.*, 2018). Additionally, transdermal studies have already proved that mycotoxins can penetrate through the skin, thus contributing for a health risk (Boonen *et al.*, 2012). These studies measured the kinetics of AFB1, OTA, fumonisin B1, citrinin, zearalenone and T-2 toxin and demonstrated that OTA have the highest permeation while AFB_1 the lower permeability rates (Boonen *et al.*, 2012). Moreover, in a study developed in poultry slaughterhouses intending to assess occupational exposure to AFB_1, it was shown that dermal route was probably responsible for the exposure found in the workers performing the poultry evisceration and the fact that workers were with their hands always in contact with water facilitated the dermal absorption (Viegas *et al.*, 2016).

The Usefulness of HBM in the Case of Mycotoxins Exposure Assessment

In the food safety approach, exposure assessment is conventionally performed combining consumption and occurrence data. However, it is well known that these methods are centered on some assumptions and uncertainties, even when probabilistic approaches are used. Considering the characteristics of HBM studies and type of data provided, it is possible to assume that exposure assessment performed through this direct approach contributes to overcome some of the limitations already identified such as the collection of consumption data, the heterogeneity of food contamination, the influence of culinary processing, and the interindividual variability (De Boevre *et al.*, 2013; Assunção *et al.*, 2016; de Nijs *et al.*, 2016; Zidek *et al.*, 2017; Martins *et al.*, 2019). In human biomonitoring studies of mycotoxins, it is important to include data collection on both food consumption and urine biomarkers, allowing simultaneously the identification of the main food contributors and validation of exposure, respectively (Martins *et al.*, 2020). As referred by Martins *et al.* (2020), the link between HBM data and food consumption data is more successful for mycotoxins with short half-lives because the moment of food survey are most of the times in accordance with urine sampling. Turner *et al.* (2008) compared data obtained for 24 h urine (urinary DON) with intake data from the UK National Diet and Nutrition Survey through a multiple linear regression model. Within this study it was possible to conclude that whole meal bread, pasta, fruit pies, buns/cakes, and white bread were contributors for exposure to DON and the respective increase in exposure when 50 g of each food were consumed (Turner *et al.*, 2008).

Recently, Viegas *et al.* (2018) extensively reviewed the available literature for occupational exposure to mycotoxins and the use of HBM to assess exposure (Viegas *et al.*, 2018). From this review, it was possible to conclude that the outputs of HBM studies conducted worldwide point out for the fact that mycotoxins can represent a risk in occupational settings (De Santis *et al.*, 2019). Furthermore, in recent studies developed in one industrial bakery and in swine farms the use of HBM allowed to recognize that even if workers are exposed through food consumption to mycotoxins, occupational exposure adds and contributes to the total exposure (Viegas *et al.*, 2018, 2019). In the same studies, HBM also revealed that exposure occurs as a mixture of mycotoxins, for both workers and general population. This is comprehensible since, besides the presence of multiple mycotoxins in the workplace environment, this is also a common feature of food commodities (Viegas *et al.*, 2019).

Overall, the use of HBM tools to assess exposure to mycotoxins in occupational context or by food consumption is an area of increasing research. Although many constraints still exist, the advances on knowledge of toxicokinetics of these compounds should be considered a step forward in this area. The HBM approaches to evaluate exposure to mycotoxins represent an adding value by providing more detailed information and making possible to understand exposure characteristics. Altogether, it will give support to risk management measures and policy actions, in order to reduce this exposure to the minimum possible.

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Mycotoxins as Endocrine Disruptors – An Emerging Threat

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Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi and one of the most relevant group of food contaminants (Bennett and Klich, 2003). These toxins are considered ubiquitous and can affect the food chain in the stages of production, harvest, storage, and processing (Vettorazzi and López de Cerain, 2016). They are associated with several health outcomes in human and animal health such as, carcinogenic, immunotoxic, nephrotoxic, neurotoxic, teratogenic, and hepatotoxic effects (Wu *et al.*, 2014; De Ruyck *et al.*, 2015; Mally *et al.*, 2016; Ostry *et al.*, 2017) and constitute a concern from the public health and economic perspectives. To prevent these issues, many standards and regulation had been put in place (Eskola *et al.*, 2018, 2019).

Some mycotoxins have been pointed out as compounds presenting endocrine disruptive activity. According to the World Health Organization and the United Nations Environment Programme (UNEP), an endocrine disruptor (EDC) is a substance that alter one or more function of the endocrine system and consequently cause adverse effects in an intact organism, its progeny and a (sub)population (UNEP and WHO, 2012). EDCs may alter physiology during the whole life span of an individual, from fetal development to adult growth (Gore *et al.*, 2015), and with different degrees of evidence, could be associated to several health effects. These effects are also dependent on both the level and timing of exposure being especially critical when exposure occurs during development (UNEP and WHO, 2012).

Considering the human exposure through oral intake, EDCs are classified in four categories: EDCs with bioaccumulation ability (e.g., polychlorinated biphenyls – PCBs, polybrominated flame retardants, perfluorinated chemicals), compounds used for food production (e.g., pesticides), chemicals present in food due to contact materials (e.g., BPA), and endocrine-active substances naturally present in food (e.g., genistein; mycotoxins) (Diamanti-Kandarakis *et al.*, 2009; Ribeiro *et al.*, 2017).

The human exposure to EDCs is a public health concern and several attempts have been made in order to harmonize concepts and methodologies for hazard, safety, and risk assessment (EFSA, 2013; ECHA, EFSA and JRC, 2018).

Considering that endocrine-active substances naturally present in food as mycotoxins constitute an emerging threat to public health and the recent data evidencing human exposure to these compounds, here the authors intend to gather and systematize key information regarding mycotoxins considered EDCs.

Zearalenone

Main Characteristics

The *Fusarium* mycotoxin ZEN, considered the parent compound, belongs to a family of compounds sharing the ring system of a macrocyclic β-resorcylic acid lactone (RAL). It is produced by *Fusarium* species such as *F. graminearum, F. culmorum, F. equiseti, and F. verticillioides* (Steinkellner *et al*, 2019). Modified forms of ZEN have been described mainly in food from plant origin, due to the biotransformation performed by plants and/or fungi (EFSA, 2016). These compounds formed by conjugation with sulfate (ZEN-Sulf) and glucose (ZEN-Glc) molecules are important because they are hardly detected by the routine food analysis (Rychlik *et al.*, 2014).

Zearalenone is stable, does not degrade at high temperature and exhibits fluorescence under ultraviolet (UV) light. The structure and shape of zearalenone and its metabolites resembles endogenous estrogen, the 17β-estradiol (Kowalska *et al.*, 2016).

Metabolism of Zearalenone

Following ingestion and absorption, ZEN is metabolized mainly by enterocytes and hepatocytes (Molina-Molina *et al.*, 2014). The biotransformation of ZEN occurs in two major pathways. The phase I is characterized by the reduction of the keto group to an alcoholic hydroxyl group to yield the stereoisomeric compounds α-zearalenol (α-ZEL) and β-zearalenone (β-ZEL). Reduction of the olefinic double bond of ZEN leads to zearalanone (ZAN), and reduction of both the olefinic double bond and keto group to α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) (Steinkellner *et al.*, 2019). The phase II is characterized by the conjugation of the parent compound and/or the phase I metabolites with glucuronides or sulfate groups in order to become polar and facilitate the urinary excretion. The conjugated metabolites are excreted in the bile and entero-hepatic recirculation and finally eliminated with urine (Molina-Molina *et al.*, 2014). Fitzpatrick *et al.* (1988) analyzed the metabolism of ZEN and found α-ZEL to be the major

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Table 1 Relative potency factors for ZEN, metabolites, and modified forms as defined by EFSA

Compound	*Relative potency factor*
β-ZEL; β-ZEL-Glc, β-ZEL-Sulf	0.2
ZEN, ZEN-Glc, ZEN-Sulf, cis-ZEN, cis-ZEN-Glc, cis-ZEN-Sulf, cis-β-ZEL, cis-β-ZEL-Glc, cis-β-ZEL-Sulf	1.0
ZAN, ZAN-Glc, ZAN-Sulf	1.5
β-ZAL, β-ZAL-Glc, β-ZAL-Sulf	2.0
α-ZAL, α -ZAL-Glc, α -ZAL-Sulf	4.0
cis-α-ZAL, cis-α-ZAL-Glc, cis-α-ZAL-Sulf	8.0
α -ZEL, α -ZEL-Glc, α -ZEL-Sulf	60

Abbreviations: cis-ZEN, cis-zearalenone; cis-ZEN-Sulf, cis-zearalenone-sulfate; cis-β-ZEL, cis-β–zearalenol; cis-β-ZEL-Glc, cis-β-zearalenol-glucoside; cis-β-ZEL-Sulf, cis-β-zearalenol-sulfate; cis-α-ZAL, cis-α-zearalanol; cis-α-ZAL-Glc, cis-α-zearalanol-glucoside; cis-α-ZAL-Sulf, cis-α-zearalanol-sulfate; ZAN, zearalanone; ZAN-Glc, zearalanone-glucoside; ZAN-Sulf, zearalanone-sulfate; ZEN, zearalenone; ZEN-Glc, zearalenone-glucoside; ZEN-Sulf, zearalenone-sulfate; β-ZEL, β-zearalenol; β-ZEL-Glc, β-zearalenol-glucoside; β-ZEL-Sulf, β-zearalenol-sulfate; β-ZAL, β-zearalanol; β-ZAL-Glc, β-zearalanol-glucoside; β-ZAL-Sulf, β-zearalanol-sulfate; α-ZAL, α-zearalanol; α-ZAL-Glc, α-zearalanol-glucoside; α-ZAL-Sulf, α-zearalanol-sulfate; α-ZEL, α-zearalenol; α-ZEL-Glc, α-zearalenol-glucoside; α-ZEL-Sulf, α-zearalenol-sulfate.
Note: EFSA, 2016. Appropriateness to set a group health-based guidance value for zearalenone and its modifiedforms. EFSA Journal 14 (4). doi:10.2903/j.efsa.2016.4425.

metabolite in the feces and the conjugated glucuronides to be the major metabolites in the urine (Fitzpatrick *et al.*, 1988). The extents and rates of various metabolic reactions are important because the metabolites of ZEN vary in their estrogenic potential (Mukherjee *et al.*, 2014).

Recently, Slobodchikova *et al.* (2019) performed an experiment to generate phase I and phase II (glucuronidation) metabolites of mycotoxins (including ZEN) using standard *in vitro* microsomal incubation protocol. Regarding ZEN, for phase I there were reported a variety of ZEN oxidized metabolites and for phase II the glucuronidation pathway was confirmed as the predominant metabolic pathway. Phase II reactions resulted in various glucuronide forms of parent toxin, its metabolites of phase I reactions and double glucuronide forms (Slobodchikova *et al.*, 2019).

Mode of Action and Toxicity

The human exposure to ZEN is not associated with acute episodes. However, the chronic exposure is of potential concern due to the structural similarity of ZEN with 17β-estradiol. This characteristic allows the binding of ZEN to estrogen receptors with high binding affinity and thereby exerting estrogenic effects (Mally *et al.*, 2016).

Regarding the estrogenic activity of ZEN, metabolites and modified forms, an important aspect is the establishment of the relative potency factors for each compound. Overall, the limited number of studies using the uterotrophic effect as endpoint for estrogenicity showed that phase I metabolites of ZEN retain estrogenic activity. The α-isomers are invariably much more estrogenic than the corresponding β-isomers, and exceed the estrogenicity of ZEN. The orientation of the hydroxyl group at the aliphatic ring proved to have a pronounced effect on the estrogenic activity, with α-ZEL being more estrogenic than β-ZEL and ZEN (Metzler *et al.*, 2010). The estrogenic potency of α-ZEL *in vivo* is about 62 times higher than ZEN and one order of magnitude lower than that of the most potent endogenous estrogen 17β-estradiol (EFSA, 2016). Reduction of the olefinic double bond of ZEN, leading to ZAN, virtually does not change the hormonal activity (EFSA, 2016). The EFSA CONTAM Panel proposed relative potency factors based on the relative estrogenicity potencies determined in the uterotrophic assay and given on a molar basis (**Table 1**).

In 2011, the EFSA CONTAM Panel identified the estrogenic activity of ZEN as the critical effect with female pigs being the most sensitive to this effect. Based on a No Observed Effect Level (NOEL) in pigs of 10.4 μg kg^{-1} bw day^{-1} and considering an uncertainty factor of 40 (4 for interspecies differences in toxicokinetics because humans were considered not to be more sensitive than the female pig, and 10 for intra-human variability), a TDI of 0.250 μg kg^{-1} bw day^{-1} was established (EFSA, 2011). In 2016, the EFSA CONTAM Panel considered the effects of ZEN, its metabolites and modified forms as additive and proposed a group TDI for all these related compounds of 0.250 μg kg^{-1} bw day^{-1} (EFSA, 2016).

The mechanisms by which ZEN influences the synthesis and secretion of mammalian sex steroid hormones were recently reviewed by Zheng *et al.* (2019) and Rai *et al.* (2019). These mechanisms include: Interference in feedback that mediate FSH and LH production in pituitary gland, the inhibition of viability of Leydig and granulosa cells that produce sex steroids, the oxidative stress by stimulating the leakage of electrons and increasing the levels of free radicals, the induction of cell apoptosis, and the influence on mitochondrial functions (Rai *et al.*, 2019; Zheng *et al.*, 2019).

Due to its ability to induce cell proliferation, some studies assessing the carcinogenicity of ZEN have been performed. The experimental studies were performed in animals with results showing induction of hepatocarcinoma (mice), prostate gland inflammation, mammary gland cysts, and increased hepatocellular vacuolization (rats) (NTP, 1982). Regarding studies in humans, scarce data are available. Belhassen *et al.* (2015) found a potential correlation between development of breast cancer and exposure to ZEN by obtaining results (adjusted OR = 1.54, 95% CI = 1.10–2.77) that suggested a potential role of α-ZAL in the risk of developing breast cancer. However, the data available is limited and further assessments should be performed. Due to lack of evidence regarding the carcinogenic effect of ZEN, it is classified in group 3 of IARC (not classifiable up to its carcinogenicity to humans) (IARC, 1993).

Another recent issue is the transfer of contaminants through placental barrier and consequently the early-life exposure to these substances. Recently Warth *et al.* (2019) performed an *ex vivo* experiment and obtained results suggested that there is *in utero* exposure to ZEN, α-ZEL, and ZEN-14-sulf. These results highlighted the need for further assessments regarding the early-life exposure.

Occurrence of Zearalenone in Food

ZEN is produced by fungi of the genus *Fusarium*, which attacks crops during its growth in the field as well as during storage period. Humans can be exposed to ZEN directly, by contaminated food, or indirectly, through products derived from animals exposed to mycotoxins (Marin *et al*, 2013). ZEN production is favored by high humidity and low temperature conditions and it simultaneously occurs with deoxynivalenol and less frequently with aflatoxins (Alshannaq and Yu, 2017), thus contributing for the issue of co-occurrence of mycotoxins (Alassane-Kpembi *et al.*, 2016).

ZEN is not removed during processing and affects the entire human food chain through a range of food products including cereals, meat, milk, wine, beer, dried fruits, and spices (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015; Ribeiro *et al.*, 2015; Abrunhosa *et al.*, 2016; Lee and Ryu, 2017).

Considering the health effects associated to human exposure to ZEN, and aiming to protect the consumers' health, the European Commission established maximum admissible levels for the occurrence of ZEN in foods. These levels are laid down in Regulation 1881/2006 and ranged from 20 to 200 $\mu g\ kg^{-1}$ (European Commission, 2006a), according to the considered food matrix. Considering the possibility of human indirect exposure and the economic burden, the European Commission produced a recommendation of guidance levels for the occurrence of ZEN in feed, ranging from 100 to 3000 $\mu g\ kg^{-1}$ (European Commission, 2006b). In China, the mL for ZEN has been regulated at 60 $\mu g\ kg^{-1}$ in wheat, wheat flour, corn, and corn flour (NHC and CFDA, 2017). In USA there are no maximum admissible levels established for the occurrence of ZEN in food and feed.

Alternaria Toxins

Main Characteristics

Alternaria fungi, where the most predominant is *A. alternata*, are a common plant pest in cereals, oilseeds, fruits, and vegetables (Arcella *et al.*, 2016). *Alternaria* fungi do not only contaminate harvests they can also spoil foods in the fridge-temperatures. *Alternaria* species produce more than 70 phytotoxins and some of them have been chemically characterized as mycotoxins to humans and animals. These metabolites can be grouped into several categories and seven main different groups exist: Nitrogen-containing compounds, steroids, terpenoids, pyranones, quinones, phenolics, and others (Lou *et al.*, 2013) (**Fig. 1**).

The group of pyranones is the most studied because important mycotoxins as alternaric acid (AltA), alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT) are included in this group. Few dibenzopyranones (two benzene rings) are produced by *Alternaria* fungi but they are the most important toxic compounds because AOH and AME are included in this group.

Nitrogen-containing metabolites are classified in three different groups: amines and amides, cyclopeptides, and other nitrogen-containing metabolites. Important *Alternaria* mycotoxins as tenuazonic acid (TeA) and altersetin are classified as amines and amides. Tenuazonic acid is considered the most acute toxic *Alternaria* mycotoxin (Asam and Rychlik, 2013). Altersetin toxicity has not been widely studied, but its presence caused the inhibition of topoisomerase II human cells (Jarolim *et al.*, 2017).

Quinones are derived from aromatic compounds as benzene or naphthalene resulting in a fully conjugated cyclic dione structure. Two different quinones groups are produced by *Alternaria*: Anthraquinones and perylenequinones. Macrosporin A and altersolanol A are considered the most important anthraquinones because of their presence in food and toxicity. Macrosporin A can be commonly found in food products; for instance 79% of wheat samples analyzed in Nigeria (Egbontan *et al.*, 2017) and 57% of the analyzed nuts samples were positive for macrospoprin A occurrence (Varga *et al.*, 2013). Perylenequinones are characterized by a pentacyclic-conjugated chromophore. Large amount of perylenequinones are produced by *Alternaria* genera and some of them can be toxic. The most important toxic perylenequinones compounds produced by *Alternaria* are Altertoxin (I, II, and III), alterperylenol and stemphyltoxin (I, II, and III). They are less common than anthraquinones but they are the *Alternaria* toxins with the strongest mutagenic properties (Fleck *et al.*, 2012, 2016). However, there are a lack of data about perylenequinones presence in food and it is not clear if these mycotoxins may be a concern for public or animal health.

Phenolic metabolites from *Alternaria* fungi have phenolic group in the structure. Up to now, toxic phenolic compounds for humans or animals produced by *Alternaria* fungi have not been identified. Resveratrol can be produced by some *Alternaria* species and it can reduce the risk of some human diseases as cancer and cardiovascular disease (Shi *et al.*, 2013).

Mode of Action and Toxicity

The most important *Alternaria* mycotoxins due to their harmful effects are alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), altenuene (ALT), tentoxin (TEN), and alter toxins (ATX) (Ostry, 2008). *Alternaria* mycotoxins are well known to have estrogenic activity, however, this will be always lower than ZEN estrogenic activity (Dellafiora *et al.*, 2018).

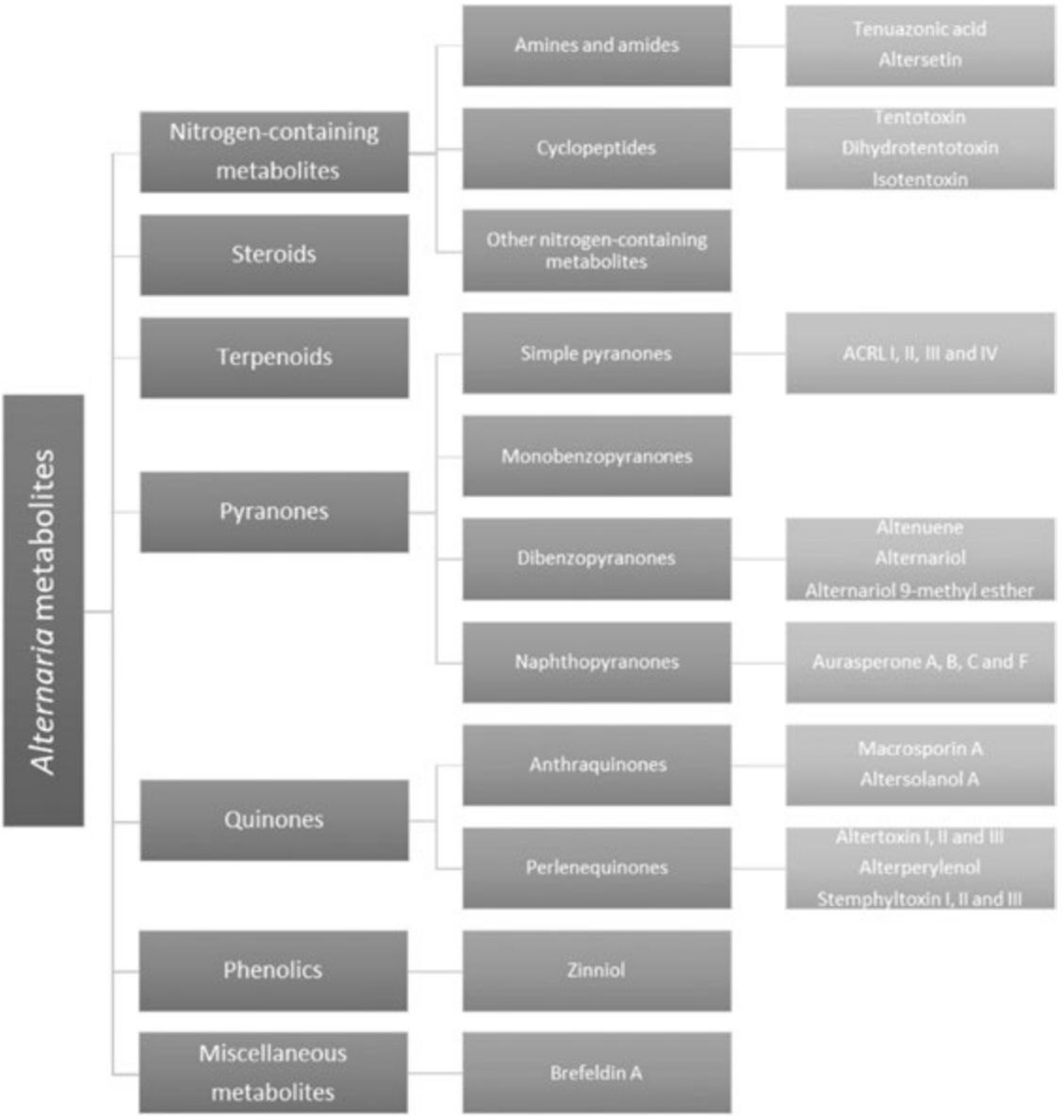

Fig. 1 Classification of *Alternaria* metabolites with the most important mycotoxins for each group.

Some *Alternaria* mycotoxins and several phase I and II metabolites of them are able to bind and activate estrogen receptors (ER) because they act as mimetic of 17-β-estradiol (Lehmann *et al.*, 2006; Dellafiora *et al.*, 2018), and AME has the strongest estrogenic activity (Dellafiora, Dall'Asta and Galaverna, 2018). For instance, AOH replaced estradiol from isolated recombinant ER-α and -β, acting as pure ER agonist. The AOH affinity was approximately 10,000 and 2500 times less than the endogenous hormone 17β-estradiol for the ER-α and ER-β, respectively (Frizzell *et al.*, 2013). *Alternaria* causes the increase of progesterone receptor expression as a synergistic effect of *Alternaria* estrogenic potency, so, hormone levels can be affected due to the *Alternaria* presence. Thus, an increase of progesterone and estradiol production was detected after cells were exposed to AOH. On the other hand, testosterone and cortisol were not affected. Some *in vitro* studies concluded that *Alternaria* toxins exert genotoxic, mutagenic, and carcinogenic properties. Thus, AOH and AME induced DNA strand breaks at concentrations $\geq$ 1 μM and had mutagenic potential at $\geq$ 10 μM (Brugger *et al.*, 2006; Fehr *et al.*, 2009). AOH and AME are also cytotoxic because human intestinal cells viability (HCT 116 and Caco-2) were reduced after exposure to AOH and AME. For cells viability, the synergistic effect of AOH and AME was again observed because the toxic effect was higher when both mycotoxins were present (Bensassi *et al.*, 2015; Fernández-Blanco *et al.*, 2016). The carcinogenic effects of *Alternaria* toxins has not been tested in *in vivo* studies; however, *Alternaria* mycotoxins were believed to be one of the causes of human esophageal cancer (Zhen *et al.*, 1991). Hence, due to their possible harmful effects, *Alternaria* toxins are of concern for public health.

Although *Alternaria* toxins have a significant toxicity, relevant *in vivo* toxicological data are not available and consequently an appropriate risk assessment is still lacking. Additionally, the co-occurrence of *Alternaria* mycotoxins with other mycotoxins that could cause a synergistic toxic effect is a possible scenario, as it was observed for AOH and ZEN in an *in vitro* study (Crudo *et al.*, 2019).

Occurrence of *Alternaria* Toxins in Food

The presence of *Alternaria* toxins in food has not been widely studied as other mycotoxins. However, the interest of *Alternaria* presence in food products increased and several studies showed that *Alternaria* toxins are common in food (Escrivá *et al.*, 2017). Cereals, vegetables, fruits, grains, and wine are the food items where the occurrence of *Alternaria* toxins is reported (Aichinger *et al.*, 2019). The frequent co-occurrence of multiple *Alternaria* toxins was also described in many other foods, including peppers. As an example, (Gambacorta *et al.*, 2019) analyzed samples of fresh, dried, grounded, and fried sweet pepper, wherein AOH, AME, TeA, and TEN were found together (limit of quantifications in the low μg kg^{-1} range). In particular, TeA was detected in all samples, while AOH was detected in 86%, 43%, 100%, and 14% of fresh, dried, grounded, and fried products, respectively.

AOH and AME are frequently found in combination (Vaquera *et al.*, 2016) as they share a common biosynthesis pathway (Saha *et al.*, 2012). Due to its large presence in food products, EFSA did a dietary exposure assessment for AOH, AME, TeA, and TEN to the European population using 15,563 analytical results from 4249 selected samples (Arcella *et al.*, 2016). The highest mean levels of AOH were reported for some grains in particular for buckwheat and oats. AOH was also present in diverse samples of tomato-based products such as tomato puree and tomato sauce. The presence of AME was lower and it highest occurrence was found in samples of tree nuts and oil seeds, in particular chestnuts and sesame seeds. TeA was reported in high concentrations in samples of tomatoes and several tomato-based products and an average concentration of 351.2 μg kg^{-1} was found in tomato soup (dried soup) (Arcella *et al.*, 2016). EFSA assessed the dietary exposure from all the analytical data gathered and toddlers and children presented the highest dietary exposure to *Alternaria* mycotoxins. Considering the upper-bound approach results, toddlers and infants presented a mean exposure of 1614 and 1490 ng kg^{-1} bw day^{-1} to TeA, 71.6 and 54.4 ng kg^{-1} bw day^{-1} to AOH, 38.8 and 34.4 ng kg^{-1} bw day^{-1} to AME, 33.4 and 30.1 ng kg^{-1} bw day^{-1} to TEN, respectively. Cereal-based food for infants and young children was considered the main contributor to the high dietary exposure to TeA (Arcella *et al.*, 2016).

Although *Alternaria* toxins are common in food samples, currently there is not any regulation for the occurrence in food and feed in any region of the world. As *Alternaria* metabolites are toxic compounds and they are common in food, a regulation to establish maximum admissible limits should be established in the coming years.

Metabolism of *Alternaria* Toxins

As *Alternaria* toxins group is formed by highly diverse chemical structures, it is also difficult to predict a similar metabolism pattern for all of them. Contrary to other mycotoxins, scarce information exists about *Alternaria* mycotoxins metabolism. *In vitro* and *in vivo* studies agreed that AOH and AME are hydroxylated at various positions. AME was metabolized to six different compounds (dihydroxy-AME, 8-hydroxy-AME, 10-hydroxy-AME, 4-hydroxy-AME, 2-hydroxy-AME, methyl-hydroxy-AME) while AOH was metabolized to five different hydroxyl compounds (8-hydroxy-AOH, 4-hydroxy-AOH, 10-hydroxy-AOH, 2-hydroxy-AOH, and methyl-hydroxy-AOH) (Pfeiffer *et al.*, 2007). *In vitro* studies performed with rat and human cell livers from phase I caused the apparition of four different hydroxylated altenuene compounds (8-hydroxy-altenuene, 10-hydroxy-altenuene, 4-hydroxy-altenuene, and stereoisomer of 4-hydroxy-altenuene). Moreover, 8-hydroxy-altenuene was identified as the predominant metabolite while the other three metabolites (10-hydroxy-altenuene, 4-hydroxy-altenuene, and stereoisomer of 4-hydroxy-altenuene) were formed in much smaller amounts (Pfeiffer *et al.*, 2009). All the studied species followed a similar pattern, with 8-hydroxy-altenuene appearing as the predominant compound. Phase II metabolism leaded to the formation of glucuronide metabolites and $>$ 75% of 8-hydroxy-altenuene was conjugated to glucuronide acid (Pfeiffer *et al.*, 2009). Apparently, human fecal microbiota is not able to metabolize AOH, AME, and ALT (Lemke *et al.*, 2016).

Alternaria mycotoxins can be metabolized to several other compounds and some of them could be the predominant in biological fluids. However, metabolite compounds have not been analyzed *in vivo* samples. There is a need to check the metabolization of *Alternaria* mycotoxins *in vivo* samples to perform a correct biomonitoring program.

Biomonitoring and Toxicokinetic Studies

Although traditionally performed combining consumption and occurrence data, the use of this method to assess human exposure could be associated to some uncertainties that a biomonitoring approach could solve. Over the last years, biomonitoring has been considered a powerful and promising tool to assess the exposure to contaminants. Traditional biomonitoring studies of internal exposure through urine or plasma analysis of targeted chemicals or metabolites are useful to link exposures to health outcomes. There are still some gaps to be able to link the mycotoxin intake with the mycotoxin concentration analyzed in the biological fluids. To solve these gaps, it is necessary to unravel the mycotoxin metabolism and the toxicokinetics (Vidal *et al.*, 2018).

In recent years, several studies tried to develop toxicokinetics models in humans for different mycotoxins, and ZEN and *Alternaria* mycotoxins have not been an exception. For ZEN, there was a first study performed with only one adult volunteer (Mirocha *et al.*, 1981). ZEN was administrated by oral dose (100 mg) and the urine was collected during 24 h and analyzed

Table 2 Presence of zearalenone and its metabolites in the biological samples analyzed from 2015 to 2019

Matrix	*Mycotoxins*	*N (%)*	*LOD (ng mL^{-1})*	*Average conc. (ng mL^{-1})*	*Max. Conc. (ng mL^{-1})*	*Average conc. (ng g^{-1} creatinine)*	*Max. Conc. (ng g^{-1} creatinine)*	*Country*	*Reference*
Urine	α-ZEL	1(0.4)	0.06	5.0	5.0	4.3	4.3	Belgium	(Heyndrickx *et al.*, 2015)
	β-ZEL-glucuronide	2(0.8)	0.12	0.8	1.0	0.9	1.1		
	α-ZEL-glucuronide	–	–	–	–	–	–		
	ZEN-14-glucuronide	–	–	–	–	–	–		
	β-ZEL	–	–	–	–	–	–		
	ZEN	–	–	–	–	–	–		
Urine	ZEN	–	0.15	–	–	–	–	Haiti	(Gerding *et al.*, 2015)
	α-ZEL	4(3)	0.125	1.46	2.49	0.97	–		
	β-ZEL	–	0.125	–	–	–	–		
	ZAN	–	0.375	–	–	–	–		
	ZAN-14-glucuronide	–	0.125	–	–	–	–		
	α-ZEL-glucuronide	–	2.5	–	–	–	–		
	ZEN-14-glucuronide	–	1.875	–	–	–	–		
	β-ZEL-glucuronide	–	2.50	–	–	–	–		
	ZEN	–	0.15	–	–	–	–	Germany	
	α-ZEL	–	0.125	–	–	–	–		
	β-ZEL	–	0.125	–	–	–	–		
	ZAN	–	0.375	–	–	–	–		
	ZAN-14-glucuronide	–	0.125	–	–	–	–		
	α-ZEL-glucuronide	–	2.5	–	–	–	–		
	ZEN-14-glucuronide	–	1.875	–	–	–	–		
	β-ZEL-glucuronide	–	2.50	–	–	–	–		
	ZEN	–	0.15	–	–	–	–	Bangladesh	
	α-ZEL	–	0.125	–	–	–	–		
	β-ZEL	–	0.125	–	–	–	–		
	ZAN	–	0.375	–	–	–	–		
	ZAN-14-glucuronide	–	0.125	–	–	–	–		
	α-ZEL-glucuronide	–	2.5	–	–	–	–		
	ZEN-14-glucuronide	–	1.875	–	–	–	–		
	β-ZEL-glucuronide	–	2.50	–	–	–	–		
Urine	ZEN	92(37)	–	0.09	–	0.14	–	Sweden	(Wallin *et al.*, 2015)
	α-ZEL	53(21)	–	0.13	–	0.19	–		
	β-ZEL	45(18)	–	0.10	–	0.13	–		
Urine	ZEN	13(100)	2	31	90	28	61	Germany	(Föllmann *et al.*, 2016)
	α-ZEL	6(46)	10	16	75	14	38		
	β-ZEL	3(23)	10	8	21	8	26		

(*Continued*)

Table 2 Continued

Matrix	*Mycotoxins*	*N (%)*	*LOD (ng mL^{-1})*	*Average conc. (ng mL^{-1})*	*Max. Conc. (ng mL^{-1})*	*Average conc. (ng g^{-1} creatinine)*	*Max. Conc. (ng g^{-1} creatinine)*	*Country*	*Reference*
Urine	ZEN	–	3	–	–	–	–	Spain	(Rodríguez-Carrasco *et al.*, 2017)
	α-ZEL	–	2	–	–	–	–		
	β-ZEL	–	4	–	–	–	–		
	α-ZAL	–	8	–	–	–	–		
	β-ZAL	–	8	–	–	–	–		
	ZAN	–	8	–	–	–	–		
Urine	ZEN	36(91)	0.002	0.09	0.42	–	–	Sweden	(Mitropoulou *et al.*, 2018)
	α-ZAL	21(53)	0.01	0.13	1.83	– –			
	β-ZAL	18(45)	0.01	0.08	1.33	– –			
Serum	ZEN	41(85)	0.07	0.087	–	–	–	USA	(Mauro *et al.*, 2018)
	α-ZEL	3(6)	0.07	0.001	–	– –			
	β-ZEL	17(35)	0.07	0.089	–	– –			
	ZAN	15(31)	0.07	0.102	–	– –			
	Zeranol	8(17)	0.07	0.005	–	– –			
	Taleranol	4(8)	0.07	0.001	–	– –			
Urine	ZEN	214(71)	0.05	0.24	3.7	–	–	China	(Li *et al.*, 2018)
	α-ZEL	3(1)	0.13	0.035	0.52	– –			
	β-ZEL	12(4)	0.20	0.082	2.6	– –			
	ZAN	66(22)	0.10	0.017	2.1	– –			
	α-ZAL	–	0.13	–	–	– –			
	β-ZAL	–	0.07	–	–	– –			
Urine	ZEN	98(82)	0.001	0.75	19.99	–	–	Nigeria	(Šarkanj *et al.*, 2018)
	α-ZEL	5(4)	0.001	1.27	2.52	– –			
	β-ZEL	7(6)	0.003	0.88	2.74	– –			
Urine	ZEN	60(100)	0.01	0.10	0.28	0.09	–	Germany	(Ali and Degen, 2018)
	α-ZEL	60(100)	0.01	0.16	0.45	0.16 –			
	β-ZEL	60(100)	0.01	0.05	0.20	0.04 –			
Plasma	ZEN	16(6)	0.03	0.157	0.418	–	–	China	(Fan *et al.*, 2019)
	ZAN	3(1)	0.03	0.260	0.346	–	–		
Urine	ZEN	16(6)	0.03	0.146	0.311	0.166	0.342		

	ZAN	21(8)	0.03	0.342	1.82	0.431	2.24		
Urine	ZEN	62(100)	0.01	0.028	0.084	0.114	–	Bangladesh	(Ali and Degen, 2019)
	α-ZEL	62(100)	0.01	0.198	0.346	0.993	–		
	β-ZEL	11(18)	0.01	0.013	0.022	0.023	–		
Urine[a]	ZEN	45(48)	0.20	0.17	3.98	0.28	7.59	Portugal	(Martins *et al.*, 2019)
	ZAN	–	0.15	–	–	–	–		
	α-ZEL	–	0.61	–	–	–	–		
	β-ZEL	–	0.91	–	–	–	–		
	α-ZEL-glucuronide	–	1.40	–	–	–	–		
	β-ZEL-glucuronide	–	1.72	–	–	–	–		
	α-ZAL	–	1.12	–	–	–	–		
	β-ZAL	–	1.60	–	–	–	–		
	ZEN-14-glucuronide	15(16)	0.30	0.17	25.70	0.18	39.78		
	ZEN-14-sulfphate	–	2.60	–	–	–	–		
Urine	ZEN	54(57)	0.20	1.30	11.51	1.01	8.28		
	ZAN	–	0.15	–	–	–	–		
	α-ZEL	5(5)	0.91	2.70	8.60	2.14	6.14		
	β-ZEL	–	0.61	–	–	–	–		
	α-ZEL-glucuronide	–	1.40	–	–	–	–		
	β-ZEL-glucuronide	–	1.72	–	–	–	–		
	α-ZAL	–	1.12	–	–	–	–		
	β-ZAL	–	1.60	–	–	–	–		
	ZEN-14-glucuronide	15(16)	0.30	0.15	25.70	0.13	12.42		
	ZEN-14-sulfphate	–	2.60	–	–	–	–		

[a]Urine samples collected during 24 h.

Abbreviations: ZAN, zearalanone; ZEN, zearalenone; ZEN-14-glucuronide, zearalenone-14-glucuronide; ZEN-14-sulfphate, zearalenone-14-sulfphate; α-ZEL, α-zearalenol; β-ZEL, β-zearalenol; α-ZEL-glucuronide, α-zearalenol-glucuronide; β-ZEL-glucuronide, β-zearalenol-glucuronide; α-ZAL, α-zearalanol; β-ZAL, β-zearalanol.

Table 3 *Alternaria* mycotoxins detected in human biological fluids

Matrix	*Mycotoxins*	*N (%)*	*LOD (ng mL^{-1})*	*Average conc. (ng mL^{-1})*	*Max. Conc. (ng mL^{-1})*	*Average conc. (ng g^{-1} creatinine)*	*Max. Conc. (ng g^{-1} creatinine)*	*Country*	*Reference*
Urine	Tenuazonic acid	6(100)	0.2	6.8	17.3	5.8	10.3	Germany	(Asam, Habler and Rychlik, 2013)
Urine	Tenuazonic acid	48(100)	0.2	6.58	44.4	8.13	63.8	Germany	(Hövelmann *et al.*, 2016)
	Allo-tenuazonic acid	46(96)	0.04	1.25	5.72	1.52	10.1		
Urine	Alternariol	8(6.7)	0.01	0.06	0.20	–	–	Austria	(Šarkanj *et al.*, 2018)
Urine[a]	Alternariol	27(29)	0.4	0.28	24.55	0.37	16.94	Portugal	(Martins *et al.*, 2019)
	Alternariol monomethyl ether	–	0.5	–	–	–	–		
Urine	Alternariol	12(13)	0.4	0.21	9.91	0.18	4.28	Portugal	
	Alternariol monomethyl ether	–	0.5	–	–	–	–		

[a]Urine samples collected during 24 h.

by ZEN, α-ZEL, β-ZEL. The urine analysis showed that it was not possible to detect ZEN, α-ZEL, β-ZEL without a previous β-glucuronidase treatment. After β-glucuronidase treatment, ZEN (13.27 μg mL^{-1}) followed by α-ZEL (12.94 μg mL^{-1}) were the predominant compounds found in urine. β-ZEL was also detected (4.63 μg mL^{-1}). However, since ZEN, α-ZEL, and β-ZEL were detected after β-glucuronidase treatment, it means that all detected forms were in the glucuronide form. The urine samples were gathered at three different times (6, 12, and 24 h). All three compounds showed a similar excretion pattern with lower concentrations in the first 6 h after ZEN administration, 3.71 μg mL^{-1} (ZEN) and 2.97 μg mL^{-1} (α-ZEL), without detection of β-ZEL. The second period of collection (from 6 to 12 h after oral administration) achieved the highest rate of mycotoxin extraction for all three analyzed mycotoxins: 6.87 μg mL^{-1} of ZEN, 6.00 μg mL^{-1} of α-ZEL, and 2.66 μg mL^{-1} of β-ZEL. Finally, from 12 to 24 h after ZEN administration, the concentration recovered started to decrease 2.69 μg mL^{-1} of ZEN, 4.02 μg mL^{-1} of α-ZEL, and 1.97 μg mL^{-1} of β-ZEL. The glucuronide forms present in the analyzed urines could be ZEN-14-glucuronide, ZEN-16-glucuronide, ZEN-14,16-diglucuronide, α-ZEL-14-glucuronide, and β-ZEL-14-glucuronide. Another toxicokinetic study of ZEN was performed using only one volunteer again (Warth *et al.*, 2013). They monitored urinary excretion of ZEN after the unique volunteer was exposed to ZEN at a dose of 0.167 μg kg^{-1} bw via a naturally contaminated diet for four consecutive days. Following enzymatic hydrolysis, the concentration of total ZEN (as the sum of conjugated and unconjugated ZEN) was measured in 24-h urine samples collected before, during and after ZEN exposure. ZEN was reported to be present mainly in the form of its glucuronide (ZEN-14-glucuronide). During ZEN exposure, concentration of total ZEN in 24-h urine samples ranged from 0.30 to 0.59 ng mL^{-1}, which corresponded to an excretion rate of 7.0%–13.2% of the administered dose within 24 h. Other metabolites such as α-ZEL and β-ZEL were not analyzed (Warth *et al.*, 2013). From the few existing information about ZEN toxicokinetics in humans, it seems clear that glucuronides forms of ZEN and α-ZEL are the predominant compounds in the urine (Martins *et al.*, 2019). The predominance of glucuronides form of ZEN and α-ZEL agrees with the *in vitro* results.

Recent years, several studies analyzed ZEN and its metabolites in biological fluids (**Table 2**). *In vitro* studies have been able to detect more than 30 different ZEN metabolites (Yang *et al.*, 2017). There are mainly products from hydroxylation, reduction, and glucuronidation but they are only small fraction of total ZEN excretion. The metabolites α-ZEL, β-ZEL 8-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide are the predominant forms *in vitro*. In addition, ZEN, α-ZEL, and β-ZEL can be mutually transformed in liver microsomes. Although ZEN is metabolized to an amalgam of ZEN forms, biomarker-analysis in urine should focus on free ZEN, α-ZEL, β-ZEL, and some of the most common hydroxylation and glucuronidation products like 8-OH-ZEN, 13-OH-ZEN, 15-OH-ZEN, and ZEN-14-glucuronide (Vidal *et al.*, 2018).

ZEN, α-ZEL, and β-ZEL and their glucuronide forms are the most analyzed metabolites in biological samples and these are the predominant compounds in urine. Thus, (Martins *et al.*, 2019) who analyzed the major quantity of ZEN metabolites in urine (10 different compounds, **Table 2**), found only presence of ZEN, ZEN-14-glucuronide and α-ZEL. Other studies found presence of other ZEN compounds; however, their concentrations were normally low and with small percentage of positive samples.

Few toxicokinetics with *Alternaria* mycotoxins have been performed. Moreover, as *Alternaria* mycotoxins are structurally very different among them, the results cannot be compared. Firstly, important differences in absorbance have been detected among *Alternaria* mycotoxins. Therefore, AOH and AME had a low systemic absorption and they were mainly excreted by feces (>85%) and they were scarcely recovered in the urine (<10%) after 24 h of oral administration in rats (Schuchardt *et al.*, 2014; Puntscher *et al.*, 2019). It is recommended to non-target analysis of AOH and AME metabolites in blood and urine to perform biomonitoring of AOH or to study its metabolism. The low absorption of AOH and AME points out that they should be found in feces, moreover, they could be in free form because apparently human fecal microbiota is not able to metabolize AOH and AME (Lemke *et al.*, 2016). Šarkanj *et al.* (2018) detected for the first time AOH in human urine samples: 6.7% of analyzed samples (n=120) from Nigeria were positive for AOH with an average concentration of 0.06 ng mL^{-1} (Šarkanj *et al.*, 2018). Martins *et al.* (2019) detected AOH in 29% (n=94) of the analyzed Portuguese samples, with a median concentration of 0.28 ng mL^{-1}. The larger AOH detection in Portuguese samples may be due to collection of 24-h urine samples (Martins *et al.*, 2019). Future biomonitoring studies should focus on hydroxylated and glucuronide metabolites, specially, phase II metabolites that could be the expected predominant compounds (Pfeiffer *et al.*, 2009).

TeA has a higher percentage of absorption; in a study performed with two human volunteers, > 87% of orally administered TeA was recovered in the free form in urine within 24 h (Asam *et al.*, 2013). As TeA is mainly excreted in free form in the urine, it was detected in two different studies were human urine samples from Germany were analyzed (**Table 3**). All the analyzed samples were positive for TeA presence (Asam *et al.*, 2013; Hövelmann *et al.*, 2016).

Therefore, apart from AOH and AME, other *Alternaria* mycotoxins as TeA and STX should be included in the biomonitoring programs due to their large presence and toxicity. However, elucidating the toxicokinetics is necessary for all *Alternaria* mycotoxins to perform an accurate biomonitoring program.

There is a clear need for further validation of ZEN and *Alternaria* toxins and their metabolites as urinary biomarkers of mycotoxins exposure by determining urinary excretion rates in a larger number of individuals to take into account possible inter-individual differences caused by different factors as gender and age.

Conclusions and Future Perspectives

Considering the available reviewed literature, it is important to highlight the adverse health effects that exposure to *Alternaria* mycotoxins and zearalenone may pose to human health. The estrogenic effects are a major concern particularly in early-life and

pre-pubertal stages of development; however, the effects of both mycotoxins go far beyond and possible cytotoxic, immunotoxic, and carcinogenic effects are attributed to these mycotoxins. Several gaps are identified; the knowledge on toxicokinetics of both mycotoxins is still very incomplete and the development of future studies to unravel the metabolism in human body is of high importance. A more complete information on metabolism makes possible not only the use of biomonitoring tools to accurately assess the exposure but also a proper risk assessment. Additionally, the interaction of mycoestrogens with other potential EDCs from other sources should be assess in order to have a deeper knowledge on the burden related with the exposure to these compounds, supporting the establishment of preventive measures that ensure public health.

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Fungi in Milk and in Dairy Products

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Introduction

Fungi are commonly found in the environment. They are eukaryotic, gram-positive, heterophilic living microbes (Pal, 2014). They are carcinogenic, cytotoxic, neurotoxic, immunosuppressive, they can cause allergic reactions and "sick building syndrome – SBS". The most popular fungi found in food are species of the genus of *Aspergillus* (*A. ochraceus*) – cereals, peanuts, coffee beans; *Penicillium* (*P. expansum*, *P. notanum*, *P.chrysogenum*, *P.cyclopium*) – stale bread, cheese, apples, citrus; *Fusarium* (*F. poae*, *F.graminearum*, *F.sorotrichoides*, *F.oxysporum*, *F.solani*) – cereals and crops (Chróst, 2016). (**Tables 1** and **2**).

Also from the medical perspective, reduction of the fungi occurrence in foods is important – fungi can produce mycotoxins that are toxic to humans – they are hepatotoxic, nephrotoxic, neurotoxic, teratogenic, estrogenic, have immunosuppressive effects, can damage the digestive system, can cause cancer and reproductive problems.

The main reason of products contamination by fungi are a warm climate and inadequate cooling (Pal, 2014). Contamination of products by fungi can also come from air, surfaces, ingredients and environment. Fungi contamination can be visible in the form of colonies or so-called "toad skin" or "cat hair". This is caused by fungi of the genera *Galactomyces geotrichum* and *Mucor* spp. (Garnier *et al.*, 2017b).

Some of the researches estimate that 5%–10% of all produced food in the world is lost because its spoilage by fungi (Buehler *et al.*, 2017; Garnier *et al.*, 2017a; Buehler *et al.*, 2018).

Milk and dairy products are a major type in few diets habits in many countries, therefore is very important to monitor and protect these products from contamination (Bouillon *et al.*, 2019).

Table 1 Fungi and mycotoxins which they can produce

Mycotoxin	*Fungi*	*References*
Aflatoxins (B_1, B_2, G_1, G_2)	*Aspergillus flavus, Aspergillus parasiticus, Aspergillus nominus*	Pitt (2000)
	Aspergillus flavus, Aspergillus parasiticus, Aspergillus nominus, Aspergillus pseudotamarii, Aspergillus bombycis, Aspergillus ochraceus	ELbagory *et al.* (2014)
	Aspergillus flavus, Aspergillus parasiticus	Hymery *et al.* (2014)
	Aspergillus ochraceus, Aspergillus flavus, Aspergillus parasiticus, Aspergillus nominus	Chróst (2016)
	Aspergillus flavus, Aspergillus parasiticus	Sengun *et al.* (2008), Becker-Algeri *et al.* (2016), del Palacio *et al.* (2016), Moubasher *et al.* (2018)
Ochratoxin A	*Penicillium verrucosum, Aspergillus ochraceus, Aspergillus niger, Aspergillus carbonarius, Penicillium viridicatum*	Pitt (2000)
	Aspergillus ochraceus, Aspergillus melleus, Aspergillus niger, Aspergillus sulfurous, Aspergillus awamori, Penicillium verrucosum, Penicillium crysogenum, Penicillium nordicum	Becker-Algeri *et al.* (2016)
	Aspergillus ochraceus, Penicillium verrucosum,	Chróst (2016)
Trichotecenes	*Fusarium graminearum*	Pitt (2000)
	Trichotecium, Trichoderma, Cephalosporium, Myrothecium, Stachybotrys, Cylindrocarpon, Verticimonosporium, Phomopsis	Becker-Algeri *et al.* (2016)
Fumonisins	*Fusarium moniliforme, Fusarium proliferatum*	Pitt (2000), Chróst (2016)
	Fusarium proliferatum, Fusarium verticillioides, Fusarium fujikuroi, Fusarium globosum, Fusarium oxysporum	Becker-Algeri *et al.* (2016)
	Fusarium species	Piątkowska *et al.* (2018)
Zearalenone	*Fusarium graminearum*	Pitt (2000)
	Fusarium graminearum, Fusarium culmorum Fusarium solani	Chróst (2016)
	Fusarium graminearum, Fusarium culmorum	Becker-Algeri *et al.* (2016)
	Fusarium species	Piątkowska *et al.* (2018)
Citrinin	*Penicillium citrinum, Penicillium viridicatum, Penicillium expansum, Penicillium verrucosum, Aspergillus carneus, Aspergillus niveus, Aspergillus terreus*	Hymery *et al.* (2014)
Toxin T-2, HT-2	*Fusarium sporotrichioides*	Becker-Algeri *et al.* (2016)
	Fusarium graminearum, Fusarium poae, Fusarium solani	Chróst (2016)
	Fusarium species	Piątkowska *et al.* (2018)

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Table 2 Maximum levels of mycotoxins in milk and dairy products

Mycotoxins	*Milk and milk products*	*maximum levels [μg/kg]*
Aflatoxin M_1	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	0.05
	Infant formulas and follow-on formulas, including infant milk and subsequent milk	0.025
Ochratoxin A	Dietary products for children (e.g., milk)	0.5

Note: (EC) No 1881/2006, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuff.

Fungi and Yeasts in Milk

Milk for human consumption is most commonly derived from cows – almost 83% of world milk production (Pal, 2014; Becker-Algeri *et al.*, 2016; Conte and Panebianco, 2019), but milk can be also obtained from a variety of animal sources such as goat (2%), sheep (1%) and buffalo (13%) (Becker-Algeri *et al.*, 2016), donkey (less than 0.1% and it is used as a substitute of breastmilk) (Sarno *et al.*, 2012; Conte and Panebianco, 2019) and human for human consumption. Cow's milk can be consumed directly as well as a component of dairy products and milk powder. Goat milk has been gaining benefits as an alternative to cow's milk, because it is less likely to cause allergies. It has also higher levels of iron and fat content. A soft curd is formed with it, which is more digestible. Cheeses made from goat's milk are popular in the Mediterranean and southern regions of Europe (Quigley *et al.*, 2013).

Sheep's milk is rarely drinking raw, and it is often used to make sheep's cheese (Quigley *et al.*, 2013; Spanamberg *et al.*, 2014).

Buffalo milk is consumed mostly in India and Pakistan. It is not common in Europe, but it is important in some Mediterranean countries, where it is utilized for making a traditional mozzarella cheese (Quigley *et al.*, 2013).

In the countries of the Mediterranean region, donkey milk and products based on it are very popular. It has low fat and protein content, while high lactose content (Malissiova *et al.*, 2015; Gross *et al.*, 2019).

Raw milk is very good environmental medium for the fungal growth, because it has a specific composition: it contains proteins, carbohydrates, minerals, fat and vitamins (Lawan *et al.*, 2012; Elhaj *et al.*, 2014). Microbial contamination of milk and milk products is mostly caused by unhygienic conditions, human factors, air, surfaces, supplier ingredients and milking environment on a farm (Cooke and Brazis, 1968; Pal, 2014; Garnier *et al.*, 2017b; Buehler *et al.*, 2018). Fungi are also commonly found in the dairy farms, therefore they can be present as a natural contaminants in raw milk. However this kind of fungi are mostly heat sensitive, thus they are not a direct source of fungi contamination in dairy products made of them (Buehler *et al.*, 2017). Raw milk contains a lot of nutrients for people in any age (especially it is consumed by infants and in childhood), however it has a neutral pH and high water content, which enables the growth and development of many microorganisms including fungal species (Vahedi *et al.*, 2013; Gulbe and Valdovska, 2014; Amaechi and Adiele, 2015; Von Neubeck *et al.*, 2015; Becker-Algeri *et al.*, 2016).

In addition, the occurrence of fungi in raw milk is affected by physiological condition of animals, breeding conditions and even the weather (Delavenne *et al.*, 2011; Quigley *et al.*, 2013). The presence of fungi is also affected by certain climate and vegetation features, as well as geographical location (Pešić-Mikulec *et al.*, 2005).

Additionally, dairy cattle suffer for mastitis, which can be caused by yeast like *Candida* spp. This is an inflammatory reaction of the udder. This infections are really common and also the most costly disease in dairy industry. Milk from cows with these infections has higher somatic cell count lowering the quality of milk. As a result, milk originating from the dairy cattle suffering from mastitis is not marketable (Dworecka-Kaszak *et al.*, 2012), since can contain flakes, clots, blood or has another color (Zastempowska *et al.*, 2016). Environmental factors have an important influence on fungal growth. Although there are some species that grow in specific seasons and in particular conditions, they have also a capability of living for the whole year (Pešić-Mikulec *et al.*, 2005). Also microbiota of milk can vary from other milk farms (Lavoie *et al.*, 2012).

Due to fungal contamination milk mycotoxins can be also present. Many studies indicate the presence of zearalenone in cow's milk (Gajęcka and Gajęcki, 2014; Becker-Algeri *et al.*, 2016), in bovine milk (Becker-Algeri *et al.*, 2016), in ruminants' milk (Zastempowska *et al.*, 2016), aflatoxin M_1 in cow's milk (Pitt, 2000; Sengun *et al.*, 2008; Jankovic *et al.*, 2009; Becker-Algeri *et al.*, 2016; Decontardi *et al.*, 2018), in bovine milk (Becker-Algeri *et al.*, 2016), in human breast milk (Sengun *et al.*, 2008; Zastempowska *et al.*, 2016) and fumonisins in bovine and human milk (Zastempowska *et al.*, 2016). (**Table 3**).

Fungi and Yeasts in Dairy Products

Dairy products are products derived from processed milk. It is consumed every day by millions people in the world, so the risk of a potential food-borne contamination is a big concern for people (Lawan *et al.*, 2012).

As a result of milk fermentation by bacteria, yeast or fungi can produce many products such as cheese, butter or yogurts (Moreira *et al.*, 2001). However, also the contamination of milk can have an influence to the physical and organoleptic characteristics of milk. On the other hand, characteristics of milk may affect the quality and shelf life of dairy products (Spanamberg *et al.*, 2014). Therefore, contamination of milk has an impact on the contamination of dairy products made of it.

Kefir is a symbiotic combination of microorganisms – lactic acid bacteria, yeast, sometimes acetic acid bacteria along with natural polysaccharides and protein matrix. Kefir grains are elastic, slimy, white to pale yellow with irregular structure, usually

1–3 cm long (Garofalo *et al.*, 2015; Bengoa *et al.*, 2018). Research conducted by Garofalo *et al.* in Italy, showed the presence of yeast *Dekker anomala* found in kefir (Garofalo *et al.*, 2015).

Butter is made from milk, cream or both. It is one of the popular varieties of dairy product because of its high nutritive value (El-Diasty and Salem, 2009).

Yogurts are milky products that are produced using probiotics strains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* susbsp. *bulgaricus* (Maïworé *et al.*, 2019). In fact, yogurts are less contaminated by fungi than cheeses (Boor *et al.*, 2017).

Cheeses are known as a first class food products because of high content of proteins (casein), fat, calcium, magnesium, potassium, phosphate, minerals and vitamins (mostly vitamin D) (El-Diasty and Salem, 2009; El-Badry and Raslan, 2016; Hameed, 2016). Cheeses have a very complex microflora that is divided into two groups: starters and secondary flora. Starters are

Table 3 The occurrence of fungi in different countries

Country	*Farms type and number*	*Results*	*Conclusion*	*Reference*
Spain (Europe)	50 samples from commercial lot	Fungi: *Aspergillus, Penicillium, Myrothecium, Paelomyces, Yeast: Torulopsis candida*	Contamination caused by poor hygiene in manufacturing practices during packaging	Medina and Jordano (1993)
Republic of Benin (Africa)	42 samples from 12 farms and 6 markets	Molds and yeast were detected but no data about isolates are available	Contamination caused by poor hygiene during milking	Farougou *et al.* (2012)
Hungary (Europe)	297 samples from 4 geographical locations: lowlands, hill-mountainous, alluvial plains by the river and sub-mountainous and part by the river	The most frequent were fungi: *Fusarium* (hilly mountains in spring), *Penicillium* (hilly mountains in summer as well as sub-mountainous and part by the river in winter) and *Cladosporium* (sub-mountainous and part by the river in autumn)	The percentage of molds depending on geographical location and season of the year	Pešić-Mikulec *et al.* (2005)
Latvia (Europe)	547 samples from 14 organic farms from different regions of Latvia	Main detected fungi were of genera *Mucor, Absidia, Aspergillus, Penicillium, Geotrichum, Rhizopus, Apophysomyces, Trichoderma, Scopulariopsis, Cladosporium, Chateomium, Cunningchamellas, Fusarium* and *Sepedonium*	Occurrence of fungi depends on the location and season of the year	Gulbe and Valdovska (2014)
Quebec (Canada)	19 dairy farms from 6 region of Quebec	The most abundant species were: *Debaryomyces hansenii*. Nevertheless, there were also isolates of *Candida* (*C. glaebosa, C. parapsilosis, C. zeylanoides, C. tropicalis, C. rugosa, C. apicola, C. catenulata, C. glabrata, C. intermedia, C. pararugosa, C. silvae*), *Cryptococcus, Galactomyces, Issatchenkia, Nakazawaea, Pichia, Rhodotorula, Trichosporon, Trichothecium, Yarrowia, Lichtheimia corymbifera, Acremonium, Fusarium, Eurotium, Cladosporium, Penicillium, Gibberella, Phaeosphaeria, Phoma* and few other (but with no significant significance).	Microbiota of milk can vary from other farms. In most cases	Lavoie *et al.* (2012)
Poland (Europe)	66- quarter-milk from 44 cows with clinical or subclinical mastitis on small farms in Łomża or near Warsaw	*Candida parapsilosis, Candida krusei, Candida lusitaniae, Candida famata, Candida guilliermondii, Candida tropicalis,Candida albicans, Cryptococcus, Trichosporon, Saccharomyces, Rhodotorula, Geotrichum*	The number of mammary gland infections caused by *Candida* species has been increasing in the recent years are	Dworecka-Kaszak *et al.* (2012)
Algeria (Africa)	304 cows of different breeds	*Candida, Trichosporon, Rhodotorula Saccharomyces*	*Candida* spp can cause the mammary gland infections	Ksouri *et al.* (2015)
Poland (Europe)	Cows with clinical symptoms of mastitis on small farms in Łomża or near Warsaw	*Candida, Trichosporon, Geotrichum,*	*Candida* is an etiological factor for mastitis	Krutkiewicz *et al.* (2011)
Spain (Europe)	20 farms from Castilla La Mancha region – ewe's milk	The most frequently species was *Geotrichum candidum.* Also were detected *Fusarium, Cladosporium, Aspergillus, Penicillium, Trichosporon asahii, Lichtheimia ramosa*	*Geotrichum candidum* is the natural member of the microbiota in raw milk, *Trichosporon asahii, Lichtheimia ramosa* are an etiologic agents of mastitis	Marín *et al.* (2015)
Sudan (Africa)	160 camel's milk samples originating from different farms in Bahri (Khartoun North)	Fungi were not isolated. However, the samples were really contaminated by bacteria.	Camel milk should be pasteurized and heated before drinking. The udder and the worker's hands should be washed before milking	Elhaj *et al.* (2014)

Table 4 The occurrence of fungi in different countries

Country	*Dairy products type and number*	*Results*	*Conclusion*	*Reference*
Slovenia (Europe)	14 curd samples, 13 soft cheese samples, 13 semi-hard cheese samples	*Geotrichum, Aspergillus, Mucor, Fusarium, Moniliella, Penicillium*	The curd samples were more contaminated than cheese samples. It was probably caused by improper processing hygiene conditions	Torkar and Vengušt (2008)
Italy (Europe)	54 crust samples	*Penicillium, Aspergillus*	*Aspergillus* spp and *Penicillium* spp are important members of the grana cheese	Decontardi *et al.* (2018)
Serbia (Europe)	23 total samples of raw milk, infant food, pasteurized milk, whey, yogurt	Mainly *Geotrichum candidum* in samples of raw milk, infant food, pasteurized milk and in yogurt. *Aspergillius* was dominant in whey samples	It is important to take prophylactic measures to prevent factors of mycotoxin produce	Jankovic *et al.* (2009)
Egypt (Africa)	5 samples of raw milk, 5 samples of plain yogurt, 5 samples of kareish cheese, 5 samples of butter	In all products (mostly): *Aspergillus brasiliensin*, *Aspergillus flavus*, *Cladosporium sphaerospermum, Mucor circinelloides, Penicillium chrysogenum*, *Candida metapsilosis, Candida orthopsilosis. Candida parapsilosis, Candida zeylanoides, Cyberlindnera jadinii, Debaryomyces hansenii, Galactomyces candidus, Kazachstania unispora, Kluyveromyces marxianus, Myerozyma guilliermondii, Pichia exigua, Rhodotorula mucilaginosa, Trichosporon asahii*	The contamination of this products can come from environment	Moubasher *et al.* (2018)
Egypt (Africa)	Kareish cheese, Damietta cheese, processed cheese, 50 samples each	Kareish cheese*: Aspergillus fumigatus*, *Aspergillus niger, Penicillium corylophilum, Cladosporium cladosporidis.* Damietta cheese: *Rhizopus* spp., *Syncephalastrum* spp., *Paecilomyces* spp,. *Aspergillus versicolour*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terrus*, *Penicillium corylophilum*, *Cladosporium cladosporidis, Nigrospora* spp. Processed cheese: *Paecilomyces* spp,. *Aspergillus fumigatus, Aspergillus niger*, *Aspergillus terrus*, *Penicillium corylophilum, Cladosporium cladosporidis, Ulocladium* spp All kind of cheese: *Candida albicans, Candida krusei Debaryomces hansenii*	Cheese which is treated by Natamycin can have extended shelf-life	Hameed (2016)
USA (North America)	hard, very hard, semi-hard and semi-soft cheese, soft fresh cheese (cottage cheese, mozzarella, feta, ricotta, cream cheese), washed rind cheese and Spanish cheese	*Debaryomyces hansenii, Galactomyces candidus, Candida parapsilosis, Candida sake, Candida batistae, Kluyveromyces lactis, Pichia kudriavzevii, Yarrowia lipolytica*	Differences of yeasts and fungi isolated can be observed between cheeses type. It is difficult to learn if species are present due to the starter culture or the contamination from other sources like environment, workers etc.	Banjara *et al.* (2015)
Great Britain (Europe)	British blue-veined cheese Stilton (one – 7.56 kg)	*Penicillium roqueforti, Yarrowia lipolytica, Debaryomyces hansenii, Kluyveromyces lactis, Trichosporon ovoides*	The results showed that microflora in Stilton cheese is different in the sections of it	Gkatzionis *et al.* (2014)

China (Asia)	Hurood and Jueke cheese, also raw milk (two batches each)	*Kluyveromyces*, *Pichia*, *Geotrichum*, *Galactomyces*, *Torulaspora*, *Candida*, *Naumovozyma*, *Hanseniaspora*	From the same region but different time, microflora is different	Gao *et al.* (2017)
Serbia (Europe)	3 traditional Serbian cheese	*Debaryomyces hansenii*, *Kluyveromyces lactis*, *Candida zeylanoides*, *Trichosporon ovoides*, *Candida pararugosa*, *Torulaspora delbrueckii*, *Galactomyces geotrichum*, *Yarrowia lipolytica*	Different detection methods can detecting different species	Šuranská *et al.* (2016)
France (Europe)	hard cheese (48 samples), butter (1 sample), cream (2 samples), cream cheese (8 samples), pasteurized milk (2 samples), yogurt (4 samples), fresh cheese (6 samples), yogurt drink (3 samples)	Fungi species: Hard cheese: *Cladosporium sphaerospermum*, *Mucor (circinelloides, racemosus, spinosus)*, *Penicillium (antarcticum, brevicompactum, chrysogenium, commune, discolor, nalgiovense, nordicum, palitans, roqueforti, solitum)*, *Thamnidium elegans*, *Debaryomyces hansenii*, *Sporodiobolus salmonicolor*, *Yarrowia lipolytica* Butter: *Cladosporium phyllophilum* Cream: *Penicillium* (*adametzioides, charlsii*), *Meyerozyma guilliermondii* Pasteurized milk: *Cladosporium halotolerans*, *Penicillium charlesii* Yogurt: *Penicillium* (*bialowiezense, solitum*), *Candida parapsilosis*, *Meyerozyma guilliermondii* Fresh cheese: *Candida parapsilosis*, *Kluyveromyces marxianus*, *Pichia fermentans*, *Rhodotorula mucilaginosa*, *Trichosporon asahii*, *Yarrowia lipolytica*. Cream cheese: *Didymella pinodella*, *Exophiala* sp., *Penicillium (fellutanum, palitans)*, *Candida inconspicua*, *Cryptococcus pseudolongus*, *Galactomyces geotrichum*, *Kluyveromyces lactis* Yogurt drink: *Didymella pinodella*, *Penicillium (commune, solitum)*, *Candida intermedia*, *Galactomyces geotrichum*	Despite the used of preservatives the contamination of dairy products is still present	Garnier *et al.* (2017b)

lactic acid bacteria, the function of which is to produce lactic acid during fermentation of lactose. The secondary flora includes bacteria and fungi that might contribute to the development of organoleptic properties (Marín *et al.*, 2015). Yeasts can support the function of starter culture of lactic acid bacteria through the assimilation of lactate and formation of alkaline metabolites. The main supporting function of yeasts are the formation of aroma compounds and flavoring activity. These yeasts can be e.g.,: *Yarrowia lipolytica*, *Debaryomyces hansenii*, *Kluyveromyces lactis* (Šuranská *et al.*, 2016).

The production area has a significant impact on the cheese making process. For example, specific environmental conditions are associated with traditional cheese production, which has characteristic aroma, texture and taste (Panelli *et al.*, 2013).

Although fungi are important in pasteurized milk, especially when they are used for the production of cheese and other dairy products. A feature characteristic of some types of ripening cheese with fungi is extensive proteolysis and lipolysis (Torkar and Vengušt, 2008). Contamination of dairy products by fungi can come from the environment, air, equipment, handlers, packaging materials, farm workers, cheesemakers, brine, walls or shelves of ripening rooms (Lavoie *et al.*, 2012; Pal, 2014; Zastempowska *et al.*, 2016; Buehler *et al.*, 2017; Moubasher *et al.*, 2018). The spoilage of dairy products can even originate from water, milk or inadequate infrastructures for milk transportation to the processing facilities (Amaechi and Adiele, 2015; El-Badry and Raslan, 2016).

Fungi are sometimes visible on the surface of butter, cheese and old cream. They often grow in large quantities in the form of a fluffy growth. Dark patches inside the butter suggest the presence of *Cladosporium*, and the most common fungi on cheese is *Penicillium* (ELbagory *et al.*, 2014; Pal, 2014).

Dairy products, like yogurts and cheeses, are more susceptible to spoilage by fungi and yeasts, because there are a lot of strains that grow at low temperatures and low pH (El-Diasty and Salem, 2009; Boor *et al.*, 2017; Buehler *et al.*, 2017; Moubasher *et al.*, 2018). In addition, there is a lot of kind of cheeses that have reduced water activity and also high concentration of salt, thus they have good conditions for some species of fungi to grow (Buehler *et al.*, 2017). Moreover, yeast can assimilate organic acids (such as succinic, lactic or citric) and they are also resistant to cleaning and disinfecting agents (ELbagory *et al.*, 2014). In the cheese production, fungi and yeasts can be also undesirable, because they change the flavor, make an off-odor, make an anomalous textures, discolorations, deformation of the packets containing the cheese and accumulate mycotoxins (Bullerman, 1981; Sengun *et al.*, 2008; ELbagory *et al.*, 2014; Marín *et al.*, 2015; Hameed, 2016; Garnier *et al.*, 2017b; Moubasher *et al.*, 2018). In cheeses, sometimes a source of fungi can be the cheese rind, because during cheese slicing, fungi can be transferred to cheese surface (Garnier *et al.*, 2017a). Yeasts can produce alcohol and CO_2, resulting in yeasty taste of these dairy products (Moubasher *et al.*, 2018). Also yeast can come from brine, surface, equipment, or ingredient contaminations (Garnier *et al.*, 2017b). However, there are a lot of cheeses, such as in blue-veined varieties (e.g., Roqueford or Cabrales) and surface mold-ripened cheeses (e.g., Brie or Camembert), in which the actions of fungi are necessary to develop an appearance, texture and flavor profile (Marín *et al.*, 2015). These fungi are: *Penicillium camemberti* and *Penicillium roqueforti* (Hymery *et al.*, 2014). Also fungi and yeasts are added to cheeses to provide a characteristic consistency, aroma and prolong the shelf life (Lavoie *et al.*, 2012; Banjara *et al.*, 2015; Moubasher *et al.*, 2018). This fungal species added to dairy products are: *Debaryomyces hansenii*, *Candida catenulata*, *Galactomyces geotrichum*, *Kluyveromyces marxianus*, *Mucor lanceolatus*, or *Saccharomyces cerevisiae* (Garnier *et al.*, 2017a). Also these fungi are: *Aspergillus*, *Cladosporium*, *Geotrichum* and *Trichoderma* (Hymery *et al.*, 2014). These cheeses are mostly blue-veined, such as the French Bleu and Roquefort, the Italian Gorgonzola, the English Stilton, the Danish Danablue or the Spanish Cabrales, Picón Bejes-Tresviso, and Valdeón (García-Estrada and Martín, 2016; Ropars *et al.*, 2017). In addition, *Geotrichum candidum* is added to Camembert and Brie (Dupont *et al.*, 2017). In fact, the presence of *Penicillium roqueforti* contributes to the aerobic conditions that can result in a blue-vein appearance (Gkatzionis *et al.*, 2014; Ropars *et al.*, 2017; Yunita and Dodd, 2018).

In 1997, the study was conducted in Brazil. Samples of yogurt were collected from local manufactures and supermarkets from South of Minas Gerais. In tested samples, following species were isolated: *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, *Hansenula* spp., *Mrakia frigida*, *Candida parapsilosis*, *Debaryomyces castellii*, *Candida maltose*, *Schizosaccharomyces pombe*, *Candida mogii*, *Kluyveromyces marxianus*. The *Mrakia frigida* was the first time reported in yogurts. The authors concluded that warmer weather and inadequate refrigeration are the main reasons for higher levels of contamination (Moreira *et al.*, 2001). (**Table 4**).

Conclusions

Contaminated milk and dairy products are available almost all over the world. This paper shows that various kinds of fungi can be present in different types of milk and dairy products. Since now, up to 100 of fungi species have been identified as being responsible for dairy products spoilage. For example, *Penicillium* spp. are mainly isolated from hard and semi-hard cheeses, but they are also found in all other product types, including fresh, blue-veined, mould-ripened, soft- and semi-soft cheeses, butter, yogurt, milk as well as in buffalo, goat, or ewe's milk cheeses (Garnier *et al.*, 2017a). Also in milk samples, there were detected several fungi like *Aspergillus*, *Penicillium*, *Myrothecium*, *Paelomyces*, *Mycogene*, *Mucor*, *Absidia*, *Geotrichum*, *Rhizopus*, *Apophysomyces*, *Trichoderma*, *Scopulariopsis*, *Cladosporium*, *Chateomium*, *Cunningchamellas*, *Fusarium*, *Sepedonium*, *Lichtheimia*, *Malassezia* and yeast like *Candida*, *Cryptococcus*, *Galactomyces*, *Issatchenkia*, *Nakazawaea*, *Pichia*, *Rhodotorula*, *Trichosporon*, *Trichothecium*, *Yarrowia*, *Saccharomyces*. In addition, the most abundant species in dairy products are fungi such as: *Geotrichum* (cheeses, yogurts), *Penicillium* (cheeses, yogurts, butter, cream), *Aspergillus* (cheeses, whey, yogurt, butter), *Mucor*, *Cladosporium* (cheeses, yogurts, butter), *Fusarium*, *Moniliella*, *Rhizopus*, *Syncephalastrum*, *Paecilomyces*, *Nigrospora*, *Thamnidium* (cheeses) and yeasts: *Dekker* (kefir), *Kluyveromyces*, *Galactomyces*, *Candida* (cheeses, yogurts, butter, cream), *Debaryomyces*, *Cyberlindnera*, *Kazachstania*, *Myerozyma*, *Pichia*, *Rhodotorula*, *Trichosporon* (cheeses, yogurts, butter), *Saccharomyces*, *Hansenula*, *Mrakia*, *Schizosaccharomyces*, *Meyerozyma* (yogurts), *Yarrowia*, *Torulaspora*, *Naumovozyma*, *Hanseniaspora* (cheeses), *Cryptococcus* (cream).

Determining the sources of fungi in products consumed daily is extremely important, because it helps to prevent future contaminations. It will also estimate any associated potential health risk (ELbagory *et al.*, 2014), especially because there is a lot of factors which can cause the growth of fungi and yeasts.

Preventive measures that should reduce the occurrence of fungi contamination on these products are very important for an economic reason – contaminated products are not suitable for sale (El-Badry and Raslan, 2016; Vimont *et al.*, 2019). Fungi can change the characteristics of milk, cheese, yogurt, etc, thus products can became not tasty.

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Profile of Fungi in Dietary Supplement, Based on Plant Raw Material

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Introduction

Herbal plants are used as everyday products and as raw materials in the pharmaceutical industry for the production of, among others, dietary supplements for the use and treatment of diseases; however, they may not meet the quality, safety, and efficacy requirements of the treatment (World Health Organization, 2001,2005). According to the WHO, herbal medicines are defined as herbal products in the medicines category in a national drug regulatory framework, therefore they may include "herbs", "herbal materials", "herbal preparations", and "finished herbal products"/"herbal medicinal products" (World Health Organization, 2007). The World Health Organization (2014–2023) confirms the use of phytotherapy is popular and is the main source of health care primarily in countries with less availability of conventional medicine, but also in developed countries or as complementary therapy used by about 70% of Australians and one-third of Americans (Complementary Medicines Australia, 2017). Global market for medicinal plants is constantly growing, for 2016 it was valued at 71.19 billion USD by Hexa Research (2017) and Rocha-Miranda and Venâncio (2019). The medicinal plant consists of many active chemicals, such as mucilage, polyphenols, polysaccharides, etc., which may modify the effects of the active principles. Thus, it is believed that some natural remedies may have toxic effects on the body or act as an agonist or antagonist of the active substance (Dar *et al.*, 2013). Therefore, toxicity tests for pre-assessment of the efficacy of plant extracts are so important (Hewawasam *et al.*, 2016).

Herbal plants are exposed to different types of pollution, from chemical to microbiological, which have negative impact on human body. Examples of potential sources of contaminants as well as manufacturing stages, during which they may be detectable, are shown in **Table 1**.

Dietary supplements are foodstuffs and they must comply with the definition of food in Regulation (EC) No 178/2002 of the European Parliament and of the council of 28 January 2002 (European Commission, 2002), according to which: "food (foodstuff) means any substance or product, processed, partly processed or unprocessed, intended for human consumption or the consumption of which by people can be expected." Current legal regulations do not have guidelines for ingredients of dietary supplements other than vitamins and minerals, which means that the market for these products is characterized by considerable diversity. According to a report from the European Commission (SEC, 2008), the number of substances used in the production of dietary supplements is about 400, of which 50% of the dietary supplements market in the European Union are vitamins and minerals (**Table 2**). Dietary supplements contain mostly plant raw materials traditionally used in Europe, including pharmacopoeia raw materials; however, they should be in supplements at doses much lower than the therapeutic ones.

Under current law, trade of a dietary supplement does not require authorization, as is the case with medicinal products. Therefore, most of these products are not tested and their use is monitored poorly or not at all. The cause of negative impact of dietary supplements on a body may be contamination by various mold fungi during collection of plant ingredients, use, packaging, and distribution. Almost 80% of deaths in Poland result from the so-called civilization diseases, i.e., in the study of cardiovascular diseases – approx. 48%, malignant cancers – approx. 24%, and injuries and poisonings – approx. 7% (Ałtyn *et al.*, 2018). Organizations dealing with the issue of food, i.e., the Food and Agriculture Organization (FAO), The World Health Organization (WHO), and the EU Food Safety Commission (EFSA) see the risk of using fungi as quite realistic, which is why committees have been flooding in recent years to assess the toxicity of them in foodstuffs. World market of medicinal herbs in 1985 was estimated to ~ $17 billion, while current market analysis shows that by 2023 it will increase to ~ $111 billion (average annual growth rate of 7%–8%). Factors that increase the demand include the cost of insurance-based medical care in some areas (America), growing desire of people in industrialized countries to take over their own health, and increasingly older population in these countries (Market Research Futures, 2018).

Occurrence of Fungi in Plants and Dietary Supplements – A Global Problem

Molds form a group of about 100,000 species and are widespread in nature. They are one of the most common environmental pollutants, due to their high ability to multiply under favorable conditions, on virtually any substrate (Ahmad *et al.*, 2014). It is estimated that about 25% of the biomass share is mold (Hussein and Brasel, 2001). From an ecological point of view, they play an important role in the natural circulation of matter, because they contribute to the degradation of biomass making it again useful as a nutrient. At the same time, however, as pathogens they are important in crops (especially cereals), causing a decrease in yield and a decrease in their quality.

Mold infection of raw materials and products (especially plant ones) and, as a result, the mycotoxin load is not uniform every year, therefore the problem in question is eagerly silenced, hence it is still underestimated. The most important types of mold fungi include: *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Claviceps* spp. They can be formed either by primary phytopathogenic species of fungi (e.g., *Fusarium graminearum*), already on plants growing in the field, or during storage of foodstuffs, raw materials and feed, under conditions favorable for their development (e.g., *Penicillium* spp).

Table 1 Classification of major contaminants and residues in herbal medicines

General classification	*Group*	*Subgroup*	*Specific examples*	*Possible sources*
Chemical contaminants	Toxic and hazardous materials	Toxic metals and non-metals	Lead, cadmium, mercury, chromium (arsenic, nitrite)	Polluted soil and water, during cultivation/growth, manufacturing process
		Persistent organic pollutants	Dioxin aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex	Polluted air, soil and water, during cultivation/growth
		Radionuclide	Cs-134, Cs-137	Air, soil, water during cultivation/growth
		Biological toxins	Mycotoxins	Post-harvest processing, transportation and storage
			Bacterial endotoxins	Post-harvest processing, transportation, and storage
Biological contaminants	Microorganisms	Bacteria	*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* species, *Shigella* species, *Escherichia coli*	Soil, post-harvest processing, transportation and storage
		Fungi	Yeast, molds	Post-harvest processing, transportation, and storage
	Animals	Parasites	Protozoa–amoebae, Helminths–nematoda	Soil, excreta; organic farming/cultivation, manufacturing process
		Insects	Cockroach and its parts	Post-harvest processing, transportation, and storage
		Others	Mouse excreta, earthworms, acarus	Post-harvest processing, transportation, and storage
Solvents	Organic solvents	Acetone, methanol, ethanol, butanol	Soil and water, Turing cultivation/growth, Manufacturing process	
Residues				
General classification	Group	Subgroup	Specific examples	Possible sources
Agrochemical Residues	Pesticides	Insecticides	Carbamate, chlorinated hydrocarbons, organophosphorus	Air, soil, water, during cultivation/growth, postharvest processing
		Herbicides	2,4-D, 2,4,5-T	Air, soil, water, during cultivation/growth, postharvest processing
		Fungicides	Dithiocarbamate	Air, soil, water, during cultivation/growth
	Fumigants	Chemical agents	Ethylene oxide, phosphine, methyl bromide, sulfur dioxide	Post-harvest processing
	Disease control agents	Antiviral agents	Thiamethoxam	During cultivation
Residual solvents	Organic solvents	Acetone, methanol, ethanol, butanol	Manufacturing process	

Note: Stages of production, at which contaminants are detectable – (1) medicinal plants; (2) herbal materials; (3) herbal preparations; (4) finished herbal products.
World Health Organization, 2007. WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. Geneva: World Health Organization.

Table 2 The highest tolerable intake level of some selected nutrients

Nutrient	*Acc. to SCF/EFSA*[a]	*According to IOM*[a]	*According to EVM*[b]
Biotin (μg)	–	–	900 (GL in food)
Folic acid (μg)	1000(Ref. only to folic acid supplements)	1000	1000 (GL in food)
Nicotinic acid (mg)	10	35 (niacin)	17 (GL in supplements only)
Nicotinamide (mg)	900	35 (niacin)	560 (GL)
Vitamin B_6 (mg)	25	100	10 (SUL in food)
Vitamin C (mg)	–	2000	–
Vitamin A (retinol) (mg)	3000 (does not refer to post-menopausal women)	3000	1500 (GL)
B – carotene (mg)	–	25 (ref. to smokers)	7 (SUL in supplements only)
Vitamin D (μg)	50	50	25 (GL in food)
Vitamin E (mg)	300	1000	800 (SUL in foods)
Calcium (mg)	2500	2500	1500 (GL in food)
Phosphorus (mg)	–	4000	250 (GL in food)
Magnesium (mg)	250 (Ref. to supplements)	350	400 (GL in food)
Iron (mg)	–	45	17 (GL in food)
Zinc (mg)	25	40	25 (SUL in foods)
Copper (mg)	5 (does not refer to women during pregnancy and breastfeeding)	10	10 (SUL)
Fluoride (mg)	7 (Ref. to children above 8 years and adults	10	–
Iodine (μg)	600	1100	500(GL in addition to food)
Selenium (μg)	300	400	450 (SUL)
Molybdenum (μg)	600	2000	–

[a]Determined as UL – the highest tolerable level.
[b]It is a GL value – tolerable level or as SUL value – the max safe level.
Note: Krasnowska, G., Sikora, T., 2011. Suplementy diety a bezpieczeństwo konsumenta. Żywność. Nauka. Technologia. Jakość 4 (77), 5–23.

Diseases called mycoses (aspergillosis, mucormycosis, penicilliosis) are included in the direct disease effects caused by fungi. They cause generalized and organ (invasive) mycoses as well as allergies. Fungal allergens (spores and fragments of fungi) are transferred with air bioaerosol. After entering the respiratory system, as well as remaining outside the body, especially in people and animals susceptible to allergies, they cause responses such as bronchial asthma, conjunctivitis, allergic rhinitis, up to alveolitis (Grajewski, 2006).

Some mold species are able to produce mycotoxins in the material to such an extent that negative effects may occur not only on the yield and quality of the raw material, but also on human and animal health. The insight that this group of natural, structurally very different toxic substances is formed by molds, is slowly occurring not only in pistachios, peanuts or dried figs, as well as in local crops or the human habitat (damp housing) and can be a cause of poisoning, slowly breaks through. Both own research and conducted by global scientific centers confirm that dangerously high levels of molds contamination are present in herbs, medicinal plants, as well as dietary supplements, which can pose a threat to human health (Grajewski, 2006).

Since the middle of the 20th century, researchers have identified the presence of fungi in herbs and dietary supplements. One of the first reports on the fungal spoilage of plant-based drugs was showed by Matsushima *et al.* (1958) and Kallings *et al.* (1966). Hitokoto *et al.* (1978) report of molds contamination of herbal plants in 1978. In their study, the results showed that *Aspergillus* and *Penicillium* species were predominant, but *Rhizopus*, *Mucor*, *Cladosporium*, and *Aureobasidium* spp. were also found in a few samples. Molds contamination of herbal drugs from Japan and India was also reported by Yamazaki *et al.* (1980)). Results of their work showed that 17 out of 50 samples were contaminated. *Aspergillus* and *Fusarium* species (54% and 24%, respectively) were the most frequent from Indian drugs. Halt (1998) analyzed the level of molds in medicinal plants and herbal tea. The dominant fungi detected were *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Absidia*, *Alternaria*, *Cladosporium*, and *Trichoderma* species. In 81 different samples, divided into 3 groups: final products (EP), raw material (RM), and during harvest (SH), degree of fungal contamination as well as the occurrence of natural *Aspergillus flavus* were examined. In all samples, the number of fungi did not exceed the international mandatory limits. The dominant species were *Aspergillus* (100%), *Penicillium* ($<$50%), and to a lesser extent *Fusarium* (5.6%). *A. flavus* was present in all three groups of samples (Rizzo *et al.*, 1998). Research conducted by Efuntoye (1996, 1999) in terms of the occurrence of fungi in sun dried herbal drugs from Nigeria showed that twenty-eight fungal species were isolated, with *A. niger*, *Aspergillus flavus*, *Fusarium verticillioides*, *Trichoderma viride*, *Penicillium expansum* and *Mucor fragilis* being the dominant ones.

In research conducted by Bugno *et al.* (2006), 10 types of fungi were identified in medicinal plants from Brazil, where 55% of samples exceeded the maximum limit of the fungi number set by the US Pharmacopoeia (10^2 cfu g^{-1}). The genus *Aspergillus* was the most common (90%) followed by *Penicillium* (40%). Also Singh *et al.* (2008) studied fungal contamination in six medicinal plant samples, viz. *Adhatoda vasica* Nees, *Asparagus racemosus* Linn., *Evolvulus alsinoides* Linn., *Glycyrrhiza glabra* Linn., *Plumbago zeylanica* Linn., and *Terminalia chebula* Retz. A total of 858 fungal isolates were detected from the analyzed samples, of which the Aspergillus genus was the most dominant causing infection of most raw materials. Also Ahmad *et al.* (2014) reported that in 70% of medicinal plant samples, the level of molds exceeded the permissible limits determined by the United States Pharmacopeial Convention Inc. (2005). These results are in agreement with findings of Abou-Arab *et al.* (1999) and Abou Donia (2008). Results

of Jahani *et al.* (2013) work showed the fungal contamination of barberry, with the high frequency of *Aspergillus*, *Penicillium* genus. Findings of Filipiak-Szok *et al.* (2016) showed that in the dietary supplements, the sum of molds and yeasts was lower than 69 cfu g^{-1}. Ting *et al.* (2013) showed molds contamination in traditional herbal medicines. *Bacillus* spp. was the most prevalent molds, and was detected from 7 of 8 samples. The least commonly recovered was the *Clostridium* spp. and *Staphylococcus* spp., detected only in 2 samples. In study by Sharma *et al.* (2014), a total of 16 fungal species belonging to 7 different genera, were isolated from the collected samples. *Aspergillus* was recorded as the most dominant genus with 9 species. *A. niger* was the most predominant species with occurrence frequency of 63.33%.

Tournas *et al.* (2006) analyzed 46 ginseng supplement samples including Siberian ginseng root, Chinese ginseng herb and root, and American ginseng root. Seventy eight percent of the ginseng herb, 100% of the Siberian, 56% of the Chinese, and 48% of the American ginseng root samples showed fungal contamination. The highest contamination (4.3×10^5 cfu g^{-1}) was observed in the locally grown American ginseng root and the lowest (<100 cfu g^{-1}) in ginseng extract. Fungi found in ginseng herb were *A. alternata*, *Aspergillus niger*, *Aspergillus*, *Cladosporium* and *Penicillium* spp., *Eurotium chevalieri*, *Rhizopus* spp., and yeasts. In 2009, research conducted by Tournas showed the presence of mold and yeast in herbal products and dietary supplements. The most common molds belonged to the genera *Aspergillus* spp., *Penicillium* spp., and *Eurotium* spp. The highest amount of mold and yeast was found in alfalfa supplements (5.6×10^6 cfu g^{-1}), while the lowest in ginger supplements (1.0×10^2 g^{-1}). Subsequent research conducted by Filipiak-Szok *et al.* (2016) concerned the contamination of dietary supplements and plant extracts, such as ginseng. Test results confirmed a high degree of contamination by fungi (*Cladosporium* spp., *Eurotium* spp., *Aspergillus* spp., *Rhizopus* spp., and *Penicillium* spp.). Rajeshwari and Raveesha (2016) studied herbal preparations for the presence of microorganisms, including molds. Eighteen herbal substances were tested, including Ashwagandha. Only in 6 of the tested materials, there was the presence of fungi. The most commonly isolated molds belonged to the genus *Aspergillus* spp. (20%) and *Penicillium* spp. (17%). *A. niger* was the most common mold species followed by *A. flavus*. In 2018, Su *et al.* (2018) analyzed eight RHs (e.g., Chinese yam, American ginseng, ginseng, astragalus, polygala, bupleurum, and liquorice), i.e., 48 samples in total (6 samples for each herb). Results showed that all 48 samples were contaminated by molds, with level from 1.08 to 4.391 log cfu g^{-1}, but samples did not exceed the maximum limit of 10^5 cfu g^{-1} (equivalent to 5 log cfu g^{-1}) set by the WHO for raw medicinal plants.

Green coffee and its extracts are included in dietary supplements that support the slimming process. Previously, they were not included in products endangering human health and life, because they were not consumed in this form. Research conducted by Twarużek *et al.* (2015) showed significant contamination of green coffee samples. The test material was dominated by molds of the genus *Aspergillus* spp. (63%) and *Penicillium* spp. (25%), while coffee extract was mainly contaminated with Penicillium spp. (72%). Research of Haas *et al.* (2013) concerned the content of fungi in Pu-Erh tea – red tea originating in the Yunnan province in southwestern China. It is used as a herbal dietary supplement supporting weight loss, lowers cholesterol and protects against caries. A total of 19 types of fungi were identified in this material, including 31 species, including *Aspergillus acidus* and *Aspergillus fumigatus*, or *Zygomycetes* and *Penicillium*. Also the results of research by Zhao *et al.* (2010) confirm the occurrence of fungi in all samples tested, in the range from 1.6×10^3 to 1.16×10^5 cfu g^{-1}, and 62 isolates were identified as belonging to 41 species from 19 genera, including 13 species of *Aspergillus* and 7 *Penicillium* species and 21 species of other genera. Due to the presence of fungal species such as *Aspergillus fumigatus*, *Penicillium* spp., *Trichoderma* spp., and *Fusarium culmorum*, etc., which can secrete toxic metabolites, further monitoring of the hygienic state of Pu-erh tea products is necessary. Kostecka and Bojanowska (2013) analyzed fresh basil, lemon balm and mint, as well as dried herbs, herbal teas, and dietary supplements. Molds were present in all samples, with the dominant genera: *Aspergillus* and *Fusarium*. Also in the samples there were genera of *Alternaria* and *Penicillium*, followed by *Mucor*, *Rhizopus* and *Uclodium* sp. Similar results were reported by Wójcik-Stopczyńska and Jadczak (2007) and Wójcik-Stopczyńska *et al.* (2010) in the study of whom, herbs were most frequently colonized by fungal genera of *Alternaria*, *Botrytis*, *Penicillium*, *Fusarium* and *Aspergillus*. One hundred forty-four samples belonging to 48 kinds of dried medicinal plants collected from various markets in the city of Mansoura in the province of Dakahlia were examined for the natural occurrence of mold. Among the analyzed samples, 36 species and 1 variety belonging to 11 genera were isolated. The genus *Aspergillus* (91.7%) was the most common type of fungus followed by *Penicillium* (68.8%). The most common species of fungi were *Aspergillus niger*, *Penicillium chrysogenum*, *Aspergillus flavus var columnaris*, and *Aspergillus flavus*, the incidence of which were 75%, 56.3%, 41.7% and 33.3%, respectively (Fatma *et al.*, 2017). One-hundred-fifty dry samples from Egypt, basil, mint, marjoram, chamomile, and fennel were analyzed for contamination by fungi and yeast. The highest number of molds was found in mint, basil, marjoram, fennel and chamomile, in descending order, respectively, while the highest number of yeast was found in chamomile, mint, fennel, basil, and marjoram, respectively. *Aspergillus* spp. *Fusarium* spp., *Penicillium* spp. *Muco*r spp. A *Humicola* spp. were more frequently detected. Thirty samples of fennel among mold and yeast were also analyzed and it was clear that the number of yeast and mold did not exceed 10^5 cfu g^{-1}. The greatest contamination by mold was present in the case of dry mint, basil, marjoram, fennel and then chamomile. (in descending order). In contrast, the largest count of yeast was present in chamomile, dry mint and fennel. Basil and marjoram showed 10^2 cfu g^{-1} for yeast (Abol-Ela *et al.*, 2011).

In contrast, research conducted by Aiko and Mehta (2016) showed an average content of fungi in stevia samples at the level of 3.5×10^2, where dominated the genus *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp. Keter *et al.* (2017) analyzed samples collected from Eldoret, Mombasa. In total, 88.2% of powders and 64.3% liquids from Eldoret were contaminated, while oils and control substances (conventional drugs, chlorphenamine, and paracetamol syrups) were not contaminated. Mombasa herbal powders were more contaminated at 84.2% and liquids at 66.7%, tablets 66.7%, while oils, capsules, and controls were not contaminated. Dlugaszewska *et al.* (2019) analyzed samples ($n = 106$) were had a mixed fungal contamination pattern with more than one genus present, with the dominant genera isolated from DS and RM, respectively: *Aspergillus* spp. (45.0%; 47.0%), *Alternaria* spp.

(26.3%; 23.0%), *Penicillium* spp. (17.0%; 10,0%), *Fusarium* spp. (1.0%; 7.0%), *Cladosporium* spp. (3.0%; 3.0%). In studies conducted by Chen *et al.* (2020), the presence of fungi was detected in 48 samples of 13 different medicinal herbs. In all, 83.3% of all samples were contaminated with fungi. Sixty-nine strains were determined, of which four were found to be dominant: *Aspergillus* spp. (26.1%), *Penicillium* spp. (24.6%), *Rhizopus* spp. (14.5%), and *Trichoderma* spp. (11, 6%). Gajewska and Głowacka (2017) conducted a study, the purpose of which was to determine the level of contamination with dried filamentous fungi of dried herbs and spices available in organic stores and hypermarkets in Poland: garlic (*Allium sativum*), dill (*Anethum graveolens*), parsley (*Petroselinum crispum*), basil (*Ocimum basilicum*), oregano (*Origanum vulgare*), tarragon (*Artemisia dracunculus*), thyme (*Thymus vulgaris*), marjoram (*Origanum majorana*), nutmeg (*Myristica fragans*), curry (*Helichrysum angustifolia*), turmeric (*Curcuma longa*), black pepper (*Piper nigra*), sweet pepper (*Capsicum annuum*). Mycological analysis showed that filamentous fungi belonging to a total of 5 genera were isolated from dried herbs and spices: *Aspergillus* (*A. niger*, *A. flavus*), *Penicillium*, *Mucor*, *Alternaria*, and *Cladosporium*.

Contamination of plant raw materials and dietary supplements with fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* is very dangerous both for humans and animals due to the toxic products of secondary metabolism: mycotoxins, which can cause both acute and chronic toxic effects, and even death (Food and Agriculture Organization of United Nations, 2001). Yamazaki *et al.* (1980) showed that 17 out of 50 samples were contaminated by mycotoxins. AFB_1 was the most frequently detected mycotoxin and it was detected above tolerance levels fixed by the World Health Organization. Study of Su *et al.* (2018) also reported that among 13 isolates of *A. flavus*, five ones from Chinese yam, ginseng, astragalus, and liquorice were found to produce AFB^1 and AFB^2. In the research of (Bugno *et al.*, 2006), almost 22% of isolates from medicinal plants were able to produce mycotoxins: 42.9% were aflatoxygenic strains, 22.4% were ochratoxygenic strains, and 34.7% were strains producing citrinine. Also Singh *et al.* (2008) studied aflatoxin B_1 contamination of six medicinal plant samples, viz. *Adhatoda vasica* Nees, *Asparagus racemosus* Linn., *Evolvulus alsinoides* Linn., *Glycyrrhiza glabra* Linn., *Plumbago zeylanica* Linn., and *Terminalia chebula* Retz. Also Wu *et al.* (2014) studied previously isolated *Alternaria tenuissima* strain from the of *Tribullus terrestris* L. and found it to be a producer of mycotoxins (altertoxins II and IV). Also samples belonging to 48 kinds of dried medicinal plants collected from different markets in Mansoura city, Dakahlia governorate, were examined for the natural occurrence of aflatoxins. Out of 30 medicinal plant samples screened, 19 samples (63.3%) were found to be contaminated with aflatoxins in the range of 1.5–724.6 ng g^{-1} (Fatma *et al.*, 2017). Both own research and conducted by global scientific centers confirm that in herbs, medicinal plants, as well as dietary supplements, dangerously high levels of mycotoxins are also present: aflatoxin (AF), ochratoxin A (OTA), fumonisin (FB), zearalenone (ZEN), which may pose a risk to human health (Vidal *et al.*, 2013; Trucksess *et al.*, 2009; Tassaneeyakul *et al.*, 2004; Veprikova *et al.*, 2015; Solfrizzo *et al.*, 2015; Ariño *et al.*, 2007; Arroyo-Manzanares *et al.*, 2013; Badreldin *et al.*, 2008; D'Ovidio *et al.*, 2006; Karkanis *et al.*, 2018; Koul and Sumbali, 2008; Lippolis *et al.*, 2017; Zain, 2011; Zhang *et al.*, 2018).

Conclusions

Consumers are increasingly interested not only in the health-promoting properties of medicinal plants and dietary supplements, but also in their safety for health. Results of conducted research confirm that these products are exposed to various types of mycological contaminations, which largely depend on environmental conditions. Contamination can occur at any stage of production, i.e., during cultivation, as well as during harvesting, processing, storage, and transport. Despite many benefits of taking dietary supplements, however, we must remember about their hygienic status, and that, according to the saying of Paracelsus (1493–1541), a Swiss–German doctor: everything is poison, nothing is poison, the action depends only on the dose.

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Molds in Food Spoilage

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Introduction

Food spoilage is a metabolic process that causes foods to become undesirable or unacceptable for human consumption due to changes in sensory characteristics (Sahu and Bala, 2017; Rawat, 2015). The main cause of food spoilage is invasion by microorganisms such as molds, yeast and bacteria. Fungi are ecologically, physiologically and morphologically diverse and constitute one of the largest group of organisms on earth with at least 1.5 million and more probably, close to 3 million species (Hawksworth, 2015; Quéro *et al.*, 2019). Fortunately for the food industry, the number of concern as spoilage agents in food, drink, and water are relatively few; only a few hundred, of which only about 50 are commonly encountered (Hawksworth, 2015). Among fungi, microscopic filamentous fungi (also called molds) have an important impact on human activities, either positive or negative. On one hand, they are important producers of enzymes, organic acids and antibiotics and can be used to manufacture fermented foods (Chalupová *et al.*, 2014; Chen *et al.*, 2014; Rodríguez *et al.*, 2015; Quéro *et al.*, 2019). On the other hand, they are able to spoil a large variety of food and food commodities, from raw materials to finished products. Their growth can lead to several types of product-alteration such as taste and odor problems and decay (Pitt and Hocking, 2009; Quéro *et al.*, 2019). Filamentous fungi cause important food losses and substantial economic damage (Quéro *et al.*, 2019; Thornton and Wills, 2015; Rodríguez *et al.*, 2015).

The exact causes of food losses vary throughout the world and are very much dependent on the specific conditions and local situation in a given country. In broad terms, food losses is influenced by crop production choices and patterns, internal infrastructure and capacity, marketing chains and channels for distribution, as well as consumer purchasing and food use practices. Irrespectively of the level of economic development and maturity of systems in a country, food losses should be kept to a minimum (State of Food and Agriculture, 2019; Rawat, 2015). These losses are very difficult to evaluate, because they fluctuate depending on the location, the season, and the type of product spoiled. For instance, 1%–3% losses were reported in the bakery industry, and 5%–10% losses were reported for postharvest fruits treated with fungicides (Dagnas and Membre, 2013; Rawat, 2015).

The issue of food losses is of high importance in the efforts to combat hunger, raise income and improve food security in the world's poorest countries. Food losses have an impact on food security for poor people, on food quality and safety, on economic development and on the environment. There are also environmental and resource costs associated with food spoilage and loss. If 20% of a crop is lost, then 20% of the fertilizer and irrigation water used to grow that crop was also lost (Rawat, 2015). A new FAO report launched 14 October 2019, Rome – by the UN Food and Agriculture Organization, provides insights into how much food is lost – as well as where and why – at different stages of the food supply chain, calls for informed decisions for an effective reduction and offers new ways to measure progress. FAO report provides with new estimates of food loss from post-harvest up to retail to help identify appropriate measures for an effective reduction. According to the State of Food and Agriculture (2019), globally around 14% of the world's food is lost after harvesting and before reaching the retail level, including through on-farm activities, storage and transportation (State of Food and Agriculture, 2019).

Diversity of Spoilage Fungi

Food spoilage may be defined as a process or change which renders a product undesirable or unacceptable for consumption (Nychas and Panagou, 2011). Spoilage may arise from insect damage, physical damage, indigenous enzyme activity in the animal or plant tissue or by microbial infections. Most natural foods have a limited life. Perishable foods such as fish, meat and bread have a short life span. Other food can be kept for a considerably longer time, but it decomposes eventually. Enzymes can bring about destruction of polymers in some foods, while chemical reactions such as oxidation and rancidity decompose others, but the main single cause of food spoilage is invasion by microorganisms such as molds, yeast and bacteria (Garnier *et al.*, 2017; Rawat (2015)). It has been estimated that 5%–10% of the worldwide food losses is due to the fungal spoilage (Quéro *et al.*, 2019).

Losses are the results of one or more problems occurring in the supply chain, from initial agricultural production down to the consumer level (State of Food and Agriculture, 2019). The moldy foods are the source of various food borne conditions due to gastroenteritis in human. Currently, the emphasis is put on the total quality of food, which means that not only food should be nutritionally balanced but should be microbiologically safe too (Embaby *et al.*, 2015). In this context, correct identification of food fungal spoilage is a key step to better manage the food safety and quality. An erroneous identification could lead to the risk underestimation or unnecessary counter measures (Hawksworth, 2015; Quéro *et al.*, 2019).

Filamentous fungi are also a constraint on food safety issue due to the production of mycotoxins or allergens by these molds. To avoid or reduce the mold spoilage, several hurdles can be used: reducing the water activity of the food, thermal processing, addition of preservatives, reduction of oxygen in the packaging using vacuum, oxygen scavengers or modified atmosphere

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packaging (MAP), and refrigerated storage. However, the addition of each of these hurdles can be selected for different groups of spoilage fungi (Rico-Munoz *et al.*, 2019; Rodríguez *et al.*, 2015; Thornton and Wills, 2015). To prevent mold spoilage, it is essential that the associated mycobiota be adequately isolated and accurately identified (Rico-Munoz *et al.*, 2019). The fungi groups associated with fungal spoilage are: xerophilic fungi that can grow at water activity (*aw*) below 0.85 and spoil low and intermediate *aw* foods; heat resistant fungi that survive pasteurization and baking, spoiling heat-processed products; preservative-resistant fungi that can grow in the presence of preservatives such as sorbates or benzoates and spoil beverages and foods that are preserved; anaerobic fungi that can grow under very low oxygen concentration and spoil vacuum or modified atmosphere packaged foods and psychrophilic and psychrotolerant fungi that can grow under refrigeration and spoil refrigerated foods (Pitt and Hocking, 2009; Rico-Munoz *et al.*, 2019).

Xerophilic Fungi

Xerophilic fungi are defined by the ability to grow under reduced water activity conditions, i.e., to complete their life cycles on substrates that have been dried or concentrated, in the presence of high levels of soluble solids such as salts or sugars (Pitt and Hocking, 2009; Rico-Munoz *et al.*, 2019). First defined by Scott, *aw* is related to the water available in the matrix and is generally the dominant factor in the control of food stability and spoilage (Dagnas and Membre, 2013; Pitt and Hocking, 2009). Fungi can be subdivided among two groups (Hocking, 2001; Pitt and Hocking, 2009). The first contains those that are capable of growing at or below 0.85 *aw* values, generally known as moderate xerophiles. The second group, known as extreme xerophiles, contains filamentous fungi that either cannot grow at high aw (i.e., above about 0.96–0.97 *aw*) or grow very slowly and consequently compete very poorly in high *aw* environments. Many important food spoilage fungi can be described as "moderate xerophiles" such as *Penicillium* species, some *Aspergillus* species (including species with eurotiummorph) and *Paecilomyces variotii*. The most common *Aspergillus* species (with eurotiummorph) are *A. montevidensis* (=A. amstelodami), *A. chevalieri* (=*Eurotium chevalieri*), *A. pseudoglaucus* (=*Eurotium repens*), *A. ruber* (=*Eurotium rubrum*) and *A. glaucus* (=*Eurotium herbariorum*) (Chen *et al.*, 2017; Pitt and Hocking, 2009; Samson *et al.*, 2010; Rico-Munoz *et al.*, 2019). They produce mildly heat-resistant ascospores that, under some conditions, can survive heating at 70–75°C for several minutes. These species are widely associated with deterioration of stored commodities (cereal grains, oilseeds, nuts, spices), flour baked goods (bread, fruit cakes), pet food products, dried and fermented meat and fish products, cheeses, and high sugar products such as confectionery, dried fruit, jams and conserves (Hocking, 2001). Only a few *Penicillium* species are capable of growing below 0.80 *aw* and among these are *P. brevicompactum*, *P. chrysogenum*, *P. implicatum* and other species such as *P. cinnamopurpureum* (Hocking and Pitt, 1979; Pitt and Hocking, 2009).

Among the most commonly encountered species causing spoilage of fruit are *Penicillium italicum*, associated with the blue rot of citrus, *Penicillium digitatum* causing a devastating and rapid green rot of citrus, and *Penicillium expansum* the blue rot of apples and pears. Apart from the possibility of allergic response to the enormous numbers of dry air-borne spores produced by *P. italicum* and *P. digitatum*, especially the latter, there are no special health risks associated with these species as the rotten fruit will usually be discarded. This is not always the case with *P. expansum* that produces the mycotoxin patulin (Moss, 2008). *Aspergillus* species are both tolerant of elevated temperature and reduced *aw*, and many important food spoilage species produce mycotoxins (Hocking, 2001). Some of the most xerophilic species are *A. restrictus* and *A. penicillioides*. The species most tolerant to low oxygen tensions (0.45% oxygen) is *A. candidus*.

Among the mycotoxigenic fungi, *A. flavus* and *A. parasiticus* are capable of growing at a minimum *aw* of 0.80 at 30°C. In the group of the black aspergilli, *A. niger* can grow down to 0.77 *aw* at 35°C. *Aspergillus ochraceus* can also grow at 0.77 *aw* at 25°C while *A. versicolor* has a minimum *aw* for growth of 0.78 and 0.80 (Hocking, 2001). *Penicillium* and *Aspergillus* spp. are the most common spoilage fungi, whereas *Fusarium* species are mostly responsible for significant yield losses of small grain cereals and maize (Pitt and Hocking, 2009; Quéro *et al.*, 2019). Dry foods can also be spoiled by extreme xerophiles. These fungi are those that have an absolute requirement for, rather than tolerance of, reduced *aw* (Hocking, 2001). Some of the most common fungi in this category are *Bettsia fastidia*, *B. alvei*, and *Chrysosporium xerophilum*, *Xerochrysium dermatitis*, *X. xerophilum*, *Wallemia sebi* and *Xeromyces bisporus* (Pitt and Hocking, 2009; Pitt *et al.*, 2013; Rico-Munoz *et al.*, 2019).

Heat-Resistant Molds (HRM)

Spoilage of thermally processed products by heat-resistant molds (HRM) is a widespread problem for the food industries. They are responsible for the spoilage and degradation of heat-processed fruit, fruit juices and purees, and juice-containing beverages as well as teas. They can grow at low pH and low oxygen partial pressure. These molds can survive not only at temperatures applied during pasteurization, but also the high pressure used during high-pressure processing. HRM are also able to produce several mycotoxins that pose a hazard to human health (Frąc *et al.*, 2015; Rico-Munoz *et al.*, 2019).

Ability of HRM to survive these elevated temperatures is due to the formation of highly heat-resistant spores called ascospores (Dijksterhuis, 2007). Thermal treatments will not prevent spoilage of products by heat-resistant molds (Evelyn and Silva, 2015; Evelyn *et al.*, 2016; Scaramuzza and Berni, 2014; Tranquillini *et al.*, 2017). Species belonging to the genera Paecilomyces (with byssochlamysmorph), Aspergillus (with neosartorya-morph) and Talaromyces, have been most frequently isolated from spoiled

products (Frąc *et al.*, 2015). Ascospores of the genera *Penicillium*, *Hamigera*, *Thermoascus*, *Rasamsonia* and *Monascus* are less commonly occurring. Species of the genera Hamigera and Thermoascus form ascospores that are highly heat-resistant (Rico-Munoz *et al.*, 2019).

Ascospores of HRM can be found in the ingredients (liquid and dry), packaging and the processing environment. These molds have an asexual phase, during which they produce non-heat resistant spores called conidia and a sexual phase when they produce the heat-resistant ascospores. These ascospores need a trigger such as heating or high pressure to be activated. This activation takes place during pasteurization and high-pressure processing (HPP). During activation, the thick outer layer is broken. After activation and if the conditions are favorable, the ascospore can germinate and grow, causing spoilage of the product during storage. Prevention of spoilage of heat-processed products by HRM would require reduction or elimination of the HRM ascospores contamination from raw ingredients, packaging and the processing environment (Rico-Munoz *et al.*, 2019). *Xeromyces bisporus* is able to grow at lower *aw* (0.61–0.62) than any other known organism. It produces heat-resistant ascospores and some are able to survive heating at 80°C for 10 min (Hocking, 2001; Rico-Munoz *et al.*, 2019). Ascospores of *X. bisporus* appear to be capable of surviving the baking process, leading to sporadic spoilage of baked goods, which is often not manifested until many months after production. This fungus has never been isolated from soil or decaying vegetation. The most likely sources of *Xeromyces* infections appear to be dried fruits, spices and sugary foods with low *aw* (Pitt and Hocking, 1997).

Preservative-Resistant Molds (PRM)

Preservative-resistant molds (PRM) are able to spoil products containing preservatives such as sorbic, benzoic, propionic and acetic acids and their salts (Samson *et al.*, 2004). There are two kinds of PRM: (1) the heat-resistant PRM such as *Paecilomyces variotii*, *P. niveus (Byssochlamys nivea)* and *Monascus* species (*M. ruber*, *M. pilosus*) and (2) not heat-resistant ones such as *Penicillium roqueforti*, *P. paneum* and *P. carneum*, as well as conidia (non heat-resistant spores) i.e., *P. variotii*, a very common mold in the processing and packaging environment of these products (Rico-Munoz *et al.*, 2019).

The first group produce heat-resistant ascospores that are often found in ingredients such as corn flour (Rico-Munoz *et al.*, 2019). These ascospores can survive baking and pasteurization processes. The ascospores in the ingredients can also contaminate the surfaces of the equipment before the oven or pasteurizer or filler. Ascospores can then contaminate the open products and survive the heating step. These species grow better at warmer temperatures and spoilage of preserved products by these molds usually occurs during the warmer months.

A few heat-resistant fungi such as *Paecilomyces variotii* are also preservative-resistant. Some of the preservative-resistant molds such as *Penicillium roqueforti* can grow at low oxygen concentrations as well as under refrigeration (Rico-Munoz *et al.*, 2019). In the case of the second group, the non heat-resistant group, the contamination happens after the heat step given to these preserved products since they are killed by baking or pasteurization processes (Knight and Menlove, 2006; Rico-Munoz *et al.*, 2019; Tournas, 1994). Therefore, for preserved foods such as bread to become moldy, it must be contaminated either from the air, bakery surfaces, equipment, food handlers or raw ingredients after baking during the cooling, slicing or wrapping operations (Knight and Menlove, 2006; Saranraj and Geetha, 2012). In the case of the *Penicillium* species that can grow under refrigeration such as *P. roqueforti*, spoilage of these products may happen more frequently during the colder months. Fluctuation of temperature in the packaging areas must be avoided since it can result in condensation. The condensation on surfaces (walls, ceilings, overhead piping, etc.) can be conducive for mold growth. Furthermore, moisture condensation inside the package, due to packaging of the products prior to being completely cooled, may accelerate the mold growth and spoilage (Rico-Munoz *et al.*, 2019; Saranraj and Geetha, 2012).

Anaerobic Fungi

Majority of food spoilage molds have an absolute requirement of O_2 to produce adenosine triphosphate (ATP) via the oxidative phosphorylation pathway (Pitt and Hocking, 2009), although a wide diversity of mold species are able to grow under reduced O_2 partial pressure as low as 1% (Nguyen Van Long and Dantigny, 2016). Some *Penicillium* species and many other common food spoilage fungi are inhibited only slightly when grown in nitrogen atmospheres containing approximately 1.0% oxygen (Pitt and Hocking, 2009). The growth of *Mucor ambiguus* on Koji agar and *P. roqueforti* on wheat extract agar was reported at O_2 partial pressure of 0.10% (Yanai and Kojo, 1980) and 0.14% (Magan and Lacey, 1984), respectively. *Paecilomyces variotii* produced normal colonies at 25°C under 650 mm of vacuum. Most spoilage molds appear to be sensitive to high levels of carbon dioxide (Pitt and Hocking, 2009). *Paecilomyces niveus* and *Paec. fulvus* (*Byssochlamys fulva*) were capable of growing at atmospheres containing even 60% carbon dioxide and less than 0.5% oxygen (Rico-Munoz *et al.*, 2019, Taniwaki *et al.*, 2001).

Aspergillus chevalieri and *Xeromyces bisporus* did not grow in an atmosphere of 80% CO_2 plus 20% O_2 during incubation for 60 days, or in 20% CO_2 and less than 0.5% O_2 (Taniwaki *et al.*, 2009, 2010). These atmospheres affected the viability of their spores. Neither atmosphere inhibited the growth of *Mucor plumbeus*, *Fusarium oxysporum*, *Paecilomyces fulvus*, *Paecilomyces niveus*, *Penicillium roqueforti*, *Penicillium commune* and *Aspergillus flavus*. At less than 80% CO_2 plus 20% O_2, patulin, cyclopiazonic acid and roquefortine C were produced at low levels by *Paec. niveus*, *P. commune* and *P. roqueforti*, respectively. Aflatoxin was not produced at all by *A. flavus*. *Xeromyces bisporus* was capable of growing in an atmosphere containing only 1% O_2, even in the presence of 70%–95% CO_2 but not in an atmosphere containing less than 0.5% O_2 and 20% CO_2 (Pitt and Hocking (1997)). It has been

reported that fungal growth is reduced, but rarely inhibited at CO_2 partial pressures ranging from 50% to 90% (Nguyen Van Long and Dantigny, 2016; Rico-Munoz *et al.*, 2019). Nguyen Van Long Vasseur and Couvert *et al.* (2017) confirmed that a modified atmosphere, consisting of O_2 or CO_2 partial pressure higher than 1% and 70%, respectively, was not sufficient to prevent conidial germination of the five fungal species frequently encountered as food spoilers in dairy products or, in the case of *Mucor lanceolatus* and *P. roqueforti*, utilized as cheese ripening cultures. *Mucor plumbeus*, *Fusarium oxysporum*, *Paec. fulvus*, and *Paec. niveus* are able to grow under anaerobic conditions (Pitt and Hocking, 2009; Rico-Munoz *et al.*, 2019).

Some species of *Rhizopus* and *Mucor* are frequently associated with the spoilage of fresh fruits and vegetables. *Rhizopus stolonifer* and *Mucor piriformis* are responsible for the rapid decay of soft fruits and these molds can spread rapidly, especially on commodities stored at temperatures above 20°C. *M. piriformis* has been described as a destructive pathogen of strawberries and a number of species of *Rhizopus*, especially *Rhizopus sexualis*, are pathogenic to strawberries causing a soft rot.

Psychrophilic and Psychrotolerant Fungi

Morita (1975) suggested that microorganisms that grow at 0°C or below that have an optimum temperature of 15°C and a maximum of 20°C should be called psychrophiles, while mesophilic microorganisms that grow at 0°C should be called "psychrotolerant" or "psychrotrophic" (Moyer *et al.*, 2017, Rico-Munoz *et al.*, 2019).

Microorganisms that grow at refrigeration temperatures (0–7°C) but having temperature optima above 20°C are called psychrotrophs. Psychrotrophs are defined as microorganisms that produce visible growth at 7°C $\pm$ 1°C with in 7–10 days, regardless of their optimum growth temperature. The microorganisms that are most commonly associated with refrigerated foods and cause

Table 1 Occurrence of molds in various products of food

Products	*Fungal species*	*Selected refs*
Vegetables		
Fresh vegetables	*Cladosporium, Alternaria, Penicillium, Geotrichum, Rhizopus, Fusarium, Botrytis, Colletotrichum, Aureobasidium*	Barkai-Golan and Paster (2008), Barth *et al.* (2009), Jeddi *et al.* (2014), Marinelli *et al.* (2012), Moss (2008), Tournas (2005).
Minimally processed vegetables	*Alternaria, Cladosporium, Penicillium, Geotrichum*	
Sprouts	*Alternaria, Cladosporium, Penicillium, Phoma, Fusarium, Geotrichum, Mucor, Rhizopus*	
Dairy products		
Fermented milk products	*Aspergillus, Penicillium, Alternaria, Cladosporium, Helminthosporium, Rhizopus, Acremonium, Mucor*	Banjara *et al.* (2015), El-Badry and Raslan (2016), ELbagory *et al.* (2014), Embaby *et al.* (2015), Hameed (2016), Khalifa and Nossair (2016), Sengun *et al.* (2008).
Cheese	*Penicillium, Aspergillus, Cladosporium, Geotrichum, Mucor, Trichoderma, Alternaria, Rhizopus,*	
Fruit		
Fruit juices	*Aspergillus, Penicilium, Curvularia, Fusarium, Alterneria, Colletotrichum, Geotrichum*	Alam *et al.* (2019), Aneja *et al.* (2014), Barkai-Golan and Paster (2008), Barth *et al.* (2009), Battilani *et al.* (2006), Diguta *et al.* (2015), Moss (2008).
Fresh fruit	*Penicilium, Colletotrichum, Botrytis Cladosporium, Alternaria, Epicoccum, Trichoderm, Mucor, Rhizopus*	
Dried herbs and spices	*Aspergillus Penicillium, Mucor, Alternaria, Cladosporium, Rhizopus, Paecylomyces, Absydia, Eurotium, Trichoderma*	Gajewska and Głowacka (2017), Karan *et al.* (2005), Kulshrestha *et al.* (2014); Nurtjahja *et al.* (2019).
Flour	*Penicilium, Aspergillus, Fusarium, Alternaria, Rhizopus, Acremonium, Mucor, Cladosporium*	Al-Defiery and Merjan (2015), Dec *et al.* (2015), Hakima *et al.* (2016), Halt *et al.* (2004), Plavšić *et al.* (2017), Weidenbörner *et al.* (2000).
Breakfast flakes and muesli	*Aspergillus, Penicillium, Cladosporium, Rhizopus, Mucor, Fusarium, Chaetomium, Trichoderma, Eurotium*	Ismail *et al.* (2008), Piotrowska (2013), Weidenbörner and Kunz (1994).
Bread and bakery products	*Alternaria, Aspergillus, Curvularia, Fusarium, Penicillium, Geotrichum Endomyces, Mucor, Rhizopus*	Ajith and Sunita (2018), Hakima *et al.* (2016), Nielsen and Rios (2000), Ravimannan *et al.* (2016), Saeed *et al.* (2018), Unachukwu and Nwakanma (2015), Vagelas *et al.* (2011).
Coffee	*Aspergillus, Penicillium, Cladosporium, Alternaria, Mucor, Fusarium, Rhizopous, Scopulariopsis*	Bokhari and Aly (2009), Djossou *et al.* (2015), Kusumaningrum and Rasyidah (2019), Nganou *et al.* (2014), Rahim *et al.* (2011).

food spoilage are psychrotrophs and not psychrophiles. Psychrophiles usually die at room temperature or above. (Rico-Munoz *et al.*, 2019; Vasavada and Critzer, 2015). Psychrotolerant filamentous fungi strains of *Penicillium*, *Alternaria*, *Fusarium*, *Cladosporium* and others grow exceptionally well at low temperatures and are relevant to refrigerated foods. According to Pitt and Hocking (2009), low temperatures permissive to fungal growth lie between 7°C and 0°C for some species of *Fusarium*, *Cladosporium*, *Penicillium* and *Thamnidium* (Samson *et al.*, 2004). Although psychrophilic and psychrotolerant fungi are definitely a problem in foods preserved at low temperatures, the amount of research related to this topic is small (Geiges, 1996; Gill, 2006, 2011; Gill and Lowry, 1982). In the literature, research on species of psychrophilic fungi is rare and restricted to temperatures just above zero, although the minimum for growth is generally estimated to be at temperatures slightly below 0°C (Geiges, 1996). The minimum temperature for growth of fungi may be considerably different depending on the substrate, on which the species develops, with lower permissive temperatures when growing in food compared to a conventional culture medium.

Saccomori *et al.* (2015) reported that *Penicillium polonicum* was able to germinate forming microcolonies (about 1 mm) in 2 of 5 (40%) of the plates of culture media incubated at 0°C, whereas *P. glabrum* did not germinate at this temperature. Both species formed colonies in the first week when incubated at 5°C (Rico-Munoz *et al.*, 2019).

Yeasts and molds are able to grow in a large variety of food including raw materials such as cereals, vegetables, fruits, meat, and milk, as well as processed products (Filtenborg *et al.*, 1996). Since the first detection of fungi in food, many investigations on the natural occurrence of molds in different products have been conducted. Examples of molds contaminations in food are summarized in **Table 1**.

Several traditional methods, also called traditional hurdle technologies, are implemented and combined to prevent and control such contaminations. Prevention methods include good manufacturing and hygiene practices, air filtration, and decontamination systems, while control methods include inactivation treatments, temperature control, and modified atmosphere packaging (Garnier *et al.*, 2017). Preparation, packaging, and distribution could cause increased contamination. Hence including good agricultural practices, good manufacturing practices, and Hazard Analysis and Critical Control Points, should be implemented to reduce the risk of microbial contamination from farm-to-fork and to assure safe products (Jeddi *et al.*, 2014).

Mold Spoilage Assessment

To assess mold spoilage, the appropriate methodology and media must be used. While classic mycological detection methods can detect a broad range of fungi using well validated protocols, they are time consuming and results can take days or even weeks (Rico-Munoz *et al.*, 2019). New molecular detection methods are faster, but require good DNA isolation techniques, expensive equipment and may detect viable and non-viable fungi that probably will not spoil a specific product. More research is needed on the development of detection and identification methods that are both faster and highly sensitive (Rico-Munoz *et al.*, 2019). In recent years, MALDI-TOF MS has emerged as a powerful tool to identify microorganisms and has successfully been applied to the identification of filamentous fungi, especially in the clinical context (Chalupová *et al.*, 2014; Quéro *et al.*, 2019; Rico-Munoz *et al.*, 2019).

Conclusion

Mold spoilage prevention requires knowledge of the associated mycobiota on food. Control of fungal spoilage is a major concern for industrials and scientists that are looking for efficient solutions to prevent and/or limit the fungal growth or development in dairy products. Different traditional methods, also called traditional hurdle technologies, are implemented to control such contaminations including air treatment, cleaning and disinfection procedures, heat treatment, water activity reduction by brining, refrigeration, modified atmosphere packaging, as well as the use of chemical preservatives, which are considered as food additives. More research is needed on the development of detection and identification methods that are both faster and highly sensitive.

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Mycobiota Causing Diseases in Pets

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Do Fungi Need to Be Pathogenic?

Fungal strains (understand isolates) belonging to one species are very much close to each other in their genetic toolkit. That leads to the phenomenon the particular fungal strain as so called etiologic agent of a disease can be actually coincidental if the other members of the same species live outside the human body. Thus, in contrary to bacteria where particular strains possess so called pathogenicity islands in their genomes, and they differ among themselves in virulence and antibiotic resistance, in mycology, the pathogenicity can be attributed only to the particular species not necessarily to its every single isolate (strain) (Gräser *et al.*, 2008). Pathogenicity of fungi is a potential (virtual) behaviour of them, which might not always be displayed (Ribeiro *et al.*, 2017). On the other hand, every strain behaves very actually under given circumstances, incl. causing a disease in a host present. It even can happen that due to somehow reduced virulence (over the evolution) of human-associated infectious agents the ones with highest degree of adaptation (to humans only) can almost not cause disease. Some of the pathogenic fungi have a warm-blooded hosts different from humans.

All fungi are optimally living in their natural biotop – the niche which they are much more often isolated from in comparison to other places, so called unoptimal environments. Some species are, however, able to inhabit remarkably wide variety of environments, they have a wide ecological amplitude (Pitt and Hocking, 1997; Fortin *et al.*, 2017). This ability is based on mechanisms of conversion into a metabolically silent state (chlamydospores, endoconidia etc.) and it is polyphyletic in the kingdom Fungi. The most fungi culturable in vitro do belong to this group. Anyway, there is the large group of highly specialized fungi that require special methods for being isolated. And some of clinically important fungi are exactly such organisms.

In animal and human medical mycology, one usually posts immunocompromised patients might be invaded by any fungus practically. While even the established pathogenic fungus are, perhaps, causing disease only when being implanted into the host coincidentally as they naturally occur – grow and reproduce – in the environment. That might explain e.g., the agents of deep mycoses are not transmitted from host to host unlikely pathogenic bacteria do. Though, some fungi might have developed the ability or even a preference to use warm-blooded host bodies in some parts of their life cycle. Fungi of similar morphologies may basically differ in their optimal environment what results in absolutely different isolation rates (e.g., saprophytic *Cladosporium cladosporioides* versus pathogenic *Cladophialophora bantiana*) (Perfect, 1996).

Therefore, fungi are mostly facultative pathogens – they can be found outside the host bodies, perhaps just in a dormant state, but they are able to grow and propagulate in the animal (human) body (Pana *et al.*, 2014). Only very few species (like dimorphic fungi) – obligatory pathogens - are found exclusively on mammals. But, there are also some commensal species that complete their life cycles on the body (e.g., *Malassezia*, *Piedraia*).

Properties enabling the fungus to overcome hostile environment conditions (e.g., heat, UV radiation) are called vitality factors like content of melanin, carotenes, thick cell walls, meristematic growth. In terms of animal pathogenicity, they are non-specific. These factors alone are insufficient to cause a mycosis and they do not allow fungal adaptation to mammalians (Morschhäuser *et al.*, 1996; Sterflinger *et al.*, 1999, 1998; Chechi *et al.*, 2018). Such a fungus grows on living tissue as hyphal clumps with sterile elements, and is not able to return to its normal environmental conditions anymore. Thus, it is dying with its host. The passage through the host does not have any evolutionary sense for the fungus. Selective evolutionary adaptation to the host conditions is not possible as the fungal body (thallus) is vegetative.

Anyway, there are fungi termed zoodemes which thalli in the host tissue or body fluids are actually independently propagating generations of fungal cells (e.g., yeasts with budding cells or arthroconidia, hyalohyphomycetes able to form conidia in living tissue, *Pseudallescheria* anamorph sporulating in blood). Zoodemes might be then seen as roots of evolutionary pathogenicity. The adventitious sporulation enhances invasion, like vascular penetration, so, fungus is found in blood cultures (Schiemann *et al.*, 1998; de Hoog *et al.*, 2009).

There is a dynamic balance between fungal vitality and host response: fungus wants to get optimal evolutionary fitness via maximizing reproduction and dispersion, while host needs to minimize the damage due to infection, i.e., heavy fungal growth and severe inflammation are apparent. We are speaking about pathogenic fungus. If the fungus is highly adapted to the host, it may lead to a paradox, lower virulence, and that fungus becomes adapted infectious agent, not causing infections in the end.

Dimorphic fungi have invasive zoodeme form and the hyphal environmental one. So, it is obvious some evolutionary advantage of animal association, not just the coincidental phenomenon. Apparently, the survival of the fungus will be more successful when utilizing an appropriate host. That is a sort of ecological strategy of the fungus (de Hoog *et al.*, 2009; de Moraes Gimenes Bosco *et al.*, 2019a). Virulence factors are then restricted only to properties enabling penetration and survival in living animals. We are now speaking about evolutionary pathogenesis with rather stubborn clinical picture and localization. To succeed in ecological competition with other fungi present, dimorphic fungi thus employ S- and P-strategies: S for stress-tolerance (inhabiting the unfavourable environments, deserts, shelters etc.) and P for pathogenicity, as known in *Coccidioides* or *Histoplasma* (Dix and Webster, 1995; Teixera and Barker, 2016). The host animals serve as a reservoir of the fungus, which may hide in the infected host with properly working immune responses without causing disease but it might be dispersed from the host back into the environment. The fungus possess only a gentle virulence. If the host immune system becomes impaired (its acquired cellular

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part), the fungus reactivates endogenously (Romani, 2011; Marques, 2008). The process may lead to the host death, the fungus enter to its saprobic phase and spread out of the dead body (often the case with rodent reservoirs in endemic areas of some fungi where these fungi have a continuous distribution) (Livas *et al.*, 1995; del Rocio Reyes-Montes *et al.*, 2016). *Histoplasma*´s strategy is a bit different: the fungus is environmentally occurring on animal excrements, its distribution is disjuncted. If an animal functions just as a carrier of the fungus to replace it, we speak about vector (e.g., some dermatophytes use furry animals) (Ampel, 1999). So, fungi have developed balanced strategies with known or unknown animal species where the virulence of fungus is moderate. While, human fungal infections seem to be only evolutionary dead ends.

Several hundreds of fungal species appear to be able to persist in the living animal tissue, though with the low vitality. When entering the otherwise healthy host with properly working immunity, they remain just sitting at the place of entrance (wound, airways). Innate cellular immune system of the host might be really hard working to control such an invader, thus, severe inflammation might occur. That explains chronic character of mycotic diseases. The fungus is unable to return to its common environment, that is also why it is very difficult to grow the primary culture of that fungus. From the fungal evolutionary fitness point of view, it is not a useful part, and the infection is the spill-over one (Bouhry *et al.*, 1999). In the case of immune impairment, the fungus – opportunistic pathogen – takes advantage and disseminate. Rapidly growing fungi like *Mucorales* are applying R-strategy, R for ruderal, they rapidly colonize and reproduce and disappear before other organisms enter. Fungi producing many extracellular antibiotics and toxins are working with C-strategy, C for combative, typical for aspergilli and fusaria, causing more chronic mycoses in immunocompromised hosts (Dix and Webster, 1995).

Some fungi are just a commensals on the host body, utilizing some compounds produced on the skin or its adnexes without damaging the living animal. But coincidentally, they can invade into to adjacent tissue. A typical example in animals is *Malassezia*, restricted to the vertebrate host, living from dead cells of the stratum corneum (Guillot and Bond, 1999). These species are so called autochtonous commensals as the vertebrate is their natural ecological niche. Anyway, once entering the blood stream via catheter, they are able to cause transitory fungemia. So, they might produce zoodemes, too. The fungi which are only landed on the animal body without taking any nutritional profit are stated as contaminants.

Dermatophytes might be regarded as commensals, too. They utilize keratin as the main source of carbon and nitrogen. Geo- and zoophilic *Microsporum* species do not need any living animal tissue at all. Ornamentation of their spores and fruit bodies with a lot of hairs, hooks, combs etc., allow them to remain between fur and feathers for efficient dispersion (**Fig. 1**). Some pathogenic species, esp. associated with humans (*Trichophyton*) are unable to produce any conidia as they propagulate only via skin scales containing infectious arthroconidia (Gräser *et al.*, 1999). Zoophilic dermatophytes on human hairless skin provoke strong inflammation followed by clearance. That process indicates a low level of adaptation of the fungus to an acquired immunity.

Faeces, dung and guano harbour plenty of fungi. Most of them are living on half-way digested products, not in the host gastrointestinal tissue. The fungi colonizing excrements are causing mycoses extremely rarely, despite of being in very close contact with the particular animal. The opposite is endosaprobe, the fungus with ecological niche inside the animal digestion tract. Typical example is *Candida* which can not survive longtime in the stool because of low competitive ability. This fungus has developed abilities to defend itself against the host reactions and against the bacteria co-present with it, establishing a delicate balance among each other (Bernhardt *et al.*, 1995; Wheat, 1995). There might be the same behaviour of *Pseudallescheria boydii* in water animals, commonly occurring in heavily organically polluted waters or in the soils very rich in nutrients (de Hoog *et al.*, 2009).

If a non-pathogenic fungus enter the host body, its immediately eradicated by the animal´s unspecific cellular immunity while being fully competent. The numbers of pathogenic fungal cells first increase exponentially and with some delay further growth is controlled by acquired cellular immunity (Schaffner, 1999). Immunosuppression does not have any impact on this curve. It means, the reaction of host immune system clearly distinguish between fungal pathogens and opportunists: the first ones are capable to overcome host first, unspecific defence line. Esp. fungal species living in the vicinity of animals, being in closed confrontation with their immune system, may have developed the ways to escape the natural immunity successfully. The species

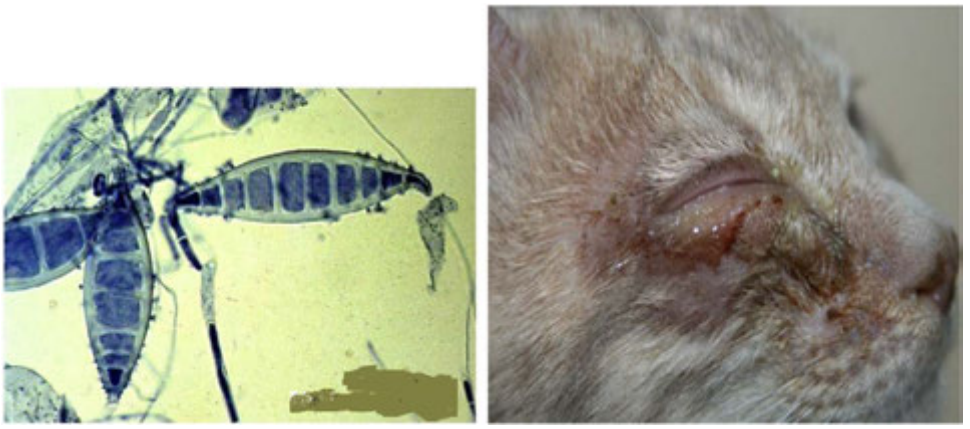

Fig. 1 *Microsporum canis* macroconidia, magnification 1,000 × easily attachable to kitten fur (with microsporidiosis).

that are primarily controlled by the acquired immunity, have reached the higher level of pathogenicity. Such species are the members of *Onygenales, Eurotiales* and *Chaetothyriales* according to phylogeny. These groups have acquired many of suitable vitality factors. It can be expected, there are some evolutionary "hot spots" in kingdom Fungi, where silent opportunists have developed and the genuine pathogenicity can be expected. The *Onygenales* containing several families of animal-related fungi, represent the highest level of pathogenic adaptation among the fungi known. They take profit of debilities of acquired immunity (Masclaux *et al.*, 1995; Sigler *et al.*, 2013; Antinori, 2014). The original character of their pathogenic evolution is most likely keratin assimilation. In the systemic of this order, each taxon seems to have found its specific way to modulate innate immunity.

Ecology of Pathogenic Fungi

Natural ecotops of fungal pathogens causing deep mycoses are presented in **Table 1** (de Moraes Gimenes Bosco *et al.*, 2019a; de Hoog *et al.*, 2000).

Microscopic Mycobiota Able to Cause Diseases in Pets, Incl. Captive Animals

The fungi proven to be infectious agents in companion animals upto now belong to many specific groups – archiascomycota, yeasts – asco- as well as basidiomycotic, dermatophytes, *Onygenales, Eurotiales* etc., as it is clear from **Table 2** (de Moraes Gimenes Bosco *et al.*, 2019a; de Hoog *et al.*, 2000; de Moraes Gimenes Bosco *et al.*, 2019b; Seyedmousavi, 2019; Ovchinnikov and Vasilyev, 2019; Moraru *et al.*, 2019; Moriello *et al.*, 2017; Schmidt, 2015; Seyedmousavi *et al.*, 2015, 2018a; Barrs and Talbot, 2014; Schwartz, 2018; Guillot *et al.*, 2018; Bosco and Bagagli, 2018; Gremiao *et al.*, 2017).

Macroorganism Histopatological Responses to Subcutaneous and Systemic (Deep) Mycoses

In the histologic mounts, specialists may encounter several typical tissue (organ) reaction pictures while being affected by pathogenic fungal growth, see **Table 3** (McKenzie, 1988).

There is also possibility to apply immunofluorescent procedures to identify fungal hyphae in the tissues affected (Thirion Delalande *et al.*, 2005) **Fig. 2**.

Human Health Risks When Working With Medically Important Fungi

According to the risk that dealing with particular fungi in the (veterinarian, medical) mycological labs might represent, mycobiota could be classified to the occupational health risk categories:

GRAS (Generally Regarded As Safe) – harmless organisms widely applied in food processing, biotechnology etc. (e.g., *Penicillium roqueforti*).

BSL-1 (BioSafety Level) – saprophytic organisms, or plant pathogens living in non-vertebrate niches, or commensals. They are able to cause superficial, uninvasive or mild infection coincidentally (e.g., *Scytalidium lignicola*).

In both groups no specific occupational safety recommendations must be fulfilled.

BSL-2 – microorganisms naturally living on non-vertebrates but relatively easily able to survive in vertebrate tissue. Pathogens causing superficial infections and opportunistic pathogens belong to this group (e.g., *Aspergillus fumigatus*).

A common good laboratory praxis in the mycological laboratory must be followed. Be aware of fungi easily aerosolized, apply always sealed Petri dishes, and prefer to work in a safety cabinet.

BSL-3 – pathogens with potential to cause deep mycoses in basically healthy macroorganism (e.g., *Onygenales*).

Table 1 Niches and reservoirs of pathogenic mycobiota in the nature

Fungus	*Natural environment*	*Animal reservoir*
Blastomyces dermatitidis	Woods	Dogs
Coccidioides immitis	Desert soil	Rodents
Cryptococcus neoformans var. *neoformans*	Woods	Pigeons
C. neoformans var. *gattii*	Woods	Koalas
Emmonsia parva	Desert soil	Rodents
Histoplasma capsulatum	Bat dung, guano	?
Paracoccidioides brasiliensis	Woods	Armadillos
Talaromyces (Penicillium) marneffei	Soil	Bamboo rat
Sporothrix schenckii	Woods	?

Table 2 Microscopic fungi isolated as causative agents of known mycoses in pets

Animal	*Type of mycosis*	*Causative agent*
Dogs	Superficial – Cutaneous	*Malassezia pachydermatis, Microsporum canis, M. cookei, M. praecox, M. audouinii, M. (Nannizzia) gypseum, M. (N.) persicolor, M. gallinae (Artroderma grubyi), Trichophyton mentagrophytes, T. erinacei, T. ajjeloi, T. terrestre, T. rubrum, T. ronsurans, T. violaceum, Epidermophyton floccosum*
	Subcutaneous – Deep	*Pythium insidiosum, Aspergillus fumigatus, A. niger, A. nidulans, A. versicolor, A. flavus, A. terreus, A. deflectus, A. flavipes, A. carneus, Penicillium* spp. *(verruculosum), Coccidioides immitis, Blastomyces dermatitidis, Histoplasma capsulatum, Paracoccidioides lutzii, Sporothrix brasiliensis, S. schenckii, Conidiobolus coronatus, Cryptococcus laurentii, C. neoformans, Acremonium hyalinulum, Bipolaris spicifera, Chrysosporium pannicola, Curvularia geniculata, C. lunata, Geomyces pannorum, Pseudomicrodochium suttonii, Scedosporium prolificans*
Cats	Superficial – Cutaneous	*Malassezia pachydermatis, M. sympodialis, M. globosa, M. slooffiae, M. nana, Microsporum canis, M. (Nannizzia) gypseum, M. (N.) persicolor, M. cookei, M. praecox, M. audouinii, M. gallinae (Artroderma grubyi),Trichophyton mentagrophytes, T. rubrum, T. ronsurans, T. violaceum, T. ajjeloi, T. terrestre, Epidermophyton floccosum*
	Subcutaneous – Deep	*Pythium insidiosum, Aspergillus felis, Aspergillus section Fumigati, A. udagawe, A. viridinutans, Coccidioides immitis, Histoplasma capsulatum, Sporothrix schenckii s. str., Bipolaris spicifera, Metarhizium anisopliae, Ochroconis humicola, Paecilomyces fumosoroseus, Staphylotrichum coccosporum*
Rodents (mice, voles, rats, rabbits, chinchillas, hamsters, shrews, guinea pigs, water rodents, squirrels)	Superficial – Cutaneous	*Trichophyton mentagrophytes, T. erinacei, Microsporum (Nannizzia) persicolor, M. amazonicum (Athroderma borellii), M. cookei (A. cajetani), M. gallinae (A. grubyi), M. gypseum*
	Subcutaneous – Deep	*Cryptococcus neoformans, Rhodotorula minuta, Arxiozyma telluris, Coccidioides immitis, Emmonsia crescens, Histoplasma capsulatum, Paecilomyces variotii, Penicillium (Talaromyces) marneffei*
Hedgehogs, moles	Superficial – Cutaneous	*Trichophyton erinacei, Microsporum persicolor*
Reptiles	Superficial – Cutaneous	*Chrysosporium (Nannizziopsis vriesii), Ch. queenslandicum, Beauveria bassiana*
	Subcutaneous – Deep	*Aspergillus fumigatus, A. niger, A. terreus, Coniothyrium fuckelii, Fusarium solani, Ochroconis humicola, Paecilomyces fumosoroseus*
Captive birds (swans, ducklings, pigeons, chicken, parrots, toucans), mammals (dolphins, monkeys, armadillos)	Superficial – Cutaneous	*Piedraia hortae, Microsporum cookei, Trichophyton flavescens, T. simii*
	Subcutaneous – Deep	*Rhinosporidium seeberi, Petriella setifera, Aspergillus nidulans, A. terreus, Cephaliophora irregularis, Chrysosporium tropicum, Coccidioides immitis, Curvularia geniculata, Histoplasma capsulatum, Paecilomyces variotii, Penicillium griseofulvum, Sporothrix schenckii*
Furry mammals	Superficial – Cutaneous	*Trichosporon asahii*
Fishes	Subcutaneous – Deep	*Ochroconis humicola, Sporothrix schenckii*
Insects	Cutaneous – Deep	*Ophiostoma stenoceras, Beauveria bassiana*

All laboratory handling must be carried out in the safety cabinet equipped with HEPA filters, without recirculation, that is used exquisitely just for the BSL-3 fungi. Complete decontamination of the work place and room must be performed after the work. Fungal cultures should be kept in test tubes, not on agar plates (Sugui *et al.*, 2014).

Serological Methods to Diagnose Systemic Mycoses (de Moraes Gimenes Bosco *et al.*, 2019b; Seyedmousavi, 2019; Barrs *et al.*, 2015)

In animal serum samples antibodies against the particular mycotic agent might be detected by mean of several methods. The oldest employed immunodiffusion assay, relative inexpensive and easy-to-perform but also of low sensitivity, has been overcome by ELISA and Western blot providing high sensitivity and specificity. More rapid tests are hemagglutination assay, also lacking high

Table 3 Typical deep mycosis markers present in histologic mounts from biological specimens

Mycosis	*Histological picture*				
	Purulence	*Granuloma*	*Fibrosis*	*Necrosis*	*Other*
Phaeohyphomycosis	–	+	–	–, w	0
Mycetoma	+	+	+	+	Sinus tract grains
Chromoblastomycosis	–	+	+	–	Hyperkeratosis, acantosis
Sporotrichosis	+	+	+	+	Asteroid bodies
Aspergillosis	+	+	–	+	0
Blastomycosis	+	+	+	–	0
Paracoccidioidomycosis	+	+	+	+	0
Coccidioidomycosis	+	+	+	–	0
Histoplasmosis	–	+	+	–	Calcifications, coin lesions
Zygomycosis (Fig. 2)	+	–	–	+	Oedema
Entomophthoromycosis	–	+	+	–	Oedema
Yeast infection (*Candida*)	+	+	–	+	0
Cryptococcosis	–	+	–	–	0

Source: W – weak, 0 – nothing observed.

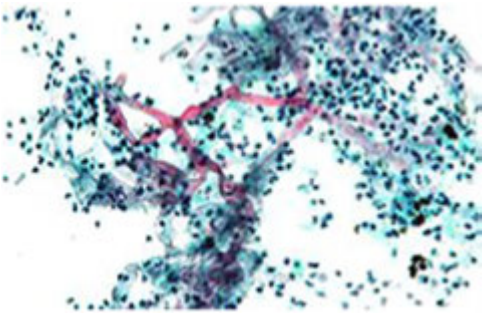

Fig. 2 Histological picture of invasive zygomycosis in sinuses. Courtesy of the histological lab of the Slovak Medical University in Bratislava.

Table 4 Antimycotics used in treatment of mycoses in social animals

Mycosis	*Antimycotic*
Pythium infections	Mefenoxam (agricultural fungicide), potassium iodide, terbinafine, itraconazole (immunotherapy with macerated fungal hyphae, photodynamic therapy, vaccination)
Superficial-Cutaneous	Lime sulphur, chlorhexidine, Insecticide lufenuron, nystatin, griseofulvin, miconazole, ketoconazole, enilconazole, itraconazole, posaconazole, terbinafin (comb. with antibiotics and glucocorticoids)
Subcutaneous-Deep	Amphotericin B, clotrimazole, miconazole, enilconazole, ketoconazole, fluconazole, itraconazole, voriconazole, posaconazole (emerging azole resistant yeasts, aspergilla) (Seyedmousavi *et al.* (2018b); Talbott *et al.* (2015))

level of sensitivity and specificity, and immunochromatography, efficient as well as rapid and convenient analytical method employed recently to confirm fungal deep fungal infection. To confirm the fungal infectious agent antigens in serum or tissue, that another promising methodic approach, successfully used e.g., *Aspergillus* galactomannan detected by ELISA.

Antifungal Therapy Applicable in Pets' Treatment

An overview of commonly administered antifungals in veterinary medicine is given in Table 4.

Chemical disinfectants are applicable topically (Zur and Elad, 2006), antimycotic drugs either topically (ointments, creams/lotions, liquids, baths) or systemically (Sim, 2016). The particular therapy is often combined with surgical removing of the affected lesion cutaneous or inside the animal body. In some diseases, successful vaccination measures are of value, e.g., against pythiosis in dogs, or experimental phototherapy (Singh and Srivastava, 2019; Mendoza *et al.*, 2003; Pires *et al.*, 2014).

Conclusion

Just a very few representatives of fungal microorganisms might be stated as true pathogens. They developed some specific strategies enabling them exploit living animals to spread themselves in their natural inhabitat. The particular animals, usually living in endemic areas of such fungi, serve as reservoirs without showing any symptoms of a mycosis. Other fungi, so called opportunistic pathogens co-exist with their animal hosts in a delicate dynamic balance with the primary target not to kill the animal. Anyway, esp. companion animals and the captive ones might show relatively high degree of sensitivity to fungal diseases, incl. the systemic mycoses, even with fatal outcome possibility. Once the animal living with humans is taken from very different natural conditions to somebody's house, it become vulnerable and prone to infectious diseases (reptiles, fishes, exotic birds or mammals) much more. On the other hand, many pathogens are crossing from animals to humans, and are able to spread quickly to new places. Though, recently taken into account mostly with viruses, the behaviour might be obvious in fungi as well, and those originated in animals continue to evolve and at some point could pose a threat to humans. Especially, the anthropogenic changes of the nature worldwide drive the emerging of new zoonotic diseases in humans as is the concept of planetary health, new scientific discipline. In the case of diseases in companion animals and pets, due to really close contact with humans, mostly children as well, the crossing pathogens must be taken very seriously as a part of preventive measures in human medicine, too (Vidal, 2020). The fungi isolated as causative agents of mycoses should be handled precautionary in the diagnostic lab. Animal mycoses of different localizations on the bodies can be treated with the same antimycotics which are nowadays applied in human medicine as well.

Potential Conflict of Interest

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Production of Native and Recombinant Enzymes by Fungi for Industrial Applications

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Introduction

Enzymes are biological catalysts that accelerate chemical reactions with high efficiency and specificity (Robinson, 2015). Almost all enzymes are protein in nature, with the exception of catalytic RNA molecules (ribozymes) that play an important role in gene expression (Robinson, 2015). Also known as biocatalysts, enzymes mediate metabolic processes in living organisms (microorganisms, plant, animals, and humans) to occur at rates fast enough to sustain life. For example, Radzicka and Wolfenden (1995) estimated that oritidine5′-monophosphate decarboxylase, an enzyme involved in nucleic acid metabolism, accelerates the decarboxylation of orotic acid reaction rate by 17 orders of magnitude, indicating that the catalytic effect of enzymes is vital for living organism. The capacity of enzymes to reduce process time and its low energy requirement has gained interest in industry.

Centuries before their biotechnological exploitation, enzymes derived from living organisms had unknowingly been used for food and alcohol production. For example, the conversion of grains and fruits to alcohol is mediated by enzymes produced by fermenting yeasts (Ray *et al.*, 2016). Attempts to extract the active agent in milk coagulation for cheese production had led to the direct use of enzymes in industrial processes. In 1874 the Danish chemist Christian Hansen used rennet extracted from calf stomach for commercial cheese production (Abdel-Fattah and Mabrouk, 1972). The late nineteenth century also witnessed the first documented use of fungal enzymes in an industrial process. Jokichi Takamine developed a method of production and extraction of amylase used for malting in brewing (Robinson, 2015; Bennett, 1998). Today, enzymes are widely used in industries including food, detergents, agriculture, chemicals, pharmaceuticals, textile and fuel (Adrio and Demain, 2014; Meyer *et al.*, 2011). There are more than 3000 different enzymes (intra and extracellular) known but only 5% (mostly extracellular) are commercially used (Marzo *et al.*, 2019; Green and Beezhold, 2011; Schwab, 2002), and over 500 commercial products worldwide are processed using enzymes (Thapa *et al.*, 2019). The enzyme global market is expected to grow at a compound annual growth rate of 6.83% during the forecast period of 2019–2024 (Kunal and Amit, 2019). Up until the 1970s, industrial enzymes were mainly produced by animals and plants. Owing to their economic, technical and/or ethical advantages over plants and animals, microorganisms are currently the preferred hosts for producing industrial enzymes. Among microorganisms used for industrial enzymes production, fungi are frequently used for producing heterologous proteins and for eukaryotic enzymes that require posttranslational modifications to be functional (Nevalainen and Peterson, 2014). This is because bacteria do not have the posttranslational modification machinery found in eukaryotes. The high degree of nutritional flexibility, metabolic versatility and the ability to produce large quantity of extracellular enzymes have made fungi the preferred hosts for the industrial production of native enzymes as well as recombinant enzymes from endogenous and heterologous organisms. Moreover, enzymes and organic acids produced by fungal species of genera such as *Aspergillus*, *Mucor*, *Rhizopus*, *Trichoderma*, *Penicillium*, *Pichia*, and *Saccharomyces* have received the GRAS (Generally Recognized As Safe) status from the Food and Drug Administration of the United States, allowing their broad use in different industrial sectors, including food and pharmaceutical industries. With recent advances in molecular genetics and genome editing, and the availability of genomic and proteomic tools, the production of recombinant enzymes has been expanded. About 90% of industrial enzymes are produced as recombinant proteins (Lara-Marquez *et al.*, 2011; Adrio and Demain, 2014). Described here is the production of native and recombinant enzymes by fungi for different industrial sectors.

Fungal Hosts for Industrial Enzyme Production

The use of fungal enzymes to convert and produce fermented foods and beverages such as bread, cheese, wine and beer, dates back centuries (Cairns *et al.*, 2018). Currently, the fungi that produce the majority of industrial enzymes of fungal origin come from a limited number of genera of which *Aspergillus* and *Trichoderma* species predominate (Frisvad *et al.*, 2018). The production organisms should preferably have a history of producing products that have received the GRAS status (US Food and Drug Administration, 2019), which means that they are known to be non-pathogenic and they do not produce metabolites that could be harmful to human and animals. This is especially important when the enzyme is used in food or drug applications. In addition to safe production, at least two other criteria need to be taken into consideration in selecting an industrial enzyme producer for development: (1) The capacity of the fungus to secrete large quantities of enzymes to the cell exterior to facilitate downstream processing; and (2) genetic tractability for production of recombinant enzymes. Fungi that are amenable to CRISPR genome-editing technology would have both short- and long-term advantages in strain development. *Aspergillus* species (e.g., *A. niger*, *A. oryzae*) and *Trichoderma reesei* have the above mentioned features and are used in industry for the production of extracellular enzymes such as alpha-amylases, glucoamylases, cellulases, pectinases, xylanases, and proteases (Frisvad *et al.*, 2018).

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Table 1 List of important commercial fungal enzymes

Enzyme	*Source*	*Action*	*Sector application*[a]
α-Amylase	*A. niger, A. oryzae*	Starch hydrolysis	Food, technical
α-Galactosidase	*A. niger, A. oryzae, T. reesei*	Remove terminal α-galactosyl moieties from polysaccharides	Food, technical
α-Glucosidase	*A. niger, A. oryzae, P. chrysogenum, T. reesei*	Hydrolysis of starch dextrin to glucose	Food, feed, technical
Aminopeptidase	*A. niger, A. oryzae, R. oryzae*	Releases amino acids from N-terminal of proteins and peptides	Food, technical
Arabinofuranosidase	*A. niger*	Hydrolysis of non-reducing arabinofuranosyl from arabinan	Food
β-Glucosidase	*A. niger, P. decumbens, P. multicolor*	Releases glucose from oligosaccharides of cellulose	Food, feed, technical
Catalase	*A. niger*	Converts hydrogen peroxide to water and oxygen	Food, technical
Cellulase	*A. niger, P. funiculosum, Rasamonia emersonii, T. reesei, T. viride*	Hydrolysis of plant cell wall cellulose to oligosaccharides	Food, feed, technical
Dextranase	*P. funiculosum*	Endohydrolysis of D-glucosidic linkages in dextran	Food
Glucoamylase	*A. niger, R. delemar, R. oryzae*	Starch hydrolysis	Food, technical
Glucose oxidase	*A. niger, P. chrysogenum*	Oxidizes glucose to gluconic acid	Food, feed, technical
Hemicellulase	*A. niger, T. reesei, T. longibrachiatum*	Hydrolysis of plant cell wall hemicellulose to oligosaccharides	Food, feed, technical
Laccase	*A. oryzae, T. reesei, Trametes hirsuta*	Oxidize phenolic substrates	Food, technical
Mannanase	*A. niger, T. reesei*	Hydolysis of mannan from lignocellulose	Food, feed
Pectinases	*A. niger*	Hydrolyzes pectin	Food, technical
Pectinesterase	*A. niger*	Catalyzes de-esterification of methoxyl group of pectin	Food, feed
Phytase	*A. niger, A. oryzae*	Hydrolysis of phytic acid to release phosphorus	Food, technical
Lipase	*R. oryzae, R. delemar, R. niveus, A. niger, A. oryzae*	Hydrolyzes triacylglycerols to glycerol	Food, technical
Proteases	*A. oryzae, A. niger*	Hydrolyzes proteins	Food
Xylanase	*A. oryzae, A. niger, T. reesei*	Hydrolyzes polysaccharide xylan into xylose	Food, feed, technical

[a]Technical refer to enzymes used in detergents, textile, leather, pulp and paper industries, synthesis of organics and production of biofuels.

Aspergillus

The genus *Aspergillus* comprises ~350 species. Two particular species, *A. niger* and *A. oryzae* are widely used in industry for their native enzymes (Berka *et al.*, 1992; Mekala *et al.*, 2008; Pandey *et al.*, 2018). They produce broad range of enzymes related to degradation of storage polysaccharides, including starch and inulin (de Vries and Visser, 2001; Wang *et al.*, 2020), as well as plant cell wall components, such as xylan, xyloglucan, cellulose, galactomannan and pectin (de Vries and Visser, 2001).

The decomposition capacity of polysaccharides has made *Aspergillus* an important cell factory for the enzyme producing industry. Enzymes from *Aspergillus* are used in diverse applications such as brewing, cheese manufacturing, wine modification, food preservation, starch degradation, juice clarification and animal feed additives. Enzymes from *Aspergillus* have limitations in processes requiring alkaline pH such as in detergents or high temperature (above 60°C) as in starch liquefaction. This may be because *A. niger* and *A. oryzae* have been evolved to thrive in acidic environment at temperatures around 30°C, and enzymes produced by them tend to have acidic pH optimum and temperature optimum below 60°C (Murphy *et al.*, 2012; Strasser *et al.*, 2015).

The industrial exploitation of *Aspergillus* started in 1894 with the patent of Takamine on the production of alpha-amylase from *A. oryzae* (Takamine, 1914). Since then different fermentation techniques have been employed for the industrial production of amylases. An optimized *A. niger* strain can produce over 40 g L^{-1} of alpha-amylase, and this can be further improved with recombinant DNA technologies (Wang *et al.*, 2016).

Trichoderma

The species of the genus *Trichoderma* are ubiquitous filamentous ascomycetes. *T. reesei* is the best-known species of the genus used in industry. The enzymatic secretion potential of *T. reesei* (*Hypocrea jecorina*) was discovered during the Second World War when the fungus destroyed the US Army equipment on the Solomon Islands. Researchers have exploited this decomposition potential to screen fungi from the Solomon Islands for their ability to degrade native crystalline cellulose. The strain of *T. reesei* designated QM6a was shown to possess the highest ability to break down cellulose and it had become the starting strain for developing hyper-producers of cellulases and hemicellulases (Bischof *et al.*, 2016). Generations of random mutagenesis of *T. reesei* QM6a have resulted in strains that hyper-produce cellulolytic enzymes; among them strain RUT-C30, a fourth-generation mutant, is able to produce about 20 g $L^{-1}$of extracellular protein and displayed cellulase activity of 15 filter paper units mL^{-1}under controlled fermentation conditions (Peterson and Nevalainen, 2012). Strains derived from *T. reesei* are considered benchmark organisms for the production of industrial cellulolytic enzymes.

Other fungi used for industrial enzymes production include species of *Saccharomyces*, *Rhizopus*, *Fusarium*, *Penicillium*, *Pichia*, *Candida*, *Mucor*, *Rhizomucor*, *Rhizopus*, *Disporotrichum* and *Humicola* (Bennett, 1998; Nevalainen *et al.*, 2005). **Table 1** summarizes the major enzymes used in industries and their production hosts.

Fermentation Technologies for Fungal Enzymes Production

Fermentation is a metabolic process that produces chemical change in organic substrates through the action of enzymes that are produced by a growing microorganism. In contrast to yeast and bacterial cells in which the division is planktonic, filamentous fungi grow by forming new cells at the tip of existing hyphae where they can form branches. The branches can grow into either elongated hyphae or clump together with neighboring branches into pellets, making fermentation of filamentous fungi challenging. Two main methods of fermentation, solid-state fermentation and submerged fermentation have been developed to adapt the different morphologies and natural habitats of fungi.

Solid-State Fermentation

Solid-state fermentation is known as the oldest fermentation technology and takes place in the absence or near absence of free water in a solid matrix that contains enough moisture to support the activity of the microorganisms (Lekha and Lonsane, 1997; Sugai-guerios *et al.*, 2015). This fermentation process mimics the natural environment of the microorganisms, especially for filamentous fungi, thus promoting high productivity. Solid-state fermentation has been used for the production of fermented food such as Koji with *A. oryzae* and for cheese making with *Penicillium roqueforti* (Ashok, 1992). However, technical limitations reduce the use of solid-state fermentation in industrial sectors at large scale. One of these limitations is to define the proper substrate for fermentation, the selection of which depends on the adherence capacity of the microorganism being used to produce the enzyme and its requirement of water activity. Adherence property and the requirement of water activity are dependent on the physiological characteristic of the microorganisms. Microorganisms with a higher water activity requirement, such as bacteria, have difficulty to perform in solid-state fermentation. Most solid-state fermentations used in the enzyme industry are based on fungi as the native producers and are not used in the production of recombinant enzymes. This technique of fermentation mimics the natural habitat of the filamentous fungi far better than submerged cultivation and may explain their high productivity. The fungal filaments in solid-state fermentation favor high penetration into the substrates and have high osmotic tolerance compared to bacteria and other single-cell microorganisms (Ashok, 1992). However, the disadvantage of solid-state fermentation is the scale-up from laboratory to commercial production (Dulf *et al.*, 2019). This is due to the large size of inoculum and surface areas that are needed for commercial production. With large inoculum size, the aeration and agitation needed for the control of parameters such as pH and temperature become difficult as they disturb the fermentation process. In addition, the recovery of the final product (enzyme) from the solid substrate at larger scale is complex and labor intensive. These short comings have reduced the application of solid state fermentation in commercial enzymes production and limited its use to bioremediation, bioleaching, biobenefication and biopulping (Arora *et al.*, 2018). In these cited industrial sectors, solid-state fermentation has the advantage of being economical (no water or less water requirement and use of raw, inexpensive material as substrate).

Submerged Fermentation

Submerged fermentation is a method of cultivating microorganisms in liquid nutrient media. For industrial production, this implies growing the selected microorganism in closed vessels, called bioreactors, which contain nutrient broths. The system design for bioreactors allows supply of oxygen as required for aerobic microorganisms and the ability to monitor and control different parameters such as pH, temperature, viscosity, dissolved oxygen, foam formation, biomass formation, substrate utilization and desired product formation (Liu *et al.*, 2019; Xiong *et al.*, 2012). The ability to control parameters online has facilitated the used of submerged fermentation where fermentation takes place in large fermenters with volumes that vary from thousands to hundred-thousand liters (Liu *et al.*, 2019; Xiong *et al.*, 2012). For example, 90% of commercial xylanases are produced using submerged fermentation (Polizeli *et al.*, 2005). Fungal submerged fermentation is often associated with high viscosity of the culture broth, which limits oxygen and mass transfer, and can affect enzyme production. However, this limitation can be reduced or eliminated with the implementation of different submerged fermentation processes. There are four types of submerged fermentation processes: batch culture, fed batch culture, continuous culture and perfusion batch culture.

Batch cultivation is the simplest mode of operation, in which the microorganisms are inoculated in fixed volume of fermentation medium for a defined time, after which the broth is collected for downstream processing. In fed batch process mode, concentrated components of the nutrients are gradually added to the batch culture. In continuous reactors, fresh medium is continuously added and broth from the bioreactor is continuously withdrawn. This allows the fermentation process to be operated for long periods. In the perfusion batch cultivation mode, there is an equal volume of addition of new medium and withdrawal of cell free broth. Each type of submerged fermentation process has advantages and disadvantages, and their use depends on the end-use of the production and the type of microorganism that is being used. For example, continuous reactors can be more productive

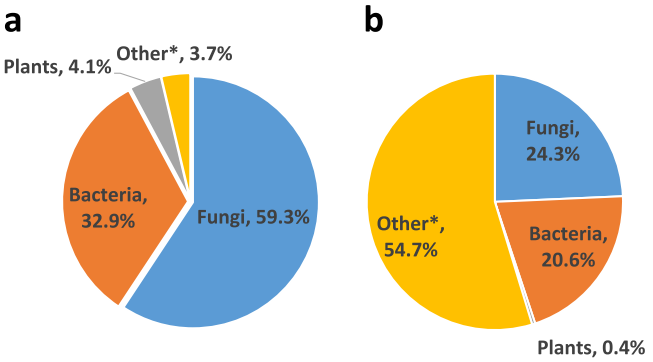

Fig. 1 Host and Origin of industrial enzymes. (a) Host for industrial enzyme production, (b) Origin of industrial enzymes. *Referred to mammalian or unknown. Reproduced from AMFEP, 2015. List of Commercial Enzymes. Available at: https://amfep.org/_library/_files/Amfep_List_of_Enzymes_update_May_2015.pdf. (Accessed January 2021).

than batch reactor and can reduce the viscosity for fungal cultivation; however the long fermentation time in the continuous process increases the risk of contamination. Submerged fermentation is more commonly used in industrial fermentation than solid-state fermentation for enzyme production due to its ability to control the fermentation parameters using defined substrates. However submerged fermentation is not practical for complex substrates such as agricultural, forestry, and food processing residues and wastes. These complex substrates need to be first processed before use by the microorganism in the bioreactors.

Application of Fungal Enzymes

Fungal enzymes have revolutionized the biotechnology industry due to their functionalities, applications, low environmental impact, biodegradability and cost effectiveness. Enzymatic processes have become preferred alternatives to chemical process. The increased research and development activity within the enzyme industry and academic laboratories is the driving force behind the market expansion (BCC, 2020). Fungal enzymes are used in industrial sectors (pharmaceutical, agricultural, food, feedstuff, cosmetics, detergent, textile, waste treatment, petroleum) as processing aids for the production of numerous products. For example, cheese, milk, yogurt, bread, cookies, fruit juice, beer, wine and meat are food products that are frequently processed with enzymes such as alpha-amylase, alpha-glucosidase, glucose oxidase, cellulases, xylanases, pectinases, proteases and lipases (Kumar and Satyanarayana, 2013; Damasio *et al.*, 2019; Ding *et al.*, 2019). The main application of enzymes in food industry is starch conversion to generate ingredients for foodstuffs. In addition, enzymes in this sector are also used to improve food flavor, texture, digestibility and the nutritional value (Kumar and Satyanarayana, 2013; Damasio *et al.*, 2019; Ding *et al.*, 2019).

Enzymes play an important role in animal nutrition and their use in this sector is expected to grow, especially for swine and poultry nutrition (BCC, 2020). The main purpose of using enzymes in feed is to improve digestibility to lessen production cost and reduce the environmental footprint (Gupta *et al.*, 2015). Enzymes such as phytases, beta-galactosidases, glucanases, xylanases, alpha-amylases and polygalacturonases are used in the feed industry (Caballero *et al.*, 2007; Walsh *et al.*, 1993). Their addition to animal feed is important for animal growth and nutrition, and the global market of these enzymes is increasing due to the increased demand for meat products. For example, the use of phytases to release phosphorous from phytate found in feed has two benefits by reducing the use of expensive inorganic phosphorus in animal diets and the environment pollution caused by excessive manure phosphorus runoff (Bohn *et al.*, 2008).

Besides their application in food and feed industries, the medical uses of enzymes notably include digestive aids with amylases, proteases and lipases (Ianiro *et al.*, 2016), anti-clotting with urokinase (serine protease) and streptokinases as fibrinolytic and thrombolytic agents (Kumar and Singh, 2004; Tharwat, 2006), and for diagnosis purpose with glucose oxidase along with peroxidase to detect the glucose level (Cherry *et al.*, 1999; Dubey *et al.*, 2017). In this area of enzyme applications, recombinant heterologous enzymes are often involved.

Enzymes used in cosmetics include among others proteinases for dermatological use and superoxide dismutase in anti-aging formula due to its protective action against oxidative stress (BCC, 2020).

Technical enzymes are used in detergents, textile, leather, pulp, paper, organics synthesis and biofuels production. The main technical enzymes are amylases, proteases, lipases, cellulases, xylanases and catalases. The important properties of technical enzymes are the ability to act at broad range of temperature and pH. For example, biofuels production would benefit from enzymes that are active at $>60^{\circ}$C for hours (reduced the risk of contamination and accelerate reaction time) whereas in detergents value can be gained from enzymes that are active at cold temperature (reduced energy input and preservation of fabrics integrity) and alkaline pH.

About 60% of commercial enzymes reported in 2015 are produced using fungi as hosts (**Fig. 1(a)**). Among the fungal production hosts, *Aspergillus* sp. are most often used (46%), followed by *Trichoderma* sp. (20%) and *Penicillium* sp. (8%) (AMFEP,

2015). In addition, among known enzymes of fungal origin, most of the native and recombinant enzymes produced are from *Aspergillus* sp. (**Table 1**, **Fig. 1(b)**). Therefore, fungi are both the predominant source of current industrial enzymes produced and the main hosts used for their production.

Commercial Native Enzymes From Fungi

Amylases

Fungal amylases as a group represent one of the main enzymes used in industry (De Souza and De Oliveira, 2010; Singh *et al.*, 2014). Alpha- and beta-amylases catalyze the hydrolysis of starch into sugars and are broadly applied in industries. Their fields of application include pharmaceutical, food, detergent, textile and biofuel industry. Alpha-amylase is an endo-1,4-α-D-glucanglucohydrolase that randomly cleaves the 1,4-α-D-glycosidic linkages between adjacent glucose units in the amylose chain (Ray, 2004; Roth *et al.*, 2019; van Lanen and LeMense, 1946; De Castro *et al.*, 2010). Beta-amylase produces maltose from starch by hydrolyzing the α-1,4-glucan linkages (Ray, 2004). Alpha-amylase from *A. oryzae* was the first microbial enzyme to be manufactured and solid-state fermentation was initially used (Bogar *et al.*, 2002; Rahardjo *et al.*, 2005). To improve productivity, the manufacture of amylases now uses submerged fermentation with *A. oryzae* and *A. niger* as the main producers. Fungal amylases, particularly those from *A. niger* have a good tolerance to low pH (Varalakshmi *et al.*, 2009; Shenolikar and Stevenson, 1982). The *A. niger* genome harbors multiple genes encoding alpha-amylases (Pel *et al.*, 2007) and the major secreted alpha-amylase has an optimal pH of 3.2–3.5. The acid alpha-amylase is known to be suitable for saccharification of starch at pH 3.5–4.0 in combination with the *A. niger* alpha-glucosidase (Shenolikar and Stevenson, 1982; Takagi *et al.*, 1969; Tokhadze *et al.*, 1975).

Proteases

Proteases (endopeptidases and exopeptidases) are a large group of enzymes that hydrolyze the peptide bonds of proteins. The endopeptidases act preferentially on peptide bonds in the inner regions of the polypeptide chain whereas exopeptidases act near the ends of the polypeptide chain. Depending on their site of action at the amino- or carboxyl-terminal of the polypeptide, they are classified as aminopeptidases or carboxypeptidases, respectively (Sabotic and Kos, 2012). Carboxypeptidases can be divided into serine peptidases, cysteine peptidases and metallopeptidases based on their active site amino acid residues or requirement for metal ions for activity. In the same manner, endopeptidases comprise serine proteases, aspartic proteases, cysteine proteases and metalloproteases (Rao *et al.*, 1998).

Proteases are used in industrial processes including detergents, textiles, leather, cheese production, baking, beer preservation, and meat tenderization (Kang *et al.*, 2014; De Souza *et al.*, 2015; Sullivan and Calkins, 2010; Vishwanatha *et al.*, 2010). They are also used in the pharmaceutical industry and represent about 60% of the total enzyme market (Mordor and Intelligence, 2018).

Xylanases

Xylanases are a group of enzymes that depolymerize xylan, the second most abundant plant cell wall polysaccharide, to xylose. Multiple enzymes are involved in the hydrolysis of xylan and they include endoxylanase, beta-xylosidase, alpha-glucuronidase, alpha-arabinofuranosidase and acetylxylanesterase (Juturu and Wu, 2012). The principal commercial sources of xylanases among mesophilic filamentous fungi are *Aspergillus* sp. and *Trichoderma* sp. The need for xylanases with a greater range of pH and temperature tolerance has directed effort for the research on xylanases producers among thermophilic fungi and bacteria (Lasa and Berenguer, 1993; Bruins *et al.*, 2001).

Submerged fermentation is the preferred method of manufacturing xylanases. However, about 10% of xylanases production is performed using solid-state fermentation (Polizeli *et al.*, 2005), which takes advantage of using cheap complex substrates, such as underutilized agricultural products, food processing wastes and forestry residues, as carbon sources. In addition, co-cultivation of different fungal xylanase producers can be used to boost the production titer. For example, a mixed culture of *T. reesei*, *A. niger* and *A. phoenicis* using solid-state fermentation has been reported to produce 2800 U $g^{-1}$of xylanases (Gutierrez-Correa and Tengerdy, 1998).

Pectinases

Pectinases are enzymes that break down pectin, a polysaccharide found in plant cell walls. Also known as pectic enzymes, pectinases are classified in three groups: the hydrolases that hydrolyze the glycosidic bonds of pectic acid (also known as polygalacturonic acid), the lyases that degrade pectic acid through an elimination reaction, and esterases that cleave the methyl ester bonds in pectin. The commercial pectinases are mixtures of these three types of enzyme activities. For economical and functionality reasons, microbial source of pectinases represents 90% of industrial pectinases. Filamentous fungi including *A. niger*, *A. oryzae*, *Aspergillus awamori*, *Aspergillus sojae*, *Aspergillus* carbonarium and *Trichoderma* sp. are known producers of pectinases with *A. niger* as the major commercial producer (Blandino *et al.*, 2001). Due to the importance of pectinases in food industry, they represent almost 25% of the global food enzyme market (Ruiz *et al.*, 2012). Pectinases are used for extraction and clarification of fruits juice and wine, textile and plant fiber processing, tea, coffee and oil extraction (Da Silva *et al.*, 2005).

Glucose Oxidase and Catalase

Glucose oxidase is abundantly produced by *A. niger* and *Penicillium chrysogenum*. Traditionally, solid-state fermentation and submerged fermentation of strain NRRL3 of *A. niger* (ATCC 9029) is the common source for the commercial glucose oxidase (Crueger and Crueger, 1990; Dubey *et al.*, 2017). However, submerged fermentation has been found to be more effective for production due to the ability to control the cultivation parameters of the technology (Dubey *et al.*, 2017). Glucose oxidase converts β-D-glucose to D-gluconolactone and hydrogen peroxide by oxidation (Primo-Martin *et al.*, 2003). D-gluconolactone is further hydrolyzed to gluconic acid. The application of glucose oxidase is mainly in food industry. The enzyme is used in baking industry for improving dough texture. Glucose oxidase is thought to possess antioxidant properties that preserve the taste and flavor of wine and beer. The antimicrobial (antibacterial and antifungal) activity mediated by glucose oxidase is exploited as preservative for milk and dairy products. However, this attributed property is dependent of the amount of catalase that is usually co-produced in the host strain. The antimicrobial action has been suggested to be primarily based on the release of hydrogen peroxide by glucose oxidase, but the presence of catalase converts hydrogen peroxide to oxygen and water (Fuglsang *et al.*, 1995). The catalase activity is exploited for textile enhancement and in various food processing (Tzanov *et al.*, 2002). For most large-scale applications, the enzymatic activities of glucose oxidase and catalase are not separated. Both may be used together to avoid net hydrogen peroxide production.

Cellulases

Cellulose is the principal component of plant cell wall and the most abundant renewable polymer on the planet. A coordinated action of multiple enzymes endoglucanases, exoglucanases, cellobiohydrolases and beta-glucosidases collectively called cellulases is required for efficient degradation of cellulose. They hydrolyze the glycosidic bonds in cellulose to release glucose (Berg *et al.*, 1988; Tomme *et al.*, 1988).

The major industrial fungal cellulases producer is *T. reesei* (Irwin *et al.*, 1993). *Trichoderma* cellulolytic enzymes are known for acting simultaneously in a synergistic manner (Reese, 1956). In addition to *T. reesei*, *Aspergillus* sp. are used for industrial cellulases production. For a complete overview of cellulases produced by *T. reesei* and *Aspergillus* sp., the reader is referred to the review of Payne *et al.* (2015).

Glucoamylase

Also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, and 1,4-α-D-glucanglucohydrolase, Glucoamylase hydrolyzes from non-reducing ends of amylose and amylopectin to glucose monomers. Glucoamylases can also cleave the 1,6-α-linkages at the branching points of amylopectin (Manjunath *et al.*, 1983). The major source of glucoamylase comes from *A. niger*. In addition, *A. awamori* and *Rhizopus* sp. are industrially used for glucoamylase production (Silveira *et al.*, 2006; Anto *et al.*, 2006). Glucoamylase together with the other amylolytic enzymes such as alpha-amylases are used in biorefineries for their capacity to liquefy starch. In addition, glucoamylase is used in the production of glucose that serves as feedstock for fermentations and in the production of high fructose sirups. It is also the key enzyme in Sake and soy sauce production. Since the production capacity of *A. niger* glucoamylase is dependent of the medium and cultivation condition and the glucoamylase market is competitive, there is continuous development in the optimization of the production method along with genetic engineering of the producing strains.

Commercial Heterologous Enzymes From Fungi

The production of fungal enzymes from native sources sometimes cannot meet the increasing market demand due to their low yields or to the high cost of the industrial fermentation processes. Recombinant enzyme production in easily cultivable and genetically tractable hosts with higher production yield can allow reduction of the costs of production. The capability of filamentous fungi to grow in a broad range of substrates, to produce large amounts of enzymes and the availability of their eukaryotic post-translational protein modification machinery make them attractive hosts for heterologous enzyme production from different sources. In addition, the availability of genome sequences and genetic engineering tools has increased heterologous enzyme production in fungi. Heterologous enzyme productivity can be increased by inserting multiple copies of target genes into the host organism, use of strong promoters, and efficient signal peptide sequence for extracellular secretion. Another advantage of heterologous protein production is the possibility to commercialize an enzyme from a toxin-producing organism by transferring the encoding gene into a safe fungal host (Piscitelli *et al.*, 2010). Although heterologous fungal enzymes can be efficiently produced, production of gene products from exogenous organisms can still result in low production yields due to poorly understood factors including protein mis-folding. For example, production levels of thermophilic bacterial xylanases in *T. reesei* have remained considerably low at about 100 mg L^{-1} even after expression optimization (Te'o *et al.*, 2000).

Genetic Strain Improvement for Native and Heterologous Enzyme Production

Wild isolates of fungi do not typically produce enzymes at levels and cost that meet industrial demands. They normally produce just enough for their own benefit and only when it is necessary. Therefore, strains improvement for enzyme production is required and is performed in industry to increase the production yield. Most of production hosts in industry have been genetically improved to increase production capacity.

Before the advent of targeted genetic engineering, genetic strain improvement was based on classical mutagenesis that used physical and chemical mutagens such as X-ray irradiation, UV irradiation, ethyl methane sulfonate and nitrosomethyl guanidine, diethylsulfate (Kun *et al.*, 2019). These mutagens act directly on the genome of the microorganism, causing damage to the DNA, and resulting in mutations. Mutants are screened to identify those displaying higher enzyme activity, indicating higher level of enzyme production. Although the results of random genetic improvement are unpredictable, success has been realized in the past using these approaches and they are still in use. An example of successful strain improvement using X-ray mutagenesis was performed in *P. chrysogenum*, to increase the production of penicillin (Salo *et al.*, 2015). In addition, random mutagenesis has been used to improve fungal enzyme production, such as for glucose oxidase (Dubey *et al.*, 2017), alpha-amylase (Aleem *et al.*, 2018), and lipase (Bassegoda *et al.*, 2012). Parasexual segregation in *A. niger* has also demonstrated to be a suitable method as exemplified by improved chymosin production (Bodie *et al.*, 1994).

Until recently, the use of molecular genetics and gene transformation tools for production of recombinant heterologous enzymes was laborious due to the lack of genomic data and genome-editing tools. Currently the ability to introduce or delete genes becomes faster with the implementation of CRISPR/Cas9 technology (Ishino *et al.*, 2018).

Heterologous protein production in fungi may often undergo extensive modifications in the secretory pathway, including proteolytic processing and glycosylation, which could reduce the production yield. Different strategies have been developed to increase the production yield of heterologous proteins that include the construction of protease-deficient strains (Liu *et al.*, 2013; van den Hombergh *et al.*, 1995), improved secretion signals (van den Hombergh *et al.*, 1995; Gouka *et al.*, 1997; Moralejo *et al.*, 1999), use of strong promoters (Moralejo *et al.*, 1999), and integration of multiples copies of the foreign DNA, among others (Nevalainen and Peterson, 2014). Studies have been conducted on glycosylation in filamentous fungi and the main mode of glycosylation is by high mannose-type of sugars addition (Deshpande *et al.*, 2008). However, further development in this field is necessary to understand the bottlenecks in heterologous protein secretion.

Fungal protein secretion is linked to the physiology of the fungus. It has been established that the majority of native and heterologous proteins are secreted at the hyphal tip. An example is the production of a heterologous barley cysteine proteinase, which showed that secretion occurs solely at the hyphal tip in *T. reesei* (Nykänen *et al.*, 1997), supporting the hypothesis that modified strains with short filaments would improve protein secretion. In some cases, however, heterologous proteins such as *Hormoconis resinae* glucoamylase P and calf chymosin are secreted from mature parts of the hyphae in *T. reesei* (Inokuma *et al.*, 1988; Joutsjoki *et al.*, 1993a,b), implying that heterologous protein secretion and localization are protein dependent.

Enzyme Discovery

The growing world population, the diminishing fossil reserves and environmental concerns have increased the demand of biobased products and technologies. For example, the demand for biofuel is expanding to reduce greenhouse gas emissions (Khanna *et al.*, 2011). Enzymes are playing a major role in sustainable, biobased technologies. For the bioeconomy to thrive, new enzyme functionalities and more efficient enzymes are required to displace chemical processes. Enzyme discovery and enzyme engineering are two approaches pursued by industry to expand the functionality of enzymes to overcome the current shortcomings.

Genome Mining and Functional Metagenomics

(1) *Genome mining*
Nucleic acid and protein databases are rich resources for discovering novel enzymes (Aharoni, 2009). To date, around 300,000 genome sequences are available in the publicly accessible databases and about 180 million protein sequences available in the UniProt database. A systematic exploration of the available genome and protein resources with relevant bioinformatic tools can help to identify proteins of interest. For example, a known enzyme can be selected from a curated database and used as starting point to search for new enzymes performing the same or similar reactions in protein databases. The gene sequences encoding the selected enzymes with predicted activities can be retrieved and used as templates to clone or synthesize the genes of interest. The cloned or synthesized genes can be introduced by gene transformation methods into desired organisms for enzyme production and characterization. Characterized enzymes can be found in curated databases that include the Carbohydrate-active enZymes (CAZy) database (Lombard *et al.*, 2014), MycoCLAP (Strasser *et al.*, 2015) and the lipase engineering database (Fischer and Pleiss, 2003).

(2) *Functional metagenomics*
Massively parallel sequencing technologies, along with development of bioinformatic tools, are becoming a rapid strategy to discover enzymes in metagenomes (Kaul and Asano, 2012). Functional metagenomics starts from extracting DNA directly from a mixed population of organisms from diverse environments. A metagenomic library constructed with the extracted DNA is then screened for the production of enzymes (Steele *et al.*, 2009). For example, more than 100 new nitrilase genes

have been discovered from diverse geographical locations such as deep ocean beds, volcanic vents, and arctic tundra by metagenomics, allowing a creation of a library of enzymes for the conversion of nitriles to carboxylic acid (Desantis *et al.*, 2002). In addition, the DNA obtained from metagenomic studies can be sequenced and potential genes that code for catalytic enzymes can be searched based on sequence similarity or protein motifs using InterProScan (Jones *et al.*, 2014).
Functional metagenomics has been useful in discovering novel cellulases in a wide variety of natural thermophilic environments, such as continental geothermal pools and hot springs (Graham *et al.*, 2011), compost (McAndrew *et al.*, 2013) and hydrothermal vents (Placido *et al.*, 2015). Thermophilic enzymes have potential for industrial applications and their discovery by metagenomics has potential impact due to the difficulty of growing thermophiles or extremophiles microorganisms in the laboratory. However, a main limiting factor for the discovery of enzymes, especially from fungal origin, by functional metagenomics is that the host organism used for the metagenomic libraries is typically a bacterium (e.g. *E. coli*), in which the success rate of functional expression of eukaryotic gene low (Duan and Feng, 2010). In addition to metagenomics, metatranscriptomics, metaproteomics and high throughput screening have shown potential for enzymes discovery. For further understanding of the method of metatranscriptomics and metaproteomics for enzymes discovery, the reader is referred to (Warnecke and Hess, 2009) and (Kleiner, 2019).

Protein Engineering Approaches

Protein engineering of a specific enzyme (e.g., cellulase or hemicellulase) is an effective approach to enhance the enzyme activity, specificity and stability to suit diverse applications. This approach involves the engineering of modified catalysts to achieve desired functions. Directed evolution and rational enzyme design are two methods often used in protein engineering (Rigoldi *et al.*, 2018).

- *Directed evolution*
  Directed evolution of enzymes consists of creating random mutations in the native enzyme. It can be performed by error prone mutagenesis (Yang *et al.*, 2017; Entzminger *et al.*, 2015), site-directed mutagenesis (Belsare *et al.*, 2017) or DNA shuffling (Lehtonen *et al.*, 2016). Directed evolution generates a library of enzyme variants. Activity screening of the library allows for the identification of enzymes with improved property. For example, directed evolution has been used to improve activity and stability of endo-beta-1,4-xylanase of *Thermomyces lanuginosus* by error prone PCR mutagenesis (Stephens *et al.*, 2007).
- *Rational design*
  Rational design implies the use of computational design and modeling programs to improve the catalytic domain, the stability or additional properties of the enzymes *in silico*. It includes phylogenetic analysis and structural comparison to heterologous enzymes, which guide the rational design (Luke *et al.*, 2007). Among rational design strategies to improve enzyme stability, the introduction of disulfide bridges and/or surface hydrogen bonds are commonly used. Disulfide bridges and surface hydrogen bonds increase protein rigidity by locking their fold in the global conformation and by increasing protein rigidity respectively (Dombkowski *et al.*, 2014). Another strategy for rational enzyme engineering involves strengthening the well-packed hydrophobic cores which are involved in preserving enzyme stability and conformational specificity (Rigoldi *et al.*, 2018). Different computational tools have been developed to assist design and engineering of enzymes; for example, Rosetta (Zanghellini *et al.*, 2006), Orbit (Dahiyat and Mayo, 1996), Metal search (Desjarlais and Clarke, 1998) and Dezymer (Hellinga and Richards, 1991). Rosetta has been used to stabilize the local interactions of surface cavities of xylanases which resulted in 15 times increase in enzyme half life at 50°C compared to the native xylanases (Joo *et al.*, 2010). Increasing disulfide bridges by rational design has improved the thermostability of lipases in *Rhizopus chinensis* (Yu *et al.*, 2012). The rational design of enzymes suffers from drawbacks such as the unavailability of knowledge of proteins structure or the failure to prevent large hydrophobic clusters on the proteins surface (Tiwari *et al.*, 2012). Novel algorithms or methods to improve rational design are in continuous development.

Conclusion

Fungal enzymes have long played an important role in food and beverage production. They are now part of many industrial sectors, including detergents, textile, feed, pharmaceuticals and chemicals. Challenges remain in using fungal enzymes in industry; for example, low yields in the production of heterologous proteins, and cost of production of functionally diverse enzymes. Continuously improving fermentation processes and developing effective expression systems for native and heterologous fungal enzyme production will be key factors in expanding the use of enzymes in replacing chemical processes to enhance industrial sustainability.

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Fungal Laccases as Biocatalysts for Wide Range Applications

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General Aspects of Laccases

Laccases are phenol oxidases (EC 1.10.3.2) belonging to the multicopper oxidases (MCO) superfamily. Discovered in 1883 in the exudates of the lacquer tree *Toxicodendron vernicifluum* (Yoshida, 1883), laccases have been isolated from plants, fungi (ascomycetes, basidiomycetes and deuteromycetes), prokaryotes and arthropods, where they are involved in different biological processes. Fungal laccases are implicated in intra- and extracellular physiological processes including morphogenesis, pigmentation, pathogenesis, delignification and detoxification (Claus, 2004; Hoegger *et al.*, 2006; Morozova *et al.*, 2007). White-rot basidiomycetes responsible for wood decay in nature are among the main laccase producers together with the litter-decomposing fungi. They secrete laccases as part of an array of oxidoreductases, with ligninolytic peroxidases as the main players, enabling the efficient biodegradation of the lignin polymer (Eggert *et al.*, 1996; Lundell *et al.*, 2010).

Fungal laccases are mainly monomeric extracellular proteins although some basidiomycete (e.g., *Pleurotus ribis*, *Pleurotus pulmonarius*, *Trametes villosa*, *Cantharelus cibarius*) or ascomycete laccases (*Rhizoctonia solani*) consist of homodimers with each subunit with M_W similar to monomeric laccases (Morozova *et al.*, 2007). Laccases typically fold in three cupredoxin-like domains, each of them with a typical β-barrel topology. The protein structure is stabilized by two disulfide bonds, one located between domains one (D1) and three (D3), and the other between domains one (D1) and two (D2) (**Fig. 1**; Rivera-Hoyos *et al.*, 2013; Hakulinen and Rouvinen, 2015).

Laccases present four copper ions in the active site that act as cofactors for their catalytic activity. Depending on their UV/visible and electron paramagnetic resonance (EPR) spectroscopy properties, these copper ions are classified as follows: Type 1 (T1) copper, EPR detectable and with a strong absorption at 600 nm, is responsible of the blue color of laccases; Type 2 (T2) copper, colorless but detectable by EPR; and a pair of type 3 (T3) copper ions, with no EPR signal due to an antiferromagnetic coupling mediated by a bridging hydroxyl ligand, and weak absorbance at 330 nm (Jones and Solomon, 2015). The T2 and T3 coppers form the tri-nuclear cluster (TNC) where O_2 is reduced to water by the four electrons taken from four molecules of substrate at the T1 site (Sekretaryova *et al.*, 2019). T1 site is located in the D3 of the protein, while the tri-nuclear cluster (T2/T3) is embed between D1 and D3, with both domains providing residues for the coordination of the three coppers (arranged in a regular triangle). The T1 copper is coordinated by one Cys and two His. In the TNC, each of the T3 coppers have three His ligands and both are connected by an OH bridge, while the T2 copper is coordinated by two His (Jones and Solomon, 2015). The highly conserved Cys-His superexchange pathway (around 13 Å) serves as via to transfer the electrons from T1 site to the TNC (**Fig. 2**; Piontek *et al.*, 2002; Jones and Solomon, 2015). A conserved Asp residue (Asp 206 according to PM1 numbering) assists with His ligand (His455 according to PM1 laccase numbering) to the concerted electro-proton transfer at the T1 site (Galli *et al.*, 2013). Besides, there are two conserved acid residues (Asp 77 and Asp 453 according to PM1 laccase numbering) located close to the TNC that have a crucial role in O_2 reduction by aiding O_2 binding and providing protons (favored at acid pH) (Jones and Solomon, 2015).

According to the reduction potential of the T1 site, laccases can be classified as low (LRPLs, $E^0 < +500$ mV, most plant and prokaryotic laccases), medium (MRPLs, E^0 +500 to around +700 mV) and high (HRPLs, E^0 from +720 to +800 mV) redox potential laccases (Pardo and Camarero, 2015a). The latter are mainly produced by the white-rot, and litter-decomposing basidiomycete fungi and are of great biotechnological interest due to their high oxidation versatility (Hoegger *et al.*, 2006). These dramatic changes in laccase's redox potential are attributed to a perturbation in the geometry of the T1 site (Augustine *et al.*, 2008) that is in part modulated by the presence of a Met as the axial ligand in plant and bacterial laccases. The distorted tetrahedral geometry of the latter enzymes is replaced by a trigonal planar geometry in fungal laccases (MRPLs and HRPLs) with a non-coordinating residue (Leu or Phe) in the axial position (Hakulinen *et al.*, 2002; Piontek *et al.*, 2002). The influence of the axial amino acid in T1 redox potential has been demonstrated in several studies (Hall *et al.*, 1999; Xu, 2002). For instance, substitution of the axial ligand Met 502 in CotA laccase from *Bacillus subtilis* ($E^0 = 455$ mV) by non-coordinating Leu or Phe increases E^0 to 515 or 548 mV, respectively (Durão *et al.*, 2006).

Eukaryotic laccases show molecular masses between 60 and 130 kDa of which 10–50% may be attributed to glycosylation, mainly N-glycosylation (Xu *et al.*, 2019). N-glycosylation takes place in the lumen of the endoplasmic reticulum and is finalized in the Golgi (Burda and Aebi, 1999; Herscovics, 1999), and might influence secretion, proteolytic susceptibility, catalytic activity or thermal stability of laccases (Madhavi and Lele, 2009).

Laccases are capable to oxidize a wide range of different compounds, preferably *o*- and *p*-substituted phenols and aromatic amines, together with N-heterocycles (indole, benzothiazol, tetrahydroquinoline, hydroxyphthalimide, naphthol, etc), heterocyclic thiols, as well as some metals and organometallic complexes. The oxidation of these compounds only requires O_2 from the air as electron acceptor and produces water as sole-by product (Gianfreda *et al.*, 1999; Polak and Jarosz-Wilkolazka, 2012; Mogharabi and Faramarzi, 2014). The promiscuous activity and low catalytic requirements turn laccases into biocatalysts of choice for many different applications.

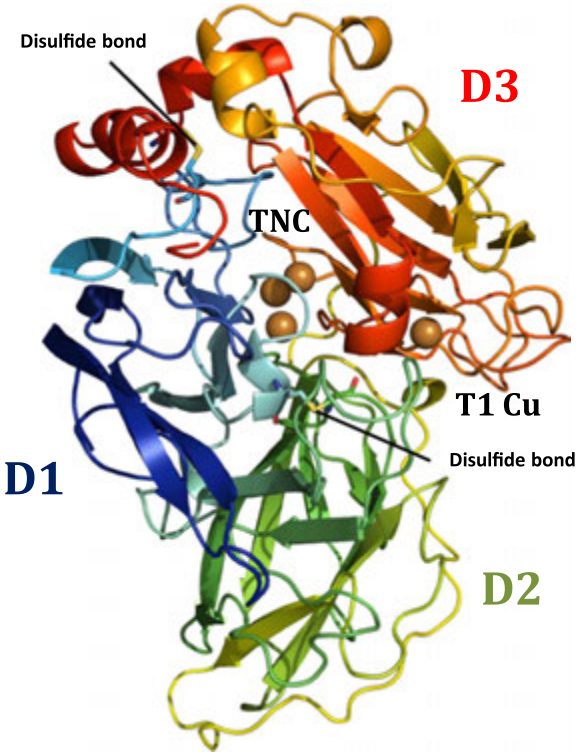

Fig. 1 Cartoon representation of the crystal structure of *Pycnoporus cinnabarinus* laccase (PDB 2XYB), showing the three cupredoxin domain (D1-D3) folding, the four catalytic coppers as spheres, and the two disulfide bridges in yellow. TNC refers to tri-nuclear cluster where oxygen is reduced to water and T1 Cu is the copper responsible for substrate oxidation.

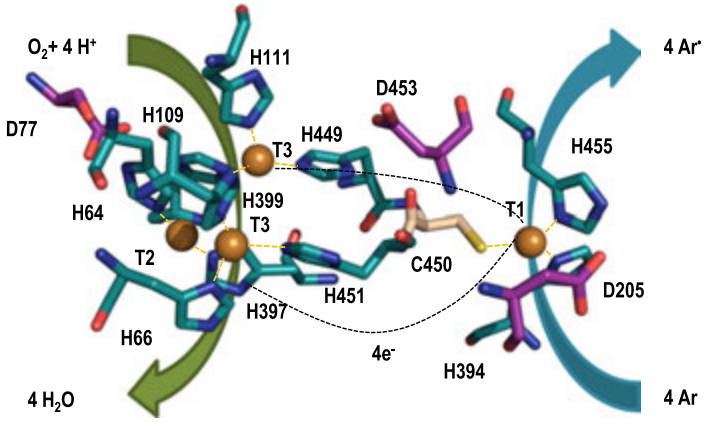

Fig. 2 Catalytic site and catalytic mechanism of laccase. His (blue) and Cys (wheat) residues coordinating the catalytic coppers and conserved Asp residues involved in proton transfer (purple) are shown. The four coppers are depicted as spheres. Residues coordinating the four catalytic coppers and electron transfer from T1 site to the tri-nuclear cluster through the triplet H-C-H are shown based on PM1 laccase structure (PDB: 5ANH).

Catalytic Site and Reaction Mechanism of Laccases

The catalytic cycle starts with the oxidation of the substrate at the T1 site. It is assumed that laccase acts as a battery, storing the electrons from four monovalent oxidation reactions, which are transferred from the T1 site to the TNC to reduce one O_2 molecule to two H_2O molecules (Jones and Solomon, 2015). Consequently, the enzyme is transformed from the resting oxidized state into

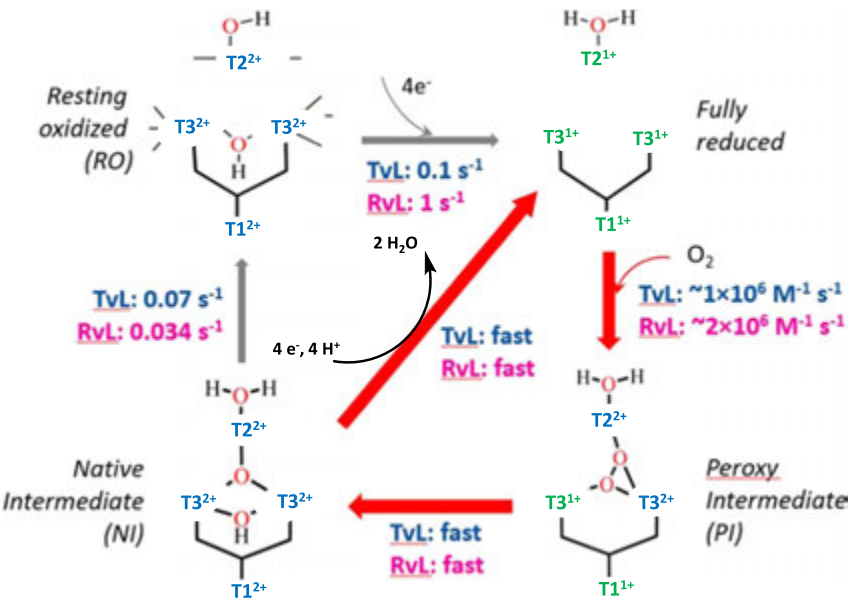

Fig. 3 Redox mechanism of the high redox potential laccase from *T. versicolor* (TvL) *vs* the low redox potential laccase from *Rhus vernicifera* (RvL). Adapted with permission from Sekretaryova, A., Jones, S.M., Solomon, E.I., 2019. O_2 Reduction to water by high potential multicopper oxidases: Contributions of the T1 copper site potential and the local environment of the trinuclear copper cluster. J. Am. Chem. Soc. 141, 11304–11314. doi:10.1021/jacs.9b05230, American Chemical Society.

the fully reduced state (**Fig. 3**). Then, the interaction of the TNC with O_2 proceeds in two consecutive steps. In the first one, two electrons, one from the T2 Cu and the other from one of the T3 Cu ions of the fully reduced enzyme are donated to the molecular O_2 generating the peroxy intermediate. In the second step, the bond O–O is broken with the donation of two more electrons from the T1 and T3 copper sites, rendering the fully oxidized native intermediate (NI) of the laccase. A proton transfer, required for the reduction of molecular O_2 to water in the TNC, takes place at the same time as the electron transfer. The total reduction of NI by the oxidation of a new set of substrate molecules and the release of two water molecules will start a new catalytic cycle or, in case of lack of more substrate, the slow decay to the resting oxidized form will take place (Yoon and Solomon, 2007; Jones and Solomon, 2015).

Laccase Mediator Systems

The oxidation capabilities of laccase can be enhanced in the presence of low molecular weight compounds that act as redox mediators once oxidized by the enzyme. In the so-called laccase mediator systems (LMS), the oxidized mediator ("stabilized radical") acts as a diffusible carrier, overcoming steric hindrances, to enable the oxidation of bulky substrates such as lignin, cellulose or starch polymers inaccessible to the enzyme, or of substrates with higher redox potentials that are not oxidized by the enzyme alone (Baiocco *et al.*, 2003; Camarero *et al.*, 2004; Kunamneni *et al.*, 2008; Cañas and Camarero, 2010). The 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), was the first compound described in the 1990s as laccase redox mediator for the enzymatic oxidation of non-phenolic lignin model compounds and the delignification and bleaching of paper pulps (Bourbonnais and Paice, 1990, 1992). Since then, the ability of other synthetic compounds such as 1-hydroxybenzotriazole (HBT), violuric acid, or 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) to act as redox mediators of laccases has been demonstrated in a range of oxidation reactions of target compounds (Call and Mücke, 1997; Galli and Gentili, 2004; Xu *et al.*, 2009; Benzina *et al.*, 2012; Rostami *et al.*, 2018). Due to the different oxidation mechanisms, the use of different mediators may lead to different products (Baiocco *et al.*, 2003). Ultimately, the use of mediators adds higher versatility to the enzyme as biocatalyst.

The industrial application of LMS has, however, two main obstacles: the elevated cost of synthetic mediators and the possible generation and discharge in the industrial effluents of toxic intermediates. Substitution of synthetic mediators by low-cost natural mediators obtained from renewable sources constitute an efficient, eco-friendly and sustainable alternative for the application of LMS in white biotechnology processes. This is the case of certain phenolic compounds derived from lignin polymer that can be obtained as bio-products from the same industrial processes of biomass conversion in which laccases could be implemented (e.g., wood pulping and pulp bleaching). These natural phenolic compounds have been successfully assayed as laccases mediators in dye decolorization, detoxification of aromatic pollutants, pitch removal, delignification and bleaching of paper pulps or to enhance saccharification in biofuel production (Camarero *et al.*, 2005, 2007, 2008; Cañas *et al.*, 2007; Gutiérrez *et al.*, 2007; Kunamneni *et al.*, 2008; Cañas and Camarero, 2010; Babot *et al.*, 2011; Hollmann and Arends, 2012; Rico *et al.*, 2014; Pardo and Camarero, 2015b).

Heterologous Expression of Fungal Laccases

Enzyme overexpression is mandatory for its commercialization as industrial biocatalyst, and constitutes a challenging task in the case of basidiomycete laccases. Examples of overproduction of wild basidiomycete laccases are barely found in the literature. One outstanding example is the gram-scale production (1.5 g l^{-1}) of *P. cinnabarinus* wild type laccase by the monokaryotic *Pyconoporus cinnabarinus* ss3 strain using ethanol as inducer (Lomascolo *et al.*, 2003). The homologous overproduction of the recombinant native laccase was later achieved (1.2 g l^{-1}) by transforming a laccase-deficient *P. cinnabarinus* monokaryotic strain with the *P. cinnabarinus* laccase gene under the control of *Schizophyllum commune* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter (Alves *et al.*, 2004). The homologous overexpression of laccase III from *Coriolus* (*Trametes*) *versico*lor under control of the GAPDH promoter was also reported, although no enzyme yields were informed (Kajita *et al.*, 2004). Homologous expression of *Coprinus cinerea lcc1* under the control of various homologous and heterologous promoters was proved as well, obtaining also the highest laccase activity (3 U ml^{-1} with ABTS) with the promoter of GAPDH from *Agaricus bisporus* (Kilaru *et al.*, 2006).

The difficult genetic manipulation of the natural producer strains, together with the lack of GRAS (generally recognized as safe) status and optimized scale-up protocols for industrial fermentation of wild laccases, discouraged the use of basidiomycete strains to engineer and produce laccases so far, drawing the attention to heterologous expression hosts (Otterbein *et al.*, 2000). To this end, optimization of laccase CDS according to the codon usage of the heterologous host, selection of adequate expression vectors and signal peptides and optimization of the expression conditions are carried out with dissimilar results.

The bacterium *Escherichia coli* is successfully used for the expression, engineering and scale up production of bacterial laccases (Alessandra *et al.*, 2010; Hämäläinen *et al.*, 2018), but the effective expression of active fungal laccases in bacteria has not been achieved so far, most likely due to differences in their post-translational processing machineries (Salony *et al.*, 2008; Ma *et al.*, 2018).

By contrast, ascomycete expression systems (both yeasts and filamentous fungi), well established for the industrial production of eukaryotic enzymes, are well suited as heterologous hosts for the functional expression of basidiomycete laccases. However, the levels of basidiomycete enzymes produced in these systems are often much lower than those of ascomycete enzymes. Some of the reasons behind this fact may be: (1) the disparities in basidiomycete and ascomycete gene models (basidiomycete genes often have more introns with less conserved start and stop sequences), (2) the sensitivity of basidiomycete laccases to ascomycete proteases (3) the induction of the repression under secretion stress (RESS) mechanism which downregulates the transcription of genes encoding secreted proteins or (4) the activation of mRNA-destabilizing proteins which may affect the heterologous mRNA stability, thereby diminishing the production of heterologous proteins (Su *et al.*, 2012; Casado López *et al.*, 2016).

The low expression yields and frequent hyperglycosylation of recombinant proteins hinder the use of the yeast *Saccharomyces cerevisiae* as industrial host (Herscovics, 1999), whereas the powerful methanol-inducible alcohol oxidase (AOX1) promoter and the high-density growth of the yeast *Pichia pastoris* (up to $OD_{600} = 500$ in bioreactor) provide, in general, higher enzyme yields. Conversely, variable enzyme expression yields are obtained depending on the characteristics of the heterologous protein, which makes this expression system weakly predictable (Cereghino, 2002; Mate *et al.*, 2013; Wang *et al.*, 2016). The highest enzyme yields ever reported (550 mg l^{-1}) for a basidiomycete laccase produced in *P. pastoris* in fed-batch fermentation corresponds to *T. versicolor* laccase using AOX1 promoter (Hong *et al.*, 2002). Constitutive promoters such as the GAPDH have also been assayed. That is the case of fed-batch fermentation of *P. pastoris* producing POXA1b laccase from the basidiomycete *Pleurotus ostreatus*, where constitutive production under GAPDH outperformed the enzyme levels obtained in AOX-induced cultures (Pezzella *et al.*, 2017). This is in agreement with results obtained with the ascomycete *Botrytis aclada* laccase (Kittl *et al.*, 2012), where the enzyme levels were about 40–60 mg l^{-1}.

Replacement of the native laccase signal peptide by the *S. cerevisiae* α-factor signal sequence is frequently applied to increase the production of the recombinant enzyme by yeast. This approach was successfully used to express, for instance, two laccases from the white-rot fungus *Physisporinus rivulosus* in *P. pastoris* (Hildén *et al.*, 2013). Moreover, the directed evolution of the laccase encoding sequence fused to the α-factor pre proleader was successfully used in several enzyme engineering works to increase the secretion of fungal laccases by *S. cerevisiae* (Bulter *et al.*, 2003; Mate *et al.*, 2010; Camarero *et al.*, 2012; Pardo *et al.*, 2012). This strategy has recently rendered outstanding laccase production yields in *S. cerevisiae* (25 mg l^{-1}) in our laboratory (de Salas *et al.*, 2019a).

Among all the host systems used for protein expression, filamentous fungi show the highest expression levels especially when the secreted protein is homologous or is from fungal origin. Even if the genetic manipulation of filamentous fungi is more complex than that of yeasts, the high expression yields of active enzyme and the low cost of the growth media (filamentous fungi show enormous nutrition flexibility) make them the preferred hosts for production of industrial enzymes (**Fig. 4**). There are several examples of basidiomycete laccases heterologously expressed in *Aspergillus*, *Trichoderma* or *Penicillium* (Abianova *et al.*, 2010). The highest expression yields (close to gram per liter) have been reported in *Aspergillus* (Couto and Toca-Herrera, 2007; Alessandra *et al.*, 2010). For instance, gram-scale production of a laccase from *Trametes* sp. C30 has been achieved in *A. niger* (Mekmouche *et al.*, 2014). The heterologous production of other native basidiomycete laccases and their variants engineered *in vitro* has also been attained in this species although at a lower scale, i.e., *P. cinnabarinus* wild-type laccase (145 mg l^{-1}, Record *et al.*, 2002) and an *in vitro* evolved variant (23 mg l^{-1}, Camarero *et al.*, 2012), and wild-type POXA1b and its 1H6C variant (13 mg l^{-1} and 20 mg l^{-1}, respectively, Macellaro *et al.*, 2014). *Aspergillus oryzae* is the preferred host for producing industrial enzymes at Novozymes (world leader company in commercializing industrial enzymes), such as the high-redox potential laccase from *T. villosa* commercialized as NS 51002 (no longer available) and the ascomycete *Myceliophtora thermophila* laccase (NS 51003). This expression system has been used for the overexpression of basidiomycete laccase variants developed in our lab by directed evolution to an industrial relevant scale in Novozymes (de Salas *et al.*, 2016, 2019b).

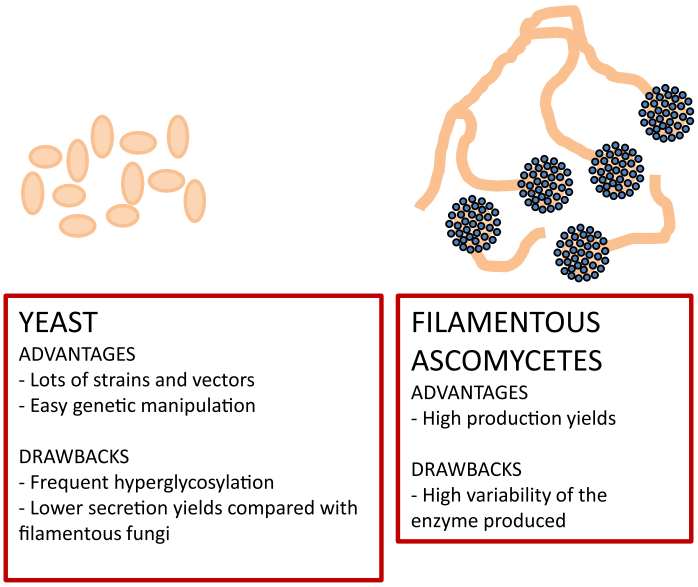

Fig. 4 Comparison of yeast and filamentous ascomycetes as heterologous hosts for laccase production.

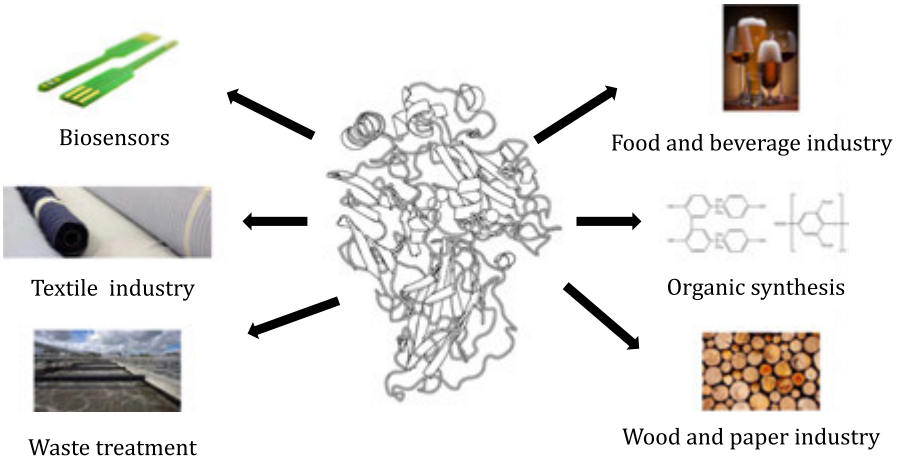

Fig. 5 Some examples of laccase applications.

Enzymes can substitute chemical catalysts in diverse reactions providing the advantages of less toxicity than chemical catalyzers and increasing the selectivity in some cases. Nevertheless, the frequent demanding conditions of the industrial processes (extreme pH, high temperature, presence of co-solvents or inhibitors, etc.), together with the recalcitrance of some substrates or selectivity requirements are main hurdles for the application of wild-type enzymes in industrial processes. Therefore, the engineering of the enzyme is often required to adjust the activity and stability of the enzyme to the operating conditions or to design new or improved activity or selectivity towards specific substrates. Although this step, together with the aforementioned overexpression of the enzyme, is indispensable to apply laccases at industrial scale, this is not within the objectives of this chapter, and there are other reviews dealing with this subject (Kunamneni *et al.*, 2008; Gonzalez-Perez *et al.*, 2012; Pardo and Camarero, 2015b).

Biotechnological Applications

Laccases, alone or in LMS, are multipurpose biocatalysts with application in a wide range of industrial processes (**Fig. 5**).

Fungal laccases are commercialized as industrial enzymes by Novozymes (Denmark), Jena Biosciences (Germany), Creative Enzymes (USA), Ecostar (India), USBiological (USA), ASA Spezialenzyme (Germany), etc. Bacterial laccases are produced and

commercialized by MetGen (Finland). So far, there are quite a few successful cases of laccases formulated and commercialized for target-applications: Denim fabric finishing (DeniLite®), paper pulp delignification and bleaching (Novozyme NS-51003, MetZyme® LIGNO™), O_2 depletion to preserve flavors from food and beverages (Flavourstar®) or treatment of wine cork stoppers (Suberzyme®). Nevertheless, there is room for the application of laccases in other industrial sectors, as demonstrated in many research studies commented below.

Pulp and Paper Industry

Due to the natural activity of laccases on phenolic lignin, one of the first applications of laccases studied was precisely related to the delignification of wood pulp in the pulp and paper industry (Bourbonnais and Paice, 1992). Laccase-mediator systems can be applied for pulp bleaching and delignification to remove the residual lignin remaining in the paper pulp after cooking. The integration of a laccase-based enzymatic stage in the totally chlorine-free (TCF) or elemental chlorine-free (ECF) bleaching sequences can improve the brightness of the pulp, reduce the use of chlorine-based harsh chemicals in ECF bleaching and, simultaneously, enhance pulp strength properties while reducing the energy consumption during refining of the pulp (García *et al.*, 2003; Camarero *et al.*, 2004; Ibarra *et al.*, 2006). Other applications of laccases related with the paper industry are: (1) fiber functionalization through oxidative coupling of low molecular weight compounds (such as phenols) to the paper surface, which enhances paper strength and resistance to water absorption or adds new properties such as antioxidant activity; (2) removal of lipophilic extractives deposited in the bleached pulps ("pitch control"); (3) deinking of secondary fibers; or (4) enzymatic treatment of the effluents from paper mills (Gutiérrez *et al.*, 2007; Widsten and Kandelbauer, 2008; Madhavi and Lele, 2009; Garcia-Ubasart *et al.*, 2011; Fillat *et al.*, 2012; Cusola *et al.*, 2015). Recent functionalization examples by LMS include the grafting of carboxymethyl cellulose and chitosan on kraft pulp fibers to obtain high-resistance papers (Ballinas-Casarrubias *et al.*, 2017) or functionalization of bacterial cellulose to produce paper-silver nanoparticles composites with antimicrobial activities (Morena *et al.*, 2019).

Textile Industry

Laccases can improve the dyeing efficiency and reduce the cost of the dyeing process by *in situ* oxidation of inexpensive precursors after their adsorption by the fabric, and aid textile bleaching by avoiding back staining by degrading the released dyestuff after dyeing or by bleaching the textile fibers from natural dyes or impurities (Vasil'eva *et al.*, 2008). Some of the first developed commercial laccase formulations are used for denim fabric finishing (fading of indigo-dyed denim) to give the characteristic gray cast (Galante and Formantici, 2003; Pezzella *et al.*, 2015). Laccases have also been used for functional modification of molecules on textile fabrics such as cotton or wool, improving properties as water repellence or resistance (Lantto *et al.*, 2004; Guimarães *et al.*, 2011; Pezzella *et al.*, 2015). *In situ* polymerization of catechol and *p*-phenylenediamine catalyzed by laccase on different fabrics can yield colored low-conductive fabrics with good fastness behavior after washing (Su *et al.*, 2019a). Moreover, this polymerization reaction has been used for coating wood, cotton and polyethylene terephthalate fabrics to provide antimicrobial properties against gram-positive and gram-negative bacteria. These enzyme-treated textile fabrics are of special interest in hospitals to reduce the spread of nosocomial diseases (Su *et al.*, 2019b).

Waste Treatment

More demanding environmental regulations have pushed laccases to new application opportunities in waste treatment and disposal (Pezzella *et al.*, 2015). Laccase can transform toxic recalcitrant compounds into less toxic and more degradable derivatives by direct dechlorinating, cleavage of aromatic rings or oxidative transformation of heterocycles and polycyclic aromatic hydrocarbons in hydroxylated intermediates (Schultz *et al.*, 2001; Cañas *et al.*, 2007; Teng *et al.*, 2019). Wastewaters from the food, textile, dye or printing industries enriched in phenols and aromatic amines can be detoxified with laccases (Lante *et al.*, 2000; Madhavi and Lele, 2009). For example, a mix of fungal enzymes containing laccases applied to winery-derived biomass (in combination with a sonication pretreatment) shortened the degradation time required with fungal degradation and produced commercially useful compounds such as gallic acid, lactic acid and glycolic acid (Karpe *et al.*, 2017). The removal of emergent organic pollutants catalyzed by laccase has also been described. For instance, deamination and demethylation of tetracycline and oxytetracycline antibiotics was recently attained with a fungal laccase (Tian *et al.*, 2020).

Synthetic organic dyes are very stable to temperature, light and microbial attack, making them difficult to degrade. The world total dye production per year is around 800,000 tons, of which at least 10% is released to the environment through wastewaters where they are a source of eutrophication and can form toxic by-products through oxidation, hydrolysis or other chemical reactions (Konstantinou and Albanis, 2004). Traditional methods for color removal from these wastewaters, such as filtration, adsorption or coagulation-flocculation, are expensive and have operational problems. Laccases can catalyze the decolorization of a wide range of synthetic organic dyes alone or in the presence of redox mediator compounds (Claus *et al.*, 2002; Gubitz *et al.*, 2002; Camarero *et al.*, 2005). The decolorization is most frequently linked to detoxification. Detoxification of phenolic azo dyes by laccase was proved by the asymmetrical breakdown of the azo linkage releasing molecular nitrogen, avoiding aromatic amine formation (Chivukula and Renganathan, 1995). Other example of azo dye degradation is the use of a laccase from *T. versicolor* for the degradation of orange 2 and acid orange 6 which degradation products were non-toxic (Legerská *et al.*, 2018).

Food and Beverages

One relevant example of laccase's application in food and beverage processing is found in the wine industry to substitute the use of SO_2 for the removal of phenolic compounds involved in the maderization of wine, a process that causes turbidity, color intensification, and aroma and flavor alterations. The polyphenols are oxidized by the enzyme, polymerized and removed by clarification. Similar uses have been proposed for the treatment of fruit juices and beer. "Flavourstar" commercial laccase manufactured by Novozymes A/S is used to remove oxygen from finished beer in order to avoid its reaction with proteins or fatty acids, which prevents the formation of off-flavor compounds (Osma *et al.*, 2010). A process that uses laccase as dough additive in baking has also been patented. This process increases the volume and softness of the baked product and at the same time increases strength, stability and reduces the stickiness of the dough, thereby improving its machinability. It has been suggested that this effect is produced by the oxidizing effect of the laccase in the dough components improving the strength of the dough gluten structures (Minussi *et al.*, 2002; Pezzella *et al.*, 2015).

Biosensors

Some optical and thermal biosensors have been reported using laccases, although electrochemical biosensors where laccase is covalently immobilized in carbon-based electrodes, to allow a direct electron transfer, has been widely demonstrated for detection of phenols with low detection limit and high sensitivity (Li *et al.*, 2012; Rodríguez-Delgado *et al.*, 2015). The performance of the biosensor clearly depends on the enzyme immobilization strategy (Casero *et al.*, 2013). A recent study combined botryosphaeran fungal biopolymer with multi-walled carbon nanotubes to immobilize laccase in the electrode, obtaining a novel biosensor for the determination of dopamine and spironolactone with good selectivity (Coelho *et al.*, 2019). As an example of how far the application of laccases in biosensors can go, a textile biosensor was constructed by functionalizing cotton yarn with a semiconducting polymer on which laccase was absorbed. It was demonstrated to monitor human health biomarkers (e.g., Tyr) in a non-invasive way, finding potential application in sport, healthcare and working safety (Battista *et al.*, 2017). Another example is the design of a pH responsive color-changing wool with conductive features through laccase-catalyzed polymerization of diaminobenzenesulfonic acid (Zhang *et al.*, 2018). In the last years, intensive research has been done on the application of laccases in enzymatic biofuel cells through their immobilization in nanocarbon cathodes modified with metal nanoparticles. This technology, with applications in biosensing, bioreactors or bioenergy conversion (Sekretaryova *et al.*, 2016; Zhang *et al.*, 2017; Rodríguez-Padrón *et al.*, 2018), offers higher redox potentials than commercial fuel cells, but it requires more development studies to be commercialized (Ghosh *et al.*, 2019).

Laccases in Organic Synthesis

The synthesis of chemical organic compounds catalyzed by laccase (with or without mediators) has attracted the attention of intensive research in the last two decades to provide a more efficient, cleaner and sustainable alternative to the use of expensive catalysts or toxic reagents.

Laccases can catalyze the synthesis of colorful organic compounds that can be used as dyes trough the oxidation of various phenolic and aromatic precursors. Catechol or indole derivatives (Ganachaud *et al.*, 2008; Kim *et al.*, 2011), phenoxazine-derived dyes such as cinnabarinic acid (Eggert *et al.*, 1995; Bruyneel *et al.*, 2008), and several azo dyes have been obtained with laccase as biocatalyst (Polak and Jarosz-Wilkolazka, 2012). The latter are the most used dyes in paper printing and textile dyeing, where they account for around 50% of the world total dye production due to their color variety (Konstantinou and Albanis, 2004). Some examples of azo dye synthesis by laccase include an azo dye with two anthraquinoinic sulfonated chromophores obtained with immobilized *Perenniporia ochroleuca* MUCL 41114 laccase (Enaud *et al.*, 2010), or the homocoupling reaction of 4-methylamino benzoic acid mediated by laccase (Martorana *et al.*, 2011). The coupling of phenylenediamine and α-naphtol catalyzed by *P. ostreatus* POXA1b laccase renders SIC-RED dye, while the coupling of resorcinol and 2,5–diaminobenzenesulfonic acid renders other colored compounds (Pezzella *et al.*, 2016; Giacobelli *et al.*, 2018). The use of 1-naphtol and 1-naphthol-8-amino-3,6-disulfonic acid as precursors catalyzed by a fungal laccase engineered in our lab also rendered a new acidic dye with excellent dyeing properties (de Salas *et al.*, 2019a).

Laccases also catalyze the synthesis of pharmaceutical compounds of high value. Some examples are the enzymatic oxidation of 4-methyl-3-hydroxyanthranilic acid to obtain actinocin, a proven antitumoral compound (Osiadacz *et al.*, 1999; Burton, 2005), or the synthesis of the powerful antitumoral drug vinblastine by oxidative coupling of katarantine and vindoline (Sagui *et al.*, 2009). These enzymes have been used to conjugate catechins and dextran to obtain drugs with anticancer activities, or to obtain new derivatives from resveratrol and the hormone β-estradiol (Nicotra *et al.*, 2004a,b; Vittorio *et al.*, 2016). The regioselectivity of some LMS in oxidation reactions saves time and money compared with chemical organic synthesis. Fungal laccases and TEMPO as mediator can selectively oxidize the primary hydroxyl group in monosaccharides and disaccharides under mild conditions for their application in different technical areas (Marzorati *et al.*, 2005). A laccase from *Trametes pubescens* and TEMPO were used for the oxidation of the primary hydroxyl group of natural glycosides amygdalin or colchicoside to the corresponding carbonyl groups (Baratto *et al.*, 2006). Laccase-TEMPO can also selectively oxidize glycerol to generate glyceraldehyde and glyceric acid (Liebminger *et al.*, 2009), or enhace the antioxidant activity of propolis, attributed to the formation of hydroquine, catechol and phloroglucinol (Botta *et al.*, 2017). More recently, it has been discovered that laccases are capable of selective phenol coupling, as a tailoring step of polyketide synthesis, in the abscense of auxiliary proteins. Polyketides are a structurally and functionally diverse class of bioactive natural compounds proven to be a rich source of pharmaceutical and agrochemical lead compounds (Fürtges *et al.*, 2019).

Amination reactions catalyzed by laccase are of interest due to the antibacterial, antifungal, antiallergenic, anti-inflammatory and anticonvulsant activities of aniline derivatives (Niedermeyer *et al.*, 2005). For example, reaction of anilines with 2,5-dihydroxybenzoic acid derivatives catalyzed by laccase renders N-analogous of corollosporines with important antimicrobial activity (Mikolasch *et al.*, 2008). Other examples are the synthesis of 5-alkylaminobenzoquinone and 2,5-bis (alkylamino) − 1,4-benzoquinone by oxidative coupling of 2-methoxy-3-methylhydroquinone and primary amines, such as octylamine, cyclooctylamine, and geranylamine (Herter *et al.*, 2011); the dimerization of salicylic esters or bisphenol A (Ciecholewski *et al.*, 2005); the stereoselective amination of racemic sec-alcohols by laccase-TEMPO (Martínez-Montero *et al.*, 2017); and the synthesis of triaminated cyclohexa-2,4-dienones using catechol and primary amines as precursors (Wellington *et al.*, 2018). Laccases also catalyze the synthesis of cyclic products using 2,5-dihydroxybenzoic acid derivatives, and aromatic and heteroaromatic amines as precursors through oxidative C–N bond formation followed by cyclization (Hahn *et al.*, 2010). Another cyclization reaction catalyzed by laccases is the bioconversion of 2´,3,4-trihydroxychalcone to 3´,4´-dihydroxyaurone. Aurones are plant secondary metabolites with significant biological activity including anti-cancer, anti-parasitic and enzyme-inhibitory activity (Zerva *et al.*, 2019).

Oxidative coupling and polymerization reactions catalyzed by laccase

The oxidative coupling of phenols or aromatic amines is one of the most useful abilities of laccases that have been learned from nature, and which are widely exploited in organic synthesis reactions. Lignification of the plant cell walls consists of the oxidative coupling of phenolic precursors, the cinnamyl alcohols, catalyzed by plant laccases and peroxidases. The electronic delocalization enables the radicals to couple at *o*- and *p*-positions through different ether and aryl-alkyl linkages to yield diverse polymeric products (type of lignins) depending on the abundance of precursors available in the plant or tissue (Boerjan *et al.*, 2003). Accordingly, two different dimeric products (with C–O or C–C bonds) are obtained from *in vitro* oxidation of ferulic acid catalyzed by laccase, while *in vitro* oxidation of sinapic acid leads to a single C-C bonded dimer (Tranchimand *et al.*, 2006; Mogharabi and Faramarzi, 2014). The laccase-ABTS system has been used for the catalysis of the oxidative coupling of heterocyclic thiols, rendering their corresponding disulfides, which have numerous applications such as drug delivery systems, vulcanizing agents or as intermediates for the synthesis of sulfinyl and sulfenyl compounds (Abdel-Mohsen *et al.*, 2013).

While the enzymatic polymerization of syringic acid or DMP is regioselective, yielding chains of poly-phenylene oxide units (Ikeda *et al.*, 1996b, 1996a), other oxidative coupling reactions can lead to non-controllable polymer structures with undesirable characteristics. Different strategies have been developed for gaining control of the process and obtaining the desired polymer structure. The presence of organic co-solvents, such as 1,4-dioxane or methanol, increases the solubility of the aromatic substrates leading in some cases to an important control over the polydispersity and polymer size (Hollmann and Arends, 2012). In addition to classical organic solvents, ionic liquids have been used as co-solvents for polymerization of phenols (Sgalla *et al.*, 2007; Eker *et al.*, 2009; Khlupova *et al.*, 2016). These chemicals can increase substrate solubility or act as enzyme immobilization matrix, enabling enzyme recycling during the reaction. It has also been observed that ionic liquids stabilize the enzyme in the presence of anionic surfactants required for example for the synthesis of polyaniline, thus increasing polymer yields (Zhang *et al.*, 2014). Regioselectivity is crucial for most polymerization reactions and it can be achieved by substrate engineering. For example, linear conductive polyaniline can be obtained without templates by blocking the *o*-position of aniline. However, this strategy yields a less conductive and electroactive polymer than the standard chemical synthesis of polyaniline (Kim *et al.*, 2007). Some compounds can act as scaffold (templates) during the polymerization reaction to avoid branch formation. These templates are molecules with generally long chain structures that direct the proper alignment of the monomers. In some cases, they are negatively charged, what serves as counter ion (dopant) for the synthesized polymer (Hollmann and Arends, 2012). While templates are often used to achieve polymerization of phenolic precursors, polyaniline synthesis is one of the most significant examples of the use of templates to aid the synthesis of a linear polymer (Kim *et al.*, 2005).

Synthesis of polyaniline catalyzed by laccase

Chemical synthesis of conductive polyaniline is relatively cheap due to the low cost of aniline and the high product yields. However, this synthesis is far from being environmentally friendly due to the elevated quantities of toxic oxidants needed and the very acidic pH of the reaction, releasing large quantities of pollutants (Shumakovich *et al.*, 2011). Even more, the polyaniline (PANI) obtained from the chemical polymerization of aniline is soluble only in some organic solvents, making the processability of the product difficult and, hence, increasing the cost of its application (Huang and Kaner, 2006).

On the other hand, the enzymatic polymerization of aniline to produce conductive PANI is environmentally friendly, avoiding the use of chemical oxidizers and allowing to increase the pH of the reaction up to 3.5. The pH of the reaction must be kept below the pKa of aniline − 4.6 at 25°C- to have a prevalence of anilinium cations in the reaction). Higher pH values implicate the prevalence of neutral aniline that yields PANI oligomers with low or none electroconductive capabilities (Sapurina and Stejskal, 2008). Also as aforementioned, only a linear polyaniline can display conductive capabilities. To obtain head to tail polymers and to avoid parasitic branch formation, a co-solvent or template must be present during the reaction (Ćirić-Marjanović *et al.*, 2017). Still some template-free synthesis studies of polyaniline using laccase as biocatalyst have been reported. Conductive cotton and bacterial cellulose have been obtained in a free template reaction by *in situ* polymerization of aniline by laccase using HBT as mediator in an ultrasonic bath (Su *et al.*, 2018; Shim *et al.*, 2019).

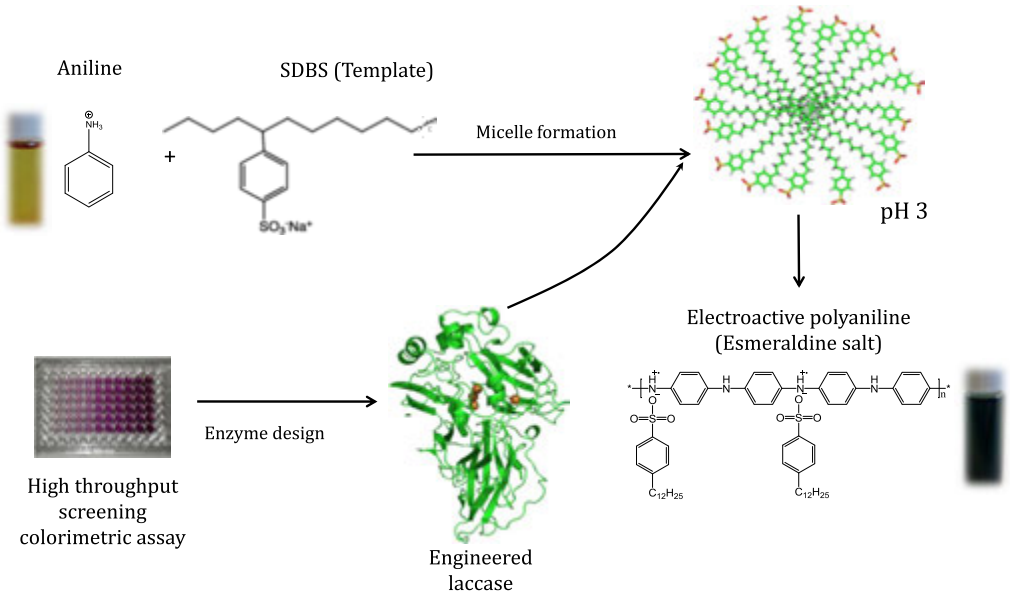

Fig. 6 Scheme of the synthesis of conductive PANI carried out in our lab using an engineered laccase and sodium dodecylbenzenesulfonate (SDBS) as template.

However, most often the template-free polymers are less defined and with an unclear protonation or oxidation states (Ćirić-Marjanović *et al.*, 2017).

Polyelectrolytes like anionic surfactants, poly(vinylphosphonic acid), sulfonated polystyrene or even DNA are commonly used as templates for the synthesis of electroactive PANI (Liu *et al.*, 1999; Nagarajan *et al.*, 2000, 2001; Karamyshev *et al.*, 2003). Among templates, anionic surfactants are preferred due their commercial availability and capability to maintain the water solubility of PANI, thus increasing its processability. These templates aggregate to form micelles over a critical micellar concentration, providing a suitable local environment for aniline *p*-coupling (Hino *et al.*, 2006). The polymerization process of aniline associated to this micellar structure yields water soluble PANI, that can be precipitated with ethanol or acetone that disaggregate the anionic surfactant micelles (Streltsov *et al.*, 2008). Different macromolecular structures can be obtained by changing the anionic surfactant during the enzymatic synthesis of PANI. Anionic surfactants usually inhibit enzyme activity by protein denaturation, although the presence of aniline protects the enzyme from the action of the surfactant (Otzen, 2011). The use of different anionic surfactants and different concentrations yields polyaniline with different spectrometric and macromolecular structures. Polyaniline with excellent electrochemical and electro-conductive properties was synthesized by using a fungal laccase variant engineered in our lab in the presence of sodium dodecylbenzenesulfonate (SDBS) as doping template (**Fig. 6**). The PANI obtained is displayed in nanofibers, which provides additional advantages over other macromolecular assemblies like a porous structure and a large surface-to-volume ratio, which are optimal properties for biosensor development (de Salas *et al.*, 2016). More recently, polyaniline's production yield has been enhanced up to 87% (w/w, referred to aniline precursor) by the additional engineering of the laccase through directed evolution and semi-rational design (de Salas *et al.*, 2019a).

Conclusions

The high redox potential of some fungal laccases boosts their capabilities to oxidize diverse aromatic compounds, which together with the use of O_2 from the air as sole catalytic requirement, make these enzymes biocatalysts of choice for many industrial sectors. Some of the first applications of fungal laccases were related to the pulp and paper industry, taking advantage of their intrinsic high activity on lignin phenols associated with their natural function in plants and fungi. Their use as biocatalyst in textile, food and beverage industries and waste treatment have also been historically targeted in many research studies. In the last two decades, new research studies emerged to open the range of laccase's applications to biosensor design or to catalyze organic synthesis reactions with promising results. Many of the examples described have been relegated over the years to the laboratory environment. The difficult overexpression of fungal laccases and need to adjust their catalytic activity and stability to specific operating conditions are main hurdles for the industrial application of these enzymes. However, new and more restrictive environmental regulations could be the catalyzer for the implementation of some of these new enzymatic processes to an industrial scale.

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Fungal Lignin-Modifying Peroxidases and H_2O_2-Producing Enzymes

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Plant Cell Wall Structure and Composition

Plant cell walls provide strength to the plant and are important for e.g., plant growth, cell differentiation, intercellular communication and water movement. They also protect plants from abiotic and biotic stresses including microbial attack (Cosgrove, 2005). Plant cell walls are mostly composed of polymeric compounds, the most abundant of which are the carbohydrates cellulose and hemicelluloses, and aromatic lignin. These polymers are arranged to a complex network that is called lignocellulose. In addition, the cell walls contain pectins, structural proteins and extractives, such as terpenes, resins and phenols. Plant cell walls have three different layers that are the primary and secondary walls, and the middle lamella, each of which have unique structure and chemical composition (Harris and Stone, 2009). Furthermore, the composition of the plant cell walls has significant differences between plant species and tissues as well as the growth phases of the plant (Eriksson *et al.*, 1990).

Lignin

The aromatic heteropolymer lignin is, after cellulose, the second most abundant biopolymer on earth. In the plant cell walls, lignin is cross-linked to hemicellulose and provides plant with strength, facilitates water transportation and protects the cell walls from microbial attack (Eriksson *et al.*, 1990; Harris and Stone, 2009). Lignin is synthesized in plant cell walls by enzymatic polymerization of phenylpropanoid monolignols, i.e., *p*-coumaryl, coniferyl and sinapyl alcohols, to *p*-hydroxyphenol, guaiacyl and syringyl subunits, respectively (Higuchi, 1990). These subunits are linked together with various C–C and ether bonds, of which the β-aryl-ether (β-O-4) linkage is the most abundant (Vanholme *et al.*, 2010). Mainly guaiacyl subunits are present in softwoods that contain 27%–32% lignin (as dry weight), while both guaiacyl and syringyl subunits exist in hardwoods where lignin forms 21%–31% (as dry weight) of the cell wall (Sjöström, 1993). Herbaceous plants contain all three lignin subunits and their lignin amount varies greatly, from 5% to 10% in grasses (Schaefer *et al.*, 1985; van Soest, 1982) up to 23% in crop straw (Buranov and Mazza, 2008). While being present in all plant cell wall layers, most of the lignin is in the thick secondary cell wall, but middle lamella has the highest lignin concentration. The heterogeneous, irregular structure of lignin with non-hydrolyzable interunit linkages makes its biological degradation difficult and also restricts obtaining complete knowledge of the chemical structure of native lignin molecules.

Plant Cell Wall Carbohydrates

Up to 40%–50% of plant dry weight is cellulose that is the most abundant polymer in plant cell walls and gives them rigidity. Cellulose is a linear homopolysaccharide of repeating D-glucose units that are joined together by β-1,4-linked glycosidic bonds (Sjöström, 1993). The linear cellulose chains are connected by intermolecular hydrogen bonds to microfibrils that contain highly organized crystalline regions and less organized amorphous regions (Ding and Himmel, 2009). Orientation of these rigid microfibrils differs between the secondary cell wall sublayers (Harris and Stone, 2009).

Hemicelluloses are branched heteropolysaccharides that are linked to cellulose microfibrils by hydrogen bonds, therefore supporting the cell wall structure (Harris and Stone, 2009). Approximately 20%–30% (as dry weight) of plant matter is hemicelluloses. Four main types of hemicelluloses are xylan, xyloglucan, β-glucan and mannan, and their backbones are built from β-1,4-linked D-xylose, β-1,4-linked D-glucose, β-1,3;1,4-linked D-glucose and β-1,4-linked mannose residues, respectively. These backbones are decorated with branched monomers and short oligomers that include D-galactose, D-xylose, L-arabinose, L-fucose, D-glucuronic acid, acetate, ferulic acid and *p*-coumaric acid (van den Brink and de Vries, 2011).

Pectins are the most complex hemicelluloses, which provide additional crosslinks between cellulose and hemicelluloses, and are mainly found in the primary cell wall and middle lamella. D-Galacturonic acid is the main monomer present in four different pectin substructures that are homogalacturonan (HGA), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen, 2008). HGA, XGA and RG-II have a linear backbone of α-1,4-linked D-galacturonic acid residues. In HGA, the backbone can be methylated and acetylated, whereas in XGA, the backbone is substituted by β-1,3-linked D-xylose residues. RG-II is a minor component of pectin and its D-galacturonic acid backbone can contain at least 12 different glycosyl residues. RG-I is the most complex pectin structure, which backbone consists of alternating D-galacturonic acid and L-rhamnose residues and have chains of L-arabinose and/or D-galactose linked to the L-rhamnose residues. In addition, RG-I can have branched polysaccharides such as arabinogalactan and arabinan as its side chains, which are often referred to as the "hairy" pectin regions. (Caffall and Mohnen, 2009).

In this chapter, we do not discuss about carbohydrate degrading enzymes of basidiomycete fungi, which have been extensively reviewed e.g., by Rytioja *et al.* (2014).

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Lignin-Modifying Fungi

Lignin-modifying fungi are the only organisms capable of efficiently mineralizing lignin into CO_2 (Blanchette, 1995). They usually belong to the phylum Basidiomycota, but some ascomycete species can also modify lignin (e.g., Xylariales species) (Worrall *et al.*, 1997). The potential of mineralization of lignin is however lower in *Xylaria* spp. than in basidiomycete fungi (Liers *et al.*, 2011, 2006). Lignin-modifying species have been broadly divided into white-rot and brown-rot fungi according to the visual appearance they cause to wood residue during decay.

White-Rot Fungi

White-rot fungi (e.g., *Phanerochaete chrysosporium*, *Dichomitus squalens*, *Trametes versicolor*) are the most efficient lignin degraders in nature (Eriksson *et al.*, 1990). They are able to degrade all structural constituents of the plant cell wall including lignin. These fungi secrete lignin-modifying enzymes such as class II heme peroxidases, laccases and H_2O_2-producing enzymes to get access to plant polysaccharides, which they can use as carbon sources (Kirk and Farrell, 1987). White-rotted wood has a soft and bleached appearance with fiber-like cellulose-enriched material (Hatakka and Hammel, 2010). In addition, black spots of manganese dioxide deposits are typically observed in the wood colonized by white-rot fungi (Daniel and Bergman, 1997; Eriksson *et al.*, 1990). Although white-rot fungi are predominantly found in hardwood, also litter-decomposing fungal species (e.g., *Agrocybe praecox*, *Stropharia coronilla*) cause white-rot type decay.

Brown-Rot Fungi

Brown-rot fungi are most commonly found on conifer trees and they all belong to Basidiomycota (e.g., *Gloeophyllum trabeum*, *Rhodonia* (*Postia*) *placenta*) (Gilbertson, 1980). In contrast to white-rot fungi, brown-rot species modify lignin through non-enzymatic breakdown. They have evolved from ancestral white-rot lineages several times with subsequent reduction in key plant biomass degrading enzymes (Arantes *et al.*, 2011; Floudas *et al.*, 2012). It is estimated that only 7% of wood decaying fungi are brown-rotters (Hatakka and Hammel, 2010). They primarily attack the polysaccharide fraction of wood, whereas lignin is only modified e.g., by demethoxylation. The residual lignin is dry, brown and has decreased strength resulting in its breaking into cubical cracks (Akhtar *et al.*, 1997; Hammel, 1997). Brown-rot fungi modify the plant cell wall by oxidative radical based system with secretion of iron-sequestering oxalate (Espejo and Agosin, 1991; Zhu *et al.*, 2016).

Oxygen-derived free radicals, such as hydroxyl radical ($\cdot OH$), generated in Fenton reaction ($Fe^{2+} + H_2O_2 -> Fe^{3+} + {}^{-}OH + \cdot OH$) diffuse into the wood. Lignocellulose is disrupted by attack of $\cdot OH$ leading to rearrangement of lignin by depolymerization, repolymerization and demethylation resulting with conformational changes in its structure (Arantes *et al.*, 2011; Goodell *et al.*, 1997).

Grey-Rot Fungi

Based on the analysis of the basidiomycete genomes, reclassification of wood rot types has been suggested (Floudas *et al.*, 2012; Nagy *et al.*, 2016; Riley *et al.*, 2014). The continuum of fungal decay strategies has been observed by species with intermediate wood decay mechanism such as *Schizophyllum commune*, *Jaapia argillacea* and *Botryobasidium botryosum*. These so-called grey-rot fungi resemble brown-rot species by lacking class II heme peroxidases, but still having diverse enzymes for cellulose degradation typical for white-rot fungi. Also some ectomycorrhizal fungi (ECM) have shown decay strategies similar to wood-decaying fungal species. Radical-based oxidation mechanism similar to brown-rot fungi has been reported in *Paxillus involutus* (Rineau *et al.*, 2012). Although most ECM have few or no class II heme peroxidases, gene repertoire of some ECM such as *Cortinarius glaucopus* and *Hebeloma cylindrosporum* suggest that they are able to produce white-rot (Bödeker *et al.*, 2014; Kohler *et al.*, 2015).

Soft-Rot Fungi

Soft-rot causing fungi are ascomycetes (e.g., *Daldinia*, *Chaetomium* and *Trichoderma* species) that mainly attack cellulose fraction of wood, but are also able to convert lignin to some extent (Hatakka and Hammel, 2010). They are mostly soil-inhabiting species, which degrade wood in wet environments. These fungi typically colonize hardwood resulting with brown, soft and sponge-like appearance. Soft-rot species are able to demethylate lignin, but relatively little is known about their enzymatic system (Osono, 2020; Venkatesagowda, 2019). Species in the Xylariaceae family have been shown to decompose wood lignocellulose by cellulases, xylanases and laccases, but they lack lignin-modifying peroxidases required for efficient mineralization of lignin (Blanchette, 1995; Liers *et al.*, 2011; Nagy *et al.*, 2016).

Table 1 Division of fungal lignin-modifying peroxidases and H_2O_2-producing enzymes addressed in this article into (sub)families of auxiliary activities (AA) in the CAZy database

CAZy (sub)family	*Enzyme name*	*Enzyme abbreviation*	*EC number*
AA2	*Class II peroxidases*		
	Manganese peroxidase	MnP	EC 1.11.1.13
	Lignin peroxidase	LiP	EC 1.11.1.14
	Versatile peroxidase	VP	EC 1.11.1.16
AA3	*Glucose-methanol-choline oxidoreductases (GMCs)*		
AA3_2	Aryl-alcohol oxidase	AAO	EC 1.1.3.7
	Glucose oxidase	GOX	EC 1.1.3.4
AA3_3	Methanol oxidase/Alcohol oxidase	MOX/AOX	EC 1.1.3.13
AA3_4	Pyranose 2-oxidase	POX, P2O	EC 1.1.3.10
AA5	*Copper radical oxidases (CROs)*		
AA5_1	Glyoxal oxidase	GLX, GLOX	EC 1.1.3.15
AA5_2	Galactose oxidase	GAO	EC 1.1.3.9
	Raffinose oxidase	RaOx	EC 1.1.3.-
	Alcohol oxidase	AlcOx	EC 1.1.3.13
AA7	*Glucooligosaccharide oxidase*	*GOOX, GOO*	*EC 1.1.3.-*

Class II Fungal Heme Peroxidases

The biological modification of lignin is possible because of a wide array of extracellular oxidoreductases produced by fungi, especially by the wood- and litter-degrading white-rot species. The main lignin-modifying enzymes, which are widespread among white-rot fungi, include lignin peroxidases (LiPs), manganese peroxidases (MnPs) and versatile peroxidases (VPs), together with other oxidoreductases (**Table 1**). These enzymes have biotechnical interest, not only because of their ability to degrade and modify lignin, but also due to their potential to oxidize other recalcitrant compounds, including both organic and inorganic compounds. Most of these enzymes are classified as auxiliary activities (AA) in the CAZy database (See "Relevant Websites section") according to their structural similarity (Lombard *et al.*, 2014). Other peroxidase enzymes possibly involved in fungal lignin degradation are members of heme thiolate peroxidase (HTP) superfamily, including e.g., chloroperoxidases and peroxygenases, and dye-decolorizing peroxidases (DyPs). Comprehensive information about these enzymes is reviewed by e.g., Hofrichter *et al.* (2015) and Linde *et al.* (2015). Furthermore, we do not address laccases in this chapter, as they are described in detail e.g., in Canas and Camarero (2010).

Besides fungi, peroxidase enzymes are found in other micro-organisms, plants and animals. The lignin-modifying peroxidases belong to class II secreted fungal peroxidases, which are members of the non-animal peroxidase superfamily (Hofrichter *et al.*, 2010). In the CAZy database, fungal lignin-modifying peroxidases belong to AA2 family. The non-animal peroxidase superfamily also includes class I intracellular non-animal peroxidases and class III classical secreted plant peroxidases (Hofrichter *et al.*, 2010; Welinder, 1992). Class II peroxidases catalyze oxidation of numerous substrates using hydrogen peroxide or organic peroxides as electron acceptor while water is produced as a by-product. In the multistep reaction, two electrons are derived from substrate molecules to reduce the enzyme and two water molecules are released. Consequently, organic radicals are formed, which rapidly undergo further chemical reactions leading to formation of oxidized or coupled (oligomeric) products (**Fig. 1**).

Manganese Peroxidase

The ligninolytic ability of manganese peroxidases (MnPs) is based on their ability to oxidize Mn(II) to Mn(III) that form chelated complexes with organic acids such as oxalate, fumarate and malate. The chelated Mn(III) serves as a freely diffusible oxidant, a mediator, which penetrates into the wood cell wall and begin the decay process by oxidizing phenolic substructures in lignin via formation of phenoxyl radicals. These radicals further react to yield various breakdown products (Hofrichter, 2002). Consequently, this further allows the invasion of enzyme molecules into wood (Blanchette *et al.*, 1997). Besides phenolic lignin substructures and dimers, the Mn(III) is able to catalyze the oxidation of phenolic substrates, such as phenols, amines, dyes (Wong, 2009). Since Mn (III) chelate is a mild oxidant, it is not capable of oxidizing non-phenolic lignin units that form up to 90% of the lignin polymer (Popp and Kirk, 1991). MnPs have been shown to oxidize these structures by some mediators or co-oxidants, such as thiols, unsaturated fatty acids and their derivatives (Bermek *et al.*, 2002; Jensen *et al.*, 1996; Kapich *et al.*, 1999; Wariishi *et al.*, 1989).

Lignin Peroxidase

Lignin peroxidase (LiP) is a ligninolytic enzyme, which is relatively nonspecific to its substrates and a wide array of oxidations, all of which are dependent on H_2O_2, have been confirmed (Hammel *et al.*, 1993; Kirk and Farrell, 1987). The efficient oxidation of nonphenolic aromatic compounds without participation of mediator molecules distinguishes LiPs from MnPs. The oxidation products of non-phenolic lignin structures are highly reactive aryl cation radicals that further undergo a variety of non-enzymatic

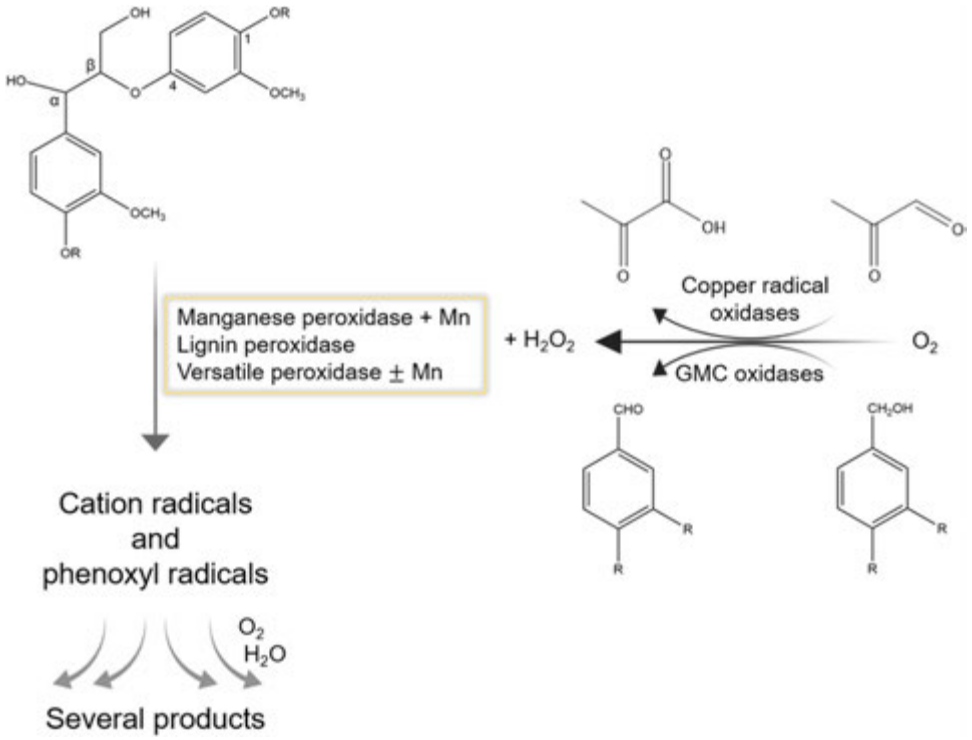

Fig. 1 The peroxidase substrate presented includes a typical ether substructure, which represents the major linkage (β-O-4 linkage, 90%) in natural lignins. Benzyl alcohol derivatives (R = H or OCH_3) are substrates for aryl-alcohol oxidase, a member of the glucose-methanol-choline (GMC) oxidases. Methylglyoxal is a substrate for glyoxal oxidase, a member of the copper radical oxidases (CROs). These enzymes have significant role in producing H_2O_2, which is the electron acceptor used by peroxidases. Schematic illustration of oxidative mechanisms involving class II peroxidases and H_2O_2-producing enzymes in the lignin modification (adapted from Cullen, D., 2013. Wood decay. In: Martin, F. (Ed.), The Ecological Genomics of Fungi. Hoboken, NJ: John Wiley & Sons, Inc. pp. 43–62.

reactions, such as cleavage of C_α-C_β side chains of lignin and demethylation (Lundell *et al.*, 1993; Schoemaker *et al.*, 1985). LiPs convert wide range of phenolic substrates to phenoxy radicals, which subsequently react to form ring-cleavage products or lead to a variety of reactions (Harvey *et al.*, 1993). The reactions result in many different products dependent on substrate structure. In addition, LiPs can oxidize several other substrates including veratryl alcohol (Hammel *et al.*, 1993).

Versatile Peroxidase

Versatile peroxidase (VP) combines the catalytic properties of MnP and LiP, and is able to oxidize their respective substrates (Ruiz-Dueñas *et al.*, 2009). Thus, similar to MnPs, VPs can oxidize Mn(II) to Mn(III), and additionally, they can oxidize non-phenolic lignin structures or substrates such as veratryl alcohol, without addition of Mn, like LiPs (Mester and Field, 1998). VPs demonstrate different pH optima for the two types of enzyme activities: the optimal pH for Mn(II) oxidation is around 5, while pH 3 is the optimum for the oxidation of aromatic compounds. These optimum pH values are close to the corresponding pH values observed for MnPs and LiPs, respectively (Martínez, 2002).

Structural Properties of Class II Peroxidases

The class II peroxidases are globular proteins with two calcium-binding sites (**Fig. 2**) and usually have eight cysteine residues forming four disulfide bonds (MnPs have five) providing additional stability for the secreted enzymes (Martínez, 2002). The active electron transfer center of the peroxidases is the iron-containing heme (protoporphyrin IX) which binds hydrogen peroxide or less reactive organic peroxides (Hofrichter *et al.*, 2010). Heme is buried in the interior of the enzyme and has access to the outer medium through two channels (Ruiz-Dueñas and Martínez, 2009). The main channel found in all class II peroxidases, is used by H_2O_2 and small molecule substrates. In the second site, Mn(II) is oxidized to Mn(III) by VP and MnP. MnPs have a Mn-binding site formed by three acidic residues near the heme propionates (Blodig *et al.*, 2001; Kishi *et al.*, 1996; Sundaramoorthy *et al.*, 1994, 2005). LiPs have a solvent-exposed tryptophan radical center, and VPs have both the Mn-binding site and solvent-exposed tryptophan (Doyle *et al.*, 1998; Mester *et al.*, 2001; Perez-Boada *et al.*, 2005; Ruiz-Dueñas *et al.*, 2009). In MnP and VP, acidic residues (Glu, Glu and Asp) form the Mn-binding site, whereas in LiP one of these residues is present (Glu), but the two others are

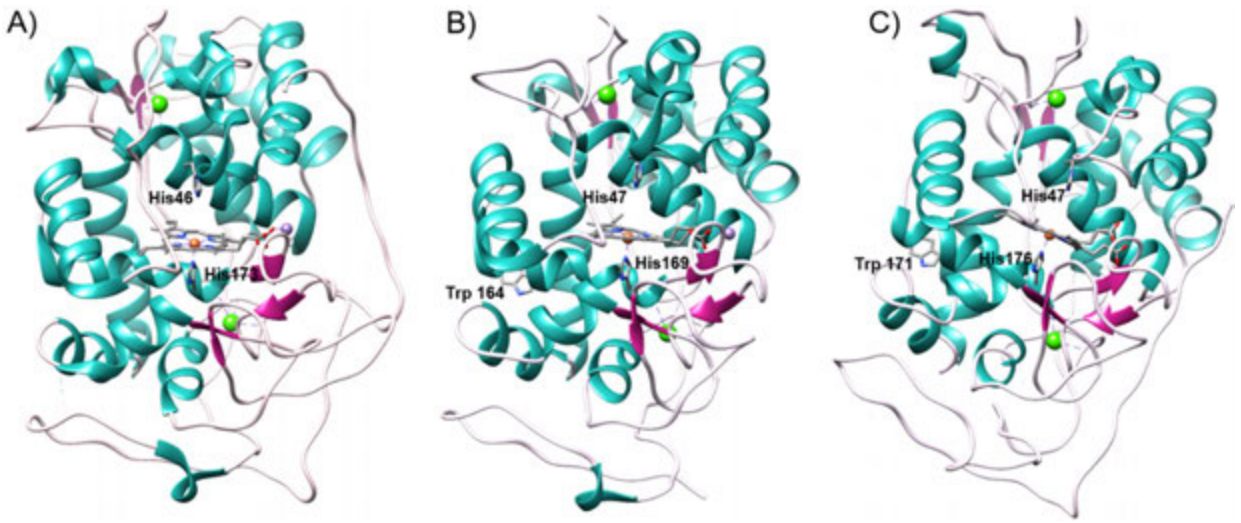

Fig. 2 Ribbon models for the molecular structures of class II peroxidases. (A) A manganese peroxidase (MnP) from *Phanerochaete chrysosporium* (image of Protein Data Bank (PDB) ID 3M5Q). (B) A versatile peroxidase (VP) from *Pleurotus eryngii* (image of PDB ID: 2BOQ). (C) A lignin peroxidase (LiP) from *P. chrysosporium* (image of PDB ID: 1LGA). The green spheres are structural Ca(II) ions in extracellular heme peroxidases. The substrate, Mn(II), near the heme, is indicated (violet sphere) for MnP and VP. Residues of catalytic solvent exposed tryptophan (VP: Trp164, LiP: Trp171) and buried heme cofactor (orange sphere: Fe) with two axial conserved histidines (MnP: His46, His173, VP: His47, His169, LiP: His47, His176) are indicated. The heme pocket of lignin-modifying peroxidases includes also other conserved residues not indicated in the figure but reviewed by Martínez. UCSF Chimera was used for graphical analysis and image generation. Reproduced from (A) Sundaramoorthy, M., Gold, M.H., Poulos, T.L., 2010. Ultrahigh (0.93 Å) resolution structure of manganese peroxidase from Phanerochaete chrysosporium: Implications for the catalytic mechanism. Journal of Inorganic Biochemistry 104, 683–690. (B) Perez-Boada, M., Ruiz-Dueñas, F.J., Pogni, R., *et al.*, 2005. Versatile peroxidase oxidation of high redox potential aromatic compounds: Site-directed mutagenesis, spectroscopic and crystallographic investigation of three long-range electron transfer pathways. Journal of Molecular Biology 354, 385–402. (C) Poulos, T.L., Edwards, S.L., Wariishi, H., Gold, M.H., 1993. Crystallographic refinement of lignin peroxidase at 2 Å. Journal of Biological Chemistry 268, 4429–4440. Martínez, A., 2002. Molecular biology and structure-function of lignin-degrading heme peroxidases. Enzyme and Microbial Technology 30, 425–444. Pettersen, E.F., Goddard, T.D., Huang, C.C., *et al.*, 2004. UCSF Chimera – A visualization system for exploratory research and analysis. Journal of Computational Chemistry 25, 1605–1612.

replaced by neutral residues (Ala and Asn) accordingly with the absence of the Mn(II)-binding site in these enzymes (Sigoillot *et al.*, 2012). The surface tryptophan of LiPs and VPs has a role in direct oxidation of the non-phenolic moiety of lignin by abstracting electrons from lignin and transferring them to heme via a long range-electron transfer (LRET) pathway (Doyle *et al.*, 1998; Mester *et al.*, 2001; Sáez-Jiménez *et al.*, 2015, 2016). This mechanism is beneficial because lignin polymers as well as various aromatic substrates cannot penetrate inside the protein to transfer electrons directly to the heme cofactor.

Evolutionary Background of Class II Peroxidases

The evolution and diversity of the class II peroxidases have been thoroughly studied in white-rot fungi. Evolutionary reconstruction and comparative genomic studies have shown that these enzymes share a non-ligninolytic peroxidase ancestor, which was further evolved so that the origin of fungal lignin biodegradation occurred in the late Carboniferous period (Floudas *et al.*, 2012; Nagy *et al.*, 2016). Duplications of the genes, possibly as a part of genome-size expansion, occurred after the appearance of the first white-rot species (Nagy *et al.*, 2016) and the evolutionary process continued and resulted in appearance of several subfamilies of ligninolytic peroxidases (Ruiz-Dueñas *et al.*, 2013). The class II peroxidases have evolved through several stages: first from MnP to gaining the catalytic tryptophan at the protein surface, thus generating VP enzymes, and following with the loss of Mn(II)-binding site resulting in the evolution of LiP enzymes (Ruiz-Dueñas *et al.*, 2013). Thus, MnPs are the most common lignin-modifying peroxidases present in the white-rot fungal genomes, while LiP encoding genes have been described from only a few genera (Floudas *et al.*, 2012).

In experimental studies, the appearance of the catalytic tryptophan (VP, LiP) has been demonstrated to increase the stability of the enzymes at low pH values, which is a key feature for enzymes acting under the acidic conditions that occur during lignin decay in nature (Ayuso-Fernández *et al.*, 2017, 2018). Besides increased stability at acidic pH, redox properties of these peroxidases have improved after the appearance of the catalytic tryptophan (Ayuso-Fernández *et al.*, 2019a). These both changes have endowed white-rot fungi with more efficient lignin degradation ability. In addition, it has been suggested that the evolutionary appearance of catalytic tryptophan residue occurred parallel to the appearance of angiosperms (hardwood trees) that have more complex and less phenolic lignin compared to gymnosperms (softwood trees) (Ayuso-Fernández *et al.*, 2019b). Overall, white-rot fungi have been specialized on angiosperm hosts during the evolution, while brown-rot fungi, without class II peroxidases, have become more generalist (Krah *et al.*, 2018).

H_2O_2-Producing Enzymes

Hydrogen peroxide is one of the key metabolites in lignocellulose degradation as it e.g., acts as an electron acceptor in the reactions catalyzed by lignin-modifying heme peroxidases (**Fig. 1**) (Martínez *et al.*, 2009). More recently, lytic polysaccharide monooxygenase (LPMO) activity was shown to be driven by H_2O_2 (Bissaro *et al.*, 2017), thus assisting in the oxidative cleavage of polysaccharides. H_2O_2 also plays an important role as a precursor for hydroxyl radical formation by Fenton reaction, which is a non-enzymatic approach for lignocellulose degradation especially in brown-rot fungi (Daniel *et al.*, 2007). In line with this, extracellular H_2O_2-producing enzymes from several AA (sub)families, representing glucose-methanol-choline (GMC) and copper radical oxidase (CRO) enzyme superfamilies (**Table 1**), are widely present in the basidiomycete white-rot, brown-rot and grey-rot fungi, as well as in ascomycete plant biomass degrading species (Ferreira *et al.*, 2015; Kjærbølling *et al.*, 2020; Riley *et al.*, 2014; Vesth *et al.*, 2018). This reflects the general importance of H_2O_2 in different fungal approaches for lignocellulose conversion, regardless of the decay type or fungal taxonomy. However, the abundance of the H_2O_2-producing enzymes encoding genes in the fungal genomes appears to be highly variable. While their exact roles in the degradation process are mostly unknown, these enzymes and the corresponding genes are often shown to be produced and expressed at the same time with lignin-modifying peroxidases when fungi are cultivated on plant biomass (e.g., Daly *et al.*, 2018; Fernandez-Fueyo *et al.*, 2012; Miyauchi *et al.*, 2017; Rytioja *et al.*, 2017). This further supports the essentiality of H_2O_2 production in fungal lignocellulose conversion. Interestingly, recent observations suggest that H_2O_2 can also be produced by laccase-catalyzed oxidation of lignin, which may provide new options for the use of redox enzymes in biomass applications (Perna *et al.*, 2020).

Glucose-Methanol-Choline Oxidoreductases

The family of glucose-methanol-choline (GMC) oxidoreductases, first described by Cavener (1992), contains structurally homologous proteins from various organisms with diverse catalytic activities (Sützl *et al.*, 2019). All GMC oxidoreductases share a common fold and possess a covalently or non-covalently bound flavin adenine dinucleotide (FAD) as a cofactor, and their structure typically contains a FAD-binding domain and a substrate-binding domain. GMC superfamily includes several fungal extracellular enzymes that catalyze the formation of H_2O_2 and are therefore likely involved in degradation of native lignocellulose polymers. These members are cataloged in AA3_2, AA3_3 and AA3_4 subfamilies in the CAZy database.

Aryl-alcohol oxidases (AAOs) are members of the CAZy AA3_2 subfamily and they catalyze the oxidation of lignin-derived compounds, such as phenolic aromatic acids and other aromatic fungal metabolites, to their corresponding aldehydes by concomitantly reducing O_2 to H_2O_2 (Hernández-Ortega *et al.*, 2012a). AAOs are monomeric, two domain containing enzymes (**Fig. 3**) that have non-covalently bound FAD in their structure (Ferreira *et al.*, 2015). These enzymes are abundantly encoded by white-rot fungal genomes, and detailed investigations have especially been concentrated in AAOs from different *Pleurotus* species (Bourbonnais and Paice, 1988; Guillén *et al.*, 2000; Sannia *et al.*, 1991).

Another representative of AA3_2 subfamily is glucose oxidase (GOX) that is structurally related to AAO and catalyzes oxidation of glucose at the C1 position to corresponding lactone by reducing O_2 to H_2O_2 (Ozimek *et al.*, 2005). GOX enzymes are less prevalent in basidiomycete fungi compared to ascomycetes (Ferreira *et al.*, 2015). Therefore, GOX has mostly been studied in

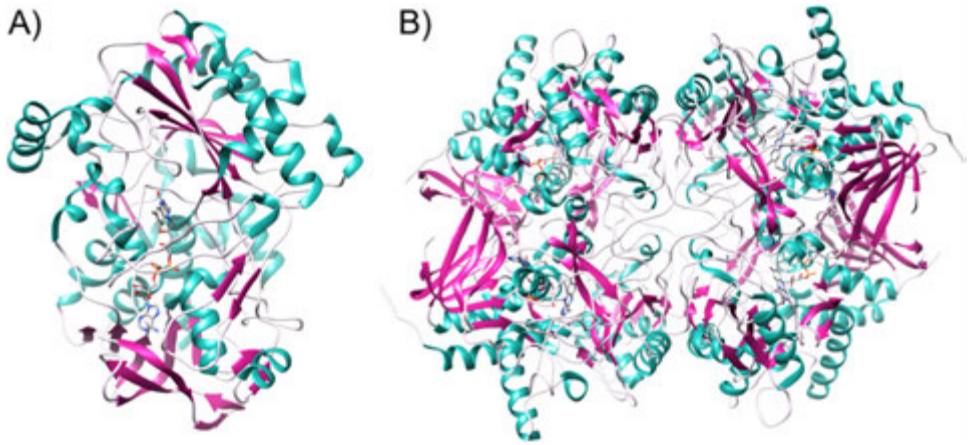

Fig. 3 Ribbon models for the molecular structures of two glucose-methanol-choline (GMC) oxidoreductases. (A) Aryl-alcohol oxidase (AAO) from *Pleurotus eryngii* (image of PDB ID 3FIM). (B) Pyranose oxidase (POX) from *Trametes multicolor* (image of PDB ID 3BG6). POX is a tetrameric protein with one FAD cofactor molecule per monomer. The FAD molecules are represented as stick models. UCSF Chimera was used for graphical analysis and image generation. Reproduced from (A) Fernández, I.S., Ruíz-Dueñas, F.J., Santillana, E., *et al.*, 2009. Novel structural features in the GMC family of oxidoreductases revealed by the crystal structure of fungal aryl-alcohol oxidase. Acta Crystallographica Section D: Biological Crystallography 65, 1196–1205. (B) Spadiut, O., Leitner, C., Salaheddin, C., *et al.*, 2009. Improving thermostability and catalytic activity of pyranose 2-oxidase from Trametes multicolor by rational and semi-rational design. FEBS Journal 276, 776–792. Pettersen, E.F., Goddard, T.D., Huang, C.C., *et al.*, 2004. UCSF Chimera – A visualization system for exploratory research and analysis. Journal of Computational Chemistry 25, 1605–1612.

ascomycete species, such as *Aspergillus niger*, especially regarding its biotechnological potential (see Section "Potential Applications of Fungal Lignin-Modifying Peroxidases and H_2O_2-Producing Enzymes"), and its role in lignocellulose degradation is not certain.

Methanol oxidase (MOX), also known as alcohol oxidase (AOX), is classified in the CAZy AA3_3 subfamily. MOX oxidizes methanol that is produced from lignin-derived phenolic substances by demethoxylation (Ander *et al.*, 1985). While the abundance of the MOX encoding genes seems to be equal in white- and brown-rot fungi (Ferreira *et al.*, 2015), it is worth to mention that MOX has especially been indicated as an important activity in brown-rot fungi (Martinez *et al.*, 2009), as MOX is possibly the main supplier of H_2O_2 for Fenton-chemistry based brown-rot wood decay. MOX enzymes have been characterized e.g., from the white-rot species *P. chrysosporium* (Nishida and Eriksson, 1987), and the brown-rot species *G. trabeum* (Daniel *et al.*, 2007).

The most distantly related enzymes of the CAZy AA3 family are pyranose oxidases (POX, P2O) that are cataloged to the subfamily AA3_4 (Sützl *et al.*, 2018). POX is a homotetramer (**Fig. 3**) and does not possess similar level of conservation of typical motifs that are present in the other AA3 family members. These enzymes catalyze oxidation of various aldopyranoses at C2 position resulting in the formation of the corresponding 2-ketoaldoses (Sützl *et al.*, 2018). POX has been investigated in detail in *P. chrysosporium* (de Koker *et al.*, 2004; Pisanelli *et al.*, 2009) as well as in another white-rot fungus, *Trametes multicolor* (syn. *Trametes ochracea*) (Leitner *et al.*, 2001). POX is widely present in wood-degrading basidiomycete fungi and it has been detected both extracellularly and in the hyphal periplasm (Daniel *et al.*, 1992).

Copper Radical Oxidases

Fungi also possess extracellular H_2O_2-producing enzymes that belong to the superfamily of copper radical oxidizes (CROs) (Whittaker *et al.*, 1996) and are classified in the CAZy family AA5. The most studied CRO of wood-degrading basidiomycetes is glyoxal oxidase (GLX) from the subfamily AA5_1, which is commonly present in these species. GLX produces H_2O_2 and carboxylic acids by catalyzing oxidation of broad array of substrates including simple aldehydes, and α-hydroxycarbonyl and α-dicarbonyl compounds, some of which are released during lignocellulose degradation (Whittaker *et al.*, 1996). Temporal correlation of GLX with lignin-modifying peroxidases in the ligninolytic cultures of *P. chrysosporium* was reported early on (Kersten, 1990; Kersten and Kirk, 1987; Kirk and Farrell, 1987). This is further supported by the clustering of LiP and CRO encoding genes in the *P. chrysosporium* genome (Martinez *et al.*, 2004).

Galactose oxidase (GAO) from the subfamily AA5_2 is another representative of CROs. GAOs oxidize several carbohydrates as well as primary alcohols to the corresponding aldehydes with the concomitant reduction of O_2 to H_2O_2. Biological function of these enzymes is unknown and their encoding genes are relatively rarely present in the fungal genomes. In addition, two subfamily AA5_2 alcohol oxidases (AlcOx) with activity towards aliphatic primary alcohols have been characterized from the plant pathogenic ascomycete species *Colletotrichum graminicola* and *Colletotrichum gloeosporioides* (Yin *et al.*, 2015). A role in plant cell wall degradation was suggested for these enzymes, although the natural substrate of them has not yet been identified. Another example of a subfamily AA5_2 member is a raffinose oxidase (RaOx) from *C. graminicola*, which uses the trisaccharide raffinose as its preferred substrate (Andberg *et al.*, 2017). Interestingly, a recent study showed that another AA5_2 paralog from *C. graminicola* oxidizes aryl alcohols to the corresponding aldehydes, thus describing AAO activity in the family AA5 (Mathieu *et al.*, 2020). More biochemically characterized AA5 members are needed to fully reveal the versatile activities of the fungal enzymes classified to this CAZy family.

Glucooligosaccharide Oxidases

Glucooligosaccharide oxidases (GOOX) are AA7 family extracellular enzymes that are primarily active on glucooligosaccharides, but their activity on xylooligosaccharides has also been shown (Vuong *et al.*, 2013). AA7 GOOXs oxidize C1 hydroxyl groups of various carbohydrates with the concomitant reduction of O_2 to H_2O_2. The biological role of these enzymes is still ambiguous, but they have been suggested to be involved in conversion or detoxification of lignocellulose-derived compounds (Levasseur *et al.*, 2013).

Potential Applications of Fungal Lignin-Modifying Peroxidases and H_2O_2-Producing Enzymes

Fungal enzymes described in this chapter have potential applications in various industries including food, polymer, and pulp and paper, as well as in bioremediation, synthetic chemistry and biosensor applications. Examples of potential applications of these enzymes in various industries are presented in **Table 2**. Enzymes utilized in applications are derived from variety of fungal sources, also other than wood-decaying fungi. However, the white-rot fungi and their peroxidases have been studied for utilization in lignin removal in the pulp and paper industry. Besides lignin, these enzymes can be used to convert variety of compounds, because of their lack of substrate specificity. The high redox potential and mode of action via the generation of radicals enables lignin-modifying peroxidases to degrade wide range of xenobiotics and organic compounds.

Various dye decolourization studies of lignin-modifying peroxidases have used either immobilized or soluble enzymes. Immobilization of enzymes using different coupling techniques usually stabilize enzymes and may enhance the activity, recovery and reuse of the enzyme (Gasser *et al.*, 2012). In addition, co-immobilization of several enzymes may be used for enhanced degradation. Peroxidases are inactivated by very high concentrations of their co-substrate H_2O_2, which may be problematic in various biocatalysis and biosensor applications. For the controlled release of H_2O_2, both LiP and MnP have been co-immobilized with GOX that continuously produces H_2O_2 upon oxidizing glucose and thus minimize inactivation of peroxidases by H_2O_2

Table 2 Examples of potential applications of lignin-modifying peroxidases and H_2O_2-producing enzymes. Some of the applications may also include other enzymes that are not indicated in the table

Application	*Enzymes*	*Fungal source*	*References*
Bioremediation			
Detection or biotransformation of recalcitrant aromatic compounds	LiP	*Phanerochaete chrysosporium*[a]	Ferapontova *et al.* (2006)
Elimination of pharmaceuticals from a municipal wastewater treatment plant effluent	VP GOX	*Bjerkandera adusta*[a], *Aspergillus niger*[b]	Touahar *et al.* (2014)
Transformation of halogenated pesticides	VP	*B. adusta*[a]	Davila-Vazquez *et al.* (2005)
Transformation of polycyclic aromatic hydrocarbons	MnP LiP	*Phanerochaete laevis*[a] *P. chrysosporium*[a]	Bogan and Lamar (1996), Hammel *et al.* (1986)
Oxidation of chlorophenols	MnP LiP	*Lentinula edodes*[a] *P. chrysosporium*[a]	Grabski *et al.* (1998) Reddy and Gold (2000)
Clinical diagnostics			
Biosensor for the measurement of galactose in serum	GAO	*Fusarium graminearum* (*Dactylium dendroides*)[b]	Kanyong *et al.* (2013)
Biosensor for the measurement of serum/subcutaneous glucose concentration	GOX	*A. niger*[b]	Poscia *et al.* (2003), Rubio Retama *et al.* (2004)
Cosmetic industry			
Skin lightening agents	LiP	*P. chrysosporium*[a]	Belinky *et al.* (2014), Draelos (2015)
Flavor and fragrance industry			
Carotenoid degradation	VP	*Pleurotus sapidus*[a]	Schüttmann *et al.* (2014)
Food industry			
Biosensor for the measurement of alcohol	AOX	*Ogataea polymorpha* (*Hansenula polymorpha*)[c] *Pichia pastoris*[c]	Alpeeva *et al.* (2005) Chinnadayyala *et al.* (2014)
Biosensor for estimation of galactose in milk and milk products	GAO	*F. graminearum* (*D. dendroides*)[b]	Sharma *et al.* (2006)
Biosensor for estimation of lactose in food	GAO	*F. graminearum* (*D. dendroides*)[b]	Sharma *et al.* (2004)
Oxidation of galactosylated polysaccharides for production of thermally stable hydrogels	GAO	*Fusarium* spp.[b]	Parikka *et al.* (2012)
Determination of glucose content in drinks	GOX	*A. niger*[b] *Penicillium vitale*[b]	Chudobová *et al.* (1996) Dudchenko *et al.* (2016)
Oxidation of D-galactose to 2-keto-D-galactose that is a precursor of a low-caloric sweetener and prebiotic food additive D-tagatose	POX	*Trametes multicolor*[a]	Salaheddin *et al.* (2009)
Removal of glucose from the egg white before spray drying	GOX	*A. niger*[b]	Sisak *et al.* (2006)
Pharmaceutical industry			
Stereoselective oxidation of secondary alcohols	AAO	*Pleurotus eryngii*[a]	Serrano *et al.* (2019)
Polymer industry			
Oxidation of hydroxymethylfurfural (HMF) to other carboxylic acid derivatives which are sources of a variety of chemicals	AAO GLOX AAO	*P. eryngii*[a] *Myceliophthora thermophila*[b] *Colletotrichum graminicola*[b]	Carro *et al.* (2014) Kadowaki *et al.* (2018) Mathieu *et al.* (2020)
Pulp and paper industry			
Biopulping: pretreatment of wood prior to the pulping	MnP	*B. adusta*[a]	Maijala *et al.* (2008)
Bleaching of wood pulps	MnP	*Trametes versicolor*[a]	Bermek *et al.* (2002)
Remediation of effluents from the pulp and paper industry	MnP LiP	*P. chrysosporium*[a]	Peralta-Zamora *et al.* (1998)
Synthetic chemistry applications			
New biomolecules by cross-linking of different substrates	VP	*P. eryngii*[a]	Salvachúa *et al.* (2013)
Textile industry			
Decolourization of malachite green dye	MnP GLOX	*P. chrysosporium*[a]	Son *et al.* (2012)
Decolourization of azo dyes from wastewater	MnP	*Bjerkandera* sp.[a]	López *et al.* (2004)
Production of H_2O_2 for textile bleaching	GOX	*A. niger*[b]	Tzanov *et al.* (2002)

[a]Basidiomycete fungus.
[b]Ascomycete fungus.
[c]Yeast.

(Qiu *et al.*, 2009; Van Aken *et al.*, 2000). Similarly, GLOX has been utilized as H_2O_2-supplying enzyme for decolorization of malachite green in combination with MnP (Son *et al.*, 2012). Besides co-immobilization, enzymes can be cross-linked for improved modification of the substrate. VP has been cross-linked with laccase and GOX, and the ability of the aggregate to eliminate a wide range of pharmaceutically active compounds in municipal wastewaters has been demonstrated (Touahar *et al.*, 2014).

The biosensors are broadly used in the clinical applications, food industry and monitoring of environmental contaminants. The first enzyme sensor was proposed by Clark and Lyons (1962), in which GOX and an oxygen electrode were employed for glucose monitoring. Ever since, extensive research has been conducted to develop GOX-based devices for self-monitoring of blood glucose levels and continuous glucose monitoring in management of diabetes (Ferri *et al.*, 2011), as well as for monitoring of glucose in food industry (Mello and Kubota, 2002). AOXs from different yeast species have been employed in various enzyme-based alcohol biosensors. These biosensors can be used to detect alcohol in several areas including forensic and clinical analyzes as well as in food and fermentation industries (Hooda *et al.*, 2018). Also peroxidases may be used in biosensors which monitor contaminants in natural environments. LiP-graphite electrode system has been developed for detection of polyphenols, lignin and lignin model compounds (Ferapontova *et al.*, 2006). The immobilized LiP demonstrated significant bioelectrocatalytic activity for the reduction of H_2O_2 by oxidizing lignin model compounds. The bioelectrocatalytical properties of VP (Munteanu *et al.*, 2005) and MnP (Ferapontova *et al.*, 2002; Petruccioli *et al.*, 2009) enzymes have also been promising. Biosensors based on the use of GAO can be used for determination of galactose in clinical chemistry, food and fermentation industries (Kanyong *et al.*, 2013; Sharma *et al.*, 2006). In addition, GAO-catalyzed oxidation of mono- and oligosaccharides, e.g., for synthesis of alcohols or amines, and production of H_2O_2 and reactive oxygen species, has been the basis of several patents and patent applications (reviewed by Parikka *et al.*, 2015).

Overall, H_2O_2-producing enzymes have been shown to be promising biocatalysts for the synthesis of valuable chemicals. Although AAO mainly catalyzes the oxidation primary benzyl alcohols, AAO variant oxidizing S-enantiomer of secondary aryl-alcohol has been obtained by protein engineering (Serrano *et al.*, 2019). The production of enantiomerically-enriched secondary alcohols is of commercial interest since chiral secondary alcohols are highly valuable building blocks (Hernández-Ortega *et al.*, 2012b). They can be used as starting materials for the synthesis of various enantiomerically pure compounds for pharmaceutical industry.

The utilization of GOX is not restricted to glucose sensors, and it is used as an analytical reagent in glucose assay kits (Giampietro *et al.*, 1982) and in clinical glucose analyzers (Heller and Feldman, 2008). In addition, GOX may be used for example in dry egg powder, wine and gluconic acid production as well as in breadmaking and as a food preservative or antimicrobial agent (Dubey *et al.*, 2017). These applications rely on the ability of GOX to remove glucose and O_2 or production of gluconic acid and H_2O_2. The diverse use has been possible since GOX is Generally Regarded As Safe and these enzymes are commercially available from several enzyme companies (Wong *et al.*, 2008). Noteworthy is that also LiP has been trademarked as Melanozyme and known on the market as Elure products (See "Relevant Websites section") of Rakuto Bio Technologies Ltd. for the treatment of hyperpigmentation and skin brightening (Belinky *et al.*, 2014; Draelos, 2015).

Concluding Remarks

Fungal degradation and conversion of plant biomass is an important process in nature. Due to this ability, fungi and their enzymes are also highly relevant for the development of bio-based economy, and they have gained increasing attention as potential candidates for several bioprocesses. However, the large-scale recombinant production of lignin-modifying peroxidases is a bottleneck that hinders the biotechnological use of these enzymes. In addition, the limitations of genetic manipulation of basidiomycetes as well as the lack of knowledge regarding the regulation of lignocellulose degradation restricts use of these fungi for homologous production of lignocellulose converting enzymes. Still, the recent developments in genome editing techniques may open up new avenues for the production of individual lignin-modifying enzymes in basidiomycetes. Protein engineering and process optimization are also needed to up-scale the potential applications of ligninolytic peroxidases and H_2O_2-producing enzymes to industrial level. Due to the multiplicity of these enzymes in fungi, elucidation of the differences in the biochemical, structural and operational properties, such as catalytic efficiency and stability, of the individual enzymes would be highly beneficial, to be able to select the most suitable candidates for specific applications.

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Fungal Peroxygenases – A Versatile Tool for Biocatalysis

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Introduction

Fungi (Mycota) represent a separate kingdom within the domain of eukaryotic organisms, which heterotrophically subsist due to the lack of chlorophyll and plastids and which do exclusively feed on dissolved nutrients (osmotrophy) because of the presence of rigid cell walls. Most fungi colonize terrestrial ecosystems and unlike their phylogenetic sister-group – the Metazoa (animals) – they are incapable of active motility (Moore *et al.*, 2011); this particularly applies to their trophic (vegetative) states, which do not serve reproduction. In consequence, "sessile" fungi cannot avoid negative effects of local (micro-environmental) conditions and must somehow counteract them *in situ*.

Fungi compete with prokaryotes in most ecosystems, in first place with heterotrophic eubacteria, such as *Bacillus* and *Pseudomonas* spp. A potential advantage of fungi over such unicellular prokaryotes is their capability of more efficiently disintegrating complex polymeric carbon-compounds including lignin-incrusted materials such as wood by means of secreted enzymes (digestion biocatalysts). The processes of decomposing such nutrients are "biochemically costly" and time-consuming, but are compensated by the intensive exchange of substances within the hyphal networks (mycelia) and plectenchyma, i.e., the rather slowly osmotically resorbed nutrients are going to be rapidly distributed within the whole fungal organism, so far there are nutrient gradients between the different parts of a mycelial colony (Moore *et al.*, 2011; Daly *et al.*, 2020). The most prominent example therefore is the substantial degradation of lignocelluloses, which is essential to maintain the global carbon cycle and cannot be accomplished by other organisms including bacteria (Floudas *et al.*, 2012). Several types of extracellularly acting enzymes are the prerequisite to get access to lignocelluloses (wood, leaf-litter, humus fractions) as carbon sources, some of which, such as manganese(II)-oxidizing peroxidases (MnPs), lignin peroxidase (LiP), aryl-alcohol oxidases (AAOs) and lytic polysaccharide monooxygenases (LPMOs), have exclusively evolved within the fungal kingdom (Note, there are a few examples of LPMOs in bacteria but they are chiefly associated with chitin degradation).

Unspecific peroxygenases (UPOs) represent another example of enzymes that exclusively occur in fungi (The few exceptions such as *Phytophthora et al.* will be discussed later), even though their natural and physiological functions have not been fully understood yet. UPOs are heme-thiolate proteins, which transfer peroxide-borne oxygen to diverse organic compounds. Regarding these properties, they resemble P450 monooxygenases (P450s) occurring in all domains of life, which, however, typically use dioxygen (O_2) and external electron donors (NAD[P]H). Unlike the P450s that are intracellular (cytosolic or membrane-bound) biocatalysts, most UPOs are extracellular enzymes, which fungi actively secrete (Hofrichter *et al.*, 2010).

Upadhyay first mentioned an unusual "alkaline lignin peroxidase" that was produced by the agaric fungus *Agrocybe aegerita* (*Cyclocybe aegerita*) in liquid culture (Upadhyay, 1995). Almost ten years later, this enzyme was described as *A. aegerita* peroxidase (AaP) and – for the time being – classified as a haloperoxidase due to brominating activities and homology of the *N*-terminus to that of chloroperoxidase [CPO = *Lfu*UPO from *Caldariomyces fumago* (*Leptoxyphium fumago*); (Ullrich *et al.*, 2004)]. Then, in 2005, it was shown that AaP is capable of hydroxylating toluene and naphthalene (Ullrich and Hofrichter, 2005), while subsequent studies demonstrated further oxyfunctionalizations, in first place of aromatic substrates (Ullrich and Hofrichter, 2007; Aranda *et al.*, 2009, 2010). Therefore, the enzyme (AaP) was re-named into aromatic peroxygenase (APO), taking also into account that oxygen transfer was favored over peroxidation (Hofrichter *et al.*, 2010). Eventually, because of diverse further oxyfunctionalisations, e.g., hydroxylation of *n*-alkanes and cycloalkanes, epoxidation of alkenes, heteroatom oxygenation of *S*- and *N*-heterocycles, it was reasonable to supplement the enzyme classification system (EC nomenclature) by a new entry: the sub-subclass of the "peroxygenases" that act with H_2O_2 as electron acceptor, one oxygen of which is incorporated into the product (EC 1.11.2.-; Hofrichter and Ullrich, 2014). Enzymes of this sub-subclass transfer oxygen from peroxides (*O*-donor) to various substrates; thus, they actually represent a special type of monooxygenase. Meanwhile, peroxygenases secreted by fungi are classified as the first entry within this sub-subclass under EC 1.11.2.1 (unspecific peroxygenase, UPO) (Hofrichter and Ullrich, 2014). Further representatives are myeloperoxidase of neutrophilic granulocytes of Chordata (EC. 1.11.2.2), plant-seed peroxygenase of Spermatophyta (EC. 1.11.2.3) or bacterial fatty-acid peroxygenase (EC 1.11.2.4) (**Fig. 1**).

Although UPOs peroxygenate various substrates, the catalyzed reactions proceed stereoselectively and often even enantioselectively. Moreover, most UPOs studied show a remarkable insensitivity towards pH, organic solvents and other physicochemical factors, which makes them interesting candidates to be used in biotechnology, organic synthesis and bioremediation.

In this chapter, we summarize and review the state of knowledge on fungal UPOs with focus on the reaction cycle and structural aspects. Furthermore, the catalyzed reactions are surveyed including selective oxyfunctionalisations. CPO (chloroperoxidase; EC 1.11.1.10, **Fig. 1**) that is actually a UPO, from the phylogenetic point of view ("*Lfu*UPO"), is considered here to some extent. However, we want to point to several comprehensive articles specifically dealing with catalytic and structural aspects of CPO (Chen *et al.*, 2008; Dembitsky, 2003; Griffin, 1992; Manoj and Hager, 2008). We also give an overview on the homologously and heterologously producible UPOs.

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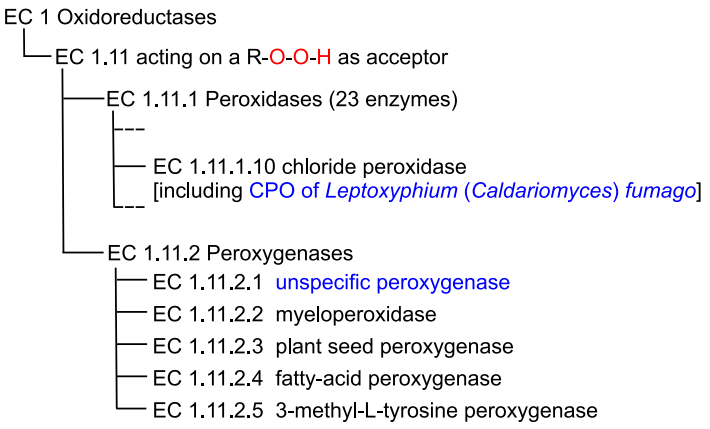

Fig. 1 Biochemical classification of oxidoreductases using peroxides (R-O-O-H) as electron acceptor (EC 1.11) including all representatives of enzymes with "H_2O_2 as acceptor, one oxygen atom of which is incorporated into the product" (= peroxygenases, EC 1.11.2). Phylogenetically related fungal enzymes are marked in blue (based on Enzyme Nomenclature: https://www.qmul.ac.uk/sbcs/iubmb/enzyme/EC1/11/).

General Aspects

The following three subarticles describe phylogenetic aspects of UPOs, their reaction cycle, and protein structure.

Phylogeny

UPOs are widely distributed within the kingdom of Fungi, whereas no *upo* genes have been detected so far in the closest relative of Fungi – the kingdom of Nucleariae ("Nucleariae are lacking chitin synthase genes division II – a gene cluster that is present in all true fungi (sometimes also named 'chitin fungi") share' (James and Berbee, 2012)), which are nowadays designated as non-fungal opisthokonts (Kiss *et al.*, 2019). Thus, the border between both kingdoms seemingly marks the limit in the occurrence of UPOs (**Fig. 2**), and hence animals (Metazoa) do not possess *upo* genes either. There are only few fungal phyla without *upo* genes or maybe, for which such genes have not yet been identified. These are all small phyla, i.e., Aphelidiomycota, Olpidiomycota, Calcarisporiellomycota or Kickxellomycota, with only eleven full genome sequences available. Larger taxonomical groups almost lacking *upo* genes are Saccharomycotina (true yeasts) and Schizosaccharomycetes (fission yeasts), which are a subphylum and a class of the Ascomycota, respectively (There is one exception: *Lipomyces starkeyi* NRRL Y-11557). These two taxa comprise highly specialized organisms with reduced genomes, which preferably dwell in sugar-rich habitats, such as nectaries or riping fruits, which likely explains their reduced genomes (Mohanta and Bae, 2015).

Apart from true fungi, *upo* genes were found in "parasitic algae" of the class of Peronosporomycetes (formerly "Oomycota" or "Oomycetes", within the supergroup of Stramenopiles). Notably, the powdery mildew (diverse species of Erysiphales, Ascomycota) and downy mildew (diverse species of the Peronosporales, Peronosporomycetes) can share the same plant hosts. Thus, an uptake of foreign DNA by horizontal/lateral gene transfer seems to be a plausible explanation for the occurrence of *upo* genes in phytoparasitic pseudo-fungi. This may have happened to other parasitic Peronosporomycetes as well, among them feared pathogens causing the potato blight (*Phytophthora infestans*) or the crayfish plague (*Aphanomyces astaci*) (Bredeweg and Baker, 2020; Szöllősi *et al.*, 2015).

The ancestral UPOs must have evolved near the base of true fungi (i.e., Eumycota; **Fig. 2**). However, the phylogeny of UPOs does obviously not fully correspond to the current fungal phylogeny. Again, this fact may be explained by horizontal gene transfer within the fungal kingdom (Szöllősi *et al.*, 2015). UPO encoding genes are divided into two families. Whereas family I contains UPOs with relative short genes resulting in theoretical mature proteins with an average molecular mass around 30 kDa (known as "short" UPOs), family II comprises larger proteins with an average molecular mass of 44 kDa (named "long" UPOs). Members of family I occur in all UPO-positive fungal phyla, while family II *upo* genes are restricted to the super-phylum of Dikarya (Ascomycota and Basidiomycota). Roughly, half of the *upo* genes in family I does not contain predictable signal peptides. Thus, family I further splits into two clades (subfamilies) of probably intracellular and extracellular UPOs. Genes without signal peptide belong to clade I.1, but almost nothing is known about this clade (except the genomic sequences) and whether they encode functional intracellular enzymes remains unclear. Clade I.2 comprises UPO sequences with signal peptides, including the characterized ascomycetous enzymes from *Leptoxyphium (Caldariomyces) fumago* (CPO) and *Chaetomium globosum* (*Cgl*UPO) as well as the basidiomycetous peroxygenases from *Marasmius wettsteinii* (*Mwe*UPO) and *Marasmius rotula* (*Mro*UPO) (Hofrichter *et al.*, 2020; Kellner *et al.*, 2016; Kiebist *et al.*, 2017; Ullrich *et al.*, 2018).

Family II forms, with regard to gene sequences, a more homogeneous clade of UPOs. It contains all "long" UPOs that are present in almost all genome-sequenced genera of Ascomycota and Basidiomycota (with the exceptions mentioned above), and all of them have predicted signal peptides. All "long" UPOs described and characterized at the protein level so far belong to basidiomycetous members of family II (**Tables 2** and **3**; Hofrichter *et al.*, 2015, 2020).

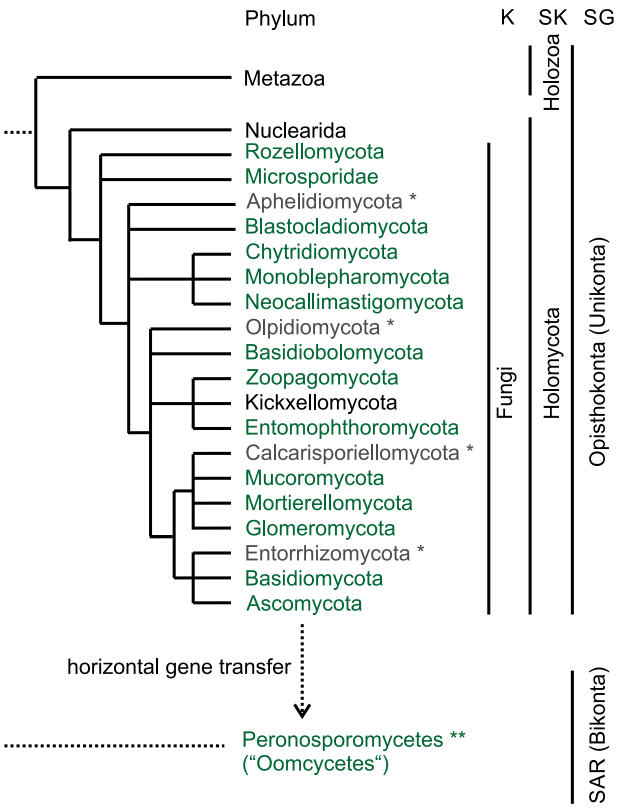

Fig. 2 Simplified phylogenetic tree of the kingdom of Fungi within the supergroup of Opisthokonta (modified according to Tedersoo *et al.*, 2018). Names marked in green represent eufungal phyla (suffix '-mycota') and pseudofungal Peronosporomycetes comprising species that have putative *upo* genes in their genomes. * Indicates small phyla without available genome data; ** the Peronosporomycetes (former "Oomycetes" or "Oomycota") are a class within the supergroup SAR (Stramelopiles-Alveolata-Rhizaria); K – kingdom, SK – superkingdom, SG – supergroup (i.e., unranked taxa above the kingdom level).

Besides the gene length, there are notable differences in the molecular structure between the UPO families. We will discuss these structural aspects later in the sub-chapter "Structural Insights".

Reaction Cycle

A first hypothetic reaction cycle of UPOs was already reported in 2007, and since then it has been steadily improved and substantiated (Ullrich and Hofrichter, 2007). Four relevant reactive UPO intermediates are involved in the cycle and two basic reaction routes can be distinguished. The reaction cycle combines one-electron oxidations known from "classic" peroxidases with the peroxide "shunt" pathway of P450 monooxygenases (P450s) (Denisov *et al.*, 2005; Dunford, 1999; Hofrichter *et al.*, 2015; Hrycay and Bandiera, 2015).

Both catalytic routes start with the UPO-resting state, in which the enzymes' heme iron is in the ferric state (Fe^{3+}) and weakly bound to a water molecule as distal ligand. When a peroxide molecule (e.g., H_2O_2) replaces water, a short-lived "pre-compound 0" (pCpd-0) is formed. Immediately the deprotonated carboxylic group of the side chain (pK_a 4.2) of a highly conserved glutamate accepts a proton from pCpd-0 under formation of a negatively charged ferric peroxo-complex (compound 0, Cpd-0). The negative charge of this important glutamate residue is stabilized by a conserved histidine (pK_a 6.0) or an arginine (pK_a 13.8) in the case of short UPOs and long UPOs, respectively (Piontek *et al.*, 2013; Ullrich *et al.*, 2018). Upon heterolytic cleavage of Cpd-0, a water molecule is released and compound I (Cpd-I) is formed (**Fig. 3**). Cpd-I corresponds to an oxo-ferryl species ($Fe^{4+}=O$) in addition with an oxidizing equivalent (cation radical, $\bullet+$) delocalized at the near heme environment. Cpd-I is the key intermediate in the reaction cycle that initiates substrate oxyfunctionalization or oxidation. The reaction sequence resembles an alternative and rarely efficient monooxygenation-route of P450s, the so-called "peroxide shunt" pathway. Unlike UPOs, the typical route of Cpd-0 formation in P450s requires two redox equivalents [mostly NAD(P)H] and dioxygen (O_2). It proceeds via three additional intermediate states: (1) an Fe^{2+}-heme-substrate complex, (2) an Fe^{3+}-superoxo complex and (3) an Fe^{3+}-peroxo-dianion complex (**Fig. 4**; Denisov *et al.*, 2005; Rittle and Green, 2010).

One of the possible UPO routes – the (per)oxygenation route – proceeds via abstraction of a hydrogen [H] from a substrate-carbon (R_3C-H), yielding a ferryl hydroxide complex (protonated compound II, HO-Cpd-II) and the corresponding substrate-radical

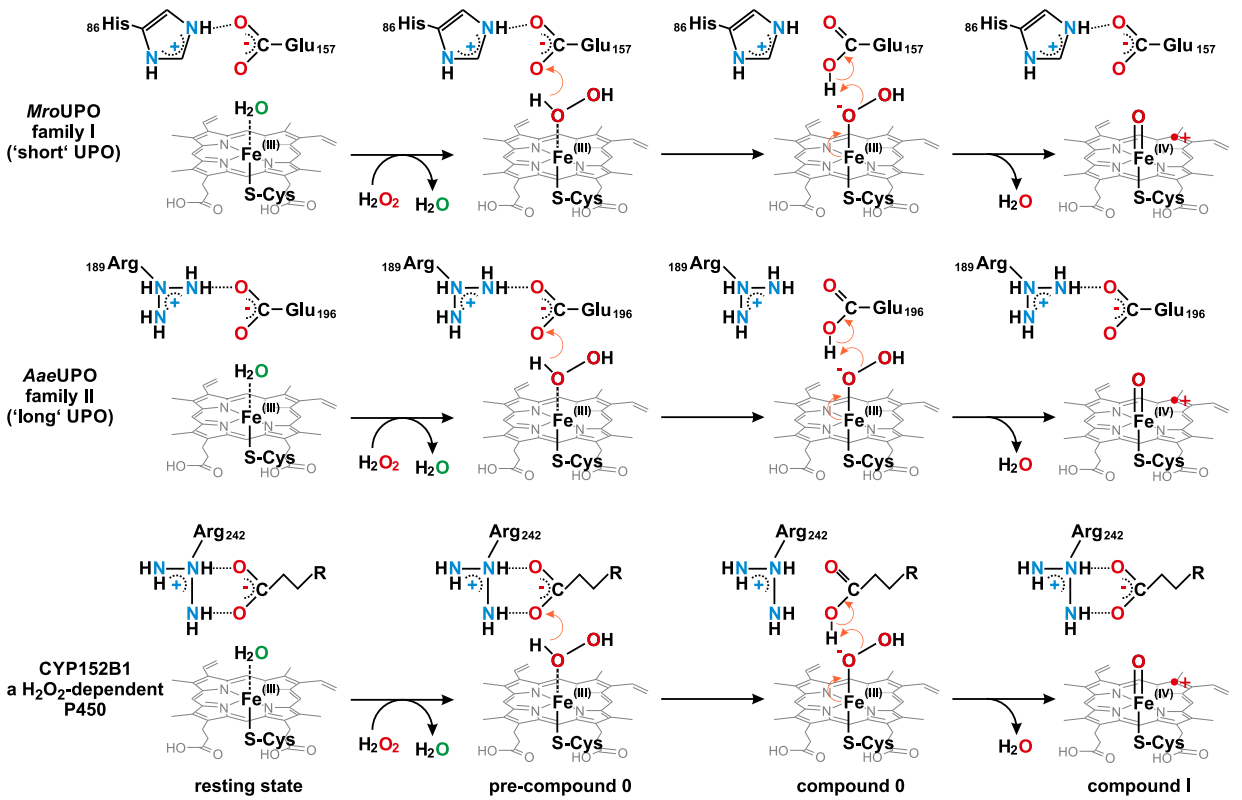

Fig. 3 Formation of compound I (Cpd-I) in UPO-family I ("short" UPOs) and UPO-family II ("long" UPOs; both classified under EC 1.11.2.1) as well as in P450-family CYP152 (fatty acid peroxygenase, EC 1.11.2.4). The graphic representations show the heme in different oxidation states and with proximal cysteine, distal ligands, acid-base catalysts (glutamate or fatty acid carboxylate) and charge stabilizers (histidine or arginine).

(R_3C•; R• in **Fig. 4**). An ensuing rebound restores the enzymes' resting state and releases the hydroxylated substrate (R_3-C-OH; ROH in **Fig. 4**) (Wang *et al.*, 2012, 2015). Additionally, in the (per)oxidative route (not shown in **Fig. 4**), Cpd-I may abstract one electron from a phenolic hydroxyl group that results in the formation of a phenoxy radical and an oxo-ferryl complex (deprotonated compound II, O=Cpd-II). The latter catalyzes a similar reaction to give a second phenoxy radical (upon proton and electron abstraction) and concomitantly returns to the resting state under release of a water molecule.

The preferred catalytic route is strongly determined by properties of the substrate (e.g., molecule size, chemical structure, functional groups) and of the particular UPO (characterized, for example, by redox potential, localization of the substrate-binding site, size and geometry of the heme channel), as well as of the reaction medium (pH, ion strength, organic co-solvent concentration) (Hofrichter and Ullrich, 2014). It should be noted that some substrate conversions start with aromatic oxygenation (route 1), resulting in the formation of phenolic compounds, which in turn will be oxidized according to route 2; this applies, for example, to benzene, naphthalene and related aromatic compounds.

One-electron oxidations (route 2) can be also accomplished by diverse peroxidases (EC 1.11.1) and oxidases (e.g., laccase, EC 1.10.3.2). The actual value of UPOs, however, with regard to synthetic applications, lies in their ability to perform challenging oxygenations using simply hydrogen peroxide.

Structural Insights

UPOs are heme-containing enzymes, in which the heme-iron is bound to a cysteine (proximal ligand). P450 monooxygenases share this feature with UPOs and hence, both can be subsumed under the heme-thiolate proteins (HTPs) (Hofrichter *et al.*, 2015; Omura, 2005). Almost all UPOs contain the characteristic "PCP motif" near the N-terminal site of the protein; only a small number UPOs is missing the first proline in this motif ("XCP motif"). In P450s, the cysteine ligand can be also part of a "XCP motif" (or another motif), but it is located near the C-terminus (Sundaramoorthy *et al.*, 1995). In contrast to P450s, all UPOs bear the aforementioned glutamate that acts as an acid-base catalyst during heterolytic peroxide cleavage. The few H_2O_2-dependent P450s (e.g., $P450_{BS\beta}$) utilize external fatty acids, e.g., palmitate, for this purpose (**Fig. 5**; Fujishiro *et al.*, 2011; Shoji *et al.*, 2007).

In UPOs, an Mg^{2+} cation (or Mn^{2+} in CPO) is located near one of the heme propionate residues (Piontek *et al.*, 2013; Sundaramoorthy *et al.*, 1995; Ullrich *et al.*, 2018). In this respect, UPOs resemble LiP (EC 1.11.1.14) and MnP (EC 1.11.1.13), which harbor a bivalent metal ion (Ca^{2+}) for protein stabilization. Furthermore, the latter enzyme uses manganese (Mn^{2+} that is

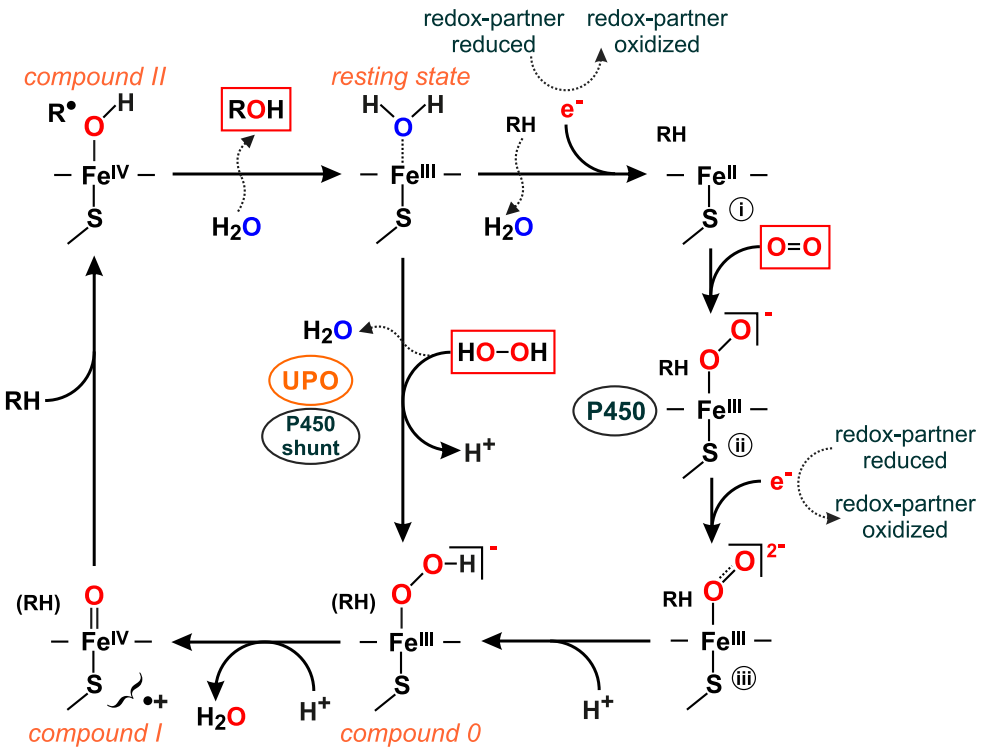

Fig. 4 Reaction cycle combining the monooxygenation routes of UPOs and P450s. The left route uses H_2O_2 as electron acceptor and *O*-donor; UPOs, P450 peroxygenases and P450s in the 'shunt' mode follow this pathway. The right route illustrates the typical P450 pathway that uses O_2 and NAD(P)H as reduced redox-partner, with following intermediates: (i) ferrous-heme substrate adduct, (ii) ferric superoxo-heme complex and (iii) ferric peroxo-heme dianion complex.

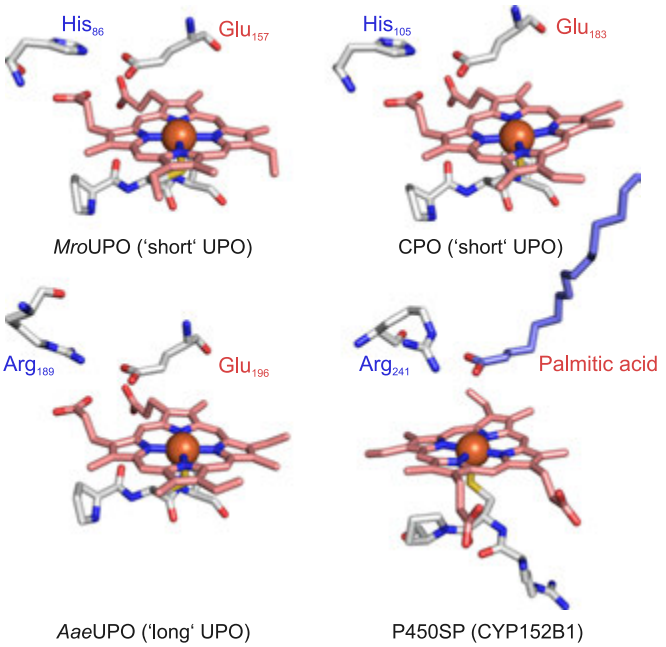

Fig. 5 Comparison of the distal sites of four different heme-thiolate proteins (HTPs), including a basidiomycetous "short" UPO (*Mro*UPO), a basidiomycetous 'long' UPO (*Aae*UPO), ascomycetous CPO (a special case of 'short' UPOs) and a bacterial fatty-acid peroxygenase (H_2O_2-dependent cytochrome $P450_{SP}$ = CYP152B1). The models show amino acid residues involved in compound 0 and compound I formation, i.e., the acid-base catalyst glutamate (Glu) and the charge stabilizers histidine (His) or arginine (Arg). In the case of CYP152B1, an external fatty acid (palmitate) replaces internal Glu. The porphyrin ring and the amino acid side chains are depicted as stick models, iron ions as red spheres. Crystal models: *Mro*UPO (pdb: 5FUJ); *Aae*UPO (pdb: 2YOR); CPO (pdb: 1CPO); CYP152B1 (pdb: 3awp).

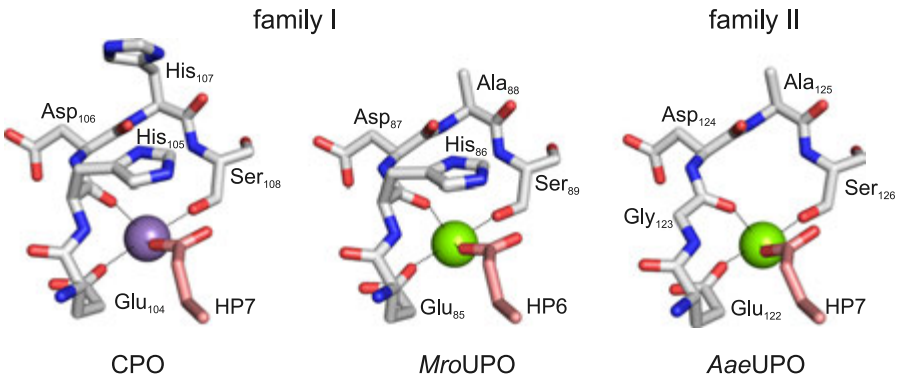

Fig. 6 Comparison of magnesium/ Mg^{2+} (manganese/ Mn^{2+}) cation binding sites of CPO and two UPOs (*Mro*UPO, *Aae*UPO), which correspond to the amino acid motifs *EHDHS*, *EHDAS* and *EGDAS*, respectively. The amino acid side chains and heme propionate residues (HP6 and HP7) are depicted as stick models, metal ions as spheres (Mn^{2+} - purple, Mg^{2+} - green). Crystal structure models: CPO (pdb: 1CPO), *Mro*UPO (pdb: 5FUJ), *Aae*UPO (pdb: 2YOR).

Table 1 Comparison of selected structural properties of four heme-thiolate proteins belonging to EC 1.11.2 (two UPOs, CPO and $P450_{SP}$ fatty peroxygenase). Data were calculated from the corresponding crystal structures deposited under 2YP1, 5FUJ, 1CPO and 3AWP, respectively

	AaeUPO	*MroUPO*	*CPO*	*$P450_{SP}$*
Native molecule	monomer	dimer	monomer	monomer
Proximal heme-ligand	cysteine	cysteine	cysteine	cysteine
Cys motif	PCP	PCP	PCP	RCP
Metal ligand	Mg^{2+}	Mg^{2+}	Mn^{2+} (Mg^{2+} also possible)	none
Chelating motif	EGD(A)S	EHD(A)S	EHD(A)S	none
C-terminal cysteine	C_{278} and C_{319} forming an internal S-S bridge	C_{227} forming an S-S bridge between two UPO monomers	none	none
Molecule size incl. glycosylation (kDa)	46[a]	32[b]	42[c]	43[d]
Sequence length in amino acids (aa)	326	236	298	415
Acid-base catalyst & charge stabilizer	glutamate arginine	glutamate histidine	glutamate histidine	palmitic acid arginine
UPO family	II (long UPO)	I (short UPO)	I (short UPO)	–
Dimensions of the heme-access channel (Å)[e]	7.8–8.6 × 10.1–10.8	8.6–9.3 × 14.9–18.9	6.3–9.1 × 7.9–9.3	11.0–12.1 × 9.5–10.1
Predominant aa-residues in the heme channel	phenylalanine	leucine, isoleucine, valine	mixture of hydrophobic and hydro-philic aa	n.d.
Heme positioning with regard to acid-base catalyst	position 1	position 2[f]	position 1	position 3[g]

[a](Ullrich *et al.*, 2004).
[b]monomeric form (Gröbe *et al.*, 2011).
[c](Morris and Hager, 1966).
[d](Matsunaga *et al.*, 1998).
[e]calculated with Pymol®.
[f]rotated by 180° with propionates facing the same direction as in position 1.
[g]starting at position 2, the propionates are rotated by approx. 145°.

oxidized to Mn^{3+}) as redox mediator, which is transiently bound at the same position as Mg^{2+} in UPOs. This again sets UPOs apart from P450s that do not contain any type of cation in the apoprotein (Denisov *et al.*, 2005; Nie and Aust, 1997; Poulos, 1993; Sundaramoorthy *et al.*, 1994). Note that Sundaramoorthy *et al.* (1995) suggested the presence of Mn^{2+} in CPO, which, however, could be Mg^{2+} as well (Piontek *et al.*, 2013).

In addition to the carboxylic group of one heme-propionate, two amino acid residues, a glutamate and a serine, and the carbonyl oxygen of the peptide bond between glycine (family II) or histidine (family I) and aspartate chelate the Mg^{2+} in UPOs (**Fig. 6**, **Table 1**). This difference in the Mg^{2+} ligands defines – along with the overall molecular mass – the two UPO families. In family I, histidine is complexed with the Mg^{2+} cation und thus, the corresponding chelate-motif is EHD(A)S. UPOs of family II, on the other hand, have a glycine at this position that is part of the EGD(A)S motif. Interestingly, the chelating histidine in family I has a further, even more important function. It functions as the charge stabilizer that assists deprotonation of H_2O_2 by the

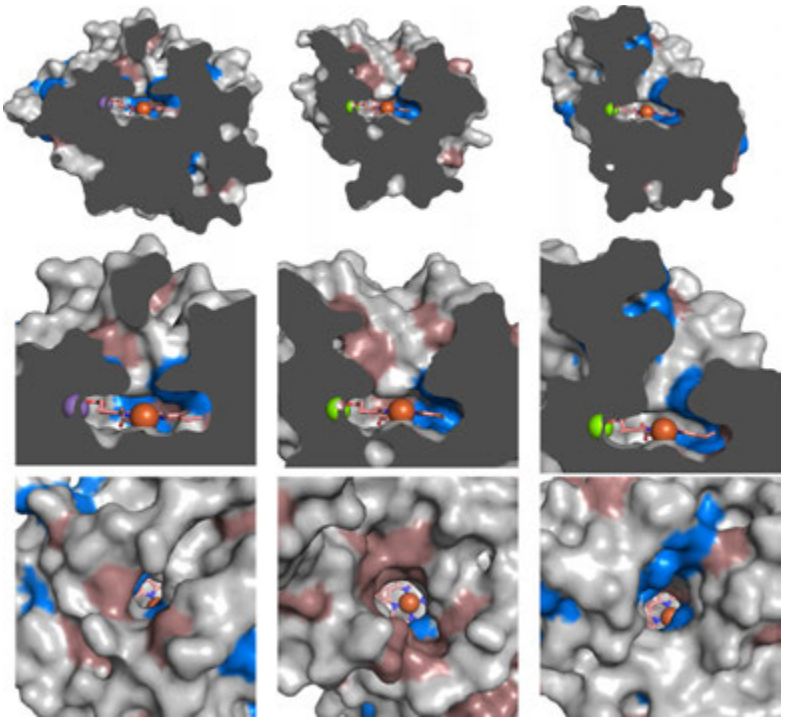

Fig. 7 Tangential cross section through 3D models (crystal structure-based) of CPO, *Mro*UPO and *Aae*UPO *(top row)* and enlarged sections of the respective active sites *(middle row)*. Adjusted graphic representations show the heme propionates at the left with the metal cations (Mn^{2+} in purple or Mg^{2+} in green). CPO has two narrow entries to the heme-access channel, while *Mro*UPO and *Aae*UPO have only one but rather wide entry. The three pictures *below* illustrate the maximum access path towards the heme in top-view. Blue color indicates phenylalanine residues, and pink color leucine and isoleucine residues.

glutamate acting as acid-base catalyst. This function of histidine (H-86 and H-105 in *Mro*UPO and CPO, respectively) is taken over by arginine (R-189 in *Aae*UPO), which is not involved in Mg^{2+} chelation, in UPOs of family II (**Figs. 3** and **5**). Due to the different polarities of histidine and arginine, this difference may affect UPO kinetics (i.e., Cpd-0 and Cpd-I formation) but not the overall catalytic cycle (**Fig. 4**).

Certainly, the molecular architecture of heme-access channels and heme-cavities is of general significance for the catalytic properties of UPOs. Their molecular dimensions and amino-acid composition determine substrate selection and binding. For example, the channel of *Mro*UPO resembles a large, laterally compressed funnel with an aperture of 9 × 18 Å. It is lined with several aliphatic amino acid residues (valine, leucine, isoleucine), which convey hydrophobicity and conformational flexibility to the channel. The heme-cavity contains, in addition to hydrophobic amino acids such as alanine and isoleucine, also hydrophilic glutamic acid. In contrast, the channel of *Aae*UPO is overall narrower, its "mouth" just measures 8 × 10.5 Å and it accommodates nine phenylalanine residues. Thus, it is not surprising that the catalytic properties and substrate spectra of UPOs can considerably vary. *Mro*UPO is able to oxidize relatively bulky and (cyclo)aliphatic substrates (e.g., steroids) whereas *Aae*UPO favors smaller aromatic substrates (e.g., naphthalene). CPO has two differently sized heme-access channels, a narrower and a wider one (Kühnel *et al.*, 2006), but even the wider channel (8 × 8 Å) is smaller than those of the other two UPOs. Furthermore, various amino acids line the channels and heme pocket in CPO (**Table 1**, **Fig. 7**), which may explain its limitation in oxidizing larger substrate molecules.

Remarkably, the heme orientation in *Aae*UPO and CPO differs from that in *Mro*UPO where it is turned by 180° ("flipped like a pancake") so that the two propionates face the same direction. In consequence, the propionate chelating Mg^{2+} switches its relative position in *Mro*UPO compared to *Aae*UPO or CPO (being position 6 (Numbers correspond to FISCHER numbering of heme carbons that are connected to the propionates (Moss, 1988)) and 7, respectively; **Fig. 6**). Whether this conformational difference has an impact on the catalytic properties of UPOs is not clear yet.

*Mro*UPO is a physiological dimer held together by a disulfide-bridge (Olmedo *et al.*, 2017). So far, we have observed this phenomenon only for short UPOs (family I) found in the agaric genus *Marasmius* (**Table 2**). Since the two monomers are covalently linked via two exposed cysteines (C-227) near the C-terminus, it is plausible that long UPOs lacking such cysteines (e.g., *Aae*UPO, CPO) cannot form dimers (instead, they form an internal disulfide-bridge near the C-terminus) (Piontek *et al.*, 2013). Whether dimerization is restricted to UPOs of the Marasmiaceae or maybe widely distributed among short UPOs, and whether dimeric UPOs may be advantageous over monomeric UPOs will have to be clarified in future structure-function studies.

Table 2 Survey on wild-type UPOs that have been purified and partially characterized

Enzyme	*Organism*	*Genes*[a]	*maximum amount*[b] *(mg L^{-1})*	*Isoforms*	*Molecular mass (kDa)*	*Crystal structure*[c]	*References*
*Aae*UPO	*Agrocybe aegerita*	18	10	3–6	45–46	2YP1	(Gupta *et al.*, 2018; Ullrich *et al.*, 2009, 2004)
CPO	*Leptoxyphium (Caldariomyces) fumago*	8[a]	600[d]	2	40–42	1CPO	(Kellner *et al.*, 2016; Morris and Hager, 1966; Pickard *et al.*, 1991; Sae and Cunningham, 1979)
*Cgl*UPO	*Chaetomium globosum*	4	44	2	36	–	(Kiebist *et al.*, 2017)
*Cra*UPO	*Coprinellus radians*	2	8	4	43–45	–	(Anh *et al.*, 2007)
*Cve*UPO	*Coprinopsis verticillata*	1	2	2	40	–	(Anh, 2008)
*Mro*UPO	*Marasmius rotula*	60	445[d]	1–6	64 (dimer)	5FUJ 5FUK	(Gröbe *et al.*, 2011; Olmedo *et al.*, 2017; Ullrich *et al.*, 2018)
*Mwe*UPO	*Marasmius wettsteinii*	52	67	1–4	61 (dimer)	–	(Ullrich *et al.*, 2018)
*Pab*UPO	*Psathyrella aberdarensis*	48	3	3	40–41	–	(Hofrichter *et al.*, 2020; Kimani, 2019)

[a]number of predicted UPO genes in the genome of the production strain.
[b]If not otherwise stated in the manuscript, values were calculated using the maximum volume activity and the finally obtained specific activity with veratryl alcohol as substrate.
[c]PDB code.
[d]600 mg L^{-1} CPO were obtained after 80 days of culturing, while 445 mg L^{-1} *Mro*UPO were the result of a 24-day cultivation.

Applicability of UPOs

For the successful implementation of UPOs in any kind of application, the following general conditions must be met: enzyme availability, enzyme stability and gentle peroxide supply. The following subarticle deals with these conditions and points out existing shortcomings.

Wild-Type UPOs

CPO (that can be regarded as the first described UPO) was originally produced in aerated and stirred submerged cultures containing synthetic CZAPEK-DOX medium. Maximum activities (MCD units correspond to the chlorination of monochlorodimedone to dichlorodimedone (Hager *et al.*, 1966)) of 5000 U L^{-1} were achieved between days 6 and 7. This activity corresponded to approx. 4 mg L^{-1} CPO protein (Morris and Hager, 1966). At that time, the versatility of CPO had not been recognized and most studies dealt with chlorination reactions. Almost four decades later, the first "true" UPO could be produced with the agaric fungus *A. aegerita* (*Aae*UPO, also known as AaP or APO, see introduction) in a soybean-based complex medium (Ullrich *et al.*, 2004). The enzyme was present in three main isoforms, which were separable by several chromatographic steps (Ullrich *et al.*, 2009). Further screenings, over more than 15 years, used various complex media and led to the discovery of more basidiomycetous (agaric) UPOs, e.g., in the families of Strophariaceae (*Agrocybe parasitica* = *Cyclocybe parasitica*, unpublished result), Marasmiaceae (*M. rotula*, (Gröbe *et al.*, 2011), and *M. wettsteinii*, (Ullrich *et al.*, 2018)) and Psathyrellaceae (*Coprinellus radians* and *Coprinopsis verticillata*), (Anh *et al.*, 2007), and *Psathyrella aberdarensis* (Melzer *et al.*, 2018; Hofrichter *et al.*, 2020). The production of an ascomycetous wild-type UPO in appreciable amounts (apart from CPO) succeeded solely in the case of the "wallpaper mold"*Chaetomium globosum* (Kiebist *et al.*, 2017).

UPO concentrations secreted by fungi in production batches were found to vary between 2 and 600 mg L^{-1} (Anh *et al.*, 2007; Anh, 2008; Blanke *et al.*, 1989; Gröbe *et al.*, 2011; Pickard *et al.*, 1991; Ullrich *et al.*, 2004; **Table 2**). Studies carried out so far have suggested that UPOs of family I are secreted at higher levels by wild-type strains than UPOs of family II. On the other hand, efforts to optimize homologous UPO production have been performed with different intensity, hence there is surely still potential to improve it further. In this context, it is also important to mention that not only the total amount of UPO protein is important but rather the specific activity towards a particular substrate, which is a measure for the "enrichment" of UPO in the culture liquid. The complex media typically used for UPO production contain varying amounts of soybean components, peptones and/or simples sugars (glucose, fructose). Though research has focused on identifying substances and conditions that specifically stimulate UPO production, it is still unknown which mechanisms trigger it. Until now, eight wild-type UPOs have been purified and characterized. **Table 2** summarizes the respective data.

Recombinant UPOs

Besides wild-type UPOs, an increasing number of recombinant UPOs has been made available. Overall, UPOs from eight different species can be heterologously expressed in fungal and bacterial hosts (*Aspergillus* spp., *Saccharomyces cerevisiae*, *Pichia* (*Komagatella*) *pastoris*, *Escherichia coli*; **Table 3**). The Danish company Novozymes A/S was the first producing a recombinant UPO from the inkcap *Coprinopsis cinerea* in *Aspergillus oryzae* and tested it for synthetic applications (Babot *et al.*, 2013; Lund *et al.*, 2016). Later, they produced also recombinant UPOs from the ascomycetous fungus *Humicola insolens* in the same host (Kiebist *et al.*, 2017). CPO from *Caldariomyces fumago* was already successfully expressed in *Aspergillus niger* in 2001 (Conesa *et al.*, 2001).

Table 3 Survey on recombinant UPO with expression systems

Enzyme	*Donating organism*	*Expression system*	*Mutant/directed evolution*	*Mass (kDa)*	*Reference*
r*Aae*UPO/n-UPO1	*Agrocybe aegerita*	*Saccharomyces cerevisiae*	No	–	(Molina-Espeja *et al.*, 2014)
r*Aae*UPO/PaDa-I	*A. aegerita*	*S. cerevisiae*, *Pichia pastoris*	Yes	51.1	(Molina-Espeja *et al.*, 2015)
r*Aae*UPO/PaDa-I-Cys	*A. aegerita*	*S. cerevisiae*	Yes	–	(Molina-Espeja *et al.*, 2019)
r*Cci*UPO	*Coprinopsis cinerea*	*Aspergillus oryzae*	No	44	(Babot *et al.*, 2013; Lund *et al.*, 2016)
r*Cgl*UPO	*Chaetomium globosum*	*A. oryzae*	No	–	(Lund *et al.*, 2016)
r*Lfu*UPO	*Leptoxyphium fumago*	*Aspergillus niger*	No	45–50	(Conesa *et al.*, 2001)
r*Cvi*UPO	*Collariella virescens*	*A. oryzae*, *Escherichia coli*	No	–	(González-Benjumea *et al.*, 2020; Lund *et al.*, 2016)
r*Dca*UPO	*Daldinia caldariorum*	*A. oryzae*, *E. coli*	No	–	(Linde *et al.*, 2020; Lund *et al.*, 2016)
r*Hin*UPO	*Humicola insolens*	*A. oryzae*	No	–	(Kiebist *et al.*, 2017; Lund *et al.*, 2016)
r*Mfe*UPO	*Myceliophthora fergusii*	*A. oryzae*	No		(Lund *et al.*, 2016)
r*Mhi*UPO	*Myceliophthora hinnulea*	*A. oryzae*	No		(Lund *et al.*, 2016)
r*Thy*UPO	*Thielavia hyrcaniae*	*A. oryzae*	No		(Lund *et al.*, 2016)
r*Pvi*UPO	*Pestalotiopsis virgatula*	*A. oryzae*	No		(Lund *et al.*, 2016)

Miguel Alcalde and co-workers successfully applied directed evolution in *S. cerevisiae* to generate UPO-mutants with improved synthetic properties, i.e. decreased peroxidative activity (mutant Pada-I). Such variants that can be produced at larger scale in *P. pastoris* (Molina-Espeja *et al.*, 2014, 2015, 2017) are useful when the envisaged substrates or products bear phenolic groups, which would easily be oxidized via one-electron oxidation to form unwanted phenoxy radicals initiating coupling reactions. Moreover, the atom efficiency regarding H_2O_2 will increase, if less by-products are formed. This effect may be of particular interest when large-scale applications will be intended in the future.

Very recently, Martínez *et al.* have developed an expression system on the basis of *E. coli* as the host. They were able to express native and mutated UPOs from Ascomycota and Basidiomycota in this system (Carro *et al.*, 2019; Linde *et al.*, 2020). However, recombinant UPOs produced in bacteria are still limited in the obtained amounts and lacking glycosylation, which may affect physicochemical properties such as water solubility and pH stability. Therefore, for the time being, eukaryotic expression systems as that of *P. pastoris* represent the method of choice to produce UPOs (Cregg *et al.*, 2009). Nevertheless, UPO properties may also change when they are expressed in S. *cerevisiae* or *P. pastoris*. For example, the molecular mass of recombinant *Aae*UPO (Pada-I) increased from 45 to 46 kDa to 51–52 kDa due to over-glycosylation (**Tables 2** and **3**). Such different glycosylation patterns could even affect kinetic parameters, which was already demonstrated for CPO and fungal aryl alcohol oxidases (Karich *et al.*, 2018).

Despite all efforts in the field of heterologous expression, there is no commercial UPO preparation available at present. It is conceivable, however, that this bottleneck will be overcome in the near future by further improving existing expression systems and by developing new expression strategies, e.g., cell-free approaches based on isolated ribosomes (Thoring *et al.*, 2019).

pH and Temperature Stability

Most UPOs can be stored at neutral pH over long periods. For example, purified *Aae*UPO dissolved in phosphate buffer (pH 7.0) and stored at 4°C has a calculated half-life of 7.3 years, which can be further extended to presumed 10.5 years and 11.1 years in the presence polyethylene glycol PEG 600 and PEG 20.0000, respectively (unpublished results). Frozen in appropriate buffers, UPOs are stable over years as well without the need of adding additional stabilizers. Even at room temperature and pH 7.0, and under reaction conditions (HMF conversion), UPOs remained stable for at least one week (Carro *et al.*, 2015). Under alkaline conditions (at pH 10.0), however, some UPOs lost their activity within minutes, e.g., *Cra*UPO and *Pab*UPO I, whereas others, e.g., *Pab*UPO II and III, remained active over several hours/days (able 2) (Anh, 2008; Kimani, 2019). Vice versa, acidic pH (3.0–5.0), was also found to negatively influence the enzyme activity, which resulted in the case of *Cgl*UPO, in a half-life of only 240 min at room temperature and a decrease in the Soret band at 420 nm (Kiebist *et al.*, 2017).

UPO Inactivation by Peroxide

Inactivation of UPOs by its co-substrate hydrogen peroxide is directly related to their intrinsic catalase side-activity, and thus dependent on the peroxide concentration in their immediate environment (reaction medium). It was shown that the pH optima of this catalase activity (*Aae*UPO, *Mro*UPO, r*Cci*UPO and CPO) are in the same range as those of their actual peroxygenating activities (Karich *et al.*, 2016; Manoj and Hager, 2001). Above or below the pH optimum, UPO activity rapidly decreases in the presence of excess peroxide (Karich *et al.*, 2016; Park and Clark, 2006). On the other hand, it turned out that the remaining peroxygenating UPO activities were higher when high levels of catalase activity were accomplished, and hence, the "true catalase reaction" of UPOs seems to be a kind of protective mechanism ("relief valve") to prevent them from inactivation (Karich *et al.*, 2016). Peroxide inactivation is thought to proceed via the formation of UPO compound III

(Cpd-III, a superoxide-heme complex), which may appear when compound II (Cpd-II) reacts with additional peroxide. The latter can emerge, out of the ordinary peroxygenase cycle (compare **Fig. 4**), after one-electron oxidation of a peroxide molecule catalyzed by compound I (Cpd-I), analogously to the action of heme-catalases (Alfonso-Prieto *et al.*, 2009; Karich *et al.*, 2016; Vidossich and Magistrato, 2014). Cpd-III in turn may react with more peroxide to form highly reactive hydroxyl radicals (HO•), which in turn oxidize the heme to form verdoheme (Karich *et al.*, 2016). This over-all mechanism is similar to the irreversible inactivation of class-II and class-III heme peroxidases (Vlasits *et al.*, 2010).

Actually, a "real substrate" (e.g., a C-H to be hydroxylated) will always compete with another peroxide (HOO-H) for Cpd-I (Karich *et al.*, 2016). In consequence, when the local/momentary peroxide concentration is kept as low as possible – being preferably even rate limiting (or at least a magnitude lower than the substrate concentration) – UPOs can be effectively prevented from acting in the catalase mode and hence from inactivation.

Peroxide Supply

Continuous slow addition of the co-substrate H_2O_2 (or organic equivalents, R-OOH) is the most effective way to counteract inactivation of UPOs (Karich *et al.*, 2017; Ullrich *et al.*, 2008; van Deurzen *et al.*, 1997b; Ullrich *et al.*, 2004). There are four general approaches to realize gentle peroxide supply: (1) 'mechanical' *ex situ* peroxide supply, (2) enzymatic in situ generation, (3) electrochemical in situ generation and (4) photochemical in situ generation (**Fig. 8**; Burek *et al.*, 2019; Karich *et al.*, 2018; Krieg *et al.*, 2011; Ullrich *et al.*, 2004). Each method has its own advantages and disadvantages that are summarized in **Table 4**.

The simplest way to feed peroxide to an ongoing UPO reaction is the supply via pipettes (repeatedly) or pump systems, e.g., syringe pumps (**Fig. 8(a)–(c)**). These methods are easy to realize, ensure fast dosing and provide high flexibility regarding the H_2O_2-concentration (Aranda *et al.*, 2009; Karich *et al.*, 2017; Ullrich *et al.*, 2004; Ullrich and Hofrichter, 2005; Ullrich *et al.*, 2008). However, the reaction volume itself limits the maximum amount of H_2O_2 that can be added. Another inevitable disadvantage consists in the basic reaction design, i.e., the local peroxide concentration in the reaction system is always highest at the point of peroxide injection where the enzyme is going to be partially destroyed (Karich *et al.*, 2016). This negative effect can be mitigated when the co-substrate level is kept at a concentration that does not harm UPO, e.g., by using a H_2O_2-sensor (van Deurzen *et al.*, 1997b).

In situ generation of the co-substrate is without doubt the best way to prevent too high local peroxide concentrations in reaction systems (Krieg *et al.*, 2011; Pereira *et al.*, 2015; Perez *et al.*, 2009). Enzymatic approaches are very efficient in generating H_2O_2, since they form peroxide equally distributed in the whole reaction space (Bankar *et al.*, 2009; Pereira *et al.*, 2015; Pesic *et al.*, 2018). Usually, oxidases are used and combined with appropriate substrates (e.g., glucose oxidase/EC 1.1.3.4 and glucose) to generate one molecule of H_2O_2 during the oxidation of one substrate molecule (Bankar *et al.*, 2009). Such a "simple" method, however, has the disadvantage of accumulating additional products (in the above case, for example, gluconolactone and gluconic acid) (Pereira *et al.*, 2015). When oxidases and UPOs share the same substrate(s), intelligent enzymatic cascade reactions may be established, as it has been the demonstrated by the stepwise oxidation of hydroxymethylfurfural (HMF) to furandicarboxylic acid (FDCA) by UPO and aryl alcohol oxidase (AAO/EC 1.1.3.7) (Karich *et al.*, 2018). Establishing such a cascade reaction requires the detailed analysis of each reaction step catalyzed by the single enzymes involved. In the case of oxidases, the performance is often limited by the availability of dioxygen (O_2) in the reaction medium. Nevertheless, such intelligent cascade approaches have several

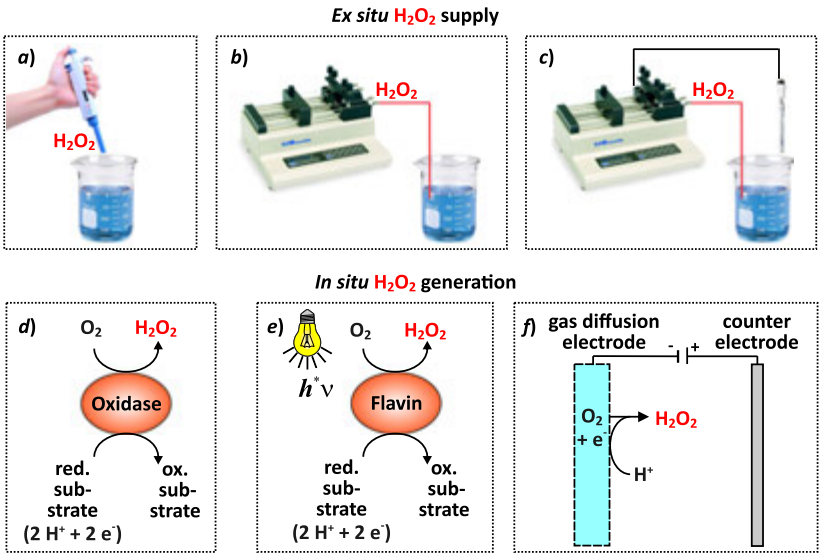

Fig. 8 Six different methods of peroxide (H_2O_2) supply in UPO reactions, *ex situ*: (a) stepwise addition by pipette, (b) syringe pump system, (c) syringe pump controlled by peroxide-sensitive electrode, and in situ: (d) H_2O_2 generation by an oxidase, (e) generation by illuminated flavins and (f) electrochemical H_2O_2 generation by a gas diffusion electrode.

Table 4 Comparison of different methods to supply UPOs with hydrogen peroxide (H_2O_2)

Method	*Equipment*	*Advantage*	*Disadvantage*	*Ref.*
Ex situ supply	Pipette	Robust and fast dosing; volume steps easily adjustable; easy handling	High local H_2O_2 concentration at the point of entry/ in-jection; limited maximum H_2O_2 amounts depending on the total volume	(Ullrich *et al.*, 2008; Ullrich and Hofrichter, 2005; Ullrich *et al.*, 2004)
Ex situ supply	(Syringe) pump	Accurate fine dosing, continuous dosing	High local H_2O_2 conc. at the point of entry; limited maximum H_2O_2 amounts depending on the total volume	(Aranda *et al.*, 2010; Karich *et al.*, 2017)
Sensor controlled *ex situ* supply	H_2O_2-sensor controlled pump (H_2O_2-stat)	Optimal H_2O_2-dosage for each reaction; technically applicable (at large scale)	Limited availability and high price of H_2O_2-sensors	(van Deurzen *et al.*, 1997b)
Photochemical in situ generation	Solely photochemical or in combination with enzymatic reactions (flavins)	Accurate fine dosing; inexpensive (if once established)	Limited availability of photochemical equipment; sluggish reaction kinetics; accumulation of by-products; O_2-limitation	(Perez *et al.*, 2009; Willot *et al.*, 2019; Zhang *et al.*, 2017, 2018)
Electrochemical in situ generation	Gas diffusion electrode	Accurate fine dosing; almost perfect distribution of H_2O_2	Limited availability of electrochemical equipment; tricky handling	(Getrey *et al.*, 2014; Horst *et al.*, 2016; Krieg *et al.*, 2011)
Enzymatic in situ generation I	Glucose oxidase	Accurate fine dosing; inexpensive; easy handling and availability	Poor atom-efficiency, accumulation of by-products (glucono-lactone); O_2-limitation	(Bankar *et al.*, 2009; Pereira *et al.*, 2015)
Enzymatic in situ generation –cascade reaction II	Different oxidoreductases (enzyme cocktail)	Accurate fine dosing, simple handling; co-solvent acts as substrate for the cascade; final by-product CO_2	Non-commercial enzymes; requires NAD^+ and additional oxidases; O_2-limitation	(Ni *et al.*, 2016; Pesic *et al.*, 2018)
Enzymatic in situ generation –cascade reaction III	Different oxidases (e.g., aryl alcohol oxidase, galactose oxidase)	Accurate fine dosing; simple handling, UPO utilizes H_2O_2 produced by oxidases, no by-products/waste	Non-commercial enzymes; requires additional oxidases; O_2-limitation	(Karich *et al.*, 2018)

advantages, e.g., inexpensive reaction design, no need of additional co-substrates, no by-products, optimal fine dosing and completeness of oxidation.

Other approaches have combined more than two peroxide-supplying enzymes and UPO, such as alcohol oxidase from *P. pastoris* (EC 1.1.3.13), formaldehyde dismutase from *Pseudomonas putida* (E.C.1.2.99.4), formate dehydrogenase from *Candida boidinii* (EC 1.2.1.2) and 3-hydroxybenzoate 6-hydroxylase from *Rhodococcus jostii* (EC.1.14.13.24). This advanced system generates three equivalents of H_2O_2 from one methanol molecule and was successfully tested in the UPO-catalyzed conversion of ethylbenzene into enantiopure *(R)*-1-phenylethanol (Fernández-Fueyo *et al.*, 2016; Ni *et al.*, 2016; Pesic *et al.*, 2018). This setup will be especially smart, if methanol is the co-solvent to enhance ethylbenzene solubility (Ni *et al.*, 2016).

Besides the enzymatic in situ generation of peroxide, two technical approaches using electrochemical or photochemical equipment have successfully been tested in oxyfunctionalization trails with *Aae*UPO (Fernández-Fueyo *et al.*, 2016; Horst *et al.*, 2016; Krieg *et al.*, 2011; Zhang *et al.*, 2017, 2018). The photochemical setup allowed the direct control of H_2O_2 generation by light intensity but required isolated flavins and reduced organic compounds for peroxide generation (Zhang *et al.*, 2018, 2017). Electro-enzymatic hydroxylation of ethylbenzene with r*Aae*UPO used electrodes to generate H_2O_2 by reduction of oxygen and facilitated total turnover numbers of up to 400,000 (Horst *et al.*, 2016). The use of electrodes to fuel UPOs may be particularly attractive in pharmaceutical synthesis and red biotechnology, since it fully prevents the formation of undesired by-products during peroxide generation.

Immobilization

One strategy to decrease the costs for enzymes is to improve their recyclability (Gao *et al.*, 2019). Immobilization is the mostly used method to achieve this. In the case of UPOs, encapsulation of the protein (*Aae*UPO, *Mro*UPO) in polymeric beads ("enzyme pearls") was demonstrated. The bead matrix consisted of cross-linked polyvinyl acetate and/or polyethylene glycol forming a three-dimensional gel network where the enzyme molecules were entrapped (Poraj-Kobielska *et al.*, 2015). A second approach reported by the same authors used hollow fiber modules that retained the UPO protein by a defined membrane system. Both protein fixation methods, however, do not represent "true" immobilization techniques, since the UPO protein was not covalently bound to the matrix. In consequence leaching effects may adversely affect enzyme performance.

Covalent immobilization of CPO using various chemical and enzymatic coupling methods has been intensely studied over several decades. In all cases, however, immobilization was accompanied by partial enzyme inactivation (Gao *et al.*, 2019; Kadima and Pickard, 1990) and there have been only few studies that reported a comparable performance of immobilized CPO compared to free enzyme (Petri *et al.*, 2004). Not much has been reported on the covalent immobilization of UPOs. Only very recently, it was

Table 5 Overview on reactions catalyzed by UPOs including exemplary substrates and particular UPOs involved

Aliphatic hydroxylation		
Substrates	*UPOs tested*	*References*
alkanes (e.g., propane, *n*-hexane, cyclohexane, cyclooctane, etc.); fatty acids (e.g., arachidic acid, oleic acid, etc.); aliphatic side chains/alkyls (e.g., toluene, ethylbenzene, etc.); steroids (e.g., cholesterol, testosterone, etc.); pharmaceuticals (e.g., propranolol, diclofenac, ibuprofen, etc.); sugar-based substrates (e.g., hydroxymethylfurfural)	*Aae*UPO; *Mro*UPO; r*Cci*Upo; *Cgl*UPO; *Cra*UPO	(Aranda *et al.*, 2018a; Babot *et al.*, 2015, 2013; Gutiérrez *et al.*, 2011; Karich *et al.*, 2018; Kiebist *et al.*, 2017; Kluge *et al.*, 2012; Peter, 2013; Peter *et al.*, 2011; Poraj-Kobielska *et al.*, 2011; Ullrich and Hofrichter, 2005)
C-C bond cleavage – a special version of aliphatic hydroxylation		
Substrates	*UPOs tested*	*References*
steroid side chain removal (e.g., cortisone, prednisone, etc.); α-oxidation of fatty acids, shortening (e.g., tetradecanedioic acid)	*Mro*UPO; *Mwe*UPO	(Olmedo *et al.*, 2017; Ullrich *et al.*, 2018)
O-Dealkylation – a special case of aliphatic hydroxylation		
Substrates	*UPOs tested*	*References*
ethers (e.g., anisole, 4-nitroanisole, 1,4-dioxane, diethylether etc.); non-phenolic lignin model dimers; isoflavones (e.g., formononetine); pharmaceuticals and drugs (e.g., naproxene, phenacetine, 4-nitro-benzodioxole, MDMA, etc.)	*Aae*UPO; *Cra*UPO	(Barková *et al.*, 2011; Kinne, 2010; Kinne *et al.*, 2009b, 2011; Poraj-Kobielska, 2013; Poraj-Kobielska *et al.*, 2011)
N-Dealkylation – a special case of aliphatic hydroxylation		
Substrates	*UPOs tested*	*References*
pharmaceuticals (e.g., tamoxifen, cocaine, morphine, flurazepam, imipramine, etc.); secondary amines (e.g., *N*-methylaniline)	*AaeUPO; Mro*UPO; *Cra*UPO	(Anh, 2008; Kinne, 2010; Poraj-Kobielska, 2013; Poraj-Kobielska *et al.*, 2011)
Epoxidation of (aliphatic) double bonds		
Substrates	*UPOs tested*	*References*
alkenes (e.g., propene, butane, trans-2-butene, cyclohexadiene, isoprene, etc.); terpenes (e.g., limonene); unsaturated fatty acids (e.g., oleic acid, linoleic acid, etc.); unsaturated aliphatic side chains (e.g., styrene, *trans*-stilbene, etc.)	*Aae*UPO; *Mro*UPO; r*Cci*UPO; *Cgl*UPO	(Aranda *et al.*, 2018a,b; Babot *et al.*, 2013; Gutiérrez *et al.*, 2011; Kluge *et al.*, 2012; Peter, 2013)
Aromatic hydroxylation – a special case of epoxidation		
Substrates	*UPOs tested*	*References*
monoarenes (e.g., benzene, chlorobenzene, toluene, anisole, 2,4-dinitrotoluene, etc.); PAH (e.g., naphthalene, benzo[*a*]pyrene, fluorene, etc.); propranolol; phenolic compounds (e.g., phenol, chlorophenol, nitrophenol, 4,6-dinitro-*o*-cresol, *p*-chloro-*m*-cresole, etc.)	*Aae*UPO; *Mro*UPO; *Cgl*UPO; *Cra*UPO	(Aranda *et al.*, 2010; Karich *et al.*, 2013, 2017; Kluge *et al.*, 2009; Kinne *et al.*, 2009a)
Aromatic dehalogenation - a special case of aromatic hydroxylation		
Substrates	*UPOs tested*	*References*
chlorinated phenolics (e.g., 4-chlorophenol, *p*-chloro-*m*-cresole, 2,4,6-trichlorophenol, etc.)	*Aae*UPO	(Karich *et al.*, 2017)
Sulfoxidation		
Substrates	*UPOs tested*	*References*
arenes with heteroatoms (e.g., dibenzothiophene, thioanisole, alkylarylsulfide, propylphenylsulfide, etc.)	*Aae*UPO; *Cra*UPO; *Cfu*PO	(Aranda *et al.*, 2009; Bassanini *et al.*, 2017; Horn, 2009; van Deurzen *et al.*, 1997a)
N-Oxidation		
Substrates	*UPOs tested*	*References*
arenes with heteroatoms (e.g., pyridine, 2-chloropyridine, 3-nitropyridine, etc.)	*Aae*UPO	(Ullrich *et al.*, 2008)
Halogenation		
Substrates	*UPOs tested*	*References*
phenol; monochlorodimedone	*Aae*UPO; *Cra*UPO; *Cfu*PO	(Anh, 2008; Anh *et al.*, 2007; Hager *et al.*, 1966; Ullrich *et al.*, 2004)
Catalase-like reaction		
Substrate	*UPOs tested*	*References*
H_2O_2	*Aae*UPO; *Mro*UPO; r*Cci*UPO; *Mwe*UPO[a]; *Cra*UPO[a]; *Cgl*UPO[a]	(Karich *et al.*, 2016)[a] unpublished

(*Continued*)

Table 5 Continued

Phenol oxidation – one-electron oxidation		
Substrates	*UPOs tested*	*References*
phenolic compounds (e.g., phenol, 2,4-dimethylphenol, hydroquinone, etc.); other peroxidase substrates such as ABTS	*Aae*UPO; *Mro*UPO; *Mwe*UPO; *Cra*UPO; *Cgl*UPO; r*Cci*UPO	(Anh, 2008; Gröbe *et al.*, 2011; Karich *et al.*, 2016; Kiebist *et al.*, 2017; Ullrich *et al.*, 2004, 2018)

[a]Refers to unpublished data.

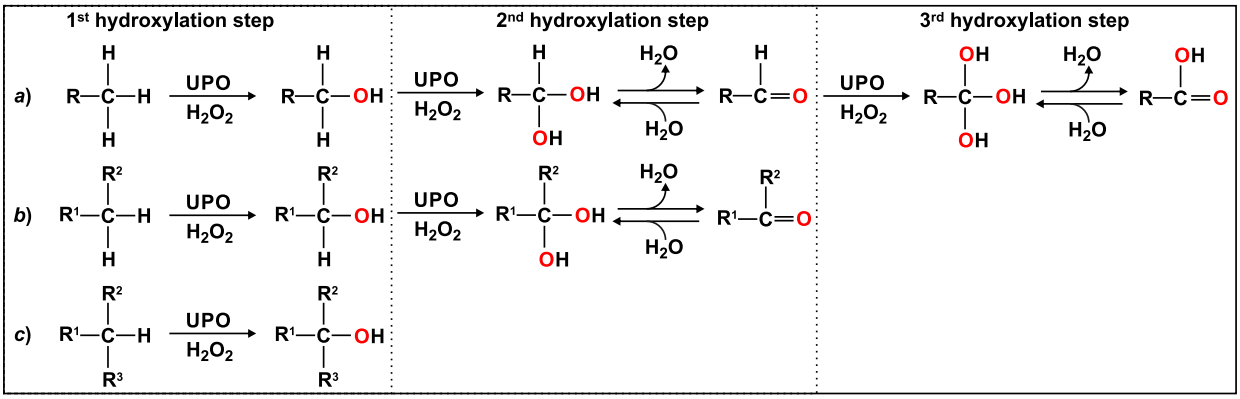

Fig. 9 Stepwise hydroxylation of *sp3*-hybridized carbons catalyzed by UPOs in dependence of abstractable hydrogen (C-H). UPOs oxidize the primary carbon of a methyl group (a) via corresponding primary alcohol and aldehyde derivatives into a carboxylic acid. Secondary carbon (b) is oxidized via a secondary alcohol into a ketone, and tertiary carbon (c) is hydroxylated to a tertiary alcohol. Aldehydes, ketones and carboxylic acids are shown in equilibrium with their corresponding hydrates.

demonstrated that UPO mutants (r*Aae*UPO) can be efficiently immobilized by the DUCI-technique (directed unique-point covalent immobilization). This structure-guided method is based on the engineering of Cys^{+}-mutants, the additional cysteine of which, at the protein surface, selectively interacts with the immobilization-carrier via a single disulfide bridge that may even ensure a controlled orientation of the enzyme (Molina-Espeja *et al.*, 2019). Such UPO mutants and the DUCI-technique could be the method of choice when industrial applications of UPOs in the pharmaceutical or fine chemical sector will be envisaged.

Reactions Catalyzed by UPOs

UPOs catalyze diverse oxyfunctionalization reactions including hydroxylations of sp3-carbon and epoxidations of sp2-carbon, some of which may undergo following reactions such as dealkylation or re-aromatization. Organic heteroatoms such as sulfur and nitrogen are subject of oxygen transfers as well, and inorganic halides are peroxygenated to hypohalites, which in turn can halogenate other molecules. This subarticle will give a concise overview on some of these reactions that are also summarized in **Table 5**.

Carbon Oxygenations

Only some UPOs (including CPO) have been demonstrated to catalyze a huge number of oxygenations of carbon atoms, which is reflected by the number of original papers (compare **Tables 2–5**) and review articles that have been published in the last two decades (Bormann *et al.*, 2015; Dembitsky, 2003; Hofrichter and Ullrich, 2006, 2011, 2014; Hofrichter *et al.*, 2015, 2020; Kiebist *et al.*, 2019; Manoj and Hager, 2008; Ullrich and Hofrichter, 2007; Wang *et al.*, 2017). Here, we focus on hydroxylations of sp3-hybridized carbons as well as on epoxidations of sp2-hybridized carbons. This includes the selective conversion of pharmaceuticals and the rather unspecific conversion of environmental pollutants. Furthermore, we discuss some peroxygenations of some naturally and physiologically important substances.

Hydroxylations (sp3)

In general, the UPO-mediated stepwise oxidation of carbon starts with the insertion of an oxygen leading to the corresponding alcohol. In the case of primary and secondary alcohols, a further hydroxylation may take place at the same carbon resulting in the formation of the corresponding aldehydes or ketones (The second peroxygenation of a carbon already bearing an alcohol functionality is also known as over-oxidation (Campestrini *et al.*, 2004; Holtmann *et al.*, 2014)), which are in equilibrium with their hydrates (*gem*-diols). Finally, aldehydes can undergo a third peroxygenation to the corresponding carboxylic acids (**Figs. 9** and **10**). These reactions strongly depend on the reaction conditions, among which the ratio between substrate and peroxide is most important. For example, Peter *et al.* (2011)

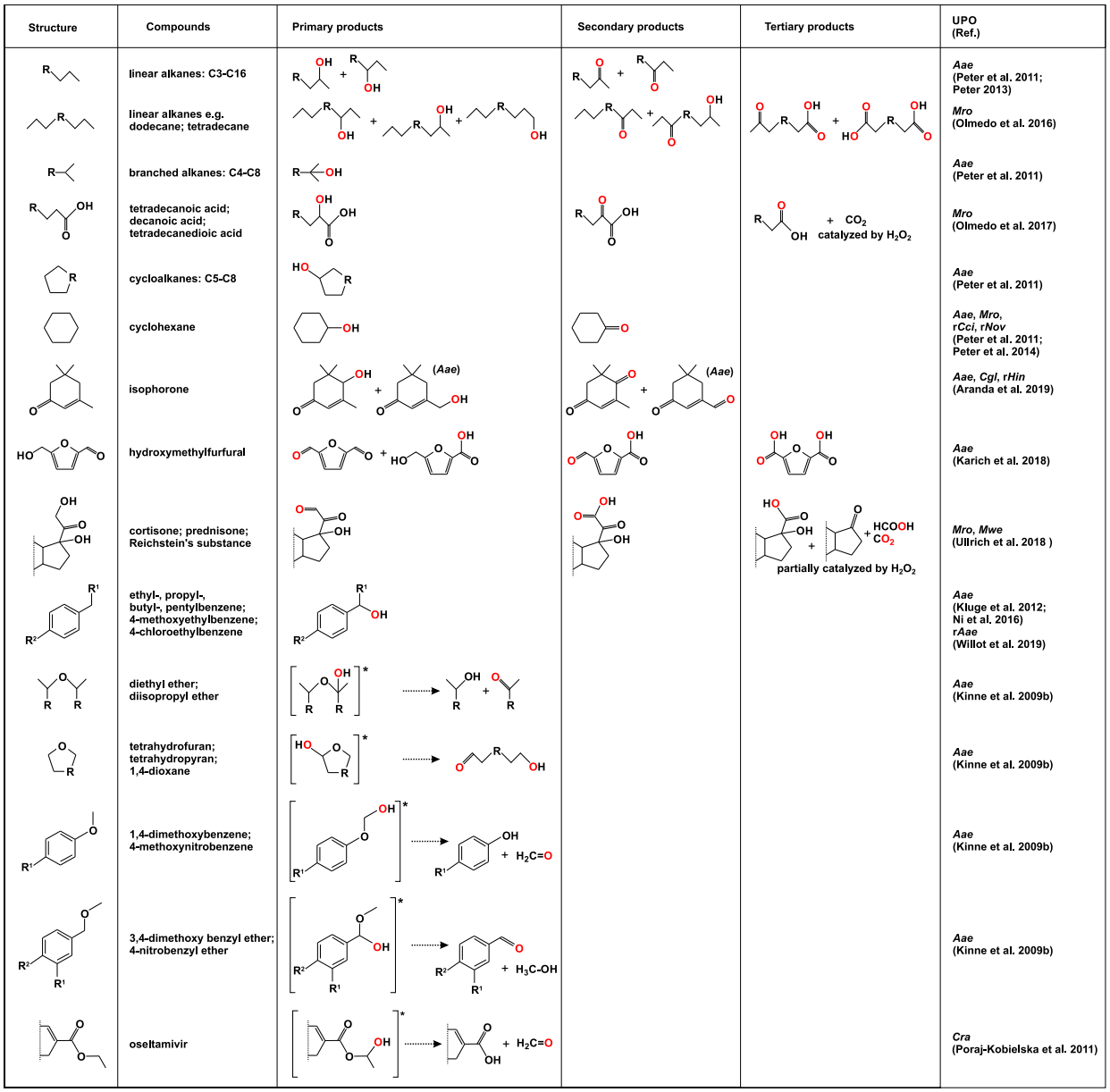

Fig. 10 Selected hydroxylations of *sp3*-hybridized carbons catalyzed by UPOs with possible consecutive reactions to secondary and tertiary products. Abbreviations of UPO-producing fungi: *Aae – Agrocybe aegerita* (r*Aae* – recombinant protein), *Cgl – Chaetomium globosum, Cra – Coprinellus radians, Mro – Marasmius rotula, Mwe – Marasmius wettsteinii,* r*Cci – Coprinopsis cinerea* (recombinant protein), r*Hin – Humicola insolens* (recombinant protein), r*Nov* – recombinant protein from Novozymes A/S of an unidentified soil mold. * Indicates unstable hemiacetal intermediates (O-C-OH) that spontaneously hydrolyze.

used an equimolar amount of peroxide in relation to the substrate concentration in the peroxygenation of alkanes with *Aae*UPO, which resulted mainly in formation of alcohols. In contrast Olmedo *et al.* (2016) used an 8- to 25-fold excess of H_2O_2 leading to a mixture of alcohols, ketones and dicarboxylic acids. Thus, just an appropriate reaction design will give the desired oxidation products, which has always to be proven beforehand for each particular UPO and substrate.

All UPOs tested so far favored the peroxygenation at secondary carbons (over terminal carbons) leading to 2- or 3-hydroxylated products. In case of *Aae*UPO, enantioselectivity for linear C4-C8 chain length was shown, starting with *S* (30%–36% enantiomeric excess = ee) from *n*-butane to *n*-pentane for 2–OH, changing to *R*- enantiomers with 50%–62% ee and 80%–100% ee from C6-C8 for 2-OH and 3-OH, respectively (Peter *et al.*, 2011). Notably, *Mro*UPO oxygenated the terminal carbon (that is unfavored due to its high C–H bond dissociation energy) of long-chain alkanes (C12-C14) when higher concentrations of H_2O_2 were applied (7–25-fold compared to *n*-alkane concentration). This led to mixtures of alcohols, ketones and acids including biotechnologically relevant dicarboxylic acids (Olmedo *et al.*, 2016).

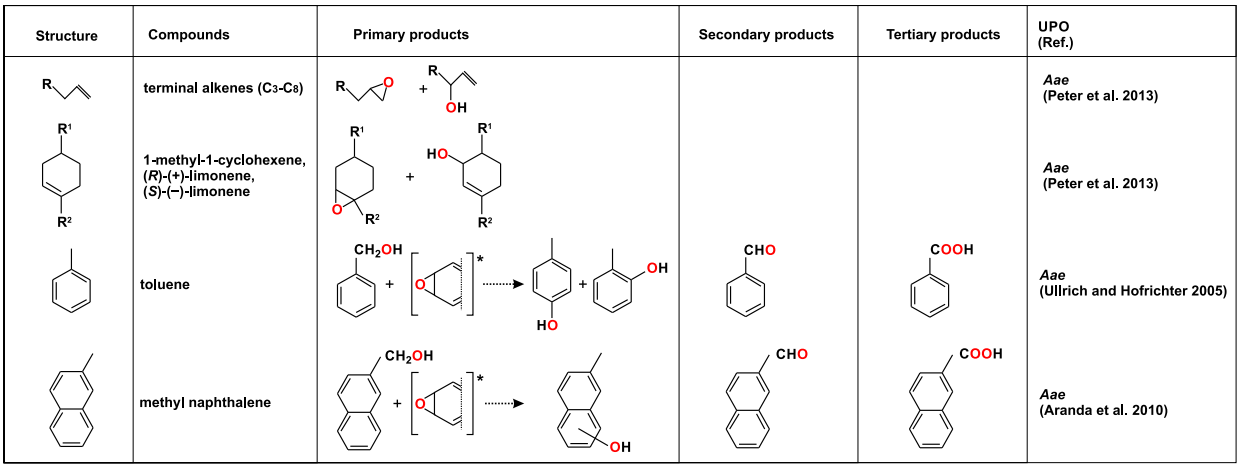

Fig. 11 Selected UPO-catalyzed hydroxylations and epoxidations of *sp3*- and *sp2*-hybridized carbons, respectively, with possible consecutive reactions to secondary and tertiary products. Note, both carbon types (*sp3*, *sp2*) are present in the same molecules. * Indicates unstable epoxide intermediates rearranging via re-aromatization and formation of phenolic products.

The peroxygenation of cyclic alkanes by *Aae*UPO was shown for C5-C8 rings yielding mainly the corresponding cycloalkanols (Peter *et al.*, 2011). With the intention to prepare cyclohexanol and cyclohexanone, four UPOs (r*Hin*UPO, *Aae*UPO, *Mro*UPO, r*Cci*UPO) were tested, among which *Mro*UPO productively utilized 87% of the added H_2O_2 to form the envisaged products with high yield (Peter *et al.*, 2014). Terminal and secondary benzylic carbons (R-CH_3, R-CH_2-alkyl), which are particularly favored targets because of the activating effects of the phenyl moiety, were oxidized either to benzyl alcohol, benzaldehyde and benzoic acid derivatives or to the corresponding (R)-1-phenyl alkanols and to smaller amounts of respective ketones (Kinne *et al.*, 2010; Fernández-Fueyo *et al.*, 2016; **Figs. 10** and **11**). The conversion of linear and branched alkanes proceeded with some regioselectivity typically yielding 2- and 3-alkanols and again small amounts of ketones. With regard to fatty acids, these positions correspond to ω-1 and ω-2, respectively. Gutiérrez *et al.* (2011) detected traces of other hydroxylation products at ω, ω-3 and ω-5 positions in stopped-flow experiments with 3,3-dimethylbutanoic acid as substrate. Just lately, Olmedo *et al.* (2017) demonstrated that *Mro*UPO catalyzes the shortening of fatty acids. This interesting reaction sequence requires the incipient peroxygenation of the C-α position, which is further oxidized to a *gem*-diol derivative that is in equilibrium with the α-keto fatty acid. A final spontaneous oxidation that may be forced by peroxide yields the decarboxylated fatty acid. By analogy with well-known β-oxidation, this route could be assigned as the α-oxidation of fatty acids. Similar consecutive peroxygenations and spontaneous oxidation were proposed for the side-chain removal from corticosteroids (Ullrich *et al.*, 2018).

The scission of ethers (*O*-dealkylation) is another reaction that is initiated by the peroxygenation of a carbon, in this case of the ether bond's adjacent methylene (R-*O*-CH_2-R′) or methyl (R-*O*-CH_3) groups. Kinne *et al.* (2009b) first demonstrated this reaction type using *Aae*UPO and diverse ether substrates including linear, branched and cyclic compounds (e.g., diethyl ether, diisopropyl ether, tetrahydrofuran). Upon hydroxylation, an unstable hemiacetal is formed (e.g., R-*O*-CH_2-*O*H-R′) that spontaneously cleaves into an alcohol (R-*O*H) and an aldehyde ($O{=}CH_2$-R′), in which the alcohol bears the oxygen of the original ether (the carbonyl is the result of water addition). Analogously UPOs perform *N*-dealkylations via hemiaminal intermediates. Among others, this was demonstrated for some pharmaceuticals and alkyl anilines using *Aae*UPO and CPO, respectively (compare **Table 5**) (Corbett *et al.*, 1979, 1978; Corbett and Chipko, 1979; Kinne *et al.*, 2010; Poraj-Kobielska *et al.*, 2011). An ester scission as special case of *O*-dealkylation was exclusively reported for the reaction of *Cra*UPO with the antiviral drug osaltemivir (Poraj-Kobielska *et al.*, 2011).

Epoxidations (sp2)

Benzene represents the simplest arene molecule and may serve as an example for the conversion of aromatic substrates by UPOs. Only *Aae*UPO was found to peroxygenate this inactivated sp2 system in substantial amounts (Karich *et al.*, 2013). The major products observed were phenol (hydroxybenzene) and dihydroxybenzenes (catechol, hydroquinone) along with smaller amounts trihydroxybenzenes. UPOs of family II (*Aae*UPO and *Cra*UPO to some extent) seemingly favor the reaction with the unsubstituted monoarene ring, while members of family II like *Mro*UPO hardly oxidize benzene. As assumed for all "aromatic hydroxylations", the reaction proceeds via an initial epoxidation to the corresponding arene oxide (epoxyarene), which is in equilibrium with the corresponding oxepine (Karich *et al.*, 2013; **Fig. 12**). Driven by the force of re-aromatization, an isomerization to the corresponding phenol follows (Karich *et al.*, 2013; Kluge *et al.*, 2009). This type of oxyfunctionalization of aromatic carbons was shown for various substituted benzenes, naphthalene and polycyclic aromatic hydrocarbons (PAH) (Aranda *et al.*, 2010; Karich *et al.*, 2017; Kluge *et al.*, 2009; Kinne *et al.*, 2010, 2009a).

The peroxygenation of alkenes (aliphatic, cyclic and benzylic derivatives) analogously results in the formation of epoxide products. However, due to a lacking driving force, i.e., re-aromatization, the rearrangement of these epoxides is not promoted and they remain relatively stable (Lin *et al.*, 2011; Peter, 2013). Epoxidation of aliphatic sp2 carbons was proven to be catalyzed by all

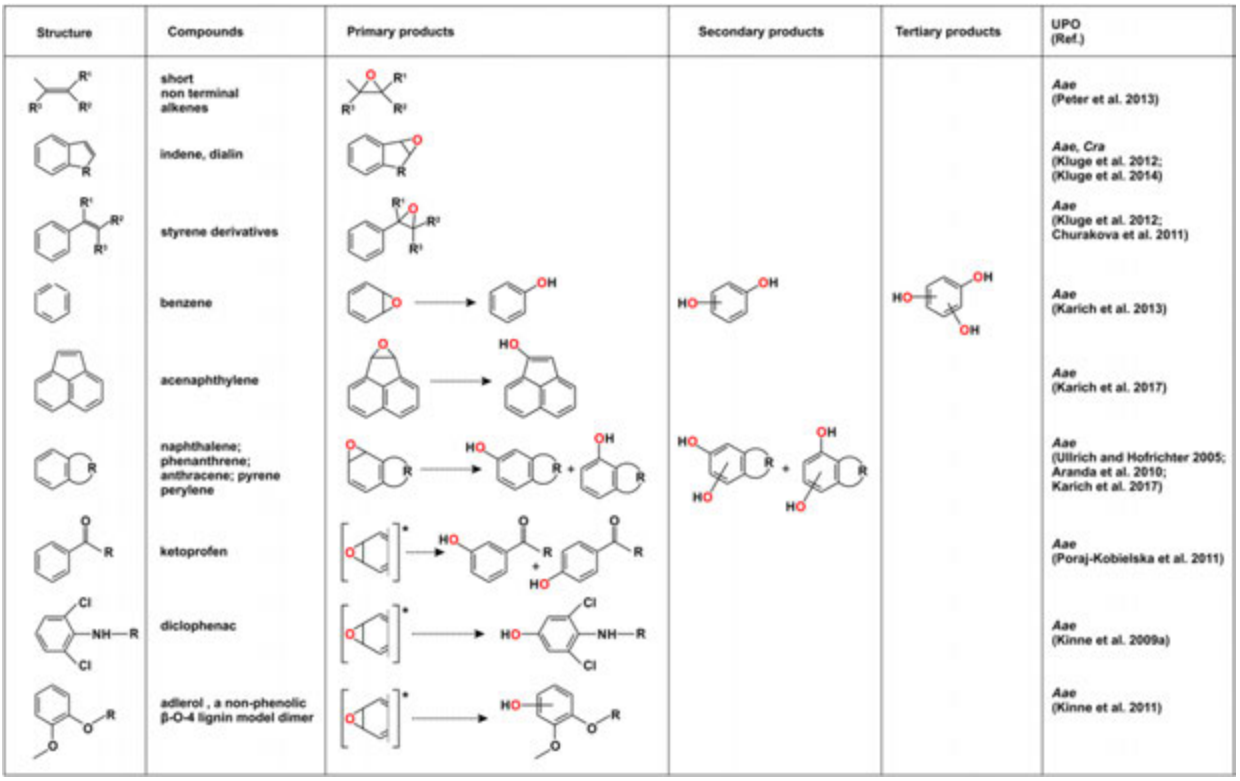

Fig. 12 Selected epoxidations of *sp2*-hybridized carbons by UPOs with possible consecutive reactions to secondary and tertiary products. For abbreviations of fungal species names, see **Fig. 10**. * Indicates unstable epoxide intermediates rearranging via re-aromatization and formation of phenolic products.

known UPOs with a high diversity of substrates, e.g., aliphatic alkenes, unsaturated fatty acids and cyclic alkenes including complex terpenes and steroids (Aranda *et al.*, 2018a, 2019; Kiebist *et al.*, 2017; Peter, 2013; Peter *et al.*, 2013; **Figs. 11** and **12**).

Alkynes (sp1)

The conversion of 1-octyne and 1-hexyne was merely shown for *Aae*UPO and led to the formation of 1-octyn-3-ol and 1-hexyn-3-ol, respectively, but the triple bond as such was not attacked. The reaction was inefficient and incomplete, which points to enzyme inactivation as in the case of P450s. Non-terminal alkynes, such as 2-hexyne or 3-hexyne, were not subject of conversion by *Aae*UPO (Peter, 2013). Probably trace amounts of highly reactive carbonyl alkenes are formed, which in turn react with the prosthetic heme of UPOs under formation of inactivating *N*-alkyls (Dexter and Hager, 1995; Peter, 2013). Similar findings were reported for the reaction of CPO with alkynes (Dembitsky, 2003).

Limitations

Limitations concerning the peroxygenation reactions of UPOs can be briefly summarized as follows: (1) steric hindrance, (2) the absence of abstractable hydrogen at the attacked carbon and (3) C–H bond dissociation energy (BDE). Unless the latter constraint, the other limitations strongly depend on the particular UPO and its structure.

Steric constraints that limit the substrate spectrum of UPOs are not surprising, since the oxygen insertion requires the direct contact of the substrate with the ferryl oxygen of the activated heme (Cpd-I). In the case of long UPOs and PAH, the current limit is given by perylene (5-ring molecule, $C_{20}H_{12}$) that was neither oxidized by *Aae*UPO nor by *Cra*UPO (Aranda *et al.*, 2010). Also, the large ring system of cyclodecane ($C_{10}H_{20}$) was not open to attack by *Aae*UPO (Peter, 2013). On the other hand, rather bulky steroids were converted depending on the substitution pattern of the sterane body. For example, cholesterol and campesterol were transformed with appreciable conversion rates (10%–100%) whereas only 2% of stigmasterol was oxidized by r*Cci*UPO, *Aae*UPO and *Mro*UPO (Babot *et al.*, 2015). 2,2,3,3-Tetramethylbutane may serve as an example of molecules that are not attacked by UPOs due to the lacking of abstractable hydrogens. The molecule contains six terminal carbons that cannot be oxidized because of the high bond energies, and the central two carbons are lacking abstractable hydrogens (Peter, 2013). Eventually, methane with its exceptional high C-H BDE of 105 kcal mol^{-1} was not converted by *Aae*UPO (Peter, 2013), which is in accordance with the BDE versus log *k* plot for *Aae*UPO Cpd-I (Wang *et al.*, 2012).

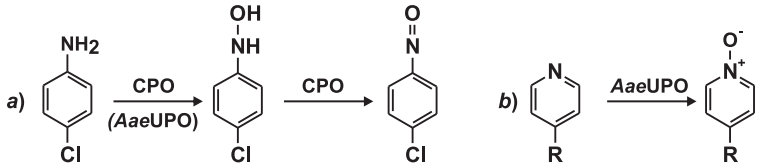

Fig. 13 *N*-oxygenations (*N*-oxidations) of (a) 4-chloroaniline via 4-chlorophenylhydroxylamine into 4-chloronitrosobenzene catalyzed by CPO (and *Aae*UPO) and of (b) pyridine derivatives by *Aae*UPO. According to Corbett, M.D., Chipko, B.R., Baden, D.G., 1978. Chloroperoxidase-catalysed oxidation of 4-chloroaniline to 4-chloronitrosobenzene. Biochemical Journal 175, 353–360. Kinne, M., 2010. The Extracellular Peroxygenase of the Agaric Fungus Agrocybe Aegerita: Catalytic Properties and Physiological Background With Particular Emphasis on Ether Cleavage. PhD thesis. International Graduate School of Zittau. Ullrich, R., Dolge, C., Kluge, M., Hofrichter, M., 2008. Pyridine as novel substrate for regioselective oxygenation with aromatic peroxygenase from Agrocybe aegerita. FEBS Letters 582, 4100–4106.

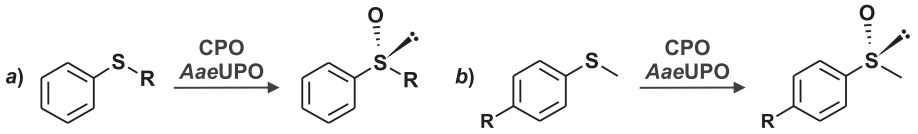

Fig. 14 Enantioselective *S*-oxygenations (sulfoxidations) of (a) aromatic thioethers (organic sulfides) and of (b) substituted thioanisoles catalyzed by CPO and *Aae*UPO. Reproduced from Bassanini, I., Ferrandi, E.E., Vanoni, M., Ottolina, G., Riva, S., *et al.*, 2017. Peroxygenase-catalyzed enantioselective sulfoxidations. European Journal of Organic Chemistry 2017, 7186–7189. Horn, A., 2009. Der Einsatz einer neuartigen Peroxidase aus dem Basidiomyceten Agrocybe aegerita am Beispiel der enantioselektiven Sulfoxidation. PhD thesis. University of Rostock. van Deurzen, M.P.J., Remkes, I.J., van Rantwijk, F., Sheldon, R.A., 1997a. Chloroperoxidase catalyzed oxidations in t-butyl alcohol/water mixtures. Journal of Molecular Catalysis A: Chemical 117, 329–337.

Oxygenation of Heteroatoms

Besides the oxygenation of C-atoms, UPOs were shown to peroxygenate organic heteroatoms, i.e., nitrogen and sulfur. UPOs will favor *N*-dealkylation over *N*-oxidation, if the nitrogen is substituted with aliphatic residues as discussed under 4.1. Otherwise, when the nitrogen is of aromatic or benzylic nature – as in pyridine or *N*-alkyl anilines – *N*-oxidation can be observed **Fig. 13** (Corbett *et al.*, 1978; Ullrich *et al.*, 2008). Pyridine and its derivatives are strongly inactivated substrates. Thus, it was not surprising that the product yields of pyridine *N*-oxides were poor in respective conversions with *Aae*UPO (Ullrich *et al.*, 2008). Interestingly, phenolic products were not observed. Maybe the heterocyclic nitrogen tends to coordinate towards the heme iron at the active site so that UPO attack favors it over carbons. *N*-oxidation of anilines was intensely studied with CPO (Corbett *et al.*, 1978; Corbett and Chipko, 1979; Corbett *et al.*, 1979), and was also observed in reactions of *Aae*UPO (Kinne, 2010). However, when comparing the oxidation of aniline and *N*-methyl-4-chloroaniline catalyzed by *Aae*UPO and CPO, respectively, it is not clear why no *N*-dealkylation product was found in the latter reaction (Kinne, 2010; Corbett and Chipko, 1979).

*Aae*UPO and CPO catalyze sulfoxidations and that of aryl-alkyl sulfides was studied in more detail, because of its intriguing enantioselectivity (Bassanini *et al.*, 2017; Colonna *et al.*, 1992; Horn, 2009; Kobayashi *et al.*, 1987). In all cases, the absolute configuration of the sulfoxides was (R) (Except in one case, where the absolute configuration was reverse due to a change in the substituent priority according to the sequence rules (Bassanini *et al.*, 2017)) and the enantiomeric excess ranged from 5% to >99% (**Fig. 14**). Whereas the product yield of the reaction was mostly higher with *Aae*UPO, the ee-values for CPO were in the same order of magnitude (Bassanini *et al.*, 2017). Besides aryl-alkyl sulfides, sulfoxidations were also observed for other substrates such as the pharmaceutical (omeprazole) and the petroleum constituent dibenzothiophene (Aranda *et al.*, 2009; Poraj-Kobielska *et al.*, 2011).

Halogenations

Chlorination reactions were first described for CPO in the early 1960s (Hager *et al.*, 1966). In the following decades, the catalytic mechanism was intensely studied (Libby *et al.*, 1982; Yamada *et al.*, 1985). CPO catalyzes a large number of chlorinations on various substrates including phenols, PAH, aromatic acids, unsaturated fatty acids and dienes (Vázquez-Duhalt *et al.*, 2001). In addition to chloride, bromide was accepted as halide source resulting in the bromination of diverse compounds (Yamada *et al.*, 1985). The mostly acknowledged reaction mechanism includes the formation of reactive hypohalites, which subsequently attack preferably sp2-hybridized carbons (Dembitsky, 2003). Hence, a well-established assay for CPO activity measurement involves monochlorodimedone (MCD) that is halogenated via hypohalite intermediates produced by the enzyme and by means of H_2O_2. The corresponding dihalogenated dimedone does not allow keto-enol tautomerism anymore, which is essential for MCD's specific UV–vis spectrum, and thus, leads to a decrease in absorbance at 290 nm (Hager *et al.*, 1966; Libby *et al.*, 1982). Unfortunately, this assay can give false positive results, caused by halogen-independent oxidation of MCD to so far unknown products (Wagner *et al.*, 2008). In addition to MCD, the halogenating activity was tested with phenol in presence of KCl or KBr. With that set-up, CPO showed high chlorinating activities yielding 4- and 2-chlorophenol as well as dichlorophenols as products. In contrast, *Aae*UPO,

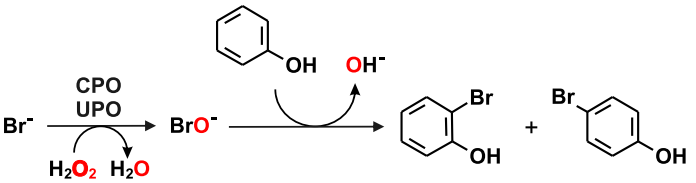

Fig. 15 Indirect bromination of phenol by CPO and *Aae*UPO resulting in mixtures of 2-bromophenol and 4-bromophenol. In the first step, bromide (Br^-) is enzymatically peroxygenated to hypobromite (BrO^-) and in the second step, hypobromite attacks the aromatic ring at the *ortho*- and *para*-positions.

*Cra*UPO and *Cve*UPO chlorinated phenol poorly and merely traces of 2-chlorophenol were detected. When bromide was provided, however, all four UPOs converted phenol into 2-bromo- and 4-bromophenol in the ratio of 1:4 (**Fig. 15**; Anh, 2008; Anh *et al.*, 2007; Ullrich and Hofrichter, 2005). Surprisingly, *Mro*UPO neither chlorinated nor brominated phenol (Gröbe *et al.*, 2011). There is obviously a high variation in the halogenation capabilities among UPOs. The molecular factors affecting the ability of UPOs to perform halogenation reactions are not well understood yet.

Conclusions and Outlook

Without doubt, UPOs belong to the most versatile enzymatic oxyfunctionalization tools and are becoming more and more relevant with respect to synthetic applications. On the one hand, their substrate spectrum is extremely broad, while on the other hand, the reactions catalyzed can be highly specific with regard to regio- and enantioselectivity. UPOs are rather stable biocatalysts, and if the peroxide is gently delivered, they will accomplish high process stability and reaction performance. These features define two major fields of (future) applications. The first one is related to the oxidation of low-value bulk chemicals and detoxification of a broad range of ecotoxicologically relevant substances, including drug residues found in municipal or pharmaceutical wastewater. For the application of UPOs in this field, the production costs of the enzymes to be applied must be as cheap as possible and they must be re-cyclable. In any case, it is recommended to optimize the envisaged reactions and the set-up regarding the supply and consumption of peroxide. The second field of application should focus on high-value products, for example, enantiopure pharmaceuticals, drug intermediates or fragrances. For this type of application, it will be important to find the UPO of choice that will catalyze the intended reaction regio- and/or enantioselectively with appreciable yields. So far, about merely twenty UPOs have been prepared and characterized, but in fact more than 4300 UPO encoding genes were identified in fungal genomes, indicating an amazing scientific and application potential for the future. A large natural pool of UPOs is available and simply waits for its exploitation. The main bottleneck that will have to be overcome is the production of UPOs at the kg and tons level. Only a global player such as Novozymes A/S may have the economic power and know-how to implement this at an appropriate scale. Besides the naturally "available" UPOs, strong potential also lies in the engineering UPOs, e.g., with regard to substrate spectrum, process stability, heterologous expression and directed evolution. Last but not least, it has to be mentioned in a UPO review again: Though we already know a lot about UPOs with regard to structure, genes, catalyzed reactions and application potential – our knowledge on their natural functions and roles in fungal physiology is rather scarce. They could be "an extracellular liver" of fungi, which helps them, as immotile organisms, to eliminate all kinds of toxins in their microenvironment or they may be part of the biochemical machinery of pathogenic fungi, which attack plant, fungal or animal hosts. Therefore, future UPO research will have to focus on more basic and ecological aspects of these fascinating enzymes, which surely will also have positive spin-off effects for applied research.

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Fungal Lytic Polysaccharide Monooxygenases (LPMOs): Biological Importance and Applications

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Introduction

For long, the enzymatic degradation of polysaccharides was thought to be exclusively catalyzed by the concerted action of multiple hydrolytic enzymes such as endo- and exo-acting cellulases. Many fungi, including the archetype cellulolytic ascomycete *Trichoderma reesei* (Gritzali and Brown, 1979), indeed produce a wide range of cellulases that find multiple industrial applications. Among these are cellulases belonging to the glycoside hydrolase family 7 (GH7) in the CAZy database (Lombard *et al.*, 2014), which are among the most powerful cellulolytic enzymes known (Teeri *et al.*, 1998) and which occur almost exclusively in fungi (Payne *et al.*, 2015).

Following an observation, in 1974, that oxygen promotes cellulose degradation by fungal secretomes (Eriksson *et al.*, 1974), which remained somewhat overlooked for decades, it was discovered in 2010 that both fungi and bacteria produce copper-dependent redox enzymes that contribute to polysaccharide conversion (Phillips *et al.*, 2011; Quinlan *et al.*, 2011; Vaaje-Kolstad *et al.*, 2010). These enzymes, currently known as lytic polysaccharide monooxygenases (LPMOs), are unique in that they have the ability to cleave glycoside bonds in a crystalline context. In other words, their action does not depend on expelling an individual polysaccharide chain from a cellulose or chitin crystal, as is the case for classical hydrolytic enzymes. In that sense, LPMOs are truly interfacial enzymes, acting at the interface between the surface of an insoluble substrate (cellulose, chitin, plant cell walls) and the surrounding aqueous medium.

Next to the discovery of LPMOs, this chapter describes multiple aspects of these fascinating enzymes. We describe the occurrence of LPMO-encoding genes and show that LPMOs are abundantly encoded in many fungal genomes, which indicates importance and possible functional versatility. Furthermore, we describe the LPMO reaction and discuss that, since this reaction depends on reducing equivalents and an oxygen source (O_2 or H_2O_2), the reaction is difficult to study and is affected by the presence of other redox enzymes. Finally, we discuss the positive impact of LPMOs on the efficiency of enzyme cocktails for processing of plant biomass, which has led to industrial application of these enzymes.

The Discovery of LPMOs and Basic Structural and Functional Information

The discovery of LPMOs was a result of studies aiming at understanding depolymerization of natural polysaccharides including cellulose and chitin (Harris *et al.*, 2010; Moser *et al.*, 2008; Vaaje-Kolstad *et al.*, 2005). The occurrence, structure and catalytic activity of proteins that later turned out to be LPMOs were studied in parallel for fungal and bacterial proteins. The first such protein described in fungal secretomes was initially referred to as cellulose-induced protein (Raguz *et al.*, 1992) or endoglucanase, due to the observation of a weak endoglucanase-like activity (Saloheimo *et al.*, 1997). The first such proteins in bacteria were proteins referred to as chitin-binding proteins (Schnellmann *et al.*, 1994). Based on these initial findings, these fungal and bacterial proteins were initially classified into the Glycoside Hydrolase (GH) family 61 (GH61) and Carbohydrate-Binding Module (CBM) family 33 (CBM33), respectively, in the Carbohydrate-Active enZymes (CAZy) database (Lombard *et al.*, 2014).

A major breakthrough came in 2005, when Vaaje-Kolstad *et al.* showed that a CBM33 protein could dramatically boost the conversion of chitin by chitinases (Vaaje-Kolstad *et al.*, 2005). It took until 2010 to discover that both CBM33s and GH61s, in fact, catalyze oxidative cleavage of polysaccharides (Phillips *et al.*, 2011; Quinlan *et al.*, 2011; Vaaje-Kolstad *et al.*, 2010). These enzymes are now collectively referred to as LPMOs and have been reclassified in the CAZy database into the Auxiliary Activity families 9 (AA9; for GH61) and 10 (AA10; for CBM33) LPMOs. The potential that LPMOs may speed up depolymerization of recalcitrant polysaccharides by an order of magnitude (Vaaje-Kolstad *et al.*, 2010) has placed LPMOs in the spotlight of research on enzymatic conversion of biomass (Chylenski *et al.*, 2019; Tandrup *et al.*, 2018).

LPMOs are secreted copper-dependent redox enzymes that share a common immunoglobulin-like β-sheet core and a distinct active site with the copper ion being coordinated by a so-called His-brace (Ciano *et al.*, 2018; Quinlan *et al.*, 2011; Vaaje-Kolstad *et al.*, 2017). The His-brace is composed of the N-terminal histidine, contributing two nitrogen ligands, one from the N-terminal amino group and one from the imidazole side chain, and another fully conserved histidine, contributing a third nitrogen ligand with its imidazole side chain. In fungal LPMOs (and LPMO-like proteins; see below), the N-terminal imidazole is methylated (Filiatrault-Chastel *et al.*, 2019; Labourel *et al.*, 2020; Lo Leggio *et al.*, 2015; Navarro *et al.*, 2014; Quinlan *et al.*, 2011), whereas the post-translational modification has not been observed in bacterial LPMOs. In between the segments forming the sheet-like core structure, LPMOs contain several insertions/loops with varying lengths that shape the substrate-binding surface around the active site. Several of these loops have been annotated (such as the "L2 loop") and are also called active site segments (Borisova *et al.*, 2015; Laurent *et al.*, 2019; Wu *et al.*, 2013).

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Based on sequence variation, LPMOs are, to date, classified into seven CAZy families: AA9–11 and AA13–16 (Levasseur *et al.*, 2013). Of 717 scrutinized fungal genomes, 531 (74%) encode one or multiple genes encoding AA9, AA11, AA13, AA14 and/or AA16 LPMOs (see below; **Table 1**). Although the structural basis of LPMO substrate specificity remains largely unknown, LPMOs show huge variation in the length and composition of the loop structures that connect their core segments, leading to variation in the catalytic surface, which likely enables different LPMOs to interact with substrates with different structures (**Fig. 1**). As of today, fungal LPMOs have been found to be able to cleave a broad range of natural polysaccharides, including cellulose, hemicelluloses, chitin and starch. The most abundant fungal LPMOs are the AA9s, known to target cellulose, β-glucans such as xyloglucan and mixed-linkage glucan, glucomannan, xylan and/or oligosaccharides (Frommhagen *et al.*, 2018). Other fungal LPMOs occur in family AA11, targeting chitin (Hemsworth *et al.*, 2014), family AA13, targeting starch (Vu *et al.*, 2019), family AA14, targeting cellulose-bound xylan (Couturier *et al.*, 2018), and family AA16, targeting cellulose (Filiatrault-Chastel *et al.*, 2019).

LPMOs act by hydroxylating one of the carbons in the scissile glycosidic bond. In other words, an oxygen atom is introduced into a C–H bond, hence the name monooxygenase. This hydroxylation destabilizes the glycosidic bond, which spontaneously breaks (Phillips *et al.*, 2011). Cellulose-active LPMOs may act exclusively at the C-1 or C-4 position, or produce a mixture of C1- and C4-oxidized products (Isaksen *et al.*, 2014; Phillips *et al.*, 2011; Quinlan *et al.*, 2011; Vu *et al.*, 2014). Initially, it was thought that LPMOs carry out a monooxygenase reaction, which implies that they use molecular oxygen and two externally delivered electrons to oxidize the substrate (**Fig. 1(E)**) (Quinlan *et al.*, 2011; Vaaje-Kolstad *et al.*, 2010; Walton and Davies, 2016). In the absence of substrate, LPMOs supplied with reductant and O_2 will produce H_2O_2 (Isaksen *et al.*, 2014; Kittl *et al.*, 2012), and this oxidase activity shows that LPMOs indeed can reduce molecular oxygen. More recently, it has been shown that LPMOs can also, and much more efficiently, catalyze a peroxygenase reaction, meaning that the enzyme employs H_2O_2 rather than O_2 (**Fig. 1(E)**) (Bissaro *et al.*, 2017, 2020; Filandr *et al.*, 2020; Hangasky *et al.*, 2018; Jones *et al.*, 2020; Kuusk *et al.*, 2018). In this case, only catalytic, rather than stoichiometric, amounts of reductant are needed to drive the reaction. One open question in the field is whether O_2-driven LPMO-catalyzed cleavage of glycosidic bonds indeed occurs, or whether apparent monooxygenase reactions are in fact limited by *in situ* generation of H_2O_2, which would then be the "true" co-substrate (Bissaro *et al.*, 2017, 2018; Wang *et al.*, 2019).

Hydrogen peroxide plays a central role in fungal metabolism, which is in part reflected by the secretomes of fungi being rich in oxidoreductases. These oxidoreductases potentially interact with LPMOs either via direct electron transfer (Tan *et al.*, 2015) or through intricate reaction networks connected by reactive oxygen species (Bissaro *et al.*, 2018; Frommhagen *et al.*, 2018). In addition, fungi secrete a broad variety of redox-active compounds, which may act as reducing agent of LPMOs (Frommhagen *et al.*, 2016, 2018; Kracher *et al.*, 2016) and interact with reactive oxygen species. This is detailed further below.

Occurrence of LPMOs and LPMO-Like Proteins in Fungi

LPMOs are abundant in the fungal kingdom, in particular in Dikarya, but are also found in early diverging fungi. In dikaryotic fungi, we find at least one copy of LPMOs belonging to the families AA9, AA11, AA13, AA14 or AA16 in 84% of Ascomycetes and 68% of Basidiomycetes (**Table 1**).

Among these, the chitin-active AA11 LPMOs are the most widespread as they occur in Dikarya and most early diverging (so-called basal) fungal lineages. Genes encoding AA11s are even found in the phylum Cryptomycota, comprising the most basal unicellular fungal species, indicating an ancient origin of these enzymes. Considering that the fungal cell wall contains chitin, it is plausible that AA11 LPMOs are involved in cell wall remodeling and growth, as has been shown recently for a membrane-bound AA11 from *Neurospora crassa* (Gonçalves *et al.*, 2019). On the other hand, the AA11 LPMOs found in entomopathogenic fungi (e.g., *Cordyceps* and *Ophiocordyceps* sp. among ascomycetes and members of the fungal order Entomophthorales, such as *Conidiobolus coronatus*, among zoopagomycetes) are potentially used to degrade the chitinous exoskeleton of insects. In two fungal species belonging to the phylum Zoopagomycota, the number of AA11 genes exceeds 20 copies in a single genome, as it is the case in the soil inhabiting fungus *Basidiobolus meristosporus* CBS 931.73 (43 copies (Mondo *et al.*, 2017)) and the entomopathogenic fungus *C. coronatus* NRRL 28638 (23 copies (Chang *et al.*, 2015)). In Dikarya, the highest number of AA11 genes is found in the ectomycorrhizal fungus *Choiromyces venosus* 120613-1 (pig truffle), which displays 14 AA11 genes (Murat *et al.*, 2018), some of which are likely to have a role in fungal cell wall remodeling.

The family AA9 is the second most widespread LPMO family in fungi. AA9 LPMOs are predominantly found in Dikarya, where 57.5% of Basidiomycetes and 76.6% of Ascomycetes have one or more AA9 genes (**Fig. 2**). A large expansion of AA9 genes has occurred in Dikarya, with an average of 12 genes in both Ascomycota and Basidiomycota. While some species carry no or only a single copy of an AA9 gene, some species have evolved to carry over 50 AA9 genes. As an example, the ascomycete plant pathogen *Verticillium longisporum* has 53 AA9 genes (Fogelqvist *et al.*, 2018), and the basidiomycete plant pathogen *Rhizoctonia solani* has 55 AA9 genes (Wibberg *et al.*, 2013). Considering that AA9 LPMOs have been shown to target a broad variety of plant polysaccharides (Frommhagen *et al.*, 2018), the complexity of plant cell walls (Lampugnani *et al.*, 2018; Somerville *et al.*, 2004) may explain the multiplicity of AA9 LPMOs in Dikarya, which predominantly comprise plant biomass degraders, plant symbionts and plant pathogens. Detailed biochemical characterization of multiple AA9 LPMOs belonging to the same organisms, such as the ascomycetes *Malbranchea cinnamomea* (Hüttner *et al.*, 2019), *Myceliophthora thermophila* (Frommhagen *et al.*, 2016), *N. crassa* (Petrović *et al.*, 2019) and *Podospora anserina* (Bennati-Granier *et al.*, 2015), indicate that AA9 LPMOs may have evolved to functionally adapt to distinct co-polymeric substructures in the plant biomass. In addition, LPMOs have been shown to react differently with a

Table 1 *The occurrence of LPMOs and LPMO-like proteins in fungi.* The table shows the number of analysed genomes within each major fungal division, the number of species containing genes encoding LPMOs (individual AA families or all AA families combined) or LPMO-like proteins (X325), and the number of those genes (range and average) for the individual genomes in each phylum. The data were generated by searching with HMM-profiles of characterized LPMOs against the UniProt Reference Proteomes database, containing 717 fungal reference proteomes. For AA9 LPMOs, HMM profiles were generated based on the sequences of 37 functionally characterized AA9 LPMOs. For the other LPMO families and LPMO-like proteins, of which only a handful of proteins have been characterized so far, HMM profiles were generated based on the sequences of 50 proteins (LPMO/LPMO-like domains only) sharing 40%–100% sequence identity with the characterized one(s)

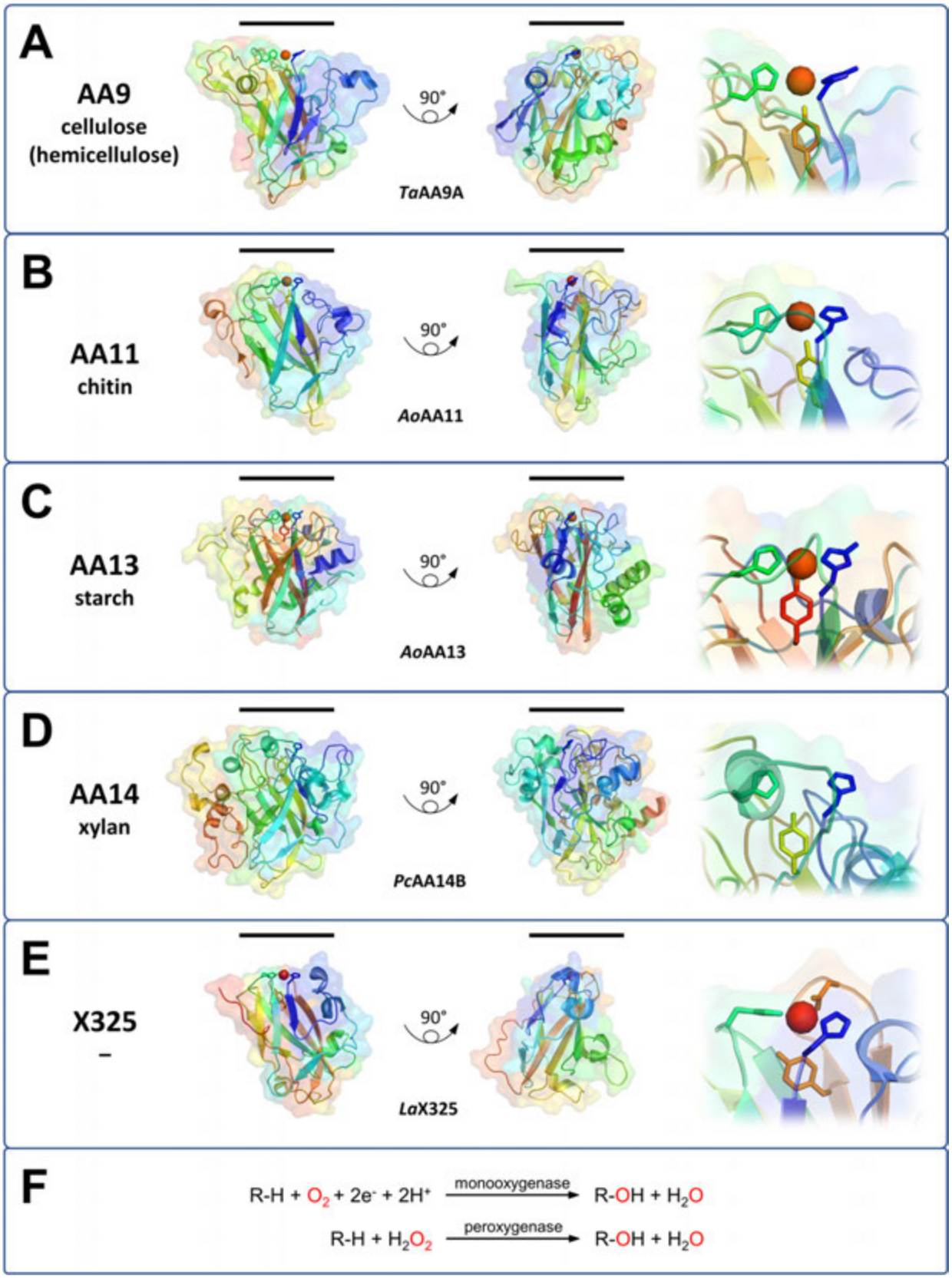
Phylum	*Species*[a]	*AA9*		*AA11*		*AA13*		*AA14*		*AA16*		*All LPMO families*		*X325*	
		Species[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]	*Species*[b]	*Gene numbers*[c]
Dikarya															
Ascomycota	462	354	1–53 (12.4 ± 11.3)	385	1–14 (3.9 ± 1.8)	173	1–3 (1.2 ± 0.5)	110	1–6 (1.2 ± 0.7)	257	1–5 (1.6 ± 0.9)	388	1–65 (17.1 ± 13.2)	380	1–7 (3.2 ± 1.1)
Basidiomycota	167	96	1–55 (12.6 ± 11.1)	28	1–8 (1.5 ± 1.4)	1	2 (2 ± 0)	102	1–19 (3.0 ± 2.1)	31	1–4 (1.8 ± 1.0)	114	2–60 (14.1 ± 12.4)	113	1–7 (1.6 ± 1.0)
Fungi incertae sedis															
Mucoromycota	29	–[d]	–[d]	10	1–10 (2.4 ± 2.9)	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	10	1–10 (2.4 ± 2.9)	25	1–5 (1.8 ± 0.9)
Zoopagomycota	14	3	1–8 (5.3 ± 3.1)	10	3–43 (13.7 ± 11.9)	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	10	3–43 (14.5 ± 12.5)	1	1
Blastocladiomycota	2	–[d]	–[d]	2	10–14 (12 ± 2)	–[d]	–[d]	1	3	–[d]	–[d]	2	10–17 (13.5±3.5)	2	2 (2±0)
Chytridiomycota	16	2	1–4 (2.5 ± 1.5)	2	1–3 (2 ± 1)	–[d]	–[d]	1	1	–[d]	–[d]	3	1–7 (3.3±2.6)	6	1–5 (3.3±1.4)
Cryptomycota	2	–[d]	–[d]	2	1–3 (2 ± 1)	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	2	1–3 (2.0±1.0)	–[d]	–[d]
Microsporidia	25	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]	–[d]

[a]The total number of scrutinized reference proteomes belonging to individual fungal species in the UniProt Reference Proteomes database.

[b]The values represent the number of reference proteomes that contain predicted proteins belonging to the indicated LPMO/LPMO-like protein family.

[c]The numbers in brackets indicate the average number of genes and standard deviation.

[d]not found.


A
AA9
cellulose
(hemicellulose)
90°
TaAA9A
B
AA11
chitin
90°
AoAA11
C
AA13
starch
90°
AoAA13
D
AA14
xylan
90°
PcAA14B
E
X325
–
90°
LaX325
F
R-H + O2 + 2e- + 2H+ monooxygenase R-OH + H2O
R-H + H2O2 peroxygenase R-OH + H2O

range of electron donors (Frommhagen *et al.*, 2016; Kracher *et al.*, 2016; Petrović *et al.*, 2019), indicating a possible adaptation to different types of redox partners, the occurrence of which may vary in distinct ecological niches.

The cellulose-active AA16 LPMOs occur exclusively in Dikarya, whereas the xylan-active AA14 LPMOs occur predominantly in Dikarya, primarily in the genomes of filamentous ascomycete fungi and basidiomycetous wood decay fungi. The gene copy number of AA14s and AA16s (on average, 1–2 per genome) is considerably lower than that of AA9s (on average, 12 per genome), which may reflect low diversity in substrate specificity among AA14s and AA16s. Accordingly, AA14 LPMOs have been shown to target recalcitrant xylan that is bound to cellulose, representing a specific substructure in the plant cell wall (Couturier *et al.*, 2018). Available data for the only AA16 characterized so far indicate activity on cellulose (Filiatrault-Chastel *et al.*, 2019). Notably, unlike the other LPMO families, AA14s are more widespread among Basidiomycetes than among Ascomycetes, with 61% versus 24% of the sequenced species carrying AA14 genes, respectively. Considering that the only fungi able to completely degrade all wood components are found in Basidiomycota (i.e., white-rot fungi), one might speculate that AA14s contribute to this ability. In addition to dikaryotic fungi, only two species from the early diverging fungi have been found to carry AA14 genes so far, the chytridiomycete *Spizellomyces punctatus* and the blastocladiomycete *Catenaria anguillulae*, with three and one genes, respectively (Mondo *et al.*, 2017; Russ *et al.*, 2016). The similarity of these genes to dikaryotic AA14s and the absence of these genes in the vast majority of early diverging fungal genomes indicate that these genes likely have been acquired by horizontal gene transfer events. However, more extensive sampling of the genomes of early diverging fungi is necessary before such conclusions can be drawn.

In addition to the LPMO families described above, the genomes of roughly one third of the sequenced Ascomycetes carry one to three AA13 LPMOs, which, based on current knowledge, act on starch (**Table 1**). The reason for the low copy number of AA13s in the genomes could be that starch is less recalcitrant and less complex than plant cell wall polysaccharides. AA13 LPMOs seem to be specific to Ascomycetes as, so far, only one basidiomycete species, *Psilocybe cyanescens*, has been found to carry AA13 genes (Reynolds *et al.*, 2018). The absence of AA13s in 166 of the 167 basidiomycete proteomes available in the UniProt Reference Proteomes database suggests that *P. cyanescens* obtained the AA13 genes by horizontal gene transfer.

In addition to catalytically active LPMOs in the CAZy AA families, LPMO-like proteins have recently been described in biomass decayers (Labourel *et al.*, 2020) and the human pathogen *Cryptococcus neoformans* (Garcia-Santamarina *et al.*, 2020). Although these LPMO-like proteins from the X325 family share a similar fold with LPMOs and contain the characteristic copper-binding histidine brace (Labourel *et al.*, 2020), no apparent catalytic activity towards polysaccharides has been demonstrated. This lack of catalytic activity could be due to the presence of an extra copper-binding ligand (**Fig. 1(E)**) that may prevent the copper from reacting with its oxygen co-substrate, as it is the case in the copper-binding protein CopC (Arnesano *et al.*, 2002; Labourel *et al.*, 2020), and/or to the absence of conserved residues around the copper site that promote polysaccharide binding. Therefore, these proteins cannot be classified as LPMOs. For completeness, it should be mentioned that a very minor fraction of proteins classified into the AA9 family carry a substitution of the N-terminal His by Arg (Lenfant *et al.*, 2017; Yakovlev *et al.*, 2012), which likely renders the protein catalytically inactive (Frandsen *et al.*, 2019).

Notably, genes encoding X325 proteins are widespread among dikaryotic fungi, occurring in 82.3% of Ascomycete and 67.7% of Basidiomycete genomes, and also occur in the early diverging fungal lineages (**Table 1**). This makes the X325 proteins the second most widespread LPMO/LPMO-like family in fungi after AA11s. The fact that we find X325 genes in all fungal divisions where filamentous growth has evolved, points to a crucial role for these somewhat enigmatic proteins. (Note that the phyla Cryptomycota and Microsporidia, for which no X325 genes have been found so far, contain strictly non-filamentous, intracellular parasites (Naranjo-Ortiz and Gabaldón, 2019)). Currently, LPMO-like proteins are hypothesized to be involved with copper homeostasis during plant biomass decay (Labourel *et al.*, 2020) or host-parasite interactions (Garcia-Santamarina *et al.*, 2020). It is likely that these X325 proteins share a common ancestor with LPMOs but evolved separately prior to the acquisition of polysaccharide cleaving ability. The fact that LPMO-like proteins and AA11 LPMOs occur in nearly all fungal phyla, including Dikarya and early diverging fungi, suggests the importance of their function in the fungal kingdom and indicates that these proteins were among the earliest to evolve from a common ancestor. Furthermore, the varying distribution of LPMO families in the phyla indicates that LPMOs belonging to different families may have evolved at various timepoints, which may indicate different selection pressures for the distinct LPMO families.

Assaying LPMO Catalysis

Characterization of the catalytic activity of LPMOs is complicated by the wide range of possible on- and off-pathway reactions that may take place when mixing reductants, O_2 and/or H_2O_2, an insoluble, not necessarily "clean" and potentially redox active

Fig. 1 Representative structures (A–E) and overall reaction (F) of fungal LPMOs/LPMO-like proteins. Panels A–E show that different LPMO families have a similar β-strand core (left and middle panels) and catalytic center (right panels), while they have evolved distinct substrate-binding surfaces that differ in length and width, as well as ruggedness (left and middle panels), to target different polysaccharides (indicated to the left). The black bars indicate the place of substrate binding for LPMOs (A–D) and the corresponding surface around the copper site for LPMO-like proteins (E). Panel F shows the two types of reaction equations that have been proposed for LPMO catalysis (Bissaro *et al.*, 2017; Vaaje-Kolstad *et al.*, 2010). PDB structures: *Ta*AA9A, 2YET; *Ao*AA11, 4MAI (incomplete structure, the only AA11 with structure); *Ao*AA13, 4OPB; *Pc*AA14B, 5NO7 (structure without active site copper); *La*X325, 6IBJ. Note that there is currently no structure for fungal AA16 LPMOs, and that methylation of His1 lacks in panels B, D and E as these recombinant proteins were not expressed in a filamentous fungal expression host but in *Escherichia coli* (*Ao*AA11 in panel B) or in *Pichia pastoris* (*Pc*AA14B in panel D and *La*X325 in panel E), which are unable to methylate the N-terminal histidine. The protein structures are shown in rainbow colors, from blue (N-terminus) to red (C-terminus); the active site copper is indicated by an orange sphere. The crystal structure of the AA14 (panel D) lacks a copper ion.

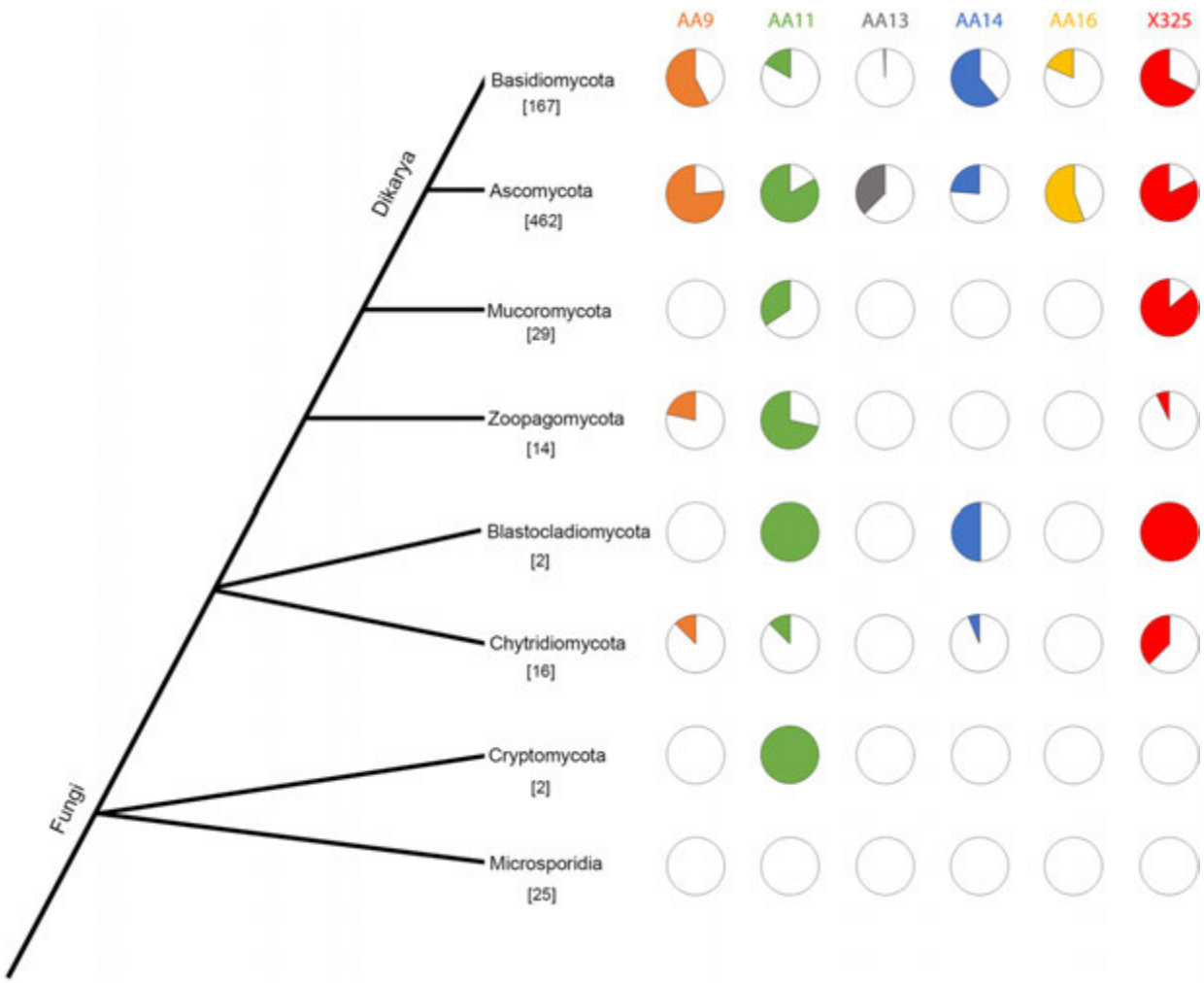


Fig. 2 The occurrence of fungal LPMOs (AA9, AA11, AA13, AA14 and AA16) and LPMO-like proteins (X325) in fungal phyla, presented on a phylogenetic tree. The numbers in brackets represent the number of individual proteomes deposited in the UniProt Reference Proteomes database for the given phylum. The pie charts show the proportion of proteomes containing one or more copies of a given protein family (in color). The range of gene copies found in phylum-specific proteomes is provided in **Table 1**.

substrate, the LPMO and, potentially, small amounts of free copper that may change during the reaction. To complicate things even further, the oxidase activity of LPMOs (see above) implies that these enzymes can synthesize their own co-substrate (Bissaro *et al.*, 2017; Wang *et al.*, 2019). Finally, LPMOs are prone to autocatalytic oxidative damage in their catalytic centers (Bissaro *et al.*, 2017) and, during an enzymatic reaction, this susceptibility may change over time as the substrate is being depleted. At lower substrate concentrations, the LPMO is more likely to engage in potentially destructive off-pathway reactions. For a detailed description of the many possible pitfalls in characterizing LPMO activity, the reader is referred to a recent review on the topic (Eijsink *et al.*, 2019) and the references therein. Here, only a few key points are briefly discussed.

Due to the abovementioned complexities, it is imperative that characterization studies are carried out with highly purified LPMOs that are free of other redox enzymes or compounds, and free of transition metals. As to recombinant production of LPMOs (Gaber *et al.*, 2020), it is important that the expression system is such that the purified enzyme contains the correct N-terminal histidine and that the resulting LPMO preparation is free of contaminating background activities, such as cellulase activity. Complete avoidance of background activity may be difficult, and, in such cases, it is very important to separate between reductant-dependent reaction products, which are LPMO products, and reductant-independent reaction products, which may be generated by background activities. It is advisable to make sure that the LPMO is copper-saturated, and one needs to use a procedure that removes remaining unbound copper after the saturation step.

Typical LPMO reactions are set up aerobically in the presence of low mM amounts of reductant, for example 1 mM ascorbic acid (Vaaje-Kolstad *et al.*, 2010). In such a setup, the reductant is consumed stoichiometrically either in a monooxygenase reaction or in reactions that generate H_2O_2, which is then used in a peroxygenase reaction (see above; **Fig. 1(E)**). Alternatively, reactions can be set up with micromolar amounts of reductant, e.g., 20 μM ascorbic acid, needed for a so-called priming reduction of the LPMO,

with addition of H_2O_2. High concentrations of H_2O_2 need to be avoided since these may lead to autocatalytic LPMO inactivation and perhaps to more unspecific generation of reactive oxygen species, which could damage any enzyme in the reaction mixture. Ideally, the concentration of H_2O_2 needs to be kept low by gradually administering it to the reaction, e.g., as done by Müller *et al.* (2018), but this may be technically challenging. *In situ* generation of H_2O_2 by adding glucose oxidase provides a possible solution (Bissaro *et al.*, 2017). Importantly, the catalytic rates observed for typical monooxygenase setups are orders of magnitude lower compared to rates observed for peroxygenase setups (ranging from 0.1 s^{-1} and much below in the former to up to 10 s^{-1} in the latter), as recently reviewed by Bissaro *et al.* (2018).

C1 oxidation of cellulose leads to the generation of lactones, which spontaneously convert to aldonic acids, whereas C4 oxidation leads to the generation of ketones, which spontaneously convert to gemdiols. These products can be identified using mass spectrometry and liquid chromatography, as extensively discussed elsewhere (see (Eijsink *et al.*, 2019), and references therein). It is important to note that C4-oxidized products are unstable at slightly alkaline pH, as discussed in (Westereng *et al.*, 2016). Of note, several groups have published LPMO activity assays that are based on the use of other substrates and other analytical methods. These methods suffer from various limitations, but can nevertheless be very useful, as discussed in (Eijsink *et al.*, 2019) and references therein.

The choice of reductant may have a major impact on the observed LPMO activity, as discussed in Section "Interplay With Other Fungal Redox Enzymes" below and in e.g. (Frommhagen *et al.*, 2016; Kracher *et al.*, 2016). It is important to note that this impact is not necessarily related to the reductants' varying abilities to reduce the LPMO, but may also relate to effects on the many possible side reactions, such as side reactions leading to the generation or consumption of H_2O_2.

Due to the many complications, including enzyme inactivation, in both monooxygenase and peroxygenase setups, there are very few studies that describe proper kinetic data for LPMOs. One notable exception is a study by Kuusk *et al.* (2018), who showed that the k_{cat}/K_m,H_2O_2 of CBP21, a chitin-active LPMO, is in the order of 10^6 $M^{-1}s^{-1}$ and some three orders of magnitude higher than the k_{cat}/K_m,O_2. Because of the occurrence of side reactions, including enzyme inactivation, progress curves for LPMO reactions are notoriously nonlinear. This obviously implies that it is impossible to draw quantitative conclusions regarding LPMO activity from single-timepoint product formation measurements.

Interplay With Other Fungal Redox Enzymes

LPMOs are copper-dependent redox enzymes and require an electron donor to reduce the active site copper from the Cu(II) to the Cu(I) state before they can perform catalysis either by using molecular oxygen or hydrogen peroxide (Bissaro *et al.*, 2017; Vaaje-Kolstad *et al.*, 2010; Wang *et al.*, 2019). In fungi, reduction of the active site copper can occur either by reducing compounds present at the fungus-plant interface, including fungal secondary metabolites, plant phenols and non-aromatic reducing agents such as ascorbic acid (Frommhagen *et al.*, 2016; Kont *et al.*, 2019; Kracher *et al.*, 2016; Westereng *et al.*, 2015), or by an enzymatic electron donor via direct electron transfer (Garajova *et al.*, 2016; Kracher *et al.*, 2016; Langston *et al.*, 2011; Phillips *et al.*, 2011; Tan *et al.*, 2015).

Early genome studies of dikaryotic fungi showed that genes encoding AA9s often co-occur with oxidoreductases belonging to the AA2, AA3 and AA5 families (Eastwood *et al.*, 2011; Floudas *et al.*, 2012). Analysis of 33 basidiomycete genomes by Riley *et al.* indicated co-evolution of AA9 genes with genes encoding AA3_2 oxidoreductases and AA2 peroxidases through gene duplication or loss, suggesting a potential interaction between these oxidoreductases (Riley *et al.*, 2014). In a later study, comparing the occurrence of oxidoreductases in 47 ascomycete and 50 basidiomycete genomes, Kracher *et al.* (2016) confirmed that AA9 LPMOs often co-occur with AA3_1 cellobiose dehydrogenases (CDH) and AA3_2 oxidoreductases, supporting a potential interplay between these enzymes. In addition, they found lifestyle-specific correlations in the occurrence of cellulolytic and lignin-active enzymes. In particular, they noted a high correlation between the occurrence of hydrolytic cellulases, AA9 LPMOs and AA3_1 CDHs in cellulose-degrading soft-rot fungi, while they noted a correlation between the occurrence of AA1 laccases, AA2 peroxidases and AA3 oxidoreductases (excluding AA3_1 CDHs) in white-rot fungi, which are capable of degrading lignin (Kracher *et al.*, 2016). Corresponding to this, gene evolution analysis by Nagy *et al.* (2016) indicated that cellulose and lignin degradation evolved separately. Ancient Dikarya and Basidiomycota had an extensive repertoire of genes encoding plant cell wall-active enzymes (including AA9 LPMOs) involved in plant cell wall polysaccharide decomposition, while they lacked key enzymes (including AA2 peroxidases) related to lignin decomposition (Nagy *et al.*, 2016). This is consistent with the hypothesis that fungi acquired the capability of lignin degradation only at a later evolutionary stage, i.e., when white-rot fungi evolved (Floudas *et al.*, 2012; Kohler *et al.*, 2015). While from an evolutionary perspective cellulose-active AA9 LPMOs seem to have evolved earlier and separately from enzymes involved in lignin degradation, there is growing evidence for the potential interplay between AA9 LPMOs and lignin-active oxidoreductases in fungal secretomes, as described here below.

Apart from evolutionary connections, AA9 LPMOs have also been found co-regulated with AA3_1 CDHs, which may be indicative of co-operativity between the enzymes (see e.g. (Miyauchi *et al.*, 2020)). Notably, transcriptional regulation of orthologous genes can differ even between species belonging to the same genus, as shown for AA2 peroxidases in three *Pycnoporus* species (Miyauchi *et al.*, 2020). This may indicate species-specific adaptation of (co-)regulation and (co-)secretion of plant cell wall-active oxidoreductases in response to distinct environmental niches. Oxidoreductases, including LPMOs, co-exist in fungal

secretomes and, consequently, are linked through reactive oxygen species, which are formed and consumed continuously as a result of their catalytic actions (Berrin *et al.*, 2017; Bissaro *et al.*, 2018). For example, hydrogen peroxide can be produced by several members of the AA3_2 oxidoreductase family, while it is consumed by both AA2 peroxidases and LPMOs. Indeed, oxidoreductases belonging to families AA1, AA3_1, AA3_2 and AA12 have been shown to drive LPMO reactions, either directly or, in the case of AA1, via redox mediators (Brenelli *et al.*, 2018; Garajova *et al.*, 2016; Kracher *et al.*, 2016; Langston *et al.*, 2011; Perna *et al.*, 2020; Várnai *et al.*, 2018).

Among the oxidoreductases, AA3_1 CDHs were the first ones shown to be able to drive oxidative cleavage of cellulose by LPMOs (Langston *et al.*, 2011; Phillips *et al.*, 2011) and have gained the most attention, which is partly related to the fact that both AA9 LPMOs and CDHs are active on cellulose. CDHs contain a central flavin-dependent AA3 domain and an N-terminal AA8 cytochrome domain that transfers electrons from the reduced flavin to the LPMO, one at a time. In addition, CDHs may contain a C-terminal cellulose-binding domain. CDHs from the ascomycetes *Humicola insolens* (Langston *et al.*, 2011), *M. thermophila* (Phillips *et al.*, 2011), *N. crassa* (Kittl *et al.*, 2012; Sygmund *et al.*, 2012), *P. anserina* (Bennati-Granier *et al.*, 2015) and *Myriococcum thermophilum* (Tan *et al.*, 2015) as well as CDH from the basidiomycete white-rot fungus *Pycnoporus cinnabarinus* (Bey *et al.*, 2013) can drive catalysis by multiple LPMOs from the same species (Bennati-Granier *et al.*, 2015; Kittl *et al.*, 2012; Sygmund *et al.*, 2012) or from other species, including a bacterial chitin-active family AA10 LPMO (Loose *et al.*, 2016).

An AA12 PQQ-dependent pyranose dehydrogenase from *Coprinopsis cinerea* (*Cc*PDH) has been shown to drive LPMO reactions, analogous to FAD-dependent CDHs (Petrović *et al.*, 2019; Várnai *et al.*, 2018). Although most fungal PDHs occur as single-domain enzymes (Takeda *et al.*, 2019), *Cc*PDH shares a similar domain architecture with CDHs, comprising an N-terminal AA8 cytochrome domain, a central AA12 dehydrogenase domain and a C-terminal CBM1 domain. It has been amply demonstrated that the CDH/PDH-driven LPMO reaction depends on direct electron transfer from the AA8 domain to the copper (Breslmayr *et al.*, 2020; Courtade *et al.*, 2016; Tan *et al.*, 2015; Várnai *et al.*, 2018) and that the CDH and LPMO reactions are coupled (Filandr *et al.*, 2020; Loose *et al.*, 2016; Petrović *et al.*, 2019). It is important to note though that the nature of the LPMO-fueling effect of CDH and PDH remains somewhat ambiguous. It cannot be excluded that the rate-limiting contribution of these enzymes to fueling the LPMO reaction relates to their low oxidase activity that leads to generation of H_2O_2. In support of this, a recent study by Kracher *et al.* has shown that LPMO activation by engineered CDH variants is proportional to their rate of H_2O_2 production (Kracher *et al.*, 2020). More generally, the latter results lend further support to the notion that H_2O_2-producing oxidoreductases in fungal secretomes can drive LPMO reactions (Bissaro *et al.*, 2018).

AA3_2 oxidoreductases, which seem to have co-evolved with AA9 genes in basidiomycetes (Riley *et al.*, 2014), have also been confirmed to drive LPMO reactions, either directly (Garajova *et al.*, 2016) or via diphenol/quinone-type redox mediators provided that the mediator is regenerated efficiently by the oxidoreductase (Kracher *et al.*, 2016). Notably, not all these oxidoreductases have the ability to drive LPMO reactions directly (Bissaro *et al.*, 2017; Filandr *et al.*, 2020; Garajova *et al.*, 2016). Two independent studies have shown that an AA3_2 glucose oxidase can drive cellulose oxidation by LPMOs belonging to the AA9 (Filandr *et al.*, 2020) and AA10 families (Bissaro *et al.*, 2017), provided that the active site copper is in its reduced state. Of note, the ability of an oxidoreductase to produce H_2O_2 may not be enough to drive the LPMO reaction, since the LPMO also needs to be reduced. This could be a plausible explanation as to why an AA3_2 aryl-alcohol oxidase was found incapable of promoting LPMO reactions despite being able to generate H_2O_2 (Garajova *et al.*, 2016).

While in artificial systems (i.e., purified enzymes acting on lignin-free cellulosic substrates in the absence of external reducing agents) some oxidoreductases cannot drive the LPMO reaction (Bissaro *et al.*, 2017; Filandr *et al.*, 2020; Garajova *et al.*, 2016), in nature LPMOs are anticipated to be in their reduced form due to an abundance of reducing compounds present at the fungus-plant interface. Notably, a substantial part of plant- or fungus-derived phenols react not only with LPMOs but also with other oxidoreductases present in the fungal secretome. AA3_2 oxidoreductases, for instance, can drive LPMO reactions by recycling diphenol/quinone-type redox mediators (Kracher *et al.*, 2016). In addition, two polyphenol oxidases have been shown to promote LPMO reactions by converting lignin-derived monophenols to diphenols, which react more readily with LPMOs (Frommhagen *et al.*, 2017). It is important to note the complex spectrum of multiple roles that lignin may play in these processes. Brenelli *et al.* have shown that low-molecular weight lignin fractions generated with laccase-mediator systems can drive LPMO catalysis (Brenelli *et al.*, 2018). Recently, lignin-derived compounds were shown to serve both as an electron donor for LPMOs, reducing the active site copper, and as a source of H_2O_2, which is generated through the reaction of these compounds with molecular oxygen (Kont *et al.*, 2019). Correspondingly, Perna *et al.* have observed that laccase-catalyzed oxidation of lignin and lignin building blocks, which generates radicals, promotes H_2O_2 production and, consequently, boosts the LPMO reaction (Perna *et al.*, 2020), thereby expanding further the network of interactions between LPMOs and other oxidoreductases. Potential interactions between LPMOs, other oxidoreductases and reactive oxygen species have been extensively reviewed by Bissaro *et al.* (2018).

As alluded to above, analysis of predicted or actual fungal secretomes may provide insight into how LPMOs and potential redox partners collaborate during plant biomass conversion. It is worth noting that such analysis also provides a powerful tool for identifying novel LPMOs with potentially crucial roles in the degradation process. Such LPMOs may belong to established families with high gene multiplicity or belong to hitherto undiscovered LPMO families (Berrin *et al.*, 2017). Indeed, several secretome studies have led to the identification of new LPMO families (Couturier *et al.*, 2018; Filiatrault-Chastel *et al.*, 2019; Sabbadin *et al.*, 2018) or redox partners of LPMOs (Frommhagen *et al.*, 2017; Garajova *et al.*, 2016). Time-resolved analysis of fungal secretomes, in particular, can give more detailed insight into the intricate network of plant cell wall-active enzymes and increase the probability

of identifying the most important LPMOs in various phases of the degradation process, as well as biologically relevant redox partners of such LPMOs (e.g. (Navarro *et al.*, 2014; Presley and Schilling, 2017)).

Applications: Cellulose Saccharification and Fibrillation

Since the discovery that *T. reesei* was responsible for degrading cotton army tents during World War II, there has been a great interest in the application of fungal cellulase systems for saccharification of cellulose-rich biomass. One possible reason for LPMOs not having been discovered earlier may be that *T. reesei* contains only few LPMO genes and that only low amounts of LPMOs are present in *T. reesei* secretomes. On the other hand, already in 1950, Reese, who lent his name to *T. reesei*, and co-workers presented the so-called C_1–C_X theory, suggesting that efficient hydrolytic conversion of cellulose by cellulases (C_X) required some sort of enzymatic activation step, catalyzed by C_1, that makes cellulose more accessible (Reese *et al.*, 1950). Combining this hypothesis with the observation, in 1974, that oxygen promotes cellulose degradation (Eriksson *et al.*, 1974) and the discovery of LPMOs in 2010 (Vaaje-Kolstad *et al.*, 2010) leads to the suggestion that LPMOs catalyze the C_1 step. More recently, Navarro *et al.* have shown early secretion of seven AA9 LPMOs of *Laetisaria arvalis* (a soil-inhabiting basidiomycete containing 16 AA9-encoding genes) in response to growth on cellulose, suggesting that some LPMOs secreted by fungi may indeed act in the first phase of the cellulose degradation process (Navarro *et al.*, 2014).

After the initial discovery of bacterial LPMO activity in 2010 (Forsberg *et al.*, 2011; Vaaje-Kolstad *et al.*, 2010), several research groups demonstrated that fungal LPMOs (then named GH61s) could cleave cellulose (Beeson *et al.*, 2012; Langston *et al.*, 2011; Phillips *et al.*, 2011; Quinlan *et al.*, 2011; Westereng *et al.*, 2011). This initiated many studies on the roles of LPMOs in saccharification of cellulose by cellulase cocktails and also resulted in the use of LPMOs in the latest generations of commercial enzyme cocktails (Johansen, 2016). In practice, this research work typically involved spiking fungal secretomes low in LPMO activity (in-house prepared or commercial) with monocomponent LPMOs. Before the catalytic activity of LPMOs was known, it had been shown that these proteins can boost the activity of chitinases (Vaaje-Kolstad *et al.*, 2005) and cellulases (Harris *et al.*, 2010; Merino and Cherry, 2007). Harris *et al.* (2010) showed that cloned and purified LPMOs from *Thielavia terrestris* and *Thermoascus aurantiacus* could increase the saccharification yield of lignocellulosic biomass by a cocktail of *T. reesei* cellulases. The first study that linked improved saccharification to LPMO activity was a 2011 study by Westereng *et al.* (2011). Here it was shown that addition of an LPMO from *Phanerochaete chrysosporium* to Celluclast, a commercial cellulase preparation, could improve the saccharification yield of cellulose by about 30%, and that this positive effect was linked to LPMO activity, which was measured as production of C1-oxidized sugars. Several LPMO-spiking studies have confirmed the positive effect of LPMOs on saccharification of cellulosic substrates by commercial enzyme preparations (Cannella *et al.*, 2012; Chylenski *et al.*, 2017; Hu *et al.*, 2014, 2013, 2015; Müller *et al.*, 2015). Interestingly, one of these studies indicated that the optimum concentration of LPMOs in a cellulase cocktail could be as high as 15% on a protein basis (Müller *et al.*, 2015) (**Fig. 3**).

Importantly, the inclusion of LPMOs in modern enzyme blends also affects process design since these enzymes require an oxygen co-substrate and reducing equivalents. This was nicely illustrated in a 2015 study by Müller *et al.* (2015), as explained in the legend of **Fig. 4**. Concerning the production of ethanol, the role of LPMOs also affects the choice between a so-called Separate

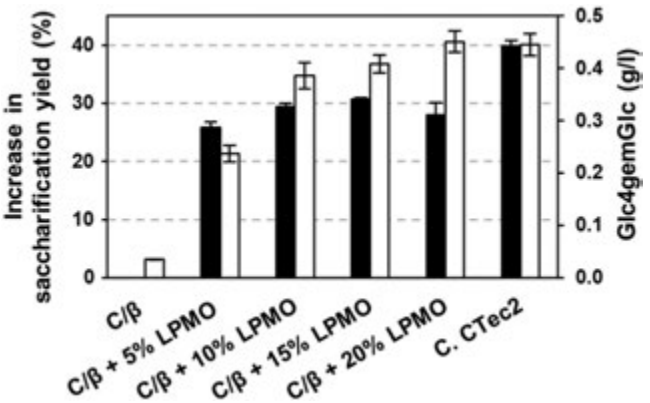


Fig. 3 Increase in saccharification yield and production of oxidized sugars upon supplying a commercial cellulase mixture with an LPMO. Steam-exploded birch wood at 100 g/L (dry matter basis) was incubated with a mixture of Celluclast, an LPMO-poor cellulase mixture, and Novozym 188, a β-glucosidase, ("C/β") for 20 h. Various fractions (0%–20%) of this enzyme cocktail were replaced by purified *Ta*AA9A (also known as *Ta*GH61A or *Ta*LPMO9A) from *T. aurantiacus*. Black bars show the increase in glucose release (in %) compared to the Celluclast/Novozym 188 mixture alone, whereas the white bars show the concentration of oxidized sugars in the product mixtures. For comparison, the results for a reaction with Cellic™ CTec2 ("C. CTec2"), a modern LPMO-containing cellulase cocktail are included. This Figure is reproduced from Müller, G., Várnai, A., Johansen, K.S., Eijsink, V.G.H., Horn, S.J., 2015. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnology for Biofuels 8, 187.

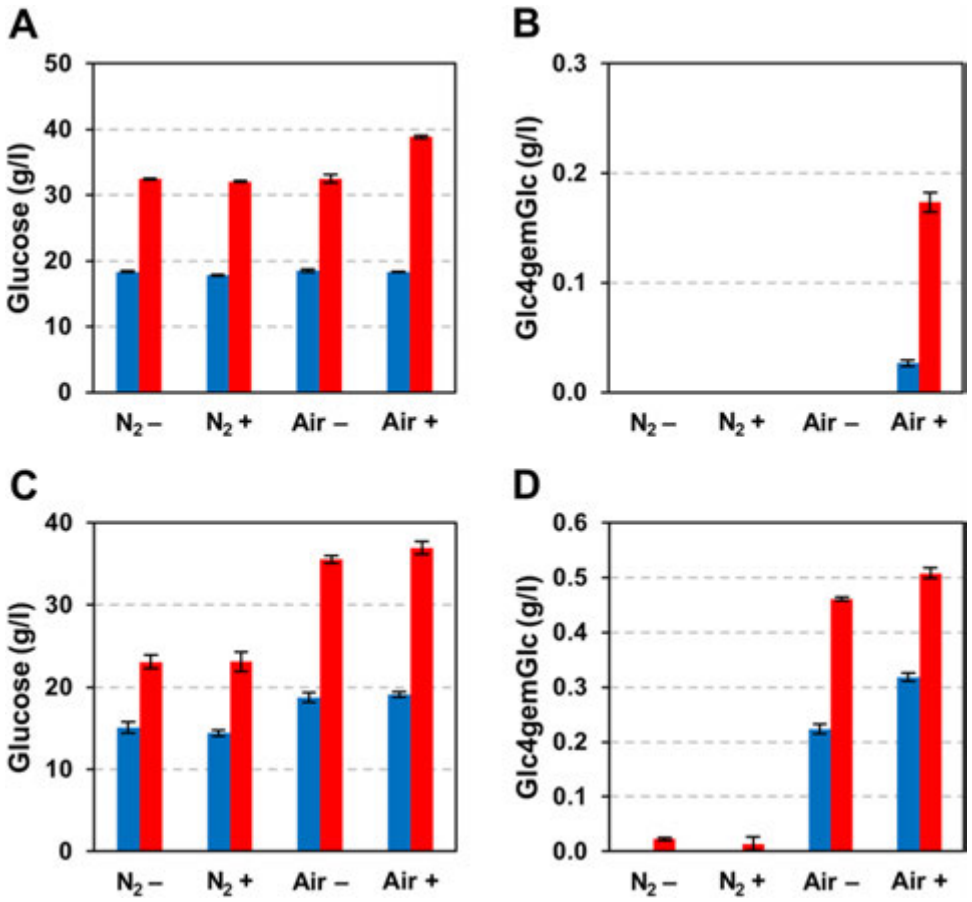


Fig. 4 Saccharification of Avicel (A, B) or steam-exploded birch wood (C, D) at 100 g/L (dry matter) under aerobic ("Air") or anaerobic ("N_2") conditions in the presence ("+") or absence ("−") of ascorbic acid. The *left panels* (A, C) show glucose concentrations at two time points, while the *right panels* (B, D) show production of the primary oxidized product, Glc4gemGlc, at the same time points. The *blue bars* represent 4 h of incubation, while *red bars* represent 18 h. Note that the presence of oxygen promotes both LPMO activity and glucose release. In the case of a "clean" substrate, LPMO activity requires an added reductant (ascorbic acid; panels A & B), whereas reduction can be provided by the substrate when the substrate is rich in lignin (panels C & D). This Figure is an adapted combined version of two figures appearing in Müller, G., Várnai, A., Johansen, K.S., Eijsink, V.G.H., Horn, S.J., 2015. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnology for Biofuels 8, 187.

Saccharification and Fermentation (SHF) process and a so-called Simultaneous Saccharification and Fermentation (SSF) process. Generally, using an SHF setup seems preferable over an SSF setup since fermenting organisms will consume O_2 in SSF and thus limit LPMO activity (Cannella and Jørgensen, 2014; Müller *et al.*, 2017).

The finding that H_2O_2 can be used to obtain efficient LPMO reactions has (obviously) been explored in optimization of biomass conversion studies. In a landmark study by Müller *et al.*, it was shown that, indeed, reactor setups with controlled addition of H_2O_2 can be very efficient (Müller *et al.*, 2018). However, so far, such positive results have only been described for clean, cellulose-rich and lignin-poor substrates. It has so far not been possible to harness the ability to speed up LPMO reactions by controlled addition of H_2O_2 in reactions with "real" biomass, as exemplified by Müller *et al.* (2018), who studied saccharification of steam-exploded birch wood. In a recent study, the approach of controlled addition of H_2O_2 to reactions with lignin-poor substrates, in this case sulfite-pulped spruce wood, was shown to be successful at demonstration scale (Costa *et al.*, 2020).

Overall, the inclusion of LPMOs in cellulase cocktails has been a great success, significantly improving enzymatic saccharification of lignocellulosic substrates. Further improvements of the current enzyme blends and processes might involve optimization of LPMO/cellulase ratios for different substrates and optimization of H_2O_2 addition regimes (or *in situ* H_2O_2 production). It is clear that the synergy between LPMOs and cellulases depends on the enzymes, the substrate and the redox conditions (Kadić *et al.*, 2019; Müller *et al.*, 2018; Tokin *et al.*, 2020).

Recent advancements in our understanding of how LPMOs accelerate saccharification of recalcitrant plant biomass by cellulases have shed light on the impact of LPMO treatment on substrate structure and, consequently, the application potential of LPMOs for surface modification and/or fibrillation of wood fibers. Initial molecular dynamics simulations by Vermaas *et al.* indicated that

accumulation of oxidized groups on the surface of crystalline cellulose by LPMO action (in particular carboxyl groups introduced by C1-oxidizing LPMOs) disrupt the structure of crystalline cellulose, reducing the decrystallization penalty for processive cellobiohydrolases (Vermaas *et al.*, 2015). Shortly thereafter, Villares *et al.* provided experimental proof, by solid-state NMR and atomic force microscopy (AFM), that the action of the C1/C4-oxidizing *Pa*LPMO9H from *P. anserina* introduces changes in the regions connecting elementary fibrils, thus promoting fibrillation at micro- and nanoscale that becomes visible after mechanical fibrillation (Villares *et al.*, 2017). Furthermore, Eibinger *et al.* showed partial detachment of cellulose chain bundles, i.e. fibrillation of cellulose, at the tips of cellulose nanocrystals upon the action of the C1-oxidizing *Nc*LPMO9F (but not of the C4-oxidizing *Nc*LPMO9C) from *N. crassa* using real-time AFM (Eibinger *et al.*, 2017). Later, Hu *et al.* (2018) confirmed that treating a cellulose pulp with the C1/C4-oxidizing *Ta*LPMO9A from *T. aurantiacus* leads to an increase in acid group content of the pulp (presumably by formation of carboxyl groups generated during C1-oxidation), which corresponded to an increase in the stability of the fiber suspension (Hu *et al.*, 2018). In addition to cellulose-active LPMOs, recently two xylan-specific AA14 LPMOs have been shown to contribute to cellulose fibrillation using AFM and transmission electron microscopy (TEM), presumably by cleaving recalcitrant xylan adhering to cellulose microfibrils (Couturier *et al.*, 2018).

There may be differences between the mechanisms that underlie apparent promotion of fibrillation by LPMOs, which could relate to regioselectivity (Eibinger *et al.*, 2017; Vermaas *et al.*, 2015; Villares *et al.*, 2017) and substrate specificity (Couturier *et al.*, 2018; Ladevèze *et al.*, 2017). Still, it is now clear that LPMOs belonging to various families, including AA9, AA10 and AA14, can promote and even induce fibrillation. Currently, endoglucanases (GH45s) are routinely applied as a fibrillation aid in industry (Marjamaa and Kruus, 2018). LPMOs, which use a fundamentally different mechanism from that of endoglucanases (i.e. combining cleavage of chains and introduction of oxidized groups at the fiber surface), provide an alternative tool for fibrillation of wood fibers and for surface modification of wood fibers (e.g. (Hu *et al.*, 2018; Koskela *et al.*, 2019; Valenzuela *et al.*, 2019)). Multiple recent studies have confirmed this potential, especially for improving mechanical fibrillation during nanocellulose production, for various materials, including cotton linters (Valls *et al.*, 2019), flax fibers (Valenzuela *et al.*, 2019), hardwood fibers (Moreau *et al.*, 2019) and softwood fibers (Koskela *et al.*, 2019). Further studies are warranted, for example to understand how the regioselectivity and substrate specificity of LPMOs relate to their plant cell wall-loosening (fibrillating) function, as well as how H_2O_2 may be used to improve the efficiency of LPMOs in fiber modification.

Future Perspectives

Ten years after their discovery, LPMOs are at the center of research on enzymatic conversion of biomass and considered a central component of fungal cellulolytic secretomes. Despite recent progress, many questions remain, for example related to the catalytic mechanism, the structural basis of substrate specificity, the degree and biological relevance of functional variation, the interplay with other redox enzymes and optimal application of these enzymes in industrial biomass processing. The plethora of LPMOs found in several fungal lineages suggests that there is a lot more to discover, including, most likely, novel functionalities. The occurrence of LPMO-like copper-binding proteins in many fungal species is intriguing and one may wonder about the possible interplay between these proteins and catalytically active LPMOs. Likewise, one may wonder about how multiple LPMOs in fungal secretomes collaborate (as shown for an AA14 and an AA9 LPMO (Couturier *et al.*, 2018)), and maybe even synergize, during biomass conversion; this aspect of LPMO multiplicity remains unexplored.

When (re-)assessing existing LPMO literature, especially literature published prior to 2018, it is important to be aware of the potentially different roles of O_2 and H_2O_2 as co-substrates and of the ongoing scientific debate regarding the role of these substrates. For example, one could ask whether the huge variation between reductants in terms of their ability to drive LPMO reactions in the presence of oxygen could be due to variation in the efficiency of *in situ* generation of H_2O_2 rather than different abilities to reduce the LPMO. In this respect, it is interesting to note the recent study by Kracher *et al.* (2020), who showed that an engineered variant of CDH with increased oxidase activity (i.e., increased production of H_2O_2) is more efficient in driving LPMO reactions compared to the wild-type enzyme.

As alluded to above, there is functional data for multiple LPMOs from the same fungal species, providing some insight into functional variation among LPMOs produced by the same organism. However, it must be noted that we currently do not have a full picture of the "LPMO potential" for any fungus; i.e. we do not know the complete catalytic potential of a complete set of LPMOs from a single fungus. Likewise, while both the distribution of genes and the properties of characterized LPMOs seem to indicate that the substrate specificities of LPMOs correlate with fungal lifestyle, there is currently too little information for substantiating this (expected) correlation.

To obtain a more complete picture of the fungal "LPMO potential", more LPMOs need to be characterized. Such characterization should include accurate (and challenging) kinetic analyses as well as thorough mapping of substrate specificity towards all potentially relevant substrates, varying from pure cellulose to a wide variety of co-polymeric structures that may occur in the plant and fungal cell walls. Further insight into the natural redox partners of LPMOs is also of great interest, one question being whether LPMOs belonging to different families or expressed during different phases of plant cell wall degradation may have different (preferred) redox partners. For example, although CDH is established as a natural redox partner of AA9 LPMOs, little is known about the natural redox partners of fungal LPMOs in the other AA families. While additional information may come from expression studies showing which LPMOs and other redox enzymes are co-expressed during growth on different substrates, functional characterization of individual enzymes is a prerequisite for further progress.

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Applications of Fungal Cellulases

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Introduction

The bio-based economy requires the use of renewable resources for the production of biochemicals and biomaterials, for which lignocellulosic biomass is becoming a valuable feedstock (Furtado *et al.*, 2014). Lignocellulose is a collective name for an inedible plant material containing polysaccharides, cellulose and hemicelluloses, and the aromatic polymer lignin. Every year, millions of tons of lignocellulosic biomass are being produced as a low-value byproduct from agroforestry, e.g., corn stover, straw, cotton seed hulls and wood sawdust, most of which end up being burned or discarded. However, when broken down to its constituent monomers, lignocellulosic biomass becomes an abundant and attractive source of fermentable sugars and aromatics, which can be used for sustainable production of valuable compounds, including biofuels and chemicals.

Cellulose, the most abundant polymer in lignocellulose (35%–50% of dry weight), is a linear polymer of glucose that can be directly fermented for the production of 2nd generation ethanol (Anwar *et al.*, 2014; El-Naggar *et al.*, 2014). Despite its simple composition, natural cellulose forms long crystalline microfibers that are cross-linked to lignin through hemicelluloses, which makes glucose extraction rather difficult. As filamentous fungi are the most prominent lignocellulose degrading microorganisms and often have high capacity for enzyme secretion, their cellulose degrading enzymes have been a focal point of intensive research. This has made fungal cellulases important industrial enzymes with applications far beyond biorefinery and led to, e.g., several commercial cellulase formulations that can be used in different industrial contexts (Kuhad *et al.*, 2011).

In this chapter, we summarize information about cellulose and cellulose-degrading enzymes produced by fungi with examples of their current applications in food, feed, brewing, textile, laundry, and biorefinery industries. Moreover, we discuss how fungal cellulases and their production could be improved to meet the requirements of the competitive biotechnology market.

Cellulose

Cellulose is the major component of lignocellulose and the most abundant renewable polymer on earth. In addition, cellulose exists in the cell walls of algae (Samiee *et al.*, 2019) and oomycetes (Fugelstad *et al.*, 2009), and is produced by certain bacterial species (Wang *et al.*, 2019). It is a water-insoluble homo-polysaccharide composed of repeated β-D-glucopyranose (D-glucose) residues that are linked by β-1,4 glycosidic bonds. The cellulose polymer contains linear glucan chains consisting of up to 25,000 D-glucose residues. Cellulose microfibrils are formed when 15–45 parallel, unbranched glucan chains are joined by intermolecular hydrogen bonds. The cellulose microfibrils are cable-like bundles that have a high tensile strength and contain crystalline, paracrystalline and amorphous regions. Of these, the crystalline conformation of cellulose is the most recalcitrant making it highly resistant to enzymatic degradation (Held *et al.*, 2014).

In plant cell walls, the cellulose microfibrils are stabilized by hydrogen bonds and are surrounded by hemicellulose polymers that provide further structural strength by linking cellulose fibers into microfibrils. Hemicelluloses are the second most abundant polysaccharides in lignocellulose and, different from cellulose, they have a highly variable structure containing several heteropolymers of different five- and six-carbon monosaccharide units, i.e., pentoses (D-xylose, L-arabinose) and hexoses (D-mannose, D-glucose, D-galactose, L-rhamnose) that additionally can be acetylated (Klemm *et al.*, 2005). The cellulose-hemicellulose matrix is cross-linked through hemicellulose with lignin, an insoluble aromatic polymer and the most recalcitrant polymer of plant biomass, which provides structural reinforcement to the plant tissue and resilience to biological and physical attacks (Lacayo *et al.*, 2013; Barros *et al.*, 2015). This molecular architecture gives enormous stability to lignocellulose and makes the exploitation and further processing of its individual polymers challenging.

Cellulose Hydrolyzing Enzymes

Many fungi are natural decomposers that feed on plant biomass. They break down the complex cellulose polymers to D-glucose through concerted enzymatic action of different cellulases. The so-called classical cellulases are hydrolyzing enzymes which cleave the β-1,4 glycosidic bond in cellulose to release D-glucose monomers, cellobiose or cellooligosaccharides, and include endo-1,4-β-D-glucanase (EGL), exoglucanase and β-glucosidase (BGL) (Kumar *et al.*, 2008). The main representatives of exoglucanases are cellobiohydrolases (CBH) that release disaccharide cellobiose from the ends of the oligosaccharides (**Fig. 1**), while EGLs (EC 3.2.1.4) randomly hydrolyze the O-glycosidic bonds in cellulose chains resulting in long glucan chains (oligomers) of different length. There are two types of CBHs, acting unidirectionally on the long chain oligomers: CBHI (EC 3.2.1.176) cleave from the reducing end and CBHII (EC 3.2.1.91) from the non-reducing end of the oligosaccharide chain. The processivity, i.e., the average number of cleavages that the enzyme performs on a cellulose chain before it dissociates from the chain, has been reported to differ between fungal CBHs. While

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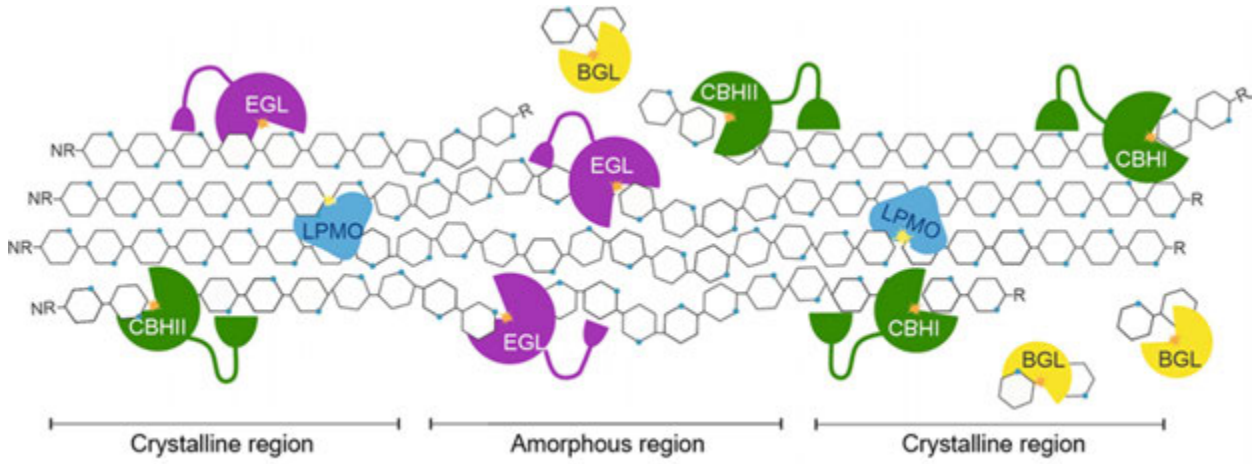

Fig. 1 Hydrolytic degradation of cellulose requires the complementary action of endo-1,4-β-D-glucanases (EGLs), cellobiohydrolases (CBHs) and β-glucosidases (BGLs). Oxidative enzymes, such as lytic polysaccharide monooxygenases (LPMOs), help to break the polymer by rendering access for CBHs and EGLs. NR = non-reducing end; R = reducing end.

processivity is possibly needed for effective degradation of crystalline cellulose, it decreases the speed of the overall hydrolysis (Igarashi *et al.*, 2011). The liberated cellobiose molecules are further hydrolyzed to D-glucose by BGLs (EC 3.2.1.21). Recently, lytic polysaccharide monooxygenases (LPMOs) that together with cellobiose dehydrogenase (CDH, EC 1.1.99.18) oxidatively cleave crystalline cellulose and render access to EGLs and CBHs, have been reported from various fungal species. We do not address LPMOs in this chapter, as they have recently been reviewed, e.g., by Chylenski *et al.* (2019) and Eijsink *et al.* (2019).

The three main cellulase enzymes belong to the glycoside hydrolase (GH) family of the Carbohydrate-Active enZYmes Database (CAZy; "see Relevant Website section"; Lombard *et al.*, 2014), in which enzymes are classified into amino acid sequence-based (sub-) families. Fungal EGLs are found in families GH5–10, 12, 26, 44, 45, 48, 51 and 74, whereas CBHs are present in GH5–7 and 48, and BGLs in GH1–3, 5, 9, 16, 30 and 39. Often these cellulases exhibit a specific two domain structure, with the catalytic domain (CD) connected by a peptide linker to the cellulose binding domain (CBD) or carbohydrate binding module (CBM). The CD contains the catalytic site of the enzyme, whereas the CBD/CBM facilitates the binding of the enzyme to its substrate.

Biotechnological Applications of Fungal Cellulases

Over many decades, cellulases have been utilized in various industrial applications making them the third most significant industrial enzymes on the global market after amylases and proteases (Menendez *et al.*, 2015). Use of enzymes is advantageous compared to chemical and mechanical processing, since enzymes are recoverable, specific, low in energy requirements and nonpolluting. In this context, cellulases can be widely applied in different industries including paper and pulp, detergent, and food and feed industries, among others (**Fig. 2**, **Table 1**). In the following sections, we will present and give examples of the biotechnology applications in which fungal cellulases are used.

Fungal Cellulases in Food, Feed and Beverage Industries

Food, feed and beverage industries are considered the major industries due to their significant contribution toward basic demands of people around the globe. The food processing industry, i.e., utilization of methods to turn fresh food into a product, is needed to retain or improve nutritional qualities of food, eliminate human health concerns and extend food storage time. In addition, processing improves food flavor, color and texture, and makes the packaging more convenient to handle (Anoop Kumar *et al.*, 2019). To achieve these targets, fungal enzymes have been successfully used in several applications involving plant-derived products. Cellulase complex - CBH, EGL and BGL - in combination with pectin and hemicellulose-acting enzymes comprise macerating enzymes that have important role, e.g., in the extraction of olive oil (Ricochon and Muniglia, 2010; Sharma *et al.*, 2015). The use of enzymes improves yield, enhances quality, increases the level of antioxidants, lowers probability of rancidity, and reduces wastage of starting material (Peres *et al.*, 2017). Additionally, as fruits in their natural form tend to rot fast, and processing them to juice or puree increases their shelf life, the addition of macerating enzymes yields better products and reduces browning (Sims and Bates, 1994). Cellulases also benefit the baking processes by reducing the size of cellulose fibers present in wheat and rye, which in turn softens the dough and reduces water absorption allowing for shorter baking times (Altunel and Ünal, 2017). Most cellulolytic enzymes used in the baking industry to break up roughage in dough originate from the ascomycete fungi *Aspergillus niger*, *Trichoderma reesei* and *Humicola insolens* (Fernandes, 2018).

Animal feed industry is another important branch in the global food industry by enabling large-scale livestock production resulting in increased availability of animal-based products to meet their rising demand. Lignocellulose biomass in form of, e.g., stover and straw

Fig. 2 Industrial fields using fungal cellulases.

Table 1 Examples of fungal species producing cellulases with industrial potential. CBH = cellobiohydrolase, EGL = endo-1,4-β-D-glucanase, BGL = β-glucosidase

Fungal species	*Examples of applications*	*Enzyme*	*References*
Aspergillus niger	Clarification of apple juice	CBH	(van den Broeck *et al.*, 2001)
	Saccharification/ethanol fermentation	EGL	(Xue *et al.*, 2018)
Aureobasidium pullulans	Improvement of wine aroma	BGL	(Baffi *et al.*, 2013)
Humicola insolens	Removal of soil particles from fiber	Alkaline cellulases	(Behera *et al.*, 2017)
Penicillium funiculosum	Biorefinery	CBH/EGL/BGL	(Ogunmolu *et al.*, 2017)
Penicillium sp.	Biofuel production	EGL/BGL	(Prasanna *et al.*, 2016)
Penicillium brasilianum	2nd generation ethanol	EGL/BGL	(Bazioli *et al.*, 2017)
Trichoderma reesei	Enzymatic saccharification of cellulose	CBHI (CEL7a)	(Divne *et al.*, 1994)
	Enzymatic wash/denim polishing	Acidic cellulase/neutral EGL	(Kuhad *et al.*, 2011)
	Bio-finishing of cotton textiles	CBHI/II	(Miettinen-Oinonen *et al.*, 2005)
	Bio-ethanol production	EGLI/CBHI	(Ellilä *et al.*, 2017)
	Paper recycling	EGLIII	(Dienes *et al.*, 2004)
	Deinking of paper waste	EGL	(Oksanen *et al.*, 2000)
Trichoderma harzianum	Degradation of cell wall of plant pathogens	EGL	(Lorito *et al.*, 1994)
	Saccharification of lignocellulosic materials	BGL	(da Silva Delabona *et al.*, 2013)
Trichoderma viride	Detergent additive	EGL	(Thapa *et al.*, 2020)
Trichoderma longibrachiatum	Biocontrol agent	EGL	(Migheli *et al.*, 1998)

has been used as animal feed for a long time, consisting mainly of low soluble carbohydrates and high content of fibers composed of cellulose, hemicellulose and lignin. Due to their symbiotic bacteria and fungi that are able to degrade cellulose and convert it into fatty acids, ruminants, e.g., cattle and sheep, can use plant biomass based feed for development and growth (Schingoethe *et al.*, 1999; Vicini *et al.*, 2003). However, the cellulose digesting ability of many mammals is very limited due to the absence of plant cell wall degrading enzymes. Thus, the major application of cellulases in the feed sector is to convert lignocellulose or plant materials into a more digestible

form. Commercial enzyme preparations, e.g., C500® (BIO-CAT, USA) that originates from *T. reesei* and *A. niger*, consist of cellulase mixtures and increase digestibility of grain and fiber by cleaving cellulose to smaller oligosaccharides and free glucose. Furthermore, various enzyme mixtures consist of both cellulases and xylanases, and break down cellulose and hemicelluloses present in the plant cell wall material. These preparations include, e.g., RONOZYME® MultiGrain, ROXAZYME® G2, RONOZYME® WX and RONOZYME® VP that are commercially available from DSM (The Netherlands). These enzymes can also be utilized to produce animal feed from other agricultural waste residues, such as rice bran, wheat straw and ficus fruit (Hatakka *et al.*, 1989; de Vries *et al.*, 2012).

The beverage industry is the largest food processing industry comprised of segments including alcoholic and non-alcoholic drinks. Approximately 90% of commercially sold ethanol is produced from natural sugars (e.g., D-glucose, D-fructose, sucrose) and starch present in plant cells (Alternative Fuels Data Center: Ethanol Production, 2019). As cellulose and hemicelluloses are composed of sugar monomers, the enzymatic breakdown of lignocellulosic biomass to fermentable sugars that can be used for production of alcohol has attracted attention.

In wine production, the addition of enzyme cocktails with a broad range of activities, such as hemicellulases, cellulase and pectinase, during the extraction process results in increased extraction and filtration rate, while consuming lower energy for the fermentation process to result in better wine stability with decreased viscosity (Villettaz, 1993; Bamforth, 2009; Espejo, 2021). Especially BGLs are used in the process of wine making, since they help releasing volatile compounds like terpenes, which are bound to glucoside fractions derived from grapes and skins, thus improving the aroma of Cabernet Sauvignon and Merlot (Francis *et al.*, 1998). One example are the BGLs from the ascomycete fungus *Aureobasidium pullulans*, which have a good activity at low temperature and seem to improve the wine aroma (Baffi *et al.*, 2013). Identification of an ethanol tolerant EGL from an *A. niger* strain isolated from a wine fermentation cellar demonstrated acidic pH optimum and good thermostability, thus having great potential for simultaneous saccharification (i.e., degradation of polymeric biomass into simple sugars) and ethanol fermentation (i.e., conversion of simple sugars into ethanol) (Xue *et al.*, 2018). Another important sector in the beverage industry includes the production and processing of juices.

Fruit juice is a product for direct consumption with the largest quantity of processed mature fruits by extraction (pressing or diffusion) of cellular content from fruit. In order to minimize the energy consumption during the extraction process and avoid incomplete extraction where part of juice is retained in pomace, enzymatic treatment is applied during the first step of fruit processing (Danalache *et al.*, 2018). Mash treatment uses pectinases in combination with cellulases and hemicellulases to produce pulps, nectars, and turbid or clear juices from various raw materials, such as apples, pears, stone fruits, berries and citrus fruits. The macerating enzymes acting on the soluble pectin and other cell wall polysaccharides allow the production of juice with lower viscosity (Bhat, 2000). Use of crude cellulases obtained from the fermentation fractions of the ascomycete species *Penicillium oxalium* and *T. reesei* for juice extraction from tomatoes resulted in enhanced yield of carotenoids with possible application as coloring agent in the food and beverage sector (Strati and Oreopoulou, 2014). Moreover, cellulase-based enzymatic hydrolysis of polysaccharides that are present in roasted coffee can be used as a cheaper and more environmentally friendly alternative to the energy-consuming thermal hydrolysis, and was tested with commercial enzyme preparations Celluclast (Novozymes A/S, Denmark), Powercell and Cellumax (Prozyn Biosolutions) in the production of instant coffee powder (Kumar, 2015).

Textile and Detergent Industries

Cellulases are one of the most common enzymes that are used in the textile industry. Novozymes A/S and DuPont (USA) are the key cellulase producers supplying these enzymes to the global market for industrial applications. Their main applications include biopolishing or biofinishing (e.g., depilling and hairiness removal) of cellulose-based fabrics, and biostoning (e.g., softening and fading) of denim. Biofinishing of cellulose-based fabrics with cellulases started in the 1980s and early 1990s. The removal of the surface fuzz from cellulose fibrils and microfibrils by enzymatic treatment results in superior hand-feel and finishes on rayon, linen and cotton knits. Decreased hairiness and increased evenness was observed after treatment of cotton yarns with purified *T. reesei* EGLs (Pere *et al.*, 2001). In addition, a fabric knitted from the enzymatically treated yarn showed decreased tendency towards pilling and higher moisture absorption (Behera *et al.*, 2017). The textile industry requires the enzymes to have process-appropriate biochemical properties, such as increased tolerance towards temperature and pH. Thermostable cellulases with many commercial applications are produced by *A. niger*, *H. insolens*, *Trichoderma viride*, *Trichoderma harzianum* and *T. reesei* (Karmakar and Ray, 2011).

Cellulases are used in the laundry and detergent industry mostly in a mixture with lipases and proteases. The ability of cellulases to remove small fuzzy fibrils from the surface of fabric leads to an improved performance of laundry detergents and results in improved color, brightness and texture. They can also enhance fabric quality, restore fabric softness and remove dirt from textiles. For example, alkaline cellulases from *H. insolens* remove soil particles present in interfibrillar spaces due to their ability to specifically and selectively interact with cellulose in the fiber space of cotton, flax, hemp and jute. In order to enhance the stability of cellulases in liquid detergents, anionic or non-ionic surfactants, citric acid or water soluble salt, proteolytic enzymes and mixtures of propanediol, and boric acid or its derivatives can be used (Behera *et al.*, 2017). In detergents, mainly EGLs originating from *T. reesei*, *T. viride*, *T. harzianum* and *A. niger* are used as additives (Thapa *et al.*, 2020).

Pulp and Paper Industry

Application of cellulases in the pulp and paper industry aims to, e.g., reduce the use of energy, improve paper quality and decrease the environmental burden caused by the pulping process. Reduced energy consumption in refining and improved pulp properties have been achieved by pretreating pulp with cellulases (García *et al.*, 2002; Kim *et al.*, 2006; Gil *et al.*, 2009). Significantly improved pulp

drainage for recycled kraft pulps was reported even at low dosage levels of *T. reesei* EGLs (Oksanen *et al.*, 2000). Also, *T. reesei* EGLs were detected to decrease pulp viscosity, but resulted in negative effect on tensile properties (Pere *et al.*, 1995).

A large number of chemicals are needed for deinking of recycled paper, making it an environmentally harmful and costly process (Virk *et al.*, 2013). To reduce the negative environmental impacts, use of cellulases, alone or together with xylanases, has been proposed as a sustainable alternative for deinking of different types of paper waste. The enzymatic treatment releases ink particles by partially hydrolyzing the fiber surface. This can also increase the relative hydrophobicity of the ink particles by removing small fibrils from their surface, which assists the separation of the particles during washing (Jackson *et al.*, 1996). In addition, the use of enzymes can lead to improvement of, e.g., dewatering and optical properties of pulp, but their excessive use may decrease paper strength and thus needs to be avoided.

The type of cellulase as well as the presence of a CBM have also been suggested to be important factors affecting the properties of enzyme-treated pulp from recycled paper (Dienes *et al.*, 2004). However, some cellulases, such as the commercial enzyme mixture Celluclast 1.5 (Novozymes A/S) derived from *T. reesei*, Iogen cellulase (Iogen, Canada) and a cellulase mixture of *T. viride* CCMI 84, have been shown to both remove ink and improve paper strength (Pala *et al.*, 2004). Cellulases from other *Trichoderma* species tested for deinking include a crude mixture of *T. harzianum* cellulases and xylanases that was able to remove ink from photocopier wastepaper pulp (Pathak *et al.*, 2015). In addition, an enzyme mixture consisting of cellulase and xylanase activities from *Trichoderma longibrachiatum* MDU-6 effectively removed chromophores, phenolics and hydrophobic compounds from newspaper pulp, but did not show significant deinking of examination or laser-printed paper pulps (Chutani and Sharma, 2016). The use of cellulases in pulp and paper industry would benefit from tailor-made enzymes and/or enzyme mixtures having more suitable biochemical properties, e.g., with respect to specificity and optimum pH.

Biorefineries

Cellulases play an essential role in converting lignocellulosic plant biomass feedstocks to biofuels and biochemicals in sugar platform based biorefineries. However, before the enzymatic depolymerization of cellulose and the subsequent fermentation or metabolic conversion of the released glucose molecules to valuable compounds, the recalcitrance of the lignocellulose matrix needs to be overcome. Thus, a pretreatment of lignocellulose either by physical, chemical or biological methods (for more details see, e.g., review by Seidl and Goulart, 2016) remains an essential step in these processes.

In biorefineries, cellulase cocktails are used, as a single cellulolytic enzyme cannot degrade the biomass efficiently enough, and often these cocktails aim to take advantage of the synergy between their monocomponent enzymes. In addition, one enzyme mixture is not optimal for different biomasses that vary in their chemical composition. Several commercial cellulase preparations are available for biorefineries and they typically have CBH as the major activity for degradation of crystalline cellulose (Champreda *et al.*, 2019). Most of the commercially available cellulases for biomass degradation are produced from derivatives of the hyper-cellulolytic *T. reesei* RUT-30 mutant strain (Peterson and Nevalainen, 2012). As *T. reesei* possesses, e.g., low BGL, hemicellulose and LPMO activities, its cellulases have been supplemented with activities from other fungi, such as those from the ascomycete species *Aspergillus awamori* (Gottschalk *et al.*, 2010), *Aspergillus aculeatus* (Suwannarangsee *et al.*, 2012), *Chaetomium globosum* (Wanmolee *et al.*, 2016), *H. insolens* (Kogo *et al.*, 2017) and *Penicillium funiculosum* (van Wyk, 1999), and the basidiomycete species *Laetiporus sulphureus* and *Pleurotus ostreatus* (Valadares *et al.*, 2016).

Other fungi used as sources for commercial cellulases applicable for biorefineries include *Talaromyces cellulolyticus* (formerly known as *Acremonium cellulolyticus*) and different *Penicillium* species. Compared to *T. reesei*, *T. cellulolyticus* produces higher BGL activity and has been shown to be effective in hydrolyzing different lignocellulose feedstocks (Fujii *et al.*, 2009). Several Penicillia have also been reported to produce enzyme mixtures that have better properties than *T. reesei* cocktails (Vaishnav *et al.*, 2018). *P. funiculosum* secretes CBH, EGL and BGL activities in well-balanced proportions (de Castro *et al.*, 2010), and CBHI from a hyper-cellulolytic *P. funiculosum* strain has been proposed as an alternative for the corresponding *T. reesei* enzyme in industrial cellulase cocktails (Ogunmolu *et al.*, 2017).

Further development of the properties and production of individual cellulases and their mixtures is still needed to make the biorefinery processes economically viable. For this, cellulases from alternative fungal and other microbial sources should be explored, particularly focusing on finding candidates that have higher specific activity and that are less susceptible to inhibition by glucose and lignin-derived compounds.

Agriculture

The applicability of fungal cellulases in agricultural biotechnology has also been explored. Supplementation of agricultural soil with cellulases has been reported to improve soil fertility by accelerating decomposition of plant residues in soil. For example, addition of commercial *T. reesei* cellulase to soil promoted degradation of rice and wheat straw as well as increased the amount of available nitrogen and phosphorous, and enhanced rice growth (Han and He, 2010). However, the use of cellulases to improve soil properties is not currently economically feasible. Other potential agricultural applications of cellulases include their use as biocontrol agents. *T. longibrachiatum* strains overexpressing an indigenous *egl1* gene have been studied as biocontrol agents against the oomycete *Phythium ultimum* that has cellulose as a major component of its cell walls. An increased EGL activity was reported to correlate with enhanced suppression of infection on cucumber seedlings (Migheli *et al.*, 1998).

Cellulases and other plant cell wall polysaccharides degrading enzymes can enhance the release and availability of bioactive compounds from plants by disrupting the structural integrity of the cell wall components. Therefore, enzyme-aided extraction provides a promising greener alternative by reducing the amount of chemicals and energy needed for the processing (Puri *et al.*,

2012). As an example, an *Trichoderma* spp. enzyme mixture containing cellulases and hemicellulases was shown to improve availability of phenolic compounds for subsequent methanol extraction from blackcurrant (*Ribes nigrum*) juice press residues (Kapasakalidis *et al.*, 2009). In addition, the use of the commercial cellulase mixture Celluclast 1.5 L (Novozymes A/S) from *T. reesei* together with the hemicellulose and pectinase mixture Peelzyme I (Novo Nordisk Ltd.) from *A. niger* and *T. reesei* enhanced recovery of polyphenols from rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) by 100% and 20%, respectively (Weinberg *et al.*, 1999). However, as, e.g., side activities in commercial enzyme preparations can reduce the yield or negatively affect the properties of the bioactive compounds, more specific enzyme cocktails tailored towards different plant materials are needed to further improve the extraction processes (Puri *et al.*, 2012; Phitsuwan *et al.*, 2013).

Improvement of Fungal Cellulases and Their Production

Even though several fungi are capable of cellulase production, the enzyme yield and amount of individual cellulase components are often not satisfactory for the above-mentioned industrial applications. Improvements in cellulase yield as well as the ability to tailor suitable ratios of the enzymes of the cellulase complex produced by fungi for individual processes is therefore important. In order to overcome these bottlenecks, different molecular engineering techniques have been successfully applied, and some examples of these are given in this section.

Forward Genetics

Forward genetics approaches that have been applied to induce genetic variation in fungi and through this improve their cellulolytic properties include UV- and chemical mutagenesis as well as adaptive evolution. For example, the use of random UV-mutagenesis yielded the hyper-cellulolytic *T. reesei* RUT-C30 strain (Montenecourt and Eveleigh, 1979). This mutant and another hyper-cellulolytic mutant *T. reesei* SS-II used in industry have recently been genome sequenced to identify genetic variations that may be involved in the high production of cellulases and thus, could contribute for further strain engineering (Peterson and Nevalainen, 2012; Liu *et al.*, 2019a). Another example of strain improvement using a combination of UV- and chemical mutagenesis is *Penicillium janthinellum* NCIM 1171 that was treated with ethyl methyl sulfonate (EMS) followed by UV-irradiation and resulted in the mutant EU1 with increased hydrolysis of microcrystalline cellulose, Avicel (Adsul *et al.*, 2007). Spore suspension of *Aspergillus oryzae* NRRL 3484 was also subjected to combination of UV-irradiation followed by chemical treatment to improve cellulase biosynthesis. As a result, a hyper-production strain UNAC4 was obtained that showed a 4-fold increase in cellulase activity compared to the wild type strain on rice straw (El-Ghonemy *et al.*, 2014). Another approach utilizing only chemical mutagens, 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) and ethidium bromide (EtBr), was used to improve cellulase activity of seven fungal strains isolated from soil. When the strains were exposed to sublethal concentrations of the mutagens for a prolonged period, stable mutants with improved cellulase production were obtained (Chand *et al.*, 2005).

An alternative approach to UV- and chemical mutagenesis is strain engineering by adaptive evolution that is based on the microorganisms' capability to adapt rapidly to various environmental conditions and enhance enzyme production (Schoustra *et al.*, 2009; Kutyna *et al.*, 2012). Based on this approach, an *A. niger* cellulase mutant was obtained resulting in a five times higher cellulose production than the parental strain (Patyshakuliyeva *et al.*, 2016). Transcriptomic analysis revealed that the expression of *noxR* encoding the regulatory subunit of the NADPH oxidase complex was reduced in the mutant compared to the parental strain. Subsequently, analysis of the *noxR* knockout strain showed the same phenotypic effect as observed for the adapted evolution mutant, confirming the role of *noxR* in cellulose degradation (Patyshakuliyeva *et al.*, 2016).

Protein Engineering

Other approaches to enhance cellulase activity include protein engineering by directed evolution, computer-guided rational and semi-rational methods, and adaptive evolution. Directed evolution is another random mutagenesis method, which requires multiple cycles of mutagenesis to generate a large library and subsequent high-throughput screening of the library for identification of the improved strains (Bornscheuer *et al.*, 2019). The rational approach creates a smaller mutant library, but is considered smarter as the design method is based on in-depth analysis of amino acid sequences and protein 3D structures, which reflects the desired enzymatic properties (Cheng *et al.*, 2015). On the other hand, the semi-rational design combines the benefits of directed evolution with computational analysis and suggests multiple, specific residues to be mutated based on prior knowledge on the structural function relationship to design "smart" libraries to engineer desired properties (Chica *et al.*, 2005). These directed evolution approaches have been utilized to improve the activity of EGLs (Liu *et al.*, 2013), CBHs (Liu *et al.*, 2014) and BGLs (Hardiman *et al.*, 2010). Even though these techniques seem to show promising results, a bottleneck is the limitation of the screening systems, where the capacity of a regular microtiter plate based screening is limited to the simultaneous screening of 10^3–10^4 variants (Longwell *et al.*, 2017).

Not only protein yield and enzymatic activity are desirable targets of improvement for industry, but also enzymes' thermostability and performance in non-conventional media. Methods like SCHEMA (structure-guided protein recombination) was successfully used to improve the thermostability of an EGL by 13°C without affecting activity (Heinzelman *et al.*, 2009; Moraïs *et al.*, 2016). In addition to thermostability, cellulase stability in non-conventional environments, e.g., in ionic liquids, seawater

with high salt concentration or organic solvents, is essential for their performance in various applications. An example of this are the ionic liquid tolerant cellulases from *T. reesei* improved via engineering of enzyme charge (Nordwald *et al.*, 2014).

Overexpression and Promoter Engineering

Homologous overexpression of *T. reesei* EGL was shown to significantly promote saccharification of pretreated corncob residues, which was even more pronounced when both EGL and BGL were overexpressed (Qian *et al.*, 2017). In *Penicillium oxalicum*, overexpression of nine *bgl* genes resulted in enhanced BGL activity and hydrolysis efficiency (Yao *et al.*, 2016). On the other hand, cellulase encoding genes from different fungi can be identified, cloned and heterologously expressed in fungal hosts to get a better combination or synergism of the corresponding cellulases. For example, a heterologously expressed BGL encoding gene from *Penicillium decumbens* led to improved cellulase activity in *T. reesei* (Ma *et al.*, 2011). In addition, cellulases from *T. reesei* have been successfully produced in the filamentous ascomycete *Ashbya gossypii* (Ribeiro *et al.*, 2010, 2013).

Another molecular engineering technique applied to improve cellulase activity is promotor engineering. CBH1 of *T. reesei* is the major component of all secreted proteins by this species. Therefore, the *CBH1* promotor has been widely used to induce hyper-production of target proteins in *T. reesei*, including BGL that is present at very low levels in *T. reesei* cellulase cocktails (Ma *et al.*, 2011; Li *et al.*, 2017). To improve promoter efficiency, binding sites within the *CBH1* promoter of *T. reesei* have been replaced with those of transcriptional activators (Zou *et al.*, 2012). When a fusion gene that consisted of *cbh1* from *T. reesei* and EGL encoding *e1* from the bacterium *Acidothermus cellulolyticus* was expressed under a control of this engineered promoter, higher enzyme activity was obtained.

Manipulation of Transcriptional Regulators

In filamentous fungi, the expression of cellulase genes is governed by several transcriptional factors (TFs) (Benocci *et al.*, 2017), thus making them attractive targets for molecular engineering to improve cellulase production. One possibility for TF manipulation is their constitutive expression. For example, the xylanase transcriptional activators Xyr1 and XLR-1 from *T. reesei* and *Neurospora crassa*, respectively, control the expression of cellulolytic and hemicellulolytic enzymes encoding genes, and heterologous expression of the constitutively active mutants of these TFs in *P. oxalicum* was shown to result in increased cellulase production in the host species (Xia *et al.*, 2019). Furthermore, a chimeric TF that contain the DNA binding domain of the cellulase transcriptional activator ClrB and the C-terminal sequence of the constitutively active mutant of the hemicellulolytic activator XlnR, was tested for cellulase production under non-inducing conditions in *P. oxalicum*. As a result, up to 7.3-fold higher cellulase activity was observed in the modified *P. oxalicum* strains compared to the parental strain (Gao *et al.*, 2017).

Increased production and activity of fungal cellulases can also be achieved through overexpression of TFs. When the XlnR encoding gene was overexpressed in the hyper-cellulolytic strain of *T. cellulolyticus*, enhancement of cellulolytic activity and expression of *cbhI* was observed (Okuda *et al.*, 2016). Simultaneous overexpression of two transcription activators, Xyr1 and Ace3, in *Trichoderma orientalis* EU7–22, was found to substantially improve the production of cellulases (Xue *et al.*, 2020). In addition, the overexpression of *xyr1* in *T. harzianum* resulted in increased hemicellulolytic activities and showed significantly improved saccharification of sugarcane bagasse (da Silva Delabona *et al.*, 2017).

Deletion or disruption of transcriptional repressors offers another strategy to improve cellulase production in fungi. A typical target for this is CreA/CRE1 that is the negative regulator of the universal carbon catabolite repression (CCR) regulatory system by which utilization of the energetically most favorable carbon sources is ensured (Benocci *et al.*, 2017). For example, deletion and truncation of *cre1* in *T. reesei* resulted in derepressed production of cellulases and xylanase (Nakari-Setälä *et al.*, 2009), and higher transcript and enzyme levels of cellulases and xylanase were detected after disruption of *creA* gene in *T. cellulolyticus* (Fujii *et al.*, 2009).

Epigenetic Engineering and CRISPR/Cas9

Another potential tool for industrial engineering is epigenetic engineering, which concerns the heritable changes in gene expression that do not involve changes in the underlying DNA sequences. Two levels of epigenetic regulation are DNA methylation and chromatin remodeling by histone modification (Waddington, 1942; Aghcheh and Kubicek, 2015). Even though there is no evidence for a regulatory role of DNA-methylation for *T. reesei*, some data is available on chromatin remodeling. While several approaches on remodeling of chromatin by transcriptional activators for improvement of cellulase expression have been discussed and several genes encoding chromatin remodeling enzymes have been identified in *T. reesei* (Gupta *et al.*, 2016; Mello-de-Sousa *et al.*, 2015), whether these genes could be improve cellulase production has not been tested yet.

Novel genome editing techniques, such as CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein-9 nuclease from *Streptococcus pyogenes*) (Doudna and Charpentier, 2014; Wang and Coleman, 2019), have already been developed for several cellulase-producing filamentous fungi, such as *A. niger* (Nødvig *et al.*, 2015), *T. reesei*, *Penicillium chrysogenum* (Pohl *et al.*, 2016), *N. crassa* (Matsu-ura *et al.*, 2015) and *Myceliophthora* sp. (Liu *et al.*, 2017; Liu *et al.*, 2019b). A gene encoding the negative regulator Cre1 has been deleted by CRISPR/Cas9 in the thermophilic species *Myceliophthora thermophila* and *Myceliophthora heterothallica* (Liu *et al.*, 2015). As a result, the deletion strains showed increased cellulase production and activities (Liu *et al.*, 2015). It is likely that there will be more examples of the use of the CRISPR technology to improve fungal cellulase production in near future.

Concluding Remarks

Fungal cellulases are important industrial enzymes that can be applied in various industrial sectors. Extensive research has concentrated on these enzymes already for decades, leading to, e.g., detailed mechanistic understanding of the action of hydrolytic cellulases (Igarashi *et al.,* 2011). However, the industrially produced cellulases originate from few fungal species and, considering that the estimated number of fungal species is between 2.2 and 3.8 million (Hawksworth and Lücking, 2017), only a very limited potential of fungal cellulases has been investigated. It is plausible to expect that efficient enzyme discovery approaches that take advantage of the ever-increasing big data together with new genome editing tools and synthetic biology approaches will provide opportunities to faster introduction of new cellulases to industrial processes and the use of tailor-made cellulases for highly specific applications.

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Further Reading

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Relevant Website

www.cazy.org
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Applications of Fungal Hemicellulases

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Introduction

Over the last few decades, microbial enzymes have gained a central role in the degradation of complex plant cell wall components either for developing an eco-friendly industrial process or in generation of value-added products from plant biomass. After cellulose, hemicellulose is the most abundant structural polysaccharide in plant cell walls making approximately 30% of its dry weight and is a perpetual by-product of agro-industries and forestry. The classification of hemicelluloses is based on the predominant monomeric sugar present in the backbone of the polymer (Dekker and Richards, 1976). Prominent hemicelluloses include xylans, xyloglucan, mannans and glucomannans. Among them, xylan, consisting of repeating units of xylose sugar linked with β-1→4 linkages, is the most abundant making up 15%–50% of cell dry weight. Xylan is classified into different categories based on the groups attached with the side chains. Homoxylan consists of xylose monomers bound by β-1→4 or β-1→3 linkages while arabinoxylans, occurring in cereals, have arabinose attached to xylose at C-2 or C-3 position. In glucuronoxylans found in hardwoods, every 10th xylose moiety is linked with 4-O-methyl-D-glucuronic acid. Acetyl xylan, found in beechwood and birchwood xylan, contains acetyl groups attached to xylose monomers. These acetyl groups of xylan help the polymer resist alkaline extraction. The last category is arabinoglucuronoxylan which is commonly present in grass where arabinose and glucuronic acid are substituents in different positions (Naidu *et al.*, 2018).

Mannan consisting of repeating mannose residues, occurs as storage polymer in some seeds and also as structural component of the plant cell wall (Krska and Larsbrink, 2020; Jana and Kango, 2020). Owing to glucose and galactose substitution, they are classified as glucomannan, galactomannan and galactoglucomannan. The hardwood glucomannan and softwood glucomannan have mannose-to-glucose ratio as 2:1 and 3:1, respectively (Soni and Kango, 2013a). The diversity and complexity of hemicellulose structure necessitates action of a wide range of enzymes for their degradation.

Fungi are natural decomposers of wood and elaborate immense number of extracellular enzymes for degrading complex polymeric substrates including hemicellulases. Over the last few decades, xylanases and mannanases sourced from different fungi have found novel and interesting applications in various industrial processes. Some major applications include bioconversion of lignocelluloses into fermentable sugars, upgrading of animal feedstock, clarification of fruit juices and wine, and facilitation of bio-bleaching of pulp (**Fig. 1**). As per the specific need of different applications such as bio-bleaching, bio-refinery, bio-scouring, de-inking, feed upgradation etc., a number of alkali-stable, acid-stable and thermostable hemicellulases of fungal origin have been characterized. According to the Association of Manufacturers and Formulators of Enzyme Product (AMFEP 2009), 60% of all the industrial enzymes are sourced from about 25 fungal genera, mainly *Aspergillus*, *Trichoderma*, *Penicillium*, *Rhizopus*, and *Humicola* (Kango *et al.*, 2019).

Fig. 1 Different industrial applications of fungal hemicellulases.

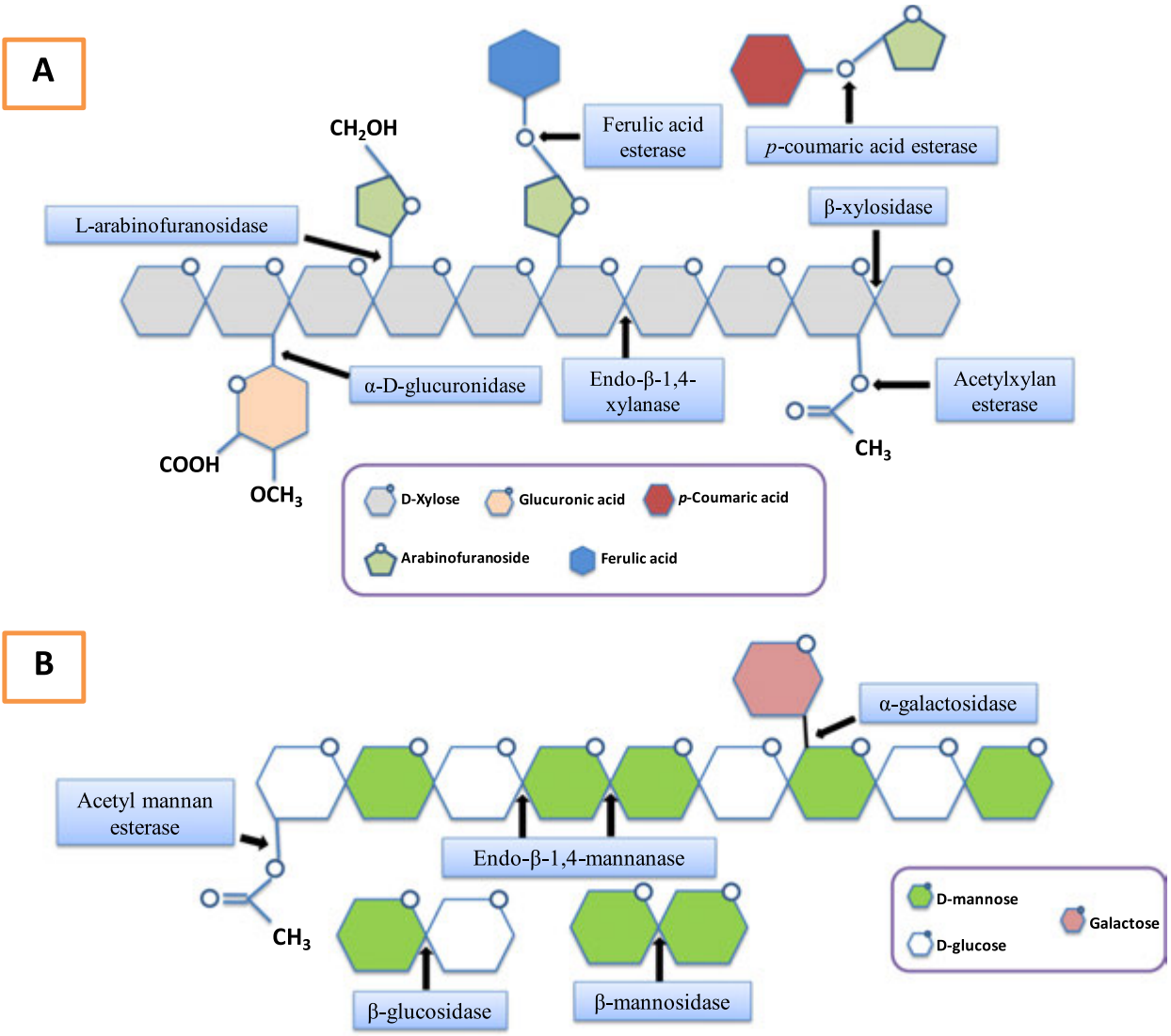

Fig. 2 Different types of fungal hemicellulases. A. Hemicellulases for complete breakdown of xylan, B. Hemicellulases for complete breakdown of mannan.

Hemicellulases are named on the basis of corresponding substrate acted upon by them, e.g., endo-xylanase, β-xylosidase, acetyl xylan esterase, endo-mannanase etc. Among them, the endo-acting enzymes, such as endo β-1→4 xylanase or endo β-1→4 mannanase, hydrolyze the internal linkages of the glycosidic bonds and disrupt the macromolecular backbone (**Fig. 2**). Being heteropolymeric, different factors like length, branching and nature of substituents in the hemicellulose, determine the efficacy of the enzyme action. In some case, de-branching or side-chain cleaving enzymes (e.g., α-galactosidase) help to disintegrate the substituents resulting in better substrate accessibility, while some de-branching enzymes act on oligomers generated after the action of endo-acting enzymes and thus assist in complete degradation of hemicelluloses. The main action of subsidiary enzymes is to cleave the groups attached with the main chain of hemicellulose structure where they exhibit co-operativity in their action (Soni and Kango, 2013b; Mäkelä *et al.*, 2018). For example, complete degradation of a complex xylan (glucuronoarabinoxylan or galactoglucuronoarabinoxylan) requires the concerted action of endo-xylanase, β-xylosidase (EC 3.2.1.37), acetyl xylan esterase (EC 3.1.1.72), α-arabinofuranosidase (EC3.2.1.55), ferulic acid esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), α-galactosidases (EC 3.2.1.23) and *p*-coumaric acid esterase (EC 3.1.1.73). Similarly, mannan hydrolysis requires β-mannosidase (EC 3.2.1.25), α-galactosidase (EC 3.2.1.22), β-glucosidase (EC 3.2.1.21) and acetylmannan esterase (EC 3.1.1.6) along with endo-β-mannanase (**Fig. 2**). Fungi are the most potent producers of extracellular lytic enzymes, including hemicellulases (Bhattacharya *et al.*, 2015). Interestingly, often a single fungus is found to produce a repertoire of hydrolases, including main chain hydrolyzing and accessory enzymes (Prajapati *et al.*, 2020). Multiplicity of endo-xylanases and endo-mannanases, seen in various fungi, suggests their versatility in degrading the hemicelluloses.

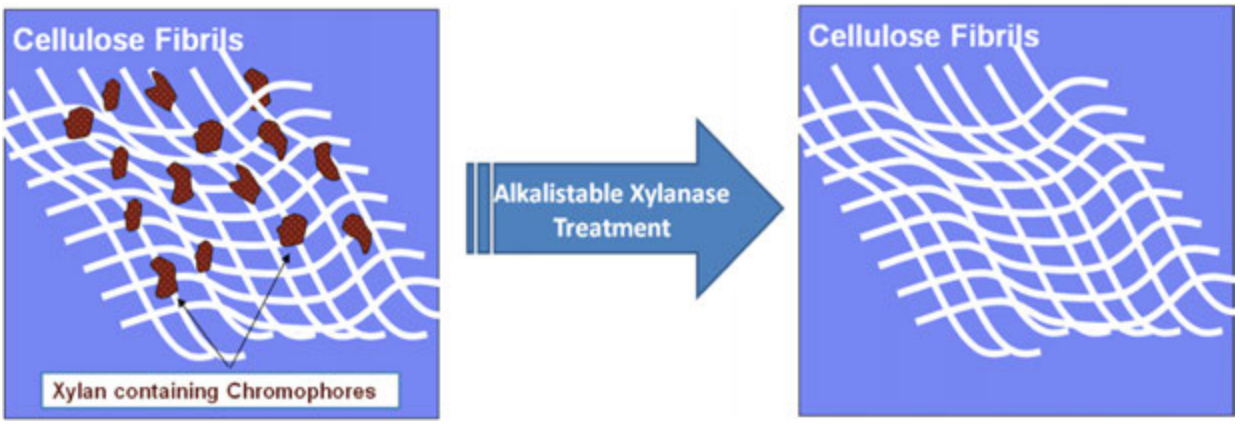

Fig. 3 In bio-bleaching, xylanase degrades xylan complex entrapped in the cellulosic fibers and enhances removal of chromophores.

Applications of Hemicellulases

Beginning with a technical application of xylanases in pulp and paper industry in bio-bleaching (Viikari *et al.*, 1994), fungal hemicellulases have found a number of applications in various industrial domains in the last three decades. Their hydrolytic activity is utilized either to remove or release unwanted xylan or mannan residues from plant biomass, poultry feed, dough, cotton etc. or to saccharify the hemicellulosic component for the release of monomeric sugars. The details of such applications are given in the following section.

Bio-Bleaching

In the kraft pulping process, pulp lignin is removed by a bleaching treatment leading to xylan precipitation on the outer surface of the cellulosic fibers (**Fig. 3**). Xylanases degrade the re-deposited xylan chromophores and enhance the bio-bleaching process (Suurnäkki *et al.*, 1997). Although bacterial xylanases offer some advantages over fungal xylanases due to their broad pH and temperature range, most of the commercial xylanases employed in the pulp industry are of fungal origin. Large scale production of thermostable fungal endo-xylanases to the level of 34.4 IU/g using *Aspergillus oryzae* has helped in making this choice (Chutani and Sharma, 2015). Different thermophilic fungal species *viz. Thermomyces lanuginosus*, *Humicola insolens*, *Malbranchea* sp., *Chaetomium thermophilum*, *Melanocarpus* sp. and *Thermoascus aurantiacus* are reported to produce thermostable endo-xylanases that may be exploited for bleaching process. In particular, thermostable cellulase-free endo-xylanase originating from *T. lanuginosus* has drawn considerable attention (Maijala *et al.*, 2012; Kumar *et al.*, 2016).

Xylanases are classified into glycosyl hydrolase (GH) families namely, GH8, GH10, GH11, GH12 and GH30, respectively (see "Relevant Websites section"). Endo-xylanases belonging to GH10 have better thermostability and high accessibility towards the xylan over GH11, which provides advantage in pulp bio-bleaching (Hu and Saddler, 2018). During the bleaching process or alkaline cooking, 4-O-methyl glucuronic acid present in xylan gets converted into hexenuronic acid which adds to the pulp yellowing necessitating its removal (Hutterer *et al.*, 2017). Endo-xylanases act on these brownish deposits and release xylan from cellulose fibers, thus leading to an increase in the whiteness of pulp. Endo-xylanase from *Penicillium oxalicum* has been shown to reduce the yellowing of wheat and poplar pulp (Zhang *et al.*, 2016). Another accessory enzyme, feruloyl esterase, augments the action of bio-bleaching xylanase by reducing the hindrance caused by side chains present in the polymer thus increasing the solubilization of lignocellulosic components (Dilokpimol *et al.*, 2016). For example, feruloylesterases from *Aspergillus terreus* have been shown to have wide range of activity on different esters making them potentially suitable for application in pulp and paper industry (Mäkelä *et al.*, 2018).

Recycling of printed paper is one of the major steps to address the environmental concerns related to perpetual cutting of trees for pulp. Removal of ink from the printed paper, e.g., newspaper, is called deinking and is the most difficult step in achieving the paper recycling. A typical enzyme-based deinking process involves pulping of the waste paper by hydrapulper followed by the addition of enzyme. Afterwards, the pulp is treated at 121°C for 15 min and then washed with excess of water to remove the ink residue. The release of ink is made easy by the degradation of hemicellulosic fibers by hemicellulases in the fibre-ink bonding region. Endo-xylanase treatment is an eco-friendly and effective intervention for removal of ink particles from the paper fibers replacing hazardous chemical treatment. The enzyme treatment is shown to cause reduction in effective residual ink concentration and kappa number while increasing the paper brightness. Treatment with cellulase reduces paper strength, while laccase causes yellowing of the paper and hence, endo-xylanase makes a suitable de-inking agent without compromising paper quality (Desai and Iyer, 2016). Endo-xylanases from fungi, namely *A. niger* (Desai and Iyer, 2016), *Trichoderma harzianum* (Pathak *et al.*, 2014), and *Trichoderma longibrachiatum* (Chutani and Sharma, 2016) are reported to be effective in deinking process. Often, the combinatorial approach involving cellulase, xylanase, amylase and lipase has been found more efficacious (Xu *et al.*, 2009; Dutt *et al.*, 2012). A crude endo-xylanase preparation from the basidiomycete, *Coprinopsis cinerea*, used for deinking also resulted in improved brightness (Dutt *et al.*, 2013).

Bio-Scouring

Cotton fiber, having multifarious applications, is the most valuable raw-material for the textile industry. Natural cotton has some hemicelluloses, lignin, fats, pectin, etc. as impurities. These impurities are removed by a pretreatment process consisting of three steps *viz.*, de-sizing, scouring and bleaching. In the scouring process, the removal of non-cellulosic substances clinging to the cellulosic fibers results in a softer and finer fabric (**Fig. 3**). Similar to the pulp treatment, conventional chemical treatments impart adverse effects on the fabric quality parameters such as fabric strength, tenacity and whiteness along with the eventual release of harmful chemicals in the environment. Endo-xylanase based bio-scouring is eco-friendly, energy saving and non-damaging to the cellulosic material. It is important to ensure that xylanase should be cellulase-free, so that the cotton polymer remains unaffected. Several fungal xylanases have been explored in the textile industry. Treatment of cellulosic fiber with endo-xylanase from *T. longibrachiatum* was shown to effectively improve bio-scouring, de-sizing and bio-finishing of the fiber without needing any additives (Abd El Aty *et al.*, 2018). In addition, combination with pectin degrading enzymes helped to improve cotton wetting ability and decreased the enzyme load in the treatment (Puranen *et al.*, 2014). In case of less fiber accessibility, EDTA is applied in the bio-scouring process because it modifies the internal structure of the polymer by removing calcium ions from pectin that reduces polysaccharide cross-bridges and steric hindrance for enzyme action. EDTA concentration is selected in such a way that it does not affect the main enzyme adversely (Choudhury, 2014).

Biorefining

The process by which different value-added products are obtained from valorization of biomass is termed as biorefining. Enormous amounts of lignocellulosic biomass generated as a waste or by-product of agro-industry, forestry etc. can serve as renewable bio-resource for generation of bioethanol, phenolics and fermentable simple sugars such as glucose and mannose (Den *et al.*, 2018). The hemicellulose portion of the lignocellulosic biomass can be degraded into simpler constituents and exploited using fungal hemicellulases. Agro-industrial waste products such as corn cob, sugarcane bagasse, copra meal, palm kernel cake and spent coffee grounds, contain high amount of hemicelluloses. In absence of appropriate technology, these are disposed instead of being valorized. Valorization of hemicelluloses usually involves two sequential steps. At first, the hemicellulose polymer is converted into simple monomeric sugars and next, these sugars are transformed into different value-added products. For example, xylan and mannan are hydrolyzed into xylose and mannose, respectively, and then further converted into bio-ethanol and xylitol using different biological or chemical methods (**Table 1**).

Pentose sugars present in hemicelluloses are not effectively utilized by the yeast *Saccharomyces cerevisiae*, which is routinely used for ethanol production at industrial level. Some other yeasts, however, are able to ferment xylose into xylitol through the enzymatic action of xylose reductase. Further, xylitol dehydrogenase converts this xylitol into xylulose, which is then fermented by the yeast into ethanol *via* pentose phosphate pathway (PPP) and glycolysis (Matsushika *et al.*, 2008). *Candida* spp. are natural D-xylose consumers and have more reduction oxidation balance during xylitol accumulation than *S. cerevisiae* (Morales-Rodriguez *et al.*, 2016). For example, sugarcane bagasse hydrolysate containing xylose was obtained from treatment with an *Aspergillus tubingensis* enzyme complex and it was converted into bio-ethanol using *Candida shehatae* (Prajapati *et al.*, 2020). One of the main problems for yeast cells is the presence of glucose repression and their sensitivity to high ethanol concentration. Yeast cells use Snf3/Rgt2 signaling pathway as a sensory cascade for detecting glucose level in the surrounding medium. The high glucose concentration controls the glucose uptake for maintaining the intracellular glucose homeostasis through this signaling pathway (Kayikci and Nielsen, 2015). Similarly, high ethanol produced in the medium inhibits the yeast growth and increases the cell death. High concentration of ethanol also influences the macromolecular biosynthesis, protein accumulation and functioning of glycolytic enzymes adversely (Stanley *et al.*, 2010).

An ascomycetous yeast, *Spathaspora passalidarum*, has been shown to ferment both xylose and glucose with high xylose fermentation efficiency due to allosteric activation of glycolysis and high affinity to NADH and xylose by xylose reductase (Long *et al.*, 2012). Similarly, some xylose fermenting white rot fungi such as *Peniophora cinerea* and *Trametes suaveolens* (Okamoto *et al.*, 2010), *Hohenbuehelia* sp. (Liang *et al.*, 2013) and *Schizophyllum commune* (Horisawa *et al.*, 2015) converted hemicellulose hydrolysate into bio-ethanol (**Fig. 4**).

Table 1 Fungal enzymes used in biorefinery industry and their final products

Substrate	*Enzyme*	*Organism*	*Final product*	*Reference*
Beechwood xylan and pretreated straw lignocelluloses	β-xylosidase, endo-1,4-β-xylanase	*Aspergillus niger*	Bioethanol	Boyce and Walsh (2018)
Wheat biomass	β-xylanase	*Fusarium* sp.	Bioethanol	Juodeikiene *et al.* (2012)
Wheat Straw	Spezyme (CP) containing xylanase	*Trichoderma reesei*	Xylitol	Liavoga *et al.* (2007)
Palm kernel cake	mannanase	–	Biobutanol	Shukor *et al.* (2016)
Wheat straw hydrolysate	–	*Aspergillus carbonarius*	Citric acid	Yang *et al.* (2016)

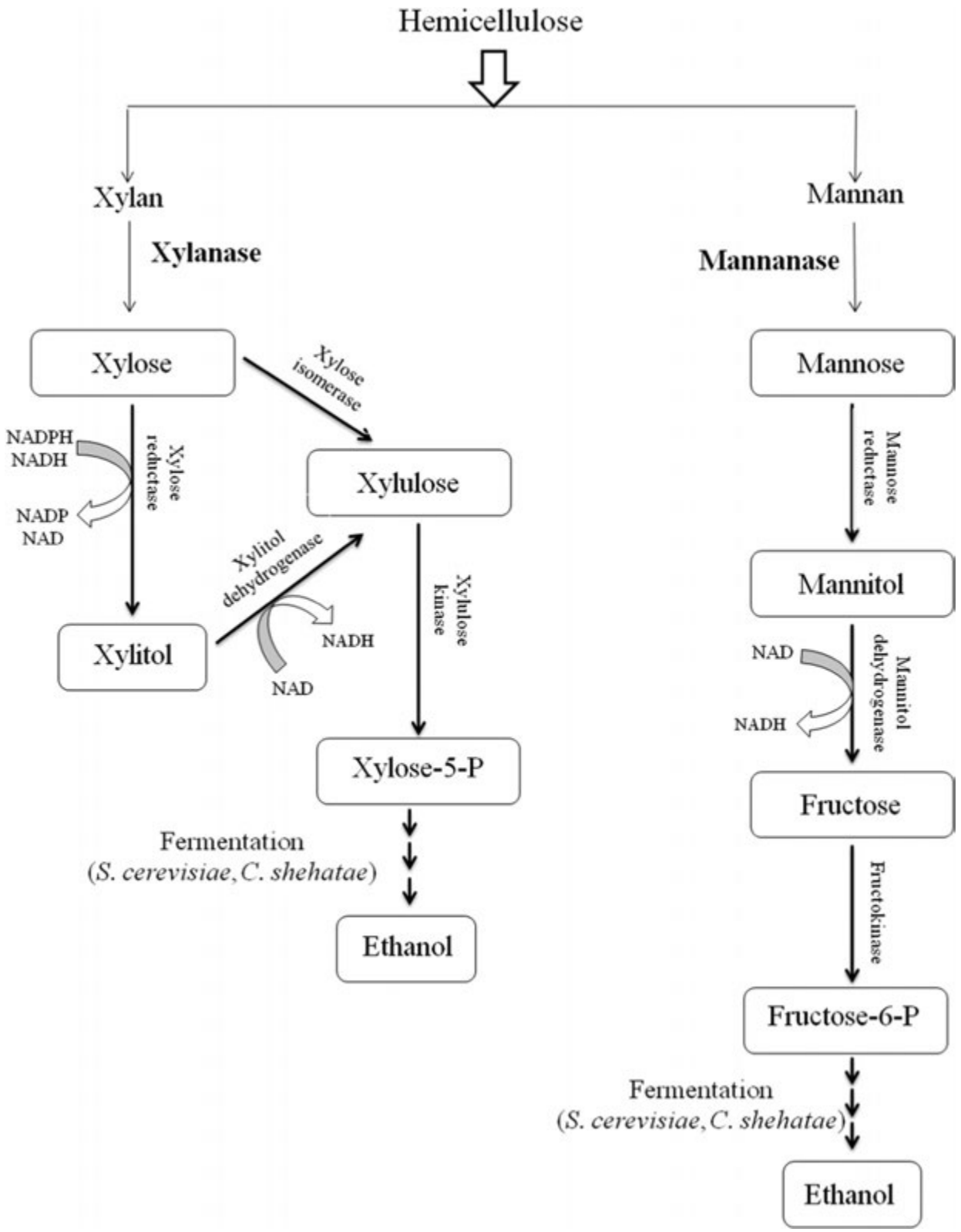

Fig. 4 Xylitol, mannitol and ethanol production from hemicelluloses (xylan and mannan) using fungal hemicellulases and yeasts in bio-refinery.

Table 2 Commercial hemicellulases used in juice industry and their fungal sources

Trade name	*Manufacturer*	*Composition*	*Source*
Pectinex Superpress	Novozymes	Pectin transeliminase, polygalacturonase, pectin esterase, hemicellulases	*Aspergillus niger*
Pectinex Ultra SP-L	Novozymes	Pectolytic and hemicellulolytic enxzymes	*Aspergillus aculeatus*
Rapidase BE Super	DSM Gist-Brocades Delft	Pectinases and hemicellulases	*A. niger*
Vinozyme G	Novozymes	Pectin lyase, polygalacturonase, hemicellulase and cellulase	*A. niger*
Cytolase 219	Danisco and Genencor	Pectinase, cellulase, hemicellulase	*A. niger and Trichoderma longibrachiatum*

Wine and Fruit Juice Clarification

In the wine and juice industry, hemicellulases such as xylanases and mannanases are mainly used for pigment extraction, minimizing haze formation, maceration of fruit skin, and overall clarification of wines and different fruit juices. Hemicellulases are used in these industries to obtain better juice quality and stability by decreasing the amount of crystallization of different carbohydrate macromolecules extracted from fruit cell walls (Beveridge and Wrolstad, 1997). In case of maceration of juice, macerating enzymes help liquefaction of fruit pulp and reduce the processing time. In addition, these enzymes also help to clarify the juice by lowering the juice viscosity (Sharma *et al.*, 2017). Hemicellulases having acid-stable character are thus required for the process. An acid stable endo-xylanase from *Gloeophyllum trabeum* decreased the mash viscosity and improved the filtration rate (Wang *et al.*, 2016a). Usually, the enzyme cocktails employed for wine and fruit juice clarification consist of pectinase blended with different hemicellulases, as they have been shown to be more efficient (Koffi *et al.*, 1991) (**Table 2**). The commercially available enzyme cocktails such as Pectinex Superpress, Pectinex Ultra SP-L, Rapidase BE and Vinozyme G. Pectinex Ultra SP-L contain hemicellulases from *Aspergillus aculeatus* or *A. niger* (Landbo and Meyer, 2004). A cellulase-free endo-xylanase belonging to GH11 from *Fusarium* sp. was shown to be effective in clarifying orange juice (Li *et al.*, 2020). A hemicellulolytic blend from *Aspergillus quadrilineatus* immobilized on aluminum oxide pellets has been shown to enhance quality of different fruit juices by reducing turbidity and improving sugar content (Suryawanshi *et al.*, 2019). Similarly, an enzyme mixture containing endo-xylanase from *A. niger* immobilized on magnetic nanoparticles has been shown to clarify papaya juice and was reusable upto 3–4 cycles (Muley *et al.*, 2018).

Oligosaccharide Generation

Due to increasing awareness regarding harmful effects of antibiotics and role of prebiotics in modulation of intestinal flora, recent years have witnessed a growing trend in the use of prebiotic oligosaccharides as nutraceuticals (Jana *et al.*, 2021). Besides conventional prebiotics like fructooligosaccharides (FOS), oligosaccharides derived from lignocellulosic biomass are also looked upon as upcoming prebiotics. Among these, xylooligosaccharides (XOS) and mannooligosaccharides (MOS) obtained from the enzymatic hydrolysis of xylan and mannans are receiving considerable attention (Suryawanshi and Kango, 2021). The controlled hydrolysis of lignocellulosic biomass using fungal hemicellulases allows the use of the agricultural waste for generation of value-added next generation prebiotic oligosaccharides (**Table 3**). Hemicellulases from *Trichoderma* and *Aspergillus* are being exploited for this purpose (Florencio *et al.*, 2016). Thermophilic fungi produce themostable enzymes that work efficiently at high temperatures thus help lowering chances of contamination. Different thermophilic fungi such as, *Myceliophthora heterothallica* (de Oliveira Simoes *et al.*, 2019), *T. lanuginosus* (Shi *et al.*, 2019), *Mycothermus thermophilus* (Ma *et al.*, 2017) and *Thermobifida fusca* (Wang *et al.*, 2015) have been shown to produce thermostable endo-xylanases, while *P. oxalicum* is reported to produce a thermostable endo-β-mannanase (Liao *et al.*, 2014). Interestingly, in many cases, end products generated by the fungal hemicellulase action are very well defined. Endo-xylanase from *A. flavus* liberated only xylobiose and xylopentose from beechwood xylan (Chen *et al.*, 2019). Similarly, *Kitosopra* sp. endo-xylanase generated only xylooligosaccharides from bagasse without any xylose formation (Rahmani *et al.*, 2019). *A. terreus* endo-β-mannanase hydrolyzed locust bean galactomannan (LBG), guar galactomannan (GG) and konjac glucomannan (KG) with mannotriose as the predominant oligosaccharide in case of LBG and GG, while mannotetraose was the prominent oligosaccharide generated from KG (Soni *et al.*, 2016). A multi-tolerant endo-β-mannanase from a well-known GRAS fungus, *Aspergillus oryzae* was able to hydrolyze different mannans into mannooligosaccharides (Jana *et al.*, 2018). Three endo-xylanases from *Penicillum chrysogenum* worked in a synergistic manner and produced XOS having different degree of polymerization (DP) (Yang *et al.*, 2019).

Guar gum is obtained from *Cyamoposis tetragonolobus*, a legume, grown mainly in India (80% of the total production) and some Asian countries (Yoon *et al.*, 2008). Fungal endo-mannanases hydrolyze guar gum into partially hydrolyzed guar gum (PHGG) that is heat tolerant, water soluble, acid-stable and taste-free dietary fiber (Niv *et al.*, 2016). PHGG consumption has been shown to alter bowel microflora favorably in animals and humans, and confer beneficial effects in treatment of irritable bowel syndrome, relief in constipation, and stimulation of growth of probiotic bacteria (Mudgil *et al.*, 2014). PHGG supplementation brings about significant increase in the population of probiotic bacteria, *Bifidobacterium* and *Lactobacillus* (Tuohy *et al.*, 2001). Guar gum contains about 38% galactose and thus its effective hydrolysis necessitates α-galactosidase activity. *P. oxalicum* produced both endo-β-mannanase and

Table 3 Fungal hemicellulases (endo-xylanases and endo-mannanases) indicated in the generation of oligosaccharides

Types	*Source*	*Substrate*	*Degree of polymerization*	*References*
Xylooligosaccharides (XOS)	*Trichoderma* species	Brewers' spent grain	X2 to X5	Amorim *et al.* (2019)
	Phoma sp.	Corn cob	X2 to X5	Wu *et al.* (2018)
	Aspergillus foetidus	Corn cob	X2 to X5	Chapla *et al.* (2012)
	Aspergillus flavus	Beechwood xylan	X2 and X5	Chen *et al.* (2019)
	A. brasiliensis	Rice rhusk	X2, X4 and X5	da Silva Menezes *et al.* (2017)
	Chrysoporthe cubensis	Oat spelt xylan	X2 and X3	de Sousa Gomes *et al.* (2017)
	Penicillium occitanis	Corncob xylan	X2 and X3	Driss *et al.* (2014)
	Mycothermus thermophilus	Beechwood xylan	X2 and X3	Ma *et al.* (2017)
	Talaromyces cellulolyticus	Beechwood xylan	X2 and X3	Nakamichi *et al.* (2019)
	Thermobifida fusca	Birchwood xylan	X2 and X5	Wang *et al.* (2015)
Mannooligosaccharides (MOS)	*Aspergillus oryzae*	Locust bean gum, Guar gum, Konjac gum	M2 to M4	Jana *et al.* (2018)
	Malbranchea cinnamomea	Locust bean gum, Konjac gum	M4	Ahirwar *et al.* (2016)
	Aspergillus quadrilineatus	Locust bean gum	M2 to M4	Suryawanshi *et al.* (2019)
	Penicillium oxalicum	Locust bean gum, Guar gum, Konjac gum	M2 to M6	Liao *et al.* (2014)

*X2-Xylobiose, X3-Xylotriose, X4-Xylotetraose, X5-Xylopentose, M2-Mannobiose, M4-Mannotetraose, and M6-Mannohexose.

Table 4 Different fungal alkali-stable xylanases and mannanases

Enzyme	*Fungal species*	*pH Optimum*	*Range of pH stability*	*References*
Endo-xylanase	*Thielaviopsis basicola*	5.5	5.0–10.0	Goluguri *et al.* (2016)
	Aspergillus oryzae	5.0	3.0–10.0	Bhardwaj *et al.* (2020)
	Aspergillus fumigatus	5.0	4.0–10.0	Deshmukh *et al.* (2016)
	Fusarium sp.	5.0	5.0–10.5	Li *et al.* (2020)
	Myceliophthora sp.	6.0	4.4–7.6	Chadha *et al.* (2004)
Endo-mannanase	*Rhizomucor miehei*	7.0	4.0–10.0	Katrolia *et al.* (2013)
	Bispora antennata	6.0	4.0–11.0	Liu *et al.* (2012)
	Humicola insolens	5.5	5.0–12.0	Luo *et al.* (2012)
	Aspergillus oryzae	6.0	5.0–10.0	Sakai *et al.* (2017)
	Penicillium freii	4.5	4.0–12.0	Wang *et al.* (2012)

α-galactosidase, and generated DP 2–7 mannooligosaccharides from guar galactomannan (Kurakake *et al.*, 2006). The formation of MOS (M3, M4) and PHGG using *A. oryzae* endo-β-mannanase has been demonstrated from guar gum (Jana *et al.*, 2018). PHGG contains higher molecular weight mannooligosaccharides and is reported to confer physiological health benefits. An endo-β-mannanase from *Rhizomucor miehei* generated PHGG at higher rates yielding 90.6% of total dietary fiber (Li *et al.*, 2017).

Detergent Additives

Easy and quick cleaning of the sticky organic dirt from clothes and dishes without using harmful chemicals is very challenging. Different stains arising from tomato sauce, coffee and tea usually contain hemicelluloses, especially mannan. Mannans are used as thickeners in various foods and due to their gelling nature the food dirt acquires very sticky nature and is very hard to clean from clothes and utensils. Fungal mannanases degrade the mannan content of the organic dirt and thus enhance the detergents' cleaning performance. Detergents are alkaline in nature and hence it is desirable to use alkali-stable mannanases. Alkaliphilic microorganisms are one of the major sources of alkali stable enzymes, however, some mesophilic microorganisms are also reported to produce alkali-stable hemicellulases (**Table 4**). Fungal species like *R. miehei* (Katrolia *et al.*, 2013), *Penicillium freii* (Wang *et al.*, 2012), *H. insolens* (Luo *et al.*, 2012) and *Biospora antennata* (Liu *et al.*, 2012) are known to produce alkaline endo-mannanase. Consequently, other alkaline active enzymes such as cellulases, amylases and xylanases can also be used with mannanases for better performance in washing.

Bakery

In the bakery industry, xylanases are used to enhance the dough specific volume, elasticity, water absorption, and improve the extensibility, softness and chewiness of the product. Xylanases which have capability to solubilize the water insoluble wheat

arabinoxylan are the most advantageous for dough treatment. Treatment of dough with endo-xylanase from *Penicillium citrinum* liberated xylan and created a continuous gluten network and the large starch molecules were found absorbed on the evenly distributed protein network (Ghoshal *et al.*, 2017). Sometimes, combination of different enzymes, including endo-glucanase, phytase, amylase and protease is applied together with endo-xylanase for achieving better dough performance. Commercial xylanase powder (Pentopan Mono BG®) from *A. oryzae* when administered with amylase and cellulase, increased dough stability, extensibility and stickiness (Liu *et al.*, 2017). A commercial thermostable endo-xylanase from *T. lanuginosus*, Novozyme 899, is widely used in baking industry to improve the crumb structure and dough stability (Chadha *et al.*, 2019). Endo-xylanase from *Myceliophthora thermophila* (previous name *Sporotrichum thermophile*) helped to increase the concentration of various metal ions in the bread dough (Bala and Singh, 2017). Similarly, endo-xylanase from *Chaetomium* sp. improved specific volume of Chinese steamed bread along with decreased crumb firmness and spread ratio (Jiang *et al.*, 2010). In addition, hardness and chewiness of Chinese steamed bread dough decreased after treatment with endo-xylanase from a salt-tolerant mangrove-isolated fungus, *Phoma* sp. (Wu *et al.*, 2018).

Animal Feed Up-Gradation

The animal feed industry is one of the fastest growing industries with an annual turnover of over USD 400 billion (see "Relevant Websites section"). Use of fungal enzymes in this industry for feed up-gradation has a well-grounded future. Animal feed contain raw cereal grains which have significant amounts of arabinoxylan and mannan as part of endosperm cell wall. These polymers impart anti-nutritional properties as they increase the viscosity of the feed and result in its low mobility in the digestive tract and colonization of pathogenic bacteria. Hemicellulose degrading enzymes are not secreted by the animals' digestive system and hence extraneous addition of fungal xylanases and mannanases helps in reducing the feed viscosity leading to improved mobility, digestion and animal growth. At the same time, the hemicellulase treatment hydrolyzes the otherwise indigestible hemicellulose fraction of animal feed into short chain carbohydrate oliogmers that enhance the growth of beneficial microbes in the gut. Acid stable fungal hemicellulases are more suitable for such applications as they perform better in the acidic condition prevalent in the digestive tract. Some acid stable endo-xylanases have been reported from *Penicillium sclerotiorum* (Knob and Carmona, 2010), *Bispora* sp. (Luo *et al.*, 2009a), *Phialophora* sp. (Zhang *et al.*, 2011) and *Aspergillus sulphureus* (Yang *et al.*, 2017). Similarly, acid stable endo-mannanases are reported from *Bispora* sp. (Luo *et al.*, 2009b), *Phialophora* sp. (Zhao *et al.*, 2010), *Penicillium pinophilum* (Cai *et al.*, 2011), *B. antennata* (Liu *et al.*, 2012), *A. niger* (Sornlake *et al.*, 2013), *G. trabeum* (Wang *et al.*, 2016b) and *Aspergillus kawachii* (Liu *et al.*, 2020). One of the commercially available heat stable commercial enzymes used in feed treatment is Ronozyme WX CT (endo-xylanase) which is derived from thermophilic *T. lanuginosus* (Nielsen *et al.*, 2008).

Conclusions and Future Perspectives

Hemicellulases, such as xylanases and mannanases, have gained importance as they have become an integral part of several commercial products such as detergents and feed additives, and industrial applications such as bio-bleaching, bio-scouring, paper deinking and prebiotic oligosaccharide generation. Fungi, being natural plant biomass decomposers, possess a variety of endo-xylanases and endo-mannanses along with accessory enzymes. The desirability of obtaining thermostable and alkali stable hemicellulases in larger amounts has triggered search for novel fungal sources and optimized production methods. Fungal hemicellulases hold a great promise in valorization of large pool of plant biomass and agro-industrial wastes like palm kernel cake, copra meal, and corn cobs for generation of value-added products like oligosaccharides, alcohols and, readily fermentable simple sugars (mannose, xylose).

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Applications of Fungal Pectinases

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Introduction

Enzymes that degrade the plant cell wall (PCW) have been extensively studied because of their central role in the plant-pathogen interaction. However, the production, properties and applications of these enzymes has gained enormous interest as a result of well-documented biotechnological potential (Lara-Márquez *et al.*, 2011; Edwards and Doran-Peterson, 2012; Latarullo *et al.*, 2016; Troiano *et al.*, 2020). Particularly, fungi well-known as producers of lytic enzymes such as pectinases, hemicellulases, and cellulases, have been widely studied because to the advantages of these organisms as models and the inherent characteristics of their lytic systems (Conejo-Saucedo *et al.*, 2010; Lara-Márquez *et al.*, 2011; Troiano *et al.*, 2020). The demand for such enzymes and their application in various industrial processes is largely due to their high efficiency in removing PCW components and their environmental benefits. Some of the advantages of fungi to produce these enzymes include their fermentation capacity, the production of large amounts of extracellular enzymes, the ease of cultivation, and the low cost of enzyme production in bioreactors (de Vries and Visser, 2001; Lara-Márquez *et al.*, 2011). In this context, most commonly used fungi are species and strains of the *Aspergillus* genus though other fungi have also been proven as efficient enzyme producers (Belda *et al.*, 2016; de Vries *et al.*, 2017; Amin *et al.*, 2019). Among these enzymes, pectinases are widely used in industrial applications such as fruit juice and wine clarification, tea and coffee fermentation, cotton bio-scouring, degumming of plant fibers, oil extraction, biofuel production, treatment of industrial wastewaters containing pectin, and synthesis of a number of value-added products (Edwards and Doran-Peterson, 2012; Kohli and Gupta, 2015; Amin *et al.*, 2019). Here we review the applications of pectinolytic enzymes produced by filamentous fungi and the strategies for their application and improvement of industrial processes.

Pectin Structure and Depolymerization

Pectin is the main component of the middle lamella of the PCW, where it is involved in functions such as adhesion between cells with active division (cell expansion), morphogenesis, porosity, ion binding, pH and ion balance, seed hydration, leaf abscission, fruit development, defense, among others (Mohnen, 2008; Lara-Márquez *et al.*, 2011). The pectin content of plant cell walls varies between dicots and nongraminaceous monocots (30%–35% dry weight [dw]), monocots such as grasses (2%–10% dw), and wood (5% dw), and different plant tissues depending of their function and stage of development (Mohnen, 2008; Voragen *et al.*, 2009). In immature fruits, insoluble pectin binds to cellulose resulting in stiffening of the cell wall. Later, during the ripening pectin is modified by plant pectinases, which soften the cell wall (Kashyap *et al.*, 2001). Pectin represents a large part of the biomass in ripe fruits and vegetables (>30%) (Hoondal *et al.*, 2002).

Pectin is mainly composed of homogalacturonan (HG; ~65%) followed by domains of xylogalacturonan (XGA; 20%–35%), and in minor proportion (~10%) rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Mohnen, 2008). HG is a domain of approximately 100 galacturonic acid residues (GalA) with α-1,4 bonds, methyl-esterifications at C-6, or acetyl-esterification at O-2 and O-3 (Mohnen, 2008). XGA is a HG backbone with 25%–75% of the GalA units substituted at C-3 with xylose and occasionally at C-4 with a second xylose residue (Mohnen, 2008). RG-I, is a heteropolymer consisting of -α-D-GalA-1,2-α-L-Rha-1,4- disaccharide units, whose GalA residues can been highly acetylated in O-2 and/or O-3, and with 20%–80% of the rhamnose (Rha) residues substituted with chains of arabinose and galactose of variable size, and occasionally with chains of fucose and glucuronic acid (Mohnen, 2008). RG-II consists of a main chain of HG with side chains formed by around 12 different sugars (arabinose, galactose, xylose, fucose, aceric acid, glucuronic acid, apiose, etc.) (Mohnen, 2008). The structural domains of pectin interact between themselves and with other molecules such as calcium, borate, polyamines, phenolic compounds, hemicellulose and cellulose (Popper and Fry, 2008).

The degree of pectin esterification can be low (<50%), medium/partial (50%–70%) and high (>70%), depending on the origin, growth and maturity stage of fruits, vegetables or tissues (Ralet *et al.*, 2003). Polysaccharides of highly-esterified pectin establish weak interactions by hydrogen bonds and hydrophobic forces through methyl groups, whereas low-esterified pectin

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Table 1 CAZy families to which fungal pectinases belong

Enzyme	*E.C. number*	*CAZy Family*
PGA	3.2.1.15	GH28
PGX	3.2.1.67	GH28, GH4
PLY	4.2.2.2	PL1, PL2, PL3, PL9, PL10
	4.2.2.9	
PEL	4.2.2.10	PL1
PME	3.1.1.11	CE8
RGAE	3.1.1. –	CE12
FAE	3.1.1.73	CE1/unclassified
RHG	3.2.1.171	GH28
RGX	3.2.1.173	GH28
RGL	4.2.2.23	PL4, PL9, PL11, PL26
RHA	3.2.1.174	GH78
GAL	3.2.1.89	GH53
	3.2.1.164	GH5, GH30
XGL	3.2.1.145	GH43
LAC	3.2.1.22	GH2, GH35
	3.2.1.23	GH42
ABF	3.2.1.55	GH2, GH51, GH43, GH62, GH2
ABN	3.2.1.99	GH43
ABX	3.2.1. –	GH93

PGA, Endo-polygalacturonase; PGX, Exo-polygalacturonase; PLY, Pectate lyase; PEL, Pectin lyase; PME, Pectin methyl esterase; AE, Acetylesterase; RGAE, Rhamnogalacturonan acetylesterase; FAE, Feruloyl esterase; RHG, Rhamnogalacturonan hydrolase; RGX, Rhamnogalacturonan galacturonohydrolase/exorhamnogalacturonase; RGL, Rhamnogalacturonan lyase; RHA, Rhamnogalacturonan rhamnohydrolase/α-rhamnosidase; GAL, Endogalactanase; XGL, Exogalactanase; LAC, α- and β-galactosidases; ABF, α-L-arabinofuranosidase; ABN, Endoarabinanase; ABX, Exoarabinanase.

makes bonds between calcium and free carboxyl groups (Willats *et al.*, 2006). When HG polymers link to each other, pectin forms a gel with a crystalline network that traps water depending on factors such as temperature, degree of methyl-esterification and acetylation, pH, sugar and calcium concentrations (Willats *et al.*, 2006).

Pectin degradation depends on several carbohydrate active enzymes (CAZymes) belonging to different glycoside hydrolase (GH), polysaccharide lyase (PL), and carbohydrate esterase (CE) families of the CAZy database (See Relevant Website Section) (**Table 1**). Polygalacturonases (PGs) break glycosidic bonds between GalA residues and are classified as endo-PG (PGA) and exo-PG (PGX) (**Fig. 1(A)**) (de Vries and Visser, 2001). Pectate lyases (PLYs) and pectin lyases (PELs) degrade polygalacturonate and esterified pectin from HG, respectively, through β-elimination at the non-reducing end of pectin (**Fig. 1(A)**). PLY activity is specific for non-esterified pectin and Ca^{2+}-dependent whereas PEL activity is specific for methyl-esterified pectin and Ca^{2+}-independent. Arg^{236} in PEL seems to play a similar role as Ca^{2+} (Jayani *et al.*, 2005). The acetyl, methyl and feruloyl residues of the pectin are removed by pectin methylesterases (PMEs), acetylesterases (AEs), rhamnogalacturonan acetylesterases (RGAEs), and feruloyl esterases (FAEs) (**Table 1**, **Fig. 1(A)**). It is known that the efficiency of the degradation of pectin by PGs and PLYs is largely dependent on the activity of PMEs (de Vries and Visser, 2001).

The rhamnogalacturonan hydrolase (RHG) break the α-D-GalA-1,2-α-L-Rha bonds in RG-I, while rhamnogalacturonan lyase (RGL) degrades α-L-Rha-1,4-α-D-GalA bonds in RG-I (**Fig. 1(B)**). Rhamnogalacturonan rhamnohydrolase/α-rhamnosidase (RHA) and rhamnogalacturonan galacturonohydrolase/exorhamnogalacturonase (RGX) break the α-D-GalA-1,2-α-L-Rha bonds in rhamnogalaturonan oligosaccharides from the nonreducing end (**Fig. 1(B)**) (de Vries and Visser, 2001).

Accessory enzymes acting on the side chains of RG-I and RG-II include endogalactanases (GALs), exogalactanases (XGLs), α- and β-galactosidases (LACs), α-L-arabinofuranosidases (ABFs), endoarabinanases (ABNs), and exoarabinanases (ABXs) (**Table 1**, **Fig. 1(A)**) (de Vries and Visser, 2001; de Vries, 2003).

Pectinase Production

Fungi produce pectinolytic enzymes for diverse purposes. The differences in enzyme production and specialization in depolymerization of substrates could be influenced by fungal lifestyle, environment and host. For instance, phytopathogenic fungi secrete pectinases during plant infection to overcome the PCW, the principal barrier for infection and invasion of host tissues. In fact, polygalacturonases are considered as virulence factors (Nakajima and Akutsu, 2014; Villa-Rivera *et al.*, 2017) .

Saprotrophic fungal strains with pectinolytic capacity have been isolated from a variety of environments rich in lignocellulosic biomass such as forest soils (Banakar and Basaiah, 2012), municipal wastes (Banu *et al.*, 2010), agricultural waste, leaf litter,

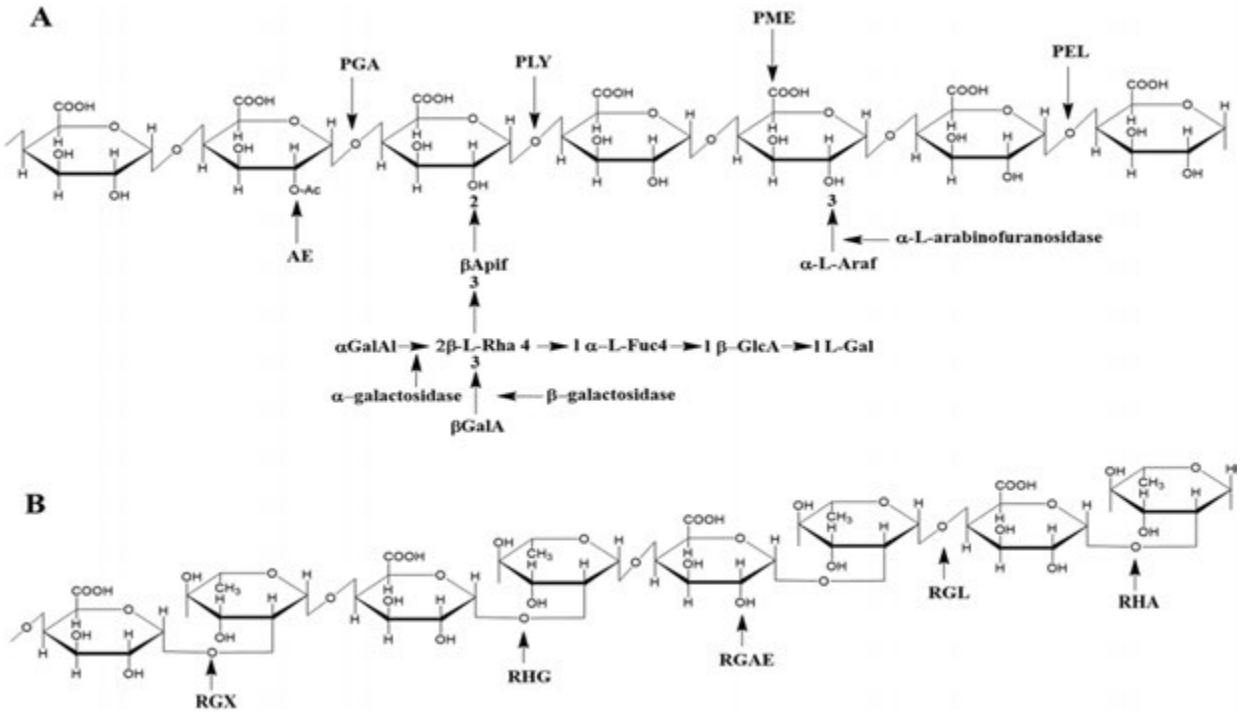

Fig. 1 Structure of pectin and sites of attack by pectinolytic enzymes. (A) pectinases that degrade homogalacturonan (HG) backbone and rhamnogalacturonan II (RG-II) side chains. PGA: Endo-polygalacturonase; PLY: Pectate lyase; PME: Pectin methyl esterase; PEL: Pectin lyase; AE: Acetylesterase. (B) pectinases that degrade rhamnogalacturonan I (RG-I). RGX: Rhamnogalacturonan galacturonohydrolase/exorhamnogalacturonase; RHG: Rhamnogalacturonan hydrolase; RGAE: Rhamnogalacturonan acetylesterase; RGL: Rhamnogalacturonan lyase; RHA: Rhamnogalacturonan rhamnohydrolase/α-rhamnosidase. Adapted from Mohnen, D., 2008. Pectin structure and biosynthesis. Current Opinion in Plant Biology 11, 266–277.

farmyard manure and cow dung (Anisa *et al.*, 2013), composts (Handa *et al.*, 2016), and decomposing fruits (Barman *et al.*, 2015; Amilia *et al.*, 2017). A wide variety of agro-industrial wastes that results from citrus fruit processing and pollute the environment, have been used as substrate for cheap pectinase production at large scale. These residues are easily accessible, have a low cost and their degradation constitutes an ecofriendly method that generate diverse monomers useful for different biotechnological purposes, thus contributing to reduce waste accumulation and pollution (Amin *et al.*, 2019).

Species of *Aspergillus* have been exploited commercially and are considered the principal hemicellulase and pectinase producers (Amin *et al.*, 2019; Troiano *et al.*, 2020). Due to wide biotechnological uses of these enzymes, new fungal strains able to produce higher pectinase amounts are explored. Also, recent efforts are focused on the improvement of fermentation conditions for increasing fungal growth and pectinase production. Accordingly, the amount of substrate, nitrogen and carbon sources, particle size of substrate, temperature, pH, inoculum size, mixing frequency, and time of incubation are among the factors evaluated (**Table 2**) (Amin *et al.*, 2019).

A diversity of pectin-containing agro-wastes such as groundnut oil, mustard oil, neem oil, black and green gram peels, wheat bran, finger millet waste, pearl millet residues, broken rice, banana peels, apple pomace, orange and lemon peels or mixtures of them have been tested as carbon sources for fungi (**Table 2**). Peels of fruits have been shown to be the best substrate for pectinase production due to their high pectin content. Moreover, addition of pectin to mixtures of natural substrates such as wheat bran + potato starch, or wheat straw + extracts of mosabi, orange, and lemon peels, remarkably increases pectinase production by *Aspergillus niger* (Akhter *et al.*, 2011; Khan *et al.*, 2012). Finally, it has been demonstrated that a pretreatment of orange peels using microwave and surfactants enhance the production of extracellular enzymes including pectinases in *Aspergillus japonicus* (Li *et al.*, 2015c).

Improvement of fermentation media for optimum pectinase production have been carried out in several fungal species and strains (**Table 2**). Solid and submerged state of fermentation have been tested with good results; conveniently, it has been reported that solid state fermentation could be less affected by catabolic repression than submerged cultures, and therefore produce higher amounts of pectinase (Sandhya and Kurup, 2013). A strategy implemented to decrease glucose content in the medium and therefore minimize catabolic repression was to co-culture *A. niger* GJ-2 with *Saccharomyces cerevisiae*, yielding a considerable increase of PG activity (Zhou *et al.*, 2011). The statistical Response Surface Methodology (RSM) approach applied in optimization of *Penicillium oxalicum* pectinase production using citrus peel in submerged fermentation, showed that temperatures of 35–39°C had more influence than NH_4Cl concentration (0.5–1.5 g l^{-1}) in enzyme production (Li *et al.*, 2015b). RSM application in *Aspergillus ibericus* pectinase production using citrus peel in submerged fermentation, showed a relationship between substrate concentration, temperature, pH, and incubation time for the optimal production of enzyme. After, immobilized purified fungal pectinase on functionalized nanoporous activated carbon, with a maximum immobilization capacity of 3360 U g^{-1}, showed

Table 2 Fermentation conditions for fungal pectinase production

Species	*Enzyme*	*Medium*	*Carbon source*	*Nitrogen source*	*Yield*	*Temperature (°C)*	*pH*	*Time of incubation*	*Reference*
Aspergillus tamarii	PGX	Solid	Mango peels	NA	141.0095 U g^{-1}	40–70	5	3, 6, and 9 days	Amande (2013)
	PEL			NA	5670.50 U g^{-1}	60	7.5	6 days	
Aspergillus niger	PGX	Submerged	Orange waste peel	NA	117.1 ± 3.4 μM ml^{-1} min^{-1}	30	5.5	ND	Ahmed *et al.* (2016)
A. niger MTCC 281	PGX	Submerged	Bhimkol banana peel	$(NH_4)_2SO_4$ 0.04%	6.6 U ml^{-1}	32.37	ND	65.82 h	Barman *et al.* (2015)
A. niger LFP – 1	PGX	Solid	Pomelo peel	NH_4NO_3 1.2%	8.9 U g^{-1}	30	ND	5 days	Darah *et al.* (2013)
Rhizopus sp. C4	PGX	Solid	Orange peel	NH_4NO_3 0.43%	11.63 IU ml^{-1}	30	ND	7 days	Handa *et al.* (2016)
Penicillium oxalicum PJ02	PGA	Solid	Orange peel	NH_4Cl 1 g L^{-1}	0.62 U ml^{-1}	36.5	ND	ND	Li *et al.* (2015b)
	PGX				36.88 U m^{-1}		ND	ND	
Aspergillus ibericus	PGX	Submerged	Citrus pectin	$(NH_4)_2SO_4$ 1.4 g L^{-1}	69.9 U m^{-1}	40	4	120 h	Mahesh *et al.* (2016)
Aspergillus oryzae	PLY	Solid	Lemon peel waste	NA	875 U ml^{-1}	40	5	3 days	Koser *et al.* (2014)
Fusarium sp. PSTF1	PGX	Submerged	Fructose	$NaNO_3$ 2 g L^{-1}	81.97 μmol ml^{-1}	28	6	72 h	Purnachandra and Saritha (2015)
A. niger LB – 02-SF	PGX	Submerged	Citrus pectin	$(NH_4)_2SO_4$ 5 g L^{-1}	14 U ml^{-1}	28	2.6	22 h	Reginatto *et al.* (2017)
Penicillium digitatum	PEL	Solid	Lemon peels	$(NH_4)_2SO_4$ 0.05 g L^{-1}	527.47 U ml^{-1}	30	8	5 days	Siddiqa *et al.* (2018)
Penicillium notatum	PEL	Solid	Wheat bran, Maltose	$(NH_4)_2SO_4$ 1%	1875 U gds^{-1}	30	6	120 h	Siddiqua *et al.* (2015)
Aspergillus flavus	PGX	Solid	Pineapple	NA	15.55 U ml^{-1}	35	5.5	72 h	Thangaratham and Manimegalai (2014)
Aspergillus tubingensis	PGX	Solid	Sucrose	$NaNO_3$ 3%	15.9 U ml^{-1}	35	4	6 days	Tai *et al.* (2013)

PGA, Endo-polygalacturonase; PGX, Exo-polygalacturonase; PEL, Pectin lyase; PLY, Pectate lyase; NA, No added; ND, No determined; gds, Grams of dry substrate.

Table 3 Biochemical characteristics of pectinases purified from fungi

Species	*Enzyme*	*MW (kDa)*	*Optimum pH*	*Optimum Temperature (°C)*	K_M	*Vmax*	*Reference*
Aspergillus parasiticus	PLY	41[a]	4	50	10.86 mg ml^{-1}	1493 U mg^{-1}	Yang *et al.* (2020)
Aspergillus niger	PEL[b]	40[a]	4.5	50	ND	ND	He *et al.* (2018)
Aspergillus giganteus	PEL	71[a] 63[c]	8.5	50	4.6 mg ml^{-1}	1428.6 U mg^{-1} min^{-1}	Pedrolli and Carmona (2014)
Penicillium digitatum	PEL	ND	5	35	0.3 μmole ml^{-1}	100 U ml^{-1}	Siddiqa *et al.* (2018)
Penicillium notatum	PEL	ND	8	65	ND	0.487 μmol min^{-1}	Siddiqua *et al.* (2015)
Fusarium decemcellulare MTCC 2079	PEL	45 ± 01[a]	9	50	0.125 mg ml^{-1}	ND	Yadav *et al.* (2014)
Fusarium lateritum MTCC8794	PEL	16	10	40	0.79 mg ml^{-1}	0.57 IU	Yadav *et al.* (2017)
Leucoagaricus gongylophorus	PGX	39[c]	5	60	0.65 mg ml^{-1}	1800 μm min^{-1} mg^{-1}	Adalberto *et al.* (2016)
Rhizoctonia solani AG2–2	PG	55[a]	4.4	30	3.5 mg ml^{-1}	ND	Al-Rajhi (2013)
A. niger MTCC478	PGX	124	4	50	2.3 mg ml^{-1}	ND	Anand *et al.* (2017a)
Aspergillus flavus MTCC 7589	PGX	29[a]	5	40	2.08 mg ml^{-1}	ND	Anand *et al.* (2017b)
Penicillium oxalicum	PGA	38[a]	5	60–70	1.27 mg ml^{-1}	5504.6 U mg^{-1}	Cheng *et al.* (2016)
Aspergillus sojae	PGA	47[a]	3.3–5	50	0.134 mg ml^{-1}	9.6 μmol mg^{-1} min^{-1}	Fratebianchi *et al.* (2017)
Neosartorya fischeri	PGX[b]	72[a]	3.5	65	2.9 mg ml^{-1}	ND	Li *et al.* (2015a)
	PGA[b]	34[a]	4.5	70	1 mg ml^{-1}		
Aspergillus niveus	PGX	102.6	4.0–6.5	50	6.7 mg ml^{-1}	230 U mg^{-1}	Maller *et al.* (2013)

[a]Molecular mass calculated by SDS-PAGE.
[b]Recombinant protein.
[c]Molecular mass calculated by gel filtration.
PLY, Pectate lyase; PEL, Pectin lyase; PGA, Endo-polygalacturonase; PGX, Exo-polygalacturonase; ND, No determined.

better thermal and storage stability than free pectinase (Mahesh *et al.*, 2016). Also, nylon scouring pad cubes have been used as a matrix of support for immobilization of *A. niger* HFD5A-1 cells, increasing pectinase production to about 335% in submerged fermentation systems (Ibrahim *et al.*, 2014). Another strategy to increase PG activity in *Aspergillus sojae*, was the addition of 20 g l^{-1} of aluminum oxide microparticles to shake flask fermentation medium, resulting in 2.2-fold higher levels of activity than control, a result that could be important to pectinase production in large scale bioreactors (Karahalli *et al.*, 2017).

Improvement of a medium for solid state of fermentation consist in the use of a column-tray bioreactor containing 70% of moisture and substrate particle size of 0.7–2 mm. When implemented in *Aspergillus* and *Penicillium* strains, this process resulted in high levels of fungal biomass and pectinase production (Ruiz *et al.*, 2012). On the other hand, a pilot scale packed bed bioreactor with up 30 kg of dry sugarcane bagasse was implemented in *A. niger* CH4 solid state fermentation, and may represent a low cost alternative for pectinase production (Pitol *et al.*, 2016). Finally, it has been reported that the implementation of intermittent agitation provide uniformity across the packed bed bioreactors, increasing pectinase production in solid state fermentation (Finkler *et al.*, 2017).

As pectinases have important industrial applications, they have been purified and characterized from native or heterologous systems. Some of their biochemical properties are summarized in **Table 3**, where it can be seen that molecular mass, optimum pH and temperature of characterized pectinases are very variable. Thermostable and thermophilic enzymes typically are the most useful for industrial applications. However, there are applications where low temperatures favor the quality of the final product. Additionally, the use of active enzymes at low temperatures eliminate the need to heat the system and decrease the energy required (Adapa *et al.*, 2014; Li *et al.*, 2017). Therefore, fungi capable of producing these types of enzymes are constantly being sought for use in biotechnological processes.

Biotechnological Applications

Juice Production

The viscosity in ripe fruits is a problem that hinders the filtration process producing a low yield and retention of molecules and pigments that contributes to the flavor and properties of juices (Adapa *et al.*, 2014). Juice extraction produces suspended insoluble particles formed by negatively charged pectin associated with proteins that prevents their precipitation (García, 2018). Filtration, decantation and centrifugation methods are common practices used for clearing or depectinization of juice extracts (Domingues *et al.*, 2012; García, 2018). However, the application of pectinases improves the quantity of the extracted juice, releases positively charged molecules, which aggregate and precipitate, makes liquefaction more efficient, increases the amount of soluble solids, reduces viscosity, facilitates filtration and clarification, increases acidity (citric acid) and the mount of anthocyanin and total

Table 4 Effects of pectinase treatment of some fruit juice extracts

Fruit	*Fungus*	*Enzyme*	*Application effect*	*Reference*
Apple	*Aspergillus awamori*	PGX	93% increased transmittance 38% viscosity reduction	Dey and Banerjee (2014)
Orange	*Aspergillus niger*	PGX PEL PME	38.2% turbidity and color reduction 31.6% viscosity reduction	Salim *et al.* (2018)
Banana	*Penicillium oxalicum*	PGA	31.76% viscosity reduction, 6% transmittance increase	Cheng *et al.* (2017)
Lemon	*Penicillium occitanis*	PGX	77% viscosity reduction 47% turbidity reduction	Maktouf *et al.* (2014)
Mango	*P. oxalicum*	PGA	5% transmittance increase	Cheng *et al.* (2017)
Pineapple	*A. niger*	PGX	1.5%–0.8% turbidity reduction	Tochi *et al.* (2009)
Blue berry	*A. niger*	PG	1.7%–20.4% transmittance increase 1.9%–4.8% sugar content increase.	Kant *et al.* (2013)
Papaya	*P. oxalicum*	PGA	78.36% viscosity reduction 43.3% transmittance increase	Cheng *et al.* (2017)
Grape	*Talaromyces leycettanus*	PGA PGX	140% efficiency in pectin degradation 14%–82% transmittance improvement	Li *et al.* (2017)

PGA, Endo-polygalacturonase; PGX, Exo-polygalacturonase; PEL, Pectin lyase; PME, Pectin methylesterase.

carbohydrates, and increases color intensity and phenolic content (Sharma *et al.*, 2017; García, 2018). Then, the combined use of enzymatic degradation and filtration or centrifugation methods is a useful common practice (Sharma *et al.*, 2017; García, 2018). Some effects of pectinase addition to juice extracts such as change in clarity, viscosity, and turbidity are shown in **Table 4**. Juice fruits with high soluble pectin content such as banana or papaya are difficult to press, so the use of pectinases decreases viscosity and allows pressing and obtention of a better amount of juice (Garg *et al.*, 2016). In cases such as bitter gourd juice, which is considered medicinal with anticancer, antidiabetic, antimalarial, and anti-inflammatory properties, incubation with *A. niger* pectinases increases the amount of juice, allow the release of bioactive molecules such as phenolics, vitamins, minerals, α-amylase and α-glucosidase inhibitors (considered as antidiabetic), important for medicinal action (Deshaware *et al.*, 2017).

Wine Production

Pectinases can be used in several steps of the wine production process, for instance, addition to the pulp after fruit trituration, and before and after fermentation. Commonly, enzymatic preparations containing pectinases and cellulases known as liquefaction mixtures are added to the pulp, where they contribute to the degradation of plant tissue, the breakdown of the cell wall, and the release of pigments and phenolics from the grape skin (Sieiro *et al.*, 2012; Nighojkar *et al.*, 2019). Before and during fermentation pectinases allow sedimentation of suspended particles resulting in more clear wines, and their application after fermentation promotes flocculation and precipitation of pectin particles, microorganisms, and proteins (Nighojkar *et al.*, 2019). Addition of enzymatic mixtures during wine production also improve characteristics such as the presence of phenolic components, anthocyanins, catechin, color intensification and stabilization, aroma etc. (Sieiro *et al.*, 2012).

Olive Oil Extraction

Pectinases and other PCW degrading enzymes can increase oil production from olives and improve its organoleptic characteristics (Hadj-Taieb *et al.*, 2012). In olives, which have high content of pectin and hemicellulose, the use of enzymatic preparations of *Penicillium occitanis* with pectinase activity, and enzymatic extracts from *Trichoderma* sp. with cellulase and xylanase activities render good results. Enzymatic treatment increases the amount of olive oil extracted by 1.5%; moreover, the synergistic effect of cellulase, xylanase, and pectinase activities increase the content of polyphenols and pigments (Hadj-Taieb *et al.*, 2012). Similarly, the use of

an extract with pectinolytic activity from *Aspergillus giganteus*, increases the extraction of olive oil by 15.3%, and decreases viscosity and turbidity (70%) (Ortiz *et al.*, 2017).

Tea and Coffee Production

Typically, tea production uses mechanical maceration, but partial breaking of the cells in leaves can result in low quality tea. Pectinase treatment improves the characteristics of tea, because degradation of pectin improves the release of important components such as theaflavin, thearubigen, and caffeine (Murugesan *et al.*, 2002). When applied to the tea maceration process, raw pectinase extracts obtained from *Aspergillus indicus*, *Aspergillus flavus*, and *Aspergillus niveusdurante*, increase its quality by 5% (Angayarkanni *et al.*, 2002). The pectinases from *A. niger* and *Mucor circinelloides* used in the tea fermentation process increase the amount of phenolic compounds and quality of the beverage (Thakur and Gupta, 2012).

Coffee production uses a fermentation process to remove the mucilage layer of the internal mesocarp, which may contain up 2.8% pectin. The use of *A. niger* pectinases reduces the process time, while maintaining grain quality (Murthy and Naidu, 2011).

Extraction of Phytopigments

Some analyses have shown that the use of commercial mixtures of pectinesterase, polygalacturonase, and pectin lyase from *A. niger* in the degradation of plant tissues improves the pigment extraction and avoids oxidation (Çinar, 2005). In addition, mixtures of cellulase and pectinase have been used for the extraction of carotenoids from orange peel, sweet potato, carrot, red capsicum and pumpkin, among others (Çinar, 2005; Nath *et al.*, 2016). Pectinases have also been used to obtain tomato lycopene, a high-value carotenoid for the pharmaceutical, cosmetic and food industry (Choudhari and Ananthanarayan, 2007), as well as for the extraction of antioxidant phenols and anthocyanins from black currant juice press residues (Landbo and Meyer, 2001). Recently, the use of *Aspergillus tamarii* pectinases for the production of lycopene and anthocyanins from agro-industrial waste has been reported (Shanmugavel *et al.*, 2018).

Bio-Scouring of Cotton

The cotton fiber used in the textile industry contains about 10%–12% of pollutant material, such as lipids, pectin, organic acids, proteins, etc. Since these molecules affect the characteristics of the cloth, they are removed with highly polluting alkaline washes (Hartzell and Hsieh, 2016). Alternatively, the bio-scouring process of cotton fiber and other fibers used in textile manufacturing, with mixtures of lytic enzymes is used to remove all non-cellulosic components, with low energy consumption and highly reduced pollution (Garg *et al.*, 2016). Typically, the bio-scouring process is carried out using mixtures of pectinases and cellulases, or only pectinases. Alkaline pectinase is the most suitable enzyme to produce better cloth characteristics, such as absorption, whiteness, higher tenacity and hydrophilicity (Besegatto *et al.*, 2018). On the other hand, the use of acidic and neutral pectinases are alternatives to chemical treatment due to ecological benefits, since it is not necessary to neutralize the wastes (Pušić *et al.*, 2015).

Commonly, fungal pectinases from *Penicillum italicum*, *Aspergillus fumigatus*, and *Fusarium* sp., among others, are used in the bio-scouring processes (Baracat-Pereira *et al.*, 1993; Brühlmann, 1995; Beg *et al.*, 2000).

Biofuel Production

The use of agricultural and forestry wastes as sources of simple sugars for fermentation, is an alternative in the production of bioethanol, instead of the sugar cane and corn currently used (Zhao *et al.*, 2012). In the process of bioethanol production, complex biomass such as lignocellulose is pre-treated for a more efficient digestion, allowing a better enzymatic conversion of polysaccharides into monomeric sugars whose fermentation by yeast produces bioethanol (Jørgensen *et al.*, 2007). It is believed that pectinase treatment is not fundamental in lignocellulose saccharification, since the pectin content is low in lignocellulosic tissues such as agricultural and forestry wastes. However, pectin can play a role as a binder and its function in the middle lamella as an adhesive and porosity regulator can interfere with the hydrolysis of the other polymers and the release of sugars (Jarvis *et al.*, 2003; Xiao and Anderson, 2013; Latarullo *et al.*, 2016).

Despite of being an attractive alternative to the use of corn grains or lignocellulose for bioethanol obtention, pectin rich (12%–35% dw) agro-industrial wastes such as sugar beet pulp, citrus waste and apple pomace, among others, are rarely used. These wastes from the food industry are advantageous as they are cheap and the partially degraded biomass does not require a special treatment prior to enzymatic degradation (Edwards and Doran-Peterson, 2012).

Direct fermentation of sugars produced by saccharification of lignocellulose represents an excellent alternative, but yeasts such as *S. cerevisiae*, which is capable of alcoholic fermentation, and *Kluyveromyces marxianus*, are unable to use pentoses and galacturonic acid (Edwards and Doran-Peterson, 2012). Therefore, there is currently a search for yeast strains capable of fermenting xylose and arabinose of rhamnogalacturonan I, which represents 18%–21% of dry weight in sugar beet pulp. For saccharification and fermentation, the sequential use of *S. cerevisiae* and bacteria such as *Escherichia coli* or *Erwinia chrysanthemi* has been proposed (Edwards and Doran-Peterson, 2012).

Recently it has been shown that enzymatic treatment of *Agave americana* biomass with *A. niger* pectinases remarkably improves saccharification compared to commercial enzymatic mixtures of cellulases and xylanases, without the need of prior treatment and with total conversion of polysaccharides to simple sugars (Wang *et al.*, 2019).

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Relevant Website

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Fungal Biotechnology: Fungal Amylases and Their Applications

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Introduction

Starch is a glucose polymer that is synthesized via photosynthesis in the chloroplasts of plants. It accounts for a large fraction of the biomass and serves as a reserve energy compound stored in plant organs such as tubers, seeds and roots (Norouzian *et al.*, 2005). Starch is metabolized through photorespiration, which requires amylases to break down the starch to glucose moieties. Amylases are glycoside hydrolases (EC 3.2.1) that hydrolyze the O-glycosyl compounds in starch and are found in animals, plants and microbes, with the preferred industrial source being microorganisms (Sun *et al.*, 2010).

Amylases are one of the oldest enzymes to be used commercially, dating back to 1984 when the α-amylase from *Aspergillus oryzae* known as "Taka diastase" was used in the pharmaceutical industry for digestive disorders (Gupta *et al.*, 2003). Since then, α-amylases have been extensively applied in several industries, including the bread and baking industry for improved volume, color and texture (Hamer, 1995).

Over the last few decades, starch-hydrolyzing enzymes have replaced the use of acid hydrolysis and are desirable for many different industrial uses due to their high degree of substrate specificity. Compared to chemical modification, the treatment of starch using enzymatic hydrolysis has several advantages, including better process control, higher product yields, faster reaction times at lower temperatures and cleaner effluents (Miao *et al.*, 2018). However, only a small number of bacterial and fungal strains meet the criteria for commercial amylase production, e.g., strains of *Bacillus* spp., *Aspergillus* spp. and *Rhizopus* spp. (Cereia *et al.*, 2006; Zeng *et al.*, 2011).

This chapter describes the different types of amylases, their properties, industrial applications and production processes, as well as predictions for the future amylase market.

Starch, the Natural Substrate for Amylases

Starch typically consists of a mixture of amylose and amylopectin molecules and the relative ratio of these polymers determines the diverse chemical properties of starch granules (van der Maarel *et al.*, 2002). Amylose (representing 10%–30% of the granule mass) is a linear polymer consisting of up to 6 000 glucose units linked by α-1,4 glycosydic bonds (de Souza and de Oliveira Magalhães, 2010). Amylose adopts a single helical structure that is randomly positioned within the amorphous lamella and is linked to the functional properties of starch. In contrast, amylopectin is a branched polymer that consists of short linear chains of 10–60 glucose units joined by α-1,4 bonds and α-1,6 linked side chains of 15–45 glucose units (Streb and Zeeman, 2012).

Starch in its native state is referred to as raw or unmodified (granular) starch. Raw starch granules have a densely compacted polycrystalline structure that resists enzymatic hydrolysis (Lin *et al.*, 2011). The starch granules are comprised of alternating semi-crystalline and crystalline shells and a large part of the amylopectin that forms the semi-crystalline shells is in the double-helical form, rather than the crystalline form. Starch is hydrolyzed into simpler carbohydrates called dextrins, with the dextrose equivalent (DE) indicating the fraction of the glycosidic bonds that has been hydrolyzed. For example, maltodextrin (DE 10–20) is an oligosaccharide used as a filler and thickener in the food industry, corn syrups (DE 30–70) are used as sweeteners, and dextrose (DE 100) is available as commercial glucose prepared by the complete hydrolysis of starch (Fellows, 2016).

At low temperatures ($<50^{\circ}$C), granular starch is in its solid phase and insoluble in aqueous media (Cinelli *et al.*, 2015). However, starch granules undergo irreversible gelatinization when heated above a specific temperature. The starch gelatinization temperature is determined by the distribution and chain length of the amylopectin (Jane *et al.*, 1999) and is characterized by the phase transition of starch granules from an ordered to a disordered state (Ubwa *et al.*, 2012). The breakage of the hydrogen bonds results in an uptake of water and swelling of the molecules, which leads to the disruption of the starch crystalline structure. Leaching of the amylose from the starch molecule takes place, resulting in the formation of soluble starch that is easy to hydrolyze (Daniel *et al.*, 2000). The conditions for starch gelatinization are determined by the plant type, degree of amylopectin cross-linking, as well as the salt, sugar and lipid content. Most A-type starches, which generally occur in cereal starches (such as wheat and corn), and are formed in warm and dry conditions (Ai and Jane, 2015). In contrast, B-type starches (B-type crystallites) are found in tubers, bananas and high-amylose starches, they are formed in cold and wet conditions and exhibit a lower gelatinization temperature (T_0 of 58.2°C) than A-type starches (Liu, 2005).

The hydrolysis of raw starch is significantly slower than that of soluble starch and higher enzyme loadings are required for its hydrolysis. When the four most economical native starches were compared for their susceptibility to hydrolysis, they were ordered (in diminishing order) corn $\geq$ wheat $>$ cassava $>$ potato (Szymanowska-Powałowska *et al.*, 2012). Inefficient catalysis may result from various factors, including the physical architecture (degree of branching), the molecular structure of the solid substrate and the molecular configuration of the enzyme. Other factors that influence the hydrolysis of starch in its heterogeneous phase include enzyme diffusion in the medium, substrate accessibility, substrate recognition leading to adsorption, formation of the enzyme-substrate complex and catalytic action (Gallant *et al.*, 1997; Tawil *et al.*, 2011).

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Role of Amylases in Starch Hydrolysis

Fungal starch hydrolyzing enzymes are found predominantly in the glycoside hydrolase (GH) family 13, with several examples occurring in the GH14 and GH15 families. Isoamylases (EC 3.2.1.68) and α-amylases (EC 3.2.1.1) are endo-type enzymes in the GH13 family that display α-1,4–cleavage activity and are responsible for the liquefaction of starch. The degradation of pullulan by pullulanases (EC 3.2.1.41, GH13) produces maltotriose (Gomes *et al.*, 2003), whereas isoamylases and pullulanases cleave α-1,6-bonds, thus converting amylopectin to amylose and oligosaccharides with 25–30 glucose residues. Exo-amylases (such as β-amylases, EC 3.2.1.2; GH14) only cleave α-1,4-glycosidic bonds, whereas glucoamylases (EC 3.2.1.3; GH15) and α-glucosidase (E.C. 3.2.1.20; GH13/GH31) display both α-1,4–cleavage and α-1,6–debranching activities (Sun *et al.*, 2010; Okuyama *et al.*, 2017). Compared to α-glucosidases that act better on short maltooligosaccharides, glucoamylases have a preference for hydrolyzing long-chain polysaccharides. The β-amylases hydrolyze the oligosaccharides from the non-reducing ends with the release of maltose residues (Tiwari *et al.*, 2015).

The GH13 family features 22 different substrate specificities involving α-glucosidic linkages, which include hydrolases, transglucosidases and isomerases (Møller *et al.*, 2016). The glycogen-debranching enzyme (GDE) plays a key role in carbohydrate metabolism and is highly conserved in animals and fungi (Nakayama *et al.*, 2001; Zhai *et al.*, 2016). The GH13_25 GDEs are bifunctional enzymes that exhibit both α-1,4-glucanotransferase (EC 2.4.1.25) and α-1,6-glucosidase (EC 3.2.1.33) activities. Zhai *et al.* (2016) expressed the *Candida glabrata* GDE (CgGDE) in *Escherichia coli* and the crystal structure showed four domains with an elongated structure, with the transferase and glucosidase domains being separated by two (middle) domains. The structures of the transferase and glucosidase active sites indicated that they are highly selective towards substrate branch length. The GDE transferase activity was similar to reactions catalyzed by members of the GH13 family, while the C-terminal end of the CgGDE displays an $(\alpha/\alpha)_6$-barrel structure, similar to members of the GH15 family. However, the glucosidase part of GDEs was novel and has been classified into the new GH133 family (Møller *et al.*, 2016).

Complete starch degradation requires the hydrolysis of both α-1,4- and α-1,6-glycosidic bonds. Four groups of starch-converting enzymes confer this activity and include endo-amylases, exo-amylases, debranching enzymes and transferases, with enzymes from the first two groups being used for commercial starch hydrolysis. A schematic representation of the chemical structure of amylopectin and amylose and the respective sites for hydrolysis by the different types of amylases is shown in **Fig. 1**.

Depending on the starch composition and origin, α-amylases and glucoamylases would be sufficient for the complete degradation of amylose to D-glucose. The α-amylases would generate oligosaccharides, whose ends are degraded by glucoamylases to release D-glucose. This implies that organisms producing these two enzymes would be able to hydrolyze amylose into

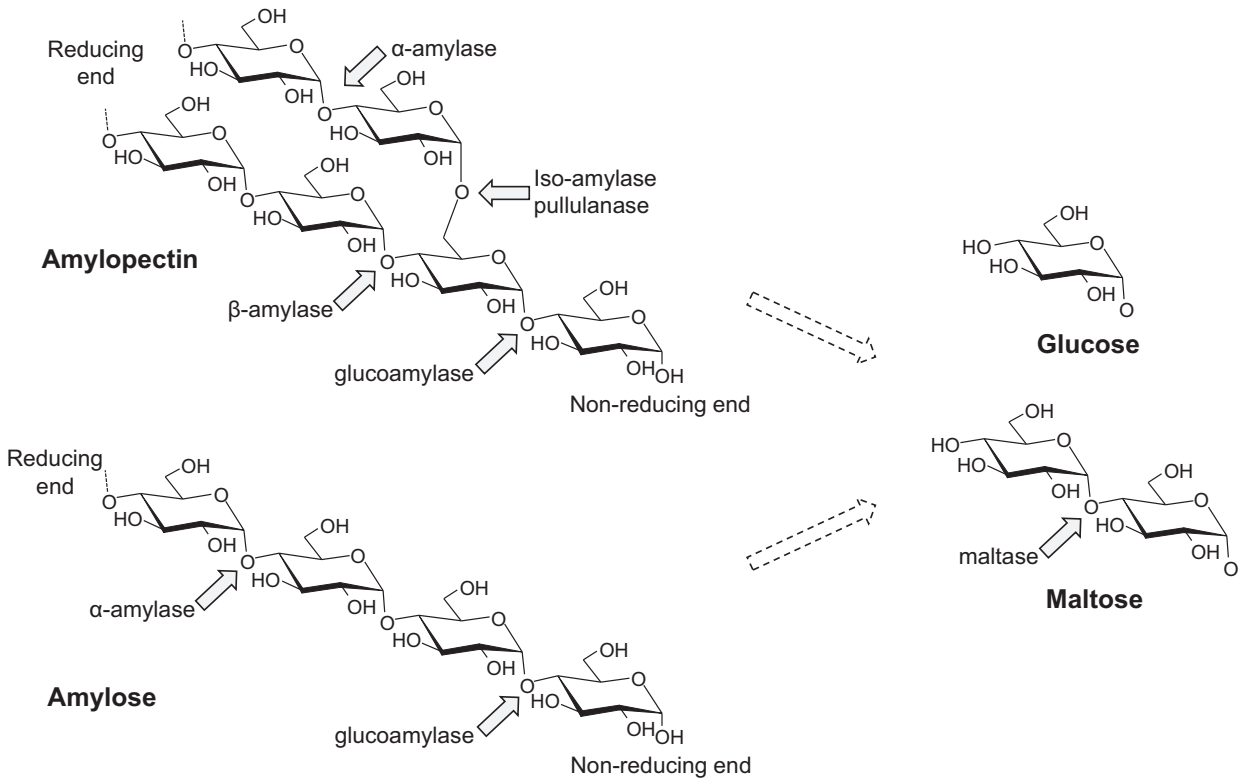

Fig. 1 Chemical structure of amylopectin and amylose chains and the sites for hydrolysis by the different types of amylases.

D-glucose, but not necessarily utilize all the starch components (Peterson, 1998). Given the importance of these two enzymes, they will be discussed in more detail in the following sections.

Scanning electron microscopy (SEM) can be used to observe the hydrolysis of starch granules (Myburgh *et al.*, 2019). The two types of hydrolysis associated with native starch granules and α-amylase are 'centrifugal' (associated with regions and layers) and 'centripetal' (surface to center). Both these occur in corn, rice and wheat starch granules, whereas only centrifugal hydrolysis has been observed in potato starch granules (Sarikaya *et al.*, 2000). When α-amylases hydrolyze the interior linkages on the surface of starch granules, they provide the substrate for glucoamylases that hydrolyze the shorter dextrins from the non-reducing ends of the starch molecule to glucose. This centripetal type of hydrolysis results in the formation of small holes (pits), which allow the α-amylases to enter the interior of the starch molecule where they preferentially hydrolyze amorphous regions prior to crystalline areas. Pit formation is often referred to as "Swiss cheese hydrolysis" and is a characteristic manner in which cereal starch is degraded (Sujka and Jamroz, 2007). The synergistic action of the α-amylase-glucoamylase combination results in the complete degradation of raw starch into glucose units (Sun *et al.*, 2010). Wong *et al.* (2007) suggested that the ratio of α-amylase and glucoamylase activities can vary from 3:1 to 1:3, but efficient hydrolysis would only be possible if the α-amylase is in excess and there are sufficient activity levels for both enzymes (Görgens *et al.*, 2015).

α-Amylases

The GH13 family contains 42 subfamilies with fungal α-amylases (EC 3.2.1.1) grouped in subfamily GH13_1 (Chai *et al.*, 2016). This group comprises the largest family of glycoside hydrolases, with the majority of enzymes acting on starch, glycogen and related polysaccharides. An α-amylase consists of a single polypeptide chain that is folded into three domains (A, B, and C). Domain A is the catalytic domain, domain B has an irregular structure and domain C is believed to stabilize the catalytic site of the enzyme by protecting the hydrophobic patch (Singh and Kayastha, 2014). Most of the enzymes have an active site cleft between domains A and B where a triad of residues (Asp, Glu, and Asp) perform the catalysis. The α-amylases hydrolyze the internal α-1, 4-bonds of amylose and amylopectin at random, producing maltodextrins with a length of 10–20 glucose residues, as well as maltose and free glucose (van Zyl *et al.*, 2012).

α-Amylases play a dominant role in carbohydrate metabolism and have entirely replaced the use of chemical hydrolysis in the starch-processing industry (Gupta *et al.*, 2003; de Souza and de Oliveira Magalhães, 2010). α-Amylases were the first enzymes produced for use on a commercial scale and the global α-amylase market is expected to reach USD 1.6 billion by 2020, growing at a compounded annual growth rate of 12.5% from 2016 to 2020, with the baking industry predicted to be the fastest-growing market at 15.0% per annum (Amylase market see "Relevant Websites section").

The molecular mass of α-amylases varies from 10 to 210 kDa, but microbial α-amylases are usually 50–60 kDa (Gupta *et al.*, 2003). Most α-amylases display maximum activity in the pH range of 4.5–7.0 (van Zyl *et al.*, 2012); thermostable acidic α-amylases are preferred for the industrial hydrolysis of starch since starch slurry has a pH around 4.5 (Sharma and Satyanarayana, 2013). In contrast, alkaline amylases are desirable for the detergent and food industries (Das *et al.*, 2004), thus prompting searches for microbial strains producing these α-amylases. From an economic and technical perspective, thermostable α-amylases are beneficial as they allow for higher operational temperatures. However, recombinant α-amylases with optimum activities at 30–37°C will increase the rate of starch hydrolysis at the growth temperature of the yeast host strains (Carrasco *et al.*, 2016).

Amylases fulfill a wide range of applications and have been investigated for over 30 years to identify the important determinants for enzyme function (Ju *et al.*, 2019). Both the DNA and amino acid sequences of several α-amylases have been included in comparative studies (Imanaka and Kuriki, 1999; Janaček *et al.*, 1997). It revealed large variation in the length of the open reading frames (Kajiwara *et al.*, 1997) and assisted functionality studies of its different domains (MacGregor *et al.*, 2001), e.g., unraveling the evolutionary origin of the α/β-barrel of α-amylase (Farber and Petsko, 1990; Farber, 1993). A selection of these enzymes and some of their key properties are summarized in **Table 1**.

Glucoamylases

Glucoamylases (glucan α-1,4-glucosidase, EC 3.2.1.3) are grouped in GH15 family and are exo-acting enzymes that catalyze the hydrolysis of α-1,4- and α-1,6-glucosidic linkages to release the inverted β-D-glucose from the non-reducing ends of starch (Chen *et al.*, 2012). As indicated in **Table 1**, glucoamylases are produced by a wide range of microorganisms, but enzymes for commercial applications are mainly produced by filamentous fungi as they secrete large quantities of the enzyme. The industrial production of glucoamylases has focussed on *Aspergillus niger*, *Aspergillus awamori* and *Rhizopus oryzae* due to the stability of their enzymes (Lin *et al.*, 2007).

These enzymes are mainly used for the production of glucose syrup, high-fructose corn syrup and bioethanol, and have the ability to degrade large oligosaccharides containing up to 90% α-l,6-linkages. The pH and temperature optima of glucoamylases are generally in the range of 4.5–5.0 and 46–60°C, respectively, and they are relatively stable at higher temperatures (van Zyl *et al.*, 2012). A few thermophilic strains produce a glucoamylase with an optimum temperature of 70°C, such as *Talaromyces emersonii* (now *Rasamsonia emersonii*), *Halvina lanuginosa* and *A. niger* IMDCC No. 120 (James and Lee, 1997). The molecular mass of glucoamylases can vary from 55 kDa (raw starch-degrading glucoamylase from *Saccharomycopsis fibuligera*) to about 300 kDa (glucoamylase from *Saccharomyces cerevisiae* var. *diastaticus*) (Hostinová and Gašperík, 2010).

Table 1 Properties of a selection of fungal and yeast amylases

	Amylase	*pH optimum*	*Temperature optimum (°C)*	*Starch digestion*	*Reference*
Filamentous fungi					
Aspergillus awamori	α	4.8–5.0	50	Raw	Bhella and Altosaar (1985)
A. awamori	G	5.8	45	Soluble	Pestana and Castillo (1985)
Aspergillus fumigatus	α	6.0	55	Raw	Singh *et al.* (2014)
Aspergillus niger	G	4.0–4.5	70	Raw	Nielsen *et al.* (2002)
Aspergillus phoenicis	Crude enzyme[a]	4.5–5.0	60–65	Raw	Benassi *et al.* (2013)
Aspergillus tubingensis[b]	α	4.0	60	Raw	Viktor *et al.* (2013)
A. tubingensis[b]	G	4.5	70	Raw	Viktor *et al.* (2013)
Aureobasidium pullulans[b]	G	4.5	60	Raw	Li *et al.* (2011)
Lentinula edodes[b]	G	4.6	50	Raw	Wong *et al.* (2005)
Penicillium brevicompactum	α	5.0	30–50	Raw	Balkan and Ertan (2010)
Penicillium chrysogenum	α	5.0	30–40	Soluble	Balkan and Ertan (2005)
Penicillium oxalicum[b]	G	4.5	65	Raw	Xu *et al.* (2016)
Talaramyces emersoni	α	4–5.0	55–70	Raw	Bunni *et al.* (1989)
T. emersoni[b]	G	4.0–4.5	70	Raw	Nielsen *et al.* (2002)
Thermomyces lanuginosus	G	5.0	70	Raw	Thorsen *et al.* (2006)
Yeast					
Lipomyces starkeyi[b]	α	6.0	40	Raw[c]	Kang *et al.* (2004)
Lipomyces kononenkoae[b]	α	3.5	60	Raw	Eksteen *et al.* (2003b)

[a]Native glucoamylase secreted into the growth media.
[b]Recombinant enzyme production.
[c]Punpeng *et al.* (1992).
Note: α – α-Amylase, G – Glucoamylase.

Starch Binding Domain (SBD)

Carbohydrate-binding modules (CBMs) are non-catalytic ancillary domains that function independently of the catalytic domain and are often present in glycoside hydrolases. CBMs that have an affinity for insoluble raw starch are generally referred to as SBDs (Peng *et al.*, 2014). The SBD is usually composed of about 100 amino acid residues and is present in a number of amylolytic enzymes. It is comprised of several β-strand segments forming an open-sided, distorted β-barrel structure and it is connected to the catalytic domain by a glycosylated linker region (Juge *et al.*, 2002; Barchiesi *et al.*, 2015).

The SBD plays a fundamental role in granular starch hydrolysis by performing several simultaneous functions. It binds to the starch molecules and thereby increases the concentration of the substrate at the catalytic site. A strong correlation between raw starch hydrolysis and the adsorption of amylases to raw starch granules has been described for bacterial, yeast and fungal α-amylases and glucoamylases (Mitsuiki *et al.*, 2005). The SBD may also disturb the structure of the starch by disrupting polysaccharide chain interactions on the granule's surface, which enhances the amylolytic rate (Santiago *et al.*, 2005; Barchiesi *et al.*, 2015).

Sequence-based classification divides SBDs into ten CBM families, namely 20, 21, 25, 26, 34, 41, 45, 48, 53, and 58 (Peng *et al.*, 2014). The diverse CBM20 family has been shown to function in raw starch binding with two starch-binding sites (SBS) that are positioned on the exterior of the active site area (Cockburn *et al.*, 2014), presumably with different functions. It has been reported that site 1 is probably used as the initial starch recognition site, whereas site 2 is associated with the subsequent binding to the starch polymer (Sorimachi *et al.*, 1997). The CBM20s are grouped based on their amino acid sequences, substrate binding specificities and position in the protein (middle, N- or C-terminal) (Barchiesi *et al.*, 2015). CBM20s are predominantly associated with starch-active proteins from GH families 13, 14, 15 and 77, but GH families 14, 31, 57, 97, and 119 also contain the CBM20 (Møller *et al.*, 2016). Microbial amylases that are involved in raw starch metabolism often contain an SBD at the C-terminus of the protein (Latorre-García *et al.*, 2005), except for the α-amylase from *Thermoactinomyces vulgaricus* and the glucoamylase from *R. oryzae* that contain an N-terminal SBD (Santiago *et al.*, 2005).

Application of Amylases

As indicated in **Fig. 2**, amylases play a key role in industry with applications ranging from the production of high-fructose syrups, corn bioethanol, detergents, textile and paper industries, food industries (baking and chocolate industry) to newer biopharmaceutical applications (Gupta *et al.*, 2003; Saini *et al.*, 2017). It is thus not surprising that amylases represent 25%–30% of the global enzyme market and have the largest market share of all enzyme groups (de Souza and de Oliveira Magalhães, 2010; Saranraj and Stella, 2013). The value of the starch enzyme and starch bioethanol markets alone was estimated to be worth $2.24 billion in 2018 (Mehta and Satyanarayana, 2016). A selection of these applications are discussed in more detail in the following sections.

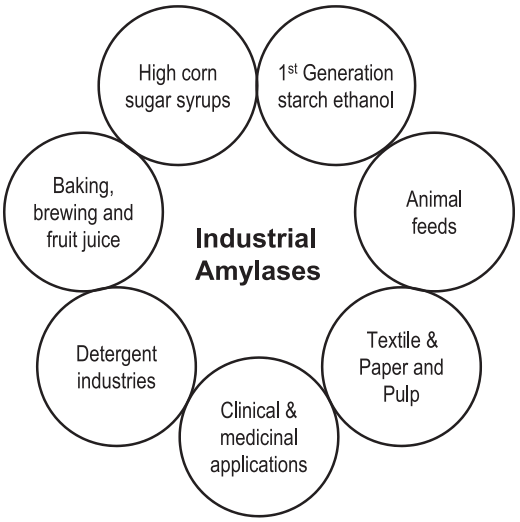

Fig. 2 Amylases are used in a variety of industrial applications, ranging from food to transport fuel.

Production of High-Fructose Corn Syrups

The production of high-fructose corn syrups (HFCS) represents the largest application of starch in the beverage (beer) and soft-drinks markets. The production of HFSC comprises of three steps: liquefaction, saccharification and isomerization. During liquefaction, starch is heated in a jet cooker to 105–110°C for a short period and then partially hydrolyzed at 95°C using a thermostable α-amylase that produces shorter oligosaccharides by an endo-wise random disruption of the large molecules (Fujii *et al.*, 1988). After cooling to 55–60°C and pH adjustment to 4–5, saccharification of the liquefied starch is performed with a fungal glucoamylase to generate glucose-rich dextrins. In a third step, about half of the glucose is converted to fructose with the aid of glucose (xylose) isomerase. The bulk of HFCS is used in the production of soft-drinks (Mehta and Satyanarayana, 2016), but glucose-rich syrups are also used as a supplement in high-gravity beer brewing to ensure high ethanol concentrations (Casey *et al.*, 1984).

Biofuel Production From Starch

After high-sugar syrups, first-generation ethanol production from hydrolyzed starch is the second-largest industrial application of amylases. The first two steps are equivalent to the production of high-sugar syrups, namely liquefaction with thermostable α-amylases, followed by saccharification with glucoamylases. First, the starch slurry is gelatinized in a jet cooker at 100–105°C for 5 min, followed by a liquefaction step using thermostable α-amylase at high temperatures (>95°C for up to 3 h). The saccharification step involves cooling of the slurry to 60°C and the addition of a glucoamylase to release the fermentable sugars (Białas *et al.*, 2014; Mehta and Satyanarayana, 2016; Robertson *et al.*, 2006). The sugars are subsequently fermented to bioethanol, which is distilled and used as a gasoline extender.

The conventional process of cooking the starch during liquefaction has several disadvantages, such as the high energy input (up to 30%–40% of the ethanol value) required for gelatinization, and pH adjustment steps (Lim *et al.*, 2003). Commercial α-amylases function optimally at pH 6–6.5 and the pH of the fermentation broth needs to be lowered to pH 4–5 before glucoamylase addition and fermentation (Robertson *et al.*, 2006). The use of granular/raw starch-hydrolyzing enzymes can eliminate the high temperatures required for starch gelatinization and improve the efficiency of starch-to-ethanol conversion using simultaneous saccharification and fermentation (SSF) (Gohel and Duan, 2012). There are several commercial granular starch hydrolyzing enzyme (GSHE) cocktails available that can be used in combination with a fermenting yeast. These amylases can hydrolyze the raw starch at relatively low temperatures; 48°C is recommended for SSF, after which the fermentation broth is cooled to 30°C prior to yeast addition (Görgens *et al.*, 2015).

Food and Beverage Markets

Mass production of bread in the baking industry faces several challenges, such as proper leavening to provide volume, improved texture and taste, as well as improved shelf-life. Amylases have been used since the mid-1900s to address these challenges. The addition of α-amylase allows for the hydrolysis of smaller starch particles during bread leavening, thus reducing the viscosity of the dough. This allows for proper bubble formation during the release of CO_2 by the bakers' yeast and releases additional sugars for the yeast, thus prolonging the fermentation phase. The volume of the bread increases, the texture improves and the prolonged fermentation can also contribute to the taste. The addition of amylases also limits drying of the bread and firming of the crumb during storing – a process called staling. However, it is important not to overdose with α-amylase, which can result in a sticky dough that counters the positive effects. More recently, further emphasis has been placed on the use of debranching amylases, such

as pullulanases, that generate dextrins with a degree of polymerization (DP) of 4–9, which is ideal for anti-staling and avoiding stickiness (Goesaert *et al.*, 2009; Gupta *et al.*, 2003; Tiwari *et al.*, 2015).

Several beverage industries rely on amylases. Chocolate slurries are rich in starch, which negatively affects the application of chocolate syrups. Amylases are added to remove excessive dextrins and thus lower the viscosity to improve the flow properties of chocolate syrups in confectionery mixtures (Tiwari *et al.*, 2015). Apple juice contains up to 15% starch, which can slow down filtration due to membrane fouling and cause high viscosity and hazing of the juice. The addition of amylases can address these problems and assist in clarifying the juice (Carrín *et al.*, 2004). Barley α-amylase is used in the brewing industry to maximize the release of fermentable sugars and the Milliard reaction to ensure the characteristic color of wort during beer making. Although the addition of exogenous amylases can be advantageous, it is not allowed in the beer industry. Germination of barley during malting is therefore precisely controlled to provide sufficient endogenous amylases for the production of wort (Bamforth, 2009). Fungal amylases are also extensively used in the preparation of oriental foods like tempeh, soy sauce, koji stage, idli and many other fungal foods (Saini *et al.*, 2017).

Amylase in Animal Feeds

Amylases have found application in the feeds of monogastric animals, such as chickens and pigs. The starch component in the typical corn-soy diet of broiler chickens is the primary source of energy to promote fast growth. However, the inevitable presence of non-starch polysaccharides (e.g., arabinoxylan) in the diet can impede starch utilization. Moreover, the high feed rate of modern broilers can cause limited starch digestion despite the production of endogenous amylases by hatchlings. The addition of exogenous amylases to assist in starch digestibility has been shown to positively impact energy release and growth in broiler chickens. By supplementing with additional enzymes, such as xylanases to hydrolase the arabinoxylan, it reduces the viscosity of the feed in the stomach and enhances feed conversion (Stefanello *et al.*, 2017, 2015).

For piglets, the most stressful phase is directly after abrupted weaning from the sow. Their digestive tracts have to adapt from frequent milk feeding to ad-libitum feeding (free feeding) on solid foods, mostly plant-based feeds in dry format. Under- or overfeeding could affect the integrity of the gut microflora or proliferation of pathogenic bacteria. The addition of exogenous hydrolases helps with the adaptation to the more complex plant-based feeds. However, since piglets produce their own endogenous amylases, the effect of amylase addition to the feed of weaning piglets has a marginal effect and is not as dramatic as, for example, the addition of phytase enzymes (Torres-Pitarch *et al.*, 2017).

Detergent Industries

Traditional chemical detergents used for cloth- and dishwashing are too harsh for delicate clothes, as well as dangerous when accidentally ingested. The introduction of enzymes, such as proteases, amylases and mannanases allows for environmentally-friendly detergents for automatic washing in much milder conditions, including cold water for clothing. More than 90% of modern detergents contain amylases for the removal of starchy stains from potatoes, gravies, chocolate and custards (de Souza and de Oliveira Magalhães, 2010; Gupta *et al.*, 2003). However, amylases for the detergent industry should be thermostable and able to function at an alkaline pH (Singh *et al.*, 2016).

Textile and Paper-and-Pulp Industries

Starches are used in the textile and paper-and-pulp industries to enhance the production and the quality of end-products. Weaving exerts mechanical stress on fabric yarns, which are usually covered with a starch coat to facilitate weaving. However, this starch coat has to be removed from the fabrics (a process called desizing) before the fabric can be bleached and colored. Amylases are used to selectively remove the starch by converting them to soluble dextrins without attacking the yarn fibers (de Souza and de Oliveira Magalhães, 2010; Gupta *et al.*, 2003). Similarly, starch is incorporated during papermaking to enhance the stiffness and strength of paper, whilst providing a superior finishing that enhances the writing and erasability properties. The starch has to be of a constant high DP and low viscosity to ensure uniform coating. Amylases are therefore used to modify the raw starch to the correct consistency required for coating (de Souza and de Oliveira Magalhães, 2010; Gupta *et al.*, 2003; Tiwari *et al.*, 2015).

Clinical and Medicinal Applications

Since Stocks (1916) suggested that amylase activity in the blood and urine of patients could indicate pancreatic enzyme production, the amylase test has been used as a diagnostic test for pancreatic disorders. In recent years, newer applications for amylases in pharmaceuticals have been established. A slow release of compounds is required for effective drug delivery; an example is the use of cross-linked amylose (CLA) compressed with drug powder for slow-release, which can be controlled by the ratio of CLA to drug powder. The compressed drugs are stable in the dry formulation and require rehydration of the CLA after ingestion before amylases can degrade the CLA/drug matrix (Dumoulin *et al.*,1999).

Production of Industrial Amylases

Enzyme are industrial catalysts and they need to tolerate the relatively harsh conditions that are often associated with industrial processes. The pH profile, pH stability and thermostability of enzymes are important factors to be considered in the development of enzyme production processes (de Souza and de Oliveira Magalhães, 2010). Industrially important enzymes have traditionally been produced using submerged fermentation, where different parameters (e.g., pH, temperature, aeration, oxygen transfer, and moisture) can be controlled. Solid-state fermentation systems offer a promising alternative, since they resemble the natural habitat of several microorganisms (de Souza and de Oliveira Magalhães, 2010; Sundarram *et al.*, 2014). Bulk enzyme production requires minimal downstream processing and is often used as crude preparations, whereas enzyme applications in pharmaceutical and clinical sectors require high purity amylases (de Souza and de Oliveira Magalhães, 2010).

The suitability of enzymes for large-scale production is influenced by the yield, stability and production costs (Naidu and Saranraj, 2013; Das *et al.*, 2011). Emerging genetic engineering tools have facilitated the use of recombinant amylases that further promoted their use in industrial applications (Muralikrishna and Nirmala, 2005), in particular with the aim of obtaining enzymes with desired or enhanced characteristics (Abdel-Fattah *et al.*, 2013). Furthermore, low-value agricultural residues have gained much interest as an inexpensive substrate for recombinant enzyme production (Kim and Dale, 2004).

Recombinant Production of Amylases

Various amylases have been cloned and expressed in bacterial and yeast strains for a number of reasons, including understanding enzyme characteristics, increasing enzyme titers and developing enzymes with novel properties (Sivaramakrishnan *et al.*, 2006; Miao *et al.*, 2018).

Recombinant strains have also been constructed to express highly active amylases for use in industrial biofuel production (Cripwell *et al.*, 2019a). The main challenge in engineering a robust amylolytic yeast is the ability to hydrolyze raw starch at high concentrations, while simultaneously fermenting the hydrolyzed sugars to ethanol (Favaro *et al.*, 2015). Compared to laboratory strains, industrial strains have valuable characteristics, including enhanced tolerance to ethanol, acids and sugars. A well-used transformation platform for the heterologous expression of foreign genes in *S. cerevisiae* uses high-copy 2-micron plasmids that can be maintained at around 10–50 copies per cell. However, industrial *S. cerevisiae* strains cannot be genetically engineered using these plasmids as plasmid stability requires the use of selectable markers and thus the need to maintain selection pressure (Shi *et al.*, 2016). Stable chromosomal integration is thus required to engineer robust industrial *S. cerevisiae* yeast strains to produce extracellular amylases (Favaro *et al.*, 2013).

An in-depth understanding of heterologous gene expression in *S. cerevisiae* has been generated over the last few decades with a wide range of genetic tools available for strain development and improvement. In addition, the genetic competence of *S. cerevisiae* strains to accept foreign DNA has facilitated targeted chromosomal manipulations. However, the lack of native starch-hydrolyzing enzymes in *Saccharomyces* species has limited its application in the starch bioprocessing industry (Eksteen *et al.*, 2003a), with the exception of the amylolytic *Saccharomyces diastaticus* strain (Adam *et al.*, 2004).

Amylolytic Strains for Biofuel Production

Constructing a starch-degrading yeast requires the genetic engineering of industrial yeast strains that can produce high levels of heterologous amylolytic activity. This strain would theoretically lead to higher ethanol production from starch without requiring pre-treatment of the substrate (Mukerjea *et al.*, 2006). It would be beneficial if the different amylases originate from the same organism, thus complementing one another thanks to evolutionary development and compatibility. Screening for novel amylolytic enzymes is thus essential to generate a sufficiently large collection of raw starch-degrading glucoamylases and α-amylases that can be heterologously produced by fermenting yeast strains. Ideally, these recombinant enzymes should have the characteristic raw starch-binding sites within the SBD. Different strategies have been employed to produce amylases in *S. cerevisiae*, with cell-surface display and consolidated bioprocessing among the favorites.

The use of a cell-surface display technique has allowed for the expression of amylase genes on the surface of a cell through linkage with a genetically fused anchor protein that keeps the amylase cell-bound. To construct yeast strains with the ability to hydrolyze starch as a substrate for bioethanol production, the cell-surface display technique can be combined with metabolic engineering approaches to manipulate target pathways within yeast cells. This would ensure that bottelnecks in sugar utilization do not negatively affect ethanol productivity and could result in the construction of novel microbial factories for the production of value-added compounds (Tanaka and Kondo, 2015). Several groups have produced ethanol from starchy biomass using this engineering approach, for example, Shigechi *et al.* (2004) co-displayed the *R. oryzae* glucoamylase and bacterial *Streptococcus bovis* α-amylase to produce ethanol directly from raw corn starch.

Consolidated bioprocessing (CBP) combines enzyme production, substrate hydrolysis and glucose fermentation into a one-step process using a single organism, and is used for bioethanol production at low temperatures (van Zyl *et al.*, 2012). Amylolytic strains for efficient CBP of natural starchy biomass were first described by Favaro *et al.* (2015) when codon-optimized variants of the *Thermomyces lanuginosus* glucoamylase (*TLG1*) and *Saccharomycopsis fibuligera* α-amylase (*SFA1*) genes were δ-integrated into two *S. cerevisiae* strains with promising industrial traits. However, the challenge remains to construct strains that can produce ethanol concentrations

comparable to the >20% v/v ethanol reached in the conventional starch-to-ethanol process (Görgens *et al.*, 2015; Walker and Walker, 2018). Currently, there is no industrial process that uses a recombinant amylolytic yeast strain (producing both an α-amylase and glucoamylase) for starch CBP. Although commercially available glucoamylase-producing *S. cerevisiae* strains are available, they lack the α-amylase required for the liquefaction of starch. There is thus a strong demand for a drop-in CBP yeast that simultaneously expresses raw starch α-amylase and glucoamylase-encoding genes for complete starch (raw) hydrolysis.

Several studies have screened raw-starch fungal amylase-glucoamylase gene combinations in laboratory *S. cerevisiae* strains. The *amyA* α-amylase and *glaA* glucoamylase genes from *Aspergillus tubingensis* (Viktor *et al.*, 2013), as well as amylases originating from *Aspergillus terreus* (Sakwa *et al.*, 2018), *Aureobasidium pullulans*, *Neosartorya fischeri*, *Rhizomucor pusillus*, *T. emersonii*, *Talaromyces stipitatus*, and *Thermomyces lanuginosus* (Cripwell *et al.*, 2019a) have been cloned and expressed in *S. cerevisiae* Y294 laboratory strains, with varying abilities to degrade raw starch under fermentation conditions.

Recently, Cripwell *et al.* (2019b) integrated α-amylase and glucoamylase genes originating from *T. emersonii*, into the Ethanol Red™ industrial *S. cerevisiae* strain and demonstrated efficient conversion of raw corn starch to ethanol under high substrate loadings. Myburgh *et al.* (2019) further demonstrated that this amylolytic strain could be used for the fermentation of broken rice. These breakthroughs represent significant progress towards constructing CBP strains that produce fungal amylases for the biofuel industry and demonstrate the use of different raw starch substrates as feedstock.

Searching for Novel Amylases

Although a number of amylases have been identified from various microbial sources (Sun *et al.*, 2010), there is still a large number of unknown or poorly characterized microorganisms in nature and uncharacterized proteins from sequencing projects that could be explored for improved amylase production (Helbert *et al.*, 2019). In addition, several approaches can be pursued to improve the properties of (known) enzymes that would render them more effective as biocatalysts. These include protein engineering using saturated mutagenesis, error-prone PCR and gene shuffling coupled to high-throughput screening (Miao *et al.*, 2018). Metagenomic approaches and metatranscriptomic analyses can assist with the search for amylases with higher enzyme yields, activity and stability. Furthermore, recent advances in bioinformatics have made it possible to identify new enzymes through database mining of available sequencing data (Li *et al.*, 2012). For example, Pel *et al.* (2007) described the genomic DNA sequence of *A. niger* strain CBS 513.88, a filamentous fungus widely used in industry to produce enzymes and organic acids. Yuan *et al.* (2008) combined the sequencing data from the genome of *A. niger* CBS 513.88 with microarray experiments to identify novel enzymes. They reported 17 previously unknown enzymes from the GH13, 15 and 31 families with at least eight genes that encode α-amylases. When these genes were expressed in *Pichia pastoris*, the recombinant AmyG and AmyE α-amylases exhibited acid-stability and high levels of maltotriose being produced, making them potential candidates for applications in both the food and starch processing industries (Wang *et al.*, 2018).

Metatranscriptomic analysis is another approach that provides a direct and efficient sequence-based method to mine for novel enzymes. Yi *et al.* (2018) successfully obtained a thermostable α-amylase from Chinese Nong-flavor liquor. The enzyme displayed high stability at 60°C, acidic pH optima at 5.0–5.5 and was more efficient towards amylopectin hydrolysis, compared to amylose hydrolysis. Thermostable enzymes are attractive catalysts for many biotechnological processes and play a crucial role in the conventional starch to ethanol process.

Future Outlook

The global market for industrial enzymes was estimated to be around $4.2 billion in 2014 (Singh *et al.*, 2016) and due to a large demand for industrial amylases by various industries (specifically dairy and baking), it has been predicted that the global α-amylase baking enzyme market will reach $382.4 million by 2025 (Global amylase market see "Relevant Websites section"). There are several factors that are driving the growth of the amylase market, with food quality and environment-friendly manufacturing process being high on the list (Food enzyme market see "Relevant Websites section").

Biotechnological advances have positively benefited the production of new and improved amylases, thus increasing the global demand for these enzymes. The search for novel amylases is a continuous process (Mobini-Dehkordi and Javan, 2012) with a growing demand for industrial amylases for applications in beverage production (milkshakes, juices, wine and beer), pharmaceuticals and detergents (Global amylase market see "Relevant Websites section"). Furthermore, advances made with modified enzymes are expected to propel the use of amylases on a global scale since amylase properties can be tailored for improved performance under industrial conditions.

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Applications of Fungal Inulinases

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Introduction

Inulin: General Introduction, Distribution, Annual Production and Availability

The global inulin market is expected to exceed USD 2.5 billion by 2023. In 2015, the global market size for inulin production and consumption was estimated at over 250 kg tonnes and is likely to exceed 480 kg tonnes by 2023 with an increase of 8.5% (See Relevant Websites section). The European inulin market size was projected over 125 kg tons in 2015 and is expected to gain a moderate increase in upcoming years. The highest gain in inulin market that of more than 10% during 2016–2023, was estimated in Asia Pacific due to a boost in consumption of functional foods and dietary fibers particularly in India, Australia, and China.

Inulin, a fructan, is a storage polysaccharide mainly composed of a fructofuranose backbone connected by β $(2\rightarrow1)$ glycosidic linkages with a terminal glucose entity (Kango and Jain, 2011). A $(1 \leftrightarrow 2)$-bonded α-D-glucosyl group forms the terminal end of the inulin backbone (**Fig. 1**). It is the second most abundant plant storage carbohydrate polymer after starch and categorized as non-digestible carbohydrate as its fiber does not get digested in the colon. The fructose chain length varies from 2 to 60 monomeric units in an inulin molecule. When inulin is composed of a maximum of 10 fructose units, it is referred to as inulooligosaccharide (IOS) or fructooligosaccharide (FOS) or oligofructose. Inulin is found in several tropical and temperate plants as a major storage polysaccharide (**Table 1**). Apart from the prominently known inulin sources such as chicory, Jerusalem artichoke and dahlia, several common fruits, vegetables and cereals like onion, garlic, leek, tomato, banana, wheat, rye and barley are also some sources of inulin (Mensink *et al.*, 2015).

Inulinases: Characteristics, Production and Heterologous Expression

(1) *Types of inulinases*

Based on the type of the end products generated by their action, inulinases are classified into fructose generating exo-inulinases and oligosaccharide generating endo-inulinases.

(a) *Exo-inulinase (EC 3.2.1.80; β-D-fructanfructohydrolase or EC 3.2.1.153; β-(2,1)-D-fructanfructohydrolase)*: Exo-inulinase (EC 3.2.1.80) catalyzes the hydrolysis of terminal and non-reducing $(2-1)$-linked β-D-fructofuranose residues in fructans, while the other exo-inulinase (EC 3.2.1.153) hydrolyzes the terminal, non-reducing $(2-1)$ and $(2-6)$-linked β-D-fructofuranose residues in fructans and generates fructose (**Fig. 2**). Another relevant enzyme, sucrase (EC 3.2.1.26; β-D-fructofuranoside fructohydrolase or invertase) is also involved in the hydrolysis of terminal non-reducing β-D-fructofuranoside residues in β-D-fructofuranose and sucrose.

(b) *Endo-inulinase (EC 3.2.1.7; 1-β-D fructanfructanohydrolase)*: Endo-inulinases are also referred as 1-β-D fructanfructanohydrolases that catalyze hydrolysis of $(2\rightarrow1)$-β-D-fructosidic linkages within the main chain of inulin or polyfructose (**Fig. 2**).

Inulinases (exo and endo) and related enzymes such as fructosyltransferase, levan hydrolase and β-fructofuranosidase are categorized in glycoside hydrolase (GH) family 32 in the Carbohydrate-Active EnZymes (CAZy) database (Lombard *et al.*, 2014; Trollope *et al.*, 2015).

(2) *Structural characteristics of inulinases*

In the Enzyme Commission classification, inulinases belong to the group glycosidases (EC 3.2.1) of the glycosylases sub-class (EC 3.2). Crystal structure data of a fungal endo-inulinase, INU2, from *Aspergillus ficuum* has a 3-D structure sharing five β-strands organized as two β-sheets in a C-terminal β-sandwich domain. The N-terminal domain of the structure is composed of a 5-fold β-propeller catalytic domain with four β-sheets. The structure revealed that it has an extra inulin binding pocket formed by a conserved amino acid motif, W-M(I)-N-D(*E*)-P-N-G, and two loops. This structure includes Trp40, which plays a critical role in the catalysis of third unit of inulin and inulin-like substrates (Pouyez *et al.*, 2012). Inulinases and related enzymes have WMN(D/E)PN, RDP, SVEVF and EC (P) motifs (**Fig. 3**) as their conserved domains in the primary structure showing the involvement of conserved amino acid sequences in their catalytic mechanism (Park *et al.*, 2003; Nagem *et al.*, 2004; Kango and Jain, 2011). Exo-inulinases produced by the yeasts *Kluyveromyces marxianus* and *Pichia guilliermondii* are devoid of the SVEVF motifin their conserved domain (Liu *et al.*, 2013). Inulinase from *K. marxianus* Y-303 had 27% α-helices, 28% β-sheets and 45% unordered structures in its secondary structure (Holyavka *et al.*, 2016). The catalytic action of most of the glycoside hydrolases, including inulinase, involves two acid residues that act as nucleophile and proton donor. Site-specific mutagenesis and chemical modification procedures unraveled the catalytic functions of yeast inulinase carried out by

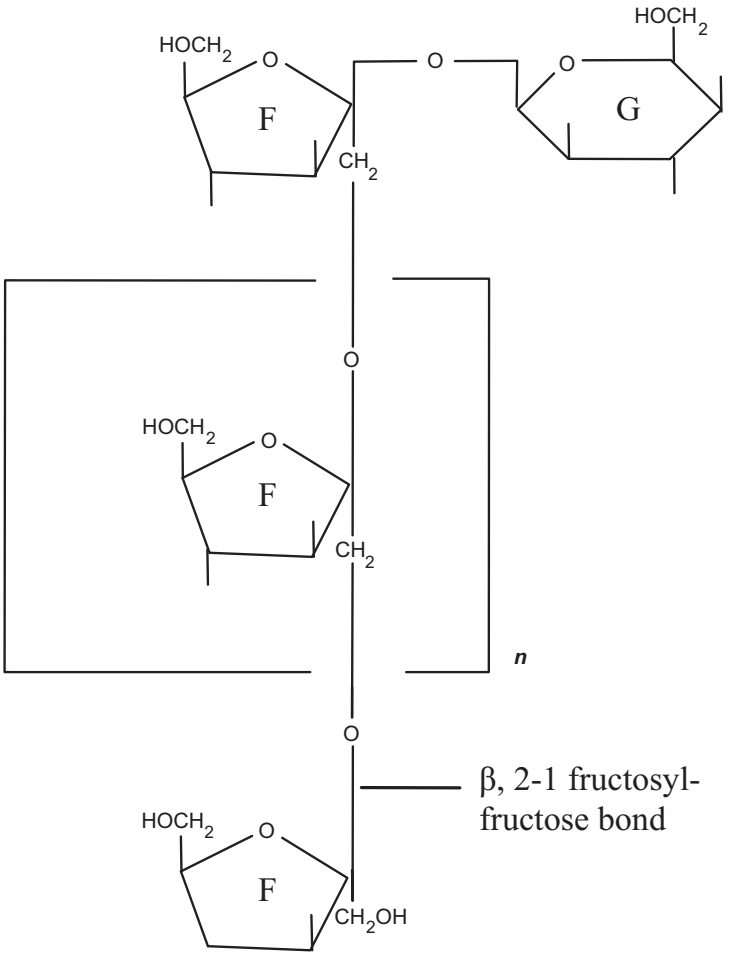

Fig. 1 Structure of inulin (Vandamme and Derycke, 1983). F-Fructose, G-Glucose.

Table 1 Some common sources of inulin

Source	*Plant part*	*Inulin content (%)*
Onion	Bulb	2–6
Jerusalem artichoke	Tuber	14–19
Dahlia	Tuber	9–12.5
Chicory	Root	15–20
Leek	Bulb	3–10
Garlic	Bulb	9–16
Artichoke	Leaves-heart	3–10
Banana	Fruit	0.3–0.7
Rye	Cereal	0.5–1
Barley	Cereal	0.5–1.5
Dandelion	Leaves	12–15
Burdock	Burdock	3.5–4.0
Camas	Bulb	12–22
Murnong	Root	8–13
Yacon	Root	3–19
Salsify	Root	4–11
Sweetleaf	Leaves	8–23
Suma	Roots	11.45

Note: Modified from Kango, N., Jain, S.C., 2011. Production and properties of microbial inulinases: Recent advances. Food Biotechnology 25, 1532–4249.

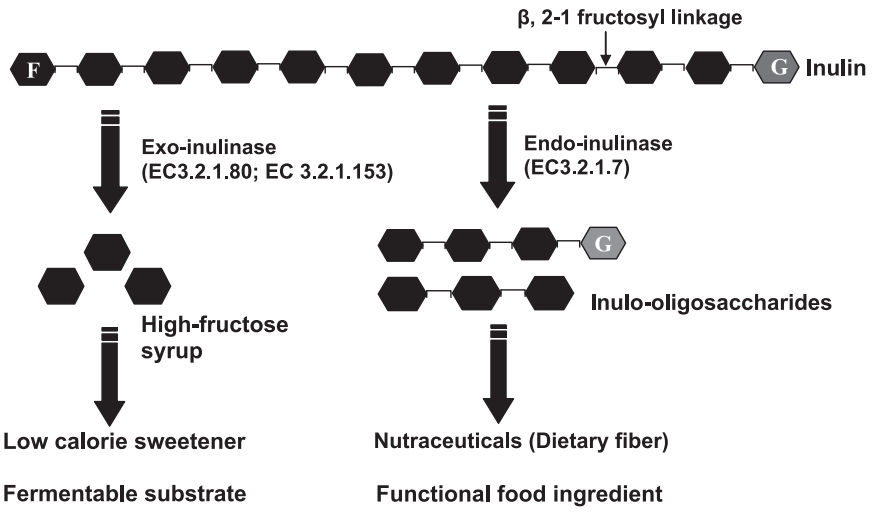

Fig. 2 Inulin being degraded by microbial exo- and endo-inulinase enzymes. Action of exoinulinase liberates fructose from the macromolecule while endoinulinase produces inulo-oligosaccharides. F-Fructose, G-Glucose. Modified from Kango, N., Jain, S.C., 2011. Production and properties of microbial inulinases: Recent advances. Food Biotechnology 25, 1532–4249.

the Asp23 and Glu204 amino acids belonging to the conserved part of WMN(D/E)PN and EC(P) sequences (Reddy and Maley, 1990).

(3) *Production and properties of inulinases*

A number of fungal and yeast strains have been reported to secrete high titers of extracellular inulinases. Several *Penicillium* spp. sourced from Jerusalem artichoke fields were found to produce inulinases, among which *Penicillium subrubescens* FBCC 1632 was selected as an efficient inulinase producer (Mansouri *et al.*, 2013). Recombinant inulinases sourced from *A. ficuum*, *Aspergillus niger* and *Penicillium janthinellum* were expressed in the yeast, *Pichia pastoris* resulting in high yield of inulinases (Chen *et al.*, 2013; He *et al.*, 2014; Chen *et al.*, 2015). Some other yeast such as *K. marxianus*, *Cryptococcus aureus*, *Meyerozyma guilliermondii* and *Zygosaccharomyces cerevisiae* are also reported to produce inulinases (Rawat *et al.*, 2017a). *Penicillium oxalicum* produced 322.1 U/gds (per gram dry substrate) exo-inulinase when grown on carrot pomace (Singh *et al.*, 2018), whereas *Aspergillus awamori* produced 2.2 U/mg exo-inulinase when grown on asparagus root extract medium (Rawat *et al.*, 2015a). *Aspergillus fumigatus* and some *Penicillum* spp. were able to produce exo- and endo-inulinases during growth on dahlia or asparagus root and tuber extract medium (Rawat *et al.*, 2015a).

Exo-inulinases are also produced by several fungi, yeast and bacteria, among which Aspergilli are recognized as a prominent source of exo-inulinases. Several reports ascertained the use of agro-waste or plant biomass for inulinase production by fungi (Das *et al.*, 2019a). Utilization of inulin-rich plant biomass for inulinase production is a cost-effective and eco-friendly approach. *A. niger* is well known for the production of high titers of exo-inulinase in solid state fermentation (SSF). It has been reported to produce 11.13 U/gds of exo-inulinase on Jerusalem artichoke and bean, while 137.2 U/gds and 200 U/gds when grown on rice bran and banana peels, respectively (Narayanan *et al.*, 2013; Al-Dabbagh and Mahmood, 2015). Other members of this genus, *Aspergillus parasiticus*, *Aspergillus terreus* and *Aspergillus versicolor* are reported to produce exo-inulinase on sugarcane bagasse, artichoke leaves, garlic wastes, chicory roots and orange rinds, respectively (Abd El Aty *et al.*, 2014). *Penicillium brevicompactum* produced 1.24 U/gds and 0.66 U/gds exo-inulinase on artichoke leaves and garlic waste, respectively, while *Penicillium rugulosum* produced 239 U/gds exo-inulinase on copra waste substrate (Abd El Aty *et al.*, 2014; Dilipkumar *et al.*, 2014). Other than filamentous fungi, some yeasts such as *Cryptococcus*, *Geotrichum*, *Kluyveromyces* and *Saccharomyces* are well known sources of exo-inulinase. *C. aureus* produced 420 U/gds exo-inulinase on wheat bran and rice husk substrates (Sheng *et al.*, 2009). Other studies suggested that *K. marxianus* is among the best yeast for exo-inulinase production on low-cost substrates. *Kluyveromyces* S120 was reported to produce 409.8 U/gds inulinase on wheat bran, while another *K. marxianus* strain produced 106.72 U/gds and 21.23U/gds activities on coarse wheat bran and corn flour substrate, respectively (Selvakumar and Pandey, 1999; Xiong *et al.*, 2007). Another isolate of *K. marxianus* produced 463 U/gds inulinase on sugarcane bagasse (SCB), cane molasses and soybean bran while it produced 586 U/gds inulinase on mixed SCB and soybean meal (Mazutti *et al.*, 2010). Dimorphic yeast, *Geotrichum candidum*, resulted in a similar level of 412 U/gds inulinase when grown on leek powder (Canli and Kurbanoglu, 2012).

(4) *Inulinase assay and characterization*

It is important to understand some important aspects of the inulinase assay with regard to the choice of substrate, as inulin from various sources (e.g., chicory and dahlia inulin) shows variability. The amount of enzyme that catalyzes formation of 1 μmol of fructose in one minute from inulin (usually 0.5–1.0% w/w chicory inulin) is defined as one inulinase unit, while the invertase or fructofuranosidase unit is defined as the amount of enzyme that generates 1 μmol of fructose or glucose from sucrose (Kango, 2008; Rawat *et al.*, 2015a). Exo-inulinases catalyze the exo-hydrolysis of the

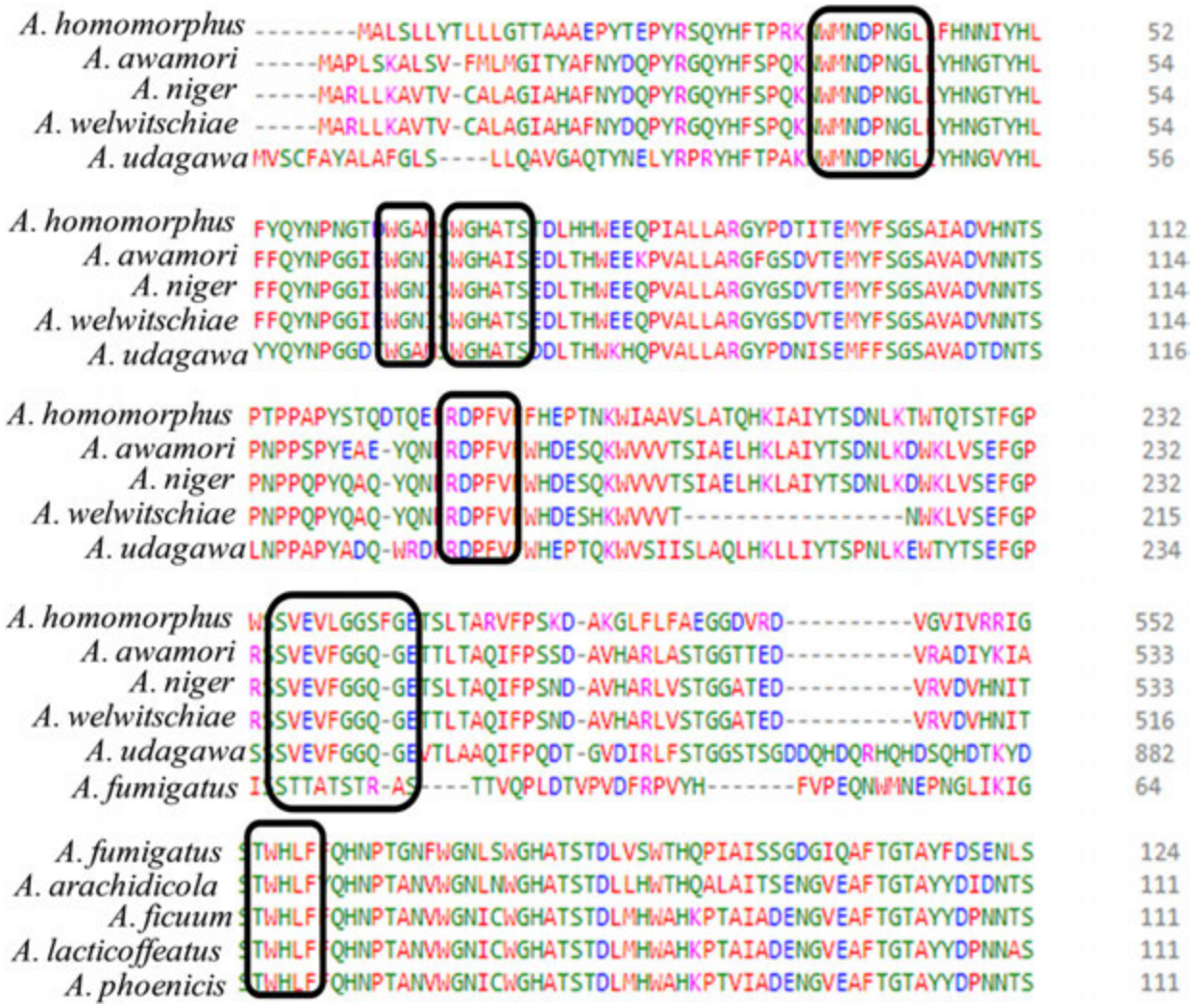

Fig. 3 Multiple alignment of some *Aspergillus* inulinases amino acid sequences. Sequences have been selected from GenBank and aligned using CLUSTAL W software for multiple alignment. Sources of fungal inulinases: *Aspergillus lactoffeatus* CBS 101883 *(PYH56754.1)*; *Aspergillus phoenicis (RDK37757.1)*; *Aspergillus niger (EHA25512.1)*; *Aspergillus fumigatus (KEY80659.1)*; *Aspergillus awamori* exo-inulinase *(CAC44220.1)*; *Aspergillus udagawae (GAO81637.1)*; *Aspergillus arachidicola (PIC87873.1)*; *Aspergillus ficuum (CAA07345.1)*; *Aspergillus homomorphus (RAL14360.1)*; *Aspergillus welwitschiae* exo-inulinase *(RDH30424.1)*.

inulin molecule and, therefore, also show invertase activity while, endo-inulinase lacks this activity. Usually, 0.5–1.0% inulin (Chicory) in citrate buffer (pH 5.0 at 50°C) is used for the inulinase assay (Rawat *et al.*, 2015a). This assay is based upon estimation of the fructose equivalents generated from the inulin polymer, but does not distinguish between exo- and endo-inulinases. These can be distinguished on the basis of end-product analysis of hydrolysis using thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) (Rawat *et al.*, 2015a). The hydrolytic activity of exo-inulinase can be distinguished from invertase by the determination of I/S ratio that is the relative activity towards inulin and sucrose. Higher value of I/S ratio indicates greater affinity of the hydrolase towards inulin as compared to sucrose.

(5) *Cloning and over-expression of inulinase*

As for other commercial enzymes, cloning and heterologous expression of exo- and endo-inulinases in bacterial and yeast hosts for enhanced yield has been attempted by several workers. Yeasts play an important role as expression host as they have the ability to grow faster than filamentous fungi and can produce high amounts of eukaryotic or processed proteins. Exo-inulinase from *A. niger* was co-expressed with a gene from *Saccharomyces cerevisiae* named *hac1*, which increases protein folding and secretory pathways of eukaryotic proteins from the endoplasmic reticulum, in *P. pastoris*. The growth of the recombinant *P. pastoris* strain resulted in 95% inulin conversion into fructose (Chen *et al.*, 2016). Endo-inulinases from *Lipomyces starkeyi* and *Aspergillus arachidicola* have also been cloned and expressed in *Escherichia coli* (Bao *et al.*, 2019; Jiang *et al.*, 2019).

Applications of Inulinases

High Fructose Syrup Production

Inulin is abundantly stored in many plants such as chicory, Jerusalem artichoke, dahlia and dandelion (**Table 1**). Due to the abundance and renewability of this plant fructan, it can be readily utilized for generation of fructose and FOS for food, beverages and pharmaceutical industries (Singh and Singh, 2017a). Major applications of inulinases in diverse industries are compiled in **Table 2**.

Fructose is a low-calorie and generally regarded as safe (GRAS) sweetener having a two-fold higher sweetness index and half the calorific value compared to sucrose (Singh *et al.*, 2017b; Kamble *et al.*, 2019). Fructose production using traditional methods involves either sucrose hydrolysis by invertase into equimolar mixtures of glucose and fructose (Chen *et al.*, 2016), or production of glucose-fructose syrup, using starch hydrolysis by glucoamylase and subsequent conversion into fructose by glucose isomerase. The maximum achievable yield by these methods is only 45%–50% of fructose in the syrup (Chen *et al.*, 2016). In comparison to conventional methods of fructose generation from starch using α-amylase, glucoamylase and isomerase, fructose generation using exo-inulinases yields up to 90%–95% fructose from raw inulin sources (Hughes *et al.*, 2017). These procedures also require purification of fructose using ultra-filtration and crystallization (Guthrie and Morton, 2000). In contrast, inulin is a linear polymer of fructose and therefore, fructose generation using fungal inulinases is a single step process. Biocatalytic hydrolysis of inulin by exo-inulinase to generate fructose units is considered as an efficient, direct, economic and eco-friendly approach (Singh *et al.*, 2018).

Major exo-inulinase producers belong to the genera *Aspergillus*, *Fusarium*, and *Penicillium* and application of their enzymes has been reported to result in high fructose syrups from inulin (Rawat *et al.*, 2015a; Magadum and Yadav, 2018). Among yeasts, *Kluyveromyces* spp. is known to be a prolific source of exo-inulinase (Liu *et al.*, 2016; Bae *et al.*, 2018).

Inulin rich plants, such as Jerusalem artichoke and dahlia are gaining increased industrial attention, because these are economical, abundant and renewable sources of this fructan. Exo-inulinases convert plant inulin into fructose, an easily fermentable monosugar, which can be used for production of a number of products such as citric acid, ethanol, butanol, biodiesel, and lactic acid (**Fig. 4**) (Gao *et al.*, 2015).

Fructose generation from enzymatic hydrolysis of plant inulin is gaining interest due to its cost-effective generation using agro-wastes. Many plants listed in **Table 1** have sufficient amounts of inulin, which yields fructose when hydrolyzed either chemically or by fungal inulinases. Enzymatic hydrolysis is preferable as it yields pure fructose syrup and indiscriminate products due to acid or alkali treatment are avoided. Both roots and stems of some inulin rich plants were used as raw material for fructose generation (Holyavka *et al.*, 2018). *A. niger* was used for saccharification of *Agave tequilana* fructans to fructose (Huitrón *et al.*, 2013). Alginate immobilized exo-inulinase from *A. niger* was tested for fructose generation from dahlia inulin and dandelion tap root extract in a packed bed bioreactor. Continuous generation of fructose (>90% w/w yield) from *Taraxacum officinale* tap root extract and chicory inulin using *A. niger* exo-inulinase in a packed-bed reactor has been demonstrated (Rawat *et al.*, 2017b). Various inulin sources such as Jerusalem artichoke root, chicory root, asparagus root, dahlia tubers and agave juice have been used for enzymatic generation of fructose using fungal and yeast inulinases (Garcia-Aguirre *et al.*, 2009; Singh *et al.*, 2018). Asparagus and chicory root extracts hydrolyzed by *Aspergillus tubingensis* inulinase in a batch reactor resulted in 14.25 g/L fructose generation (Trivedi *et al.*, 2015) while kuth (*Saussarea lappa*) root powder upon treatment with *A. niger* inulinase in a continuous fermentation system produced 68 g/L of fructose (Yewale *et al.*, 2013).

Production of Prebiotic Fructooligosaccharides

Fructooligosaccharides (FOS) are well known prebiotics as they have tremendous capacity to selectively nourish beneficial gut microbiota. Some FOS naturally occur in plants such as Jerusalem artichoke, dahlia with inulin, fructose and other sugars (Rawat *et al.*, 2017b). Inulin type FOS or inulooligosaccharides (IOS), consist of fructose units linked with β-2,1 glycosidic linkages with a terminal glucose unit, while the other type is composed of only fructose (Fn or FOS) molecules linked by β-2,1 glycosidic linkages. FOS can be obtained either by hydrolyzing naturally occurring fructan (inulin) into oligofructose; Fn type FOS (DP > 9) by endo-inulinase, or by transfructosylation of sucrose into oligofructose containing terminal glucose; GFn type FOS (DP < 4) by fructosyltransferase (Ganaie *et al.*, 2014; Bali *et al.*, 2015; Choukade and Kango, 2019).

Several endo-inulinases have been reported from fungal strains among which endo-inulinase from *A. niger* is well explored and also commercially exploited to generate inulin type FOS (Kango and Jain, 2011; Li *et al.*, 2013; Vallejo-García *et al.*, 2019). Endoinulinase from *A. niger* has been reported to produce inulotriose (F3), inulotetraose (F4) and inulopentaose (F5) from inulin produced by the bacterium *Leuconostoc citreum*. The enzyme yielded 30 g/L of FOS and 10 g/L of free fructose in 4 h from 40 g/L bacterial inulin (Vallejo-García *et al.*, 2019). Kawee-Ai *et al.* (2019) have reported that conversion of sucrose (300 g/L) to FOS (0.71 FOS g/g substrate) using *A. niger* inulinase can be expedited by conducting the reaction under high pressure (300 MPa). An endo-inulinase from *A. arachidicola* generated DP3 and DP4 oligosaccharides at 60°C (Jiang *et al.*, 2019). Inulinase from *L. starkeyi* produced FOS mixture containing 26% DP3, 34% DP4, and 23% DP5 oligosaccharides from 5% (w/v) inulin (Bao *et al.*, 2019). A mixture of exo-inulinase prepared using *A. niger* and commercial endo-inulinase (Megazyme) in a ratio of 1:3 resulted in 33% hydrolysis of low DP agavins (from *Agave angustifolia*) at 60°C and generated 10% of different DP FOS with DP7 and DP8 as major products (Huazano-García and López, 2018).

Table 2 Applications of fungal inulinases in generation of value-added products

Applications	*Organism*	*Enzyme*	*Product (s)*	*Yield*	*Reference*
High Fructose Syrup (HFS)	*Aspergillus flavus* *Aspergillus terreus*	Exo-inulinase	HFS	–	(Das *et al.*, 2019b)
		Exo-inulinase	HFS	92% bioconversion of sucrose using Ca-alginate immobilized inulinase	(Magadum and Yadav, 2018)
	Kluyveromyces marxianus	Immobilized exo-inulinase on KU – 2 matrix cation-exchanger/ Adsorption	Fructose	–	(Holyavka *et al.*, 2019)
Single Cell Oil (SCO)	*Rhodotorula paludigena*	Inulinase	Mannitol esters of 3-hydroxy C14, C16 and C18 fatty acids with variable acetylation	48.5 g/L of polyol esters of fatty acids and16.9 g/L of intracellular lipid in 156 h	(Wang *et al.*, 2019)
	Yarrowia lipolytica X2	Inulinase	Fatty acid	42% (w/w) in 78 h	(Shi *et al.*, 2018)
	Y. lipolytica X + N8	Inulinase	$C_{15:0}$ (0.28%), $C_{16:0}$ (14.26%), $C_{16:1}$ (10.06%), $C_{17:1}$ (1.46%), $C_{17:0}$ (0.27%), $C_{18:2}$ (8.77%), $C_{18:1}$ (53.10%), $C_{18:0}$ (8.82%), $C_{20:0}$ (0.42%), $C_{22:0}$ (0.43%), $C_{24:0}$ (2.14%),	48% (w/w) in 78 h	
	Recombinant oleaginous yeast *Aureobasidium melanogenum*	Inulinase	$C_{16:0}$ (29.95 ± 0.2%), $C_{16:1}$ (2.13 ± 1.0%), $C_{18:0}$ (6.06 ± 0.4%), $C_{18:1}$ (35.58 ± 4.0%), $C_{18:2}$ (25.98 ± 0.3%)	66% (w/w) in 120 h 0.12 g/g of consumed inulin	(Li *et al.*, 2019)
Oligosaccharides (IOS or FOS)	*Aspergillus niger*	Endo-inulinase (Novozym 960)	Inulotriose (F3), inulotetraose (F4), inulopentaose (F5)	approximately 30 g/L of FOS and 10 g/L of free fructose were produced from 40 g/L of bacterial inulin in 4 h.	(Vallejo-García *et al.*, 2019)
	Y. lipolytica recombinant strain	Endo-inulinase	DP 3–5 FOS	0.912 ± 0.012 g_{FOS}/g_{Inulin} in 2 h	(Mao *et al.*, 2019)
	Aspergillus arachidicola	Endo-inulinase	DP3 and DP4	–	(Jiang *et al.*, 2019)
	Lipomyces starkeyi	Endo-inulinase	DP 3–5	26% DP3, 34% DP4 and 23% DP5	(Bao *et al.*, 2019)
Bioethanol	*Saccharomyces cerevisiae*	Exo-inulinase	Bioethanol	85.2 g/L in 72 h	(Khatun *et al.*, 2016)
	K. marxianus	Exo-inulinase	Bioethanol	105 g/L at 37°C 97 g/L at 40°C	(Charoensopharat *et al.*, 2015)

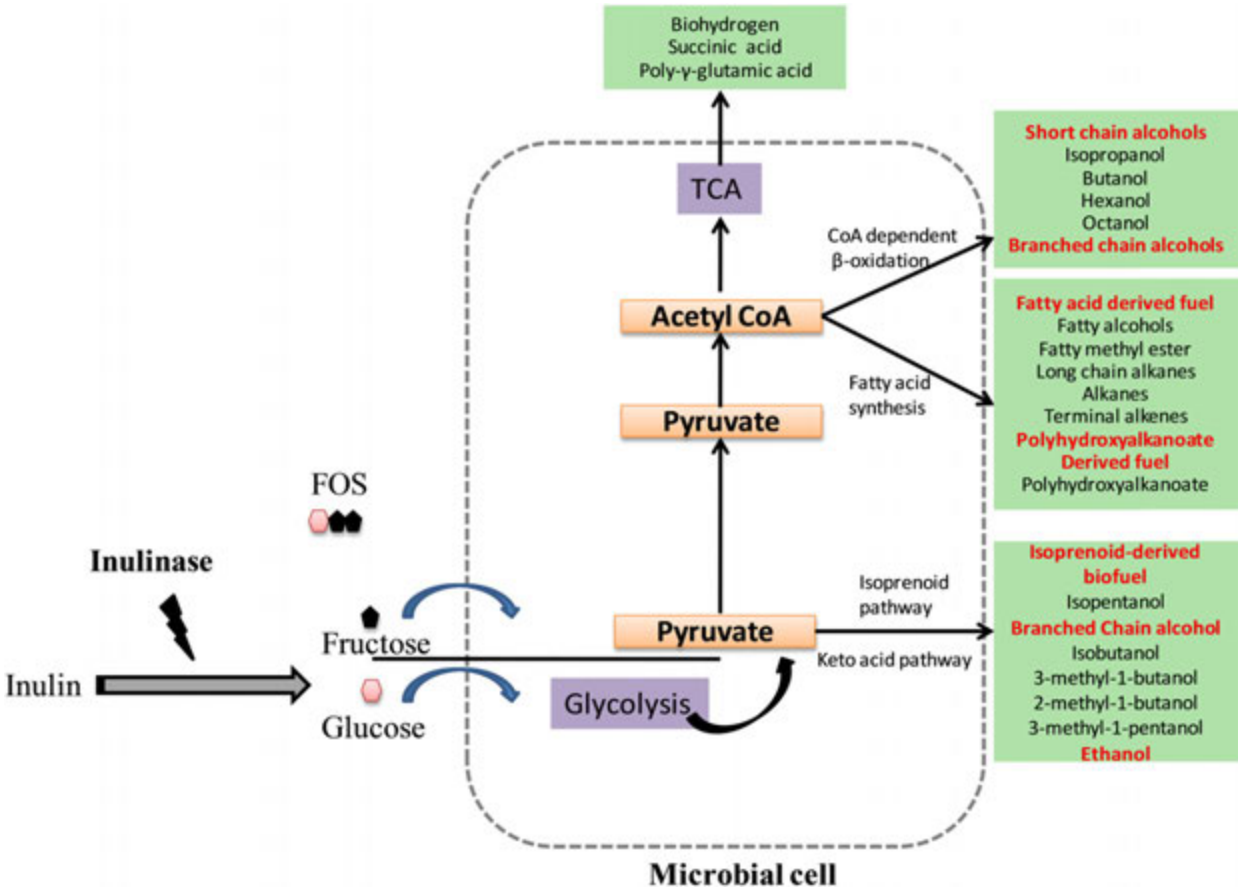

Fig. 4 Metabolism of fructose generated by inulin hydrolysis and generation of various value-added products.

A number of fungi belonging to genera *Aspergillus*, *Penicillium*, *Fusarium*, *Arthrinium*, and *Kluveromyces* have been shown to produce inulinase that generate inulooligosaccharides from chicory or Dahlia inulin (Rawat *et al.*, 2015a,b). Agavins or the fructans derived from Agave (*A. angustifolia*) have been exploited to generate branched fructooligosaccharides (*a*-FOS) using a commercial endo-inulinase from *A. niger* (Huazano-García and López, 2018). Cloning and overexpression of fungal inulinases has been successfully attempted in both bacteria and yeast. *A. arachidicola* endo-inulinase gene expressed in *E. coli* resulted in 88.4% IOS generation from 10 g/L inulin (Jiang *et al.*, 2019). Endoinulinase gene from *A. fumigatus* was cloned and over expressed in *P. pastoris* which resulted in the generation of IOS of DP 3, 4, 5, and >5 (Chen *et al.*, 2015). Recombinant endo-inulinase from *Fusarium oxysporum* was expressed in *P. pastoris* and used to generate FOS from inulin (8%) to 90.5±5% DP 3–8 FOS (Yang *et al.*, 2016). Mao *et al.* (2019) developed a high inulinase yielding *Yarrowia lipolytica* strain expressing a fusion gene comprising inulin binding module from the bacterium *Bacillus macerans* and endo-inulinase from *A. niger*. The engineered *Y. lipolytica* generated FOS (DP3–5) with a yield of 0.912 ± 0.012 g_{FOS}/g_{Inulin} in 2 h.

Bioethanol Generation

Ethanol is the most popular biofuel that can be produced using monosugars generated from renewable plant biomass. A number of plants have been reported to contain inulin as their storage polysaccharide (Rawat *et al.*, 2017a). Fructose generation from inulin hydrolysis using exo-inulinase and its subsequent ethanolic fermentation by yeasts is viewed as a cheap and eco-friendly approach for bioethanol generation (Chi *et al.*, 2011). Coffee wastes containing pulp and mucilage were utilized to produce ethanol by *K. maraxianus* Km7 strain by the catalytic action of inulinase, with a maximum ethanol yield of 10 g/L (Galindo-Leva *et al.*, 2016). Exo-inulinase producing yeasts, such as *K. marxianus* and *S. cerevisiae* are used for consolidated bioprocessing (CBP), in which hydrolysis of an inulin rich feedstock is coupled with fermentation of fructose to ethanol. Fructose is a readily fermentable sugar and is efficiently converted to ethanol by yeasts (Chi *et al.*, 2009). Plant biomass containing inulin such as Jerusalem artichoke tubers, Dahlia tubers and Asparagus roots can be utilized as raw material for fructose generation, which could further be utilized for large scale ethanol production. Simultaneous saccharification and ethanol generation of Jerusalem artichoke tuber powder using *K. marxianus* resulted in 104.83 g/L ethanol at 37°C (Charoensopharat *et al.*, 2015). Similarly, *S. cerevisiae* 6525

generated 84.3 g/L ethanol from 240 g/L dry powder of Jerusalem artichoke tubers in 72 h with a bioethanol yield of 0.453 or 88.6% (Wang *et al.*, 2015).

Single Cell Oil (SCO) Production

Biodiesel production from greener sources is gaining increasing interest and as a result, utilization of oleaginous SCO generators has gained considerable attention in recent years (Wang *et al.*, 2018). Microorganisms utilize plant sugars and produce oil in their cells similar to plant oils and are, therefore, suggested as alternate sources of oil (Liang and Jiang, 2013). Biodiesel is more advantageous as compared to traditional fuel due to its renewable, non-toxic and biologically degradable nature (Zeng *et al.*, 2017). Several yeasts, such as *Rhodotorula*, *Yarrowia*, *Aureobasidium*, *Candida*, *Cryptococcus*, *Trichosporon*, *Lipomyces*, *Papiliotrema*, *Rhodosporidium*, and *Pichia* have been reported to produce single cell oil (Li *et al.*, 2019). SCO production using lignocellulosic materials is comparatively expensive due to mandatory pretreatment. Inulin, on the other hand, can easily be converted into fructose monomers by the action of exo-inulinases, which can be further utilized by oleaginous yeasts for SCO accumulation in their cells. *Rhodotorula paludigena* produced 48.5 g/L of polyol esters of fatty acids mainly composed of mannitol esters of 3-hydroxy C14, C16, and C18 fatty acids with variable acetylation and accumulated 16.9 g/L of intracellular lipids from inulin in 156 h in batch fermentation (Wang *et al.*, 2019). An oleaginous yeast, *Y. lipolytica*, accumulated 48.3% (w/w) oil and produced 13.3 g/L dry cell mass in 78 h from inulin, while 50.6% (w/w) oil and 14.6 g/L dry cell mass was accumulated from Jerusalem tuber extract in 78 h (Zhao *et al.*, 2010b). *Aureobasidium melanogenum* 9–1 produced 28.5 g/L of crude heavy oils in the medium containing 120.0 g/L glucose. Interestingly, the strain also produced 30.2 g/L of crude heavy oils in medium containing 160.0 g/L inulin. A transformant of this strain expressing exo-inulinase from *K. marxianus* could yield 43.0 g/L of crude heavy oils and cell dry weight was 24.7 g/L in 120 h of fermentation (Xin *et al.*, 2017).

Citric Acid and Lactic Acid Production

Global citric acid production has reached 2 million tonnes in 2015 and is expected to increase by 3.7% annually (See Relevant Websites Section). It is mainly used as food acidulant to provide protection against oxidative deterioration of food flavoring agents (Rakicka *et al.*, 2019).One of the most important criteria for industrial production of organic acids is the use of low-cost substrates for economic production. These raw materials should be inexpensive, renewable and non-toxic for industrial application. In this regard, inulin containing plant biomass presents itself as a promising substrate for industrial production of organic acids. Citric acid (200 g/L) production from pure inulin using *Y. lipolytica* with a yield of 0.85 g citric acid per gram of inulin has been demonstrated (Rakicka *et al.*, 2019). Another strain of *Y. lipolytica* produced 105.2 g/L citric acid from 200 g/L pure inulin (Rakicka *et al.*, 2016), while another *Y. lipolytica* strain produced 84 g/L citric acid and 1.8 g/L of isocitric acid from 10% inulin in 214 h when grown in a 5 L batch fermenter (Liu *et al.*, 2013).

Single Cell Protein and Tequila Production

Single cell protein (SCP) has variety of applications in nutraceutical industries due to high protein and essential amino acid content. Recombinant *Y. lipolytica* expressing an exo-inulinase gene from *K. marxianus* origin accumulated 47.5% protein in a 4% inulin medium in 72 h (Cui *et al.*, 2011). *C. aureus* G7a and its mutant Z114 were found to accumulate 59.1% and over 61% total protein when grown on inulin rich Yacon extract for 45 h at 37°C (Zhao *et al.*, 2010a).

Tequila, a fermented product of blue agave (*A. tequilana*) is a Mexican beverage that originated in Jalisco, Mexico. This beverage is the distilled product of fermented fructan rich agave juice. Enzymatic hydrolysis of agave with inulinases is an environmentally friendly approach to obtain tequila from agave juice and inulinase from *A. niger* has been employed for this purpose (Huitrón *et al.*, 2013).

Immobilized Inulinases

Enzyme immobilization is one of the best approaches for their cost-effective application and achieving higher stability and efficiency under harsh industrial environment (Singh *et al.*, 2017c). Generally, inulin hydrolysis is carried out at or over 60°C to obtain high fructose yields and to avoid any microbial contamination. Immobilized exo-inulinase from *K. marxianus* on KU-2 matrix (a cation-exchanger) showed optimal activity at 70°C (Holyavka *et al.*, 2019). Another anion exchanger matrix, AV-17–2P, was used to immobilize *K. marxianus* inulinase and was reusable up to 10 times (each cycle of 20 min) with no significant loss in activity (Holyavka *et al.*, 2018). Glutathione reductase coated gold nanoparticles (GSHAuMNPs) were also employed as covalent immobilization matrix to immobilize commercial inulinase resulting in 83% conversion of inulin with high amounts (98%) of fructose generation. The immobilized enzyme was recyclable upto 10 cycles (Mohammadi *et al.*, 2019). An exo-inulinase from *Aspergillus tamarii* was immobilized on kaolinite clay using glutaraldehyde as a cross-linker. The enzyme showed enhanced affinity towards the substrate and was thermostable at 65°C for 6 h (Garuba *et al.*, 2018). Immobilized inulinase from *A. niger* showed enhanced storage stability up to 28 days when immobilized on Eupergit® C matrix using *bis*[4(2-hydroxy-3-methacryloyloxypropoxy)phenyl] as cross-linker *via* sulfide and divinylbenzene cross-linking. The enzyme produced fructooligosaccharides ranging from DP2 to DP6 (Trytek *et al.*, 2015).

Conclusion

Fungal inulinases have received considerable interest among researchers for their role in food and nutraceutical industries in the production of HFS and prebiotic FOS. Inulinases have paved the way for value-addition of abundant inulin rich plant material e.g. conversion of Jerusalem artichoke tuber powder to SCO, organic acids, alcohol etc. The strategy of molecular cloning and heterologous expression of fungal inulinases in yeasts has allowed consolidated bioprocessing for simultaneous saccharification and fermentation of inulin into ethanol, citric acid, lactic acid etc. Current trends in fungal inulinases suggest that they have very high potential in exploiting plant inulin for generation of HFS and bioethanol.

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Fungal Proteases: Current and Potential Industrial Applications

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Introduction

Filamentous fungi have been widely used for a long time as cell factories for the production of high value compounds, metabolites and enzymes. Particularly, fungi are known to produce a broad range of hydrolytic enzymes such as lignocellulose degrading enzymes, lipases and proteases. These enzymes are extensively studied and explored for various industrial applications. Proteases represent a large and complex group of enzymes that catalyze the hydrolysis of peptide bonds in proteins (López-Otín and Bond, 2008). They are structurally and functionally diverse and vary in their substrate specificity, catalytic mechanism, nature of their active site, stability, optimum pH and temperature (López-Otín and Bond, 2008). They are secreted by all living organisms and are involved in carrying out and regulating various complex biological processes and signaling cascades. They play an important role in processes such as cell growth, cell division, apoptosis, signal transduction, sporulation and germination, viral protein synthesis, protection against harmful peptides, degradation of incorrectly folded proteins and autolysis (Rao *et al.*, 1998). The major role of extracellular proteases is in nutrition due to their involvement in the hydrolysis of proteinaceous substrates. The degradation of long polypeptides into smaller peptides and amino acids contributes to their uptake by the cell.

Besides their involvement in essential biological functions, proteases are used in numerous industrial processes representing the most dominant enzyme sales worldwide (Giansanti *et al.*, 2016; Li *et al.*, 2013a; Rao *et al.*, 1998). Proteins are degraded enzymatically using proteases or chemically in industrial settings. Protein hydrolysates produced using chemical catalysts often contain undesirably modified amino acids. Moreover, chemical degradation is not environmentally friendly and can result in uncontrollable reactions. Using proteases avoids these disadvantages. Proteases have high reaction rates that can be easily controlled. They are also sustainable, highly specific and can be easily inactivated once the desired degree of modification is achieved (López-Otín and Bond, 2008; Rao *et al.*, 1998). Therefore, production of proteases has gained interest in scientific and industrial communities.

Proteases obtained from animals, plants, and, particularly, microorganisms represent a large and important industrial section. Plant proteases include papain, bromelain, keratinases, and ficain (Rao *et al.*, 1998). Their extraction is a time-consuming process and depends on many factors such as climate conditions, viability and availability of land. Examples of proteases from animal origin are trypsin, chymotrypsin, pepsin and rennin (Rao *et al.*, 1998). These enzymes are produced at smaller quantities when compared to the production of proteases from plants and microorganisms. The use of animals as a source of proteases is dependent on availability of livestock and is regulated by ethical policies.

For the reasons mentioned above, microorganisms represent an excellent source of proteases and are main producers of commercially available proteases. The vast diversity, fast growth, easy to manipulate, and simplified downstream processing are advantages of using microorganisms for the economical and efficient bulk production of proteases in comparison with using plant or animal cells (Contesini *et al.*, 2018; de Souza *et al.*, 2015). Microbial proteases have a broad spectrum of industrial applications such as dairy and meat processing, brewing, detergents, leather and pharmaceutical industries. The commercial protease cocktail Alcalase was the first detergent protease which was initially obtained from a strain of the bacterium *Bacillus subtilis* (Chouyyok *et al.*, 2005). Another example of a bacterial protease is a thermostable zinc endopeptidase thermolysin that is obtained from *Bacillus thermoproteolyticus* and has optimum catalytic activity up to 70°C and potential in the biopharmaceutical industry (Inouye *et al.*, 2007; O'Donohue *et al.*, 1994). Bacterial proteases show high reaction rate and temperature stability, while fungal proteases are diverse, substrate specific and effective over a wide pH range (Rao *et al.*, 1998). Examples of fungal species reported to produce proteases include the ascomycetes *Aspergillus sojae* (Gerelt *et al.*, 2000; Ke *et al.*, 2018), *Aspergillus oryzae* (Gerelt *et al.*, 2000; Yue *et al.*, 2019) and *Penicillium chrysogenum* strain Pg222 (Benito *et al.*, 2003; Omrane Benmrad *et al.*, 2018; Zhu *et al.*, 2009), and the zygomycete *Rhizomucor miehei* (Soltani *et al.*, 2016; Sun *et al.*, 2018).

In this chapter, proteolytic enzymes from filamentous fungi and their characteristics will be discussed in relation to their current and potential industrial applications including food, pharmaceutical and detergent industries, leather and fabric processing, waste management, and research practice. The focus is also on different classes of fungal proteases, their mode of action and their role in fungal physiology.

Fungal Proteases

Filamentous fungi are extensively studied for protease production because of their ability to grow on various substrates. Production of proteases by fungi is strain dependent and influenced by a wide range of factors including pH, carbon and nitrogen sources, temperature, aeration, requirement of metal ion, agitation, etc. (Rao *et al.*, 1998). Based on the optimum pH, proteases can be grouped into acidic (pH 2.0–6.0), neutral (pH 6.0–8.0) and alkaline (pH 8.0–13.0) proteases (Rao *et al.*, 1998; Sabotič and Kos, 2012). Acidophilic and alkalophilic fungi, often originating from high acidic or alkaline environments, are able to produce

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Table 1 Main groups of proteases, their properties and examples of fungal sources

Class	*Active site*	*Optimal pH range*	*Fungal source*	*Reference*
Aspartic protease	Asp-Asp	3 – 5	*Endothia parasitica*	(Sardinas, 1968)
			Mucor miehei	(Sternberg, 1972)
			Mucor pusillus	(Zhang *et al.*, 2015; Tonouchi *et al.*, 1986)
			Mucor baciliformis	(Areces *et al.*, 1992; Venera *et al.*, 1997)
			Penicillium oxalicum	(Hashem, 2000)
Serine protease	Ser-His-Asp/Glu	7 – 11	*Arthrobotrys oligospora*	(Minglian *et al.*, 2004)
			Aspergillus clavatus	(Hajji *et al.*, 2007)
			Penicillium chrysogenium	(Omrane Benmrad *et al.*, 2018)
			Tritirachium album	(Yang *et al.*, 2016)
			Thermoascus aurantiacus	(Li *et al.*, 2011)
Metalloprotease	Glu-Try	7 – 9	*Armillaria mellea*	(Healy *et al.*, 1999; Kim and Kim, 1999, 2001)
			Coprinopsis cinerea	(Lilly *et al.*, 2008)
			Microsporum cani	(Brouta *et al.*, 2002)
			Pleurotus ostreatus	(Joh *et al.*, 2004; Nonaka *et al.*, 1997)
Glutamic protease	Glu-Asp	2 – 3.5	*Phanerochaete chrysosporium*	(Sims *et al.*, 2004)
			Talaromyces emersonii	(O'Donoghue *et al.*, 2008)
Cysteine protease	Cys-His-Asp	2 – 3	*Botrytis cinerea*	(Liu *et al.*, 2019)
Threonine protease	Thr	6.5 – 7.5	The role is mostly unknown	

acidic and alkaline proteases, which have great potential in industrial applications. For instance, in the detergent industry where the pH of cleaning products usually ranges from pH 9.0–12.0, alkaline proteases are used (Maurer, 2004; Niyonzima and More, 2015a,b; Saeki *et al.*, 2007). Examples of fungal species that are able to produce alkaline proteases include the ascomycetes *Myceliophthora* sp. (Zanphorlin *et al.*, 2010), *Aspergillus tamarii* (da Silva *et al.*, 2018) and *Fusarium* sp. (Ueda *et al.*, 2007), and the zygomycete *Rhyzopus oryzae* (Negi *et al.*, 2020). A number of fungi, e.g. the ascomycetes *Neurospora crassa* (Guo *et al.*, 2011), *A. oryzae* (Vishwanatha *et al.*, 2010; Yue *et al.*, 2019) and *Penicillium duponti* (Emi *et al.*, 1976), and the basidiomycetes *Phanerochaete chrysosporium* (Dass *et al.*, 1995) and *Trametes sanguinea* (Tomoda and Shimazono, 1964), produce acid proteases which are used in food industry, as digestive aids and have potential in ethanol production.

Besides being grouped by their activity under acidic, neutral, or alkaline conditions, proteases can be also divided into exopeptidases, i.e., aminopeptidases and carboxypeptidases that act on the on the N-terminus or C-terminus of the peptide chain, respectively, and cleave one or a few amino acids, and endopeptidases that target internal peptide bonds (López-Otín and Bond, 2008; Rao *et al.*, 1998). Based on the catalytic mechanism of proteases, they are classified into six classes: aspartic, cysteine, glutamic, metallo, serine, and threonine proteases (López-Otín and Bond, 2008; Rawlings *et al.*, 2010). To attack the peptide bond of the substrate, aspartic, glutamic and metalloproteases utilize an activated water molecule as a nucleophile, while in the cysteine, serine and threonine proteases, an amino acid residue (Cys, Ser, or Thr, respectively) located in the active site acts as the nucleophile (López-Otín and Bond, 2008; Rao *et al.*, 1998). The MEROPS database represents the biggest collection of known proteases, their inhibitors and protease peptide substrates, and it is constantly being updated (Rawlings *et al.*, 2016). **Table 1** summarizes the main groups of proteases based on the mechanism of catalysis and shows examples of their fungal sources.

Genomic studies demonstrated that genes encoding aspartic, cysteine, serine and metalloproteases comprise > 75% of all genes encoding extracellular proteases in the genomes of fungi (Nguyen *et al.*, 2019). Interestingly, experiments with fungal pure cultures and genome analysis showed that aspartic proteases were the dominant proteases in the extracellular protease pool of most fungal species (Caldwell, 2005; Rineau *et al.*, 2015; Shah *et al.*, 2013; Theron and Divol, 2014; Vranova *et al.*, 2013). The pH range of aspartic protease activity is usually between pH 3 and 5 (Hsiao *et al.*, 2014; Kumar *et al.*, 2005; Vishwanatha *et al.*, 2010). Several genes encoding extracellular fungal aspartic proteases have been cloned and the corresponding enzymes purified for industrial use (Gomi *et al.*, 1993; Horiuchi *et al.*, 1988; Jarai *et al.*, 1994; Purushothaman *et al.*, 2019; Takenaka *et al.*, 2019; Tonouchi *et al.*, 1986; vanKuyk *et al.*, 2000).

Serine proteases have been also extensively studied in fungi, especially those which are involved in host-fungus interactions (Bagga *et al.*, 2004; Geremia *et al.*, 1993; Jashni *et al.*, 2015; Monod *et al.*, 2002; Reddy *et al.*, 1996; Williams *et al.*, 2003). For instance, subtilisin-like serine proteases are secreted by many fungal pathogens during penetration and colonization of a host (Bagga *et al.*, 2004; Jashni *et al.*, 2015; Ke *et al.*, 2018; Li *et al.*, 2010; Monod *et al.*, 2002). In industry, serine proteases are widely used as detergents (Vojcic *et al.*, 2015). They are also applied for research purposes such as protein degrading agents, e.g., during nucleic acid purification (Proteinase K from the basidiomycete *Tritirachium album*) (Yang *et al.*, 2016). Serine proteases purified from fungi are mainly neutral or alkaline proteases (Minglian *et al.*, 2004; Wang *et al.*, 2007; Zanphorlin *et al.*, 2010).

Metalloprotease is another class of proteases which plays an essential role in fungal pathogenesis (Yike, 2011). These enzymes require divalent metal ions (Mg^{2+}, Mn^{2+}, Co^{2+}, or Zn^{2+}) for their activity and are inhibited by compounds binding these metals,

Fig. 1 Current and potential industrial applications of fungal proteases.

such as the chelating agent ethylenediaminetetraacetic acid (EDTA). Zinc-dependent metalloproteases such as fungalysin are the most common metalloproteases in fungi. For instance, fungalysins from human and animal fungal pathogens promote pathogenicity by degrading structural proteins such as elastin and keratin (Brouta *et al.*, 2002; Markaryan *et al.*, 1994; Sirakova *et al.*, 1994). In plant pathogens, fungalysins act as a virulence factor, which is required for degradation of the plant chitinases and, consequently, promote infection (Ökmen *et al.*, 2018; Pan *et al.*, 2020; Sanz-Martín *et al.*, 2016). An unusually high number of fungalysins were found in the genome of the model basidiomycete *Coprinopsis cinerea*, a non-pathogenic fungus (Lilly *et al.*, 2008). The eight predicted *C. cinerea* fungalysins were compared with fungalysins from other fungi and further divided into two groups (Lilly *et al.*, 2008). Seven predicted *C. cinerea* fungalysins formed a group with similarity to fungalysins of the basidomycete *Laccaria bicolor* (Lilly *et al.*, 2008), while one of them was most similar to fungalysins from the basidiomycete *Ustilago maydis* and the ascomycete *Aspergillus fumigatus* (Lilly *et al.*, 2008). However, the functions, specificity, and regulation of fungalysins in non-pathogenic fungi remain largely unknown. Interestingly, several research studies have also characterized and purified metalloproteases with fibrinolytic activity and potential use in medicine from various basidiomycetes (Healy *et al.*, 1999; Joh *et al.*, 2004; Kim and Kim, 1999, 2001; Nonaka *et al.*, 1997; Wu *et al.*, 2011). Most of the purified metalloproteases are neutral or alkaline proteases.

The majority of the studies on fungal proteases are focused on the above described aspartic proteases, serine proteases and metalloproteases. The least characterized classes of proteases in fungi are extracellular cysteine and glutamic proteases, and the role of fungal threonine proteases is mostly unknown. Based on the pH at which these proteases are active, cysteine and glutamic proteases are categorized as acidic proteases, while threonine protease as neutral proteases (Madala *et al.*, 2010; Rao *et al.*, 1998). Functionally characterized fungal cysteine proteases are intracellular enzymes, for instance, cysteine protease from the ascomycete *Botrytis cinerea* plays crucial roles in mycelial growth, conidiation, sclerotial development and virulence (Liu *et al.*, 2019). Glutamic proteases are a distinct class from the previously described proteases. A comparative genomics approach was used to evaluate the conservation and evolutionary distribution of glutamic acid protease encoding genes in 23 fungal genomes (Sims *et al.*, 2004). This study demonstrated that glutamic proteases were present in all tested ascomycetes. Moreover, a large number of glutamic proteases were also found clustered together in the basidiomycete *P. chrysosporium* genome (Sims *et al.*, 2004). In another research study, glutamic protease from the thermophilic fungus *Talaromyces emersonii* was isolated and characterized (O'Donoghue *et al.*, 2008). This protease had maximum hydrolysis at 50°C and pH 3.4, and its activity was essential for growth of *T. emersonii* (O'Donoghue *et al.*, 2008). Potential industrial applications of fungal cysteine, glutamic acid and threonine proteases have been poorly investigated in contrast to the aspartic proteases, serine proteases and metalloproteases.

Fungal Proteases in Industry

Proteases of fungal origin are used in a large variety of biotechnological applications, mainly in food industries such as brewing and cereal processing, cheese making, baking, production of protein hydrolysates (Liu *et al.*, 2018; Ozturkoglu-Budak *et al.*, 2016; Setoguchi *et al.*, 2019; Wang *et al.*, 2005). Fungal proteases also have extensive applications in pharmaceutical industry as potential drug targets, diagnostic biomarkers or as therapeutic agents. They are also envisaged to have potential in leather and fabric processing, waste treatment, bioremediation processes and detergent industry. Finally, they represent important tools for numerous research applications in molecular biology. **Fig. 1** provides a summary of the current and potential applications of fungal proteases in industry.

The physical and chemical characteristics of fungal proteases have been explored and described in detail. Strains producing high amounts of proteases were isolated, studied through extensive screening, characterized, and further improved through genetic modifications (Jarai *et al.*, 1994; Liu *et al.*, 2018; Papagianni and Sergelidis, 2014; Setoguchi *et al.*, 2019; Sircar *et al.*, 2015; Starzyńska-Janiszewska *et al.*, 2015). A large number of protease-producing fungal strains belong to the Ascomycota and the Mucoromycota including *Aspergillus* (Liu *et al.*, 2018; Starzyńska-Janiszewska *et al.*, 2015; Takenaka *et al.*, 2019), *Penicillium* (Emi *et al.*, 1976; Papagianni and Sergelidis, 2014; Zhu *et al.*, 2009), *Fusarium* (Juntunen *et al.*, 2015; Ueda *et al.*, 2007), *Trichoderma* (Deng *et al.*, 2018; Zhang *et al.*, 2017), *Thermoascus* (Li *et al.*, 2011; Merheb-Dini *et al.*, 2009), *Rhizopus* (Hsiao *et al.*, 2014; Kumar *et al.*, 2005) and *Mucor* (Kangwa *et al.*, 2018; Tonouchi *et al.*, 1986). It has also been shown that proteases from the Basidiomycota have unique properties with potential in various applications (Inácio *et al.*, 2015). However, in comparison to the bacterial proteases, fungal proteases are less studied and commercially used (Tsiatsiani and Heck, 2015). Thus, with the wide diversity of fungi and their abilities to adapt to different carbon and nitrogen sources by secreting an arsenal of extracellular enzymes, the research of fungal proteases is an area that warrants more exploration.

Proteases for the Food Industry

The use of proteases in the food industry has a large variety of applications which are linked to the nutritional and functional value of the products. They play an essential role in modifying the functional properties of proteins during the cheese making process and the viscoelastic properties of dough in baking. Proteases are used in the brewing industry, processing of cereals, obtaining protein hydrolysates, production of seasonings and improvement of the quality of protein-rich food.

Dairy

The main application of proteases in the dairy industry is the coagulation of milk during the production of cheese. The processing of milk was earlier mostly performed by using milk-coagulating calf rennet which is a mixture of the proteases chymosin and pepsin (Rothe *et al.*, 1976). A rising demand for cheese production led to shortage of calf rennet and the increased ethical issues associated with its use by cheese manufacturing industries. Therefore, mining for alternative enzymes from microbial sources for milk clotting has been a focus for researchers and cheese producers. Fungal acidic aspartic proteases are extensively used in large-scale commercial settings gradually replacing calf rennet in cheesemaking process (Mandujano-González *et al.*, 2016). These proteases produced by fungal species such as *Mucor miehei* (Sternberg, 1972), *Mucor pusillus* (Tonouchi *et al.*, 1986; Zhang *et al.*, 2015), *Mucor baciliformis* (Areces *et al.*, 1992; Venera *et al.*, 1997), *Endothia parasitica* (Sardinas, 1968), and *Penicillium oxalicum* (Hashem, 2000) have been purified and their enzymatic properties have been explored in detail. Fungal proteases appear to be more proteolytic and heat-stable than calf rennet resulting in non-specific proteolytic activity which leads to a loss of casein fragments (Mandujano-González *et al.*, 2016). High levels and high thermal stability of proteases are associated with bitterness in cheese after storage (Habibi-Najafi and Lee, 1996). Therefore, comprehensive research has been performed to improve fungal proteases (Vishwanatha *et al.*, 2010; Zhang and Zhao, 2010; Yegin *et al.*, 2012; Zhang *et al.*, 2015). This resulted in the development of enzymes mixtures with low levels of nonspecific proteases and which are inactivated during pasteurization (Feijoo-Siota *et al.*, 2014).

Cow's milk also contains about 30 proteins which can potentially induce allergic reactions or sensitization (Tsabouri *et al.*, 2014). The main milk allergens are whey proteins and αs-casein (El-Agamy, 2007). Proteases are able to efficiently hydrolyze proteins and, consequently, eliminate these major milk allergens (Lee, 1992). Fungal proteases are increasingly used for this application (Ena *et al.*, 1995; Lakshman *et al.*, 2011). However, extensively hydrolyzed peptides have bitter taste which is a major drawback of using proteases (El-Agamy, 2007).

Baking

Fungal proteases play an important role in baking process. Wheat flour is a typical component of dough and contains insoluble protein gluten. Gluten determines functional properties of dough such as viscoelastic properties and facilitates dough to rise during the baking process (Heredia-Sandoval *et al.*, 2016). The addition of proteases that partially hydrolyze gluten can accelerate the dough making process by decreasing the mixing time of the dough and results in the higher loaf volumes (Rao *et al.*, 1998). A protease from *A. oryzae* has been used to modify gluten in wheat flour during the baking process (Rao *et al.*, 1998). It has been also shown that *A. oryzae* protease has potential to be used as an additive in the formulation of gluten-free bread, for example, bread made with rice flour. Treatment of rice flour with *A. oryzae* protease improved the characteristics of the dough (Hamada *et al.*, 2013). It had higher viscosity, slower sedimentation of the flour particles and increased loaf volume (Hamada *et al.*, 2013).

Brewing

Processing of cereals such as barley or rice is a fundamental part of brewing. It involves several enzymes with (hemi)cellulolytic and proteolytic activities. Cell wall components of cereals are degraded by cellulases and hemicellulases, while protein-based materials are degraded by proteases during the malting process. Proteases release and increase the amount of alpha-amino nitrogen which is important for the growth of yeast that performs the alcoholic fermentation. For instance, acid proteases from *A. oryzae* are used to hydrolyze proteins from the steamed koji rice during the fermentation of sake (Kitano *et al.*, 2002; Shindo *et al.*, 1998). Acid proteases from fungal origin have been investigated for their abilities to degrade proteins in the haze fraction

formed during production of various beverages. It has been demonstrated that the addition of a proline specific protease from *Aspergillus niger* during the fermentation phase of beer brewing prevented chill-haze formation in bottled beer (Lopez and Edens, 2005). Furthermore, acid proteases from *Aspergillus saitoi* are used to hydrolyze the proteins that cause turbidity during the production of fruit juices and alcoholic beverages (Sumantha *et al.*, 2006).

Protein hydrolysate production

Proteins are essential elements in food and, when hydrolyzed, represent a valuable source of functional molecules. Protein hydrolysates generated by proteases are widely used as food additives. Animal derived proteins (gelatin, casein, whey proteins) and plant derived proteins (soy, rice, maize proteins) are sources of protein hydrolysates with potential application in the functional food industry. Bioactive peptides derived from proteolysis possess antioxidant, antihypertensive, antimicrobial, and immunomodulatory activities. Protein hydrolysates also react with other food elements and create various flavors. For example, the reaction between reducing sugars and amino acids called Maillard reaction enhances caramel-like flavors, soy sauce-like odors, umami and mouthful tastes and reduces bitterness (Li *et al.*, 2013b; Lingnert and Eriksson, 1980; Liu *et al.*, 2012). Protein hydrolysates are used in a variety of foods, such as soups, stock cubes, broth, savory sauces, canned meat and ready-made meals. Fungal proteases from species representing the genera *Aspergillus*, *Rhizopus*, and *Penicillium*, are extensively applied to produce protein hydrolysates for food and functional food industries (Agrawal *et al.*, 2004; de Castro and Sato, 2014; Lima *et al.*, 2015; da Silva *et al.*, 2019a; Sparringa and Owens, 1999; Su *et al.*, 2011).

Proteases for the Pharmaceutical Industry

Proteases are a major focus for therapeutic applications as potential drug targets, diagnostic biomarkers or as therapeutic agents (Turk, 2006). Common molds, in particular *Aspergillus fumigatus*, can cause life-threatening symptoms in patients with weakened immunological systems or asthma (Denning *et al.*, 2014; Hohl *et al.*, 2005). Serine proteases appear to play an important role in the development of these symptoms (Basu *et al.*, 2018; Tai *et al.*, 2006). For instance, serine proteases may induce epithelial barrier dysfunction in asthma through cytoskeletal rearrangements (Druey *et al.*, 2020). Another example is an *A. fumigatus* alkaline serine protease found in patients diagnosed with cystic fibrosis which is a lung disease characterized by chronic progressive airway infection (Cowley *et al.*, 2017). Research studies indicate that fungal protease activities may be used as potential drug targets to develop novel therapeutic agents for the treatment of infections caused by fungi to improve patient health (Cowley *et al.*, 2017; Druey *et al.*, 2020). *A. fumigatus* secreted proteases have also been investigated as novel diagnostic biomarkers for diagnosis of fungal infection (Kniemeyer *et al.*, 2016; Moloney *et al.*, 2016; Watson *et al.*, 2011).

The wide diversity and specificity of fungal proteases make them a unique source in developing effective therapeutic agents. Fibrinolytic proteases are important enzymes that may act as thrombolytic agents in the therapeutic treatment of thrombosis (blood clotting) (da Silva *et al.*, 2019b; Lee *et al.*, 2005). The formation of fibrin, the main protein component of blood clots, and its adhering to the blood vessels' epithelial wall are responsible for cardiovascular diseases, such as high blood pressure, ischemic heart diseases, valvular heart disease and stroke (Corte *et al.*, 2011). Fungi have been shown to be a good source of proteases with fibrinolytic activities and have increasingly attracted pharmaceutical and medical interests. Fibrinolytic proteases from several fungal species, for example, the zygomycete *Mucor subtilissimus*, the ascomycetes *A. oryzae* and *Fusarium* sp., and the basidiomycetes *Pleurotus ostreatus* and *Schizophyllum commune* have been reported (da Silva *et al.*, 2019b; Liu *et al.*, 2014; Lu *et al.*, 2010; Nascimento *et al.*, 2016; Shirasaka *et al.*, 2012; Ueda *et al.*, 2007).

Another application of therapeutic proteases includes digestive enzymes preparations. The use of digestive drugs is mainly directed at correcting enzyme efficiencies. Digestive enzyme preparation is a mixture of three enzyme classes: lipases, amylases and proteases. Proteases derived from species in the genus *Aspergillus* are widely used as a component of digestive preparations (Rao *et al.*, 1998; Sugiura *et al.*, 1976). For instance, acid protease and alkaline protease from *A. oryzae* are included in the Amano Enzyme protease preparation (Yang *et al.*, 2017). *A. oryzae* proteases from this enzyme preparation were shown to be responsible for the bifidogenic effect in rats which may prevent against various disorders such as colon and liver diseases, allergies and insulin resistance (Yang *et al.*, 2017).

Proteases for Leather and Fabric Processing

The main building block of leather are collagens, keratins, glycoproteins and lipids which are all enzymatically biodegradable. Therefore, enzymes play an important part in the leather processing industry (Thanikaivelan *et al.*, 2004). Soaking, dehairing, bating, and tanning are principle steps of the leather processing (Thanikaivelan *et al.*, 2004). The process of soaking is crucial to make the raw hide swell and restore its moisture. Alkaline protease treatment is used during this step to remove globular proteins and to hydrolyze non-collagenous proteins of leather. This results in improved water absorption and accelerated penetration of chemicals. Alkaline proteases are also widely applied in the dehairing process to enhance the degradation of the epidermal structure of the hide. Bating is a process where proteolytic enzymes hydrolyze and remove a non-collagen protein, which makes the leather flexible and elastic (Rao *et al.*, 1998). Various combinations of microbial proteases together with trypsins have been used in leather processing. The potential use of fungal proteases from *Aspergillus flavus*, *A. tamarii* and *Aspergillus parasiticus* in leather processing has been demonstrated (Anitha and Palanivelu, 2013; da Silva *et al.*, 2016; Malathi and Chakraborty, 1991). Application of proteases for soaking, dehairing and bating is more environmentally friendly and more efficient when compared with traditional methods which involve the use of hazardous chemicals.

In addition to the application of proteases in the leather processing industry, they also have a valuable role for the silk industry. Silk is formed by two proteins: sericin (22%) and fibroin (76%) (Ki *et al.*, 2009; Wang and Zhag, 2011). Fibroin filaments are the main component of a textile fiber. Sericin protein is a protective and gum gluing layer over the fibroin. Degumming is a removal of sericin during silk processing and plays a key role in achieving strength, softness and a typical shiny and elegant aspect in the finished fabric. This process consumes high energy and water and has an impact on the environment. Thus, application of proteolytic enzymes has gained a lot of attention in recent years. Several fungal proteases have been investigated to develop enzyme-based silk degumming process (Eom *et al.*, 2020; Freddi *et al.*, 2003; More *et al.*, 2013).

Other Applications of Fungal Proteases

Proteases of fungal origin have potential in a variety of industrial applications. In recent years, fungal proteases gained an interest in industrial portentous waste management in industries like poultry, fishery, and leather (Hathwar *et al.*, 2011; Majumder *et al.*, 2015). For instance, poultry-processing factories produce millions of tons of feathers annually as a waste product (Rodrigues Marcondes *et al.*, 2008). The recalcitrant and fibrous protein β-keratin is a main component of feathers, therefore, keratinolytic enzymes produced by fungi may have an important role in feather decomposition. The ascomycete fungi *Alternaria tenuissima*, *Acremonium hyalinulum*, *Curvularia brachyspora*, *Beauveria bassiana* and *Chrysosporium keratinophilum*, and the basidiomycete *Termitomyces clypeatus* showed high keratinolytic activities and have potential use in biotechnological processes involving keratin hydrolysis (Majumder *et al.*, 2015; Rodrigues Marcondes *et al.*, 2008; Singh, 2002). Furthermore, fungal proteases may contribute significantly to the reduction of wastewater generated by distillery and agricultural industries (Morimura *et al.*, 1994; Salgado *et al.*, 2016).

The use of proteases in the detergents is the most dominant industrial application of proteases. These proteases are mainly derived from bacterial sources. The most characterized and commercialized for use in detergents are alkaline serine proteases subtilisins produced by *Bacillus licheniformis, Bacillus pumilus* and *Bacillus subtilis* and high alkaline proteases from *Bacillus clausii* and *Bacillus halodurans* (Rao *et al.*, 1998). Several studies have demonstrated that fungal alkaline proteases were also suitable for inclusion in detergent composition (Hajji *et al.*, 2007; Niyonzima and More, 2015a,b; Omrane Benmrad *et al.*, 2016).

Proteolytic enzymes also have applications in life sciences, particularly in molecular biology. They are used in peptide synthesis, cell isolation and tissue dissociation, cell culturing, antibody fragment production, structure-function relationship elucidation and nucleic acid isolation (Morihara, 1987; Mótyán *et al.*, 2013; Rao *et al.*, 1998). The most widely used proteolytic enzyme in nucleic acid purification is the Proteinase K obtained from the basidiomycete fungus *T. album* (Dattagupta *et al.*, 1975; Yang *et al.*, 2016).

Conclusion and Future Prospective

Proteases are a unique class of enzymes involved in diverse physiological, biochemical and regulatory processes in all living organisms and represent immense commercial importance. Microorganisms are a preferred source of proteases because of their fast growth, accessibility to genetic manipulation and easiness in cultivation. Microbial proteases have been extensively used in detergent and food industries, and as therapeutic agents. Fungi possess a wider variety of proteases than bacteria. A common source of commercially available fungal proteases is *A. oryzae* which is able to produce acid, neutral, and alkaline proteases. Fungal proteases have broad substrate specificity and are active over a wide range of pH. Proteases of fungal origin have a large variety of biotechnological applications. They are used in food industries including brewing and cereal processing, cheese making, baking and production of protein hydrolysates. Fungal proteases are also applied in pharmaceutical industry where they are used as potential drug targets, diagnostic biomarkers or as therapeutic agents. Moreover, they have also found their use in leather and fabric processing and in many research applications particularly in the field of molecular biology. Many studies have also shown that fungal proteases have potential in waste treatment, bioremediation processes and detergent industry. The use of proteases for commercial applications is recognized as sustainable and an environmentally friendly technique.

This chapter provided an overview of the applications of fungal proteases (**Fig. 1**). Hydrolysis of proteins by fungal extracellular proteases for their growth, reproduction, sporulation and germination presents a good example of the implementation of a diverse and efficient fungal system into a well-designed industrial process. Use of fungal proteases for various applications has a long history and presents great importance for human society. Since we are moving towards a biobased renewable and sustainable society, these applications will certainly expand and will grow exponentially in the future. Proteolytic enzymes from fungal origin has already implemented in many industries, however the ongoing search for new and better protease from fungi, assisted by developing technologies, promises to provide more specific, thermostable and versatile proteases.

Large number of genome data generated from advanced post-genomics era and constantly increasing number of fungal genomes indicate that fungi possess high genetic potential with respect to the putative genes encoding proteases (Grigoriev *et al.*, 2014; Iqbal *et al.*, 2018; Muszewska *et al.*, 2017). While usually only well-studied protease producing fungi (e.g. *A. oryzae, A. niger*) were used as enzyme factories, currently many other fungal species have been demonstrated to have high potential in producing proteases suitable for many industrial applications (da Silva *et al.*, 2019a,b; Liu *et al.*, 2014; Nascimento *et al.*, 2016). Fungal genomes can be explored for novel proteases and their activities, but can also be mined for orthologous enzymes from related fungi that may have favorable properties.

The exploitation of fungal biodiversity and the discovery of new fungal strains with abilities to produce high level of proteases has been a great deal of interest in the biotechnological potential of these enzymes. For example, exploring new fungal strains that inhabit harsh environments such as extreme temperatures (hot springs and the Arctic), extreme pH (acidic or alkaline), high pressure, and high salinity. Fungi from these environments have adapted to thrive in these extreme ecological niches. As a result, they are able to produce unique enzymes that are active and stable under harsh conditions in which mesophilic enzymes could not survive. Such unique proteases have a great economic potential in many industrial processes, including agricultural, chemical, food and pharmaceutical applications.

The development of screening technologies, computational design, synthetic biology, and crystallography will allow characterization, engineering and production of proteases with tailored and improved physicochemical properties. It will increase the future potential for their current and novel applications that are faster, more accurate, specific and environmentally friendly.

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Multifarious Applications of Fungal Phytases

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Introduction

Phytic acid (*myo*-inositol hexakisphosphate) or phytate in its salt form is the main organic storage form of phosphorus (60%–80% of total) in plant tissues such as legumes, cereal grains, oilseeds, and pollen (Lott *et al.*, 2000; **Fig. 1**). Phytic acid acts as an anti-nutritional factor because of its ability to chelate cations, like Ca^{2+}, Zn^{2++}, Fe^{2+} and Mg^{2+}, and make them unavailable, to form complexes with starch and proteins that interferes with their digestion, and to inhibit the activity of digestive enzymes (Harland and Morris, 1995).

Phytase (*myo*-inositol hexakisphosphate phosphohydrolase) catalyzes the removal of phosphate from phytic acid or its salts (phytates) in a stepwise manner by cleaving five of the six phosphates (Wyss *et al.*, 1999; Konietzny and Greiner, 2002). The first reports of phytase activity from rice grains (Suzuki *et al.*, 1907) and an ascomycetous fungus *Aspergillus niger* (Dox and Golden, 1911) were published more than 100 years ago. Phytases are generally classified based on their structure and catalytic mechanism of cleavage of the phosphate from phytic acid as Histidine Acid Phosphatases (HAPs), β-propeller phosphatases/alkaline phytases (BPPs), purple acid phosphatases (PAPs), and dual-specificity phosphatases/protein tyrosine like phosphatases (DSPs/PTLPs). HAPs have been reported in numerous fungi such as *Cladosporium* sp., *Aspergillus niger*, *A. awamori*, *Fusarium oxysporum* and *Debaryomyces castellii* (Gontia-Mishra *et al.*, 2014; Jatuwong *et al.*, 2020). The structure of BPPs is similar to a six-bladed propeller (Ha *et al.*, 2000); these are alkaline phytases active at pH > 7.0 and found mainly in bacteria. Recently, the first alkaline fungal phytase was reported from *Arthrobotrys oligospora* (Hou *et al.*, 2020). PAPs belong to a special group of acid phosphatases (APases), which hydrolyze phosphate from phosphomonoesters and anhydrides with an optimum pH below 7.0 (Schenk *et al.*, 1999). They are most widespread in plants and also found in bacteria, fungi, and animals (Gontia-Mishra and Tiwari, 2013). PTPLP, also known as cysteine phytase, has the CX5R (S/T) domain in their active center, which participates in hydrolysis and are active in the pH range between 4.0 and 6.0. PTPLPs were first isolated from bacteria like *Selenomonas ruminantium* and *Megasphaera elsdenii*, which dwell in the rumen of ruminants (Puhl *et al.*, 2008, 2009). Recently there is also a report of neutral phytase from *Penicillium* sp. C1 (Ya-nan *et al.*, 2020).

Depending on the carbon atom where phosphate hydrolysis is initiated, phytases have also been classified as 3-, 5-, and 6-phytases (Mullaney and Ullah, 2003; Sun *et al.*, 2017). The 3-phytases (EC 3.1.3.8), which begin the hydrolysis from the phosphate group on C3 atom of phytic acid, are present in many bacterial and fungal species, while the 6-phytases (EC 3.1.3.26), which first hydrolyze the bond on C6 position, are present in plants and *Escherichia coli*. The 6-phytases perform complete dephosphorylation of the substrate, whereas the final product of hydrolysis with 3-phytases is L-*myo*-inositol-2-phosphate (Wodzinski and Ullah, 1996; Wyss *et al.*, 1999). 5-Phytases (EC 3.1.3.72), which start the reaction from the cleavage of the phosphate on C5, have also been identified, for example in the bacterium *Selenomonas* (Puhl *et al.*, 2008).

In nature, phytases are produced in a wide range of plant and animal tissues, and microbes (Vohra and Satyanarayana, 2003). Of immense commercial significance (reduction of phytate in foods and feeds, aquatic pollution abatement, soil amendment and therapeutics) are phytases from filamentous fungi such as *Aspergillus*, *Myceliophthora*, *Mucor*, *Penicillium*, *Rhizopus* and *Trichoderma* (Dailin *et al.*, 2019). A phytase from *Aspergillus ficuum* NRRL 3135 has been most commonly used commercially (Chelius and Wodzinski, 1994). Two methods of fermentation, Solid State Fermentation (SSF) and Submerged Fermentation (SmF), are employed for the production of fungal phytases (Han *et al.*, 1987; Shivanna and Venkateswaran, 2014). In the most common method, SSF, agricultural residues and other cheap plant biomass substrates like citrus peels, wheat bran, wheat straw, soybean meal and rice bran are used for the production of phytases from filamentous fungi such as *Aspergillus flavus*, *A. ficuum*, *A. niger*, *Thermomyces lanuginosus* and *Myceliophthora thermophila* (previous name *Sporotrichum thermophile*) (Singh and Satyanarayana, 2006; Awad *et al.*, 2014; Huang *et al.*, 2018). Using SmF, phytases can be produced from *Aspergillus carbonarius*, *A. fumigatus*, *A. niger*, *A. oryzae*, *Cladosporium* spp.,

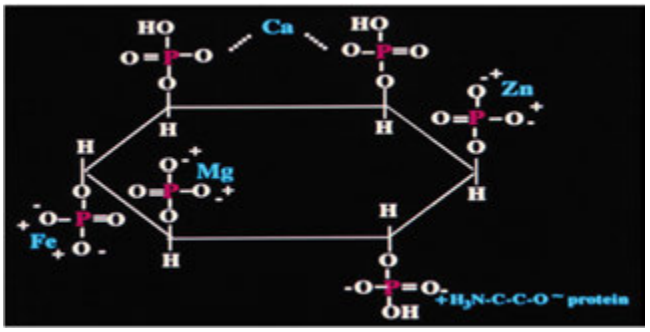

Fig. 1 Structure of phytic acid showing six phosphate groups bound to cations and protein.

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Mucor piriformis, Rhizopus oligosporus and *M. thermophila* (Howson and Davis, 1983; Singh *et al.*, 2011). Yeasts such as *Arxula adeninivorans, Candida krusei, Pichia anomala* have also been reported to possess phytase activity (Kaur *et al.*, 2007; Olstorpe *et al.*, 2009). Phytase from the yeast *Pichia anomala* has been produced in submerged fermentation using Yeast extract-Peptone-Dextrose (YPD) medium (Vohra and Satyanarayana, 2002a) and cane molasses medium (Kaur and Satyanarayana, 2005).

In order to study their biochemical properties as well as structural and functional relationships, various chromatographic methods have been employed to purify phytases (Vohra and Satyanarayana, 2002b; Singh and Satyanarayana, 2009; Ranjan and Satyanarayana, 2016). Thermostability of phytase is desirable so that it can withstand temperatures as high as 70–90°C during the feed pelleting process (Doyle and Erickson, 2006; Dersjant-Li *et al.*, 2015). Resistance to proteases and broad range pH stability are important to withstand conditions in the gastrointestinal tract (Walton and Gray,1979; Schroder *et al.*, 1998). Fungal phytases are active in the optimum temperature range of 37–67°C. Most fungal phytases are active at acidic pH in the range of 2.0 and 6.0 and belong to a class of HAPs (Vohra and Satyanarayana, 2003; Singh *et al.*, 2011), but phytases of some fungi such as the basidiomycete *Agaricus bisporus* and mucoromycete *Rhizopus microsporus* var. *microsporus* have optimum activity at pH 5.0–8.0 and 9.5, respectively (de Oliveira Ornela and Guimaraes, 2019). A phytase from *P. anomala* is optimally active at 60°C and pH 4.0 (Vohra and Satyanarayana, 2001). This enzyme is highly thermostable and acid stable, with a $T_{1/2}$ of seven and eight days at 60°C and pH 4.0, respectively, thereby suggesting its applicability in animal feeds (Vohra and Satyanarayana, 2002b).

The first recombinant phytase was produced by expressing the *phyA* gene of *A. fumigatus* in *A. niger* (Rebello *et al.*, 2017). The recombinant phytase was highly thermostable and remained active over a wide pH range. A phytase gene from the thermophilic fungus *M. thermophila* was cloned and expressed heterologously in *E. coli* and *Pichia pastoris* (Ranjan *et al.*, 2015; Singh *et al.*, 2018; Maurya *et al.*, 2017). The phytase from the yeast *P. anomala* was successfully over-expressed heterologously in other yeasts like *Hansenula polymorpha* and *P. pastoris* (Kaur *et al.*, 2010; Joshi and Satyanarayana, 2014).

Improvement in the enzyme characteristics of phytases has extensively and successfully been achieved using enzyme engineering (Chen *et al.*, 2015). Amelioration of phytase thermostability, specific activity, pH stability and protease resistance can be achieved by protein engineering. As mentioned before, stability of phytase at high temperatures is desirable to withstand feed pelleting conditions (Doyle and Erickson, 2006; Dersjant-Li *et al.*, 2015). In order to withstand conditions in the gastrointestinal tract, phytase must be stable in a broad pH range with a high specific activity (Schroder *et al.*, 1998). Hence, protein engineering has been extensively used to enhance thermostability of HAP, a commercially very important phytase. Improved thermostability of *A. niger phyA*, *E. coli appA* and *appA2*, and *A. fumigatus* phytase has been attained by protein engineering methods such as protein glycosylation, random mutation and site-directed mutagenesis (Mullaney *et al.*, 2010; Vasudevan *et al.*, 2019). In view of the multifarious applications of fungal phytases (**Fig. 2**), this review focuses on the potential biotechnological applications of these enzymes.

Applications of Phytases

Applications in Foods and Animal Feed

The most important biotechnological application of phytases is in the nutrition of monogastric animals and humans. Monogastrics are not able to breakdown phytates to liberate inorganic phosphorus due to lack of phytase, thus preventing their

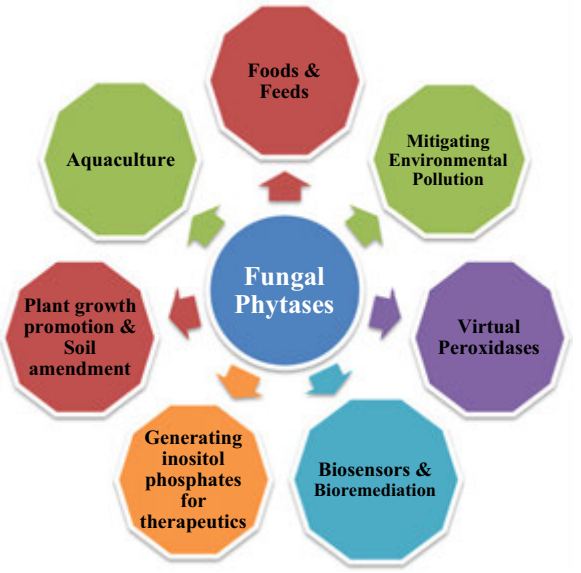

Fig. 2 Multifarious applications of Fungal Phytases.

assimilation and concomitant reduction in the anti-nutrient phytate content of foods and feeds. The supplementation of phytase has, therefore, emerged as one of the most attractive and effective additives in foods and feeds.

Foods

The presence of phytates in plant-based foods, including flours, is well documented (Greiner and Konietzny, 2006). A large part of our global population tends to suffer from mineral (mainly, iron and zinc) deficiencies due to the ingestion of phytate rich plant diets (Bentley *et al.*, 1997; Tatala *et al.*, 1998). Studies in humans have conclusively shown that iron and zinc absorption from a meal corresponds inversely to its phytate content (Nävert *et al.*, 1985). The dietary phytase gets inactivated during cooking, resulting in poor phytate digestion that affects mineral absorption in the small intestine. Various food processing and preparation methods, like germination, soaking, cooking and fermentation, lead to only a partial reduction in the phytate content of the processed materials.

Phytases have been found to be useful as a food additive during fermentation, food processing and bread making, where they play an important role in human nutrition by degrading phytate during the processing as well as in the gastrointestinal tracts. During bread making, phytase improves proofing time, volume, and crumb structure. A phytase from *A. niger* is used in making dough that results in enhanced degradation of phytates (Turk and Sandberg, 1992), better iron absorption in humans (Sandberg *et al.*, 1996), increased bread volume as well as improved crumb texture (Haros *et al.*, 2001). Rosell *et al.* (2009) reported that *A. niger* phytase could be used in the frozen storage of bread to overcome the detrimental effects of bran on the mineral bioavailability. When bread was supplemented with a phytase of the thermophilic ascomycete *M. thermophila*, improvement in the bread quality and reduction in phytate content was reported (Singh and Satyanarayana, 2008a). This phytase has been shown to release inorganic phosphate from calcium, magnesium and cobalt phytates (Singh and Satyanarayana, 2010), and effectively dephytinize sesame oil cake and soymilk (Singh and Satyanarayana, 2006, 2008b). There are several studies on phytase activity in baker's yeast (Greiner *et al.*, 2001; Haraldsson *et al.*, 2005), whereby the researchers have emphasized the role of yeast phytase in bread making. During whole meal bread making, reduction in phytate levels were attained due to the activities of both wheat and yeast phytases (Turk *et al.*, 1996). The use of high yeast levels and fermentation times have been suggested to result in high-fiber bread with lower phytate content (Harland and Harland, 1980). Turk *et al.* (2000) studied two commercial strains of baker's yeast with the ability to express phytase activity and reported that they could rapidly degrade all the *myo*-inositol hexakisphosphate within 24 h. A native high-phytase *S. cerevisiae* strain has been suggested to be suitable for the production of food-grade phytase and for the direct use in food production (Veide and Andlid, 2006). The supplementation of whole wheat dough with a *P. anomala* phytase that was over-expressed in *P. pastoris* resulted in effective dephytinization of bread without any change in texture (Joshi and Satyanarayana, 2015a).

Apart from bread, phytases from fungi have also been used for reducing phytates from other plant derived foods. The level of phytic acid in *Icacina mannii*, a shrub used as a source of starch-based food during periods of famine in Africa, was reported to reduce 90% after six days of fermentation with *S. cerevisiae* (Antai and Nkwelang, 1998). Chapathi, the traditional unleavened flat bread that contains phytic acid and is prepared from whole wheat flour is the staple food in parts of India and some surrounding Asian countries. A mutated strain of the yeast *Candida versatilis* was used for the preparation of chapathi dough with up to 45% reduced phytic acid level (Bindu and Varadaraj, 2005). The purified recombinant phytases from *M. thermophila* and *P. anomala* were also useful in dephytinization of Indian baked breads Tandoori and Naan (Ranjan *et al.*, 2015; Joshi and Satyanarayana, 2015a). The phytase from *S. cerevisiae* MTCC 5421 efficiently dephytinized pearl millet flour (94%), and completely refined wheat flour as well as removed phytate from wheat-based (Naan) and pearl millet-based (Rabadi) fermented products (Roopashri and Varadaraj, 2015). Similarly, baker's yeast as a phytase source for reduction in phytic acid content in Tarhana, a traditional Turkish fermented and dried cereal food, was investigated (Bilgicli *et al.*, 2006).

Filamentous fungi commonly used in oriental food fermentation have been examined for their ability to produce phytase. Tempeh, a popular oriental fermented food made from soybeans, is inoculated with *Rhizopus oligosporus* in the koji process, where the fermentation improves the digestibility, vitamin content and flavor of soybean (Fardiaz and Markakis, 1981). Since phytate binds to proteins, protein isolates from soybeans are rich in phytate. Thus, phytases can be applied in the production of phytate-free soybean milk because of a high level of phytate (0.56%) in soymilk (Anno *et al.*, 1985; Khare *et al.*, 1994). The cell-bound phytase of *P. anomala* effectively dephytinized soymilk (Kaur and Satyanarayana, 2010). Besides dephytinization, the recombinant *P. anomala* phytase also fractionated allergenic glycinin from soy protein, which can be potentially useful for producing specialty foods for people allergic to specific components of soy protein (Joshi and Satyanarayana, 2014). The role of an *A. ficuum* phytase in legume dephosphorylation has also been investigated. It was shown to result in 78% reduction in phytate content of soybean meal after 15-h treatment (Han and Wilfred, 1988).

Animal feeds

Animal feeds, mainly hog and poultry diets, are plant based, and approximately two-thirds of the phosphorus of feedstuffs is in the form of phytate phosphorus (Tyagi *et al.*, 1998). Under most dietary conditions, this phytate phosphorus is unavailable to the monogastric animals (Nelson, 1967), and therefore inorganic phosphate supplementation is required, which adds to the cost of the feed due to the limited rock phosphorus supplies. Moreover, the unutilized phytate is excreted in the manure that causes problems in terms of phosphorus pollution. The phytates also chelate several important minerals, thereby reducing their availability to the animals. Phytases are therefore incorporated into the feed of non-ruminants or monogastric animals. Besides reducing the need of phosphorus supplementation and making trace minerals available, the degradation of *myo*-inositol hexakisphosphate yields *myo*-inositol, an important growth factor. This improves the digestibility and the nutritional value of the feeds, besides enhancing

growth rate of monogastric animals and mitigating the level of phosphorus excretion, thus minimizing the chances of environmental pollution. The nutritional value as well as the environmental significance of the use of fungal phytases in releasing phosphorus from plant-based animal feeds has been well documented (Kaur *et al.*, 2007; Singh and Satyanarayana, 2015).

Based on *in-vitro* feed experiments, it has been found that phytases with broad substrate specificity, for example the acidic phytases from *A. niger* and *A. terreus*, are better than those with narrow substrate specificity for animal nutrition (Wyss *et al.*, 1999). An ideal phytase for the poultry feed industry would be the one that withstands acidic pH and protease enzymes in the stomach and small intestine where phosphorus absorption takes place, cost-effective in production, and resistant to elevated temperatures (from 65 to 80°C) that are encountered during feed pelleting (Lei and Stahl, 2001). The most widely used phytases in animal feeds are HAPs. Supplemental phytase improves calcium, zinc, and iron utilization by animals (Lei *et al.*, 1993; Jondreville and Dourmad, 2005). Several feed trial experiments with poultry as well as swine have confirmed the possibility of replacing inorganic phosphorus (Pi) supplementation by using microbial phytase in phytate-rich diets for monogastric animals, and it has been reported that 500–1000 units of phytase can replace approximately 1.0 g Pi supplementation and reduce the total phosphorus excretion by 30%–50% (Yi *et al.*, 1996; Kemme *et al.*, 1997).

The phytase from *Aspergillus* was the first one studied to reduce the phytate content in poultry feeds (Nelson *et al.*, 1968, 1971). However, it was not until 1991 that the first phytase feed enzyme became commercially available. The *A. niger* phytase under the trade name of Natuphos (BASF) was approved for use as a feed additive in a number of Asian, European and North and South American countries, including Canada and the United States (Wodzinski and Ullah, 1996). There are various studies conducted on application of *A. niger* phytases in animal feeds. For example, the addition of *A. niger* phytase to maize and soybean meal-based diets led to improvement in growth and bone mineralization in broiler chicks with better relative retention of phosphorus and calcium (Ahmad *et al.*, 2004). Similarly, supplementing diets of pigs with *A. niger* phytase resulted in increased body weight and higher phosphorus content in their bones (Sands *et al.*, 2009). The waste tea fungal biomass produced during black tea fermentation, containing various nutrients and phytase, was supplemented as a dietary ingredient for broiler chicks, which led to increased feed consumption and body weight (Murugesan *et al.*, 2005). The phytase from *A. niger* van Teighem efficiently hydrolyzed phytate phosphorus in several commercial livestock feeds (Vats *et al.*, 2009). There was enhanced growth performance of broilers whose phosphorus-deficient diets were supplemented with phytase from *A. niger* and evaluated till 35 days after hatching (Srikanthithasan *et al.*, 2020). Very recently, a novel fungal beta-propeller phytase has been reported from the nematophagous fungus *Arthrobotrys oligospora* with a potential application in animal feed, based on its utility in releasing phosphorus from soybean meal and minerals from durum wheat and finger millet flour (Hou *et al.*, 2020).

The phytase from the yeast *Saccharomyces castellii* CBS 2863 successfully reduced the amount of phytate content in wheat bran and glandless cotton flour, thus proving the strain's efficacy in the pretreatment of animal feeds (Segueilha *et al.*, 1993). Matsui *et al.* (2000) reported that dietary yeast phytase improved bioavailability of phosphorus in growing pigs. However, its efficacy was less than that of *A. niger* phytase. The supplementation of cell-bound phytase of *P. anomala* in the feed of broiler chicks resulted in their improved growth, better phosphorus retention in their body and reduced excretion of phosphorus in the feces (Vohra and Satyanarayana, 2003), and was found to be economically viable as a chick feed supplement (Vohra *et al.*, 2006).

Following first commercialization attempts by Gist-Brocades Europe (1993–1994), several companies, like Alko Inc (Finland), Alltech (USA) and BASF (USA), started the industrial scale production of phytase marketed under the trade names Finase, Allzyme and Natuphos, respectively, and successfully utilized them in feed applications. All these originated from filamentous fungi. The Food and Drug Administration (FDA) of USA has provided phytase preparations with GRAS (Generally Regarded As Safe) status (Wodzinski and Ullah, 1996). The production of phytases at industrial scale is expensive, which limits their widespread use as a feed additive.

Phytases from transgenic plants and animals

Generating transgenic plants and animals expressing microbial phytase is a novel way to enzymatically reduce the phytate content in feed and food. By overexpressing mainly HAP encoding (Lung *et al.*, 2005) genes, several phytase-transgenic plants have been created (Mullaney *et al.*, 2000). Such plants aid in reducing phytic acid in seeds and grains used for animal and human consumption. The high phytase activity in fodder breaks down phytate, thereby facilitating the assimilation of phytate phosphorus from the fodder (Ige *et al.*, 2010). This approach also liberates the chelated metal ions like iron and zinc from phytate, thus making them available for absorption by humans (Lung *et al.*, 2005). There are several reports on the expression of phytase in transgenic plants. A comprehensive list of transgenic phytase crops and source of enzyme along with their experimental results is presented in **Table 1**. In most of these cases, mainly *phyA* from *A. niger* was expressed in the transgenic plants meant for feed additives. An improved growth rate resulted from supplementation of broiler diets with transgenic seeds (Pen *et al.*, 1993). Hamada *et al.* (2005) reported high-level expression of *Schwanniomyces occidentalis* phytase in transgenic rice plants, which could be stored as silage without any loss of activity until use as a feed additive (Hamada *et al.*, 2006). Based on these reports, the production of phytase through bio-farming using transgenic technology seems to be a promising approach.

The *app*A gene from *E. coli* was overexpressed in the salivary gland of pigs to generate the best-known phytase-transgenic animal, Enviropig (Golovan *et al.*, 2001a). This novel technology led to a 75% reduction in fecal phosphorus excretion in these animals. Thus, the activation of phytases in the saliva of transgenic animals facilitated the assimilation of phytates from the fodder. On similar lines, phytase-transgenic animals including fish, such as the Japanese medaka (*Oryzias latipes*) (Hostetler *et al.*, 2005), and mice (Golovan *et al.*, 2001b) have been developed. These studies suggested the possibility of developing poultry varieties with improved phytate digestion.

Table 1 Transgenic phytase crops and their sources

Phytase source	*Type of* phy *gene*	*Plant system*	*Achievement*	*References*
Aspergillus awamori	*phyA*	Soybean	Improved thermostability	Gao *et al.* (2007)
Aspergillus ficuum	*phyA*	Alfalfa	Alternative to commercial phytase	Ullah *et al.* (2002)
	phyA	Potato	Alternative to commercial phytase	Ullah *et al.* (2003)
	AfphyA	Soybean	Improved phosphorus absorption efficiency	Li *et al.* (2009)
	phyA	Tobacco	Proved to be equal to commercial phytase	Ullah *et al.* (1999)
Aspergillus fumigatus	*fphyA*	Tobacco	Showed thermostability	Wang *et al.* (2007)
	phyA	Wheat	Broiler growth improved	Brinch-Pedersen *et al.* (2006)
Aspergillus japonicus	*phyA*	Wheat	Increased bioavailability of iron and zinc in grains, enhanced phosphorus uptake	Abid *et al.* (2017), Mohsin *et al.* (2017)
Aspergillus niger	*phyA*	Arabidopsis	Improved phosphorus acquisition from soil	Mudge *et al.* (2003)
	phyA	*Brassica napus*	Improved phosphorus acquisition from soil & alternative to commercial phytase	Wang *et al.* (2013)
	phyA, phy2A	Maize	Phosphorus availability increased	Drakakaki *et al.* (2005), Chen *et al.* (2008)
	phyI	Rice	Enhanced inorganic phosphates	Liu *et al.* (2006)
	phyA	Sesame	Improved phosphorus acquisition	Jin *et al.* (2004)
	phyA	Soybean	Alternative to commercial phytase	Li *et al.* (1997)
	phy, phyA	Tobacco	Improved phosphorus acquisition from soil, improved broiler growth, proved to be an alternative to commercial phytase	Pen *et al.* (1993), Verwoerd *et al.* (1995), George *et al.* (2005, 2009), Giles *et al.* (2018)
	phyA	Wheat	Phytate degraded to lower forms	Brinch-Pedersen *et al.* (2000), Geetha *et al.* (2019)
Peniophora lycii	*phyA*	Tobacco	Improved phosphorus acquisition from soil	George *et al.* (2009)
Schwanniomyces occidentalis	*phy*	Rice	Increased shelf-stability	Hamada *et al.* (2005)

Applications in Aquaculture and Fish Farming

Feed costs constitute up to 70% of the total fish production costs in aquaculture (Rumsey, 1993). The anti-nutritional effect of phytates is more problematic in fish due to their short gastrointestinal tracts (Richardson *et al.*, 1985), which in turn hinders the use of plant-derived protein in fish feed. Phytase is thus useful as a means to promote the use of low-cost plant meals in the aquaculture industry and to maintain the acceptable phosphorus levels in water. The use of phytases in fish feed has increased during the past a few decades, mainly in response to concerns over aquatic phosphorus pollution (Cao *et al.*, 2007). It is well documented that the use of microbial phytase in fish feed can enhance the bio-availability of phytate-bound phosphorus and nitrogen, and thus less phosphorus is discharged into the aquatic environment (Rodehutscord and Pfeffer, 1995).

The effect of commercial phytase (Natuphos) from *A. niger* on common carp (*Cyprinus carpio*) was investigated by Schafer *et al.* (1995), which resulted in improved utilization of phytate phosphorus, reduced phosphorus secretion as well as enhanced body weight and body phosphorus content. Transgenic Japanese medakas (*O. latipes*) producing *A. niger* phytase were created to examine the feasibility and efficacy of producing fish capable of degrading phytate (Hostetler *et al.*, 2005). Most of the reports on phytase application in aquaculture are using the commercially available fungal phytases. However, yeast and fungal phytases provide the added advantage in fresh water and marine aquaculture due to their probiotic role. When plant based low phosphorus diets for Nile tilapia (*Oreochromis niloticus*) were supplemented at 750 U kg^{-1} with an engineered phytase SP1002, having improved properties such as an elevated pH optimum, increased stability against proteolytic activity and heat, produced by a genetically modified strain of *Hansenula polymorpha*, significantly improved digestibility of protein, calcium and phosphorus was observed (Liebert and Portz, 2007). The cell-bound phytase of *P. anomala* was also found to be a useful supplement to plant-based feed for marine as well as freshwater fish (Vohra *et al.*, 2011). During fish feeding trials on juvenile milkfish *Chanos chanos*, soybean meal was supplemented with the cell-bound phytase and used to replace fishmeal. This resulted in positive effects on growth of the fish, suggesting the use of *P. anomola* phytase in environment-friendly mass culturing of milkfish (Hassan *et al.*, 2009).

Phytases in Plant Growth Promotion and Soil Amendment

Around 10%–50% of the total organic phosphorus content in the soil is present as phytic acid (Mullen, 2005). This is not readily available to plants because it complexes with cations or adsorbs to various soil components. Hence, there is a need to mobilize this phosphorus source (Richardson *et al.*, 2001). The presence of plant growth-promoting fungi in the plant rhizosphere improves the availability by solubilizing/mineralizing phosphorus (Zhang *et al.*, 2016; Hossain *et al.*, 2017; Singh and Satyanarayana, 2010). Several extracellular phytase producing fungi like *A. flavus*, *A. fumigatus* and *A. rugulosus* that inhabit the rhizosphere have been investigated for

their role in promoting plant growth by improving phosphorus nutrition (Tarafdar and Rao, 1996). Also, species of *Trichoderma* and *Penicillium* have been reported to increase the extractable organic phosphorus in alkaline soils (Gaind and Nain, 2015). These studies clearly indicated that fungal phytase supplementation to soils lead to enhanced plant growth and productivity levels.

Another approach to improve the utilization of phytate phosphorus in soils is to generate transgenic plants expressing phytase. This would help in reducing dependency on phosphorus fertilizers in agriculture. Such transgenic plants secrete extracellular phytases into the rhizosphere that leads to an increased level of phosphorus availability for plants. Both fungal and bacterial phytase genes have been used to develop transgenic plants. The *A. niger* NRRL 3135 *phyA* gene was expressed in *Arabidopsis* aiming to secretion of the transgenic phytase into the rhizosphere. This resulted in a transgenic *Arabidopsis* line with improved phosphorus nutrition and growth compared to the control (Richardson *et al.*, 2001). Transgenic *Brassica napus* that expressed either the *A. niger phyA* or *appA* gene were created, which showed improved biomass yield in the presence of phytates as the only source of phosphorus (Wang *et al.*, 2013). Experiments aimed at obtaining plants with enhanced phytase activity have also been carried out with mustard, tobacco, soya, wheat, sweet potato, and canola (Balaban *et al.*, 2017). Certain properties of phytase are important in determining phytate hydrolysis such as its isoelectric point (pI) (George *et al.*, 2007; Lei *et al.*, 2013). A phytase of *Peniophora lycii* is more effective in hydrolyzing phytate than *A. niger* phytase, as the former has a lower pI (3.6) compared to the latter (5.0), thus allowing the *P. lycii* phytase to remain more soluble in the soil environment. Phytases with a low pI are more effective in hydrolyzing phytates in acidic soil, and in basic soil, phytases with high pI values are preferable. Thus, phytase genes sourced from various fungi have been used to create transgenics of tobacco, soybean, wheat and several other plants (**Table 1**), which further helps in mitigating the need for phosphorus fertilizers in agriculture as the release of such extracellular phytases from the roots of transgenic plants lead to an increased level of phosphorus and minerals available for plants, thereby improving overall plant growth and creating favorable conditions for neighboring plants (Reddy *et al.*, 2013).

Utility of Phytases in Environmental Pollution Mitigation

Phosphorus is an essential nutrient for living systems, however, too low or too high levels of this nutrient can cause problems. As discussed earlier, phytase supplementation in animal feeds is meant to overcome the problem of underutilization of phytates and the cost of Pi addition. The unutilized phytate phosphorus tends to accumulate in the environment as fecal matter (Mullaney *et al.*, 2000). This unutilized phytate is degraded by the phytases produced by soil and water-borne microbes, due to which the excess Pi runs off into water bodies that causes eutrophication and algal growth (Vohra and Satyanarayana, 2003; Kaur *et al.*, 2007). The pre-treatment of animal feeds with phytase has benefits of increasing availability of Pi, helping in nutrition and at the same time mitigating the problem of phosphorus pollution of aquatic environments. Lei *et al.* (1993) showed that the addition of PhyA from *A. niger* to a corn-soybean based feed with low phosphorus content enabled weanling pigs to utilize dietary phytate, whereby the retention of dietary phosphorus by pigs was increased by 50% and the simultaneous fecal excretion of phosphorus was lowered by 42%. The supplementation of feed of broiler chicks with cell-bound phytase of *P. anomala* not only resulted in improved growth and better phosphorus retention in the body, but there was also reduced excretion of phosphorus in the feces (Vohra *et al.*, 2006). This cell-bound phytase was also found to be a useful supplement to plant-based feed for environment-friendly cultivation of marine (*Chanos chanos*) and freshwater (*Labeo rohita, Clarias batrachus*) fish with diminishing excretion of phosphorus in the water bodies (Hassan *et al.*, 2009; Vohra *et al.*, 2011). Phytases were first introduced as animal feed supplements for environmental reasons, but soon their other nutritional and health benefits were realized.

Use of Phytases in Generating Specific Inositol Phosphates With Therapeutic Applications

There is an ever-increasing interest in inositol phosphates due to their role in transmembrane signaling and mobilization of calcium from intracellular reserves (Billington, 1993). The inositol phosphate derivatives find use as enzyme stabilizers (Siren, 1986a), enzyme substrates for various metabolic studies, inhibitors of enzymes and thus, as potential drugs, and as chiral building blocks (Laumen and Ghisalba, 1994). Certain *myo*-inositol phosphates also have beneficial health effects. The physiological functions are dependent on the number and position of the phosphate groups on the *myo*-inositol ring. The use of specific inositol triphosphates as pain killers has also been proposed (Siren, 1995). The esters of inositol triphosphate have been shown to exert significant inhibitory effects on retroviral infections including HIV (Siren, 1998). Certain *myo*-inositol phosphates have been suggested to have specific beneficial health effects such as reducing the risk of heart disease (Potter, 1995), renal stone formation (Modlin, 1980) and certain cancers (Graf and Eaton, 1993; Shamsuddin and Vucenik, 1999).

The sequential hydrolysis of *myo*-inositol hexakisphosphate by phytases releases various lower inositol phosphates, which are useful for studying their physiological effects. An efficient bioreactor with immobilized phytase could be advantageous for producing various lower isomers of phytate. Quan *et al.* (2003) immobilized cells of phytase producing *Candida krusei* in Ca-alginate gel beads for the preparation of various inositol phosphates, and the pure isomers were then isolated by ion-exchange chromatography.

Phytases can also be used to prepare novel specific phytate derivatives (Greiner and Konietzny, 1996). The phytase from *S. cerevisiae* was used for generating D-*myo*-inositol 1,2,6-triphosphate, D-*myo*-inositol 1,2,5-triphosphate, L-*myo*-inositol 1,3,4-triphosphate and *myo*-inositol 1,2,3-triphosphate (Siren, 1986b). Chemical synthesis of these compounds is difficult, requiring protection and deprotection steps (Billington, 1993). Thus phytases, which convert phytic acid to specific lower *myo*-inositol phosphates, would be useful for industrial production of the specific *myo*-inositol phosphate derivatives. Among the advantages of enzymatic hydrolysis over chemical synthesis are the stereospecificity and mild reaction conditions.

Table 2 Some examples of commercial fungal phytases

Organism of origin	*Commercial name of the product*	*Company*
Aspergillus awamori	Avizyme®	Finnfeeds International
Aspergillus niger	Natuphos®	BASF
	Allzyme®	Alltech
	Finase® P/L	AB Vista
Penicillium funiculosum	Rovabio®	Adisseo
Peniophora lycii	Ronozyme® P	Novozymes/DSM

Virtual Peroxidases Derived From Phytases and Their Uses

Peroxidases are enzymes that catalyze a wide variety of oxidations with hydrogen peroxide being the main oxidant. Considering the structural similarity of the active site of vanadium-dependent haloperoxidases, fungal phytases and acid phosphatases, a semisynthetic peroxidase was designed (Van de Velde *et al.*, 2000). In this enzyme, vanadate ion was incorporated into the active site of *A. niger (ficuum)* NRRL 3135 phytase, which resulted in the transformation of native phosphohydrolase activity of phytase into virtual peroxidase, which could catalyze enantio-selective oxidation of prochiral sulfides. It was observed that only histidine acid phosphatases with the active site sequence 'RHGXRXP' could function as a peroxidase when vanadate ion was incorporated into the active site. Vanadate serves as a phosphate analog and can inhibit the phytate hydrolyzing activity of phytase, thus making the enzyme behave like a vanadate-dependent haloperoxidase (Ullah *et al.*, 2011). The recombinant phytases of *P. anomala* and *S. thermophile* were also demonstrated to display haloperoxidase activity after insertion of vanadate into their active sites (Joshi and Satyanarayana, 2015b; Ranjan and Satyanarayana, 2016). The vanadate haloperoxidases have multifarious applications like in immunoassays, diagnostics, and as anti-microbial agents.

Other Applications

A novel application of phytases is in different biosensors. As phytic acid plays an important environmental role in poultry industry and has a health aspect in food industry, a phytase-based biosensor was developed for simple, one step quantitative detection of phytic acid based on the sequentially acting enzymes, *i.e.*, phytase and pyruvate oxidase. This was the first report of the use of phytase in biosensors (Mak *et al.*, 2004). Thereafter, a yeast cell (*Arxula adeninivorans*) based assay using phytase was developed for the detection of estrogenic activity in wastewater (Hahn *et al.*, 2006). The photometric assay enabled estrogen determination in sewage samples within 30 h. Another innovation was the use of the yeast *Candida melibiosica* strain 2491 producing a high phytase, as a biocatalyst in microbial fuel cell, by which the assimilation of inorganic phosphates was coupled with the electrochemical process (Hubenova and Mitov, 2010). Organophosphorus pesticides are chemically phosphoric acid esters which act on pests by inhibiting acetylcholine esterase activity. These compounds are not easy to eliminate from the environment, therefore leading to their bioaccumulation. Recently, phytase from *A. niger* NCIM 563 was discovered that actively degrades organophosphorus pesticides like chlorpyrifos, monocrotophos and methyl parathion (Shah *et al.*, 2017). This field can be explored further for its potential in the management as well as control of environmental phosphorus pollution. Ionic liquid mediated self-assembled phytase nanospheres were shown to have anti-tumor properties that could be enhanced in efficiency by adding platinum ions on their surface and loading them inside the anti-tumor drug curcumin that is present in turmeric, an Indian spice (Soni *et al.*, 2015). These nanospheres containing phytase I from *A. niger* are promising in therapeutics for development of multifunctional drug-delivery systems as they exhibit 25% anti-cancer effect that improved to 90% in platinum-phytase-curcumin nanospheres. The platinum-phytase spheres are also amenable as an efficient green and reusable phytate degradation system.

Commercially Available Phytases

Phytases are playing a significant role in the feed and food industry. In 2015, phytase had a highest revenue share of 83.6% of the food and feed industry (See Relevant Website Section) and accounted for annual sale of US$ 350 million. The first commercialized phytase called Natuphos was derived from *A. niger* and launched by BASF (Ludwigshafen, Germany) in 1991 (Haefner *et al.* 2005; Ariza *et al.* 2013). This was followed by the commercial product Ronozyme® P, a 6-phytase from *Peniophora lycii*. For producing phytase on a commercial scale, fungal hosts are preferred, because they can produce high enzyme titers and introduce post-translational modifications like N-linked glycosylation (Guo *et al.*, 2008; Greiner and Konietzny, 2012) that can result in improved thermostability (Wu *et al.*, 2014), a property desirable for phytase. Examples of phytases used in industrial scale and some of their commercial products are listed in **Table 2**.

Conclusions

Several fungal phytases seem to be suitable for various biotechnological applications due to their broad substrate spectrum along with thermostability, acid-stability and versatility in terms of applicability (conventional as well as novel).

Phytases enable enzymatic green phosphate generation and they are especially useful in animal nutrition, due to the increasing concerns about problems related to phosphorus pollution in regions of intense animal farming. However, their potential applications in various other industries including human nutrition, soil fertility, management of environmental phosphorus pollution as well as novel uses in other fields, makes them enzymes with high overall value. In addition, many phytase producing filamentous fungi and yeasts are GRAS organisms or have probiotic potential, making them safe for use. Also bioengineering is enabling the use of phytases by generating phytate hydrolyzing transgenic plants and animals. Simultaneously, the discovery of new fungal phytases with higher production levels and desirable characteristics for specific applications is required.

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Modification of Plant Carbohydrates Using Fungal Enzymes

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Introduction

A main driver of terrestrial carbon-recycling is the continuous decay of (dead) plant biomass, mainly mediated by the combined activities of highly dynamic fungal communities (Lladó *et al.*, 2017; López-Mondéjar *et al.*, 2018; Ontl and Schulte, 2012). Even related fungi from similar habitats have been shown to produce complementary enzyme-sets acting on the same substrate, which points to an evolutionary selection of efficient communities (Benoit-Gelber *et al.*, 2017; Benoit *et al.*, 2015; Hu *et al.*, 2011). Fungal enzymes are also major biocatalysts in many food and biotechnology applications, and essential for the transition from conventional chemical processes to biorefineries (Dilokpimol *et al.*, 2018; Frommhagen *et al.*, 2018; Himmel *et al.*, 2007; Straathof, 2014). Nevertheless, selection of the right fungal strain and most optimal enzymes for specific applications remains challenging and will be discussed in detail in this chapter.

Plant biomass is largely composed of cell wall material mostly originating from primary and secondary cell wall layers (Carpita, 2000). In general, polysaccharides are the most abundant cell wall components, next to structural proteins and aromatic lignin (Pauly and Keegstra, 2008). The primary cell wall surrounds the cell and is the major part of the plant cell wall in fruits and vegetables. This layer is mainly composed of cellulose microfibrils embedded in a network of pectin and various hemicelluloses, i.e., xyloglucan, xylan or mannan (Cosgrove, 2005). Exact compositions vary for example per source, tissue type or environmental growing conditions. The secondary plant cell wall is deposited on the outside of the plasma membrane surrounding the cell lumen, and on the inside of the primary cell wall. This secondary cell wall represents the main part of the dry matter of lignocellulosic feedstocks. Examples of the latter are grass-like agricultural by-products or hard- and softwoods.

It should be noted that the ratio of primary versus secondary cell wall components differs for example per botanical source and tissue type, and that certain types of biomass (i.e., sugar beet pulp, rape seed meal) do not fit in a strict system defining only "primary cell wall rich fruits and vegetables" versus "secondary cell wall rich grasses and woods".

The secondary cell wall contains cellulose and hemicellulose, in addition to the heterogenous aromatic polymer lignin (Boerjan *et al.*, 2003; Cosgrove, 2005; Meents *et al.*, 2018; Pauly and Keegstra, 2008). Hemicellulose is mainly represented by (acetylated) xylan in grasses and hardwoods or mannan in softwoods (Ebringerová *et al.*, 2005; Gírio *et al.*, 2010; Scheller and Ulvskov, 2010).

This chapter aims to give an overview of the structures of most occurring plant cell wall polysaccharides and corresponding fungal plant cell wall modifying enzymes with a focus on plant cell wall degradation.

Carbohydrate Active Enzyme Classification

Since the early search for enzymes, to benefit products and usually greener processes for the food or biorefinery industry, carbohydrate active enzymes have received elaborate attention (Frommhagen *et al.*, 2018; Gírio *et al.*, 2010; Himmel *et al.*, 2007; Jonathan *et al.*, 2017; Pauly and Keegstra, 2008). As more and more enzymes were discovered, varying in their origin, substrate specificity and mode-of-action, soon the need for a classification to improve selection and optimization of enzymes became evident. With the increasing amounts and easiness to obtain genome-based information many enzymes could be recognized based on gene level. Although this gene information does not provide data on actual enzyme functions, in 1998 it provided the basis for classification with the foundation of the so-called Carbohydrate-Active enZYmes database (see "Relevant Websites section"; Lombard *et al.*, 2014). The CAZy database describes families of structurally-related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds. Currently, five enzyme classes are defined in CAZy, each containing multiple families: Glycoside Hydrolases (GHs), Glycosyl Transferases (GTs), Polysaccharide Lyases (PLs), Carbohydrate Esterases (CEs) and Auxiliary Activities (AAs). Within the amino acid sequence similarity-based classification, families represent mutual biochemical mechanisms and protein structure information rather than actual substrate specificity. The latest developments comprise establishment of subfamilies, which are expected to better represent substrate specificity (Dilokpimol *et al.*, 2018).

GHs are by far the largest CAZy class and GH-candidates are widespread throughout the fungal kingdom (see "Relevant Websites section"; Lombard *et al.*, 2014). Additionally, many food, biomedical and biorefinery applications built on the use of various GHs, and, therefore, this class comprises the most biochemically and structurally characterized enzymes. GHs are considered to mainly follow either inverting or retaining mechanisms (Davies and Henrissat, 1995; McCarter and Withers, 1994). A distinct difference for retaining over inverting enzymes is the formation of a glycosyl enzyme intermediate, and, consequently, a second reaction step (Davies and Henrissat, 1995; McCarter and Withers, 1994). In the case that in this second step, instead of water leading to hydrolysis, another carbohydrate molecule acts as acceptor, transglycosylation takes place (Bissaro *et al.*, 2015). Obviously, at high carbohydrate concentrations, the equilibrium will be directed towards transglycosylation with minor hydrolysis

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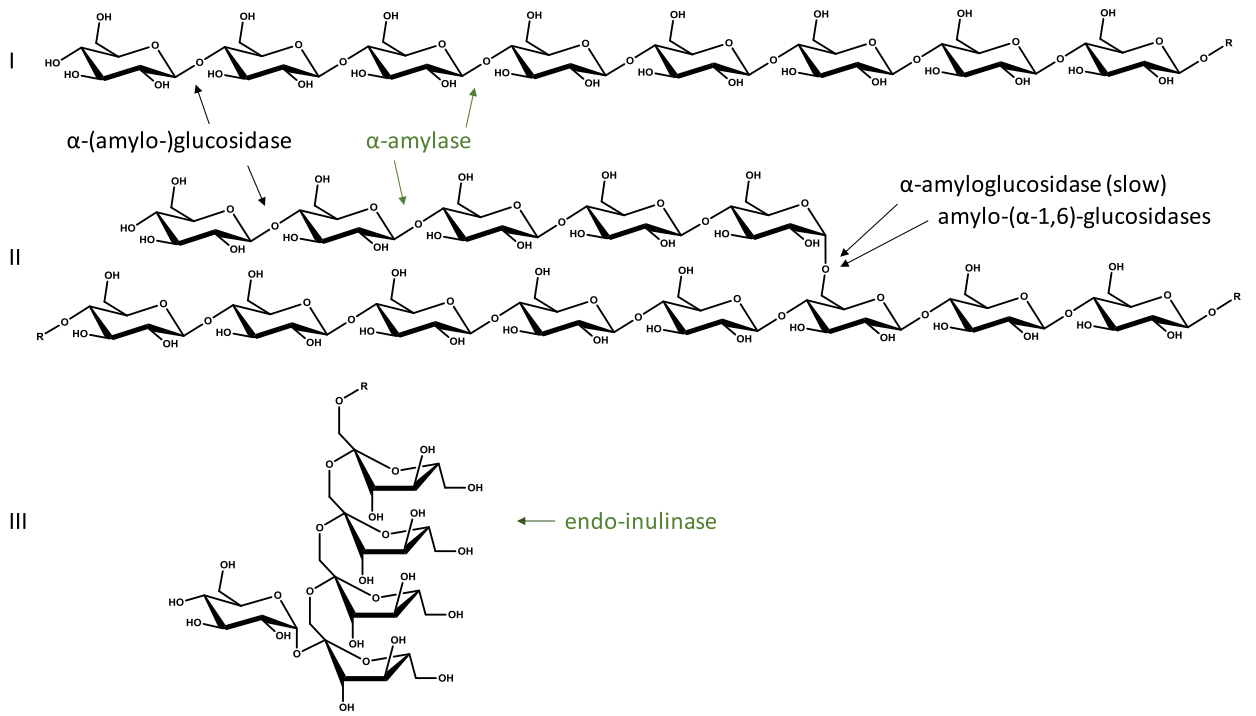

Fig. 1 Amylose (I), amylopectin (II) and inulin (III) structures and corresponding fungal hydrolytic enzymes. In green enzymes active towards the backbone of the polysaccharide are indicated, while in black exo-active and substitution removing enzymes are shown.

taking place. In contrast, GTs use "activated" sugar phosphates as glycosyl donor and, therefore, are out of the scope of this chapter (Lairson *et al.*, 2008).

PLs are distinct from GHs as PLs specifically cleave uronic acid-containing polysaccharides, while they act via a β-elimination mechanism resulting in unsaturated (oligomeric) products, having a double bond in the non-reducing end residue, and in a new reducing end (Benen and Visser, 2003; Lombard *et al.*, 2014).

CEs catalyze the de-*O*- or de-*N*-acylation of substituted carbohydrates (Biely, 2012; Nakamura *et al.*, 2017). Two classes of substrates for carbohydrate esterases are to be considered: those in which the sugar plays the role of the "acid", such as pectin methyl esters and those in which the sugar behaves as the alcohol, such as in acetylated xylan (Benen *et al.*, 2003; Benen and Visser, 2003; Nakamura *et al.*, 2017). The most common CE reaction mechanism is the Ser-His-Asp catalytic triad catalyzed deacetylation, also found in classical lipases and serine proteases (Benen *et al.*, 2003; Rauwerdink and Kazlauskas, 2015).

Catalytic proteins listed in AA families are redox enzymes, which are assumed to work together with other CAZymes. These AA families have been introduced after the discovery of the lytic polysaccharide monooxygenases (LPMOs) around 2010 (Lombard *et al.*, 2014), and classification and characterization of these AA members is still in its infancy.

Fungal Plant Carbohydrate Degrading Enzymes and Their Substrates

Two main plant storage polysaccharides are starch and inulin and their enzymatic degradation, which is relatively well understood, is highly relevant for many food and agro-industrial processes. In contrast, understanding the wide variety of plant cell wall polysaccharide structures and fungal enzymes to degrade these structures remains a major challenge. Therefore, this chapter shortly reviews starch and inulin and their enzymatic degradation, while the main part of this chapter will review plant cell wall polysaccharide structures and the fungal enzymes needed to degrade them.

Starch and Inulin Degrading Glycoside Hydrolases

As just mentioned, starch and inulin are considered natural storage carbohydrates, which form an energy reserve for plants. Many grains (i.e., corn, wheat) and tubers (i.e., potato) contain about 60%–80% of starch on a dry matter (DM) basis (Bach Knudsen, 1997). Relatively high amounts of inulin can be found in various roots, in particular chicory roots (10%–15% of DM) (Schaafsma and Slavin, 2014).

Starch is composed of two homoglycans, amylose and amylopectin (van der Maarel *et al.*, 2002). Amylose is a linear polysaccharide composed of α-(1→4)-linked glucosyl residues, while amylopectin has additional α-(1→6)-branching points (**Fig. 1**). Fungal starch degrading GHs comprise α-amylases in GH13 (Saranraj and Stella, 2013), debranching amylo-(α-1,6)-glucosidases in GH133, amyloglucosidases in GH15 and some α-glucosidases in GH31 (**Fig. 1**; **Table 1**). The large GH13 family is further

Table 1 Selected glycoside hydrolase (GH) families, and lytic polysaccharide monooxygenases (LPMOs) in auxiliary activity (AA) families taking part in starch, inulin, pectin, cellulose and hemicellulose degradation[a]. Only CAZy families with over five relevant fungal members are indicated

Major substrate	*Enzyme*	*CAZy (sub-)family*[a]
Starch	α-Amylases	GH13_1, GH13_5, GH13_32
	Amylo-(α − 1,6)-glucosidases	GH133
	Amyloglucosidase	GH15
	α − 1,4-glucosidases	GH31
	LPMOs	AA13
Inulin	Inulinases; exo-inulinases	GH32
Cellulose	Endo-β − 1,4-glucanases	GH5, 7, 12, 45
	Cellobiohydrolases	GH6, 7
	β − 1,4-glucosidases	GH1, 3
	LPMOs	AA9
Xyloglucan	Endo-β − 1,4-glucanases	GH5, 12, 16, 74
	β − 1,4-glucosidases	GH1, 3
	β − 1,4-galactosidases	GH2, 35
	α-Xylosidases	GH31
	α-Fucosidases	GH29, 95
	LPMOs	AA9
Xylan	Endo-β − 1,4-xylanases	GH5, 10, 11, 30, 43
	β-Xylosidases	GH3, 30, 43
	α-Arabinofuranosidases	GH3, 43, 51, 54, 62
	α-Glucuronidases	GH67, 115
	Acetyl xylan/feruloyl esterases	CE1
	Glucuronoyl esterases	CE15
	LPMOs	AA14
Mannan	Endo-β − 1,4-mannanases	GH5, 26, 134
	β − 1,4-mannosidases	GH1, 2, 5
	LPMOs	AA9
Pectin	(Rhamno-)galacturonan hydrolases	GH28
	Unsaturated rhamnogalacturonan hydrolase	GH105
	α-Rhamnosidases/rhamnogalacturonan α-L-rhamnohydrolases	GH78
	α-Arabinofuranosidases; endo/exo-arabinanases; arabinoxylan Arabinofuranohydrolase	GH3, 43, 51, 54, 93, GH62
	Endo-β − 1,4-galactanases	GH53
	β − 1,4-galactosidases	GH2, 35
	β-Xylosidases	GH3, 30, 43
	Pectin lyases	PL1
	Pectate lyases	PL1, 3, 9
	Rhamnogalacturonan (exo-) lyases	PL4, 11, 26
	Pectin methyl esterases	CE8
	Rhamnogalacturonan acetyl esterases; pectin acetyl esterases	CE12
	Feruloyl esterases	CE1

[a]Based on CAZy (CAZy.org; Lombard *et al.*, 2014).

divided in over 40 subfamilies, and while originally fungal α-amylases were mostly classified in subfamily GH13_1, recent work showed their presence in subfamilies GH13_5 and GH13_32 as well (Stam *et al.*, 2006; Janíčková and Janeček, 2020). Debranching pullulanases have been suggested to classify in GH13_12–14, GH13_39 and GH13_41, thoroughly reviewed by Møller *et al.* (2016), but so far no fungal candidates have been suggested (Xu *et al.*, 2013). In fungi, the amylo-(α-1,6)-glucosidases from GH133 might be important for debranching purposes.

Candidates from all three fungal starch degrading GH families are wide-spread, and are for example found in *Aspergillus niger*, *Aspergillus oryzae*, *Podospora anserina* and *Rhizopus oryzae* (van den Brink and de Vries, 2011). The α-amylases are known to endo-act, meaning that they can cleave the glycosidic linkages inside the α-(1→4)-linked glucan backbone resulting in dextrins of various degree of polymerization (DP). In general, α-amylases cannot degrade the backbone in the vicinity of an α-(1→6)-branching

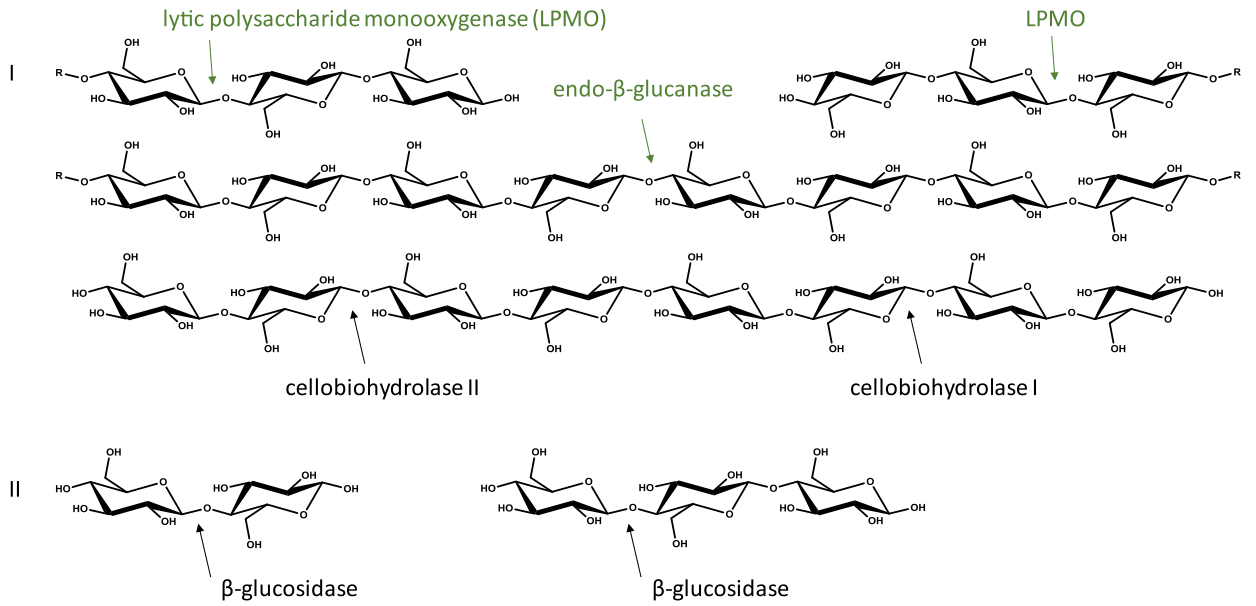

Fig. 2 Cellulose (I), cellobiose and cellotriose as example of cello-oligosaccharides (II) with corresponding fungal hydrolytic and oxidative enzymes. In green enzymes active towards the backbone of the polysaccharide are indicated, while in black exo-active and substitution removing enzymes are shown. For the detailed mechanism of lytic polysaccharide monooxygenase (LPMO) see **Fig. 6**.

point. Amyloglucosidases and α-glucosidases release one glucosyl residue per cleavage from the chains ends, a process that is known as exo-active (Jonathan *et al.*, 2015; Warren *et al.*, 2015). Amyloglucosidases can also act towards the α-(1→6)-linked branching points, albeit at a slower rate compared to cleavage of the α-(1→4)-linkages (Jonathan *et al.*, 2016). Most characterized fungal amyloglucosidases act from the non-reducing end (Jonathan *et al.*, 2016; Karim and Tasnim, 2018).

The name inulin is used for a group of heterogenous polysaccharides, built from a terminal glucosyl residue coupled to (1→2)-linked fructosyl residues ranging in DP from 2 to >100 (**Fig. 1**; Schaafsma and Slavin, 2014). Inulin can be hydrolyzed by either acid hydrolysis or endo-inulinases into smaller oligomers. Inulin molecules with a DP lower than 10 are also considered as fructo-oligosaccharides. Both inulin and fructo-oligosaccharides are not digestible by the human digestive enzymes and, therefore, considered as dietary fiber (Raninen *et al.*, 2011). Fungal inulin degrading enzymes are mostly found in the GH32 family, for example (exo-)inulinases (**Table 1**). Fungi vary considerably in abundance of GH32 enzymes, for example multiple GH32 inulinase encoding genes are present in the genome of various *Aspergillus* strains, while these are absent in other fungi, such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (van den Brink and de Vries, 2011). GH32 enzymes are retaining enzymes, which for some of the (putative) fungal GH32 members might result in transglycosylation activity forming fructan-polysaccharide mixtures (Lasseur *et al.*, 2009).

Cellulose Degrading Glycoside Hydrolases

Cellulose is the main polysaccharide in plant cell walls of all photosynthetic higher plants and algae. The structure is a homogeneous linear polymer of β-(1→4)-linked glucan chains, which form microfibrils via hydrogen bonds and van der Waals forces (Jarvis, 2003). Alternate (rotated by 180°) glucosyl units are linked to form a flat ribbon (**Fig. 2**) tightened by hydrogen bonds (Jarvis, 2003). Dependent on the source, cellulose varies in crystalline forms and the microfibrils differ in length, i.e., a very high DP (14,000 – 15,000), in for example the secondary cell wall of cotton, or a shorter DP (between 500 and 8000) in primary cell walls (Atalla and Vanderhart, 1984; Mohnen *et al.*, 2008). Crystalline regions alternate with amorphous regions as a result of a variability on hydrogen bonding or interaction with other polymers, such as hemicelluloses or lignin (Atalla and Vanderhart, 1984).

The degradation of cellulose is catalyzed by the action of three different classes of cellulases: endoglucanases (endo-β-1,4-glucanases), exo-active cellobiohydrolases (CBHs) and β-1,4-glucosidases (BGLs) (Bischof *et al.*, 2016; Lynd *et al.*, 2002; Reese, 1956). The latter hydrolyze soluble cello-oligosaccharides into glucose, acting from the non-reducing ends, and are predominantly found in the GH1 and GH3 families (**Table 1**; Lynd *et al.*, 2002). Endoglucanases degrade internal β-(1→4)-linked glucan bonds in cellulose, and fungal candidates are distributed over the GH families 5, 7, 12 and 45. Cellobiohydrolases cleave off cellobiose (DP2) from β-(1→4)-linked glucan chain from either the reducing (CBHI) or non-reducing (CBHII) ends and fungal candidates are found in the GH7 and GH6 families, respectively (Lynd *et al.*, 2002). CBHs are considered to be important for the hydrolysis of, in particular, crystalline parts of cellulose (Teeri, 1997), while endo-glucanases prefer acting towards amorphous regions (Béguin and Aubert, 1994). Notably, the classification of cellulose degrading enzymes into these categories is oversimplified since endoglucanases have already been shown to cleave cellulose randomly or processively and CBHs also vary in their mode-of-action from strictly exo-acting to highly processive (Payne *et al.*, 2011). That might also be the reason that fungi usually possess multiple

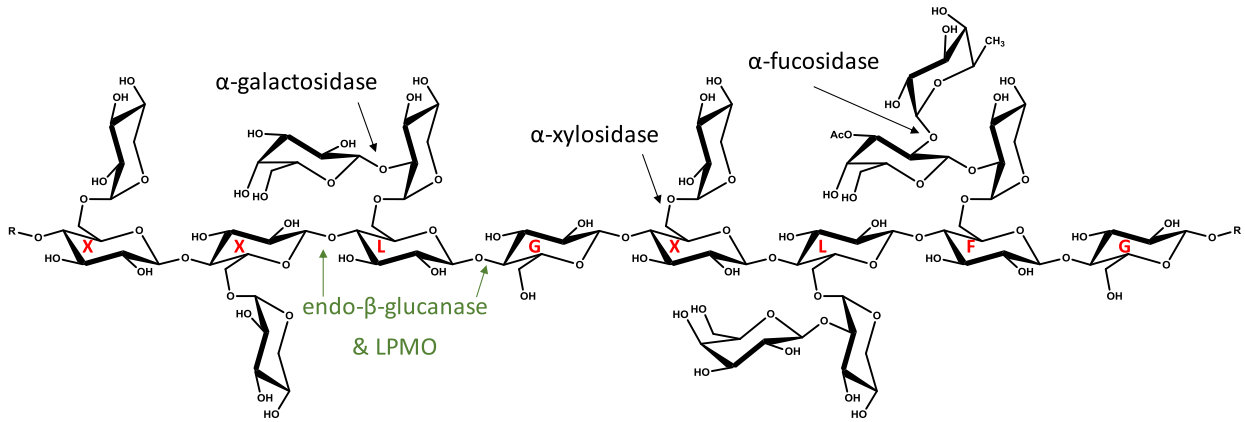

Fig. 3 Xyloglucan and corresponding fungal hydrolytic and oxidative enzymes. In green enzymes active towards the backbone of the polysaccharide are indicated, while in black exo-active and substitution removing enzymes are shown. For the detailed mechanism of lytic polysaccharide monooxygenase (LPMO) see **Fig. 6**. Letters (in red) in the structure indicate letter-code nomenclature for the (decorated) glucosyl units according to Fry *et al.* Reproduced from Fry, S.C., York, W.S., Albersheim, P., *et al.*, 1993. An unambiguous nomenclature for xyloglucan-derived oligosaccharides. Physiologia Plantarum 89, 1–3.

endoglucanase, CBH and BGL candidates (van den Brink and de Vries, 2011). For example, *Aspergillus niger* has five endoglucanase (GH5 and 12), four CBH (GH6 and 7), and 13 BGL (GH1 and 3) candidates, while *Myceliophthora thermophila* C1 comprises in total over 50 cellulose degrading enzymes (van den Brink and de Vries, 2011; Verdoes *et al.*, 2007).

It has been shown that CBHs are inhibited by their product cellobiose, which obviously hinders complete saccharification of cellulose (Bischof *et al.*, 2016). Therefore, cellulase mixtures are often supplemented with BGLs. Most commercial cellulase cocktails are enriched in the powerful *T. reesei* cellulases. Notably, these mixtures are often supplemented with BGLs from other fungi, i.e., like various *Aspergilli* species, as the BGLs of *T. reesei* are mostly membrane bound and, hence, not excreted by the fungus (Sørensen *et al.*, 2013). Another bottleneck for efficient cellulose degradation is the glucose inhibition of BGLs, hence, candidates able to maintain high conversion at high glucose concentration are aimed at (Sørensen *et al.*, 2013).

Other enzyme or protein classes that are involved in the degradation of cellulose are, for example, expansins, swollenins and lytic polysaccharide monooxygenases (LPMOs). These proteins are less studied and summarized as non-hydrolytic cellulose active proteins (NHCAPs) (Ekwe *et al.*, 2013). In particular, LPMOs possess a different catalytic mechanism compared to classical cellulose hydrolases, which is explained in detail later in this chapter.

Xyloglucan Degrading Glycoside Hydrolases

Xyloglucans are mostly found in the primary cell walls of higher plants (Ebringerová *et al.*, 2005; Gírio *et al.*, 2010; Hayashi, 1989; Vincken *et al.*, 1997). Contents based on dry matter vary, for example, the primary cell wall of poplar is composed of up to 6% (w/w) of xyloglucan (Park *et al.*, 2004), while those of *Arabidopsis thaliana* comprise between 20% and 25% (w/w) of xyloglucan (Ebringerová *et al.*, 2005). Xyloglucan consists of a backbone of β-(1→4)-linked glucosyl residues being α-(1→6)-substituted with xylosyl residues. For the majority of xyloglucan structures, the xylosyl substituents can be further extended with galactosyl and fucosyl residues (**Fig. 3**), while further substitution with arabinosyl residues is particularly found for solanaceous xyloglucans. The (substituted) glucosyl backbone units of xyloglucans occur in a repetitive pattern, which are indicated with a one-letter coding system code (G, X, L, …) for an eased identification and characterization (**Fig. 3**; Fry *et al.*, 1993).

The backbone of xyloglucan is cleaved by endo-β-1,4-glucanases, which are present in the GH5, 12, 16 and 74 families (Kubicek *et al.*, 2014). These enzymes are widespread in the fungal kingdom, and usually plant cell wall degrading fungi possess multiple candidate genes for xyloglucan degradation in their genome (Kubicek *et al.*, 2014; van den Brink and de Vries, 2011). In particular, xyloglucan active endo-β-1,4-glucanases of family GH74 have recently been shown to be further dividable into five subgroups via structure based phylogenetic analysis (Arnal *et al.*, 2019). Although in GH74 mainly bacterial candidates are found (see "Relevant Websites section"; Lombard *et al.*, 2014), fungal members seem to co-cluster (Arnal *et al.*, 2019). The subgroups coincide with a difference in mode-of-action and regio-specificity: a major subgroup hydrolyzed the xyloglucan backbone non-specifically at G or X (i.e., non-processive), while another subgroup seem to correlate to a more processive xyloglucan degradation (Arnal *et al.*, 2019; Vincken *et al.*, 1997). Note that only few endo-β-1,4-glucanases are characterized to date as xyloglucan specific enzymes, not showing activity towards other substrates such as β-glucan or cellulose (Pauly *et al.*, 1999; Vincken *et al.*, 1997). Further, β-glucosidases (GH1 and GH3), if not hindered by the xyloglucan substitution, can cleave off (non-substituted) glucose of xylogluco-oligosaccharides, like for cellodextrins (**Fig. 2**). Such β-glucosidases have more freedom to act after activity of or in synergy with various xyloglucan side chains removing enzymes, such as α-xylosidases (GH31), α-galactosidases (GH27 and GH36) and α-fucosidases (GH95) (**Table 1**; de Vries and Visser, 2001; Hayashi, 1989).

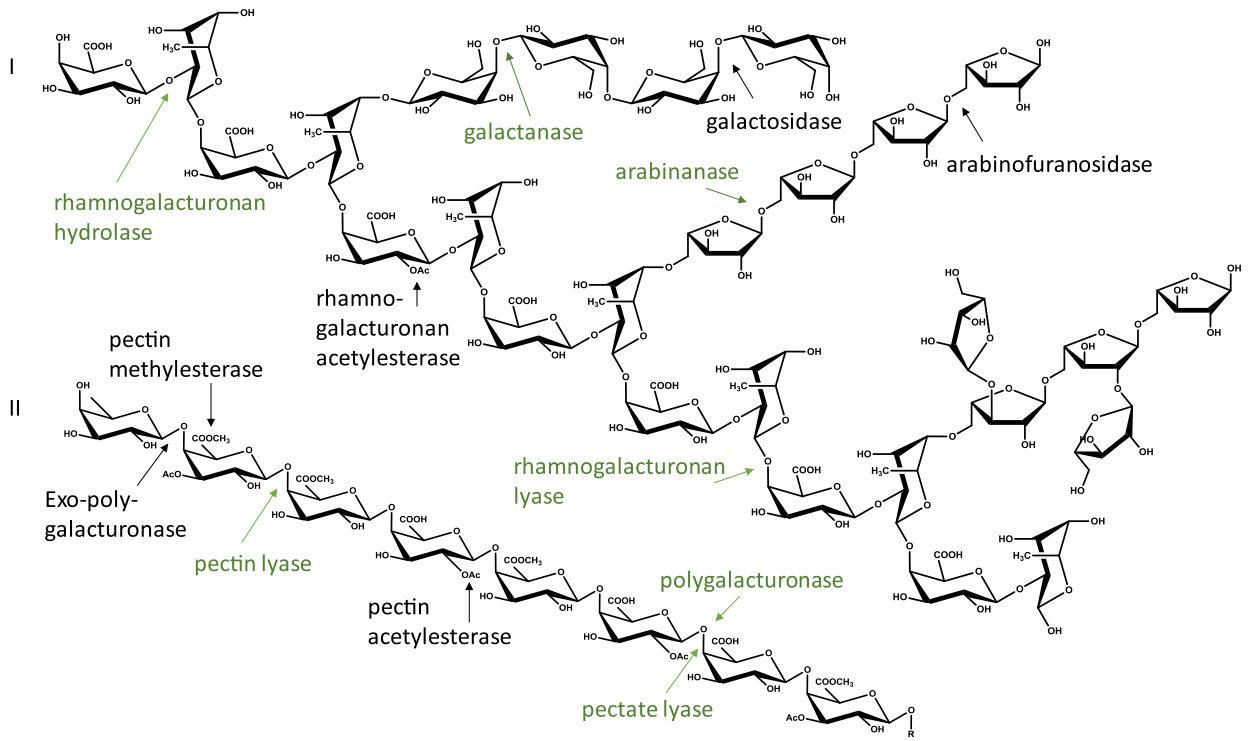

Fig. 4 The main pectic structures: substituted rhamnogalacturonan I (RG-I; I) and homogalacturonan (HG; II), including corresponding fungal hydrolytic enzymes and lyases. In green enzymes active towards the backbone of the polysaccharide are indicated, while in black exo-active and substitution removing enzymes are shown. Ferulic acid esters have been shown to occur on the pectic arabinan and galactan side-chains, but their generic character has yet to be defined. Therefore, ferulic acid esters and ferulic acid esterases are not depicted in the figure.

Like for cellulose, activity of non-hydrolytic LPMOs towards xyloglucan has been shown resulting in chain-cleavage, which is further discussed below.

Pectin Degrading Glycoside Hydrolases and Polysaccharide Lyases

Pectin mostly occurs in plant primary cell walls and comprises a variety of polysaccharide structures. Predominant pectic structures are homogalacturonan (HG) and substituted rhamnogalacturonan I (RG-I) with a lower abundance of heavily substituted rhamnogalacturonan II (RG-II) (**Fig. 4**; Ralet *et al.*, 2009; Voragen *et al.*, 2009; Wong, 2008). HG has a backbone of α-(1→4)-linked galacturonic acid residues, which can be esterified at the carboxyl group at C6 with methanol. HGs from specific sources, such as from potato or sugar beet, have been shown to be esterified at *O*2 and/or *O*3 of the galacturonic acid with acetic acid (Ralet *et al.*, 2009; Voragen *et al.*, 2009). In plants, pectin is highly methyl esterified, which decreases during ripening due to endogenous methyl esterase activity. In the RG-I backbone, rhamnosyl (Rha) residues alternate with galacturonic acid (GalA) via [→4)-α-GalA-(1→2)-α-Rha-(1→] linkages. Largely depending on the source, 20%–80% of the Rha residues in RG-I are (1→4-) substituted and the length of the side-chains vary from DP1 to >40 (Ralet *et al.*, 2009; Voragen *et al.*, 2009; Wong, 2008). Side chains consist mainly of arabinans or β-(1→4)-linked (type I) (arabino-)galactans (**Fig. 4**). Arabinans are built from a chain of α-(1→5)-linked arabinosyl residues, which can be further substituted at the *O*2 or *O*3 with arabinosyl residues. Arabinogalactans constitute a β-(1→4)-linked galactosyl backbone, which is further ramified at the *O*3 and/or *O*6 with variable levels of arabinosyl and/or galactosyl residues (Ralet *et al.*, 2009; Voragen *et al.*, 2009; Wong, 2008). Next to HG and RG-I, pectin can be composed of RG-II and xylogalacturonan (XGA). Rhamnogalacturonan II (RG-II) is a structurally very complex element, composed of a GalA-backbone and substituted with rare sugars such as 2-*O*-methylxylosyl, 2-*O*-methylfucosyl and aceric acid (= 3-C-carboxy-5-deoxy-L-xylofuranose) residues (O'Neill *et al.*, 2004). RG-II is present in all plants and its levels are typically low. In XGA, GalA residues are substituted with, β-(1→3)-xylosyl residues (O'Neill *et al.*, 2004). The presence of XGA does not seem to be universal, but has been reported for many reproductive tissues of apple, watermelon, onion, potato and pear (Voragen *et al.*, 2009).

Fungal enzymatic HG and RG-I degradation relies on activities from both GH and PL families (**Fig. 4**), while enzymes playing a significant role for degradation of the complex RG-II structure are, so far, not well defined. Fungal HG and RG-I backbone degrading hydrolases are mostly found in GH28 as either polygalacturonases (PGs) for HG or rhamnogalacturonan hydrolases (RGHs) for RG-I. For a complete degradation of the pectin backbone, also exo-acting enzymes like exo-PGs, RG-rhamnohydrolases (GH78) and RG-galacturonohydrolases (GH138) are required (Gilbert *et al.*, 2008). As the backbone degrading endo- and exo-acting hydrolases are largely hindered by backbone substitutions, accessory enzymes are essential for complete degradation.

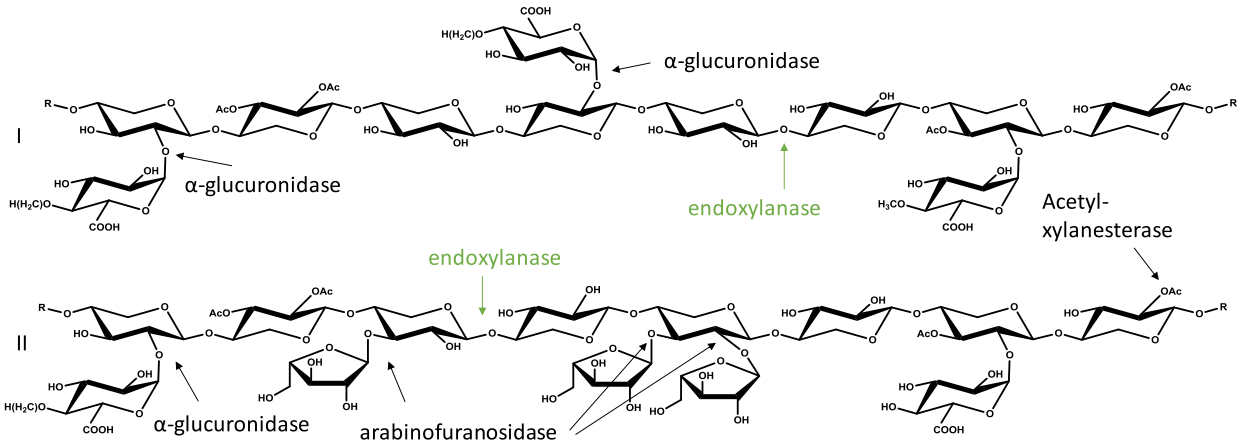

Fig. 5 Xylan and corresponding fungal hydrolytic enzymes. In green enzymes active towards the backbone of the polysaccharide are indicated, while in black exo-active and substitution removing enzymes are shown. Hydroxycinnamic acid substituents, such as ferulic acid, are not depicted in this figure, as these structural compounds are only generic for grass xylans. Hence, ferulic acid esterases are not included as well.

Examples of such accessory enzymes, produced by fungi, are pectin methylesterases (CE8) and rhamnogalacturonan acetylesterases (CE12). In addition, a whole array of arabinan degrading GHs (GH3, 43, 51, 54, 62, 93) and galactan degrading GHs (GH3, 30, 43, 53) are available from fungi to fully degrade pectin side chains. Feruloyl esterases (some of which are members of CE1, but most are not included in the CAZy family system) are active towards the feruloyl-ester that is present on the pectic arabinan and galactan side-chains found in various sources like sugar beet and potato (Kühnel *et al.*, 2012; Ralet *et al.*, 1994; Wefers *et al.*, 2014). Enzymes like xylogalacturonan hydrolase and specific exo-polygalacturonases, which can cleave the xylogalacturonans backbone rather than remove the xylosyl substituents, can be found in GH28, whereas RG-II degrading enzymes have hardly been reported in literature (Gilbert *et al.*, 2008; Zandleven *et al.*, 2005).

Fungi usually produce various members of the above mentioned GH families, although many of these enzymes have not yet been biochemically characterized. Such a plethora of pectin degrading enzymes (per fungal strain) shows the complexity of plant pectic structures resulting in the requirement for a large variety of enzymes needed for complete degradation. To give an example, *A. niger* produces seven different endo-PGs, of which each shows distinct kinetic properties, sensitivity for HG methyl esterification and processivity capabilities (Bussink *et al.*, 1992; de Vries *et al.*, 2017; de Vries and Visser, 2001). The same fungus has six RHGs in its genome, of which two are characterized showing different product profiles resulting from degradation of substituted RGs indicating a different substrate specificity of these enzymes (de Vries *et al.*, 2017; Suykerbuyk *et al.*, 1997).

In addition to the hydrolytic backbone degrading enzymes, pectin (PL1) and pectate lyases (PL1, 3, 9) cleave the methyl esterified HG backbone and non-esterified HG, respectively, while rhamnogalacturonan lyases (PL4, 11) act towards the RG backbone. The lyases act via a β-elimination mechanism, resulting in cleavage and double bond formation between the C4 and C5 of the non-reducing end product. These fungal lyases have been characterized to a limited extent (Benen and Visser, 2003; Limberg *et al.*, 2000; Remoroza *et al.*, 2014). A characterized PL4 rhamnogalacturonan lyase from *Aspergillus aculeatus* preferably cleaves the glycosidic linkage next to the fourth residue counted from the reducing end L-rhamnosyl residue and is severely affected by backbone acetylation and the presence of longer side chains that are attached to the neighboring rhamnose (Mutter *et al.*, 1994).

The PL formed unsaturated double bond could possibly be hydrated and cleaved by enzymes that are members of GH105 (unsaturated rhamnogalacturonan hydrolases) (Lombard *et al.*, 2014). Unfortunately, the characterization of these hydrolases from fungi is lacking, although several putative candidates have been identified.

Characterized enzymes are not only relevant for assessing (new) industrial applications, as discussed at the end of this chapter, but they can also serve as tools to characterize pectic structures via so-called enzymatic fingerprinting (Limberg *et al.*, 2000; Remoroza *et al.*, 2014).

Xylan Degrading Glycoside Hydrolases

Xylans are a diverse group of polysaccharides, although all have a backbone of β-(1→4)-linked xylosyl residues. Cell wall xylans can comprise 20%–30% (w/w) of the dry matter content in dicots, such as hardwoods, and up to 50% (w/w) in cereals and grasses (Ebringerová *et al.*, 2005). The latter monocotyledonous heteroxylans are further substituted by arabinosyl residues at generally *O*3, or both *O*2 and *O*3 backbone xylosyl positions, and are therefore named as "arabinoxylan" (**Fig. 5**; Kabel *et al*, 2002a,b; Rogowski *et al.*, 2015). To a lesser extent, these xylans can be substituted via *O*2 of the xylosyl residues by (4-*O*-methyl-) glucuronosyl and/or acetyl groups, via the *O*2, *O*3 or both positions ((acetylated)glucuronoarabinoxylan (GAX)) (**Fig. 5**; Kabel *et al*, 2002a,b; Rogowski *et al*, 2015; Smith *et al*, 2017). The arabinosyl substituents can be further esterified at their *O*5 position to ferulic or diferulic acid and, although in much lesser extent, to *p*-coumaric acid (Appeldoorn *et al.*, 2013). Glucuronoxylan (GX) is

found in hardwoods, whereby the xylan backbone is ramified with α-(1→2)-linked 4-*O*-methyl-glucuronosyl residues and is highly acetylated (Kabel *et al.*, 2003; Rogowski *et al.*, 2015).

The xylan backbone is degraded by endo-β-1,4-xylanases (mainly in GH10, 11, 30; **Fig. 5**) and the released xylo-oligosaccharides are further degraded by β-xylosidases (GH3, 43, 54; **Table 1**). In particular for GH10 and GH11 endo-xylanases, it has been shown that due to the enzyme and its characteristic catalytic site structure, they vary in mode-of-action towards decorated xylans, i.e., arabinoxylan and glucuronoxylan (Kolenová *et al.*, 2006; Kormelink *et al.*, 1993a; Pell *et al.*, 2004; Pollet *et al.*, 2010; Verbruggen *et al.*, 1998). To be more specific, members of the GH10 family, such as endo-xylanase I from *Aspergillus awamori*, can cleave directly next to a glucuronosyl or arabinosyl substituted xylosyl residue at the non-reducing end site (Kolenová *et al.*, 2006; Kormelink *et al.*, 1993a,b; Kormelink and Voragen, 1993; Pell *et al.*, 2004; Verbruggen *et al.*, 1998). Enzymes from this GH10 family have a "salad bowl" like shape and are able to bind substituted xylan in such a way that catalytic cleavage on the non-reducing end directly next to the substituted xylosyl is favored (Pell *et al.*, 2004). In contrast, the activity of members from GH11, recognized by their half open right hand shape-like structure, towards substituted xylan is more hindered and, therefore, their catalysis result in both larger oligosaccharides and oligosaccharides having a free (non-substituted) xylosyl residue on the non-reducing end (Kormelink *et al.*, 1993a,b; Pollet *et al.*, 2010; Verbruggen *et al.*, 1998). In addition to xylan substitution, also xylan solubility might affect the catalytic performance of GH10 and GH11 endo-xylanases differently, as for example has been shown for endo-xylanases from *M. thermophila* C1 (van Gool *et al.*, 2013).

To accommodate xylan degradation, fungi produce, in addition to the endo-xylanases, usually a variety of xylan-debranching enzymes. As most plant xylans are branched with arabinosyl or (4-*O*-methyl-) glucuronic acid residues or acetyl esters or a combination hereof, three main debranching enzyme classes are α-arabinofuranosidases (e.g., family GH43, 51, 54 etc.), α-glucuronidases (e.g., family GH67 or GH115), and acetylxylan esterases (CE1) (**Table 1**) (Biely *et al.*, 2014; Ryabova *et al.*, 2001; Sørensen *et al.*, 2006; van den Brink and de Vries, 2011). Esterases are the least specific from these three groups, although characterized members of CE1 vary in their ability or preference to attack single substituted xylosyl residues, *O*2- or *O*3-acetylated, and double substituted xylosyl residues, acetylated at both *O*2- and *O*3-positions (Neumüller *et al.*, 2015). In addition, some CE1 members are shown to act as feruloyl esterases, which is not further discussed in this chapter (Dilokpimol *et al.*, 2016). Note that acetyl xylan esterases can have a specificity for acetylated xylan over other acetylated polysaccharides, such as acetylated pectin (Biely *et al.*, 2014).

Many of the GH51 arabinofuranosidases cleave off arabinose from xylosyl residues having only one arabinosyl substituent (*O*3-linked) (Murciano Martinez, 2016; Sørensen *et al.*, 2006). Recently, in the case of the bacterial XacAbf51, for the first time a GH51 member shows the ability to cleave off *O*3-linked arabinosyl from xylosyl residues having also the *O*2-position coupled to an arabinosyl residue (Dos Santos *et al.*, 2018). So far, for fungal enzymes, this feature has only been described for a few GH43 members (Sørensen *et al.*, 2006). Like for GH51 arabinofuranosidases, GH62 candidates have been shown to hydrolyze arabinose from single substituted xylosyl residues (Wilkens *et al.*, 2017). While in the same family candidates were found to be active towards both arabinoxylan and pectic L-arabinan, their activity towards the former polysaccharide was generally higher than for L-arabinan. The recently suggested subfamily classification of GH62, might further distinct substrate specifies within this family, although, hereto, more GH62 candidates will need to be characterized (Wilkens *et al.*, 2017).

α-Glucuronidases are another example for which their structure, conserved in either the GH67 or the GH115 family, steers the ability to cleave off glucuronic acid substituents only from specific positions (Rogowski *et al.*, 2014). In basidiomycetes, GH115 prevails over GH67, which is for example shown by genomes of 38 different basidiomycetes in which only genes encoding GH115 are described (Rytioja *et al.*, 2014). For ascomycetes, like for various *Aspergillus* strains, genes encoding both GH115 and GH67 α-glucuronidases are apparent (Rytioja *et al.*, 2014).

The GH67 glucuronidases have frequently been shown to be able to release both glucuronic acid and 4-*O*-methylglucuronic acid, but only from non-reducing end xylosyl residues, i.e., in oligosaccharides (Murciano Martínez *et al.*, 2016). GH115 glucuronidases, although only a few fungal members have been described in literature, have the same ability, but are significantly more active against substrates in which the xylosyl residues, decorated with (4-*O*-methyl-)glucuronic acid, is flanked by one or more xylose residues (Chong *et al.*, 2011; Murciano Martínez *et al.*, 2016; Rogowski *et al.*, 2014). Interestingly, some of these GH115 glucuronidases, but not all, are active towards both oligomeric and polymeric glucuronoxylan structures (Murciano Martínez *et al.*, 2016; Rogowski *et al.*, 2014).

Mannan Degrading Glycoside Hydrolases

Mannans and glucomannans mainly consist of β-(1→4)-linked mannosyl units, which can be *O*2 and *O*3 acetyl-esterified, as found in coffee beans, ivory nut, but also sugar beet and soy bean (Singh *et al.*, 2018). Next to these mannosyl units, the backbone can also contain glucosyl units as reported for cell wall glucomannans, and for glucomannans present as storage polysaccharides (e.g., konjac tuber) (Berglund *et al.*, 2018). Galactomannans having a varying level of galactosylation of the mannan backbone can be found as storage polysaccharides in seeds from the locust bean tree, guar tree and cassia tree (Wielenga, 2009).

In cell walls of softwoods, *O*-acetylated (galactogluco)mannan comprises up to 25% of the wood dry weight (Berglund *et al.*, 2018). Hardwoods contain less mannan (3%–5%), which is in general not galactosylated, but may be acetylated (Gilbert *et al.*, 2008).

Enzymes that are involved in the degradation of mannans are β-mannanases (e.g., family GH5, 26, 134) and β-mannosidases (GH1, 2 and 5; **Table 1**) (Gilbert *et al.*, 2008; Shimizu *et al.*, 2015), and these enzymes have been described in full detail by others (Ademark *et al.*, 2001; Aulitto *et al.*, 2019; Von Freiesleben *et al.*, 2018).

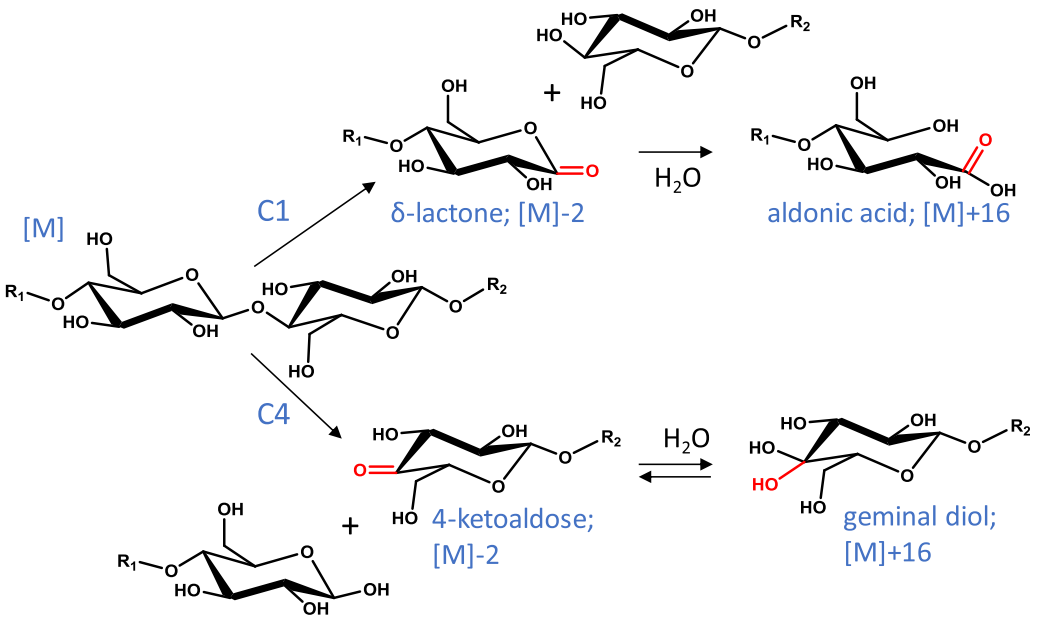

Fig. 6 General reaction of C1- and C4-oxidizing LPMOs, resulting in cleavage of the glycosidic linkage and either δ-lactones or 4-ketoaldoses are formed (Quinlan *et al.*, 2011). The δ-lactones convert in water to aldonic acids, while 4-ketoaldoses are, in water and pH dependent, in equilibrium with their geminal diol form (Bey *et al.*, 2013; Phillips *et al.*, 2011; Quinlan *et al.*, 2011).

Lytic polysaccharide monooxygenases (LPMOs)

The discovery of lytic polysaccharide monooxygenases (LPMOs), oxidizing polysaccharides resulting in subsequent glycosidic bond cleavage, has majorly revisited the concept of enzymatic (ligno-)cellulose degradation (Vaaje-Kolstad *et al.*, 2010). In contrast to the GHs discussed above, LPMOs require oxygen (or hydrogen peroxide) and an electron donor to result in oxidative cleavage (Kracher *et al.*, 2016; Bissaro *et al.*, 2017). Although LPMOs seem to have relatively low turnover numbers, they increase the substrate accessibility for other enzymes and have been shown to boost the activity of hydrolytic enzyme cocktails (Müller *et al.*, 2015). Therefore, nowadays, LPMOs are considered essential and even indispensable assets for (ligno-)cellulose conversions (Frommhagen *et al.*, 2015, 2018).

The first two LPMO families, created in the CAZy database around 2013, were classified as Auxiliary Activities (AA; AA9 and AA10), while before that year LPMOs were erroneously classified as GH61 (or CBM33 with mainly bacterial candidates) as their oxidative mechanism was not yet established (Quinlan *et al.*, 2011). Fungal AA9 LPMOs have been shown to result in cellulose chain cleavage due to oxidation of the glucosyl residues (**Fig. 6**) at the C1 position (EC 1.14.99.54, lytic cellulose monooxygenase (C1-hydroxylating)) or at the C4 carbon position (EC 1.14.99.56, lytic cellulose monooxygenase (C4-dehydrogenating)) (Frommhagen *et al.*, 2016; Phillips *et al.*, 2011). In addition, certain fungal members of the AA9 family have no strict so-called regioselectivity, resulting in both C1- and C4-oxidised oligosaccharides (Frommhagen *et al.*, 2016; Hemsworth *et al.*, 2015; Li *et al.*, 2012; Vaaje-Kolstad *et al.*, 2017). AA10 LPMOs, mainly comprising bacterial and no fungal candidates, act towards chitin (EC 1.14.99.53, lytic chitin monooxygenase) and cellulose (both EC 1.14.99.54 and EC 1.14.99.56) (Jensen *et al.*, 2019). Within the past years, new LPMO families have been added, all containing fungal members: AA11 active towards chitin and, possibly, cellulose (so far only one fungal member has been characterized, which is only active towards chitin (Hemsworth *et al.*, 2014)), AA13 active towards starch (EC 1.14.99.55 lytic starch monooxygenase). Most recently, AA families (AA14–16) have been added with so far zero, one or two biochemically characterized fungal members, hence, substrate specificities and regioselectivity have yet to be further explored for these families (see for example reviews from Chylenski *et al.*, 2019; Frommhagen *et al.*, 2018; Tandrup *et al.*, 2018).

In general, the catalytic activity of LPMO relies on a surface-exposed mono-copper-dependent active site. The copper ion is coordinated by three nitrogen atoms that are part of the histidine-brace composed of two conserved histidine residues, of which one is the N-terminus (Frandsen *et al.*, 2016; Quinlan *et al.*, 2011; Tandrup *et al.*, 2018). This N-terminal histidine is usually methylated in homologously expressed fungal LPMOs (Li *et al.*, 2012; Quinlan *et al.*, 2011). In contrast, bacterial LPMOs and LPMOs produced in heterologous hosts, such as the methylotrophic yeast *Pichia pastoris*, do not have the additional methylation at the N-terminal histidine (Hemsworth *et al.*, 2015; Li *et al.*, 2012). The presence of methylation of the N-terminal histidine has been suggested to improve the stability of LPMOs (Petrović *et al.*, 2018). However, LPMO members with or without methylation of the N-terminal histidine have been active (Borisova *et al.*, 2015; Westereng *et al.*, 2011), indicating that the role of this N-terminal methylation remains unclear.

In this section, we will further mainly discuss the largest fungal LPMO family to date, being CAZy family AA9, active towards β-(1→4)-linked glucosyl bonds in polysaccharides and, to a certain degree, in oligosaccharides. The first fungal AA9 LPMO with

shown C1- and C4-regioselectivity, or in this case nonselectivity, was *Ta*GH61 from *Thermoascus aurantiacus* (Quinlan *et al.*, 2011; **Fig. 6**). Such C1-oxidation of glucosyl residues in cellulose leads, after cleavage of the glycosidic bond, to the formation of an unstable δ-lactone (− 2 Da), which is converted into an aldonic acid in the presence of water (+ 16 Da) (Frommhagen *et al.*, 2016; Sun *et al.*, 2020a; Vaaje-Kolstad *et al.*, 2010; **Fig. 6**). Oxidation of the C4-carbon of the glucosyl residues in cellulose results, after cleavage, in the formation of a more stable 4-ketoaldose (− 2 Da) that might tautomerizes into a geminal diol (+ 16 Da) depending on the pH (Quinlan *et al.*, 2011; Sun *et al.*, 2020a; Westereng *et al.*, 2016). Currently, the number of AA9 LPMOs with biochemically analyzed C1/C4-regioselectivity is growing rapidly (Frommhagen *et al.*, 2018).

A main difference with GH structures is that AA9 LPMOs have no substrate binding pocket, but have a distinct flat binding surface around the exposed active site copper atom (Borisova *et al.*, 2015; Frandsen *et al.*, 2016; Tandrup *et al.*, 2018). The exact amino acid residues responsible for the substrate binding, however, are still unknown. Nevertheless, it can be expected that specific substrate recognition and regioselectivity of LPMOs relate to how the LPMO's flat binding surface binds to the substrate (Borisova *et al.*, 2015; Li *et al.*, 2012). Depending on the substrate specificity, these surface exposed binding loops (segments) comprise various hydrophilic and aromatic residues (Laurent *et al.*, 2019). The fact that AA9 LPMOs not only oxidatively cleave cellulose, but certain fungal AA9 members also act towards soluble cellooligosaccharides (Isaksen *et al.*, 2014) and hemicellulosic xyloglucan (Borisova *et al.*, 2015; Frommhagen *et al.*, 2018), shows that substrate binding is the result of the presence of specific loops or segments rather than binding to single amino acids (i.e., tyrosines). Even xyloglucan active AA9 LPMOs have been described to act towards xyloglucan independent of the side chains attached to the β-(1→4)-linked glucan backbone, like *Fg*LPMO9A and *Gt*LPMO9A-2 (Kojima *et al.*, 2016; Nekiunaite *et al.*, 2016), whereas others oxidize xyloglucan more specifically, such as *Nc*LPMO9C and *Mt*LPMO9J (Agger *et al.*, 2014; Kadowaki *et al.*, 2018; Laurent *et al.*, 2019; Sun *et al.*, 2020b).

Overall, more and more LPMOs will become biochemically characterized, for their substrate specificity and regioselectivity, to further contribute to the understanding of their function in nature and for commercial applications. As many fungi are known to comprise a large number of LPMOs in their genome, for example 22 putative AA9 LPMOs for *M. thermophila* C1 (Berka *et al.*, 2011), and between 9 and 33 for 18 reported basidiomycetes, for example 33 for *Coprinopsis cinerea* (Rytioja *et al.*, 2014), a further diversification in function of AA9 LPMOs can be expected.

Relevance for Industry

Many drawbacks of chemical transformation processes used in various industries can be overcome by introducing an enzymatic conversion process (reviewed by i.e., Chapman *et al.*, 2018; Alkorta *et al.*, 1998; Singh *et al.*, 2016). For example, nonspecific (chemical) reactions might result in undesired side-products, while enzymes often catalyze reactions with a high specificity. In addition, enzymes are known to react well at moderate temperatures, hence, processes might run at lower temperatures and/or lower pressures than otherwise needed to drive reactions. Such lower energy costs are beneficial both from a commercial and environmental point of view. A similar reasoning can be applied to processes needing harsh or even hazardous conditions, i.e., high temperatures, pressures, use of toxic catalysts, and acid or alkaline catalysts. Obviously, to enable application in food products applied enzymes should be food grade or "Generally Recognized As Safe" (GRAS), which is, at least in the European Union, not needed if the enzymes are only used in the process and do not end up in the final food product (Sewalt *et al.*, 2016).

Moreover, with the current possibilities of recombinant DNA technology and protein engineering, it became possible to target production of specific enzymes or combinations of enzymes by genetically modified commercial microbial production hosts. Such engineering developments have led to improvements in the stability, economy, specificity, and overall application potential of industrial enzymes. Hence, seen the above-mentioned benefits of using enzymes, it is not surprising that the number of commercial applications of enzymes is increasing every year (Chapman *et al.*, 2018).

Five major world markets for industrial enzymes can be distinguished: house hold care (i.e., detergents), food and beverages, biorefinery, feed and various technical applications (Chapman *et al.*, 2018; Singh *et al.*, 2016). In particular in the food and beverages, biorefinery and feed industry, carbohydrate active enzymes play a major role.

In the food and beverage industry, carbohydrate active enzymes have contributed to i.e., safer processes, higher yields and products with improved quality. Well-known examples are (1) the implementation of starch degrading enzymes to convert starch into dextrins and glucose for confectionery purposes, replacing hazardous highly acidic processes (Monteiro de Souza and de Oliveira Magalhães, 2010; van der Maarel *et al.*, 2002); (2) pectinases in the fruit juice industry for clarification reactions and higher yields (Alkorta *et al.*, 1998); (3) in the baking industry amylases and/or xylanases can be used to improve the dough stability, texture, volume and color, and may contribute to a prolonged freshness of breads (Markets-reports, 2014; Singh *et al.*, 2016). Feed digestibility has been shown to be improved when (hemi-)cellulose or pectin degrading enzymes, depending on the composition of the feed, are added to the feed (de Vries and Gerrits, 2018; de Vries *et al.*, 2012; Pustjens *et al.*, 2014; Torres-Pitarch *et al.*, 2019). Moreover, released pectic structures and formed non-digestible oligosaccharides are considered to contribute to the health of the animals due to either direct interactions with the gut tissue resulting in immunomodulating effects (Meijerink *et al.*, 2018; Sahasrabudhe *et al.*, 2018) or due to their growth stimulation effects of beneficial intestinal microflora (Akbari *et al.*, 2017; Tian *et al.*, 2017). The latter role has also been attributed to (human) milk oligosaccharides (Albrecht *et al.*, 2010), protecting babies and young animals in their first years and possibly beyond (Difilippo *et al.*, 2016). Currently, in infant milk formula, transglycosylating galactosidase produced galacto-oligosaccharides are added to take over the function of these milk

oligosaccharides that are yet too complex to produce at commercial scale (Alizadeh *et al.*, 2016; Difilippo *et al.*, 2016; Elwakiel *et al.*, 2018; Ladirat *et al.*, 2014a,b).

Biorefinery processes aim to upgrade agricultural biomass or food industrial side-streams, for example by their conversion into biofuels and biochemicals. Hereto, the polysaccharides need to be converted, mostly enzymatically, into (fermentable) glucose and other monosaccharides (Kabel *et al.*, 2017). In this chapter, it has been reviewed that such biomass streams are largely composed of cellulose and hemicellulosic xylan (i.e., bran, straw, stover), while other industrial by-products are richer in xyloglucan and pectin (i.e., sugar beet pulp, potato peel pulp). Their conversion requires not only a defined and well-combined set of, preferably synergetic, enzyme activities, but biorefinery processes will also benefit from robust (i.e., thermostable) enzyme cocktails (Haki and Rakshit, 2003).

To further boost the number of commercial applications of enzymes and to improve existing (fungal) enzyme cocktails, fungi and their enzymes represent an unlimited pool of possible candidates (Benoit *et al.*, 2015; de Vries *et al.*, 2017; van den Brink and de Vries, 2011). However, although many fungal genomes have been sequenced and annotated, and many genes seem to putatively encode carbohydrate active enzymes (Lombard *et al.*, 2014), only a relatively low number of candidates have been biochemically characterized. Future enzymatic applications, hence, depend on the push to characterize carbohydrate active enzymes, not only for their environmental activity and stability, but also for their exact mode-of-action on natural plant polysaccharides.

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Further Reading

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Production of Oligosaccharides by Fungi or Fungal Enzymes

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Introduction

Carbohydrates are classified according to their degree of polymerization (DP), which is the number of units composing the molecule. According to IUPAC classification, they can be divided in monosaccharides (DP 1), oligosaccharides (DP 2–9) and polysaccharides (DP$\geq$10) (McNaught, 1996).

Based on the physiological properties, carbohydrates can be classified as digestible or non-digestible. Normally, non-digestible oligosaccharides are classified as prebiotics, and according to the International Scientific Association for Probiotics and Prebiotics (ISAPP), a prebiotic is a substrate that is selectively utilized by host microorganisms to confer health benefits (Gibson *et al.*, 2017). They remain undigested in the upper gastrointestinal tract, i.e., they are resistant to hydrolysis by human saliva, gastric and pancreatic juices, and are not absorbed in the small intestine, reaching the colon where they are available for utilization by probiotics (Meyer *et al.*, 2015).

Fungi are especially attractive producers of enzymes for the production and commercialization of oligosaccharides. They are able to secrete the enzymes, which allows for greater stability and makes the production process significantly cheaper (Lange, 2017). Besides the use of free fungal enzymes, oligosaccharides can be produced directly by the mycelium or by immobilized fungal cell and enzymes (**Fig. 1**).

This chapter emphasizes the main types of oligosaccharides and their properties, natural sources, modes of production and potential industrial applications.

Fructooligosaccharides

Fructooligosaccharides (FOS) are oligomers of β(2-1)-linked D-fructose molecules with a maximum DP of 10. FOS can contain one molecule of glucose in their structure to form a linear chain linked to fructose (GFn) or only fructose (Fn) (Yildiz, 2011). GFn are mainly derived from the transfructosylation of sucrose while Fn are derived from inulin hydrolysis (Dominguez *et al.*, 2014). The smallest FOS is kestose (1-kestose, β-D-fructofuranosyl-(2$\rightarrow$1)-β-D-fructofuranosyl-(2$\rightarrow$1)-α-D-glucopyranoside). In addition to kestose, nystose (one glucose and three fructose molecules) and fructosyl nystose (one glucose and four fructose molecules) are the most common GFn molecules (**Fig. 2**). They are also referred as GF2, GF3, and GF4, respectively. Regarding Fn, it is denominated inulo(latin of polymerization degree)ose, such as inulotriose, inulotetraose, etc. (Yildiz, 2011).

The enzymes involved in FOS production are mainly invertases and inulinases. Invertase or β-fructofuranosidase (EC 3.2.1.26) catalyzes the hydrolysis of sucrose to produce an equimolar solution of fructose and glucose. In high sucrose concentrations, some β-fructofuranosidases can have transfructosylating activity, transferring fructose to sucrose, producing GFn. The β-fructofuranosidase with this specific activity is named fructosyltransferase (EC 2.4.1.9) (de Almeida *et al.*, 2018). Endoinulinases (EC 3.2.1.7) hydrolyze internal β-2,1 fructofuranosidic linkages of inulin releasing mainly inulotriose, inulotetraose and inulopentose as products (Singh *et al.*, 2020).

In industry, the most common process for FOS production is sucrose biotransformation by transfructosylation catalyzed by β-fructofuranosidases from the ascomycete fungal species *Aspergillus*, *Aureobasidium* or *Penicillium* (USDA, 2015; Sánchez-Martínez *et al.*, 2020). After enzymatic reaction, the products pass through downstream processes, including removal of reducing sugars (using the yeast *Pachysolen tannophilus*), decolorization and removal of organic impurities by activated carbon, clarification by microfiltration, purification using ion-exchange to remove color and ash components, and evaporation and concentration to yield FOS sirup. Concentrated FOS sirup is pasteurized and can be packaged as sirup or submitted to a spray drying process to be sold as a white powder (FDA, 2015; Soni and Tsai, 2016).

In 1984, Meiji Seika Co. in Japan was the first to succeed in the commercial production of FOS. They used invertase from *Aspergillus niger* to produce the products Neosugar® and Meioligo® (Yun, 1996; Dominguez *et al.*, 2014). Nowadays, several FOS are commercialized as prebiotics. Some are derived from sucrose, such as NutraFlora®, Actilight®, Profeed®, Oligo-sugar and Beneshine™P-type, while others are derived from inulin, like Oraft®, Fibrulose®, Frutalose® and olifructinesp® (Dominguez *et al.*, 2014).

Industrial processes can be performed via a batch system using a soluble enzyme, or a continuous process using immobilized enzyme or whole cells (Sánchez-Martínez *et al.*, 2020). Examples of fungi and fungal enzymes as well as their products are shown in **Table 1**.

The continuous process using immobilized enzymes or cells is an alternative to the use of free enzymes. Immobilized biocatalysts are more stable, can be removed from the reaction medium and be reused many times, reducing the final cost of the enzyme and enabling greater purity of the final product (Sheldon and van Pelt, 2013). Industrial use of immobilized enzymes for

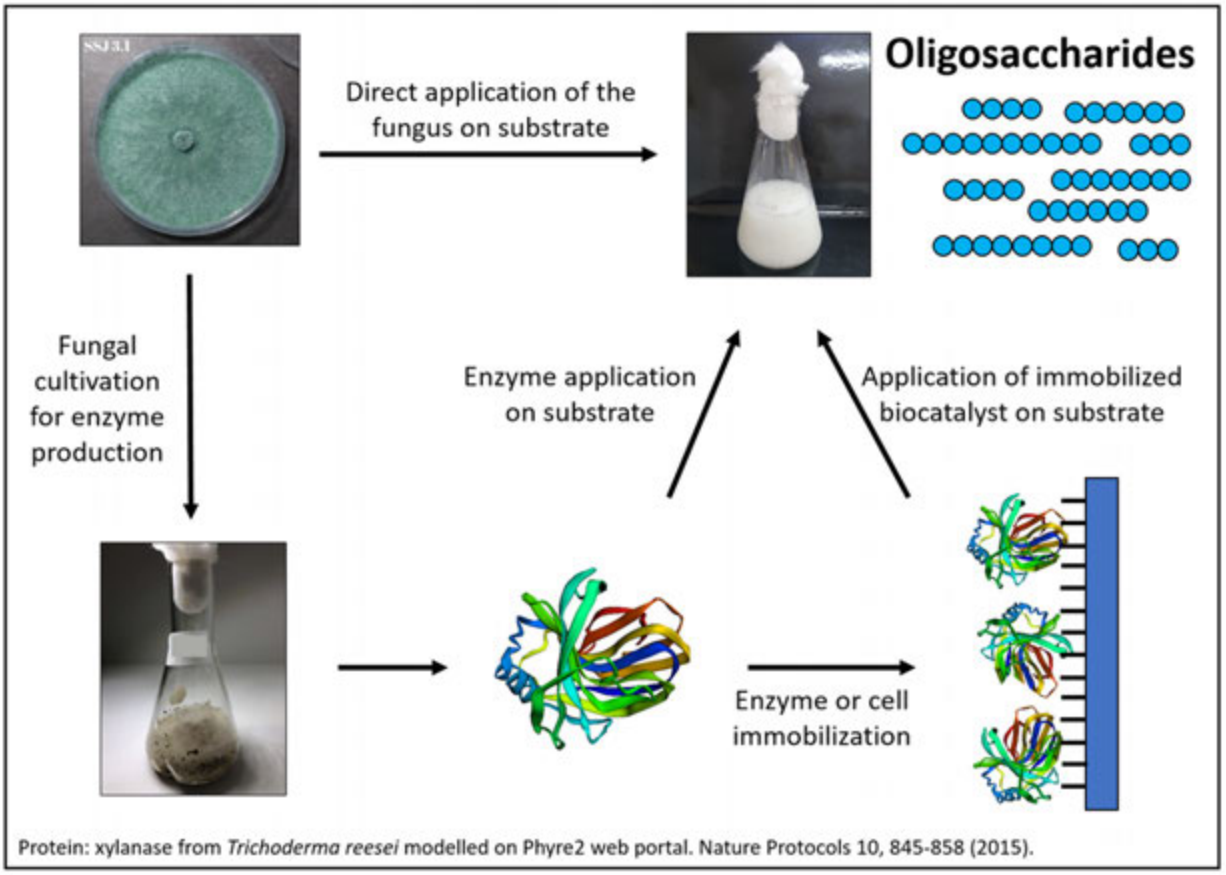

Fig. 1 Oligosaccharide production using fungi or fungal enzymes.

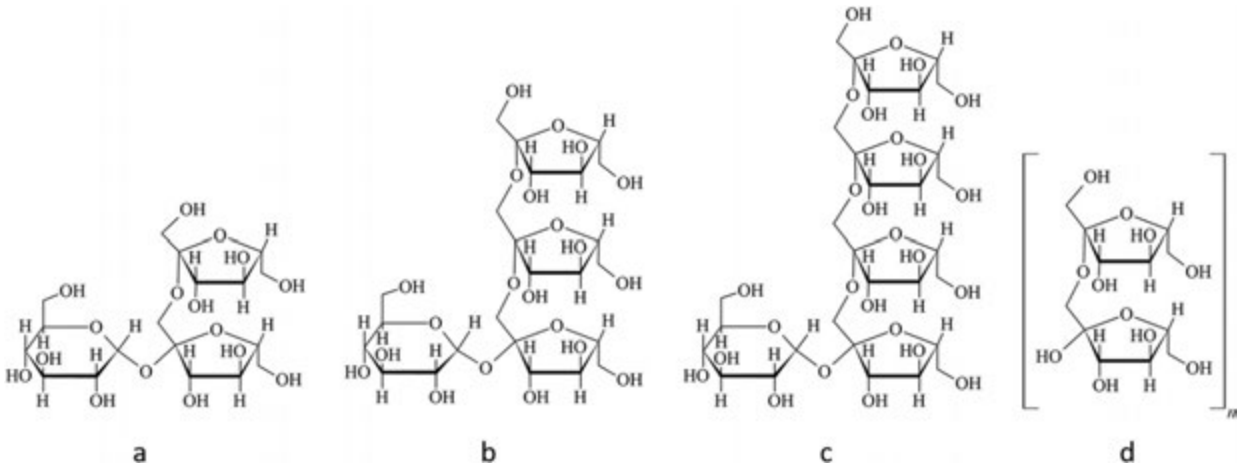

Fig. 2 Structures of kestose (a), nystose (b), fructosyl nystose (c) and the Fn-type fructooligosaccharide. $n \leq 5$.

FOS production has been reported using calcium alginate and polystyrene divinylbenzene ion exchange resin as a support (GRAS, 2017). An alternative is the use of mycelium with mycelia-bound enzymes. This approach minimizes activity loss during operation and allows for establishing a continuous process, as can be done with immobilized enzymes (Zambelli *et al.*, 2014).

The main application for FOS is in the food industry. They are low calorie and non-cariogenic sweeteners. As components of functional foods, FOS have "generally recognized as safe" (GRAS) status from the Food and Drug Administration (FDA) in the USA (Smith, 2002). FOS can also be used in bakery products, in acidophilus milk, creams, desserts, yogurt, ice cream, sauces and

Table 1 Production of fructooligosaccharides by fungal enzymes or mycelium

Enzyme	*Organism*	*Immobilization material*	*Substrate*	*Main product*	*Yield (%)*[a]	*Reference*
β-fructofuranosidase	*Aspergillus thermomutatus*	–	Sucrose 200 g/L	GF2, GF3	–	(Tódero *et al.*, 2019)
β-fructofuranosidase	*Aureobasidium melanogenum*	–	Sucrose 300 g/L	GF2, GF3, GF4	63	(Aung *et al.*, 2019)
β-fructofuranosidase	*Penicillium citrinum*	–	Sucrose 30% (w/v)	GF2, GF3	37	(Tashiro *et al.*, 2017)
Inulinase	*Aspergillus niger*	–	Sucrose 1.2 mol/L	GF3, GF2, GF4	60	(Karboune *et al.*, 2018)
Inulinase	*Aspergillus tritici*	–	Inulin 10% (w/v)	GF2, GF3, GF4	19	(Singh *et al.*, 2020)
Inulinase	*Penicillium citrinum*	–	Inulin 5% (w/v)	GF2, GF3	–	(Rawat *et al.*, 2015)
Mycelium	*Aspergillus tamarii*	–	Sucrose 50% (w/v)	GF2, GF3	55	(Choukade and Kango, 2019)
Mycelium	*Cladosporium cladosporioides*	–	Sucrose 600 g/L	GF2, GF3, GF4, 6-kestose, neokestose, blastose	56	(Zambelli *et al.*, 2014)
Mycelium	*Penicillium citreonigrum*	–	Sucrose 20% (w/v)	GF2, GF3	54	(Nobre *et al.*, 2019)
β-fructofuranosidase Pectinex® Ultra SP-L	*Aspergillus aculeatus*	Fe_3O_4–Chitosan-Magnetic Nanoparticles	Sucrose 700 g/L	GF2, GF3	14	(de Oliveira *et al.*, 2020)
β-fructofuranosidase	*Aspergillus japonicus*	Amberlite IRA 900	Sucrose 60% (w/w)	GF4, GF3, GF2	53	(Bedzo *et al.*, 2019)
Inulinase Fructozyme	*Aspergillus niger*	Polyurethane	Sucrose 15% (w/v)	GF3, GF4, GF2	31	(Kuhn *et al.*, 2016)
Inulinase Novozym 960	*Aspergillus niger*	Polyhydroxybutyrate (PHB) nanofibres and microfibers	Inulin 15% (w/w)	F3, F2, GF4, F4, GF3	–	(Beran *et al.*, 2016)
Immobilized cell	*Aspergillus aculeatus*	Calcium alginate	Sucrose 600 g/L	GF2, GF3, GF4	65	(Huang *et al.*, 2016)
Immobilized cell	*Penicillium expansum*	Synthetic fiber	Sucrose 20% (w/v)	GF2, GF3, GF4	60	(Mussatto *et al.*, 2012)
Immobilized cell	*Aureobasidium pullulans*	Calcium alginate	Sucrose 770 g/L	GF2, GF3, GF4	57	(Jung *et al.*, 2011)

[a]Yield refers to sucrose conversion considering total FOS production. G: glucose. F: Fructose.

several other food products. They can improve the organoleptic quality and provide a better-balanced nutritional composition (FDA, 2015; Sánchez-Martínez *et al.*, 2020).

FOS are considered prebiotics and their use in infant formulas is due to their positive effects on gastrointestinal function. They are reported to increase the fecal *Bifidobacterium* content to improve problems such as constipation in children that were not breast fed (Souza *et al.*, 2018). Several studies have related FOS consumption to decreased fasting blood glycaemia levels in rodents with a high-fat diet, obesity and diabetes (Le Bourgot *et al.*, 2018). FOS intake in humans has been reported to cause a significant reducing in postprandial glucose and insulin concentrations. Therefore, FOS is an interesting alternative for sugar in diets (Respondek *et al.*, 2014).

Xylooligosaccharides

Xylooligosaccharides (XOS) are sugar oligomers formed by xylose units bound via β-(1→4) linkages and they are called xylobiose (2 monomers), xylotriose (3 monomers), xylotetraose (4 monomers), xylopentose (5 monomers), xylohexose (6 monomers) and so on (**Fig. 3**) (Samanta *et al.*, 2015).

XOS are commonly found in fruits, vegetables, bamboo shoots, milk and honey and they can be commercially generated from hydrolysis of xylan (Antov and Đorđević, 2017; Mano *et al.*, 2018). Xylan is one type of hemicellulose that is the second most abundant polymer in nature and represents about 20%–40% of the dry weight of lignocellulosic materials (Malgas *et al.*, 2019). Hemicelluloses are hetero-polysaccharides composed of different sugars and are classified according to the main sugar residue of their backbone to xylans, xyloglucans, mannans, glucomannans and β-glucans (Scheller and Ulvskov, 2010).

Xylan is composed of a backbone of β-1,4-linked xylose units with diverse substituents that include different side chain carbohydrates and acids (Nordberg *et al.*, 2018). Therefore, xylan structures vary in degree of polymerization, monomeric units and types of linkages and the side groups result in branched XOS with diverse biological properties (Aachary and Prapulla, 2011; Samanta *et al.*, 2015). Commercial XOS have been produced from xylan derived from barley hulls, brewery spent grains, corn cob, corn stover, rice hulls, wheat bran, wheat straw, oat spelt and other plant biomass (Akpinar *et al.*, 2007; Jain *et al.*, 2015).

The production of XOS on an industrial scale from lignocellulosic materials rich in xylan can be performed by chemical procedures using steam, acid or alkaline solutions, enzymatic hydrolysis, or a combination of both methods (Akpinar *et al.*, 2007; Mussatto and Mancilha, 2007; Samanta *et al.*, 2015). Enzymatic degradation of xylan is more advantageous for XOS production

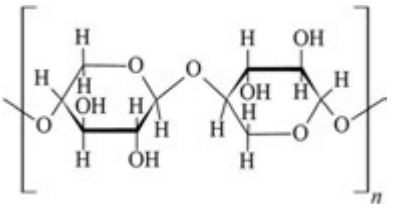

Fig. 3 Structure of xylooligosaccharides. n ≤ 5.

Table 2 Fungal endoxylanases applied for xylooligosaccharide (XOS) production

Fungus	*Substrate*	*Main XOS*[a]	*Reference*
Aspergillus flavus	beechwood xylan	X2 and X5	(Chen *et al.*, 2019)
Aspergillus luchuensis (=*A. foetidus*)	corncobs	X2, X3, X4 and X5	(Chapla *et al.*, 2012)
Trichoderma inhamatum	xylan from oat spelts	X2, X3, X4	(da Silva *et al.*, 2015)
Penicillium funiculosum	arabinoxylan from wheat	X4, X5 and X6	(Lafond *et al.*, 2011)
Talaromyces amestolkiae	birchwood xylan	X2 and X4	(Nieto-Domínguez *et al.*, 2017)

[a]X2, X3, X4, X5, X6 are referred to xylobiose, xylotriose, xylotetrose, xylopentose and xylohexose, respectively.

since enzymes show high specificity, no generation of undesirable byproducts and side reactions, no requirement of special equipment and facilitated recovery (Long *et al.*, 2018; Chen *et al.*, 2019; Wu *et al.*, 2019). Endoxylanases, β-xylosidases and some debranching enzymes are employed for XOS production (Jain *et al.*, 2015). However, XOS yields are dependent on the source of xylan, enzymatic activities and incubation conditions such as pH, temperature and hydrolysis time (Samanta *et al.*, 2015).

Moreover, for xylan degradation by enzymes, a pretreatment must be performed on the lignocellulosic material to allow for a better access of the enzymes to the xylan backbone (Samanta *et al.*, 2015; Long *et al.*, 2018). In this case, xylan is often extracted from lignocellulosic sources using alkaline solutions at a moderate temperature for two to three hours (Akpinar *et al.*, 2007; Linares-Pasten *et al.*, 2016). Extraction performed with hot water under high pressure is another relatively common method used before enzymatic hydrolysis (Immerzeel *et al.*, 2014).

Endoxylanases (EC 3.2.1.8) act on the xylan backbone, cleaving internal β-1,4-glycosidic linkages between xylopyranosyl residues and their products may include xylobiose, xylotriose, xylotetraose, and longer and/or branched XOS (Malgas *et al.*, 2019). β-Xylosidases (EC 3.2.1.37) are exo-acting enzymes that degrade short xylooligosaccharides, mainly to liberate xylose (Yan *et al.*, 2008). Therefore, the XOS produced by endoxylanases are further hydrolyzed into xylose by β-xylosidases and a endoxylanase without any β-xylosidase activity is desired for XOS production (Long *et al.*, 2018; Chen *et al.*, 2019). Some studies have reported enzymatic synthesis of XOS by the application of the transxylosylation activity of β-xylosidases (Aachary and Prapulla, 2011), i.e., the formation of larger xylooligosaccharides from small oligosaccharides by transfer reactions (Benassi *et al.*, 2013).

Most reports on the enzymatic production of XOS are related to fungal enzymes, especially endoxylanases. Considering an industrial scale, endoxylanases are mainly produced by *Aspergillus* sp. and *Trichoderma* sp., although *Penicillium* strains have been considered as good candidates to produce these enzymes (Pal and Khanum, 2011; Nieto-Domínguez *et al.*, 2017). **Table 2** shows some examples of fungal endoxylanases applied for XOS production.

The attention given to XOS production has increased since these compounds show potential as prebiotics and the recommended XOS dose is 0.12 g/kg of body weight in adults (Oku and Nakamura, 2002). XOS are associated with several health benefits. They are able to improve bowel function (Jain *et al.*, 2015; Samanta *et al.*, 2015; Antov and Đorđević, 2017) and absorption of minerals, especially calcium, by the intestine (Swennen *et al.*, 2006; Nieto-Domínguez *et al.*, 2017; Wu *et al.*, 2019). Furthermore, XOS are associated with minimizing the risks of colon cancer (Swennen *et al.*, 2006; Jain *et al.*, 2015) and the cytotoxic effect on human leukemia cells (Ando *et al.*, 2004). XOS are also related to regulation of insulin secretion from the pancreas (Samanta *et al.*, 2015) and positive effects on type II diabetes mellitus (Sheu *et al.*, 2008), as well as lowering cholesterol (Sheu *et al.*, 2008; Wu *et al.*, 2019). Moreover, they play an important role as antioxidants and in immune modulation (Nieto-Domínguez *et al.*, 2017; Wu *et al.*, 2019).

Chito-Oligosaccharides

Chito-oligosaccharides (COS) are sugars with a degree of polymerization less than 20 produced by partial hydrolysis of the polymers chitosan or chitin. Chitin is a polymer of N-acetyl-D-glucosamine (GlcNAc) linked by β,1–4 bonds and chitosan is the product of partial or complete deacetylation of chitin, composed of mixtures of D-glucosamine (GlcN) and GlcNAc (**Fig. 4**). COS can show different degrees of polymerization (DP), degrees of acetylation (DA) and pattern of acetylation (PA), especially depending on the method by which they were produced. These characteristics have a direct influence on their properties. COS with

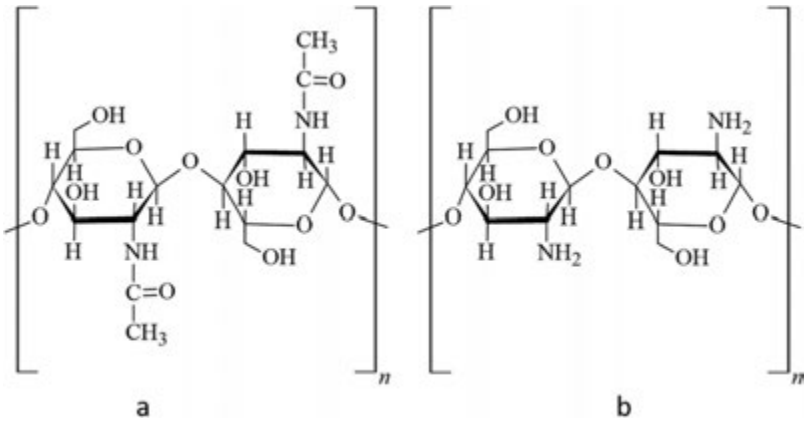

Fig. 4 Structures of chito-oligosaccharides derived from chitin (a) and chitosan (b). n ≤ 10.

DP less than 10 are generally soluble in water while for COS with DP greater than 10 it depends on the DA and the pH of the solution. The viscosity of COS decreases with lower molecular weight (Liaqat and Eltem, 2018; Aljbour *et al.*, 2019).

The most common source of chitin to produce COS are crustaceans. To produce chitosan, chitin is deacetylated in an alkaline solution or enzymatically by chitin deacetylase (3.5.1.41). Chitin deacetylase releases chitosan with specific patterns of acetylated (A) and deacetylated (D) saccharides (Naqvi *et al.*, 2016). The chitin deacetylase from the nematophagous fungus *Pochonia chlamydosporia* initially deacetylates pentamers at the penultimate GlcNAc residue from the non-reducing end (ADAAA), and then deacetylates the next residue producing di-deacetylated pentamer (ADDAA) (Aranda-Martinez *et al.*, 2018). The basidiomycete fungus *Coprinopsis cinerea* has been show to produce three chitin deacetylases (CDA1, CDA2, CDA3). The three enzymes have activities on chitobiose and CDA3 presented the highest activity against chitopentaose. CDA1 removed an acetyl group from the non-reducing end, CDA2 from the reducing end and CDA3 was able to act at either the reducing end or the nonreducing end (Bai *et al.*, 2020).

For enzymatic production of COS, chitinases are the most specific enzymes. Endochitinases (E.C. 3.2.1.14) randomly hydrolyze chitin within the chain to produce low molecular weight oligosaccharides (Kidibule *et al.*, 2018). The chitinase from the ascomycete *Trichoderma harzianum* hydrolyzed colloidal chitin and produced a fully acetylated series of COS (DP 1–4), mainly disaccharide. When chitosan was the substrate, the most abundant product was trisaccharide, presenting the acetylated residue at the reducing end (DDA) (Kidibule *et al.*, 2018).

Enzymes that preferably hydrolyze chitosan instead of chitin are named chitosanase (EC 3.2.1.132). Several fungi have been reported to produce chitosanase, including *Aspergillus fumigatus* (Zhou *et al.*, 2020), *Penicillium janthinellum* (Nguyen *et al.*, 2014), *Trichoderma koningii* (da Silva *et al.*, 2012) and *Fusarium solani* (Shimosaka *et al.*, 1993). These enzymes show differences with regards to their mode of action (endo or exo) and their specificity in degree of deacetylation of the chitosan. The enzyme from the ascomycete *Alternaria alternata* showed specificity for chitin hydrolysis, however the activity was even greater for partially deacetylated chitins. Due to this standardization of hydrolysis sites, the enzyme was denominated chitinosanase (Kohlhoff *et al.*, 2017).

Other non-specific enzymes like cellulases, lysozyme, protease and amylase were also effective for chitin depolymerization (Lv *et al.*, 2016; Öhlknecht *et al.*, 2017). Because commercial cellulases are more available than chitinases and chitosanases, COS are commonly produced using cellulases. Cellulases from *A. niger* (Xie *et al.*, 2010), *Trichoderma longibrachiatum* (Tegl *et al.*, 2016) and from *Trichoderma viride* (Wu and Tsai, 2004) showed to be effective for chitin depolymerization.

Production of COS from low molecular weight chitosan directly by fungi was reported for a strain of *Aspergillus* sp. This method avoided additional steps of enzyme extraction and purification. The fungus produced a heterogeneous oligomer mixture of COS with DP(s) of 2–9 with DA 16.67%–66.67% (Embaby *et al.*, 2018).

Chitin and/or chitosan can also be extracted from the cell wall of ascomycete, zygomycete, basidiomycete and deuteromycete fungi (Pochanavanich and Suntornsuk, 2002). This approach is an alternative to obtain COS from a non-animal source. Fungi are routinely used in industry to produce proteins, organic acids and secondary metabolites, and their wastes can be used for COS production. The mycelium of the zygomycete *Rhizopus oligosporus* was hydrolyzed by chitinases from the archaeon *Pyrococcus furiosus* and *T. viride* and a chitosanase from the bacterium *Streptomyces* sp. Digestion by chitosanase achieved 67% digestibility, while chitinases from *P. furiosus* and *T. viride* yielded 35% and 36%, respectively (Mahata *et al.*, 2014).

Among the biological applications attributed to COS, the antimicrobial effect obtained by fungal enzymes can be highlighted. Antimicrobial effects of COS have different results depending on COS molecular weight, degree of acetylation, concentration and mainly the target microorganism. COS produced by chitosanase from *Aspergillus fumigatus* effectively inhibited the growth of several phytopathogenic fungi, i.e., *Botrytis cinerea*, *Fusarium graminearum*, *Alternaria alternate*, *Magnaporthe grisea*, *Erysiphe cichoracearum* and *Alternaria solani*. The COS solution consisted on a mixture of disaccharides, trisaccharides and tetrasaccharides (Zhou *et al.*, 2020). Besides the antimicrobial effect, COS can also activate immune response to pathogens on plants, which opens the possibility for their use as a biocide in agriculture. In rice, the chitin-elicitor binding protein, the receptor that starts signal transduction and triggers immune response, specifically binds long-chain COS, such as heptamer-octamer (Hayafune *et al.*, 2014).

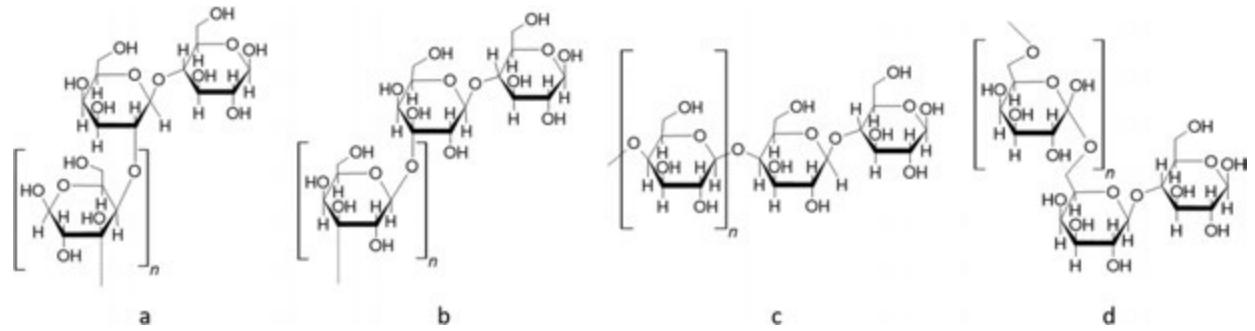

Fig. 5 Structures of galacto-oligosaccharides with β(1,2) (a), β(1,3) (b), β(1,4) (c) and β(1,6) (d) linkages. $1 \geq n \leq 6$.

In contact with *Staphylococcus aureus*, COS presenting DP > 12 caused clustering and later lysis of the cells (Li *et al.*, 2014). When the inhibitory effect of COS on the bacteria *Escherichia coli* (gram negative) and *Listeria monocytogenes* (gram positive) was compared, *E. coli* was more sensitive (Sánchez *et al.*, 2017).

Galacto-Oligosaccharides

Galacto-oligosaccharides (GOS) are soluble sugars derived from the conversion of lactose by β-galactosidases (Canfora *et al.*, 2017). β-D-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), most commonly known as lactase, catalyzes the hydrolysis of lactose $\beta(1 \rightarrow 4)$ linkages to produce glucose and galactose. This enzyme also shows transgalactosylation activity, which means the ability to transfer the galactose from lactose cleavage to a galactose from another lactose, yielding GOS (Park and Oh, 2010). GOS are short-chain carbohydrates formed by two to seven molecules of galactose and one terminal glucose molecule (**Fig. 5**) (Otieno, 2010; Torres *et al.*, 2010). The final products show several types of glycosidic linkages such as β(1,2), β(1,3) β(1,4) and β(1,6), and branched glucose residues may occur (Macfarlane *et al.*, 2008; Otieno, 2010). GOS naturally occurs in mammalian milk, especially in human milk, and to a smaller extent in cow's milk (Alander *et al.*, 2001).

β-Galactosidases are one of the most useful enzymes in the dairy industry to hydrolyze lactose to produce lactose-free milk products, in addition to GOS production (Kim *et al.*, 2004a,b). GOS can also be produced by chemical synthesis, but enzymatic synthesis is preferred (Otieno, 2010). Dairy products such as cow's milk, but mainly whey-derived lactose and pure lactose solutions, are the most used for GOS production by β-galactosidases (Macfarlane *et al.*, 2008; Otieno, 2010).

Because β-galactosidases are inhibited by the products galactose and glucose, the major difficulty for GOS production is the complete hydrolysis of lactose (Kim *et al.*, 2004a,b). The transgalactosylation reaction for GOS production normally occurs at high lactose concentration (Hsu *et al.*, 2007), and when GOS are produced glucose inhibition is more influential because galactoses are consumed in the process. Therefore, β-galactosidases that show low inhibition by glucose are more desirable for GOS production (Kim *et al.*, 2004a,b; Park and Oh, 2010). Moreover, an increase in GOS production is observed when β-galactosidases are mixed with glucose oxidases, which remove glucose as a byproduct (Hsu *et al.*, 2007; Park and Oh, 2010).

GOS are one of the most common oligosaccharides used as food ingredients (Tokošová *et al.*, 2015). The GOS production yield depends on lactose concentration, but also the reaction temperature, enzyme source and activity and the product length (Otieno, 2010; Meyer *et al.*, 2015). Furthermore, different microorganisms yield different oligosaccharide compositions in terms of degree of polymerization (Otieno, 2010; Tokošová *et al.*, 2015).

β-Galactosidases produced by fungi are interesting because of their extracellular character, higher thermal tolerance and lower pH-interval of activity (Gargova *et al.*, 1995). An extracellular β-galactosidase from *Aspergillus lacticoffeatus* was able to produce GOS when either whole cells or the crude enzyme was used as a biocatalyst (Cardoso *et al.*, 2017). When several fungal species for β-galactosidase activity and GOS production were tested, the ascomycetes *Aspergillus brasiliensis*, *Aspergillus restrictus*, *Aspergillus uvarum*, *Penicillium brevicompactum*, *Penicillium italic* and *Penicillium spinulosum*, the zygomycete *Mucor* sp. and the basidiomycete *Trametes versicolor* were shown to be able to produce GOS (Silvério *et al.*, 2018). An immobilized β-galactosidase from the ascomycete fungus *Talaromyces thermophilus* showed the ability to produce GOS with a maximum yield of 34% when the degree of lactose conversion was approximately 80% (Nakkharat and Haltrich, 2006). The same β-galactosidase from *T. thermophilus* showed high GOS production with high lactose concentrations and at high temperature (Nakkharat *et al.*, 2006). Thermostable β-galactosidases are associated with high transgalactosylation yields (Park and Oh, 2010).

The increased attention given to GOS is related to their prebiotic activities, with potential application in the food industry (Torres *et al.*, 2010; Silvério *et al.*, 2018), but also for the cosmetic and pharmaceutical industries (Krutmann, 2009) and the feed and pet food industries (Torres *et al.*, 2010). They are associated with improving defecation and ammonia elimination, colon cancer prevention, mineral adsorption stimulation, and lowing cholesterol and lipids (Swennen *et al.*, 2006; Park and Oh, 2010). Compared to other non-digestible sugars, GOS show low cariogenicity, low caloric values and low sweetness, as well as better physicochemical characteristics, including stability in low pH and at high-temperature conditions (Otieno, 2010). GOS have a

GRAS status in the United States, a non-Novel Food status in the EU, and they are recognized as foods for specific health use (FOSHU) in Japan (Torres *et al.*, 2010). Infant milk formulas and specific food for elderly or hospitalized people are good options for GOS inclusion (Alander *et al.*, 2001; Otieno, 2010). It is reported that the intake of 10 g per day of GOS is considered sufficient to cause a bifidogenic effect, and the establishment of a *Bifidus* microflora in the intestine of breast-fed infants is related to the presence of GOS in human milk (Mussatto and Mancilha, 2007). GOS have been commercialized by some companies such as Yakult Honsha and Nissin Sugar Manufacturing from Japan, Corn Products Intl. from the United States, and Borculo Domo Ingredients and Clasado from Europe, and in addition to the differences in purity of GOS, there are also differences in the types of linkages present in these oligosaccharides (Torres *et al.*, 2010).

Final Considerations

Interest in oligosaccharides has increased over the last years mainly due to their use as ingredients in functional foods. Maintenance of a balanced intestinal flora has been related to the beneficial effects as anti-obesity and anti-cancer. In this context, the use of prebiotics alone or in combination with probiotics is of great interest to the health field. In addition, several effects such as antioxidant, anti-inflammatory and antimicrobial are correlated to oligosaccharides. The biological effect has been shown to depend on the oligosaccharide structure. Carbohydrate analysis techniques such as high performance liquid chromatography, high performance anion exchange chromatography, mass spectrometry and infrared spectroscopy have provided a more accurate analysis of structures and a better understanding of the correlation between structure and biological effects. The search for new fungi able to produce oligosaccharides is always an important alternative, since more stable and specific enzymes can be found. Recombinant DNA technologies for the improvement or overexpression of enzymes are also important approaches for obtaining enzymes. These innovations, as well as the improvement of bioreactors and enzymatic immobilization techniques, are increasing the efficiency and viability of industrial oligosaccharide use.

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Metabolic Modeling of Fungi

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Introduction

Fungi have become one of the most important microorganisms in biotechnology. The biotechnological sector use fungi as cell factories to produce food and beverages (Dupont *et al.*, 2017), organic acids (Sahasrabudhe and Sankpal, 2001), enzymes (Sharma *et al.*, 2017a,b; Walia *et al.*, 2017) and bioactive compounds (Keller *et al.*, 2005; Kuck *et al.*, 2014). In spite of the interest, their full metabolic potential is still unknown (Kjærbølling *et al.*, 2019). Metabolism can be defined as the complete set of chemical reactions that occur in living organisms in order to maintain life, *i.e.*, a network of interconnected and interdependent chemical reactions where a metabolite is transformed through a series of intermediates reactions into another metabolite that can be used by the cell. Genome-scale metabolic models (GEMs) are used as *in silico* representation of *in vivo* metabolic reactions. GEMs allow researchers to determine the characteristics of an organism and understand, to some extent, what it is capable of doing based on its genetic information (Kitano, 2002). The first GEM model was created for the bacterium *Haemophilus influenzae* shortly after the first genome was published (Edwards and Palsson, 1999). The first GEM of the ascomycete yeast *Saccharomyces cerevisiae* was published in 2003 (Förster *et al.*, 2003b), while the development of filamentous fungal metabolic networks was delayed until the publication of their genomes. In 2008, the first GEMs of filamentous fungi, *Aspergillus niger* (Andersen *et al.*, 2008), *Aspergillus nidulans* (David *et al.*, 2008) and *Aspergillus oryzae* (Vongsangnak *et al.*, 2008) were published. From then on, the number of GEMs has increased (Brandl and Andersen, 2015), but not as fast as genomes have been sequenced (Leitch *et al.*, 2018).

On average, a typical fungal GEM contains 1138 unique metabolic reactions and 1050 ORFs (Brandl and Andersen, 2015), however, on average, fungal genomes contain more than 10,000 genes (see "Relevant Websites section"). Due to the large amount of data to consider, a full analysis of a metabolic network is only possible using computational approaches (Vlassis *et al.*, 2014). The reconstruction process starts constructing a network. Based on their functional annotation, a metabolic function is assigned automatically to each gene in the genome, after which a curator reviews the features of each reaction and the genome annotation. Only then, the network is transformed into a matrix, constraints are applied and the resulting GEM is tested. GEMs are constantly changing and curators are learning from the new conditions tested. Simulated results are compared against experimental data to improve the accuracy of the GEM (Kim *et al.*, 2012). The final GEM is released in the standardized SBML format (Systems Biology Markup Language format) (Hucka *et al.*, 2003).

GEMs have countless applications, among them, metabolic engineering, hypothesis-driven discovery, studying multi-species relationships, network property discovery or exploring omics data (Oberhardt *et al.*, 2009). In particular, fungal GEMs have been mainly used for exploring omics data and *in silico* strain engineering (Brandl and Andersen, 2015) **Fig 1**.

In the following sections, we review metabolic modeling reconstruction, analysis and future perspectives. We present an overview of the process to reconstruct a network with special attention for manual curation. We describe different methods to analyze a model and the integration of omics data in the analysis. We show how GEMs have been applied successfully to predict the behavior of different yeast and filamentous fungi. Finally, we discuss advances to construct GEMs for all sequenced genomes in an automatic manner at a faster rate and improving the quality.

Reconstruction of a Genome-Scale Network

The goal of the genome-scale network reconstruction (GENRE) is to include all the enzymes encoded in the genome (Ruppin *et al.*, 2010). GENREs are built up in a bottom-up manner starting with the annotation data of the genes and the associated enzymatic reactions (Cottret *et al.*, 2018). Gene-protein-reaction (GPR) associations determine the set of metabolic reactions encoded in the genome.

In the past, metabolic networks were step-by-step defined based on the reactions identified *in vitro* (Kornberg and Madsen, 1958) (Elzainy *et al.*, 1973; Kuninaka, 1957). Cellular metabolism is more complex and flexible than we often assume (Klein *et al.*, 2017; Koivistoinen *et al.*, 2012; Mojzita *et al.*, 2012). The current methodology used to generate a reliable metabolic network starts with an automatically generated draft based on the sequence similarities between gene annotation and metabolic databases, followed by an extensive manual curation of reactions and GPR mapping done by experts (Hartleb *et al.*, 2018). The process repeats itself until biomass production is achieved. Network reconstruction is a dynamic process in which the experts learn from the simulations and inconsistencies often result in changes in the networks and regular updates. For example, this is illustrated by the publication in 2019 of Yeast8 (Lu *et al.*, 2019), an improved version of the original *S. cerevisiae* metabolic network published in 2003 (Förster *et al.*, 2003b).

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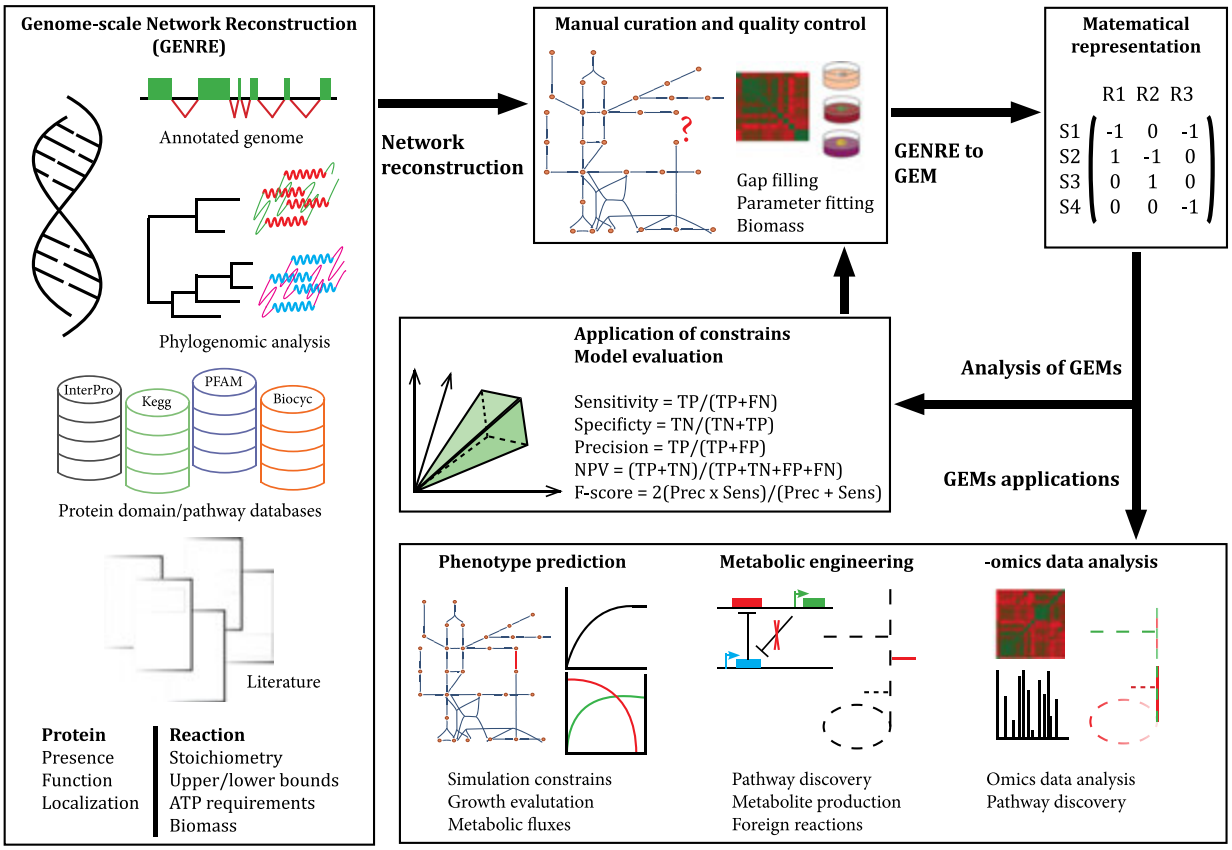

Fig. 1 Overview of network reconstruction process and uses of fungal GEMs.

To expedite the GENRE generation process, automatic and semi-automatic algorithms have been developed. In-depth analysis and comparison of current software programs can be found at (Faria *et al.*, 2018; Mendoza *et al.*, 2019). Although the available tools use different algorithms to reconstruct a network, the process follows a similar design: draft reconstruction, manual curation and biomass production. Nevertheless, additional data might be required and the order of the steps described below could be altered (for a more comprehensive explanation on network reconstruction refer to (Thiele and Palsson, 2010), steps 1–37).

Automated Draft Reconstruction

A draft version of the network is constructed based on similarity searches between the sequences found in the genome annotation and metabolic databases. The genome annotation is the keystone to build GPR associations and it defines the gene properties in which the draft reconstruction and curation relies. Even though reannotation of associated genes might occur during the reconstruction of the network, the quality of the annotation should be considered beforehand and, if necessary, improved accordingly (Chain *et al.*, 2009). McDonnell *et al.* (2018) described the step-by-step methodology followed for the curation of the *A. niger* NRRL 3 genome, the only manually curated fungal genome (Vesth *et al.*, 2018).

Algorithms use genome annotation key words like gene ontology (GO) categories (Ashburner *et al.*, 2000; The Gene Ontology Consortium, 2019) or enzyme commission (EC) numbers (NC-IUBMB and webb, 1992) and the information in metabolic databases, automatically annotated and/or manually curated, such as KEGG (Ogata *et al.*, 1999), BioCYC (Karp *et al.*, 2019) or BRENDA (Schomburg *et al.*, 2002) (for an extended list referred to (Blazeck and Alper, 2010)) to associate enzymes and metabolic reactions. Published models can also be used as source of reactions (Aite *et al.*, 2018; Henry *et al.*, 2010; Machado *et al.*, 2018; Olivier, 2018; Wang *et al.*, 2018). The metabolic reactions in the GENRE are described using metabolites and stoichiometry information provided from databases. The first draft generated automatically based on sequence similarity may include a high fraction of inaccurate reactions and GPR associations. In spite of the latest developments, manual curation of the draft is needed to obtain a more accurate network.

Manual Curation of the Draft

In order to easily and efficiently assess the large amount of information included in the draft reconstruction, curators use a confidence score system. This confidence score can be quantitative or qualitative. The minimum information included

should indicate at least if there is no available evidence, evidence for gene function (gene annotation), indirect evidence for biochemical reaction (presence of metabolites, transporters), direct and indirect evidence for gene function (knockout or overexpression experiments) or direct evidence for enzyme function and biochemical reaction (protein structure solved experimentally, biochemical assays) and a reference to the source.

According to the software used to generate the draft, different automatic rules apply to decide if an instance (either reaction or GPR association) should be included into a draft. Independent of the criteria used by each software to add a particular element, curators must decide for each instance whether to keep it or not based on literature, comparative analysis and available experimental data. If an instance is kept, support information and a score need to be added accordingly (Samal *et al.*, 2017). The downside of this method is that only known reactions are identified and species-specific reactions which are not in the databases will not be included. However, the bright side is that reactions already identified on related species are included.

The curation process starts by deciding if a pathway and its reactions are likely to be present in the metabolic network and agree with the organism under study. For example, *A. niger* and *A. nidulans* (Khosravi *et al.*, 2015), shared the D-galactose oxidoreductive pathway with the exception of one reaction for which a gene can be assigned in both species. As a result, the algorithm adds both pathways to the network. Based on literature and experimental data the curator should remove the pathway not supported by the data while adding the information to the correct pathway. In addition, for each reaction, substrates, cofactors, stoichiometry, directionality, localization and enzyme activators and inhibitors as well as the identifiers need to be confirmed and assigned a confidence score. Metabolite identifiers can be obtained from different databases such as ChEBI (Hastings *et al.*, 2013), KEGG (Ogata *et al.*, 1999), PubChem (Kim *et al.*, 2018) or database-independent identifier such as SMILES (Weininger, 1988) or InCHI identifiers (Heller *et al.*, 2013). Known spontaneous reaction should be added only if there is at least one metabolite connecting them to the network.

Once a reaction has been validated, predicted GPR associations need to be confirmed. Unfortunately, due to the lack of experimental data, after curation most of the GPR associations are based on sequence similarity or motif databases such as InterPro (Mitchell *et al.*, 2018) or PFAM (El-Gebali *et al.*, 2018) (included in the gene annotation) and/or to characterized proteins in other species. Eventually, expression and proteomic data can improve the annotation. Other problem that curators face when assigning GPR associations is assigning protein complex (multiple genes – one protein), isozymes (multiple proteins – one function) and promiscuous enzymes (one protein – multiple functions) (Machado *et al.*, 2016), as well as localization of each gene. In addition, in fungi, one of the biggest challenges when assigning GPR associations is defining transporters. Experimental characterization of transporters is a big challenge (Diderich *et al.*, 1999; Forment *et al.*, 2006; Forment *et al.*, 2014; Jørgensen *et al.*, 2007; Kruckeberg *et al.*, 1999; Sloothaak *et al.*, 2015) and *in silico* analysis have shown the complexity of the transport systems (Peng *et al.*, 2018; Sloothaak *et al.*, 2015).

Once GPR associations have been curated and pathways have been set up, the gaps in the network can be identified. A gap is a missing connection between two metabolites, either the reaction or the enzyme catalyzing the reaction is unknown, and usually it prevents the formation of biomass or known metabolic capabilities in the metabolic reconstruction such as secretion of known metabolic products. An algorithm that automatically include reactions to fill these gaps is called a gap-filling algorithm, and very often, reconstruction tools include gap-filling algorithms as part of their pipeline but not all of them. Pan and Read (2018) described and compared the latest gap-filling algorithms. Different strategies such as revising genome annotation, expression data, database information and comparative genomics are used as input data for gap-filling algorithms. These approaches were used during the generation of genome-scale metabolic network for *A. oryzae* (Vongsangnak *et al.*, 2008), *A. nidulans* (David *et al.*, 2008), *A. niger* (Andersen *et al.*, 2008; Brandl *et al.*, 2018; Lu *et al.*, 2017), *Trichoderma reesei* (Castillo *et al.*, 2016) and *Penicillium chrysogenum* (Agren *et al.*, 2013).

Biomass Formation and Energy Requirements

The final reaction in the metabolic network is the biomass reaction, a generic term for growth. This reaction is not connected to an enzyme and is the sum of many biochemical reactions needed to synthesize known biomass constituents (protein, RNA, DNA, lipids and carbohydrates) and their contribution to the overall cellular biomass. Therefore, the reaction is tailor made for each organism (Agren *et al.*, 2013; Andersen *et al.*, 2008; Castillo *et al.*, 2016). Different methods based on experimental data and omics data have been generated to calculated the biomass composition (Chan *et al.*, 2017; Gonzalez *et al.*, 2010; Santos and Rocha, 2016).

An additional element needs to be added to the biomass reaction to consider growth associated maintenance: energy drain in the form of ATP. ATP acts as a cofactor in many reactions in which stoichiometric coefficients are known. However, ATP is also required during the assembly of polymers or maintenance of gradients, processes resulting in growth (growth associated maintenance), as well as non-growth associated maintenance, which is the ATP required to stay alive. In both cases, stoichiometric coefficients are unknown. Besides, stoichiometry of ATP production through oxidative phosphorylation also needs to be determined (Baart and Martens, 2012). Unknown ATP data can be determined experimentally as described by Van Gulik (2010).

Nutrients are needed to grow a microorganism *in vivo*. In a metabolic model the nutrients are defined by the metabolites encoded in the media formulation and their maximum or minimum uptake are represented with constraints on the metabolic network. The *in silico* media composition determines the metabolites that can be transported inside the cell and those that need to be produced by the network. Changes in the media composition affect biomass reaction and therefore the expected biomass yield. The media composition is important when modeling, especially when studying gene deletion. If a metabolite is required for growth but the enzyme catalyzing the reaction is not present, no growth would be predicted unless the metabolite is supplied in the media. Therefore we can conclude that the gene encoding the enzyme is essential for the network. However, if biomass production is positive, we could predict several scenarios such as a metabolite is not essential for growth, it can be

produced by an alternative pathway or there is more than one enzyme associated to the reaction. For engineered strains, whether is activating or increasing the yield of a pathway, introducing a new pathway from a different species, flux-balance, accumulation of secondary metabolites and/or production of alternative products can be predicted as well (Brandl *et al.*, 2018; Castillo *et al.*, 2016; Pitkänen *et al.*, 2014).

Approaches to Analyze Genome-Scale Metabolic Reconstructions

The process of metabolic refinement ends, according to the protocol for generating high-quality genome-scale metabolic reconstructions (Thiele and Palsson, 2010), with a network able to generate biomass. This reconstruction can be transformed into a GEM, *i.e.*, a mathematical structure in the form of a stoichiometric matrix S, where each row represents a metabolite, columns, metabolic reactions and entries of which represent stoichiometric coefficients. A common approach to model a GEM is Flux Balance Analysis (FBA) (Orth *et al.*, 2010). FBA is a mathematical formulation that allows one to find flux distributions while maximizing an objective function and satisfying mass balance constraints. In mathematical terms,

$$\begin{aligned} &Max\ \ Z \\ &Subject\ \ to\ \ S \times v = 0 \\ &v_l \leq v_i \leq v_u \forall i = 1 \ldots n \end{aligned} \tag{1}$$

where Z is the objective function, very often the biomass reaction, which represents the specific growth rate [h^{-1}] if the flux values v_i represent specific fluxes, in [mmol h^{-1} gDW^{-1}]; v_i is the flux through reaction i, v_l and v_u are the lower and upper bounds for that reaction, respectively, n is the number of reactions of the reconstruction and S is the stoichiometric matrix. FBA returns the maximal value of Z, which is unique, and a corresponding set of fluxes through the network. The latter may not be unique (see later).

Generally, immediately after the reconstruction, FBA can be used to test the accuracy of the GEM predictions. Some widely used strategies to validate a GEM include

(1) Single gene deletion analysis: each gene of the network is knocked-out, reactions depending on the gene are removed from the network and growth is predicted. This obviously requires a set of deletion strains to compare the predictions against – such as for *S. cerevisiae*.
(2) Qualitative prediction of growth on different carbon sources or media conditions.

In both cases, predictions are compared with experimental data and accuracy scores are calculated:

Sensitivity	$TP/(TP+FN)$
Specificity	$TN/(TN+FP)$
Precision (PPV)	$TP/(TP+FP)$
Negative predicted value (NPV)	$TN/(TN+FN)$
Accuracy	$(TP+TN)/(TP+TN+FP+FN)$
F-score	$2(precision \times sensitivity)/(precision + sensitivity)$

In addition, a quantitative prediction of growth on different carbon sources or media conditions can be performed. In this case, specific growth rates are calculated on different scenarios and compared with experimental values. These types of analyses provide insight into the metabolic potential of the organism.

Other Commonly Used Approaches to Study GEMs

Dynamic flux balance analysis (dFBA)

dFBA (Mahadevan *et al.*, 2002; Varma and Palsson, 1994) is an extension of FBA and it calculates metabolite concentrations in time, so batch or feed-batch cultures can be simulated. There are two approaches for dFBA: Static Optimization Approach (SOA) (Varma and Palsson, 1994) and Dynamic Optimization Approach (DOA) (Mahadevan *et al.*, 2002). The first approach, which is implemented in the COBRA toolbox, involves the definition of time intervals and solving instantaneous linear problems at the beginning of each interval, followed by the integration over the interval. In contrast, the second approach involves optimization over the entire time period, solving just once a non-linear problem. The main advantage of the first approach is that it scales well with size as it only involves linear problems, while the advantage of the second approach is that it provides increased flexibility in the definition of constraints and objective function. For example, DOA allows the formulation of a dynamic objective function.

Flux variability analysis (FVA)

In this analysis (Gudmundsson and Thiele, 2010; Mahadevan and Schilling, 2003), each reaction of the network is maximized and then minimized while keeping the specific growth rate at optimal value. A span is calculated for each reaction such that modelers are given the feasible space for each solution, for example to study the uniqueness of an optimal FBA-based flux distribution. FVA

is also useful when the network is constrained with flux data collected during experiments and the corresponding feasible flux space is computed with FVA (Wiback *et al.*, 2004) (this is genome-scale variant of metabolic flux analysis).

Reduced cost analysis

It is often useful to identify which reaction rate constraints limit the objective function. Reduced costs are sensitivity coefficients that indicates how much the objective function would change if the flux though a particular reaction is allowed to change (Palsson, 2015). Thus, these coefficients reflect how the objective function is affected by each reaction bound. Reduced costs can be scaled to the actual flux through the reaction, to not only account for stoichiometry but also the importance (in terms of flux) of a particular reaction (Teusink *et al.*, 2006).

Integration of transcriptomics

Integration of transcriptomics data into GEMs has been widely applied since 2004 (Åkesson *et al.*, 2004). The simplest way it can be achieved, is by assuming that if a particular gene is not expressed, then all the reactions that depend on that gene will not carry any flux. By integrating transcriptomics data into a GEM, a condition-specific (or generally best known as context-specific) subnetwork can be obtained. Simulations ran in this context-specific subnetwork will result in improved predictions when compared with a generic model (Åkesson *et al.*, 2004), mainly because the space of solutions becomes smaller and therefore, more specific and realistic. Several (more sophisticated) algorithms have been reviewed and tested in the past years to integrate transcriptomic data (Machado and Herrgard, 2014; Opdam *et al.*, 2017). However, in many occasions, algorithms are highly dependent on user-defined parameters (Opdam *et al.*, 2017; Richelle *et al.*, 2019a) and additional steps should be done prior to the algorithms application to obtain a better performance (Richelle *et al.*, 2019b).

Integration of proteomics

Since in general, mRNA expression levels cannot be directly correlated with protein concentrations (Lahtvee *et al.*, 2017), proteomics measurements are a more reliable source of information than transcriptomics. Different approaches to integrate proteomics measurements into GEMs have been developed. On a first approach, GEMs can be extended to also account for macromolecular gene expression, also known as Metabolism and Expression (ME) models (Lerman *et al.*, 2012; Thiele *et al.*, 2012). In these models, the mechanism of macromolecular formation is explicitly modeled and proteomic data can be easily integrated (Yang *et al.*, 2016). However, the use of ME models has been challenging because of the lack of specific software but also because they combine linear and non-linear optimization and the simulation time considerably increases (Yang *et al.*, 2018). A different approach is Enzyme-constrained models (Sanchez *et al.*, 2017). In this second approach, each reaction is constrained based on the assumption that it cannot carry more flux that V_{max}. In mathematical terms

$$v_i < V_{max} = k_{cat}[E] \tag{2}$$

In addition, from proteomic data, the total amount of protein can be obtained. Another constraint can be formulated such that the sum of all protein abundances has to be no greater than the total amount of protein in the cell. Thus:

$$\sum_{i}^{n} [E_i] < C, \quad \textit{with } n\textit{: The number of enzymes, and } C\textit{: the total amount to protein}$$

These additional constraints have two advantages: (i) they allow for more realistic behavior of the models, such as overflow metabolism, and the corresponding identification of trade-offs, and (ii) it renders the flux distribution solutions unique. We believe this is the way forward in the field, but it does require estimates of catalytic rates.

Integration of metabolomics

Although we model metabolic networks with GEMs, integration of metabolomics data is not straightforward. The most basic use of metabolomics data is incorporating uptake and secretion rates from exometabolomics. Metabolite concentrations of sugars and metabolic products are measures in chemostats in the feed and waste. Differences in metabolite concentrations can be converted to specific rates and incorporated into genome-scale models as constraints (Mo *et al.*, 2009). More advanced methods also allow incorporation of intracellular metabolite concentrations as constraints on directionality of reactions, using thermodynamic arguments (Fleming and Thiele, 2011; Salvy *et al.*, 2019), or as input to simulate metabolite concentrations over time at genome-scale (Bordbar *et al.*, 2017; Kleessen *et al.*, 2015; Willemsen *et al.*, 2015).

Multi-omics integration

Integration of different kind of metabolic data has resulted in novel metabolic insights such as discovery of mechanisms underlying protein translation or metabolic rewiring of disease states (Cho *et al.*, 2019; Ebrahim *et al.*, 2016), yet algorithms that integrate more than one omics are scarce. Recently, algorithms called TREM-Flux (Kleessen *et al.*, 2015) and REMI (Pandey *et al.*, 2019) have been developed to integrate transcriptomics and metabolomics data into genome-scale models, resulting in enhanced predictions of metabolic fluxes in comparison with the integration of just one type of data.

Metabolic engineering

GEMs can be used to explore modifications leading to higher production rates of certain compounds of industrial interest. For that purpose, several algorithms have been developed (Castillo *et al.*, 2019; Landon *et al.*, 2019; Maranas and Zomorrodi, 2016). In the simplest case, knock-out combinations are explored to redirect flux from a by-product to another (Burgard *et al.*, 2003; Patil *et al.*, 2005). In more sophisticated approaches, amplification targets can be detected (Choi *et al.*, 2010) or foreign reactions can be incorporated into the metabolic networks (Pharkya *et al.*, 2004) or knock-outs could also be combined with downregulations (decreased fluxes) and upregulations (increased fluxes) to generate strategies to create improved strains (Pharkya and Maranas, 2006; Ranganathan *et al.*, 2010). Recent efforts have been focused on integration of metabolic and regulatory networks to make predictions more accurate (Shen *et al.*, 2019; Xu, 2018).

Applications of Fungal Models

With the release of the genome sequence of *S. cerevisiae* (Cherry *et al.*, 1997; Goffeau *et al.*, 1996), the first genome-scale model of a eukaryote microorganism was built (Förster *et al.*, 2003b). Due to the conserved functions between humans and *S. cerevisiae* (Nielsen, 2019), this yeast has been subject of extensive research and several updates for the reconstruction of *S. cerevisiae* have been performed (Sanchez and Nielsen, 2015). However, GEMs for several other species of yeasts (Lopes and Rocha, 2017) and filamentous fungi (Brandl and Andersen, 2015) have also been reconstructed and they have been successfully applied in several biotechnological areas. Overall, yeasts and fungal models have been widely applied in predictions of phenotypes and to guide metabolic engineering (Castillo *et al.*, 2019; Nielsen, 2019).

Prediction of Phenotypes

GEMs have been able to accurately predict different types of experimental data. The first applications of these models were to predict, using FBA, flux distributions, growth rates, uptake rates of nutrients or secretion rates of products under different conditions. For example, the first GEM of *S. cerevisiae* (Förster *et al.*, 2003b), iFF708 was used to predict glucose uptake, production of ethanol, glycerol, succinate and CO_2 in different anaerobic glucose-limited continuous cultures with varying dilution rates (Famili *et al.*, 2003). By constraining the model with the corresponding specific growth rates (equivalent to the dilution rate in each condition), experimental uptake and secretion rates were accurately predicted. However, adding more flux constraints will indeed improve the correspondence of the model to data, but prediction more and more could result in overfitting, something to be aware of. The same model was used to predict cell viability under 599 different single gene deletions (Förster *et al.*, 2003a). This large-scale study showed a remarkable accuracy of 87.8% highlighting the model as a predictive tool useful for large screenings.

Later models have focused on improving the quality of the reconstruction, improving its predictions for gene essentiality and integrating different kind of omics such as transcriptomics, metabolomics, fluxomics, proteomics and lipidomics. For example, metabolomics data has been integrated with a GEM of *S. cerevisiae* to find reactions that are affected by genetic (gene deletions) or environmental (oxygen) perturbations (Mo *et al.*, 2009), which may shed light on affected metabolic processes or pathways. More recently, time-resolved metabolomics has been integrated with dynamic flux balance analysis to study the mechanisms underlying metabolic changes in *S. cerevisiae* (Bordbar *et al.*, 2017; Willemsen *et al.*, 2015). Altogether, these studies allowed to understand cellular adaptation of *S. cerevisiae* to environmental and genetic changes.

In spite of the advances in the predictions made with the model of *S. cerevisiae* in terms of gene essentiality and growth measurements, the Crabtree effect remains a challenge for genome-scale metabolic models to predict. Under aerobic conditions, and above a critical specific growth rate of approximately 0.3 (1/h), the metabolism of *S. cerevisiae* changes from respiration to fermentation, even though this is energetically inefficient. Genome-scale metabolic models only take this yield (stoichiometry) into account, and therefore will always predict respiration as the optimal strategy. It is not well understood why this phenomenon occurs but a protein limitation has been suggested as the main cause. Genome-scale models usually do not consider protein limitations but recently, an extended model that include constraints with regard to protein abundances, also known as enzyme-constrained model, has been developed and was able to accurately predict uptake and secretion rates at dilution rates above 0.3 h^{-1} (Sanchez *et al.*, 2017), supporting the hypothesis that the Crabtree effect is the result of protein limitation. Other approaches such as integration of thermodynamics have also resulted in enhanced metabolic predictions in different conditions (Niebel *et al.*, 2019) and it was recently shown that any additional constraints on a genome-scale metabolic network can lead to switching behavior; the challenge is to use the right experimental data to identify the relevant constraints (De Groot *et al.*, 2019).

A. niger GEM was first published in 2008 by (Andersen *et al.*, 2008). Using *i*MA873 model, they were able to predict yields of different products, oxalic acid, citric acid and gluconic acid as well as the glucoamylase enzyme. According to the authors, over-prediction of the model might have been explained by the fact that the model predicted a theoretical maximum of a conversion of 100% of carbon source. The updated model *i*HL1210 included approximately 300 new genes and 400 reactions (Lu *et al.*, 2017). *i*HL1210 was used to predict growth under 69 carbon and 30 nitrogen sources. According to the results, the accuracy of the GEM was higher on 57 carbon and 22 nitrogen sources compared to the previous model. The latest *A. niger* model *i*JB1325 was tested against 471 different conditions including carbon and nitrogen sources and gene deletions. Of the 98 test that failed, 76% were due to an unknown metabolic pathway (Brandl *et al.*, 2018). During the reconstruction of the

original model and updates, different kind of omics and comparative analysis were used to improve the annotation and identification of new pathways. Using comparative genomics and protein database information Brand *et al.* were able to identify the genes that allowed to predict growth on L-histidine as single N-source in combination with D-galactose. Furthermore, *adaE*, a new transporter in the *ada* cluster involved in secondary metabolism (Li *et al.*, 2011), was identified through co-expression analysis of individual pathways (Brandl *et al.*, 2018). Another example of comparative genomics and omics data combined with a metabolic network.

Metabolic Engineering

GEMs of Baker's yeast have been successfully applied to generate strains with improved secretion rates of compounds of industrial interest. For example, a GEM of *S. cerevisiae* was used as a tool to explore different strategies to increase the secretion rate of ethanol (Bro *et al.*, 2006). The model predicted that the flux to glycerol could be redirected to ethanol by expressing the heterologous gene *gapN*, which would indirectly provide the NAD^+ that is usually obtained through the generation of glycerol. This strategy was experimentally validated showing that genome-scale models can be used to produce improved strains. The development of more advanced algorithms for strain design and the refinement of the reconstruction of *S. cerevisiae* have allowed to successfully generate strategies leading to an increased production of several other important compounds, such as 2,3-butanediol (Ng *et al.*, 2012), 3-hydroxypropionic acid (Borodina *et al.*, 2015), amorphadiene (Sun *et al.*, 2014), patchoulol (Gruchattka and Kayser, 2015), sesquiterpene (Asadollahi *et al.*, 2009), succinic acid (Otero *et al.*, 2013), triacetic acid lactone (Cardenas and Da Silva, 2014), tyrosine (Cautha *et al.*, 2013) and vanillin (Brochado *et al.*, 2010).

In spite of the widespread use of the *S. cerevisiae* GEMs, other species of yeast which benefit from a GEM have also been used for metabolic engineering (Castillo *et al.*, 2019; Lopes and Rocha, 2017; Simeonidis and Price, 2015). *Pichia pastoris* has been successfully used as a platform for heterologous protein production (Ahmad *et al.*, 2014). A GEM of *P. pastoris*, PpaMBEL1254 (Sohn *et al.*, 2010), was used to generate hypothesis to increase the production of human superoxide dismutase (hSOD) (Nocon *et al.*, 2014). By generating strains with the proposed genetic modifications, the hSOD yield could be increased 20% in relation with the control strain. Other species of yeasts such as *Candida glabrata* (Chen *et al.*, 2013; Li *et al.*, 2014a,b), *Candida tropicalis* (Mishra *et al.*, 2016), *Aureobasidium pullulans* (Feng *et al.*, 2018), *Scheffersomyces stipitis* (Acevedo *et al.*, 2017), *Yarrowia lipolytica* (Huang *et al.*, 2018; Kim *et al.*, 2019; Mishra *et al.*, 2018) and *Rhodosporidium toruloides* (Castañeda *et al.*, 2019) have also benefit from this *in silico* approach.

Although to a lesser extent than yeasts, filamentous fungi have also benefit from *in silico* metabolic engineering algorithms to find gene targets to overproduce certain compounds. A metabolic network for *A. niger* predicted that deletion of two genes, *pdc* and *acl*, would lead to an increased production of succinic acid (David *et al.*, 2003), which was later experimentally validated (Meijer *et al.*, 2009). Other successful examples of uses of GEMs in metabolic engineering for filamentous fungi can be found for *Ashbya gossypii* (Ledesma-Amaro *et al.*, 2015a,b; Serrano-Amatriain *et al.*, 2016). Although GEMs for several other species of filamentous fungi exist (Agren *et al.*, 2013; Castillo *et al.*, 2016; David *et al.*, 2008; Dreyfuss *et al.*, 2013; Liu *et al.*, 2013; Pitkänen *et al.*, 2014; Samal *et al.*, 2017; Vongsangnak *et al.*, 2008, 2013, 2016; Ye *et al.*, 2015), they have not yet been used for metabolic engineering or their predictions to increase product formation remain to be tested, probably because of delay in the development of efficient tools for genetic manipulation in comparison with *S. cerevisiae*.

Future of Metabolic Modeling of Fungi

The availability of genomes of fungal organisms has increased dramatically due to advances in sequencing technology. Genome sequencing has increased in throughput and decreased in cost, which has enabled the advent of large-scale sequencing projects. The Fungal Genomics Initiative led by the Broad Institute have created a resource of around 50 fungal genomes (Cuomo and Birren, 2010). To broaden the diversity of sequenced fungal genomes, the Joint Genome Institute (JGI) initiated the 1000 fungal genome (1FKG) project and have produced hundreds of fungal reference genomes that cover the entire fungal tree of life (see "Relevant Websites section") (Martin *et al.*, 2011). On completion of the 1FKG project, there will be at least one reference genome for every known family of fungi. The vast genomic resources now available for diverse fungi will provide the basis for more exploration, including in the modeling and analysis of fungal metabolic models (Grigoriev *et al.*, 2014).

High-quality fungal GEMs are limited to model organisms, for which experimental data exists for manual curation. With the recent increase in the number of sequenced fungal genomes, there is a growing demand for new bioinformatics tools for GEM building and analysis. The introduction of new automated tools that facilitate the rapid generation of models has made GEMs much more accessible to the research community studying a range of fungal organisms. One such resource is KBase (see "Relevant Websites section"), which provides a platform for tools to perform metabolic modeling of fungi and integrated data analyses (Arkin *et al.*, 2018). For example, a user can construct a metabolic model from an input genome annotation based on a curated reference set of reactions that are derived from published fungal GEMs. First, orthologous proteins are identified to detect the presence of protein families. Then, reactions are propagated from the reference set based on presence of the protein family in the genome annotation to the metabolic model. The automated output is a draft model that can be further refined

manually based on literature and experimental data or using gap-filling tools also available in KBase. Other automated tools, such as Pathway Tools (Karp *et al.*, 2016), CoReCo (Pitkänen *et al.*, 2014), and RAVEN (Wang *et al.*, 2018), also offer fast generation of fungal GEMs.

Automated modeling enables large-scale analysis of GEMs of fungi within the same framework, which offers the advantage of side-by-side comparisons. These comparisons can help identify global trends in metabolic traits across fungal families (Pitkänen *et al.*, 2014) and contribute to comparative genomics analyses. However, a major challenge for these methods is the lack of accuracy for species that have greater divergence from the current reference species. High-quality GEMs still require manual curation to obtain an accurate representation. Yet, as tools continue to be improved and new ones are developed, the time to obtain informative and predictive models will be substantially decreased, and the availability of fungal GEMs will increase.

With advances in high-throughput experiments, metabolic modeling is benefitting from the integration of experimental data from a variety of genome-scale approaches. Integration of gene regulation has been applied towards metabolic modeling (Osterlund *et al.*, 2013), but improvement is still needed to accurately reflect complex forms of regulation, like post-translational modifications. Recently, bioinformatics methods are using metabolomics datasets to mine reaction databases for predicting new gene annotations and creating better GEMs (Erbilgin *et al.*, 2019). Lastly, the rise of gene editing technologies will facilitate validation and improvement of GEMs through the testing of predictions (Idnurm and Meyer, 2018). The next steps are to apply functional genomics methods more broadly to new organisms to identify the functions of unknown genes. Characterization of new genes and pathways will help us better model biology that has been previously unknown.

Conclusions

GEMs are a valuable tool to understand the intricacies of an organism's metabolism. Despite the large number of sequenced fungal species, their use is not as extensive as in other fields. The high number of species in the kingdom, lack of previous knowledge to improve manual curation, availability of experimental data for validation of the model together with the time and human resources needed to reconstruct a high-quality genome are among the reasons. As new methodologies develop to integrate omics data and comparative genomics while decreasing time and improving the quality, new metabolic models will appear to understand the most challenging and newest species with biotechnological and even medical value.

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Production of Organic Acids by Fungi

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Introduction

Low molecular weight carboxylic acids are intermediates of the primary metabolism of almost all organisms. Some of them are also commodity (bulk) products and manufactured on a very large scale to satisfy various global markets. In fact, after antibiotics and amino acids, organic acids represent the third largest category of bulk chemicals manufactured by microbial fermentation (Anastassiadis and Morgunov, 2008). Their carboxylic group(s) is/are only partially dissociated in neutral aqueous solutions, which makes them a weak acid, optimal for food applications such as flavors and preservatives. Carboxylic acids can also function as potential bio-based building blocks for polymer production and suitable platform chemicals in green chemistry. Recently, over 300 chemicals that can be produced from biomass and have the potential to be transformed into new type of useful molecules were analyzed by the U.S. Department of Energy to select the most lucrative ones. Of the top twelve such compounds, eight are organic acids, underlining the importance of this group (Werpy and Petersen, 2004). However, to successfully compete with petroleum-based chemical technologies, economic efficiency of the bio-based organic acid production processes must improve.

Why Do Fungi Accumulate Organic Acids?

The reasons why fungi have developed the ability to accumulate and excrete organic acids in high amounts is not sufficiently understood. In contrast to other fungal biotechnological products such as secondary metabolites or enzymes, prolific organic acid accumulation is not restricted to carefully selected mutants, but even wild-type strains can do it under special cultivation conditions. These are: a high concentration ($>10\%$, w v^{-1}) of a rapidly metabolizable carbon source (usually D-glucose), a high rate of aeration, and a suboptimal concentration of nitrogen and phosphorus to limit biomass formation (= growth). Obviously, these are conditions a fungus rarely encounters in its natural habitat, where it mostly feeds on polysaccharides and proteins. It has been discussed that the purpose of the extracellular accumulation of organic acids is to decrease the pH of the environment in cases of presence of excessive amounts of a carbon source in order to prevent its utilization by other competing micro-organisms (such as bacteria and yeasts), and at the same time transform the carbon source to one that is difficult for them to utilize (Blatzer and Latgé, 2017). Organic acid formation has also been interpreted as a mechanism to chelate trace metal ions, thereby facilitating their transport into the cell, but while they may indeed play such a role in at least some cases (Guerinot *et al.*, 1990; Odoni *et al.*, 2017b), this would clearly not necessitate their accumulation in such high amounts.

Biochemical Aspects of Organic Acid Accumulation by Fungi

The organic acids accumulated by fungi can be distinguished into four types: (a) monocarboxylic acids; (b) dicarboxylic acids; (c) tricarboxylic acids; and (d) sugar acids. As illustrated in **Fig. 1**, lactic acid is the only monocarboxylic acid produced by some fungi in significant amounts that is a definitive end-product of glycolysis. Gluconic acid biosynthesis is an extracellular process and its uptake competes with glucose transport. The di- and tricarboxylic acids, however, can be considered as products of incomplete aerobic D-glucose catabolism. This incomplete catabolism (a–c) has been coined by Foster (1949) as "overflow metabolism", because it is best observed in the presence of high concentrations of D-glucose. One may argue that fungi – usually feeding on polymers – are not well equipped with regulatory mechanisms to handle the metabolism of high monosaccharide concentrations. However, the analysis of metabolic control points in fungi has documented that they operate in a similar way as in other organisms. Rather certain nutritional imbalances (low nitrogen and phosphate, limitations in inorganic ions) are responsible for the overflow metabolism (Kubicek and Röhr, 1985).

Fungi are unique in other metabolic properties though: they possess a pyruvate carboxylase that is located in the cytosol (Osmani and Scrutton, 1985; Bercovitz *et al.*, 1990; Jaklitsch *et al.*, 1991), and which can utilize the glycolytically formed pyruvate to form oxaloacetate. In addition, there are cytosolically located isoenzymes of NAD-dependent malate dehydrogenase and (in Mucorales) fumarate hydratase, which together form a "reductive tricarboxylic acid (rTCA) branch" (**Fig. 1**). Since the pathway consumes 1 ATP per pyruvate (in the pyruvate carboxylase reaction) and oxidizes the NADH formed during glycolysis, one may consider it a non-energy generating pathway for D-glucose catabolism. Three of the dicarboxylic acids overproduced by fungi (oxalic acid, malic acid, fumaric acid) are intermediates of the rTCA branch. However, succinic acid and trans-epoxysuccinic acid – although only one and two enzymatic steps, respectively, away from fumarate – are not produced by the rTCA branch because the

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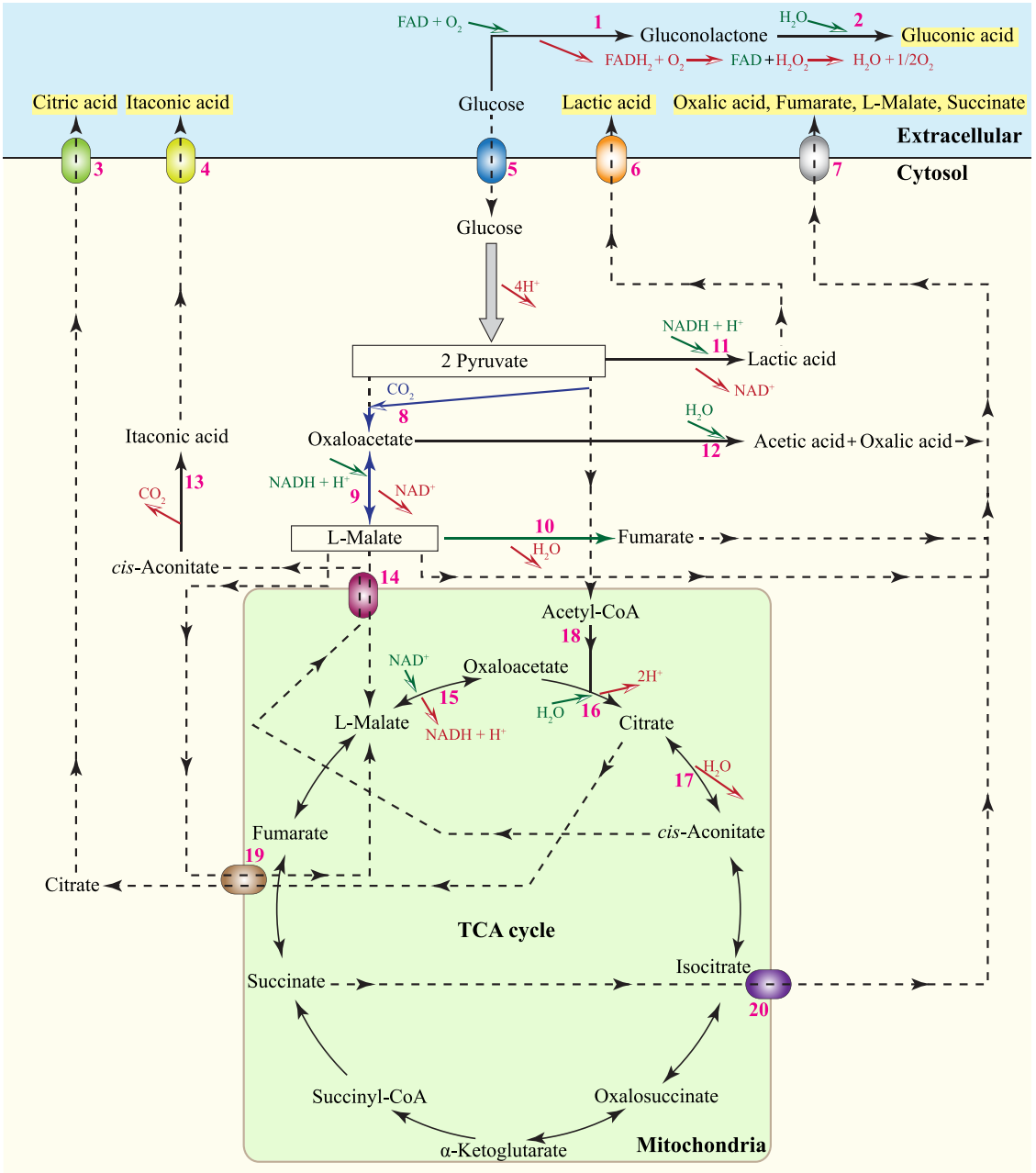

Fig. 1 Simplified biochemical pathways for organic acid synthesis in fungi. Each number indicates an enzymatic reaction catalyzed by the following enzymes: (1) glucose oxidase, (2) gluconolactonase, (3) citrate permease, (4) itaconate permease, (5) hexose permease, (6) monocarboxylic acid permease, (7) dicarboxylic acid permease, (8) pyruvate carboxylase, (9) cytosolic NAD-specific malate dehydrogenase, (10) cytosolic fumarase, (11) lactate dehydrogenase, (12) oxaloacetate hydrolase, (13) *cis*-aconitate decarboxylase, (14) mitochondrial malate/*cis*-aconitate antiport, (15) mitochondrial malate dehydrogenase, (16) citrate synthase, (17) aconitase, (18) pyruvate dehydrogenase complex, (19) mitochondrial citrate/malate antiport.

enzyme catalysing the conversion of fumarate to succinate – fumarate reductase – has not been detected in filamentous fungi yet (Yang *et al.*, 2016a). Succinate can therefore be formed only via the TCA cycle.

The major question regarding the operation of the rTCA branch is the origin of the CO_2 used by pyruvate carboxylase. During D-glucose catabolism via glycolysis, no carbon dioxide is released. Catabolic pathways that would generate CO_2 are the pentose phosphate pathway and the TCA cycle. However, in both cases CO_2 production would cost one carbon by pyruvate formed and decrease the yield by a third. Consequently, a high yield of dicarboxylic acid from glucose can only be obtained by adding extra carbon dioxide (either as $CaCO_3$ or as CO_2). As illustrated below, this principle has indeed been used in the fermentative

production of malate and fumarate. If added in the same molarity as D-glucose, the theoretical yield would then be 2 moles dicarboxylic acid from 1 mol of D-glucose.

The pyruvate carboxylase is also the key for the accumulation of the tricarboxylic acids, because the formation of citrate occurs by the citrate synthase catalyzed condensation of oxaloacetate and acetyl-CoA (Cleland and Johnson, 1954). The latter arises by oxidative decarboxylation and the released CO_2 is used as a substrate for pyruvate carboxylase. Since the biosynthesis of these two acids involves mitochondrial enzymes, export from and import into the mitochondria are another critical steps in their accumulation (Torres, 1994a,b).

The accumulation of the two tricarboxylic acids shares two additional peculiarities that are absent from the mono- and dicarboxylic acids: first, the stoichiometry of citrate (end-product of citric acid and intermediate in itaconic acid production) formation from D-glucose is the following:

$$C_6H_{12}O_6 + 0.5\ O_2 \rightarrow C_6H_8O_7 + 4\ H^+$$

The equation indicates that every mole of hexose needs an additional half mole of oxygen to get converted to citric acid. This 2 to 1 ratio is remarkable because oxygen is about 40–60,000 times less soluble in water than D-glucose (under normal conditions, the oxygen solubility is 2.56×10^{-4} mol L^{-1}, i.e., ~ 8 mg L^{-1}), and emphasizes the critical role that dissolved oxygen (and its continuous, high-rate replenishment) plays in citric and itaconic acid fermentation. It further implies that the fungus must contain a powerful system to reoxidize the NADH formed in glycolysis without ATP formation. Since fungal growth is practically at a standstill in the acid production stage, no biosynthetic demand for ATP exists, and thus high-rate carbon catabolism would result in high energy charge (= increased ATP levels). ATP is mostly generated during mitochondrial terminal oxidation, and would allosterically inhibit the glycolytic pathway. However, the electron transport chain of fungal mitochondria contains another terminal oxidase, the cyanide-resistant alternative oxidase (AOX) that uncouples mitochondrial NADH regeneration and ATP synthesis, allowing carbon catabolism and subsequent citric and itaconic acid formation to proceed unabated (**Fig. 2**). Indeed, alternative oxidase has been shown to play a crucial role in the production of both tricarboxylic acids (Kubicek *et al.*, 1980; Molnár *et al.*, 2018). This enzyme is resistant to the inhibitors of the cytochrome oxidase such as cyanide or azide and sensitive to salicylhydroxamic acid. Most importantly, alternative respiration is associated with proton translocation only at Complex I, whereas the other proton-pumping sites are bypassed. Activation of the alternative oxidase therefore significantly reduces ATP generation and, in effect, ATP inhibition over carbon catabolism (Kirimura *et al.*, 2000). However, some of the ATP generated via the standard respiratory chain will still be needed for maintaining pH homeostasis, because of the formation of 4 protons per mole glucose consumed.

Secondly, both tricarboxylic acid fermentations are extremely sensitive to the presence of trace quantities of Mn^{2+} ions, which usually have to be $< 10^{-7}$ M (Kisser *et al.*, 1980). The reason for this requirement is still not sufficiently understood. One aspect could be the fact that these limiting concentrations of Mn^{2+} ions induce a very compact morphology and small pellet size that strongly improves mass and oxygen transfer (Papagianni and Mattey, 2006; Karaffa *et al.*, 2015; Sun *et al.*, 2018). This hypothesis has, however, not yet been tested in reverse. In *Aspergillus terreus*, manganese deficiency has also been shown to trigger the expression of the alternative oxidase (vide supra). This could be due to an impairment of superoxide dismutase (a manganese-requiring enzyme), which would increase the oxidative stress, a condition known to induce alternative oxidase (Molnár *et al.*, 2018). However, this hypothesis still requires experimental verification. A deficiency in Mn^{2+} ions has also been demonstrated to lead to changes in metabolite concentrations that favor the activities of two of the regulatory steps in glycolysis (6-phosphofructokinase and 6-phosphofructo-2-kinase, which are inhibited by citrate and itaconate), but clear evidence is also lacking (Habison *et al.*, 1983; Arts *et al.*, 1987; Tevž *et al.*, 2010; Huang *et al.*, 2014). The requirement for a fermentation medium with only extremely low

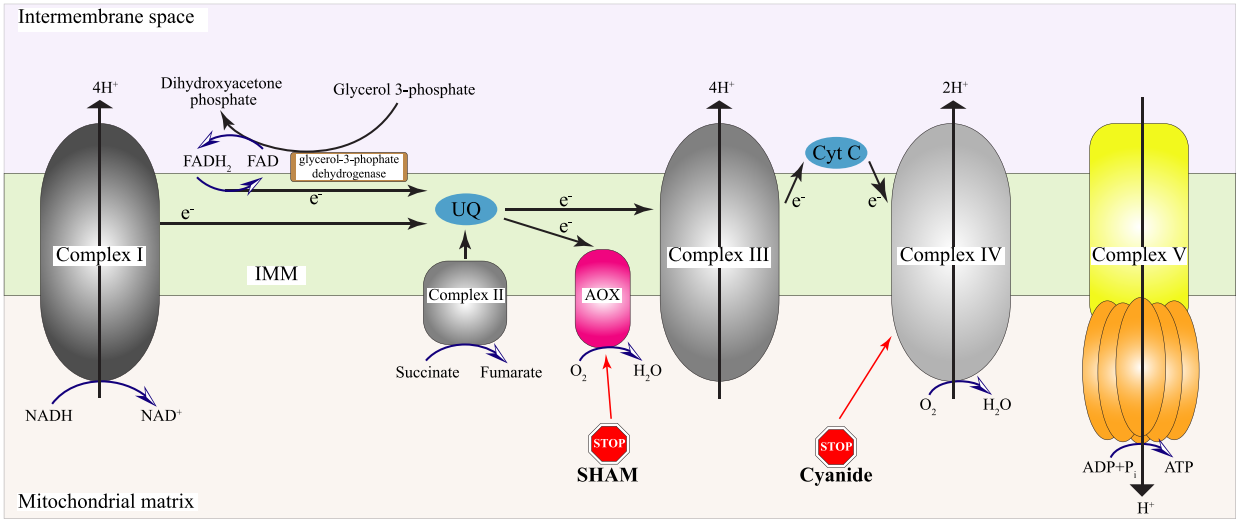

Fig. 2 Schematic overview of the mitochondrial respiratory pathways including the cyanide-resistant alternative oxidase (AOX).

concentrations of Mn^{2+} ions looks like an obstacle for production of these two acids. However, its removal by precipitation (with hexacyanoferrate), antagonization (with copper ions or lower alcohols) or addition of compounds affecting membrane permeability (quaternary ammonium salts) is well-practiced standard for over 50 years (for review see Kubicek and Karaffa, 2010). Alternatively, the recent identification of the manganese transporter of *A. niger* (DmtA), whose knock-out results in Mn^{2+} -insensitive production of citric acid (Fejes *et al.*, 2020), offers new strategies to solve this problem.

Description of the Production of Organic Acid

Below we describe the details and state of the art of the biochemistry and production of the most important fungal organic acids.

Monocarboxylic Acids

L-lactic acid

Lactic acid is a colorless, odorless monocarboxylic acid that is broadly used as an excipient in the food, cosmetics, pharmaceutical, and chemical industries (Vijayakumar *et al.*, 2008; Miller *et al.*, 2011). The L-form also serves as a building block for poly-lactic acid (PLA), a biodegradable polymer that could be set to replace petroleum-based plastics. Industrially, lactic acid can be manufactured by either through chemical synthesis or by microbial fermentation. Nowadays, the latter heavily dominates (80%–90%; Endres and Siebert-Raths, 2009). Lactic acid production traditionally uses lactic acid bacteria (*Lactobacillus* spp.) with yields approaching the theoretical maximum of 100% (w w^{-1}) of homolactic fermentation (= production of two moles of lactate from one mole of glucose, as opposed to heterolactic fermentation that produces one mole of lactate from one mole of glucose, as well as carbon dioxide and acetic acid/ethanol). A disadvantage of this process is the requirement for complex nutrients and the need to keep the pH >6 during fermentation (Hofvendahl and Hahn-Hägerdal, 2000). Therefore, lactic acid production processes have been developed with yeasts of the genera *Saccharomyces* (Colombié *et al.*, 2003; Skory, 2003b; Ishida *et al.*, 2005; Saitoh *et al.*, 2005; Valli *et al.*, 2006), *Kluyveromyces* (Bianchi *et al.*, 2001), and *Candida* expressing heterologous lactate dehydrogenase (*ldh*) genes (Osawa *et al.*, 2009; Ilmén *et al.*, 2013; Koivuranta *et al.*, 2014). This yeast-based strategy has reached industrial application and allows lactic acid production on pretreated corn starch, sugarcane or sugar beet (Miller *et al.*, 2011).

Filamentous fungi would offer the possibility of using lignocellulosic carbohydrates for production of lactic acid. Until today the only fungus known to accumulate large concentrations of lactic acid is *Rhizopus oryzae*, whose ability has been demonstrated already more than 100 years ago (Saito, 1911). Production from low-cost carbon sources such as raw starch, wheat straw, and cellulose has been reported (Tay and Yang, 2002; Park *et al.*, 2004; Vially *et al.*, 2010). Already Ward *et al.* (1938) described a fermentation process utilizing *R. oryzae*, which resulted in 63%–69% yields of L-lactic acid from chemically defined media containing 15% (w v^{-1}) glucose. The fermentation with *R. oryzae* offers several advantages over the bacterial or yeast processes: (a) a chemically defined medium with inorganic nitrogen sources can be used which simplifies product purification; (b) the fungus can metabolize high concentrations of glucose, resulting in high product concentrations; (c) enantiomerically pure L-lactic acid, necessary for food applications and preferred for PLA (poly-lactic acid, or polylactide) manufacturing can be produced. The disadvantage of *R. oryzae* is the requirement of a near-neutral pH, which – as in the *Lactobacilli* (vide supra) – compromises cost efficacy, and the by-production of ethanol, L-malic acid and fumarate (Soccol *et al.*, 1994; Magnuson and Lasure, 2004; Zhang *et al.*, 2007; Mudaliyar and Kulkarni, 2012).

The maximal theoretical yield for lactic acid production by aerobic respiration is 2 moles per mole of D-glucose, which equals a yield (g g^{-1}) of 1.0. Highest reported yields so far were 0.88 g g^{-1} (Zhou *et al.*, 1999). However, as noted above, L-lactic acid production in *R. oryzae* is accompanied by the formation of ethanol and malic acid (Meussen *et al.*, 2012). The accumulation of these by-products implies that there is a competition between lactate dehydrogenase, pyruvate carboxylase, and pyruvate decarboxylase for pyruvate, their common substrate. This has recently been quantified by determining the respective control coefficients for these enzymes (Ke *et al.*, 2015). The obligatory addition of calcium carbonate for pH maintenance helps to decrease ethanol formation, but the carbonate anion is used by the pyruvate carboxylase for oxaloacetate (and subsequent malic and fumaric acid) formation. Varying the carbonate concentrations from 0 to 30 mM showed that ethanol did not reach a minimum before fumarate and malate had begun to increase, indicating that minimizing the formation of these side products would not be possible through adjustment of carbonate concentrations alone (Longacre *et al.*, 1997). The authors therefore isolated mutants with decreased ethanol production to enable *R. oryzae* to grow anaerobically in the production phase, which eliminates metabolic flux to malate, and one such a mutant indeed reached an 86% (g g^{-1}) yield of lactic acid production. Thitiprasert *et al.* (2011) added the alcohol dehydrogenase inhibitor 2,2,2-trifluoroethanol to cultures of *R. oryzae* and obtained a higher yield of lactic acid. Skory *et al.* (1998) isolated mutants with strongly decreased alcohol dehydrogenase activity levels, but found that they still produced substantial amounts of ethanol under anaerobic conditions. Consequently, the strategy of choice must be the inhibition of the pyruvate decarboxylase. However, despite of the cloning of the genes encoding the two pyruvate decarboxylases (Skory, 2003a), no attempts to knock them out have so far been reported. In contrast, Thitiprasert *et al.* (2014) used 4-methylpyrazole, glyoxylic acid, and 3-hydroxypyruvate addition to inhibit pyruvate decarboxylase activity. Indeed, 0.1 mM 4-methylpyrazole and 1.0 μm 3-hydroxypyruvate effectively hampered ethanol production. However, because of the very broad specificity of these compounds, the activity of lactate dehydrogenase was also affected which rendered this approach not feasible.

The biochemistry of lactate synthesis by *R. oryzae* is well understood: *R. oryzae* contains three L-lactate dehydrogenases (LDH), including two NAD-dependent LDH isozymes (LdhA and LdhB; Pritchard, 1971; Yu and Hang, 1991; Skory, 2000, 2001) and a mitochondrial NAD-independent LDH (Pritchard, 1971). Notably, no D-lactate dehydrogenases are present in *R. oryzae*. The equilibrium of LdhA lies strongly on the side of lactate, whereas the equilibrium of LdhB is toward pyruvate (Skory, 2000). The results of transcriptional and enzyme activity analyses show that *ldhA* is responsible for most of the lactic acid produced by this fungus (Skory, 2000; Saito *et al.*, 2004). More recently, researchers have therefore investigated the possibility of producing L-lactic acid with *Aspergillus* spp., which can grow and produce organic acids at low pH. The drawback is that most *Aspergillus* genomes do not contain *ldh* genes, and in those who have one (e.g., *A. oryzae*) it interferes with lactic acid formation (Wakai *et al.*, 2014). The strains tested were therefore first transformed with heterologous *ldh* genes. So far, this strategy has been applied in three *Aspergillus* spp. (*A. niger*, *A. oryzae*, and *A. brasiliensis*) with *ldh* genes from different sources (*R. oryzae*, mouse, and cattle; Wakai *et al.*, 2014; Dave and Punekar, 2015; Liaud *et al.*, 2015). L-Lactic acid production was, however, much lower than in the yeast processes (generally below 50% of the theoretical yield, g g^{-1}).

Dicarboxylic Acids

Oxalic acid

Oxalic acid is probably the most widespread dicarboxylic acid that is produced by fungi, including brown-rots, white-rots, mycorrhizae, plant pathogens, and opportunistic mammalian pathogens (Dutton and Evans, 1996; Gadd, 1999). Because of its ability to strongly chelate Ca^{2+} ions, oxalate is toxic and thus no desired product in biotechnology. In fact, during citric acid production by *A. niger* oxalate can also be formed, and this has been solved by using oxalate non-producing mutants because oxalate accumulation occurs within a broad pH range (pH 2–6; although highest concentrations are observed at pH >4 Cleland and Johnson, 1956). The reason why so many fungi excrete oxalic acid is probably because it provides an easy means to acidify the medium (Dutton and Evans, 1996). In plant and human pathogenic fungi, oxalate formation has been linked to virulence. It stimulates plant lignocellulose degradation by acidification, which favors pathogen proliferation in the plant and in humans, as the calcium oxalate crystals block vessel or bronchioles (Jordan *et al.*, 1996; Nakagawa *et al.*, 1999; Lane, 2002). In brown-rot fungi – such as *Postia placenta* or *Gloeophyllum trabeum*, which combine Fenton-based oxidation and enzymatic catalysis to degrade wood – oxalate can promote Fenton chemistry by mobilizing Fe^{3+} as mono-oxalate (Presley *et al.*, 2018).

Three potential biosynthetic pathways have been proposed for oxalate formation in fungi: oxidation of glyoxylate (Balmforth and Thomson, 1984), oxidation of glycolaldehyde (Hammel *et al.*, 1994), and hydrolysis of oxaloacetate (Lenz *et al.*, 1976; Kubicek *et al.*, 1988). While the former two possibilities were proposed on the basis of enzyme activity measurements during oxalate formation, scientific proof by ^{14}C-incorporation studies and gene deletion in *A. niger* provided evidence for the origin of oxalate from D-glucose or other hexoses by hydrolysis of oxaloacetate via oxaloacetate hydrolase (Kubicek *et al.*, 1988; Pedersen *et al.*, 2000), a C(α)–C(β) bond lyase that is a member of the phosphoenolpyruvate mutase/isocitrate lyase enzyme superfamily (Han *et al.*, 2007). This mechanism has since been also confirmed in plant and human ascomycetous pathogens and basidiomycetes (Munir *et al.*, 2001; Joosten *et al.*, 2008; Chen *et al.*, 2010; Gombert *et al.*, 2011; Andrew *et al.*, 2012; Nagy *et al.*, 2012; Liang *et al.*, 2015; Presley *et al.*, 2018). Interestingly, Poulsen *et al.* (2012) identified a transcription factor (OafA) in *A. niger*, whose knock out causes an increase in the expression of the oxaloacetate hydrolase – encoding *oahA* gene at high pH, and leads to enhanced oxalate formation. At the same time, oxidative phosphorylation was down-regulated in the *oafA* mutant, indicating an indirect relationship between the two. This assumption is consistent with the recent demonstration that the overexpression of the *aoxA* (alternative oxidase-encoding) gene in *A. niger* significantly increases the rate of oxalate accumulation (Yoshioka *et al.*, 2019). These two findings are first indications that ATP-non forming NADH reoxidation, in addition to tricarboxylic acids, is also important for the accumulation of dicarboxylic acids.

L-Malic acid

L-Malic acid is produced to satisfy the increasing demand for nutritional bars and protein drinks as well as healthier functional beverages with high nutrient flavors. Yet for economic reasons, majority of the 182.6 million US$ market (2018) are still produced by chemical means. L-Malic acid production has originally been observed in *Aspergillus* spp. (Abe *et al.*, 1960; Peleg *et al.*, 1988, 1989a; Bercovitz *et al.*, 1990; Battat *et al.*, 1991) and in *R. oryzae* (Longacre *et al.*, 1997). In *Aspergillus* spp., malate can be considered as an end-product of glycolysis and the rTCA branch (*vide supra*). During a screening of nine *Aspergillus* spp., *A. flavus* (strain ATCC 13697) was detected as the best producer of L-malic acid (Bercovitz *et al.*, 1990). After optimization of fermentation parameters and the addition of $CaCO_3$, L-malate production with yields up to 128 mol percent (95 wt%) were obtained (Battat *et al.*, 1991). However, the use of *A. flavus*, which can also produce aflatoxins, is in conflict with the requirements for the safe production of food grade L-malate. Therefore, a cryptic but not aflatoxin forming species of the *A. flavus* group – *A. oryzae* (Geiser *et al.*, 1998) – was investigated for production of L-malic acid (Knuf *et al.*, 2013). Brown *et al.* (2013) further improved malic acid production from D-glucose and $CaCO_3$ with *A. oryzae* by overexpression of a native C4-dicarboxylate transporter, and the native cytosolic alleles of pyruvate carboxylase and malate dehydrogenase. A strain overexpressing all three genes accumulated 154 g L^{-1} L-malate in 164 h, corresponding to a production rate of 0.94 g L^{-1} h^{-1}. This indicates that in the presence of extra carbon dioxide, the enzymes of the rTCA pathway limit the flux rate. Liu *et al.* (2017) extended this strategy by additional heterologous expression of a phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase from *Escherichia coli* (both to support the native

pyruvate carboxylase in oxaloacetate formation), and by overexpression of the 6-phosphofructokinase encoding gene *pfkA* which was found to limit the glycolytic flux rate to malate. The final engineered *A. oryzae* strain produced 165 g L^{-1} L-malate with a productivity of 1.38 g L^{-1} h^{-1} in fed-batch culture. Most recently, the strategy of enhancing the rTCA branch was also applied to produce L-malic acid by *A. niger*: to bypass the accumulation of oxalic acid, Xu *et al.* (2019) generated an efficient malate-producing strain by deletion of the oxaloacetate hydrolase gene *oahA* which competes with malate dehydrogenase for oxaloacetate in this fungal species. Subsequent overexpression of the genes encoding pyruvate carboxylase, a cytosolic malate dehydrogenase and the C4-dicarboxylate transporter gene *c4t318* allowed production of 120.4 g L^{-1} malic acid in shake flasks, and 201.2 g L^{-1} in fed-batch fermentation.

One of the drawbacks of L-malate fermentation is the unevitable byproduction of mitochondrially produced succinate. Liu *et al.* (2018) therefore tested an alternative strategy, i.e., the engineering of the mitochondrial pathway for L-malate production in *A. oryzae*. To minimize pyruvate accumulation, they first constructed a strain that overexpressed a pyruvate carboxylase gene from *R. oryzae* both in the cytosol and mitochondria of *A. oryzae*. This strain was then further engineered by enhancing the expression of the two genes that encode the enzymes of the glyoxalate cycle, i.e., isocitrate lyase and malate synthase. In addition, they lowered the expression of citrate synthase, overexpressed the mitochondrial dicarboxylate carrier Sfc1p from *S. cerevisiae*, and finally overexpressed an NADH oxidase from *Lactococcus lactis* to decrease the $NADH/NAD^+$ ratio. This engineered *A. oryzae* strain produced 117.2 g L^{-1} L-malate with a productivity of 1.17 g L^{-1} h^{-1} from 130 g L^{-1} D-glucose and 90 g L^{-1} $CaCO_3$, while only 3.8 g L^{-1} succinate were formed.

Fumaric acid

Fumaric acid, is broadly used as a food acidulant, in the manufacture of polyester resins and polyhydric alcohols, and as a mordant for dyes. Its esters can be used in medicine to treat relapsing-remitting multiple sclerosis and have immunomodulating activities (Balak *et al.*, 2016). It is currently produced via petrochemical routes by catalytic isomerization of maleic acid in aqueous solutions at low pH (Martin-Dominguez *et al.*, 2018). Alternatively, it can be produced from D-glucose in fermentation by *R. oryzae* (Foster and Waksman, 1939; for review see Sebastian *et al.*, 2019), which later on was found to be a separate species, *R. delemar* (Abe *et al.*, 2007) which lacks the lactate dehydrogenase LdhA and therefore cannot produce lactic acid. The nutritional and physical requirements of *R. delemar* leading to maximum yields of fumarate have been examined, and like other fungal fermentations accumulating high concentrations of organic acids, high carbohydrate concentrations, a high carbon to nitrogen ratio, and addition of $CaCO_3$ are necessary to achieve high fumaric acid yields (60%–70% of D-glucose; g g^{-1}; Rhodes *et al.*, 1959). To minimize the potential by-production of ethanol, high oxygen tension is applied (Roa Engel *et al.*, 2008; Straathof and van Gulik, 2012). Proof for the biosynthesis of fumaric acid via the rTCA branch has been reported (Romano *et al.*, 1967; Overman and Romano, 1969; Osmani and Scrutton, 1985; Kenealy *et al.*, 1986; Peleg *et al.*, 1989b): an increase in the activity of the cytosolic pyruvate carboxylase was observed to correlate with glucose utilization and fumarate production in *R. delemar* (Overman and Romano, 1969). To overcome a possible limitation of the flux at this step, Zhang *et al.* (2012) overexpressed the endogenous pyruvate carboxylase (*pyc*) and also introduced the phosphoenolpyruvate carboxylase (*pepC*) gene from *E. coli*. Fumaric acid production by the *pepC* transformant increased by 26% (g g^{-1}), but the *pyc* transformants grew poorly and had low fumaric acid yields (<0.05 g g^{-1} glucose). This was probably due to the formation of large pellets that impaired oxygen transfer and consequently resulted in the accumulation of ethanol.

The cytosolic fumarate hydratase (FumR) activity which is present in *Rhizopus* spp. also increases during the production stage of the fumarate fermentation (Peleg *et al.*, 1989b). The role of the latter is, however, still unclear: its overexpression in *R. delemar* and other fungi resulted in the accumulation of more L-malate than fumarate (de Jongh and Nielsen, 2008; Xu *et al.*, 2012; Zhang and Yang, 2012), which is consistent with the fact that the equilibrium of FumR lies on the side of L-malate (Meussen *et al.*, 2012). It is likely that *R. delemar* has a specific fumarate transporter which removes fumarate from this equilibrium, but this has not been investigated so far. Interestingly, a transcriptomic and proteomic analysis has recently led to the hypothesis that during nitrogen starvation a substantial part of accumulated fumarate accumulates from the urea cycle due to amino acid catabolism (Odoni *et al.*, 2017a). The responsible enzyme – argininosuccinate lyase – is located in the cytosol, and the resulting fumarate therefore needs no further transport step to mix with the rTCA fumarate pool. This offers new strategies for the improvement of fumarate production.

Succinic acid

Succinate has been ranked within the top 12 bio-based products by the US Department of Energy (Werpy and Petersen, 2004) and hence there are numerous efforts to produce succinate biotechnologically, using recombinant anaerobic and aerobic bacteria and yeasts (Ahn *et al.*, 2016). For this reason, production by fungi was only scarcely studied. *Aspergillus carbonarius*, a member of black aspergilli was more recently introduced as a producer of L-malic acid (Weyda *et al.*, 2014), because it is known to produce citric, gluconic and malic acid from varieties of carbohydrate containing substrates, and tolerates low pH (Yang *et al.*, 2016a). Since, as outlined above, fungi do not have a cytosolic fumarate reductase, Yang *et al.* (2016b) transformed *A. carbonarius* with the *frd* gene from *Trypanosoma brucei*, which encodes an NADH-dependent fumarate reductase gene. It has been shown to be located in the cytosol and to be rate limiting for succinate accumulation by *T. brucei* (Besteiro *et al.*, 2002). To this end Yang *et al.* (2016b) used a glucose oxidase deficient strain of *A. carbonarius* that overexpressed the native *A. carbonarius* C_4-dicarboxylate transporter as a host. Impacts of the introduced genetic modifications on organic acid production were investigated in a defined medium and in a hydrolysate of wheat straw containing high concentrations of glucose and xylose. They found that overexpression of the C_4-dicarboxylate transporter alone and in combination with the *frd* gene significantly increased the production of C_4-dicarboxylic acids and reduced the

accumulation of citric acid, whereas expression of the *frd* gene alone did not result in any significant change of organic acid production profile. However, despite of the use of 80 g L^{-1} D-glucose and $CaCO_3$, respectively, the yields from D-glucose were below 30 g L^{-1}, and besides minor amounts of citric and fumaric acid, the strain produced even higher amounts of L-malic acid than succinic acid. An explanation for this could be that – as explained above – the combination of glycolysis and the rTCA branch of *Aspergillus* spp. produces neither ATP nor NADH, and fumarate reductase therefore faces a shortage of its co-enzyme.

Trans-2,3-epoxysuccinic acid (ESA)

ESA, which can be used as a good starting material for the synthesis of optically active compounds such as optically specific single β-lactam antibiotics (Yamaguchi and Nogami, 1987), was first isolated from culture filtrates of *Paecilomyces variotii*, *Talaromyces flavus* (Martin and Foster, 1955), and *A. fumigatus* (Birkinshaw *et al.*, 1945). Tracer studies showed that the epoxide results from attachment of an oxygen atom into fumarate (Aida and Foster, 1962; Wilkoff and Martin, 1963). The enzyme – likely a cytochrome P450 monooxygenase – is still unknown. Culture fluids containing ESA can be further converted to meso-tartaric acid by several bacteria and fungi including *A. fumigatus* (Martin and Foster, 1955). Meso-tartaric acid has a linear 3D structure with the carboxyl groups in "anti" position, which renders it suitable for copolymer formation with diols. The reaction from ESA to meso-tartaric acid is hydratization catalyzed by fumarase (Albright and Schroepfer, 1970). It is therefore noteworthy that not even traces of meso-tartaric acid were found in culture filtrates of those fungi that produced ESA.

Tricarboxylic Acids

Citric acid

Citric acid (2-hydroxypropane-1,2,3-tricarboxylic acid) is a weak tricarboxylic acid with pK_a values of 2.92, 4.28 and 5.21 at 25°C. Since these differences are relatively small, the pH regions in which the four ionic species exist overlap heavily. As a consequence, citric acid solutions are powerful buffers – and widely used as such – between about pH 2 and pH 8. It was first isolated by the Swedish pharmacist Carl Wilhelm Scheele in 1784 from lemon juice. Indeed, citric acid may constitute up to 8% of the dry weight of lemons, oranges, limes, and other citrus fruits, and – depending on the cultivation conditions and variations within species – its concentration may reach 0.3 M in the juices. However, being the denominator intermediate of the citric acid (a.k.a. Szentgyörgyi-Krebs) cycle, citric acid is in fact an integral part of the cell metabolism of all aerobic organisms.

Industrial citric acid production began in the mid-nineteenth century in England, utilizing citrus fruits imported from Italy and Sicily. The extracted juice was treated with calcium hydroxide to precipitate citric acid into calcium citrate, and the isolated calcium salt was then converted back to citric acid using diluted sulfuric acid. The cartel-like structure of the citrus fruit growers and growing demand however, stimulated the development of other means of production. In 1893, the German chemist Carl Friedrich Wilhelm Wehmer discovered that certain filamentous fungi (molds mostly belonging to the genus *Penicillium*, but named "*Citromyces*" back then) could produce citric acid from sugar in liquid cultures. The American chemist James N. Currie (1917) demonstrated that *A. niger* strains are far superior citric acid producers to any culture resembling Wehmer's Citromyces if grown on concentrated sugar solutions. The carbon source could be any kind of inexpensive sugary solution such as corn steep liquor, molasses or hydrolyzed corn starch. Up until World War II, citric acid manufacturing was performed by the labor intensive and relatively low-yield surface method – originally patented by Currie in 1923 – where *A. niger* was grown in shallow aluminum trays filled up with sugar and other nutrients (Doelger and Prescott, 1934). Sterile air passed over the pans supplied oxygen and served as cooling agent. The downstream process started with the separation of mycelia from the fermentation broth, and was followed by the isolation of citric acid from the cell-free medium by the calcium precipitation method described above. A submerged, batch-mode citric acid fermentation process employing mechanically agitated, aerated, high-quality steel vessels with several hundreds of m^3 volume was established in the USA in 1952, and was eventually introduced all across the world in the second half of the last century (Lockwood and Nelson, 1946; Perlman *et al.*, 1946a,b; Martin and Waters, 1952). Despite of minor differences in the upstream and downstream technology, all producers almost exclusively employ carefully selected strains of *A. niger* grown in highly optimized liquid medium. Today, citric acid is the largest-volume bulk product in biotechnology after bioethanol, with over 2.1 million tons manufactured globally in 2018. Well over half of this volume is now produced by Chinese companies. Being edible and fully biodegradable, it is most widely used as a flavoring agent and preservative in food and beverages, particularly soft drinks. It is also utilized as an emulsifying agent, while various metals are also delivered as citrate salts in dietary supplements. Apart from the food applications, citric acid is extensively used as acidifier and chelating agent in the chemical and pharmaceutical industries. As such, it dissolves rust from steel, passivate stainless steels, removes the buildup of limescale from evaporators, eliminates hard water stains from glass, as well as complexes calcium ions from blood preparations, thereby preventing clotting (Karaffa and Kubicek, 2019). Fueled by increasing demand – the global citric acid market is expected to grow at an annual rate of 4.9%, and reach US$ 3.83 billion by 2025 – the *A. niger* citric acid fermentation will most likely remain an all-important segment of industrial biotechnology in the foreseeable future (See "Relevant Website Section").

The metabolic steps leading to citric acid accumulation has been established already in the middle of the past century (Shu and Johnson, 1948; Martin and Wilson, 1951; Cleland and Johnson, 1954) and further refined in the last years (Chen *et al.*, 2014; Kirimura *et al.*, 2016). It involves the uptake of a hexose substrate from the growth medium, and predominantly glycolytic catabolism to two moles of pyruvate, their conversion into oxaloacetate and acetyl-CoA, the subsequent condensation of these two

direct precursors into citric acid in mitochondria, and finally, two consecutive, enzyme-catalyzed membrane transport steps: from mitochondria into the cytosol and through the cell membrane to the growth medium.

A massive carbon flux through the glycolytic (Embden-Meyerhof-Parnas, EMP) pathway is a fundamental prerequisite to high-yield citric acid production. However, cytosolic citrate is a strong regulator of the EMP route because it inhibits 6-phosphofructokinase and thus adjusts the rate of lipid biosynthesis (Fiechter and Gmünder, 1989). The explanation for this apparent contradiction is that under appropriate nutrient conditions, this inhibition is decisively counteracted by various positive effectors. Probably the most relevant of all is fructose 2,6-bisphosphate (Fru-2,6-P_2), whose absence renders phosphofructokinase – the key enzyme of the glycolytic pathway – practically inactive (Kubicek-Pranz *et al.*, 1990; Capuder *et al.*, 2009). On the other hand, intracellular levels of Fru-2,6-P_2 exponentially increase in the presence of high ($>10\%$, w v^{-1}) concentrations of rapidly assimilating carbon source, allowing intense glycolytic catabolism to proceed. Phosphofructokinase is also activated by elevated levels of intracellular ammonium ions resulting from the increased rate of protein degradation which, in turn, is prompted by manganese ion deficiency, one of the fundamental traits of the *A. niger* citric acid fermentation technology (Habison *et al.*, 1983). High glycolytic flux is additionally fueled by the intake of hexoses by a second, low-affinity glucose permease. In contrast to the high-affinity glucose transporter ($K_m = 0.3$ mM), this enzyme contributes to glucose uptake only at high external concentrations (Torres *et al.*, 1996; Wayman and Mattey, 2000).

The cytosolic oxaloacetate formed by the carboxylation of pyruvate is reduced to malate by the cytosolic malate dehydrogenase isoenzyme, thereby also re-oxidizing half of the glycolytically produced NADH. The cytosolic malate then enters mitochondria via a malate-citrate antiport (a tricarboxylate carrier), becomes part of the Krebs-cycle, and is thus oxidized back to oxaloacetate, one of the two direct precursors of citric acid. Citric acid formation occurs by the citrate synthase-catalyzed condensation of a CoA-activated acetyl moiety and oxaloacetate. This enzyme in located exclusively in the mitochondria, and appears to have sufficient natural capacity for citric acid overflow (Ratledge, 2000). The same is true for the other enzymes of the Krebs-cycle. Moreover, evidence proves that an intact Krebs-cycle is present throughout the citric acid fermentation, and hence theories based on the inactivation of the citric acid degrading enzymes (aconitase, isocitrate dehydrogenase) to explain accumulation are incorrect (for review, see Ruijter *et al.*, 2002). A more likely hypothesis is that the mitochondrial tricarboxylate transporter is responsible for the removal of citric acid from mitochondria (Kubicek, 1987). The provision of malate – which, as discussed above, is a counterion for the carrier – may serve as a pace maker for citrate export. In fact, an increase in the cytosolic concentration of malate precedes citrate accumulation, and gene amplification of the cytosolic malate dehydrogenase in *A. niger* has been shown to lead to increased citrate formation rates (de Jongh and Nielsen, 2008). The mitochondrial tricarboxylate carriers of higher eukaryotes generally exhibit a much higher affinity for mitochondrial citrate ($K_m = 0.027$ mM; Palmieri, 1994) than aconitase ($K_m = 3.2$ mM; Grotjohann *et al.*, 2001). This comparison suggests that the tricarboxylate carrier favorably competes with aconitase during citric acid production conditions, without any necessity for a bottleneck in the Krebs-cycle downstream of citrate.

The above-described mechanism suggest that the mitochondrial citrate/malate antiporter is a critical element of the citric acid overflow. However, knock-out of the tricarboxylate carrier gene results in a strain of *A. niger* that still accumulates citric acid with a rate of about 50% of the parental strain (Kirimura *et al.*, 2016), and the presence of other transporters for citrate is unlikely. An explanation could be that part of citrate is completely synthesized in the cytosol (Karaffa and Kubicek, 2019). This hypothesis is supported by the presence of two cytosolic isoenzymes of citrate synthase. Since pyruvate decarboxylase of *A. niger* is also located in the cytosol (see above), citrate biosynthesis would only need a cytosolic supply of acetyl-CoA. One such possibility is the oxidation of pyruvate to acetaldehyde and subsequent conversion to acetyl-CoA by an acetyl-CoA synthase (Karaffa and Kubicek, 2019). Alternatively, cytosolic acetyl-CoA can be formed by ATP-citrate lyase, whose expression is upregulated in *A. niger* under high citric acid producing conditions (Chen *et al.*, 2014). Operation of this activity involves the conversion of an ATP back to ADP and inorganic phosphate, thereby decreasing energy charge and subsequently relieving allosteric inhibition of ATP over glycolytic carbon catabolism. Both mechanisms require experimental verification though.

Export of citric acid from the cytosol to the fermentation broth is an enzyme catalyzed process. Citrate permease – like cell membrane-bound permeases in general – can transport its substrate into both directions, and under citric acid producing conditions, the *A. niger* citrate permease as an exporter has to work against a strong gradient (external concentration up to 1 M, internal concentration 4–10 mM; Habison *et al.*, 1983). The citrate permease seems to be a major bottleneck for the rate of citrate accumulation because its conditional overexpression dramatically increases the citrate production rate (Steiger *et al.*, 2019).

Itaconic acid

Itaconic acid (2-methylenesuccinic acid, 1-propene-2–3-dicarboxylic acid) is an unsaturated, weak dicarboxylic acid ($pK_a = 3.83$ and 5.41), discovered in 1837 as a thermal decomposition product of citric acid. The presence of the conjugated double bond of the methylene group allows polymerization both by addition and condensation. Esterification of the two carboxylic groups with different co-monomers is also possible (Kuenz *et al.*, 2012). These diverse properties have led to a variety of applications in the pharmaceutical, architectural, paper, paint, and medical industries such as plastics, resins, paints, synthetic fibers, plasticizers, and detergents. Recently, itaconic acid applications have penetrated the dental, ophthalmic and drug delivery fields (Hajian and Yusoff, 2015). Itaconic acid polymers could even replace the petroleum-based polyacrylic acid, which has a multi-billion dollar market (Saha *et al.*, 2019). Not surprisingly, the US Department of Energy assigned itaconic acid as one of the top 12 most promising building block chemicals for bio-based economy in 2004 (Werpy and Petersen, 2004). However, to fulfill its potential, the selling price of itaconic acid – currently at approximately \$2.0 kg^{-1} – must drop considerably. Many of the plants in developed countries

have therefore relocated to China, which now accounts for the overwhelming majority of global itaconic acid production, estimated at 42,000 tons per year (Geiser *et al.*, 2016).

Little is known about the reasons why fungi produce itaconate. Like the other organic acids, as outlined above, also itaconic acid might serve as acidifier of the environment and thus provide selective advantage for the acid-tolerant *A. terreus* over other micro-organisms. However, itaconic acid also has clear inhibitory properties: in macrophages of mammals, bacterial infection prompts the induction of a gene encoding a *cis*-aconitate decarboxylase, resulting in itaconic acid formation that inhibits bacterial metabolism as part of the immune response. The effect has been attributed to the inhibition of succinate dehydrogenase and isocitrate lyase (McFadden *et al.*, 1971), the latter being a key enzyme of the glyoxylate cycle, required for the survival of pathogens inside a host. In turn, a few strains of these bacteria have evolved to be capable of degrading itaconate (Sasikaran *et al.*, 2014). Itaconic acid also induces a transcription factor which is essential for protection against oxidative and xenobiotic stresses, and to attenuate inflammation (Kobayashi *et al.*, 2013; Bambouskova *et al.*, 2018). Whether a similar function of itaconate exists in the fungi producing it has not yet been studied.

The biosynthetic pathway of itaconic acid resembles that of citric acid, the latter acid being a direct precursor of the former. The only difference is that citric acid in *A. terreus* is further metabolized via *cis*-aconitate to itaconate by *cis*-aconitate decarboxylase (Bonnarme *et al.*, 1995). To this end, *cis*-aconitate is transported out of the mitochondria by a specific antiporter in exchange for oxaloacetate (Li *et al.*, 2011a,b). Itaconic acid – formed upon *cis*-aconitate decarboxylation – is finally secreted out of mycelia by a specific cell membrane transporter. Genes encoding these three enzymes, and a fourth one encoding a transcription factor, constitute the "itaconate gene cluster" in the *A. terreus* genome, while the cluster is notably absent in *A. niger*. Although several itaconate producers have been tested, the plant pathogenic Basidiomycete *Ustilago maydis* (the corn smut fungus) – and particularly its low pH-stable relative *Ustilago cynodontis* (Hosseinpour Tehrani *et al.*, 2019b) – seems to be the only one with a reasonable chance to become another industrial platform organism (Hosseinpour Tehrani *et al.*, 2019a). *Ustilago* has developed an alternative biochemical pathway to synthetize itaconate inasmuch as *cis*-aconitate is converted to the thermodynamically favored *trans*-aconitate by aconitate-delta-isomerase. *Trans*-aconitate is then decarboxylated to itaconate by *trans*-aconitate-decarboxylase.

Sugar Acids

Gluconic acid

Gluconic acid is a non-corrosive, non-toxic, biodegradable, weak ($pK_a = 3.86$) organic acid. It mainly occurs in plants, fruits, wine, and honey. Gluconic acid is produced from D-glucose by the oxidation of its aldehyde group (C1) to a carboxyl group. It is not to be confused with other glucose-derived acids such as glucuronic acid, where C6 is oxidized to a carboxyl group, or glucaric acid, where both C1 and C6 are carboxylic groups. In aqueous solution at neutral pH, gluconic acid forms gluconate ion that is in equilibrium with its cyclic ester D-glucono delta-lactone (1, 5-gluconolactone). The equilibrium is dependent on pH and temperature; heat and high pH increase the rate of D-glucono-delta-lactone hydrolysis. Together with its salts and the delta-lactone form, gluconic acid is included as a flavoring agent in a variety of food items such as meat, wine, and dairy products. Due to its ability to form water-soluble complexes (chelates) with di- and trivalent metal ions, it is frequently applied as counterion during therapeutic calcium and/or iron administration. For the same reason, it is also used to remove calcareous and rust deposits from metals or other surfaces. Today, gluconic acid and its derivatives have an annual market volume of approximately 80,000 tons, amounting to almost 1 billion €.

In contrast to bacterial gluconic acid formation, which is a one-step process typically catalyzed by a membrane-bound D-glucose dehydrogenase, fungal gluconic acid formation consists of two steps. The first is the direct dehydrogenation of ß-D-glucose to D-glucono-delta-lactone by glucose oxidase. This enzyme is a flavoprotein containing a FAD-cofactor which takes up two hydrogen atoms from D-glucose during catalysis and releases hydrogen peroxide. However, D-glucono-delta-lactone is unstable in aqueous solution. The second step – the hydrolysis of the lactone to its free form, gluconic acid – can therefore be either spontaneous or catalyzed by lactonase. Depending on the fungal genera, glucose oxidase is either a fully extracellular enzyme or partially cell wall-bound. In all cases, however, it requires molecular oxygen to regenerate $FADH_2$ and is inactivated at pH <3. Therefore, the fermentation has to be carefully kept above this pH value. The *goxA* gene encoding glucose oxidase is induced by high glucose concentrations and high dissolved oxygen levels, but the enzyme is competitively inhibited by the hydrogen peroxide formed during FAD recycling. To prevent this, *A. niger* also secretes several catalases that catalyze the decomposition of hydrogen peroxide to water and oxygen. Sufficient catalase activity is in fact critical during gluconic acid fermentation. The ability of *A. niger* to form glucose oxidase may be an evolutionary advantage related to competition with other micro-organisms, as the produced hydrogen peroxide has been shown to be the causal agent in combatting other fungi. However, due to the necessity of high glucose concentrations, this mechanism only applies at special conditions. In addition, glucose oxidase activity depletes the available glucose pool in exchange for the formation gluconic acid. Gluconic acid is usually a poor carbon source for micro-organisms, but happens to be a good one for *A. niger*.

At technical scale, gluconic acid is prepared by microbial fermentation of the various bacterial and fungal strains that were shown to be capable of accumulating gluconic acid, *A. niger* is the most widely used platform organism. Gluconic acid accumulation in A. *niger* cultures was first observed in 1922. The traditional gluconic acid production with *A. niger* is named "calcium gluconate process", which stems from the use of calcium carbonate for neutralization of the fermentation broth, a critical technological step to keep the extracellular glucose oxidase active. The production medium contains up to 150 g L^{-1} glucose (most frequently derived from corn or rice); further increase in the glucose concentration is hampered by the limited solubility of calcium gluconate, which

would precipitate on the mycelia and inhibit oxygen and substrate uptake. Other components of the nutrient medium – particularly phosphorus and nitrogen – are kept at low levels to prevent fungal growth (= loss of carbon source). The stoichiometry of the reaction leading to gluconic acid formation shows that on a molar basis, identical amounts of oxygen and D-glucose are needed, despite of the former being much less soluble in water. Applying overpressure (up to 3 bars) in the fermentor – which, according to Henry's law, will increase the saturation level of oxygen in the medium through increasing its partial pressure – has therefore been shown to be highly beneficial. The fermentations with almost quantitative yields ($Y_{p/s} > 95\%$ on a molar basis) are short, between 24 and 48 h. In fact, the process resembles to an enzymatic conversion rather than a microbial fermentation.

Sodium gluconate has been used as a superior alternative to the calcium gluconate process, as it enables to use even higher glucose concentrations (up to 350 g L^{-1}). Here, the pH is maintained at 6.5 by external control with NaOH, but otherwise, the process is similar to the previous one. Based on this technology a continuous process was developed, converting 35% (w v^{-1}) glucose solutions with 95% yield. Another continuous fermentation method employing the osmotolerant yeast *Aureobasidium pullulans* was also described, with the advantage of enabling extremely high (>350 g L^{-1}) initial glucose concentrations in the medium.

Engineering Aspects of Fungal Organic Acid Production

Industrially, organic acids are mostly produced by submerged fermentation in large, stainless-steel vessels of several hundreds of cubic meter volume to achieve >100 g L^{-1} yield (Roukas, 1991; Grewal and Kalra, 1995). In the case of citric acid, surface cultivation using large and flat pans is still used by some companies because it is much less influenced by the presence of manganese and morphological development (for review, see Show *et al.*, 2015). In addition, some companies – mainly in the China and South East Asia – use solid substrate fermentations (Kareem *et al.*, 2010).

High-rate provision of oxygen, as explained in the respective chapters, is essential for high organic acid yields (Grewal and Kalra, 1995). Apart from the control of oxygen transfer and the growth medium temperature, few other fermentation parameters are controlled. In some cases, additional substrate is fed before the end of fermentation.

The downstream processes (recovery and purification) may correspond to over 50% of the overall production costs for all organic acids (Cheng *et al.*, 2012). Downstream typically starts with filtering the fermentation broth to remove mycelia by sedimentation, centrifugation or filtration, after which the product in the cell-free medium is separated and purified to the desired extent. The two most traditional methods for organic acid isolation from cell-free broth are precipitation with $Ca(OH)_2$ or CaO (Heding and Gupta, 1975; Berglund *et al.*, 1998; Li *et al.*, 2016) and crystallization (Okabe *et al.*, 2009). During the former, the obtained calcium salts react with concentrated sulfuric acid and release free acid, which is further purified by active carbon absorption or ion exchange. Afterwards, the product is further concentrated and crystallized by evaporation. Advantages of precipitation are selectivity and high product purity. The three largest-market organic acids (citrate, lactate, succinate) produced by fermentation are mostly recovered by precipitation (Li *et al.*, 2016).

Crystallization of organic acids is enabled by the dependence of their solubility on the temperature: its alteration allows their concentration and subsequent crystallization, which is usually performed under vacuum.

An another frequently used separation method – chromatography – relies on the ion-exchange or adsorptive properties of resins toward organic acids (Boonkong *et al.*, 2009; Zhou *et al.*, 2011). Interactions with macroporous adsorption resins rely on the hydrogen bond and the van der Waals' force. Chromatography is typically used for the final purification of organic acids from aqueous solution, as the resin employed is highly selective. The method has low energy consumption and high capacity.

Membrane separation techniques such as microfiltration, ultrafiltration, nanofiltration, reverse osmosis (= forcing the water in the broth across a semi-permeable membrane, leaving dissolved compounds behind), pervaporation (= permeation through a membrane followed by evaporating the permeate into vapor phase), and electrodialysis have also been positively tested for organic acid recovery (Li *et al.*, 2016). However, their high capital cost and energy requirement to maintain the transmembrane flux to separate a relatively cheap product makes them uneconomical at this time.

Liquid–liquid extraction, where separation occurs based on the relative solubility of the products in two different, immiscible liquids is another means to recover organic acids (Cheng *et al.*, 2012). A non-polar organic solvent such as esters, ketones or amines takes them up first from the mother liquor which is then washed with water to recover the product. Although the conventional solvents (long-chain alcohols, esters) are not always effective due to the low distribution coefficient of some industrially relevant organic acids acid, the method can be improved by employing organophosphorus compounds, or tertiary and quaternary amines. A version of liquid–liquid extraction – reactive extraction – involves converting the product into another compound for easier extraction, and was adopted to extract lactic acid and succinic acid from broths (Li *et al.*, 2010).

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Biotechnological Advancements, Innovations and Challenges for Sustainable Xylitol Production by Yeast

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Introduction

Xylitol ($C_5H_{12}O_5$) is a five carbon sugar alcohol, also known as polyol, naturally present in small amounts in some fruits and vegetables. It presents a sweetness profile similar to sucrose with a low caloric value (2.4 kcal g^{-1}), being partially absorbed (50%) in the small intestine and metabolized in the liver with no effect on blood glucose or insulin levels. The unabsorbed fraction that reaches the distal parts of the gastrointestinal tract is degraded by intestinal microbiota leading to the formation of short-chain fatty acids (Grembecka, 2015; O'Brien-Nabors, 2011; Salli *et al.*, 2019). Due to the low-caloric content and the insulin independent-metabolism, xylitol is widely used as sweetener in food and beverage products (chewing gums, candy, fruit drinks) and also as additive in functional foods, especially applied in diabetes management. Additionally, xylitol exhibits a unique role in caries prevention. It is not metabolically used as energy source by oral flora, reducing the growth of mutans streptococci, the group of bacteria responsible for dental caries formation. In this sense, the substitution of sugar by xylitol in food along with its incorporation in dental care products (toothpaste and mouthwash) has considerable impact on dental health (Janakiram *et al.*, 2017; Maguire and Rugg-Gunn, 2003).

In addition to these advantageous properties, xylitol has been identified as one of the 12-top value added compounds to be attained from biomass and can be also used in the chemical industry as an intermediate for the synthesis of ethylene glycol, propylene glycol, glycerol, xylaric acid, and polymers (Hernández-Pérez *et al.*, 2016; Isikgor and Becer, 2015; Werpy and Petersen, 2004).

Currently, xylitol is largely used as sweetener due to the public awareness of adverse health impacts from excessive sugar consumption. As the consumers have become more aware of the link between sugar intake, and obesity and related diseases, the replacement of simple sugars in the daily diet by low-calorie sweeteners is increasing the demand of xylitol (Chattopadhyay and Raychaudhuri, 2014; Philippe *et al.*, 2014; Toews *et al.*, 2019). The global xylitol market reached US$875 million in 2019, with the chewing gum industry being the largest application segment of the market. Nevertheless, as the scope of xylitol applications is expanding, it is expected to reach US$1.37 billion by 2025 (Hernández-Pérez *et al.*, 2016).

The industrial production of xylitol started in 1975 by Finnish Sugar Co. Ltd (Finland), through chemical hydrolysis and hydrogenation of xylan. Xylan is a polysaccharide composed by β-1,4-linked xylose, abundantly present in agro-industrial residues. The procedure involves acid pretreatment of these lignocellulosic materials to generate a xylose-enriched hydrolysate. The subsequently catalytic reduction of xylose to xylitol requires an additional purification step by exchange chromatography and activated carbon, being performed at high hydrogen pressure and temperature. This procedure, in spite of using a renewable feedstock lignocellulose as raw material, is not environmental-friendly, and requires expensive equipment and large amount of energy (Dasgupta *et al.*, 2017).

Biotechnological Production of Xylitol: The Yeast's Role

Xylitol Production by Native Xylose Utilizing Yeasts

Considering the growing commercial demand for xylitol together with the safety concerns related with the costly chemical process, several research efforts have been applied over the last years for the development of biotechnological production of xylitol. Bioconversion methods based on whole cell biocatalyst, including bacteria, fungi and yeast, involve milder temperatures and pressure conditions, reducing the energy requirements and thus the overall process cost. In addition, as the microbial conversion of xylose does not require high purity, the purification steps before xylose reduction are not required, which also contribute to the reduction of production costs.

Bacteria, yeasts, and filamentous fungi are naturally capable of xylitol biosynthesis. However, there are few studies regarding xylitol production by bacteria and filamentous fungi, mainly due to the low yields and reduced final xylitol concentrations. Most studies on xylitol production with filamentous fungi (*Aspergillus*, *Byssochlamys*, *Fusarium oxysporum*, *Gliocladium*, *Myrothecium*, *Penicillium*, *Rhizopus*, and *Neurospora* sp.) reported low concentrations of xylitol with the exception of *Petromyces albertensis* that produced 39.8 g l^{-1} of xylitol after 10 days of incubation (Dahiya, 1991; Rafiqul and Mimi Sakinah, 2013). Native xylose utilizing yeasts (*Candida*, *Debaromyces*, *Kluyveromyces*) are the organism of choice for xylitol production due to their high pentose assimilation and xylose utilization capacity through the oxidoreductase pathway (**Fig. 1**). Xylose metabolism is catalyzed by two key enzymes: xylose reductase (EC 1.1.1.307) and xylitol dehydrogenase (EC 1.1.1.175). Firstly, xylose is reduced to xylitol by the xylose reductase (XR). This enzyme belongs to the aldose reductase family and generally uses NADPH over NADH as cofactor (Quehenberger *et al.*, 2019). The xylitol produced can be secreted from the cell or oxidized to xylulose by the xylitol dehydrogenase (XDH) which uses only NAD^+ as cofactor. Xylulose after phosphorylation to xylulose 5-phosphate is further metabolized through the pentose phosphate pathway (PPP). Xylitol accumulation is caused by a cofactor imbalance between the mainly NADPH-dependent XR and the NAD^+-dependent XDH.

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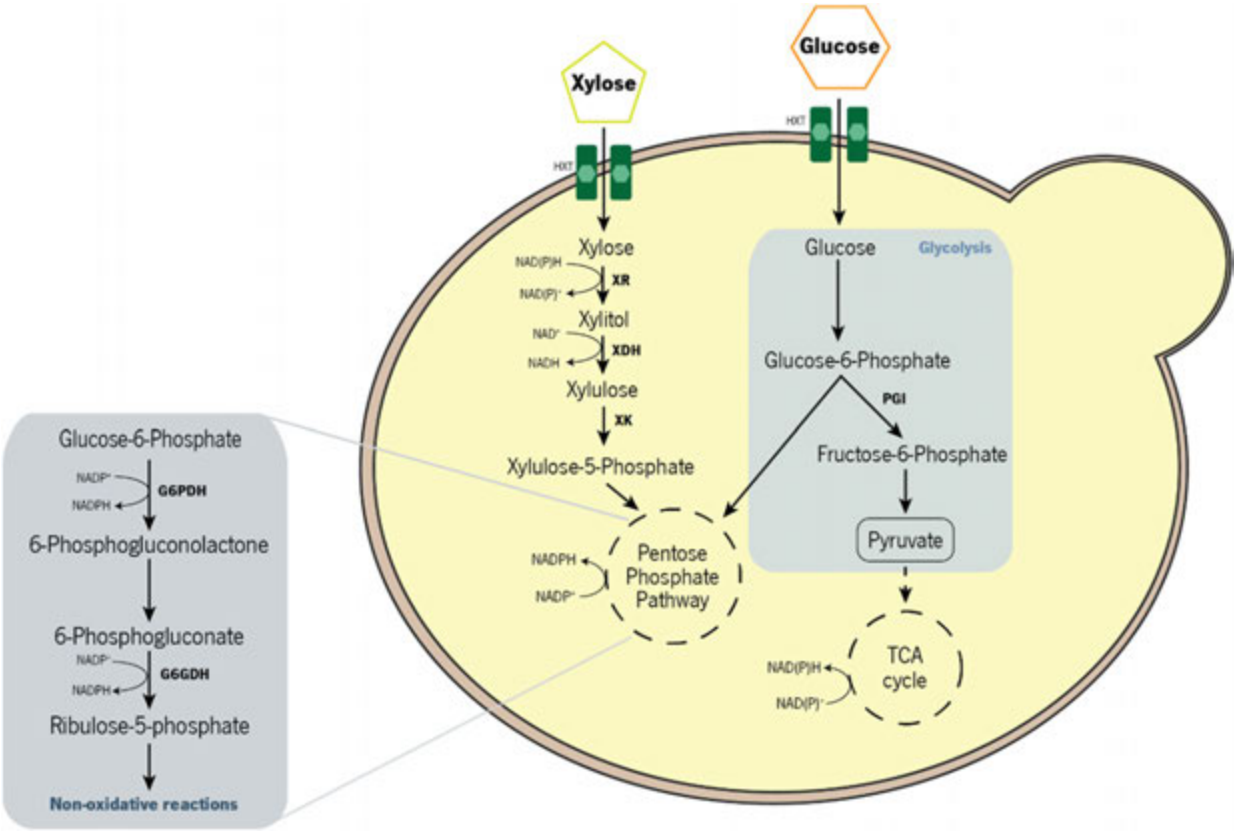

Fig. 1 Metabolic pathways involved in xylitol production by yeast.

Among yeasts, *Candida* species are the most studied for xylitol production (**Table 1**). In several tests, performed for comparison of different xylose utilizing-yeasts, *Candida* species showed better xylitol production capacity (Barbosa *et al.*, 1988; Gong *et al.*, 1981; Guo *et al.*, 2006; Misra *et al.*, 2012; Vandeska *et al.*, 1995). In fact, the higher specific activity of XR in comparison with XDH, could explain high xylitol accumulation by these yeasts (Yablochkova *et al.*, 2003). The highest xylitol productivity values were reported by *Candida tropicalis*. The optimization of culture conditions, namely aeration, initial xylose concentration and yeast extract supplementation, allowed to obtain a maximum xylitol production rate of 2.67 g l^{-1} h^{-1} by *C. tropicalis* IF0 0618 (Horitsu *et al.*, 1992). This volumetric productivity was further improved to 5.4 g l^{-1} h^{-1} in a cell recycle fermentation performed with complex medium (Kim *et al.*, 2004). Furthermore, Kwon *et al.* (2006b) reported a remarkable xylitol productivity of 12 g l^{-1} h^{-1} in cell-recycling strategy, using a submerged membrane bioreactor. In this study, *C. tropicalis* KCTC 10457 produced 182 g l^{-1} of xylitol with a corresponding yield of 0.85 g g^{-1} in a fed-batch fermentation of xylose and glucose. The glucose supplementation seems to be advantageous for the process, since it is used for yeast metabolism and also for cofactor regeneration.

The yeasts *Debaryomyces*, *Pichia*, and *Kluyveromyces* are also reported as natural xylitol-producing organisms (**Table 1**). The cultivation of *Debaryomyces hansenii* UFV-170 isolate resulted in 37 g l^{-1}, with a corresponding xylitol yield of 0.76 g g^{-1} and 1 g l^{-1} h^{-1} of productivity (Sampaio *et al.*, 2008). More recently, Himabindu and Gummadi (2015) evaluated the oxygen mass transfer coefficient in fed-batch fermentation by *Debaryomyces nepalensis* NCYC 3413, improving the xylitol yield (0.83 g g^{-1}) and final xylitol concentration (90 g l^{-1}). The use of thermotolerant yeast strains with xylose consumption ability could represent a benefit to the xylitol production process. In this regard, the yeast *Kluyveromyces marxianus* IMB2, isolated from Indian distillery, showed high-yield xylitol production (up to 0.90 g g^{-1}) but with a reduced productivity (maximum 0.24 g l^{-1} h^{-1}) (Mueller *et al.*, 2011).

Despite the innate xylitol production capacity of these yeasts, genetic and metabolic engineering strategies including optimization of xylose transport system, regulation of PPP to enhance NADPH cofactor supply and knockout or/and overexpression of XDH and XR genes have been successfully applied to yeast for improving xylitol production efficiency and were recently reviewed by Dasgupta *et al.* (2017).

Exploiting *Saccharomyces cerevisiae* as Cell Factory for the Biotechnological Production of Xylitol

As mentioned above, there is a wide range of yeast strains able to produce xylitol through the xylose reductase/xylitol dehydrogenase (XR/XDH) pathway, however, xylitol is a by-product of this pathway and the yields are limited by the use of xylose as carbon source for yeast growth and maintenance energy.

Table 1 Culture conditions and xylitol concentration, productivity and yield by native xylitol-producing yeasts

Yeast strain	*Culture conditions*		*Xylitol*			*Reference*
	pH	*Temperature*	*Maximal concentration* $g\ l^{-1}$	*Productivity* $g\ l^{-1}\ h^{-1}$	*Yield* $g\ g^{-1}$	
Candida tropicalis IFO 0618	5	30	NR	2.67	0.64	Horitsu *et al.* (1992)
Candida tropicalis ATCC 13803	6.5	30	110	5.4	0.81	Kim *et al.* (2004)
Candida tropicalis KCTC 10457	6.5	30	182	12	0.85	Kwon *et al.* (2006b)
Debaromyces hansenii UFV – 170	6	30	37	3.4	0.76	Sampaio *et al.* (2008)
Debaromyces nepalensis NCYC3413	6	30	90	0.83	0.83	Himabindu and Gummadi (2015)
Kluyveromyces marxianus IMB2	5.5	40	24	0.2	0.90	Mueller *et al.* (2011)

NR, not reported.

The yeast *Saccharomyces cerevisiae* has been used for centuries to produce food and alcoholic beverages, and today is also applied in the pharmaceutical industry for the production of biopharmaceuticals. In addition to the GRAS status (generally regarded as safe), *S. cerevisiae* is a very attractive organism for biotechnological production processes due to the vast knowledge accumulated from years of studies and its susceptibility to genetic modifications (Ostergaard *et al.*, 2000).

Considering that *S. cerevisiae* is naturally incapable of xylose consumption due to the lack of oxidoreductase pathway, the production of enzymes with XR activity enables the conversion of xylose into xylitol in a theoretical yield of almost 100%, since the produced xylitol is not further metabolized by the yeast. The main metabolic engineering strategies that have been developed for xylitol production in *S. cerevisiae* are listed in **Table 2** and are further discussed below.

The first strategy successfully applied to increase xylitol yields from xylose using *S. cerevisiae* was the production of XR, encoded by *XYL1* gene, from the xylose-fermenting *Scheffersomyces stipitis*, formerly known as *Pichia stipitis*. Since *S. cerevisiae* does not possess XDH activity, recombinant strains require a co-substrate for cell growth and regeneration of NADPH or NADH cofactors, which are essential for the enzymatic reduction of xylose by the XR (Hallborn *et al.*, 1991). Different co-substrates can be supplied to the recombinant cells for the bioconversion process. Hallborn *et al.* (1994) evaluated xylitol production using glucose, ethanol, acetic acid and glycerol as co-substrates, and only glucose and ethanol were efficiently used as carbon source. Similarly, the utilization of glucose as co-substrate improved the xylitol production by a recombinant *S. cerevisiae* harboring the *XYL1* gene from *Candida shahatae*, in comparison with the results obtained using galactose and maltose as co-substrate (Govinden *et al.*, 2001). Nevertheless, it should be taken into consideration that in *S. cerevisiae* the uptake of glucose and xylose occurs by facilitated diffusion through the same transport system (Hamacher *et al.*, 2002) and the presence of glucose competitively inhibits the transport of xylose into the cells, decreasing the xylitol productivity (Subtil and Boles, 2012). To maintain cofactor regeneration and yeast metabolism without glucose repression, a glucose-limited fed-batch fermentation strategy has been proposed for xylitol production processes. This optimized fermentation, based on a high molar ratio of xylose to glucose during the bioconversion phase, resulted in 105.2 g l^{-1} of xylitol with an improved 1.69 g l^{-1} h^{-1} productivity (Lee *et al.*, 2000). A similar strategy was further applied to compare the xylitol production by expressing the *XYL1* gene from *S. stipitis* in *S. cerevisiae* using an episomal expression vector and chromosome integration (Chung *et al.*, 2002). The high xylitol productivity and improved xylose conversion rate in the recombinant BJ3505/δXR yeast was attributed to the stable expression of the XR encoding gene by using the multicopy chromosomal δ-integration method. In addition, the chromosomal stability of exogenous XR in this recombinant *S. cerevisiae* BJ3505/δXR was demonstrated in cell-recycle fermentations, resulting in 116 g l^{-1} of xylitol and 2.34 g l^{-1} h^{-1} overall productivity (Bae *et al.*, 2004). Despite the reported high productivity, it should be noted that cell recycling processes require additional equipment such as fiber membrane filtration, which can increase the overall cost of the process in industrial-scale production.

In addition to glucose-limited fed-batch fermentation, transport engineering is an alternative to bypass glucose repression. The expression of the transporter-encoding gene *AraE* from *Bacillus subtilis* improved the xylitol productivity (2.47 g l^{1} h^{-1}) in *S. cerevisiae* expressing two copies of the *XYL1* gene from *S. stipitis* (Kim *et al.*, 2017).

Alternatively, the simultaneous utilization of cellobiose (a dimer of glucose) and xylose was proposed to prevent glucose repression since cellobiose does not inhibit xylose uptake. For cellobiose utilization, a cellodextrin transporter (*CDT1* gene) and intracellular ß-glucosidase (*GH11* gene) from the filamentous fungus *Neurospora crassa* were introduced in the engineered *S. cerevisiae* expressing the xylose reductase from *S. stipitis*. The resulting D-10-BT efficiently produced xylitol by cellobiose and xylose co-consumption (Oh *et al.*, 2013).

The availability of reduced cofactors for XR activity has been identified as an important controlling factor for xylitol production. As mentioned before, XRs strongly prefer NADPH over NADH and the main source of NADPH in yeast cells is the oxidative PPP. In this sense, several research efforts have been made to control this pathway and ensure sufficient NADPH supply for an efficient xylitol production. In the PPP, NADPH is produced from NADP+-dependent dehydrogenases: glucose-6-phosphate dehydrogenase (G6PDH) responsible for the conversion of glucose 6-phosphate to 6-phosphoglucose-lactone, and 6-phosphogluconate dehydrogenase (6PGDH) which catalyzes the conversion of 6-phosphogluconate to ribulose 5-phosphate (**Fig. 1**). The overproduction of the G6PDH protein (encoded by *ZWF1* gene) increased NAPDH regeneration, resulting in a 25% improvement of xylitol productivity compared to the recombinant *S. cerevisiae* strain only expressing the XR encoding gene (Kwon *et al.*, 2006a). Additionally, a

Table 2 Main metabolic engineering and cultivation strategies for improved xylitol production by the yeast *Saccharomyces cerevisiae* and corresponding main xylitol production parameters

Saccharomyces cerevisiae strain	*Genetic modification*	*Cultivation strategy*	*Xylitol*			*Reference*
			Maximal concentration g l^{-1}	*Productivity g l^{-1} h^{-1}*	*Yield g g^{-1}*	
GPY55–15Bα	*XYL1* (xylose reductase) from *Scheffersomyces stipitis* CBS6054	Batch	19	NR	>0.95	Hallborn *et al.* (1991)
EH13.15: pY2XR	*XYL1* (xylose reductase) from *Scheffersomyces stipitis*	Fed-batch	105	1.69	>0.95	Lee *et al.* (2000)
BJ3505/δXR	*XYL1* (xylose reductase) from *Scheffersomyces stipitis* CBS5776	Fed-batch	78	1 1.1	0.9	Chung *et al.* (2002)
BJ3505/δXR	*XYL1* (xylose reductase) from *Scheffersomyces stipitis* CBS5776	Cell-recycling fermentation	116	2.34	0.9	Bae *et al.* (2004)
Y294: pRG16	*XYL1* (xylose reductase) from *Candida shehatae*	Batch	15	NR	0.86	Govinden *et al.* (2001)
BJ3505/δXR-pKZWF1	*XYL1* (xylose reductase) from *Scheffersomyces stipitis* CBS5776; *ZWF1* (glucose – 6-phosphate dehydrogenase)	Fed-batch	86	2.0	≈1	Kwon *et al.* (2006a)
DWM-ZWF1-ACS1	*XYL1* (xylose reductase, NADPH-dependent) from *Scheffersomyces stipitis; ΔXYL* (mutant xylose reductase, NADH-preferring); *ZWF1* (glucose – 6-phosphate dehydrogenase); *ACS1* (acetyl-CoA synthetase)	Fed-batch	196	4.27	≈1	Jo *et al.* (2015)
ScpGT	*XYL1* (xylose reductase) from *Scheffersomyces stipitis, Candida tropicalis, Neurospora crassa* and *SUT1* (xylose specific transporter)	Fed-batch	19.7	0.28	≈1	Kogje and Ghosalkar (2016)
D-10-BT	*XYL1* (xylose reductase) from *Scheffersomyces stipitis; CDT1* (cellodextrin transporter); *GDH11* (ß-glucosidase) from *Neurospora crassa*	Fed-batch	93	1.50	0.98	Oh *et al.* (2013)
DXXA	*XYL1* gene from *Scheffersomyces stipitis*; *AraE* (arabinose: H^+ symporter) from *Bacillus subtilis*	Fed-batch	178	2.47	1	Kim *et al.* (2017)
PE – 2-GRE3	*GRE3* (endogenous aldose reductase)	Fed-batch	148	1.16	0.95	Baptista *et al.* (2018)

NR, not reported.

metabolic engineering approach based on the redirection of the carbon flux to the PPP by limiting the entry of a carbon into glycolysis was developed to further increase the NADPH availability in XR producing yeast. The authors have proposed the downregulation of the *PGI1* gene, coding for phosphoglucose isomerase (PGI), to reduce the conversion of glucose-6-phosphate to fructose-6-phosphate in glycolysis, resulting in accumulation of glucose-6-phosphate that can be used by G6PDH in the PPP and consequent NADPH regeneration (**Fig. 1**). The reduction of PGI activity alone had no positive effect on xylitol productivity but the simultaneous overexpression of the *ZWF1* gene in a recombinant PGI mutant strain resulted in 1.9-fold improvement compared with the parental strain expressing *XYL1* gene (Oh *et al.*, 2007).

The evaluation of xylitol production in terms of enzyme and cofactor preference was performed by engineering the industrial *S. cerevisiae* PE-2 strain (over)expressing different enzymes with XR activity: a NADPH-preferable native XR, a NADH-preferable mutant XR and the NADPH-dependent aldose reductase (encoded by the endogenous *GRE3* gene) that uses solely NADPH. The strains expressing enzymes with NADPH preference showed superior performance and the GRE3-overexpressing strain (PE-2-GRE3) was able to produce 148.5 g l^{-1} of xylitol with 0.95 g g^{-1} yield (Baptista *et al.*, 2018). The overexpression of the *GRE3* gene in *S. cerevisiae* ScpGT was also described as a better strategy for xylitol production, in comparison with the expression of the XR encoding gene from *S. stipitis*, *C. tropicalis*, and *N. crassa* (Kogje and Ghosalkar, 2016).

In a different approach, to overcome the preference for NADPH and allowing the simultaneous utilization of NADPH and NADH cofactors, a recombinant *S. cerevisiae* DWM-ZWF1-ACS1 overexpressing the *ZWF1* gene (G6PDH protein), was highly engineered for the production of both NADPH-preferable native XR and NADH-preferable mutant XR from *S. stipitis*, together with the overexpression of *ACS1* gene encoding for the acetyl-CoA synthetase. This enzyme is responsible for the conversion of acetate in acetyl-CoA that is further metabolized into the tricarboxylic acid cycle (TCA), resulting in NADH and NADPH regeneration. The co-production of the two types of XR with G6PDH and acetyl-CoA resulted in 196.2 g l^{-1} xylitol with a maximal productivity of 4.27 g l^{-1} h^{-1} (Jo *et al.*, 2015).

Table 3 Xylitol production by different yeast using detoxified and non-detoxified lignocellulosic waste as feedstock

Microorganism and genetic modifications	*Pretreatment*	*Xylitol*		*Reference*
		Maximal concentration g l^{-1}	*Yield g g^{-1}*	
(a) Fermentation using detoxified corn cob hydrolysates for xylitol production				
Debaryomyces hansenii NRRL Y – 7426	Autohydrolysis (T_{max} 202ºC, LSR 8 g g^{-1}) and posthydrolysis (125ºC, 0.5% H_2SO_4, 165 min)	62	0.73	Rivas *et al.* (2002)
Candida tropicalis	Dilute acid treatment (1% H_2SO_4, 121ºC, 30 min, LSR 8 w v^{-1})	11.89	0.58	Misra *et al.* (2013)
Candida tropicalis MTCC 6192	Dilute acid treatment (HNO_3 0.5%, 121ºC, 30 min, LSR 10 g g^{-1})	33.4	0.66	Kumar *et al.* (2018)
Candida tropicalis As 2.1776	Dilute acid treatment (1% H_2SO_4, 121ºC, 40 min, LSR 10 g g^{-1})	96.5	0.83	Li *et al.* (2012)
Candida tropicalis HDY – 02	Dilute acid treatment (1% H_2SO_4, 120ºC, 60 min) LSR 10 g g^{-1})	58	0.73	Hongzhi *et al.* (2011)
Candida tropicalis W103	Dilute acid treatment (1% H_2SO_4, 120ºC, 60 min, LSR ratio 10 g g^{-1})	68.4	0.70	Cheng *et al.* (2009)
Candida magnoliae FERMP – 16522	Dilute acid treatment (1%–3% H_2SO_4, 60 min, liquid to solid ration 10 g g^{-1})	49	0.62	Tada *et al.* (2012)
Candida sp. ZU04	Dilute acid treatment (1% w w^{-1} H_2SO_4, 110ºC, 180 min, LSR 8 g g^{-1})	34.5	0.60	Ding and Xia (2006)
Kluyveromyces marxianus YZB001	Data not supplied	11.32	0.60	Zhang *et al.* (2013)
(b) Fermentation using non-detoxified hemicellulosic hydrolysates for xylitol production				
S. cerevisiae YRH 396 (*XYL1*/*XYL2* expression and *XKS1* overexpression)	Dilute acid treatment (1% H_2SO_4, 121ºC, 40 min, LSR 10 g g^{-1})	8.17	0.45	Cortivo *et al.* (2018)
S. cerevisiae XP-RTK (*STU1* expression and *GRE3* overexpression)	Steam acid treatment (2% H_2SO_4, 2% oxalic acid) in continuous digester (6 bar, 160ºC) LSR 5–5.5 g g^{-1}. Diluted non-detoxified hydrolysate	16.3	~1	Kogje and Ghosalkar (2017)
S. cerevisiae YPH499-PIU-XR-BGL-XYL-XYN *XR, BGL, XYL and XYN expression*	Autohydrolysis (160–240°C) and 10 MPa	5.8	0.79	Guirimand *et al.* (2016)
S. cerevisiae PE – 2-GRE3 (*GRE3* overexpression)	Autohydrolysis (Tmax 205ºC, LSR 8 g g^{-1}) and posthydrolysis (125ºC, 0.5% H_2SO_4, 165 min)	29.6	0.93	Baptista *et al.* (2018)

LSR, liquid to solid ratio.

Xylitol Production From Renewable Resources by Yeast

Global climate change and fossil fuel depletion have led to policies supporting carbon emission reduction and renewable energy deployment. Wastes and biomass residues from agricultural, forestry and food activities represent a valuable resource, in contrast to appropriation of edible food crops, for sustainable production of bioenergy and biochemicals (Liu and Rajagopal, 2019). Being widely available, lignocellulosic biomass is mainly composed of three polymers: cellulose, hemicelluloses, and lignin (Palmqvist and Hahn-Hägerdal, 2000). Xylan is the most abundant polysaccharide in the hemicellulosic fraction of agro-industrial residues and hardwood that can be hydrolyzed into xylo-oligosaccharides (XOS) and xylose. In terms of fully adopting a sustainable process, different environmental friendly pretreatments have been developed to obtain xylose from xylan present in hemicellulose. Dilute acid treatment is a simple, efficient and low-cost method, commonly used for xylose recovery (**Table 3**). Nevertheless, in a biorefinery perspective that includes the valorization of whole biomass, hydrothermal pretreatment (also known as autohydrolysis or liquid hot water) is considered a promising technology for lignocellulose biomass fractionation since it allows hemicellulose solubilization and also increases the cellulose accessibility for enzymatic hydrolysis (Ruiz *et al.*, 2020). Autohydrolysis involves only water at high temperature and pressure, without adding any other chemicals. Under these conditions, hydronium ions formed by water dissociation into H_3O^+ and OH^-, catalyze hydrolysis of the xylan into oligosaccharides (XOS). These XOS can be further hydrolyzed by acids or enzymes to obtain xylose (Hu *et al.*, 2016; Rivas *et al.*, 2002), which can be used for the production of xylitol. However, pretreatment of lignocellulosic biomass also generates microbial inhibitory, such as weak acids (Guo and Olsson, 2014), furans (Larsson *et al.*, 2000), and phenolic compounds (Liu *et al.*, 2004), that negatively affect cell growth and metabolism, reducing microbial fermentative performance in terms of product yield and productivity (Palmqvist and Hahn-Hägerdal, 2000). To circumvent this bottleneck, a considerable number of physical and chemical techniques for detoxification of lignocellulosic hydrolysates have been applied to remove these inhibitory compounds prior to fermentation (Kumar *et al.*, 2020). Accordingly, several studies have been using different natural xylitol-producing yeasts (*D. hansenii, C. tropicalis, Caloptilia magnolia, K. marxianus*) to produce xylitol from detoxified lignocellulosic hydrolysates (**Table 3**). Nevertheless, the additional detoxification step implies operational costs and also results in a considerable loss of sugars, which further impairs the economics of the overall process.

For the establishment of cost-efficient lignocellulose-based xylitol production it is necessary to use robust microorganisms capable of withstanding acute stresses with improved fermentative performances. The yeast *S. cerevisiae* has developed several

mechanisms to respond to the presence of inhibitors derived from different biomass pretreatments (Cunha *et al.*, 2018). In fact, *S. cerevisiae* strains isolated from industrial harsh conditions (such as elevated temperatures, pH variations and presence of toxic compounds) have proved to be less sensitive to the negative effects caused by the presence of multiple lignocellulose-derived inhibitors (Cunha *et al.*, 2018; Della-Bianca and Gombert, 2013; Pereira *et al.*, 2014). In this sense, the use of industrial isolates that naturally present desirable traits (fast sugar consumption, inhibitor and high temperature tolerance, higher detoxification capacity) could be advantageous for xylitol production in lignocellulose-based processes. Accordingly, Cortivo *et al.* (2018) developed a strategy using substrate mixture of soybean and oat hulls for ethanol and xylitol production with an industrial *S. cerevisiae* strain. Under this biorefinery concept, the recombinant YRH 396 strain expressing the XR and XDH genes of *S. stipitis* and overexpressing the native *XKS1* gene encoding the xylulose kinase (XK), which is responsible for the conversion of xylulose to xylulose-5-P (**Fig. 1**) produced 8.17 g l^{-1} of xylitol and 2.9 g l^{-1} of ethanol through the integral utilization of both residues.

Kogje and Ghosalkar (2017) engineered an industrial *S. cerevisiae* strain to overexpress the endogenous aldose reductase with XR activity (encoded by *GRE3* gene) and a xylose specific transporter from *S. stipitis* (encoded by *SUT1* gene). The *S. cerevisiae* XP-RTK strain efficiently produced xylitol (16.3 g l^{-1}) from a non-detoxified corn cob hemicellulosic hydrolysate supplemented with glucose in fed-batch mode.

More recently, Baptista *et al.* (2018) developed an efficient and environmental-friendly strategy to produce xylitol using corn cob. In this approach, the *S. cerevisiae* PE-2 industrial strain presenting innate capacity for xylitol accumulation (Romani *et al.*, 2015), robustness features in general (Pereira *et al.*, 2011), and inhibitor tolerance, in particular (Pereira, *et al.*, 2014), was engineered by overexpression of the *GRE3* gene. To obtain a sustainable xylitol production through the valorization of whole slurry corn cob (the solid and liquid fractions obtained from the corn cob autohydrolysis pretreatment), the hemicellulosic hydrolysate (liquid fraction), after hydrolysis, was used as source of xylose, while the glucan-enriched solid phase was efficiently hydrolyzed into glucose in a saccharification and fermentation (SSF) process. The glucose released was used by recombinant *S. cerevisiae* PE-2-GRE3 cells during the bioconversion process, resulting in 29.6 g l^{-1} of xylitol with a high yield of xylitol from non-detoxified corn cob hydrolysate (0.9 g g^{-1}). This integrated process demonstrated the feasibility of using whole slurry corn cob for xylitol production.

During the last years, consolidated bioprocessing (CBP) has emerged as a promising fermentation approach for the production of bioethanol and or other valuable products from lignocellulosic biomass. The CBP system includes enzyme production, substrate hydrolysis and fermentation in a single step, reducing the number of unit operations of feedstock processing and the overall cost of the production process (Hasunuma and Kondo, 2012). The cell surface engineering technology, based on displaying functional heterologous enzymes on the cell surface, has shown to be effective for consolidated bioprocessing of lignocellulosic materials to xylitol. Guirimand *et al.* (2016) achieved the direct conversion of xylose from rice straw hydrolysate into xylitol by engineering a *S. cerevisiae* strain to produce degrading enzymes (β-glucosidase from *Aspergillus aculeatus*, β-xylosidase from *Aspergillus oryzae* and endoxylanase from *Trichoderma reesei*) on the cell surface together with the production of the cytosolic of XR from *S. stipitis*. The recombinant YPH499-PIU-XR-BGL-XYL-XYN strain produced 5.8 g l^{-1} xylitol with 79.5% of theoretical yield from xylose contained in the rice straw hydrolysate (Guirimand *et al.*, 2016). Although this cell surface engineered strain demonstrated xylitol production in a consolidated bioprocessing, the rate and yield of production are far below industrial requirements and need to be improved to achieve a successful process implementation.

Conclusions

Nowadays, xylitol applications are extended to several industrial sectors and the demand for healthy and low-calorie products by the modern consumers is a key factor to increase the xylitol market. The implemented manufacturing process is based on a costly chemical method that has high energy requirements. In this sense, the development of an environmental friendly and cost-competitive biotechnological process has received growing attention. Numerous xylose-fermenting yeasts have been investigated for the bioconversion of xylose to xylitol, but in spite of their natural xylitol production capacity, product yields are limited by the utilization of xylose for yeast growth and maintenance of energy. Alternatively, the use of engineered *S. cerevisiae* with increased production of enzymes with XR activity allows an easier control of the carbon sources for the bioconversion and for yeast metabolism. A considerable number of metabolic engineering approaches have been applied in recent years to convert *S. cerevisiae* into an efficient xylitol producer. Furthermore, to attain a fully sustainable xylitol production system based on renewable raw material utilization, the use of *S. cerevisiae* strains presents another advantage in terms of increased tolerance towards the presence of lignocellulosic-derived inhibitors, in comparison to non-*Saccharomyces* yeast. Although, the implementation of a cost-effective process for xylitol producing using renewable feedstocks hinges on overcoming challenges, such as biomass fractionation and effect of multiple microbial inhibitors/stressors present in lignocellulosic hydrolysates, understanding the mechanisms involved in cell-based bioconversion open new perspectives for the development of sustainable xylitol production.

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Biotechnology of Wine Yeasts

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A Taste of Biotechnological History

Fermented foods and beverages are not just by-products of civilization, they are central to it. Whether alcoholic beverages, such as beer and wine, were offshoots from breadmaking by the first farmers, it is clear that yeast fermentation catalyzed one of the greatest transitions in human history. From the rituals of Stone Age hunter-gathers and the origin of farming to the origin of writing and transport on wheels, one can find a possible link to yeast fermentation. All kinds of crops were fermented, and humans imbibed long before they invented the first phonetic alphabet and could count in Arabic numericals. In fact, some archeologists argue that alcoholic beverages were significant contributors to humankind's creativity, which helped foster the development of language, the arts, science and technology. They also postulate that yeast-derived alcoholic beverages inspired our hunter-gatherer ancestors to domesticate grains and fruits. The oldest firm evidence of an alcoholic beverage comes from Jiahu, China, where by 7000 BCE, a mix of rice, grapes, hawthorn berries and honey were fermented in clay jars (McGovern *et al.*, 2004).

Winemaking dates near the dawn of civilization. In the Caucasus mountains of modern-day Georgia and the Zagros Mountains of Iran as we know it today, grapes were one of the earliest fruits to be domesticated and it is estimated that wine was made as early as 7400 years ago (This *et al.*, 2006). Centuries later, with the rise of the Roman Empire, wine became the beverage of choice throughout classical antiquity. One could, therefore, think of winemaking – the fermentation of grape juice with yeast – as one of the oldest biotechnologies in the world, steeped in a long and rich history of tradition and innovation.

In today's language, biotechnology is defined as the study or application of a living organism or any of its parts to produce a product, often of commercial value. More specifically, wine biotechnology is the study of the biological fundamentals of the three main organisms involved, grapes (mostly noble varieties of *Vitis vinifera*); yeast (*Saccharomyces* and non-*Saccharomyces* species); and bacteria (malolactic bacteria of which *Oenococcus oeni* is the best known wine bacterium), as well as the fermentation and processing technologies through which grape juice is converted in to flavorsome wine. This chapter focuses on *Saccharomyces* and non-*Saccharomyces* yeast involved in modern-day winemaking.

The Rising Power of Wine Yeast

The French pioneer microbiologist, Louis Pasteur, was the first to emphatically demonstrate the role of yeasts during wine fermentation, in that it is the principal catalyst responsible for the conversion of grape-must sugars to alcohol and CO_2 (Barnett, 2000). In the mid-1800s, Pasteur isolated and noted different microorganisms co-existing during the fermentations, including different types of yeasts. Based on his drawings, two main yeasts were observed. The first was a small, lemon-shaped yeast abundant during the first stages of fermentation, which he called *Saccharomyces apiculatus* (now most probably known as *Hanseniaspora uvarum*). The second, which increased in abundance as the fermentation progressed, was a larger and rounder yeast, which Pasteur named either *Saccharomyces pastorianus* or *Saccharomyces ellipsoideus* (most likely the current *Saccharomyces cerevisiae*). Soon afterwards, the Swiss-German wine pioneer, Herman Müller, started recording the use of specially selected pure yeast cultures for his wine fermentations.

Now, many researchers use the term "yeast" to describe *S. cerevisiae* or closely-related species. This is mainly because *S. cerevisiae* is by far the most studied yeast and work on it has contributed greatly toward fundamental research. In 1996, it became the first completely sequenced eukaryote (Goffeau *et al.*, 1996). *S. cerevisiae* also has a widespread industrial role, used as a workhorse organism in a variety of fields, including the bioenergy and medical industries, as well as food and beverage production. *S. cerevisiae* is the main microbial player in modern-day winemaking. It is the chief fermenter of the sugars found in grape must and in most cases outcompetes other microorganisms during fermentation due to its prolific capacity to produce high concentrations of ethanol. Besides ethanol production, many *S. cerevisiae* strains also produce so-called killer factors, proteins or glycoproteins facilitated by cytoplasmically inherited double-stranded RNA viruses that are capable of entering susceptible strains (Becker and Schmitt, 2017; Wang *et al.*, 2015).

Many other types of yeasts are also present during fermentation, especially in the early phases, and do exert an influence on the final wine product as will be discussed later. These yeasts are often grouped under the term "non-*Saccharomyces* yeast" (NSY) or "non-conventional yeast" with most being closely related to *Saccharomyces*.

The Essence of Winemaking

Winemakers generate "must" from harvested grapes, involving a process of destemming, crushing and macerating the grapes. Once the must has been prepared, winemakers typically add sulfur dioxide (SO_2) to their must for three main reasons: (1) it is an

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antioxidant, (2) it inhibits both bacterial and many non-*Saccharomyces* yeast growth, and (3) it is a binding partner of carbonyl and keto compound like the reactive molecule acetaldehyde. Thus, this step has a profound effect on the microbial population present to conduct the fermentation and does not impact on wine strains of *S. cerevisiae*, which have an inherent resistance to SO_2. Before 1965, winemakers allowed the natural microbial population to ferment the must, largely because of the non-existent technical capabilities to manage selected yeasts. This is known as a spontaneous fermentation. Since the late 1960s, with the possibility to produce dried yeast starter cultures, commercial yeast starter cultures became available for winemakers to add at the beginning of their fermentation and were well received. When yeast is added to must it is known as an "inoculated" or "guided" fermentation. Spontaneous fermentations are still used today to promote the sense of *terroir*, but the overwhelming majority of particularly large-scale fermentations are conducted via inoculated fermentations for the sheer consistency such a fermentation provides. Spontaneous fermentations often perform sluggishly or become "stuck" meaning the microbes in the vessel has stopped their fermenting action even though a meaningful amount of unwanted grape sugars remain. Spontaneous fermentations often perform sluggishly or become "stuck" meaning unwanted grape sugars remain even though the microbes in the vessel have stopped their fermentation action. This is difficult to remedy without affecting the quality of the resulting wine. Today, many companies world-wide provide yeast starter cultures for winemakers. At the time of writing, around 200 yeast strains, belonging to the genus *Saccharomyces*, are commercialized by only seven companies working worldwide. These catalogs of different wine yeast strains (*Saccharomyces* and non-*Saccharomyces*) have been developed to assist winemakers to create a variety of styles and must compositions. Once the so-called primary fermentation is deemed finished, meaning the majority of the sugars have been converted to ethanol, another process takes place prior to bottling. Depending on the desired wine style, a secondary fermentation known as "malolactic fermentation" could take place where malic acid is converted to lactic acid (normally conducted by lactic acid bacteria) as well as fining, filtration and maturation (often done in wooden barrels).

The vessels in which fermentations takes place can range from large steel and cement tanks to as small as an Erlenmeyer flask or a test tube which would then be called a microvinification. Microvinifications are an important research tool for testing all aspects of enology, but particularly the performance of the yeast. Promising findings from these small-scale experimentations could then be used in larger scale fermentations.

The Dominant Role of Saccharomyces Cerevisiae

With the aid of genomic analyzes, we know that an important genome-related event happened approximately 100 million years ago to an ancestor of the current *S. cerevisiae* yeast in that its whole genome duplicated (WGD; Kellis *et al.*, 2004). Genomic comparisons with the yeast *Kluyveromyces waltii* (a closely-related yeast which has not undergone WGD) substantiated this claim: *S. cerevisiae* has twice the number of chromosomes (sixteen versus eight), yet similar number of genes, with *K. waltii* also showing an extensive level of synteny with *S. cerevisiae*. The WGD event resulted in both massive gene loss paired with the accelerated evolution of one of the duplicated genes. Promoter rewiring of key genes involved in fermentative and respirative growth and the expansion of the alcohol dehydrogenases culminated in the *S. cerevisiae* we have today – a yeast with the ability to make ethanol even in the presence of oxygen (Piskur *et al.*, 2006). This important feature makes *S. cerevisiae* useful in both biofuel production and the beverage industry, including winemaking. *S. cerevisiae* is one of few organisms capable of converting all the sugars in a grape must to various fermentation products, but most importantly ethanol. Ethanol acts as a preservative and impacts sensory perception in wine but is most appreciated by consumers for its hedonistic value.

Yeast are not only responsible for the conversion of grape sugars to ethanol and CO_2, but also cause a total biotransformation of the grape must, as practically all components in the must are affected by yeast metabolism. Yeast perform an equally important task in the formation of aroma-active secondary metabolites, as well as in the conversion of grape aroma precursors to varietal wine aromas (Styger *et al.*, 2011). The aroma-active compounds derived from central carbon metabolism are divided into four main categories, i.e., higher alcohols, aldehydes, medium-chained fatty acids and esters (**Table 1**). Many of the genes involved in the pathway synthesis of aroma compound production in *S. cerevisiae* have already been identified (Dzialo *et al.*, 2017; Bisson and Karpel, 2010). There are, however, many other enzymes/gene products involved in aroma production. Some gene products that are indirectly involved in the synthesis of a given aroma compound have been identified with the aid of methods connected with reciprocal-hemizygosity analysis in order to identify quantitative trait loci (Steyer *et al.*, 2012).

Higher alcohols (also known as fusel alcohols) are, from a quantitative point of view, the most abundant category of aroma compounds produced during fermentation. The biosynthesis of higher alcohols is conducted via the catabolism of amino acids known as the Ehrlich pathway (Hazelwood *et al.*, 2008). There are three enzymatic steps: (1) transaminases would deaminate the different amino acids to their corresponding α-keto acid. Depending on the amino acid, different enzymes encoded by the genes *ARO8*, *ARO9*, *BAT1* or *BAT2* would conduct the catalysis of the first step; (2) α-ketoacids are decarboxylated to their respective aldehydes – five decarboxylases have been identified in *S. cerevisiae* to conduct this action (encoded by *PDC1*, *PDC5*, *PDC6*, *ARO10* and *THI3*); and (3) alcohol dehydrogenases, encoded by *ADH1–6* and *SFA1*, would then catalyze the reduction of the aldehyde generating the higher alcohol. The higher alcohol hexanol is an important wine constituent but is derived from the grape must and not from yeast metabolism. Higher alcohols have a relatively high odor threshold but as most are in the milligrams per liter range, they do contribute to the overall bouquet of the final wine aroma (Lilly *et al.*, 2006). Most higher alcohols are not considered desirable, with the notable exception of 2-phenylethanol which imparts a flower/rose-like note to a wine. Higher alcohols do, however, act as substrates for acetate esters (van Wyk *et al.*, 2018).

Table 1 Important groups of aroma-active compound modulated by yeast in wine. Odor active values are as calculated in various aqueous solutions including beer, wine and water. ni = no information, nd = not detectable

Compound produced by yeast	*Aroma characteristics*	*Reported concentration in wine (mg/L)*	*odor threshold (mg/L)*
Higher alcohols			
Propanol	Stupefying	9–125	500–800
Butanol	Fusel odor	0.58	5
Isobutanol	Solvent	9–140	75
Active amyl alcohol	Marzipan	15–150	65
Isoamyl alcohol	Marzipan	45–490	7–300
Hexanol	Grassy	0.3–12	4–5.2
Tyrosol	Bees wax, honey-like	Trace	ni
Tryptophol	Phenol	Trace	ni
2-Phenethylethanol	Floral, rose	10–180	7.5–125
Aldehydes			
Acetaldehyde	Green, grassy	10–500	0.05
Medium-chained volatile fatty acids			
Butyric acid	Pungent	Trace	2.2–4
Isobutyric acid	Pungent	Trace	8.1
Isovaleric acid	Rancid, cheese, sweaty	Trace	0.7
Hexanoic acid	Rancid, fatty, pungent	nd–37	8–8.8
Octanoic acid	Oily, fatty, rancid, soapy	nd–41	13–15
Decanoic acid	Fatty, unpleasant, rancid	nd–54	8.2–10
Esters			
Acetate esters			
Ethyl acetate	Fruity, solvent, glue, balsamic	5–63.5	7.5–60
2-Methylpropyl acetate (isobutyl acetate)	Fruity, apple	nd–0.17	1.6
3-Methylbutyl acetate (isoamyl acetate)	Banana, fruity	0.03–5.52	0.03–0.16
2-Phenylethyl acetate	Rose, floral	nd–0.26	0.25–1.8
Hexyl acetate	Green, herbaceous, grape	nd–3.90	0.002–2.4
Ethyl esters			
Ethyl 2-methylpropanoate (ethyl isobutyrate)	Fruity, strawberry, lemon	0.01–0.48	0.001–5
Ethyl 2-methylbutanoate	Apple, strawberry, cider, anise	nd–0.03	0.0001–0.018
Ethyl 3-methylbutanoate (ethyl isovalerate)	Fruity, pineapple, lemon, anise	nd – 0.07	0.0001–1.3
Ethyl 2-hydroxypropanoate (ethyl lactate)	Fruity, milky, soapy	3.05–297.5	0.05–150
Ethyl 3-hydroxybutanoate	Fruity, green	0.05–0.58	20
Ethyl 4-hydroxybutanoate	Caramel	6.61	ni
Diethyl butanedioate (diethyl succinate)	Fruity, fermented, floral	1.21–61.11	ni
Diethyl hydroxybutandioate (diethyl malate)	Brown sugar, sweet	0.81	ni
Ethyl butanoate	Floral, Fruity, strawberry	0.07–0.53	0.001–0.4
Ethyl hexanoate (ethyl caproate)	Fruity, strawberry, green apple	0.15–1.64	0.005–0.85
Ethyl octanoate (ethyl caprylate)	Sweet, fruity, beer	0.14–2.61	0.002–0.58
Ethyl decanoate (ethyl caprate)	Oily, fruity, floral	0.01–0.70	0.012–0.51
Ethyl 3-phenylpropanoate (ethyl dihydrocinnamate)	Floral	nd – 0.003	0.002
Ethyl 3-phenylprop – 2-enoate (ethyl cinnamate)	Honey, cinnamon	nd – 0.01	0.001–0.048
Ethyl 4-hydroxy 3-methoxy-benzoate (ethyl vanillate)	Floral, sweet, vanilla	0.46	ni

Note: Values obtained from Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its importance to wine aroma – A review. South African Journal of Enology and Viticulture 21, 97–129. Sumby, K.M., Grbin, P.R., Jiranek, V., 2010. Microbial modulation of aromatic esters in wine: Current knowledge and future prospects. Food Chemistry 121, 1–16.

The aldehydes generated during amino acid catabolism are generally quickly converted to alcohols and are thus not found in meaningful levels in wine, with the only major aldehyde produced by *S. cerevisiae* metabolism being acetaldehyde (Liu and Pilone, 2000). Acetaldehyde comprises over 90% of the total aldehyde content in wine (Romano *et al.*, 1994). Pyruvate, an α-keto-acid, and the end-product of glycolysis is decarboxylated via the action of gene products encoded by *PDC1*, *PDC5*, and *PDC6* to acetaldehyde. Acetaldehyde is further converted to ethanol via the action of several alcohol dehydrogenases with Adh1p being the most prominent. Adh2 is responsible for the catalysis of the reverse reaction converting ethanol back to acetaldehyde. Acetaldehyde imparts a pleasant fruity aroma to wine at low concentrations but is considered an off-flavor when in excess providing an unpleasant grassy green note to wine. Depending on the yeast and style of winemaking levels of acetaldehyde can vary considerably ranging from 10 to 300 mg/L. Besides its role in aroma, it reacts with SO_2 forming a complex that reduces the antimicrobial and antioxidative effect of SO_2 addition. Acetaldehyde is also involved in the condensation reactions of anthocyanins with tannins which results in a stable wine pigment in red wines (Chen *et al.*, 2018).

Medium-chain volatile fatty acids are synthesized by yeasts as intermediates in the biosynthesis of long-chain fatty acids (Lambrechts and Pretorius, 2000). They do impact, to a lesser degree, the final aroma but are more important in providing the carboxylic acid moiety in the production of ethyl esters. Acetyl-CoA is considered the starting molecule in the biosynthesis of fatty acids and is formed by the oxidative decarboxylation of pyruvic acid. Two enzyme systems, acetyl-CoA carboxylase (encoded by *ACC1*) and the fatty acid synthetase complex, continue in converting acetyl-CoA to malonyl-CoA and elongating the chain of fatty acid. Depending on their solubility, fatty acids can also act as potential inhibitors and thus affect the fermentation capability of yeast.

Esters (grouped into acetate esters and ethyl esters) are arguably the most sought-after group of compounds imparting fruity and floral aromas to the wine bouquet (Sumby *et al.*, 2010). Acetate esters are derived from the condensation reaction from an alcohol and acetate-CoA whereas the ethyl esters result from the condensation reaction between an acyl-CoA (derived from medium-chain fatty acids) and ethanol. In general, esters possess a far lower odor threshold than their respective alcohol or fatty acid counterparts. The most prominent ester produced by yeast, ethyl acetate, can be considered either an acetate ester or an ethyl ester. In *S. cerevisiae*, the enzymes that drive these reactions have been identified. The two alcohol acetyltransferases Atf1p and Atf2p are chiefly responsible for acetate ester synthesis (Lilly *et al.*, 2006, 2000) with Eeb1p and Eht1p conducting ethyl ester synthesis (Saerens *et al.*, 2006). The enzyme Eat1p has also been implicated in ethyl acetate synthesis (Kruis *et al.*, 2018, 2017). The isoamyl-hydrolyzing esterase (Iah1p) has been shown to hydrolyze esters and convert them back to their respective higher alcohol (Lilly *et al.*, 2006; Fukuda *et al.*, 1998).

There are other compounds that form part of the central metabolism that could also impart flavor to the final wine product which are acetoin, 2,3-butanediol, α-acetolactate and diacetyl (Romano and Suzzi, 1996). Diacetyl, the only one with a low odor threshold, can impart an overall negative buttery note. As discussed in Romano and Suzzi (1996), there are various interconnected pathways in which *S. cerevisiae* can produce these metabolites, with diacetyl being formed either enzymatically from acetaldehyde or be converted non-enzymatically via α-acetolactate.

Sulfur-containing compounds also form a large class of wine volatiles that are extensively modulated by *S. cerevisiae*. Central to sulfur metabolism is the molecule hydrogen sulfide (H_2S), which is largely produced via the sulfate assimilation pathway during winemaking conditions (Huang *et al.*, 2017). It has a low odor threshold with a highly undesirable rotten egg aroma. Many factors contribute to excessive H_2S production, but this often occurs in grape must low in assimilable nitrogen. In general, the sulfur compounds that are intermediates in the central sulfur metabolism impart undesirable notes to the wine when excess amounts are produced (Swiegers and Pretorius, 2007; Lambrechts and Pretorius, 2000).

In contrast to the sulfur compounds discussed above, there are certain varieties of grape must that contain a significant amount of so-called varietal thiols that impart desirable notes to a wine (Ruiz, *et al.*, 2019a; Swiegers and Pretorius, 2007). The best studied varietal thiols are 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercaptohexan-l-ol, which impart box tree, broom, passion-fruit, guava and conifer aromas to a wine and have all exceptionally low odor thresholds. These compounds are often bound to a glutathione, cysteine or cysteine-glycine moieties in the must rendering them non-volatile. These bound thiols are capable of entering the yeast where functional carbon-sulfur lyases (Irc7p and Str3p) can release the thiol from its bound precursor (Holt *et al.*, 2011; Roncoroni *et al.*, 2011). There are other compounds particular to certain varieties of grape must – the most prominent are terpenes – and these are highly variable among vintages. They occur either already in free form or are bound to a sugar moiety. Although conflicting data exist, it is generally considered that *S. cerevisiae* strains do not possess the enzymatic capability to release terpenes from their bound precursors in wine, but there are proven activities from non-*Saccharomyces* which will be discussed later.

There are many other modulatory effects that *S. cerevisiae* has on transforming the grape must into wine. *Saccharomyces* has some effect on acid composition, as it can produce acetic acid (the major constituent of the volatile acidity of wine) and can have slight effects in the main wine acids, i.e., malic and tartaric acid levels. *S. cerevisiae* can also affect the color by modulating the anthocyanin composition of red wines (Benito *et al.*, 2011) and the haziness by producing pectinases (Fernández-González *et al.*, 2005; Vilanova *et al.*, 2000). Thus, the choice of yeast(s) for grape must fermentation is crucial for its impact on the final wine product.

Genomics of *Saccharomyces Cerevisiae*

Parallel to our increased understanding of the role of yeast in winemaking is the advancements in genomics. We have come a long way since the first sequencing of a yeast strain in 1996, with many companies now offering competitive pricing for the sequencing of entire genomes of organisms. The genomes of yeasts are routinely sequenced in many laboratories and it is not uncommon to find literature where the whole genome of multiple strains of yeast were eventually sequenced; either for comparative studies or to validate their findings (Borneman *et al.*, 2016; Gallone *et al.*, 2016). In addition, the development of user-friendly software to process and analyze the large amount of data generated from sequencing has made genome analysis significantly easier for researchers.

The 1996 sequence of the laboratory strain S288c (Goffeau *et al.*, 1996) along with subsequent revisions (Engel *et al.*, 2014) provided a complete map of the open reading frames in *S. cerevisiae* and enabled researchers to construct a knock-out library of this strain. The knock-out library contains null mutants of most of the non-essential genes (Brachmann *et al.*, 1998). This library has been invaluable to assign functions to genes; even extrapolating to human homologs.

The first whole genome of a wine yeast was performed on the haploid strain of AWRI1631 (a spore from the N96 wine strain; Borneman *et al.*, 2008) and currently efforts have been made to generate a knock-out library of AWRI1631 akin to the one made for S288c (Peter *et al.*, 2018). As the sequences of more wine and non-wine strains became available, a clearer picture of the

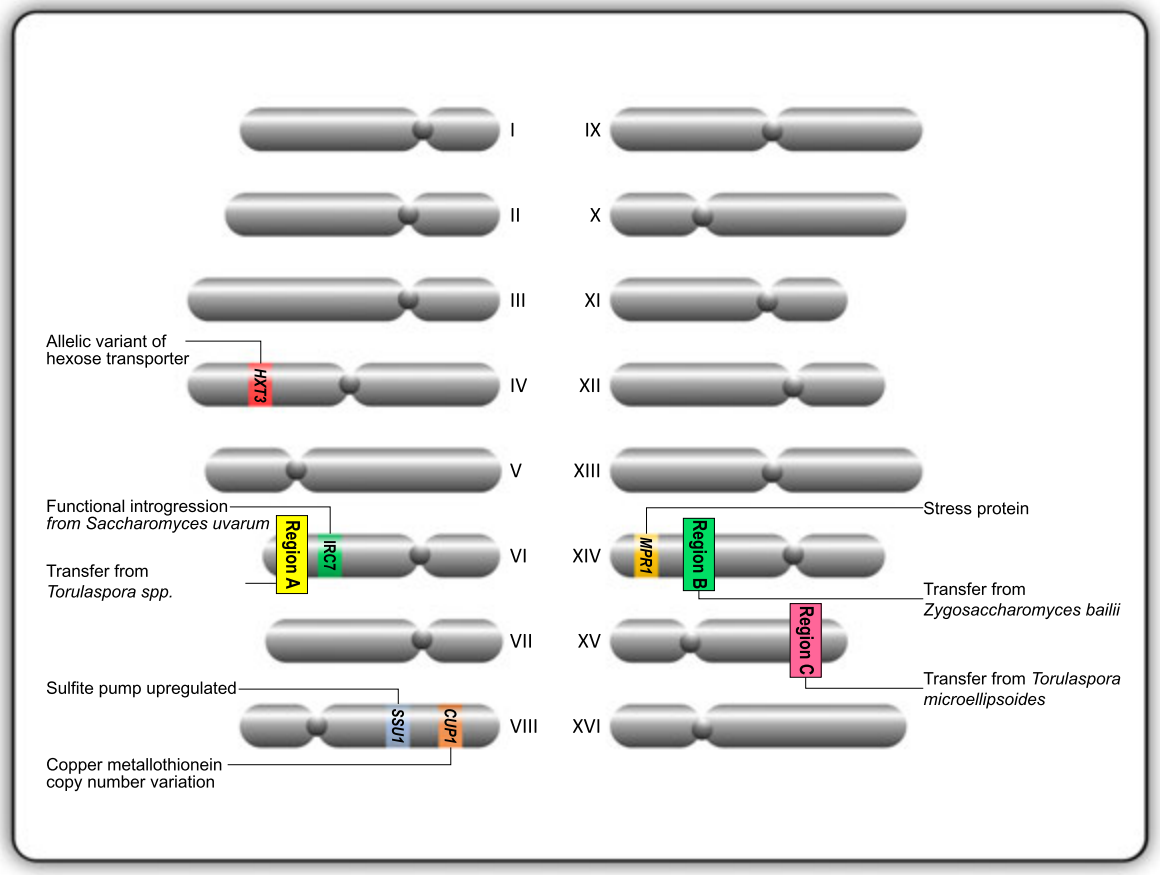

Fig. 1 Genomic features that have been identified in wine *S. cerevisiae* strains. Based on Belda, I., Ruiz, J., Santos, A., van Wyk, N., Pretorius, I.S., 2019. *Saccharomyces cerevisiae*. Trends in Genetics 35, 956–957.

genomic make-up of a wine yeast and how it differs from other *Saccharomyces* strains was revealed (Eldarov *et al.*, 2018; Borneman *et al.*, 2016; Ibáñez *et al.*, 2014; Dunn *et al.*, 2012; Hyma *et al.*, 2011; Novo *et al.*, 2009). Introgressed genes and horizontal gene transfers (HGT) played significant roles in the divergence of wine strains from other *Saccharomyces* strains. **Fig. 1** shows some of the major genomic features found in wine strains. A remarkable genomic trait that some wine strains contain are the three regions known as Region A, B and C presumably obtained via HGT from the wine yeasts *Torulaspora* spp., *ZygoSaccharomyces bailii* and *Torulaspora microellipsoides*, respectively.

Interestingly, as more genome analyzes are being conducted on *S. cerevisiae* strains it becomes evident how genomically similar many of the commercial strains are compared to the global pool of *S. cerevisiae* diversity (Borneman *et al.*, 2016).

Genetic Modification of Wine Yeast

S. cerevisiae possesses an extensive and sophisticated genetic modification toolbox with which researchers can manipulate its genome. Most of the initial development of this toolbox was undertaken on haploid laboratory strains, most notably the S288c or BY series of strains, which was the first strain whose whole genome was sequenced (Goffeau *et al.*, 1996). These laboratory strains often possess auxotrophic mutations in genes involved in nucleotide or amino acid biosynthesis and thus cannot grow in media without the presence of the specific nucleotide/amino acid. Restoration of growth occurs with the introduction of functional copies of these specific genes and can serve as selection markers for transformation. The most common auxotrophic genes are *URA3*, *LEU2*, *TRP1*, *HIS3*, *ADE2*, *LYS2*, *MET15* and *ARG2*. As *S. cerevisiae* naturally harbors multicopy episomal plasmids (2μ), many manipulations are based on this feature. Centromeric plasmids (low copy number), which contain a chromosomal-like autonomous replicating sequence (ARS) along with a part of a centromere sequence instead of a 2-micron sequence, are also common. A whole series of pRS-based *E. coli*/*S. cerevisiae* shuttle vectors (both episomal and centromeric) have been constructed and are publicly available (Tomlin *et al.*, 2001). With the notable exception of the T_{73} wine yeast strain, which has an uracil auxotrophy, wine strains are prototrophic (no mutations in the genes involved in amino acid or nucleotide biosynthesis pathways) and thus require dominant selection which often involves antibiotic resistance. Resistance cassettes against the antibiotics zeocin,

hygromycin, geneticin and nourseothricin are often used. Due to the concern over the exposure of antibiotic resistance markers in foodstuffs, non-antibiotic dominant markers have been developed which include the sulfometuron, cycloheximide, or *p*-fluorophenylalanine or high levels of $Na_2S_2O_5$ which targets the native versions of Ilv2p, Cyh2p, Aro4p and Fzf4p, respectively (Cebollero and Gonzalez, 2004). Introduction of a mutant version or the overexpression of the yeast gene would allow for the successful selection of yeast transformants. Regardless of the selectable marker used, wine yeast cannot realistically maintain plasmids with auxotrophic or antibiotic markers in a winemaking setting thus stable integration of a particular manipulation is desired. This would involve targeting regions within the genome of the yeast with little to no enological contribution for example the *HO* locus. Genomic looping out methods have also been developed with the aim to eventually remove resistance markers from the genome of transformants (Walker *et al.*, 2003).

Even though various types of protocols have been developed to introduce DNA into yeast cells (Kawai *et al.*, 2010), the overwhelming majority are based on either exposing the exponentially-grown yeast to a lithium acetate/polyethylene glycol solution followed by a heat-shock (Gietz and Woods, 2002) or electroporation (Benatuil *et al.*, 2010).

Many genes of enological relevance have been identified as has been discussed earlier. Manipulation of the yeast genes either by modulating their expression, genetically removing them, or mutating important amino acid, played a crucial role in our current understanding of the function of these genes in yeast – especially in a winemaking context. The term "self-cloned" can be applied to such a genetically modified (GM) yeast when little to no foreign DNA is present. This will be greatly improved with the advent of the precision editing techniques spearheaded by CRISPR/Cas9-based genome editing as has already been demonstrated in wine yeast (van Wyk *et al.*, 2020a; Muysson *et al.*, 2019; Vigentini *et al.*, 2017).

Many foreign or recombinant genes have been expressed in wine yeast and were subsequently used to make a wine on a laboratory-scale. Examples of the foreign genes expressed in wine yeast, and their impact of wine are shown in **Fig. 2**. Although the majority of efforts focus on improving or diversifying the aroma composition, some cases also addressed other enological issues like contamination or acidity.

Currently, two GM yeasts have been approved for use by the Food and Drug Administration (FDA) in the United States of America. The flagship GM yeast ML01 contains two heterologous genes: a malolactic conversion gene from *O. oeni* and a malate transport gene from *Schizosaccharomyces pombe*. This manipulation enabled the yeast to effectively transport malate and convert it to lactate. This was a revolutionary step in winemaking. For the first time, *S. cerevisiae* yeast could be used for meaningful malolactic fermentation and subsequently eliminate the need for lactic acid bacteria (LAB). This streamlined the winemaking process as malate-to-lactate conversion by bacteria is often marred by unpredictable outcomes. In addition, biological amines, like histamine, released by LAB are often present in the final wine product to which some individuals can have a reaction, such as a headache. This yeast has been in the development since 1997 (Volschenk *et al.*, 1997) and has shown to perform equally well during all aspects of growth kinetics as its parental strain (Husnik *et al.*, 2007). This was substantiated with subsequent transcriptome and proteome analyzes which showed negligible change compared to its parent (Husnik *et al.*, 2006). ML01 has been permitted for commercial winemaking since 2003 in the USA, and 2006 in Canada with Moldova being the only country allowing its use in Europe. The dispersal of this strain has been under scrutiny as it was shown to survive years after dissemination in a mock trial (Grossmann *et al.*, 2011) with qPCR-based methods being developed to detect its presence (Vaudano *et al.*, 2016).

The second authorized strain (ECMo01) can consistently degrade urea, which can spontaneously react with ethanol within wine fermentations to form ethyl carbamate, a suspected carcinogen to humans. The ECMo01 strain is a self-cloned strain containing an additional copy of the urea amidolyase gene *DUR1,2* which can catalyze the hydrolysis of urea (Coulon *et al.*, 2006). The additional copy is under the transcriptional control of a strong *PGK1* promoter allowing for the constitutive expression of the *DUR1,2* gene. This strain performs equally well as its parent under wine-making conditions, but markedly less ethyl carbamate is present in the final wine product. This strain has been approved for use in the USA and Canada since 2006, but little follow-up data exist on its use in practice.

In other fields, GM yeast strains have been used for industrial food applications. In Japan, self-cloned yeast strains have been developed for the use in sake production in 2001, after such strains were exempted from the Japanese government's guidelines for genetically modified food (Kitagaki and Kitamoto, 2013). Recently, Lallemand licensed a beer yeast strain expressing a lactate dehydrogenase from a lactobacillus for distribution in the USA (Rice, 2019).

The broader use of GM wine yeast has been a controversial topic of discussion (van Wyk *et al.*, 2019; Ramón and González, 2011; Cebollero *et al.*, 2007; Bisson, 2004). There are also vigorous attempts to develop new strains using breeding techniques specifically to avoid GM approaches (Pérez-Torrado *et al.*, 2015). Although the literature provides a convincing case for more large-scale trials, there is still an overall negative public sentiment towards the implementation of any kind of genetic modification in winemaking.

The Role of Non-*Saccharomyces* Yeast

As mentioned earlier in this chapter, there is a large number of yeast genera besides *Saccharomyces* present during the entire process of winemaking and are collectively called "non-*Saccharomyces* yeast" (NSY). The term "non-conventional yeast" is also commonly used to describe this group. Monitoring the dynamics and succession of the yeast population in the vineyard as well as during fermentation has been the subject of many studies (Varela and Borneman, 2017; Pretorius *et al.*, 1999). Interestingly, *S. cerevisiae* strains have an insignificant presence throughout the growth of the grape berries in the vineyard and is rarely the most dominant population at the commencement of fermentation. In **Table 2**, the yeast genera commonly isolated from vineyards and vineries are listed.

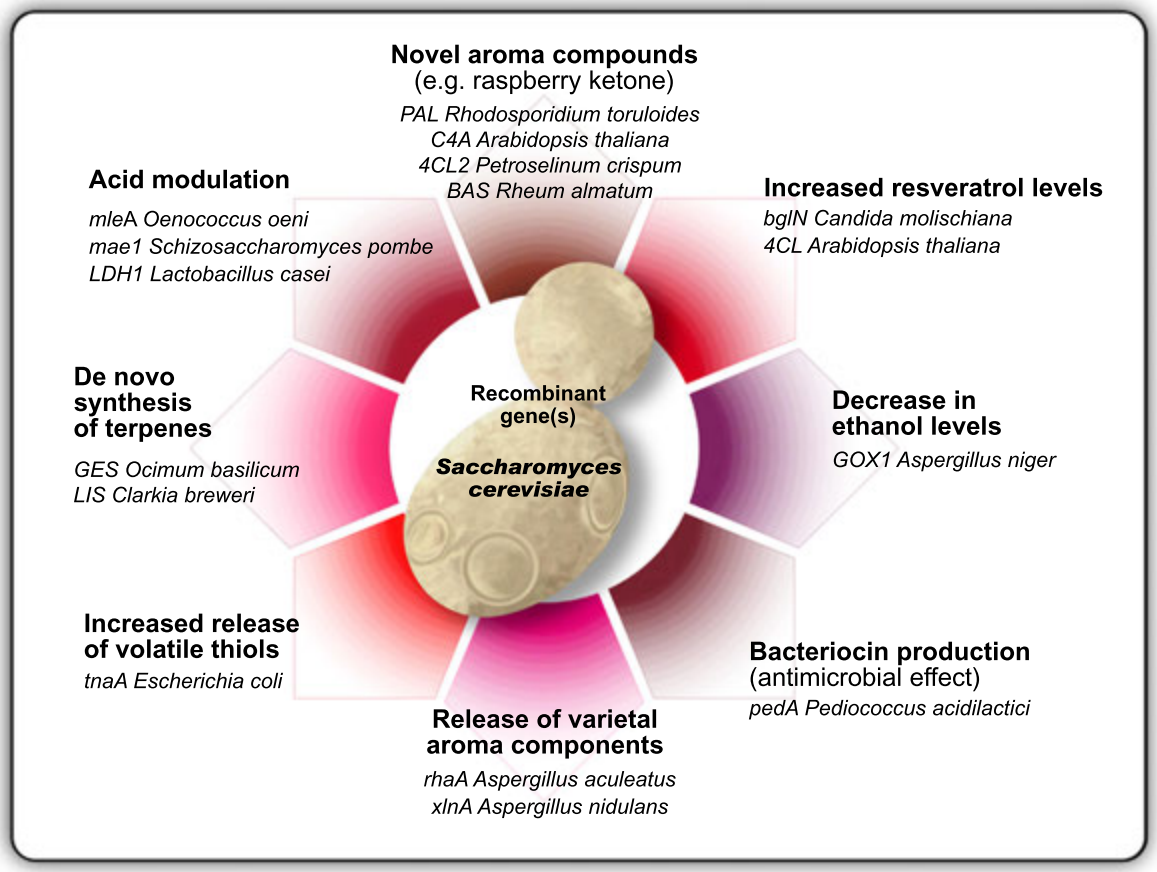

Fig. 2 Examples of foreign genes expressed in wine yeast with their subsequent enological consequence. Reference for acid modulation (Volschenk *et al.*, 2001; Dequin *et al.*, 1999); *de novo* synthesis of terpenes (Pardo *et al.*, 2015; Herrero *et al.*, 2008); increased release of volatile thiols (Swiegers *et al.*, 2009); release of varietal aroma components (Manzanares *et al.*, 2003; Ganga *et al.*, 1999); bacteriocin production (Schoeman *et al.*, 1999); decrease in ethanol levels (Malherbe *et al.*, 2003); increase in resveratrol levels (Sun *et al.*, 2015; González-Candelas *et al.*, 2000); novel aroma compounds (Lee *et al.*, 2016).

It should be noted that the taxonomy of yeast is ever-changing especially since DNA sequence analysis is now almost exclusively used for identification and classification as prescribed by the International Code of Botanical Nomenclature (McNeill *et al.*, 2012). Major overhauls in the classification of yeast occur regularly as cataloged by *The Yeast* handbook (Kurtzman *et al.*, 2011). Thus, the names of species and genera mentioned in older papers might not be in use anymore. Most relevant is *Kluyveromyces thermotolerans*, which has been reclassified as *Lachancea thermotolerans*, whereas *Candida pulcherrima*, *Candida zemplinina* and *Candida stellata* have been renamed to *Metschnikowia pulcherrima*, *Starmerella bacillaris* and *Starmerella bombicola*, respectively. As molecular techniques used to identify and distinguish between different strains become more sophisticated, strains known by a certain name often are reclassified, as was the case when some *S. bombicola* strains isolated from wine fermentations were in fact *S. bacillaris* (Csoma and Sipiczki, 2008). The teleomorph-anamorph concept which groups genera based on their sexual states are also falling out of favor and hence the *Hanseniaspora* name is preferred over the *Kloeckera* name.

At the start of most spontaneous must fermentations, yeast that are found on the berry skins, notably belonging to the *Hanseniaspora* genus, tend to dominate by sheer numbers (Martin *et al.*, 2018; Albertin *et al.*, 2016; Fleet, 2003). The composition of the yeast population shifts dramatically throughout the fermentation, as the sugar and available nitrogen levels drops, the oxygen availability decreases, the osmolarity changes and the ethanol levels rise. The addition of SO_2 to the must prior to fermentation also has a profound effect on the microbial population as it kills and limit the growth of many bacteria and NSY (Pateraki *et al.*, 2014; Constantí *et al.*, 1998). At the end of a successful fermentation, members of the *Saccharomyces* genus tend to be the dominant population.

As inoculated fermentations became a mainstay enological practice, it was a widely accepted notion that the presence of any other yeast in the fermentation would contribute toward spoilage. This is a valid argument in certain cases notably the *Brettanomyces*/*Dekkera* species which can produce 4-ethyl-phenol and other vicinal diketones which, when they reach high enough levels (over 600 μg/L), contribute unpleasant aromas to wines, such as aromas described as "barnyard", "burnt plastic", "wet animal" and "horse-sweat" commonly known as "brett" taint (Schifferdecker *et al.*, 2014). Many other yeasts can also contribute to off-flavors like *Hanseniaspora*

Table 2 Yeast genera commonly isolated from vineyards grouped in their respective classes

Ascomycota	*Starmerella*
Dothideomycetes	*Torulaspora*
Aureobasidium	*Wickerhamomyces*
Saccharomycetes	*Yarrowia*
Brettanomyces	*Zygoascus*
Candida	*Zygosaccharomyces*
Citeromyces	*Schizosaccharomycetes*
Debaryomyces	*Schizosaccharomyces*
Hanseniaspora	**Basidiomycota**
Issatchenkia	*Microbotryomycetes*
Kluyveromyces	*Rhodotorula*
Lachancea	*Sporidiobolus*
Lodderomyces	*Tremellomycetes*
Metschnikowia	*Bulleromyces*
Meyerozyma	*Cryptococcus*
Pichia	*Ustilaginomycetes*
Saccharomyces	*Rhodosporidium*
Saccharomycodes	

Note: Liu *et al.*, 2017b; Mateo and Maicas, 2016; Jolly *et al.*, 2005.

which can produce high amounts of ethyl acetate imparting a nail polish odor (Hu *et al.*, 2018b). Several other genera like *Pichia*, *Saccharomycodes* and *Zygosaccharomyces* do not necessarily contribute negatively towards the aroma profile, but they do possess spoilage characteristics and have been implicated in cloudiness or sediment formation of the final wine product (Loureiro and Malfeito-Ferreira, 2003). Risk of refermentation leading to CO_2 build-up and overpressure after bottling can also be caused by spoilage organisms.

In the past 15 years, however, new light has been cast on the role of NSY in winemaking and it is now widely accepted that NSY can provide beneficial attributes to the final wine product. Thus, substantial efforts have been directed towards employing NSY in so-called co-culturing fermentation experiments, as highlighted in several reviews (Comitini *et al.*, 2017; Mateo and Maicas, 2016; Ciani and Comitini, 2015; Jolly *et al.*, 2014; Ciani *et al.*, 2010). The term "multi-starter" or "mixed culture" fermentation can also be applied for these types of fermentations. A common motivation for using NSY is to enhance the complexity and augment the diversity of the flavor profile of wine.

Table 3 shows co-culturing experiments and it is clear from the literature that a large variety of NSYs have already been tested to assess their enological potential. There are only a few species that have repeatedly shown to provide some level of benefit to the point that commercial preparations have been developed. These are *Torulaspora delbrueckii*, *Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *Schizosaccharomyces pombe* and *Starmerella bacillaris* (**Table 4**).

There are five main benefits of employing NSYs in winemaking that have been reported in co-culturing experiments (**Fig. 3**): First, some NSY possess so-called aroma-releasing enzymes which in turn can be divided into two categories: (1) glycosidases which can release terpenes bound to sugar moieties in the must; and (2) carbon-sulfur lyases which release thiol molecules from the bound precursors. The yeasts *T. delbrueckii*, *M. pulcherrima* and *P. kluyveri* have been repeatedly implicated to perform this action of releasing aroma-active molecules from their bound precursors (Belda *et al.*, 2017b; Anfang *et al.*, 2009). A second benefit is that NSY can modulate the acid profile of the wine. For instance, *S. pombe* can efficiently convert malic acid to lactic acid, which reduces the overall tartness of wine (Mylona *et al.*, 2016). Wine fermented with *T. delbrueckii* strains often report much lower concentrations of acetic acid concentrations which, at high levels, can lead to a faulty wine (Benito, 2018). A third advantage is that many NSY tend to make more glycerol than *S. cerevisiae*, which diverts grape sugars away from ethanol production and thus co-culturing often leads to wine with lower ethanol levels (Goold *et al.*, 2017; Ciani *et al.*, 2016). Fourthly, since the regulation of the production of aroma-active compounds like esters, fatty acids and higher alcohols is different in NSYs than *S. cerevisiae*, the majority of coculturing experiments report vastly different aroma profiles including, at times, significantly higher levels of certain classes of aroma compounds resulting in a sensorially superior wine. A fifth benefit is that NSY have been shown to increase the polysaccharide and oligosaccharide content in wine – in particular mannoprotein release from cell walls. Their presence has been implicated in several positive qualities in wine including the prevention of the tartaric acid crystallization and protein haze formation along with increasing the sweetness, body and mouthfeel (Ruiz *et al.*, 2019b; García *et al.*, 2017).

There are other beneficial attributes that NSY can impart on the final wine product but have not been explored in coculturing experiments to a great extent. NSY, notably *S. pombe*, have been shown to modulate the anthocyanin structure of red wines by forming adducts of vinylphenolic pyranoanthocyanin which enhances color stability (Chen *et al.*, 2018; Benito *et al.*, 2011).

NSY can also provide selective antimicrobial activity. Just as with certain *S. cerevisiae* strains, many NSY possess so-called killer activity and have been shown to kill yeast known to cause spoilage like members of the *Dekkera*/*Brettanomyces* genus (Mehlomakulu *et al.*, 2017; Comitini and Ciani, 2011; Comitini *et al.*, 2004; Yap *et al.*, 2000). Apart from the killer toxins isolated from *T. delbrueckii* (Leticia *et al.*, 2016), and to some extent *W. anomalus* (Cecarini *et al.*, 2019), most of the other NSY studied for their killer activity in a wine setting are not known for any other enological features. These include *Candida pyralidae* (Mehlomakulu *et al.*, 2017),

Table 3 List of coculturing grape must fermentations conducted with a non-*Saccharomyces* yeast partnered with a *Saccharomyces* species along with a noteworthy outcome of the final wine. Mode of inoculations: Seq = sequential, Imm = immobilized, Sim = simultaneous

Non-Saccharomyces yeast	*Mode of inoculation*	*Noteworthy enological outcomes from mixed-culture fermentations*	*Reference*
Candida cantarellii	Sim, Seq	Higher glycerol and lower ethanol levels in Syrah wines	(Toro and Vazquez, 2002)
Candida railenensis	Seq	Increased terpene release in Muscaris wines	(van Wyk *et al.*, 2020b)
Candida sake	Sim	Increased ester content in Pedro Giménez wine	(Maturano *et al.*, 2015)
Cryptococcus flavescens	Seq	Higher glycerol and acetic acid levels in Pinotage and Sauvignon blanc wines	(Rossouw and Bauer, 2016)
Debaryomyces hansenii	Seq	Increased terpene release in Muscaris wines	(van Wyk *et al.*, 2020b)
Debaryomyces pseudopolymorphus	Sim	Increased release of terpenes in Chardonnay	(Cordero Otero *et al.*, 2003)
Hanseniaspora guilliermondii	Seq	Increased in phenylethanol in Fiano wine	(Testa *et al.*, 2020)
	Seq	Decreased acetic acid levels in Campanino wine	(Lombardi *et al.*, 2018)
	Sim	Higher levels of phenylethyl acetate in Malvasia Fina and Arinto wine	(Barbosa *et al.*, 2015)
	Sim	Increased ethyl esters in Malvasia Fina and Arinto wine	(Lage *et al.*, 2014)
	Sim	Higher levels of certain sulfurous compounds in an unspecified wine	(Moreira *et al.*, 2008)
	Sim	Increase acetate esters in Bobal wine	(Rojas *et al.*, 2003)
Hanseniaspora opuntiae	Seq	Lower ethanol yield in Pinotage and Sauvignon blanc wines	(Rossouw and Bauer, 2016)
Hanseniaspora osmophila	Imm-Seq	Increase isoamyl acetate in Verdicchio wine	(Canonico *et al.*, 2016)
	Sim	Increase in 2-phenethyl acetate levels in Bobal wine	(Viana *et al.*, 2009)
Hanseniaspora uvarum	Sim, Seq	Increase in ethyl ester levels in Cabernet Gernischt	(Hu *et al.*, 2018a)
	Sim	Increased C13-norisoprenoids, terpenes, and ethyl esters in Cabernet Sauvignon	(Hu *et al.*, 2018b)
	Seq	Increased 2-phenethyl acetate content in Bolero, Rondo and Regent wines	(Liu *et al.*, 2017a)
	Seq	Decreased ethanol levels in Pinotage and Sauvignon	(Rossouw and Bauer, 2016)
	Sim	Increase in isoamyl acetate in Negroamaro wine	(Tristezza *et al.*, 2016)
	Sim	Decreased ethanol levels in synthetic wine	(Wang *et al.*, 2016)
	Sim	Higher levels of isoamyl acetate in an unspecified wine	(Moreira *et al.*, 2008)
	Sim	Decreased ethanol levels in an unspecified wine	(Mendoza, *et al.*, 2007)
	Seq	Lower medium-chained fatty acid content in Chelva wines	(Herriaz *et al.*, 1990)
Hanseniaspora vineae	Sim, Seq	Increase higher alcohols and acetate esters in Vidal Blanc icewine	(Zhang *et al.*, 2018b)
	Seq	Higher glycerol and ester levels with lower alcohols and fatty acids in Chardonnay	(Medina *et al.*, 2013)
	Seq	Increase in 2-phenylethyl acetate in Tempranillo wine	(Viana *et al.*, 2011)
Issatchenkia orientalis	Sim	Reduction of malic acid in Campbells early wines	(Kim *et al.*,2008)
Issatchenkia terricola	Sim, Seq	Lower volatile acidity in Cabernet Sauvignon	(Shi *et al.*, 2019)
Kazachstania gamospora	Seq	Increased 2-phenethyl acetate levels in Ribolla Gialla wine	(Dashko *et al.*, 2015)
Kluyveromyces marxianus	Seq	Increased grape pectin released in Shiraz	(Rollero *et al.*, 2018)
Lachancea fermentati	Seq	Increased geraniol release in Muscat d'Alexandrie	(Porter *et al.*, 2019)
Lachancea thermotolerans	Seq	Higher levels of ethyl lactate in Riesling	(Dutraive *et al.*, 2019)
	Seq	Increased geraniol release in Muscat d'Alexandrie	(Porter *et al.*, 2019)
	Seq	Higher levels of ethyl propionate in Tempranillo wine	(Escribano-Viana *et al.*, 2018)
	Sim, Seq	Increased lactic acid in Airén wine	(Benito *et al.*, 2016)
	Seq	Lower acetaldehyde levels and more terpene release in Riesling	(Benito *et al.*, 2015)
	Sim, Seq	Higher glycerol and 2-phenylethanol levels in Sangiovese and Cabernet-Sauvignon	(Gobbi *et al.*, 2013)
	Sim	Increases in glycerol, 2-phenyl ethanol and polysaccharides in an unspecified must, lowering of pH	(Comitini *et al.*, 2011)
	Sim, Seq	Decrease in total acidity in Emir wine	(Balikci *et al.*, 2010)
	Sim, Seq	Lower levels of acetaldehyde and acetoin in Pinot Grigio	(Ciani, *et al.*, 2006)
Lodderomyces elongisporus	Sim	Lower malic acid and acetic acid levels in a synthetic wine	(Ruiz *et al.*, 2019b)
Metschnikowia fructicola	Seq	Increased isoamylacetate levels in Bolero wine	(Liu *et al.*, 2017a)
Metschnikowia pulcherrima	Imm-Seq	Lower ethanol levels in Verdicchio wine	(Canonico *et al.*, 2019a,b)
	Seq	Lower ethanol levels in Chardonnay	(Canonico *et al.*, 2019a,b)
	Seq	Higher levels of ethyl hexanoate and ethyl octanoate in Riesling	(Dutraive *et al.*, 2019)
	Sim, Seq	Higher amount of acetate esters and β-damascenone in Vidal Blanc Icewine	(Zhang *et al.*, 2018b)
	Seq	Increased volatile thiol levels in Verdejo	(Ruiz *et al.*, 2018)

Table 3 Continued

Non-Saccharomyces yeast	*Mode of inoculation*	*Noteworthy enological outcomes from mixed-culture fermentations*	*Reference*
	Sim	Higher levels of esters and lower ethanol levels in Merlot	(Varela *et al.*, 2017)
	Imm-Seq	Increase geraniol release with reduction in ethanol in Verdiccio wine	(Canonico *et al.*, 2016)
	Seq	Lower ethanol levels and higher ester content in Riesling	(Röcker *et al.*, 2016)
	Seq	Higher foam persistence in Cava wine	(González-Royo *et al.*, 2015)
	Seq	Lower ethanol levels in Chardonnay and Shiraz	(Contreras *et al.*, 2014)
	Seq	Higher levels of 2-phenethyl acetate and isoamyl acetate in Sauvignon blanc	(Sadoudi *et al.*, 2012)
	Sim	Increased 2-phenyl ethanol in an unspecified must	(Comitini *et al.*, 2011)
	Seq	Higher ester content in Debina wines	(Parapouli *et al.*, 2010)
	Seq	Increase levels of esters and higher alcohols in Muscat d'Alexandrie	(Rodríguez *et al.*, 2010)
Metschnikowia viticola	Seq	Increased β-damascenone release in Rondo wines	(Liu *et al.*, 2017a)
Nakazawaea ishiwadae	Sim	Higher amount of proteins in a synthetic wine	(Ruiz *et al.*, 2019b)
	Seq	Increased ethyl esters and acetate esters in Muscaris wines	(van Wyk *et al.*, 2020b)
Pichia fermentans	Seq	Improved total anthocyanin and tannin content in Xiang Pearl wine	(Kong *et al.*, 2019)
	Sim	Increase in medium-chain fatty acids and their corresponding esters in Ecolly wine	(Ma *et al.*, 2017)
	Seq	Increase in certain volatiles especially higher alcohols in Macabeo wine	(Clemente-Jimenez *et al.*, 2005)
Pichia guillermondii	Seq	Formation of stable vinylphenolic pyranoanthocyanin in unspecified red/white wine	(Benito *et al.*, 2011)
Pichia kluyveri	Seq	Higher levels of 2-phenethyl acetate in Riesling	(Dutraive *et al.*, 2019; Benito *et al.*, 2015)
	Sim	Increase in liberation of volatile thiols in Sauvignon Blanc	(Anfang *et al.*, 2009)
Pichia kudriavzevii	Sim, Seq	Lower volatile acidity and higher terpene levels in Cabernet Sauvignon	(Shi *et al.*, 2019)
	Seq	Lower ethanol and higher glycerol levels in Pinotage and Sauvignon blanc	(Rossouw and Bauer, 2016)
Rhodotorula mucilaginosa	Sim, Seq	Increase in presence of varietal aromas in Ecolly wine	(Wang *et al.*, 2017)
	Seq	Increase in terpene release in Fiano and Aglianico wines	(Calabretti *et al.*, 2012)
Saccharomyces uvarum	Sim	Higher glycerol and lower ethanol in Merlot	(Varela *et al.*, 2017)
Schizosaccharomyces japonicus	Imm-Seq, Sim	Lower total acidity and ethanol levels in Trebbiano	(Domizio *et al.*, 2018)
	Sim	Lower total acidity and increased polysaccharide content in an unspecified wine	(Romani *et al.*, 2018)
Schizosaccharomyces pombe	Seq	Increase in polymeric anthocyanins in Merlot	(Chen *et al.*, 2018)
	Sim, Seq	Lower malic acid, urea and ethanol in Airén wine	(Benito *et al.*, 2013)
	Imm	Reduction of malic acid and lower ethyl acetate levels in Ezerfürtű wine	(Yokotsuka *et al.*, 1993)
	Seq	Reduction of malic acid in a Seyval blanc and Aurora blend	(Snow and Gallander, 1979)
Schwanniomyces vanrijiae	Seq	Increased terpene release in Pedro Giménez	(Maturano *et al.*, 2015)
	Seq	Increased release of geraniol in Muscat	(Garcia *et al.*, 2002)
Starmerella bacillaris	Seq	Higher levels of glycerol in Barbera wine	(Englezos *et al.*, 2019, 2016a,b)
	Seq	Higher values of color intensity in Pinot Noir, Merlot, Cabernet Sauvignon and Shiraz	(Englezos *et al.*, 2018)
	Seq	Higher glycerol and lower ethanol levels in a synthetic must	(Lemos Junior *et al.*, 2018)
	Seq	Lower ethanol and glycerol levels in Kotsifali/Mantilari wine	(Nisiotou *et al.*, 2018)
	Seq	Higher concentrations of glycerol and lower acetaldehyde levels in Sangiovese red	(Lencioni *et al.*, 2016)
	Imm-Seq	Increased glycerol with a reduction in ethanol in Verdicchio wine	(Canonico *et al.*, 2016)
	Seq	Higher glycerol levels in Bovale wine	(Zara *et al.*, 2014)
	Seq	Increased glycerol and lower ethanol levels in Nero d'Avola wine	(DiMaio *et al.*, 2012)
	Sim, Seq	Reduction of acetic acid levels in Erbaluce dried grape must	(Rantsiou *et al.*, 2012)
	Sim	Higher levels of glycerol in an unspecified must	(Comitini *et al.*, 2011)
	Sim	Increased concentration of higher alcohols, ethyl esters in Macabeo	(Andorrà *et al.*, 2010)
	Seq	Increase volatile thiol release in Sauvignon Blanc	(Anfang *et al.*, 2009)
Starmerella bombicola	Imm-Seq	Higher glycerol and lower ethanol in a synthetic wine	(Milanovic *et al.*, 2012)
	Imm-Seq	Higher glycerol and lower ethanol levels in Trebbiano Toscano wine	(Ferraro *et al.*, 2000)
	Sim, Seq	Increased glycerol levels in Chardonnay	(Soden *et al.*, 2000)

(*Continued*)

Table 3 Continued

Non-Saccharomyces yeast	*Mode of inoculation*	*Noteworthy enological outcomes from mixed-culture fermentations*	*Reference*
	Imm-Seq	Higher glycerol and lower ethanol levels in an unspecified wine	(Ciani and Ferraro, 1998)
Torulaspora delbrueckii	Sim	More esters, medium chain fatty acids and higher alcohols in Chardonnay	(Shekhawat *et al.*, 2018)
	Sim, Seq	Higher amount of ethyl esters in Cabernet Sauvignon	(Zhang *et al.*, 2018a)
	Seq	Increase in varietal thiol release in Verdejo wines	(Belda *et al.*, 2017b)
	Sim, Seq	Increase polysaccharide release in Malvar wines	(García *et al.*, 2017)
	Seq	Higher maximum heights of foam in sparkling Cava	(Medina-Trujillo *et al.*, 2017)
	Sim	Higher ethanol and glycerol levels in Chenin blanc	(Ngqumba *et al.*, 2017)
	Seq	Lower acetic acid content in Chardonnay and Palomino Fino wines	(Puertas *et al.*, 2017)
	Sim	Malolactic fermentation occurring in Cabernet-Sauvignon	(Ramírez *et al.*, 2016)
	Seq	Higher volatile thiol release in Sauvignon blanc	(Renault *et al.*, 2016)
	Sim, Seq	Decreased ethanol and malic acid concentrations in Tempranillo	(Belda *et al.*, 2015)
	Seq	Lower final ethanol levels in a synthetic wine	(Contreras *et al.*, 2015)
	Seq	Improved foamability and foam persistence in Cava	(González-Royo *et al.*, 2015)
	Seq	Increase in isobutyl acetate and isoamyl acetate in Sauvignon blanc	(Renault *et al.*, 2015)
	Seq	Higher levels of diacetyl, ethyl lactate and 2-phenylethyl acetate in Tempranillo	(Loira *et al.*, 2014)
	Sim, Seq	Higher levels of both isoamyl acetate and ethyl lactate in a synthetic wine	(Taillandier *et al.*, 2014)
	Seq	Increased levels of α-terpineol and linalool in Gewürztraminer	(Jenko and Čuš, 2013)
	Sim, Seq	Increase in lactone release in Amarone wine	(Azzolini *et al.*, 2012)
	Sim	Increase in polysaccharides in an unspecified must	(Comitini *et al.*, 2011)
	Sim, Seq	Lower volatile acidity and acetaldehyde in botrytized in Semillon wines	(Bely *et al.*, 2008)
	Seq	Lower levels of acetaldehyde and acetoin in Pinot Grigio	(Ciani *et al.*, 2006)
	Sim	Lower ethanol and lower volatile acidity in Pedro Ximénez wine	(Moreno *et al.*, 1991)
Wickerhamomyces anomalus	Seq	Increase ester levels in Mazuela wine	(Izquierdo Cañas *et al.*, 2014)
Williopsis pratensis	Seq	Lower levels of malic acid content in Tempranillo wine	(Escribano-Viana *et al.*, 2018)
Williopsis saturnus	Sim	Increased ester content in Emir wine	(Tanguler, 2013; Yilmaztekin *et al.*, 2013)
	Sim	Increased active amyl alcohol in Sugraone wine	(Lee *et al.*, 2012)
Zygosaccharomyces bailii	Seq	Lower ethanol levels in Chardonnay	(Canonico *et al.*, 2019a, 2019b)
	Seq	Lower levels of malic acid content in Tempranillo wine	(Escribano-Viana *et al.*, 2018)
	Seq	Lower ethanol levels in a synthetic wine	(Contreras *et al.*, 2015)
	Sim	Higher levels of ethyl esters in Chardonnay	(Garavaglia *et al.*, 2015)
Zygosaccharomyces kombuchaensis	Seq	Fructophillic yeast, faster sugar consumer in Ribolla Gialla wine	(Dashko *et al.*, 2015)
Zygotorulospora florentina	Seq	Higher concentrations of glycerol and esters in Sangiovese red wine	(Lencioni *et al.*, 2016)

Note: Metschnikowia pulcherrima = Candida pulcherrima.
Pichia anomala = Wickerhamomyces anomalus.
Issatchenkia terricola = Pichia kudriavzevii.
Starmerella bombicola = Candida stellata.
Starmerella bacillaris = Candida zemplinina.
Hanseniaspora apiculata = Kloeckera apiculata.
Schwanniomyces vanrijiae = Debaryomyces vanriji.
Lachancea thermotolerans = Kluyveromyces thermotolerans.
Williopsis saturnus = Cyberlindnera saturnus.

Pichia membranifaciens (Belda *et al.*, 2017a), *Tetrapisispora phaffii* (originally called *Kluyveromyces phaffi*; Comitini *et al.*, 2004) and *Ustilago maydis* (Santos *et al.*, 2011). Thus, it is proposed to separately propagate and isolate the killer toxin, which could then be added to the fermentations, rather than co-culturing the NSY with *S. cerevisiae* (Mannazzu *et al.*, 2019). The mechanisms of the killer activity of these NSY strains have, in some cases, been elucidated, identifying that many bind to glucan and plasma membrane receptors, possessing cell wall degrading capabilities. Upon entry into the cell, the killer toxin can cause a variety of cell damage leading to cell arrest and apoptosis (Cecarini *et al.*, 2019; Liu *et al.*, 2015; Comitini *et al.*, 2009). It should be noted that, for unknown reasons, not all members of the *Brettanomyces*/*Dekkera* genus are equally susceptible to a specific killer toxin and are, in fact, not affected by its presence (Santos *et al.*, 2011; Yap *et al.*, 2000). Yet successful implementation of killer toxins in wine could dramatically minimize the use of pre-fermentative SO_2 additions.

It is not known whether *M. pulcherrima* produces any kind of killer toxin, but it does produce a significant amount of pulcherriminic acid – a precursor to the pulcherrimin pigment – which can have a wide-scale antimicrobial effect, absorbing iron within its

Table 4 Commercially available non-*Saccharomyces* strains (as of April 2020)

Non-Saccharomyces yeast	*Commercial name*	*Current supplier*
Lachancea thermotolerans	Concerto™	Chr-Hansen
	Lactia	Proenol
	Levulia ® Alcomeno	Cenolia
Metschnikowia pulcherrima	Excellence Bio-Naturae	Cenolia
	Flavia ®	Lallemand
	Levulia ® Pulcherrima	Cenolia
	Zymaflore ® Égide	Laffort
Pichia kluyveri	Frootzen™	Chr-Hansen
Schizosaccharomyces pombe	Atecrem 12H	Bioenologia
	ProMalic	Proenol
Starmerella bacillaris	Atecrem 11H	Bioenologia
Torulaspora delbrueckii	Biodiva™	Lallemand
	Levulia ® Torula	Cenolia
	Prelude™	Chr-Hansen
	Primaflora ® VB BIO	Cenolia
	Sihaferm Nature	Eaton
	Vinifer NS TD	Agrovin
	Zymaflore ® Alpha	Laffort
	Zymaflore ® Égide	Laffort

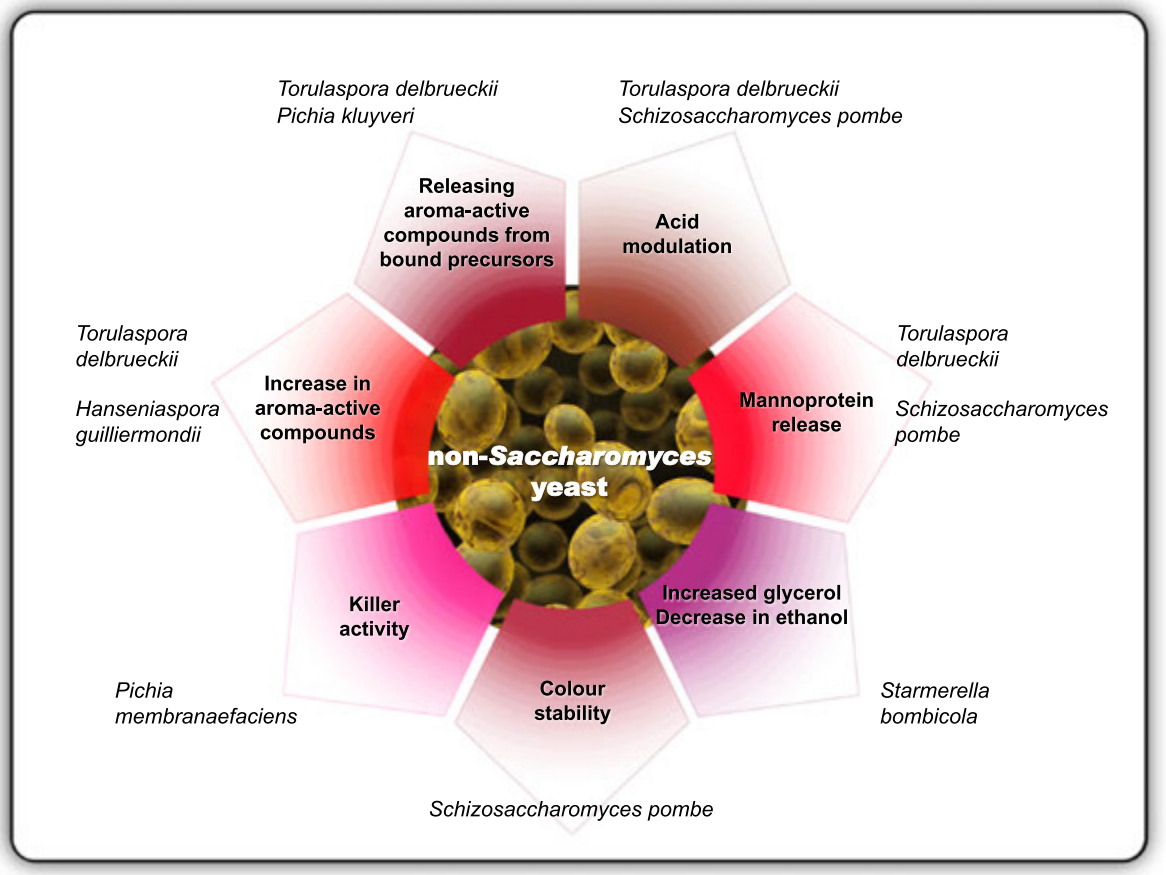

Fig. 3 Major impacts of non-*Saccharomyces* yeast (NSY) on winemaking. Noteworthy NSYs are mentioned for each influence. Other yeast examples are in **Table 3**. Color stability and killer activity are included though few examples in the literature exist of coculturing must fermentations.

environment and depleting it for other populations (Oro *et al.*, 2014). Pulcherriminic acid and pulcherrimin can also inhibit the fermentative capability of *S. cerevisiae* and are thus incompatible with certain *M. pulcherrima* strains in a co-culturing fermentation.

An important consideration for co-culturing strategies is the order should of the inoculations known as the modality of inoculations. The most popular mode is sequential inoculation where the NSY is inoculated first and ferments alone for a couple of days, before the *Saccharomyces* strain is added. Once co-cultured with *S. cerevisiae* (which turns the environment anaerobic, producing ethanol and killer toxins), many aerobic NSY strains cease fermentation when oxygen is depleted and have a negligible effect on the final wine. The other mode is known as simultaneous inoculations where the NSY and *Saccharomyces* strains are added at the same time in the beginning of the fermentation. There are couple of examples where sequential and simultaneous co-culturing have been conducted within the same experiment and it is clear that the choice can have a profound effect on the final wines being produced (Hu *et al.*, 2018a, 2018b; Zhang *et al.*, 2018b; Zironi *et al.*, 1993). Immobilized non-*Saccharomyces* cells have also been used in coculture fermentations with examples shown in **Table 2**. Cells are often encapsulated (or immobilized) within alginate beads and added to the must, often performed with a sequential type of inoculation. This method greatly improves the removal of the yeast cells and is useful if unwanted by-products are known to be produced by the NSY with long incubations like *S. pombe*, which can make excessive amounts of acetaldehyde (Yokotsuka *et al.*, 1993).

Understanding the dynamics of the interaction between two different yeast within a co-culture fermentation is complicated. On a transcriptional level, it has been shown that the non-*Saccharomyces* partner exerts their influence on the fermentative capability and nutrient acquisition of *S. cerevisiae*, as the expression of genes involved in glycolysis are dramatically modulated during a co-culture fermentations (Curiel *et al.*, 2017; Sadoudi *et al.*, 2017; Barbosa *et al.*, 2015; Milanovic *et al.*, 2012).

Regarding the genomics of NSY, a recent instructive review detailed the progress made in sequencing the important members of NSY commonly found in wine (Masneuf-Pomarede *et al.*, 2016). The overwhelming majority of the important wine-related NSYs have at least one member with a whole genome sequenced. As shown for *S. cerevisiae*, the genome sequence of NSYs (especially if multiple members are sequenced) will help untap the molecular underpinnings of their enological features. Since this review, multiple reports were published discussing and comparing the genomes sequenced of important NSYs, including the genomes of *Hanseniaspora* (Guaragnella *et al.*, 2020; Saubin *et al.*, 2020; Chen *et al.*, 2019) and *Starmerella* (Lemos Junior *et al.*, 2018). Genetic modification (GM) would be useful in validating certain gene studies but could also improve the enological properties of a specific NSY. The modification tools for the most common NSY strains are not well-developed as for *S. cerevisiae*, with only a few GM attempts of NSY described in literature, e.g., *T. delbrueckii* (Pacheco *et al.*, 2009) and *Metschnikowia* spp. (Gordon *et al.*, 2019; Nigro *et al.*, 2004). The exception is that of *S. pombe*, a yeast widely used in human disease studies and thus has a sophisticated GM toolbox (Fennessy *et al.*, 2014). As of yet, genetically modified NSYs have not been tested in a laboratory setting or any larger enological setting.

Implementations of coculturing fermentations are costly for a winemaker as two instead of one starter cultures have to be purchased. Also, as with *S. cerevisiae*, there are massive clonal variation in the performance of the same species (Escribano *et al.*, 2018). Even though the literature shows a promising future for coculturing must fermentations, considerable anecdotal evidence suggests difficulties with reproducibility. The dynamic behavior of two (or more) yeast within a fermenting vessel cannot be accurately predicted. Thus, many of these coculturing fermentations are still developed by trial-and-error methods. Nevertheless, their effects on wine are beyond dispute.

Concluding Remarks

As one the world's oldest biotechnological industries, winemaking is steeped in a rich history of tradition and innovation (Jagtap *et al.*, 2017). Despite the fact that wine is an archetypal traditional fermented beverage with strong territorial and socio-cultural connotations, business-savvy winemakers know all too well that continuous product innovation is a vital source of their competitive advantage in the marketplace (Pretorius, 2020). Continuous innovation is a fundamental business principle, which guided the wine industry's ongoing interest in Nature's treasure trove of *Saccharomyces* and non-*Saccharomyces* yeasts and how these yeasts can be further improved or used in combinations to increase the quality of the end-product.

We live in an era of transformative technologies and unprecedented scientific breakthroughs. The opportunity to harness the traditions and wisdom of the distant past and combine that knowledge with innovative technologies, such as biodigital data mining and synthetic genomics, have never been so great as now (Pretorius, 2017, 2020). Wine yeast strain development programs and the application of tailor-made blends of *Saccharomyces* and non-*Saccharomyces* stand to gain enormously from these emerging technologies. Specialized permutations of compatible *Saccharomyces* and non-*Saccharomyces* yeast strains can be differentially combined in multi-starter fermentations depending on the desired outcome from a wine quality perspective. In this way, synergistic wine yeast consortia properties and tap into the unparalleled functional versatility of *Saccharomyces* and non-*Saccharomyces* yeasts. Blending yeast starter-culture consortia for modeling purposes will require an acceleration of the current biodiversity prospecting and the purposeful hunting for non-*Saccharomyces* yeasts in those vineyards and associated wineries.

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Ethanol Tolerance and Production by Yeasts

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Introduction

In the last decades, global improvement in living standards has pushed the search for sustainable energy as alternative to the use of non-renewable fossil fuels to meet the energy requirements worldwide. In this context (bio)ethanol, also known as ethyl alcohol or chemically C_2H_5OH, is recognized as the most widely used biofuel in the transportation sector, with a long history as alternative fuel (Guo *et al.,* 2015). Additionally, ethanol is an industrially relevant chemical present in a wide range of products such as solvents, cosmetics, food and beverages, disinfectants, and pharmaceutical preparations (**Fig. 1**). Microorganisms such as bacteria and yeasts play a key role in bioethanol production by fermenting a wide range of sugars to ethanol. At the industrial level, yeasts are the preferred microorganisms for ethanol production due to their valuable properties regarding ethanol yields, tolerance, productivity, resistance to inhibitors, and their suitability to grow in very simple and inexpensive media (Mohd-Azhar *et al.,* 2017).

In this chapter, we focus on the process of ethanol production by yeasts and we highlight the most relevant physical, chemical and molecular factors affecting ethanol production and tolerance.

Ethanol Production by Yeasts During the Fermentation Process

The term "fermentation" derives from the Latin word fervere, which means to boil, to be in turmoil. It can be defined as a metabolic process in which the biochemical transformation of organic compounds, such as carbohydrates, takes places with the aid of enzymes to obtain cellular energy. Because it does not require oxygen, fermentation is classified as an anaerobic process. There are different types of fermentation, such as ethanol fermentation, in which one glucose molecule is converted into two ethanol molecules; lactic acid fermentation, in which one molecule of any six-carbon sugar is converted to two lactic acid molecules; and others, including the fermentative hydrogen production (Narayanan *et al.,* 2004; Reddy *et al.,* 2008; Wang and

Fig. 1 Schematic representation of the main industrial applications of ethanol.

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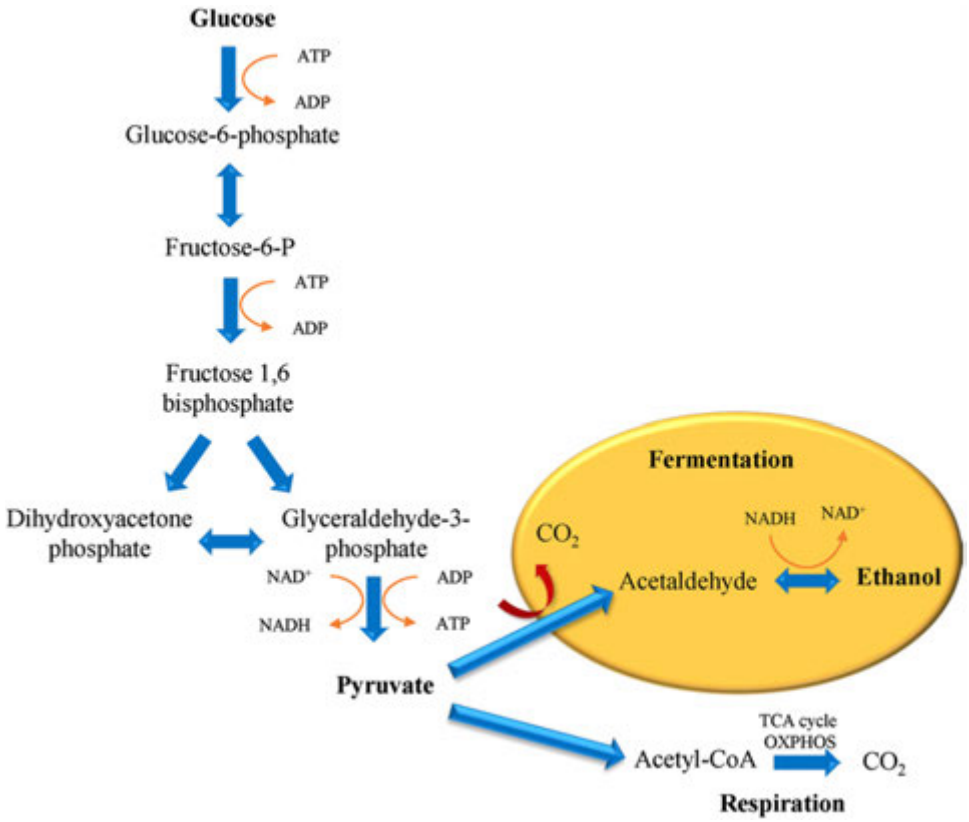

Fig. 2 Glycolysis and ethanol fermentation pathways. ATP = Adenosine triphosphate, NADH = nicotinamide adenine dinucleotide, TCA = tricarboxylic acid, OXPHOS = oxidative phosphorylation.

Wan, 2009). In this chapter, we focus on the ethanol fermentation process carried out by both *Saccharomyces* and non-*Saccharomyces* yeasts due to its relevance for many industrial applications.

Bioethanol production is based on the ability of yeasts to catabolize six-carbon molecules such as glucose, fructose, mannose, or galactose into ethanol, a two-carbon compound. The biosynthetic reactions by which yeasts convert glucose to ethanol are known as the Embden-Meyerhof-Parnas (glycolytic) pathway followed by fermentation (van Dijken and Scheffers, 1986). The glycolytic pathway occurs in the cytoplasm. Cells produce adenosine 5′-triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH) without the involvement of molecular oxygen. First, glucose is phosphorylated and one ATP molecule is consumed to obtain glucose-6-phosphate, which is then converted to fructose-6-phosphate. Fructose-6-phosphate is subsequently transformed to fructose-1,6-biphosphate with the consumption of one ATP molecule, and the resulting fructose-1,6-biphosphate is transformed into two three-carbon sugar compounds, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (**Fig. 2**). In the downstream reactions, the three-carbon sugars are transformed into pyruvate, each yielding two ATPs and one NADH. Pyruvate is located at the branch-point between respiratory dissimilation of sugars and alcoholic fermentation. In most organisms, pyruvate is transported into mitochondria, where it is converted into acetyl CoA and CO_2. The acetyl CoA is completely oxidized in the presence of O_2 into CO_2 and H_2O in the so called respiration process (Barnett, 1976; Joseph-Horne *et al.*, 2001). However, in many yeasts respiration only occurs under aerobic conditions when glucose levels are kept very low. In contrast, fermentation occurs in the mitochondria despite the presence of oxygen, represses respiration, and can use alternative carbon sources in a phenomenon known as "Crabtree effect" (de Deken, 1966). In these yeasts, pyruvate is decarboxylated to acetaldehyde and CO_2 by pyruvate decarboxylase (Hohmann and Cederberg, 1990). Acetaldehyde is then reduced to ethanol by the activity of alcohol dehydrogenase, resulting in the re-oxidation of NADH to NAD^+ (**Fig. 2**). Yeasts produce alcohol because the production of ATP is faster in fermentation compared to respiration. Besides, yeasts need to maintain the redox balance when consuming sugars under anaerobic conditions, so the $NADH/NAD^+$ ratio in fermentation is unaltered. Additionally, fermentation offers the advantage of ethanol production, which acts as an inhibitor of competing microbes that are not ethanol tolerant (Piskur *et al.*, 2006). Thus, the ability to survive and proliferate in high ethanol levels during fermentation is both ecologically important and industrially relevant trait of yeast cells (Voordeckers *et al.*, 2015).

Ethanol Fermentation by *Saccharomyces* Yeasts

Among the yeasts carrying out alcoholic fermentation, species of the genus *Saccharomyces* play a key role in the ethanol fermentation process (Belloch *et al.*, 2008). *Saccharomyces* yeasts acquired their competitive advantage in rich environments by

gaining the ability to produce and tolerate large amounts of ethanol (**Tables 1** and **2**), as well as through their ability to grow in both anaerobic and aerobic conditions (Sicard and Legras, 2011). The baker's yeast *S. cerevisiae*, a Generally Regarded as Safe (GRAS) microorganism, is a well-studied model system in biochemistry, cell biology, classical genetics, and molecular biology (Duina *et al.*, 2014), and has become the microorganism of choice for the industrial production of ethanol (Zaldivar *et al.*, 2001). *S. cerevisiae* is one of the most fermentative-prone microbial species, producing ethanol even in the presence of excess oxygen. It shows fast rates of sugar consumption and ethanol production, tolerates high levels of ethanol and organic acids, and is also one of the few yeast species that can grow under strictly anaerobic conditions (Albergaria and Arneborg, 2016). All these characteristics allow this yeast to be considered the agent of wine, bread, ale beer, and palm and sake fermentations (Lodolo *et al.*, 2008).

Besides *S. cerevisiae*, other *Saccharomyces* species used in fermentation include *Saccharomyces bayanus* (syn. *Saccharomyces uvarum*), a cryophilic yeast isolated from wine and cider fermentations (Naumov *et al.*, 2001). It is involved in lager beer fermentation and in wine making for its ability to ferment at lower temperatures, making it popular for white wine production. Moreover, *S. bayanus* contributed with the aroma attributes of Chardonnay juice and has been used in the fermentation of red grape varieties (Blazquez-Rojas *et al.*, 2012; Eglinton *et al.*, 2000). Another interesting species of this group is *Saccharomyces paradoxus*, the closest known relative of *S. cerevisiae* (Koufopanou *et al.*, 2006). *S. paradoxus* has been described as the main yeast in Croatian wines (Redžepović *et al.*, 2002) and provides an alternative approach to Chardonnay fermentation (Orlic *et al.*, 2007). Additional *Saccharomyces* species are *Saccharomyces cariocanus*, *Saccharomyces mikatae* and *Saccharomyces kudriavzevii*, which have been found in natural environments (Naumov *et al.*, 2000), but are not present in fermentative environments. However, hybridizations have allowed the generation of efficient ethanol-producing strains, such as *S. cerevisiae* x *S. mikatae* hybrids, which show similar ethanol levels to the *S. cerevisiae* parent-made wines (Bellon *et al.*, 2013). Other examples are *S. cerevisiae* x *S. kudriavzevii hybrids*, which acquired properties of both parental strains, such as good alcohol and glucose tolerance, fast fermentation performance, better adaptation to low and intermediate temperatures, and higher production of glycerol and aromatic compounds (Belloch *et al.*, 2008). Another hybrid, *Saccharomyces pastorianus* (syn. *Saccharomyces carlsbergensis*), a natural hybrid of *S. bayanus* and *S. cerevisiae* is involved in the production of lager beer being responsible for 90% of the beer market (Miller *et al.*, 2012).

Fermentation by Non-*Saccharomyces* Yeasts

While *S. cerevisiae* is undoubtedly the organism of choice for industrial production of ethanol, non-*Saccharomyces* yeasts, a group of genetically diverse microorganisms, also present high potential during the fermentation process (**Tables 1** and **2**). Non-*Saccharomyces* yeasts were initially considered as "wild" or "spoilage" yeasts, because they were often isolated from stuck or sluggish fermentations, or wines with anomalous analytical and sensorial profiles. However, nowadays the importance of non-*Saccharomyces* yeasts in spontaneous fermentation has been demonstrated since they contribute positively to the definition of the sensory quality of alcoholic beverages (Belda *et al.*, 2017). Highly diverse yeast populations, mainly belonging to the genera *Pichia*, *Candida*, *Hanseniaspora*, *Metschnikowia*, *Issatchenkia* and *Kluyveromyces* are often described in the initial stages of fermentation (Zott *et al.*, 2008), while sometimes other yeast species such as *Torulaspora*, *Saccharomycodes*, *Dekkera*, *Zygosaccharomyces* and *Schizosaccharomyces*, are also observed (Fleet, 2008).

It has been shown that non-*Saccharomyces* strains can be detected (ranging from 10^3–10^5 cells/mL) throughout wine fermentation (Jolly *et al.*, 2003). However, most of them are soon overtaken by the strongly fermentative *S. cerevisiae* strains until the process ends (Albergaria and Arneborg, 2016). Non-*Saccharomyces* yeasts exert their metabolic activity contributing to the fermentative process (Pretorius, 2000) by bringing some specific enological characteristics. They produce high levels of aromatic compounds such as esters, higher alcohols and fatty acids, which contribute to the sensory quality of alcoholic beverages (Viana *et al.*, 2008). Furthermore, they can ferment at low oxygen concentrations, allowing the increase of biomass and decrease in ethanol yields, a strategy that can be used to reduce the ethanol content of wines produced in co-culture with *S. cerevisiae* (Contreras *et al.*, 2014). Thus, to improve the chemical composition and sensory properties of wine, the use of non-*Saccharomyces* together with *Saccharomyces* strains as part of mixed and multistarter fermentations has been proposed as an alternative to spontaneous fermentation (Romano *et al.*, 2003).

Several non-*Saccharomyces* yeasts have already been used in fermentation procedures. For example, wines of the grape varieties Sauvignon Blanc, Chenin Blanc and Muscat d'Alexandrie have been obtained by sequential fermentation with *Metschnikowia pulcherrima* (anamorph *Caesalpinia pulcherrima*) and *S. cerevisiae*, showing higher quality scores than control wines obtained by fermentation with *S. cerevisiae* alone (Varela *et al.*, 2016). *Hanseniaspora* species, such as *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*), generally show low fermentative capacity, but are important in the production of wine volatile compounds. *Hanseniaspora vineae* (formerly *Hanseniaspora osmophila*) and *Hanseniaspora guilliermondii* have been reported to produce increased amounts of 2-phenyl-ethyl acetate associated with "rose", "honey", "fruity" and "flowery" aromas (Viana *et al.*, 2011). Other non-*Saccharomyces* species such as *Torulaspora delbrueckii* (teleomorph of *Candida colliculosa*), co-inoculated with *S. cerevisiae*, are used for the production of Sauvignon Blanc and Chenin Blanc wines (Jolly *et al.*, 2003). The co-inoculation of *T. delbrueckii* and *S. cerevisiae* simultaneously and/or sequentially also increased aroma in Vino Santo and Amarone wines (Azzolini *et al.*, 2015). *Schizosaccharomyces pombe* was used in mixed fermentations with *S. cerevisiae* to remove malic acid and total acidity in Airen grape juice (Benito *et al.*, 2012) and has also been used to partially remove gluconic acid after fermentation of Pedro Ximenez wines (Peinado *et al.*, 2004). Fermentation of Sangiovese grape must with *Lachancea thermotolerans* (*Kluyveromyces thermotolerans*)/*S. cerevisiae* produced wines that scored higher in "spicy" and "acidity" attributes than *S. cerevisiae* wines (Gobbi *et al.*, 2013). In addition, *Kluyveromyces marxianus* is involved in the production of the traditional Mexican spirits tequila and mezcal using *Agave tequilana* (Flores *et al.*, 2013; Lopez-Alvarez *et al.*, 2012).

Table 1 Ethanol production of *Saccharomyces* and non-*Saccharomyces* yeasts

Strain	*Substrate (concentration, g/L)*	*Time (h)*	*Temperature (°C)*	*Ethanol production (g/L)*	*References*
Saccharomyces					
S. cerevisiae	Glucose (50–200)	18–94	30	5.1–91.8	Vallet *et al.* (1996)
S. cerevisiae	Sucrose (100)	24	30–35	25.0–50.0	Pinal *et al.* (1997)
S. cerevisiae	Molasses (1.6–5)	24	30	5.0–18.4	Ergun and Ferda-Mutlu (2000)
S. cerevisiae	Sucrose (220)	96	28	96.7	Çaylak (1998)
S. cerevisiae	Amarone grapes (320)	696	15–18	148.1	Tosi *et al.* (2009)
S. cerevisiae	Coffee grounds (22)	24	30	11.7	Mussatto *et al.* (2012)
S. bayanus	Amarone grapes (320)	888	15–18	138.1	Tosi *et al.* (2009)
S. bayanus	Glucose (240)	45	30	118.0	Amin and Verachtert (1982)
S. paradoxus	Mannitol (20)	120	30	7.3	Ota *et al.* (2013)
S. paradoxus	Glucose (20)	24	30	10.2	Ota *et al.* (2013)
S. pastorianus	Glucose (100)	16	30	44.5	Fujii *et al.* (2001)
Non-Saccharomyces					
Candida utilis	Glucose (100)	18–94	30	44.4	Vallet *et al.* (1996)
Kluyveromyces fragilis	Coffee grounds (22)	24	30	1.2	Mussatto *et al.* (2012)
K. fragilis	Glucose (20–120)	18–94	30	48.9	Vallet *et al.* (1996)
Kluveromyces marxianus	Glucose (100)	18–94	30	44.4	Vallet *et al.* (1996)
K. marxianus	Sugar cane juice supplemented with 8% sucrose	48	37	75.4	Limtong *et al.* (2007)
Kuraishia capslata	Mannitol (20)	120	30	2.6	Ota *et al.* (2013)
Ogataea glucozyma	Mannitol (20)	120	30	2.1	Ota *et al.* (2013)
Pichia angophorae	Glucose (20)	24	30	11.5	Ota *et al.* (2013)
Pichia stipitis	Coffee grounds (22)	30	30	10.5	Mussatto *et al.* (2012)
P. stipitis	Giant reed (33.4)	96	30	8.2	Scordia *et al.* (2012)
Pichia kluyveri	Agave tequilana juice (120)	48	33	86.4	Amaya-Delgado *et al.* (2013)
Pachysolen tannophilus	Glucose (0–25) Xylose (0–25)	100	30	7.8	Sánchez *et al.* (1999)
Schizosaccharomyces pombe	Cassava starch (95)	66	32	72.1	Choi *et al.* (2010)

Table 2 Ethanol tolerance of *Saccharomyces* and non-*Saccharomyces* yeasts

Strain	*Time (hours)*	*Ethanol tolerance (% v/v)*	*References*
Saccharomyces			
S. cerevisiae	48	15	Belloch *et al.* (2008)
S. cerevisiae var. *capensis*	48	9.1	Aguilera *et al.* (2006)
S. cerevisiae var. *cerevisiae*	48	6.9	Aguilera *et al.* (2006)
S. bayanus	48	12	Belloch *et al.* (2008)
S. paradoxus	48	12	Belloch *et al.* (2008)
S. kudriavzevii	48	5	Belloch *et al.* (2008)
S. cariocanus	48	12	Belloch *et al.* (2008)
S. mikatae	48	10	Belloch *et al.* (2008)
Non-Saccharomyces			
Candida stellata	48	11	Gao and Fleet (1988)
Kloeckera apiculata	48	6.5	Aguilera *et al.* (2006)
K. apiculata	48	9	Gao and Fleet (1988)
Pichia membranifaciens	48	6.3	Aguilera *et al.* (2006)
Torulaspora delbrueckii	48	2.7	Aguilera *et al.* (2006)

Industrial Process for Bioethanol Production

The process of bioethanol production by yeasts largely depends on the feedstock used, which is normally pretreated to facilitate the subsequent processes. There are four methods currently applied during pretreatment, which are physical (i.e., milling), chemical (i.e., acid/alkaline hydrolysis), biological, and physicochemical (i.e., steam) (Mohd-Azhar *et al.*, 2017). After pretreatment, hydrolysis takes place. During hydrolysis, feedstocks are broken down into fermentable sugars through two possible methods: (1) acid hydrolysis, which requires high temperatures and low pH, generating inhibitory by-products; and/or (2) enzymatic hydrolysis, which is performed at less severe conditions and has the potential for total conversion of biomass to

glucose (Khawla *et al.*, 2014). After hydrolysis, the fermentation process follows (see Section "Ethanol Production by Yeasts during the Fermentation Process"). Fermentation can be performed in batch, where substrates are only provided at the beginning of the process without changing the medium; repeated batch, which involves repeated cycles of fermentation by re-inoculating the cells from one batch fermentation broth to the next batch; continuous mode, carried out by constantly adding substrates, culture medium and nutrients; and fed-batch, which is a combination of batch and continuous mode (Mohd-Azhar *et al.*, 2017).

Several factors affect the production of bioethanol, such as temperature, sugar concentration, pH, dissolved O_2, and fermentation time, which are carefully regulated throughout the fermentation process (Fakruddin *et al.*, 2012; Mohd-Azhar *et al.*, 2017). Temperature directly affects yeast's growth rate. The ideal temperature ranges from 20 to 35°C, and higher temperatures may lead to the generation of heat stress and lower ethanol titers. Initial sugar concentration has also been considered as a key factor in ethanol production, since an increase of sugar concentration directly correlates with an increase in the fermentation rate. However, sugar excess can cause steady fermentation rates. pH also affects ethanol production since it directly affects yeast growth. The optimum pH in fermentation ranges from pH 4.0–6.0, and higher pH is often associated with reduced ethanol yields. Aeration was also shown to influence fermentation. A high agitation rate positively correlates with high ethanol production by increasing the amount of sugar consumption and reducing growth inhibition by ethanol (see below). Finally, the fermentation time also affects ethanol yields. Short fermentation times result in partial fermentation due to inadequate growth of microorganisms, whereas long fermentation times may have toxic effects on microbial growth, particularly in the batch mode due to ethanol accumulation (Fakruddin *et al.*, 2012; Mohd-Azhar *et al.*, 2017).

Molecular Basis of Fermentation-Associated Stress

During fermentation, yeast cells are exposed to several stresses, including osmotic, ethanol, heat, and oxidative stress. Initially, cells deal with osmotic stress due to high sugar concentrations, while at the end of the fermentation process high ethanol concentration is the most important stress that inhibits yeast growth and metabolism, leading to the end of ethanol production (Auesukaree, 2017). The cellular response to fermentation-associated stresses involves interplay of several hundreds of genes in complex networks. As an example of this, Rossignol and collaborators have reported a change in the expression of more than 2,000 genes during *S. cerevisiae* fermentation (Rossignol *et al.*, 2003).

To deal with the different fermentation-associated stresses, yeast cells undergo several genetic responses. The osmo-regulatory response involves the activation of the high osmolarity glycerol (HOG) pathway (Saito and Posas, 2012). When yeast cells are exposed to hyperosmotic shock, the HOG pathway is stimulated and the stress-activated protein (SAP) kinase Hog1 is phosphorylated (Tatebayashi *et al.*, 2020). Activated Hog1 regulates the activities of the transcriptional activators Hot1, Smp1, Msn1, Msn2, and Msn4, and the repressor Sko1. These transcription factors bind to the so called stress response elements (STREs) (Saito and Posas, 2012). The STREs also mediate induction of transcription by heat shock, nitrogen starvation and oxidative stress (Schüller *et al.*, 1994) leading to the transcriptional activation of downstream genes related with a broad range of functional categories, including glycerol synthesis, protein biosynthesis, amino acid metabolism, nucleotide metabolism, transport, cell cycle and growth, lipid metabolism, fatty acid and ergosterol metabolism, membrane and cell wall organization, and proline and tryptophan biosynthesis (Ma and Liu, 2010).

Other groups of osmo-inducible genes include those involved in the protection against oxidative damage and protein denaturation, such as genes encoding antioxidants (*CTT1*, *GRE3*, *TRX2*, *TTR1*) and chaperones (*HSP12*, *HSP26*, *HSP42*, *HSP70*, *HSP90*, *HSP104*) (Auesukaree, 2017). Heat shock proteins (HSPs) are produced together with compatible solutes such as trehalose (Verghese *et al.*, 2012) which also plays a role in preventing protein denaturation and aggregation through its function as a protein stabilizer. It binds to unfolded proteins to maintain them in a partially-folded state until being refolded by molecular chaperones (Singer and Lindquist, 1998). The expression of trehalose metabolism-related genes including *TPS1*, *TPS2*, and *NTH1*, is rapidly up-regulated under heat stress, resulting in an increase of intracellular trehalose levels (Kitichantaropas *et al.*, 2016). In agreement, the *TPS2* gene has been shown to be required for tolerances to heat, oxidative, osmotic, and ethanol stresses (Auesukaree *et al.*, 2009).

At the end of the fermentative phase, a transient growth arrest (diauxic shift) occurs in yeasts, in which major changes in gene expression enable cells to grow throughout respiration. Using the ethanol accumulated during the fermentative growth induces the production of reactive oxygen species (ROS) (Du and Takagi, 2007). In order to deal with ROS production, the superoxide dismutase (SOD) converts superoxide anion into hydrogen peroxide, which is then reduced to water and oxygen by catalase, glutathione peroxidase and thioredoxin peroxidase (Culotta *et al.*, 2006). Yeast cells have three glutathione peroxidases, Gpx1, Gpx2, and Gpx3, which reduce phospholipid hydroperoxides, suggesting their role in protecting membrane lipids from peroxidation (Avery and Avery, 2001).

Many other genes associated with oxidative stress are upregulated under the control of Yap1p and Skn7 transcription factors. Yap1p and Skn7 coordinately control the transcription of oxidative stress-responsive genes including *SOD1* and *SOD2*, the thioredoxin encoding gene *TRX2*, the thioredoxin reductase-encoding gene *TRR1*, the peroxiredoxins encoding genes *TSA1* and *AHP1*, as well as *GPX2*, which encodes a glutathione peroxidase (Morano *et al.*, 2012).

Ethanol Stress and Tolerance

During fermentation, high ethanol concentrations cause an increase in fluidity and permeability, and consequential decrease in the yeast membrane integrity, which induces an increased passive influx of ions across the membrane triggering cytosolic acidification

(Charoenbhakdi *et al.*, 2016). Consequently, yeast cells show an elevation of H^+ATPase activity in plasma membrane (Fujita *et al.*, 2006) due to up-regulation of *PMA1* and *PMA2* genes (Fernandes and Sa-Correia, 2003). Additionally, to maintain membrane integrity and to modify membrane fluidity, some studies have identified changes associated to membrane composition, including changes in the balance of ergosterol and unsaturated fatty acids (UFAs). The predominant mono-UFAs are the palmitoleic and oleic acids, which are obtained by the desaturation of the saturated palmitic and stearic acids, respectively. This reaction is catalyzed by a membrane desaturase encoded by the gene *OLE1* (Ma and Liu, 2010), which is up-regulated during fermentation.

High ergosterol content in yeasts is associated with higher ethanol tolerance (del Castillo-Agudo, 1992). Deletion mutants of *ERG2*, *ERG3*, *ERG5*, *ERG6*, *ERG24*, and *ERG28* involved in ergosterol biosynthesis showed defective growth of yeast cells under ethanol stress (Auesukaree *et al.*, 2009). Furthermore, the cell wall integrity pathway has been reported to play an important role in controlling the transcriptional adaptive response to cell wall stress, leading to a remodeling of the cell wall architecture in order to improve its resistance (Orlean, 2012). The *BCK1* and *SLT2* genes encoding mitogen activated protein (MAP)3K and MAPK, respectively, have also been reported to be essential for ethanol tolerance. Upon ethanol exposure, the expression of specific cell wall-remodeling genes, including *FKS2*, *CRH1*, and *PIR3* (encoding -1,3-glucan synthase, chitin transglycosylase, and O-glycosylated cell wall protein, respectively) are induced (Udom *et al.*, 2019).

On the other hand, trehalose accumulation has been reported to be crucial for improved yeast growth under ethanol stressing conditions by alleviating ethanol-induced membrane permeabilization (Mansure *et al.*, 1994). The expression of trehalose metabolism-related genes such as *TPS1*, *TPS2*, and *NTH1* are up-regulated upon ethanol exposure. Trehalose is also involved in the close interplay with HSPs in stabilizing protein structures through its functions in protein binding and reduction of water activity (Jain and Roy, 2009).

Some amino acids, including proline, tryptophan and arginine, also show protective effect against ethanol stress. The expression of the *PUT4* gene encoding a high affinity proline transporter was strongly induced upon ethanol exposure (Kaino and Takagi, 2008). Proline has been shown to play a role in stabilizing proteins and membranes, and in inhibiting protein aggregation during refolding (Ignatova and Gierasch, 2006), suggesting a role in protecting yeast cells against the adverse effects of ethanol on proteins and membranes. In addition, proline has the ability to scavenge several ROS including hydroxy radicals and superoxide anions (Kaul *et al.*, 2008). In the case of tryptophan, the deletion of genes encoding tryptophan biosynthesis-related enzymes, i.e., *TRP1*, *TRP2*, *TRP3*, *TRP4* and *TRP5*, resulted in hypersensitivity to ethanol stress, whereas the overexpression of these genes, especially *TRP1* and *TRP5*, and the tryptophan permease-encoding gene *TAT2*, increased ethanol tolerance after tryptophan supplementation (Yoshikawa *et al.*, 2009). Arginine has also been reported to have a protective effect against ethanol-induced damage on cell wall, plasma membrane and intracellular organelles, possibly due to its role in inhibiting ROS generation (Cheng *et al.*, 2016). Together with the up-regulated genes described above, many other genes have been reported to be down-regulated during ethanol tolerance (Chandler *et al.*, 2004), indicating significant remodeling processes in response to ethanol stress.

Therefore, and despite extensive studies, ethanol tolerance is a complex, polygenic trait influenced by multiple alleles that may show complex interactions and may differ between strains (Snoek *et al.*, 2016). A better understanding of the genetic mechanisms underlying stress tolerance will contribute to generate strains for efficient ethanol fermentation and industrially optimized processes.

Yeast Improvement for Higher Ethanol Production and Tolerance

The main problems associated with the production of ethanol during the fermentation process is alcohol toxicity and growth inhibition of yeast cells, resulting in low alcohol titers in the fermentation broth (Jia *et al.*, 2010). In order to overcome these problems, several approaches have been applied, which include both the classical (non-GMO) optimization and the genetic engineering of yeasts for a higher ethanol production and tolerance.

Classical (Non-GMO) Optimization of Yeasts

Evolutionary engineering

A successful way to obtain strains with increased ethanol production and tolerance is evolutionary engineering, also referred to as adaptive, directed, or experimental evolution. It relies on the basic principles of (natural and/or induced) genetic variation and subsequent strain selection. Basically, a population of cells is grown under continuous selection for the phenotype of interest for many generations. Over time, random mutants will arise and these mutants will be selected for their improved phenotype (Steensels *et al.*, 2014). Strategies based on adaptive evolution are attractive, since they generate improved non-GMO strains that can be used in the industry in the short-term (Cadiere *et al.*, 2011) without going through all the legislation and restrictions associated to GMOs. Some *S. cerevisiae* strains obtained by this technique showed increased ethanol production and tolerance, and higher osmotic and temperature tolerance than their parental strains under laboratory, pilot-scale and industrial fermentation conditions (Basso *et al.*, 2008; Cadiere *et al.*, 2011; Chen and Xu, 2014). Sonderegger and collaborators reported *S. cerevisiae* mutants with up to 19% more ethanol production that the parental strain (Sonderegger and Sauer, 2003), and serial *S. cerevisiae* batch cultures increased the production from 2.5% to 10% (v/v) ethanol compared to parental strain-derived cultures (Dinh *et al.*, 2008).

UV and chemical mutagenesis

In addition to evolutionary engineering, random mutagenesis has also been widely applied to obtain mutants with improved characteristics. Among the classical methods, physical mutagens such as ultraviolet (UV) radiation and chemical agents, including N-methyl-N'-nitro-N nitrosoguanidine (NTG) and ethyl methane sulfonate (EMS), have been shown to be efficient to obtain strains with improved biotechnological properties (Parekh *et al.*, 2000). Studies have described the use of UV to improve ethanol production, tolerance and yield in yeasts, especially in *S. cerevisiae* (Revin *et al.*, 2018). Furthermore, some mutants have shown an increase of osmotolerance and improvement of thermotolerance compared to the wild type (Unaldi *et al.*, 2002). As examples, *Candida shehatae* and *Pichia stipitis* mutants obtained by UV mutagenesis showed better fermentation of xylose and glucose than the parental strain (Li *et al.*, 2012; Watanabe *et al.*, 2011), whereas irradiation of *K. marxianus* produced mutants with higher ethanol yield (0.51 g ethanol/g glucose, and 0.48 g ethanol/g galacturonic acid) than the wild type (0.43 g ethanol/g glucose, and 0.34 g ethanol/g galacturonic acid) at 46°C (Hughes *et al.*, 2013). EMS has been used to obtain *S. cerevisiae* mutants with a production of bioethanol 17.3% higher than the wild type (Mobini-Dehkordi *et al.*, 2008), and the UV irradiation followed by NTG mutagenesis allowed obtaining *K. marxianus* strains with 11% (v/v) of ethanol tolerance compared to the 8% (v/v) of the corresponding parental strain (Pang *et al.*, 2010).

Protoplast fusion

Another non-GMO technique used to improve alcohol productivity in yeasts is protoplast fusion (Steensels *et al.*, 2014). It involves the generation of new strains by allowing recombination between genomes of different parental strains, followed by selection of the cells showing the desired traits (Xin *et al.*, 2020). Using this technique, interspecific or even intergeneric crosses can be obtained. Since meiosis is not required, protoplast fusion can be used to increase the ploidy of the strains, which in some cases increases productivity (Attfield and Bell, 2003). Protoplast fusion is an important tool for the improvement of industrial yeast strains. For example, improvement has been reported for the fusion between the strain *S. cerevisiae* Q, frequently used as beer producer, with *S. cerevisiae* L. The Q/L fusant shows higher ethanol tolerance (14% v/v) than the strain Q (10% v/v) (Xin *et al.*, 2020). Other examples include protoplast fusion of *S. cerevisiae* with *Candida shehatae* followed by UV mutagenesis, resulting in a significant increase in ethanol production at 42°C, with a 90% fermentation efficiency (Pasha *et al.*, 2007). Protoplast fusion between *S. cerevisiae* and *K. marxianus* enhanced ethanol production and temperature tolerance using cheese industry waste (whey) as substrate (Ramasamy *et al.*, 2010). Although protoplast fusion offers great advantages, a strong disadvantage is that many of the hybrids are mitotically unstable and chromosomal loss or dissociation often occurs (Attfield and Bell, 2003).

Genome shuffling

Genome shuffling is also used to improve complex phenotypes in a fast and relatively easy manner (Zhang *et al.*, 2002). By applying repeated rounds of genetic recombination (either by protoplast fusion or mass mating) genome shuffling aims to combine different mutations in the same cell, leading to additive or synergistic effects (Santos and Stephanopoulos, 2008). Frequently, mutants generated by classical methods (UV or chemical mutagenesis) are screened for the phenotype of interest, and cells showing phenotypic improvement are used as a starting population for multiple rounds of genome shuffling. As examples, *S. cerevisiae* mutants generated by EMS or UV mutagenesis followed by genome shuffling showed remarkably enhanced tolerance to ethanol and osmolarity, increased metabolic rate, and increased ethanol yield compared to the wild type (Shi *et al.*, 2009). Similarly, after four rounds of genome shuffling, *C. krusei* improved its ethanol tolerance and production (Wei *et al.*, 2008).

Genetic Engineering of Yeasts

Although non-GMO techniques are preferred from an industrial point of view, non-GMO techniques are often limited to phenotypes that allow efficient selection and may involve a risk of improving one trait at the expense of others. In this context, directed genome manipulation offers a greater potential for the generation of strains with improved characteristics with no (or very few) side effects. Genetic engineering for yeast improvement in the context of ethanol fermentation comprises the manipulation of individual genes or metabolic pathways to increase the rate of sugar conversions to ethanol and ethanol tolerance.

Among the different molecular alternatives available, gene transfer, overexpression and/or gene deletions are the most applied approaches for the rational manipulation of yeast strains aiming at higher ethanol production and tolerance. Regarding ethanol production, deletion of *GPD1* and *GPD2*, two abundant NAD-dependent glycerol-3-phosphate dehydrogenases involved in glycerol synthesis in *S. cerevisiae*, increased ethanol production. Mutants *gpd1*Δ and *gpd2*Δ produced 0.456 and 0.478 g ethanol/g glucose, respectively, compared to the parental strain which produced 0.399 g ethanol/g glucose (Guo *et al.*, 2009). Deletion of *FPS*, which encodes a glycerol channel protein, alone or in combination with *GPD2* and/or *GPD1*, and together with the overexpression of the glutamate synthase-encoding gene *GLN1* and the glucose transporter *GLT-1* also significantly increased ethanol production (Cao *et al.*, 2010; Xiong *et al.*, 2014). Additionally, transfer of the glucoamylase gene of the zygomycete fungus *Rhizopus oryzae* to *S. cerevisiae* was reported to increase ethanol production up to 5% in this model yeast (Yang *et al.*, 2011).

The introduction of more precise genome editing techniques, such as Clustered Regularly Interspaced Short Palindromic Repeats-associated protein-9 nuclease (CRISPR/Cas9) (Doudna and Charpentier, 2014), has greatly improved the efficiency of traditional genetic engineering of yeast. For instance, the (over)expression of the cellulase-encoding gene *sestc*, isolated from the snail *Ampullaria gigas* Spix, under the regulation of the basidiomycete fungus *Agaricus biporus* glyceraldehyde-3-phosphatedehydrogenase (*gpd*) promoter

was achieved by CRISPR/Cas9 in *S. cerevisiae*. The resulting mutant showed 37.7-fold higher ethanol production than that of the wild type when using orange peel as substrate (Yang *et al.*, 2018). Besides, the disruption of the alcohol dehydrogenase (*ADH2*) gene by CRISPR/Cas9 allowed the introduction of a frameshift mutation in the *ADH2* locus resulting in *S. cerevisiae* strains in which ethanol yield improved up to 74.7% compared to the native strain (Xue *et al.*, 2018).

It is now generally accepted that most cellular phenotypes are multigenic traits. As a result, engineering a desired phenotype would be enormously facilitated by simultaneous multiple gene modifications (Alper and Stephanopoulos, 2007). For this, global Transcription Machinery Engineering (gTME) provides an option for multiple gene manipulation by randomly introducing mutated copies of global transcriptional factors, which control the transcription of a large set of genes (Alper *et al.*, 2006). Through mutagenesis (via error-prone PCR mutations), gTME allows for global perturbations of the transcriptome, so that improved phenotypes can be elicited quickly and effectively (Li *et al.*, 2018). Through gTME, a *S. cerevisiae* strain with three separate mutations in the *SPT15* gene, which encodes the TATA-binding protein, in one component of the RNA polymerase II and in the transcription factor D (TFIID) complex, was obtained. In the mutant, the transcription pattern of hundreds of genes changed and the strain produced 69% more ethanol than the wild type (Alper *et al.*, 2006). Xue *et al.* produced *S. cerevisiae* mutants from a wild type wine yeast, and the mutations in the *SPT8* (suppressor of ty insertions 8) gene, resulted in 8.9% higher ethanol tolerance and 10.8% higher ethanol production compared to the wild type (Xue *et al.*, 2019).

Regarding ethanol tolerance, it is important to highlight that the genetic basis of stress tolerance in yeast cells is very complex, and manipulation at the molecular level to improve stress tolerance still faces many difficulties (Zhao and Bai, 2009). Despite this, several examples demonstrated the successful improvement of yeast tolerance to high ethanol concentrations. For instance, deletion and/or the inactivation of the vacuolar acid trehalase (*ATH1*) gene in *S. cerevisiae* caused high survival rates under various stresses, including ethanol (Jung and Park, 2005).

Conclusions and Future Perspectives

Since the beginning of recorded history, a strong relationship between humans and ethanol has been developed, likely due to its presence in wine and many beverages, and due to its industrial relevance. Yeasts are the organisms of choice to produce ethanol at the industrial level, and strain improvement for high ethanol production and tolerance is of great interest to the scientific community and the industry.

Despite both classical optimization methods and genetic engineering tools have allowed the improvement of yeasts for a more efficient ethanol fermentation process, demands for increased productivity, wider substrate range utilization, as well as changes in consumer preferences lead to a greater interest in further improving the currently available industrial strains. In this context, there is an urgent need to continue exploring the genetic capacity of various natural and/or industrial strains for a better understanding of the ethanol fermentation process, and the physiological and biochemical basis of yeast stress tolerance. Finally, the use of different omics data (proteomics, transcriptomics and metabolomics) together with the implementation of new genetic manipulation techniques and synthetic biology approaches might aid in the development of robust yeast strains for bioethanol production.

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The Biosynthesis of Fungal Secondary Metabolites: From Fundamentals to Biotechnological Applications

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Introduction

Secondary metabolites (SMs) are natural products synthesized mainly by bacteria, fungi and plants. They are molecules of low molecular weight with diverse chemical structures and biological activities. The name secondary metabolite originates from the initial observation that their production is not necessary for the growth and reproduction of organisms, in contrast to primary metabolites which include lipids, amino acids, carbohydrates and nucleic acids. However, SMs are far from being secondary and the term "specialized metabolites" is emerging to describe them. It is now accepted that SMs play key roles in the survival of the organisms that produce them because SMs determine interactions within their environment. Nowadays, SM production is a major research field for organic chemists, molecular biologists and bioinformaticians alike. In this review, we will focus on SMs produced by fungi to introduce the fundamental concepts of their biosynthesis, present how the genomics era has impacted the study of fungal SMs, and report the biotechnological tools that have been developed to engineer SM biosynthetic pathways.

Fungi Produce Bioactive Secondary Metabolites That Impact Human Societies

Fungal Secondary Metabolites in Human Daily Life

Fungal SMs have impacted human societies both positively and negatively since ancient times. The episode known as Saint Anthony's fire in 1039 was an outbreak of ergotism, a disease caused by the consumption of rye that we know now was infected by the fungus *Claviceps purpurea* (van Dongen and de Groot, 1995). This disease is actually caused by SMs called ergot alkaloids that are secreted by the fungus. Many other so-called mycotoxins have been reported and their presence in food and feed is strictly regulated and monitored. The most frequently encountered mycotoxins worldwide are produced by *Fusarium* species on cereals and include deoxynivalenol (DON), fumonisin and zearalenone (Biomin mycotoxin survey see "Relevant Websites Section"). Aflatoxins produced by *Aspergillus* species are also an important threat, which are famous to have caused in England the death of 100,000 turkeys that consumed contaminated peanut meal (Richard, 2008).

Although some fungal SMs are mycotoxins, many others exhibit beneficial activities to humans. Hundreds of fungal species, mostly basidiomycetous edible mushroom-forming species, have been used in traditional Chinese medicine for thousands of years. Their beneficial properties are linked to the production of mixtures of SMs with diverse biological activities, for example against cancer, diabetes and cardiovascular diseases (Hyde *et al.*, 2019). In the Western world, fungal SMs have revolutionized modern medicine more than once (**Fig. 1**). The most famous example is the discovery of the first large-spectrum antibiotic penicillin produced by *Penicillium rubens* (formerly *P. chrysogenum*; (Houbraken *et al.*, 2011)). Penicillin belongs to a family of compounds known as beta-lactams, which still accounts for the largest antibiotic market size. The discovery of cyclosporin A produced by *Tolypocladium inflatum* brought another revolution in the second half of the 20th century: this immunosuppressive SM allowed successful organ transplantation (Colombo and Ammirati, 2011). The statin family of compounds, used as cholesterol-lowering agents, was also discovered in the 1970s and several compounds of fungal origin, like monacolin K, actually represent the largest market of drugs, reaching an annual turn-over circa US$50 billion (Hyde *et al.*, 2019). Fungal SMs find many other applications in diverse fields, including agriculture (e.g., strobilurin fungicides are all derived from SMs produced by the basidiomycetous mushroom *Strobilurus tenacellus* (Bartlett *et al.*, 2002)) and industry (e.g., *Monascus purpureus* is exploited for the production of red rice powder in Asia because it produces red pigments like rubropunctamine (Mukherjee and Singh, 2011)).

Since the 1990s, the development of natural products into marketed active molecules has entered a so-called discovery void (**Fig. 1**) because the same molecules of natural origin were rediscovered, which turned companies to redirect their efforts towards chemical synthesis of analogs of existing compounds (Li and Vederas, 2009). Despite having provided key compounds to human societies, the

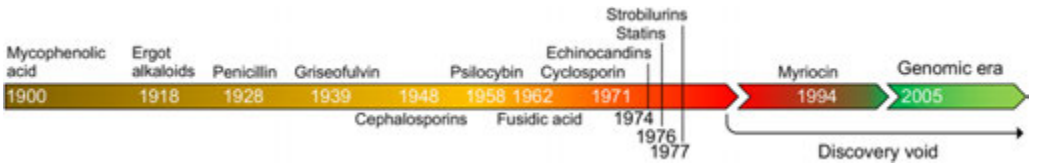

Fig. 1 Discovery of fungal secondary metabolites with commercial application. Since the end of the 1980s, we have entered a so-called discovery void due to companies stopping the prospection of natural products and investing in chemical synthesis. Since the release of the first genomes of filamentous fungi at the beginning of the 21st century, the genomic era has revived interest in fungal secondary metabolites.

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fungal kingdom had been neglected in the search for natural products. But the genomic era promises to bring fungi in the spotlight again, especially to find solutions to the emergence of multi-resistant bacterial and fungal pathogens (Harvey *et al.*, 2015). Academic laboratories and small and medium enterprises have initiated new exploitation of the fungal kingdom for bioactive compounds, and these initiatives represent the beginning of a long-term research effort.

Fungal Secondary Metabolites in Fungal Daily Life

While fungal SMs exhibit biological activities that are useful or detrimental to humans, these activities are most often side effects and do not reflect the biological function for the organisms that produce them. It is now well accepted that SMs have important functions in the interactions between fungi and their environments, especially by contributing to the colonization of specific ecological niches. Fungal pigments like dihydroxynaphthalene (DHN) melanin and cladofulvin provide protection against environmental stresses such as UV light, desiccation and extreme temperatures (Langfelder *et al.*, 2003; Dadachova *et al.*, 2007; Griffiths *et al.*, 2018). Fungal SMs are particularly important for pathogens to establish disease. DHN melanin protects human pathogens from the immune system (Chai *et al.*, 2010; Thywißen *et al.*, 2011), while this SM is required for certain plant pathogens to mechanically penetrate plant tissues (Chumley and Valent, 1990). Toxic SMs are particularly well known to contribute to plant infection. Host-specific toxins (e.g., HC-toxin, AK-toxin) produced by *Cochliobolus* and *Alternaria* species determine their host range by targeting plant cultivars that carry a sensitivity gene (Stergiopoulos *et al.*, 2013). Non-specific toxins are more widely distributed and also contribute to the virulence of many fungi. One of the most studied class of toxins are perylenequinones, photoactive toxins that make *Cercospora nicotianae* pathogenic on tobacco (Choquer *et al.*, 2005; Ebert *et al.*, 2019). More recently, it is becoming clear that SMs produced by plant pathogens play more diverse and subtle roles in virulence which are not straightforward to study (Collemare *et al.*, 2019). Symbiotic fungi also appear to rely on the production of SMs to establish a mutualistic interaction with their hosts. The ectomycorrhizal fungus *Laccaria bicolor* secretes terpenes that were shown to be required for symbiosis establishment (Ditengou *et al.*, 2015), while the symbiont *Epichloë festucae* produces indole alkaloid SMs that protect its ryegrass host from herbivorous animals (Schardl *et al.*, 2013). Such a defensive biological function can be extended to antimicrobial compounds produced by fungi when they compete with other microorganisms living in the same ecological niche (O'Brien and Wright, 2011). This function was recently experimentally proven with bikaverin and beauvericin produced by *Fusarium fujikuroi* against the bacterium *Ralstonia solanacearum* (Spraker *et al.*, 2018) and with lagopodin B produced by *Coprinopsis cinerea* in response to the presence of bacteria (Stöckli *et al.*, 2019).

The Fundamentals of Secondary Metabolite Production in Fungi

A Diversity of Biosynthetic Enzymes

Fungal SMs are classified into a few major chemical classes depending on the precursors (either acyl-coAs, amino acids, or prenyl diphosphates) and enzymes used for their biosynthesis (Keller, 2019). Polyketides, non-ribosomal peptides, terpenes and indole alkaloids are synthesized by core enzymes named polyketides synthases (PKSs), non-ribosomal peptide synthetases (NRPSs), terpene cyclases (TCs) and dimethylallyltryptophan synthases (DMATSs), respectively (**Fig. 2** and **Fig. 3(A)**). These core enzymes are characterized by highly conserved domains that exhibit diverse catalytic activities. TCs and DMATSs are enzymes which are composed of one or two conserved domains (Schmidt-Dannert, 2015). In contrast, PKSs and NRPSs are multidomain mega-enzymes with complex catalytic activities (**Fig. 3(A)**).

In fungi, two types of PKSs are found: iterative type I PKSs and type III PKSs. Type I PKSs are mega-enzymes with several domains that are used iteratively to elongate the polyketide backbone mostly from acetyl- and malonyl-CoA (Cox, 2007; Herbst *et al.*, 2018). Type I iterative PKSs are further classified into three groups depending on the presence of specific conserved domains (**Fig. 3(A)**). Non-reducing PKSs (nrPKSs) produce aromatic compounds while reducing (rPKS) and partially reducing (prPKS) enzymes produce reduced polyketides due to the presence of one or several reducing conserved domains (dehydratase (DH), keto-reductase (KR) and enoyl-reductase (ER) domains) (Cox, 2007; Herbst *et al.*, 2018). The release of the polyketide chain from the PKS can be performed through different mechanisms depending on the presence of a final thiol-esterase (TE) or reductase (R) domain (Cox, 2007). Certain PKSs do not carry such a releasing domain and have co-evolved with other enzymes, including beta-lactamases and hydrolases, to release the polyketide chain (Cox, 2007; Griffiths *et al.*, 2016). While progress has been made towards understanding the programming of iterative type I PKSs, predicting the compound they will produce in terms of acyl-CoA specificity, chain length, cyclisation and reduction steps, remains challenging.

Type III PKSs are small enzymes consisting of a single keto-synthase (KS) domain (**Fig. 3(A)**). They catalyze the iterative condensation of a starter fatty acyl-coA and several extender units, mostly malonyl-CoA (Shimizu *et al.*, 2017). Type III PKSs show considerable flexibility, accepting a wide range of starter unit, from short to long linear or cyclic acyl-coA, resulting in the production of a variety of compounds (Shimizu *et al.*, 2017; Kaneko *et al.*, 2019). Compared to type I PKSs, fungal type III PKSs have been neglected enzymes and more research effort is needed to understand how they function.

NRPSs are large multi-modular enzymes, each module usually consisting of three conserved domains (**Fig. 3(A)**): adenylation (A; recruits an amino acid), condensation (C; catalyses the peptide bond) and thiolation (T; carries the peptide chain) domains (Singh *et al.*, 2017). Eventually, amino acid epimerization and N-methylation domains can be found within modules

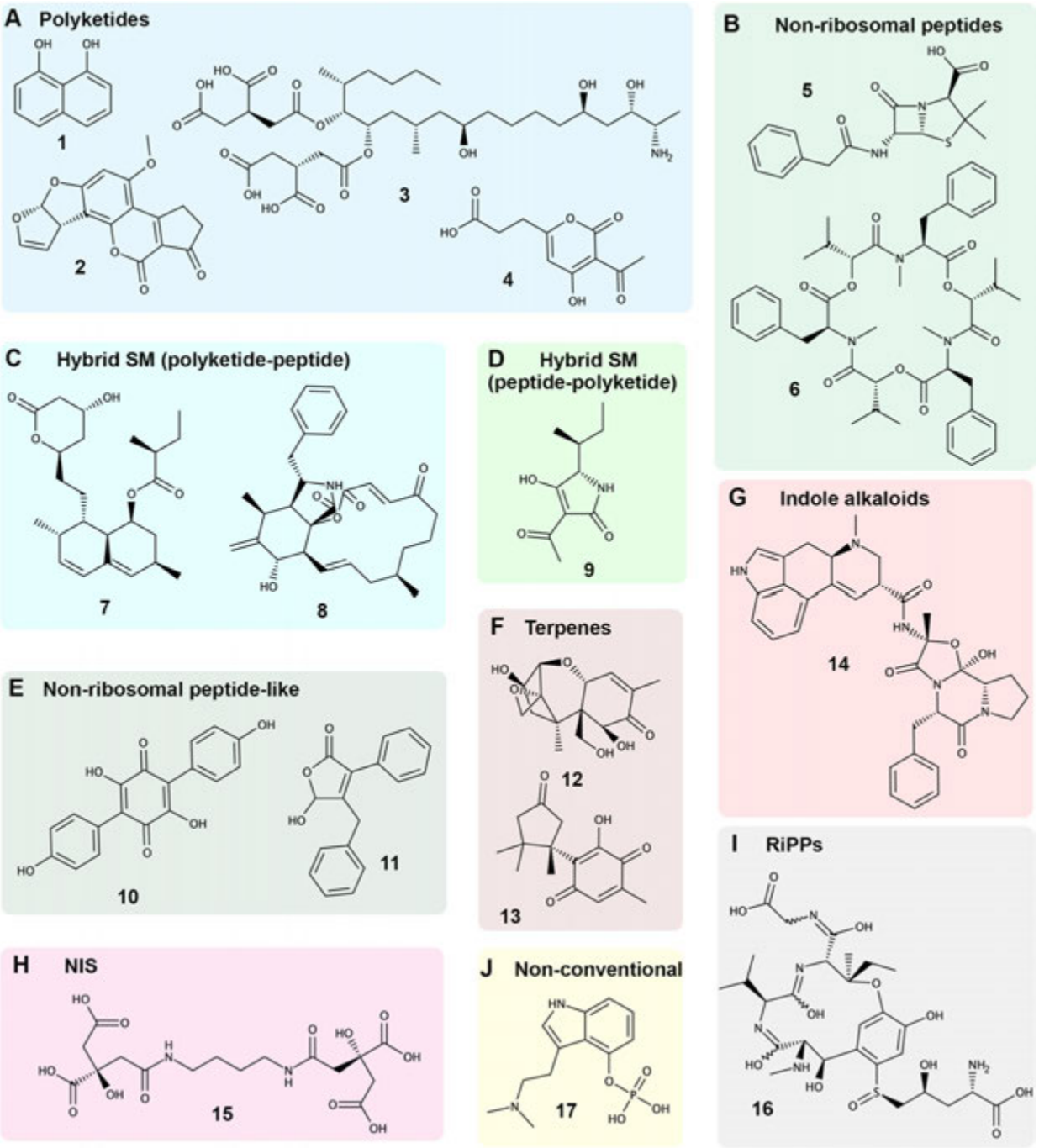

Fig. 2 Diversity of chemical structures observed for fungal secondary metabolites (SMs). Examples of fungal secondary metabolites. (A) polyketides (1) 1,8-dihydroxynaphthalene; (2) aflatoxin B1; (3) fumonisin B; (4) cyperone B1, (B) non-ribosomal peptides (5) penicillin G; (6) beauvericin, (C) hybrid polyketide-peptides (7) monacolin K; (8) cytochalasin A, (D) hybrid peptide-polyketides (9) tenuazonic acid, (E) non-ribosomal peptide-like (10) atromentin; (11) microperfuranone, (F) terpenes (12) deoxynivalenol; (13) lagopodin B, (G) indole alkaloids (14) ergotamine, (H) NRPS-independent siderophore (NIS) (15) rhizoferrin, (I) Ribosomally synthesized and post-translationally modified peptides (RiPPs) (16) ustiloxin, and (J) unconventional compounds (17) psilocybin.

(Singh *et al.*, 2017). Often, the NRPS organization ends with a single C domain that is responsible for the release of the peptide chain through cyclisation (Zhang *et al.*, 2016). In many cases, NRPS modules are sequentially used, so that the number and order of the modules determine the length and nature of the peptide. However, some fungal NRPSs are iterative and contain modules that reuse the A domain of another module to incorporate a new amino acid (Schwecke *et al.*, 2006; Yu *et al.*, 2017). Similarly to fungal PKSs, it is possible to classify NRPSs into large phylogenetic groups (Bushley and Turgeon, 2010), but predicting which

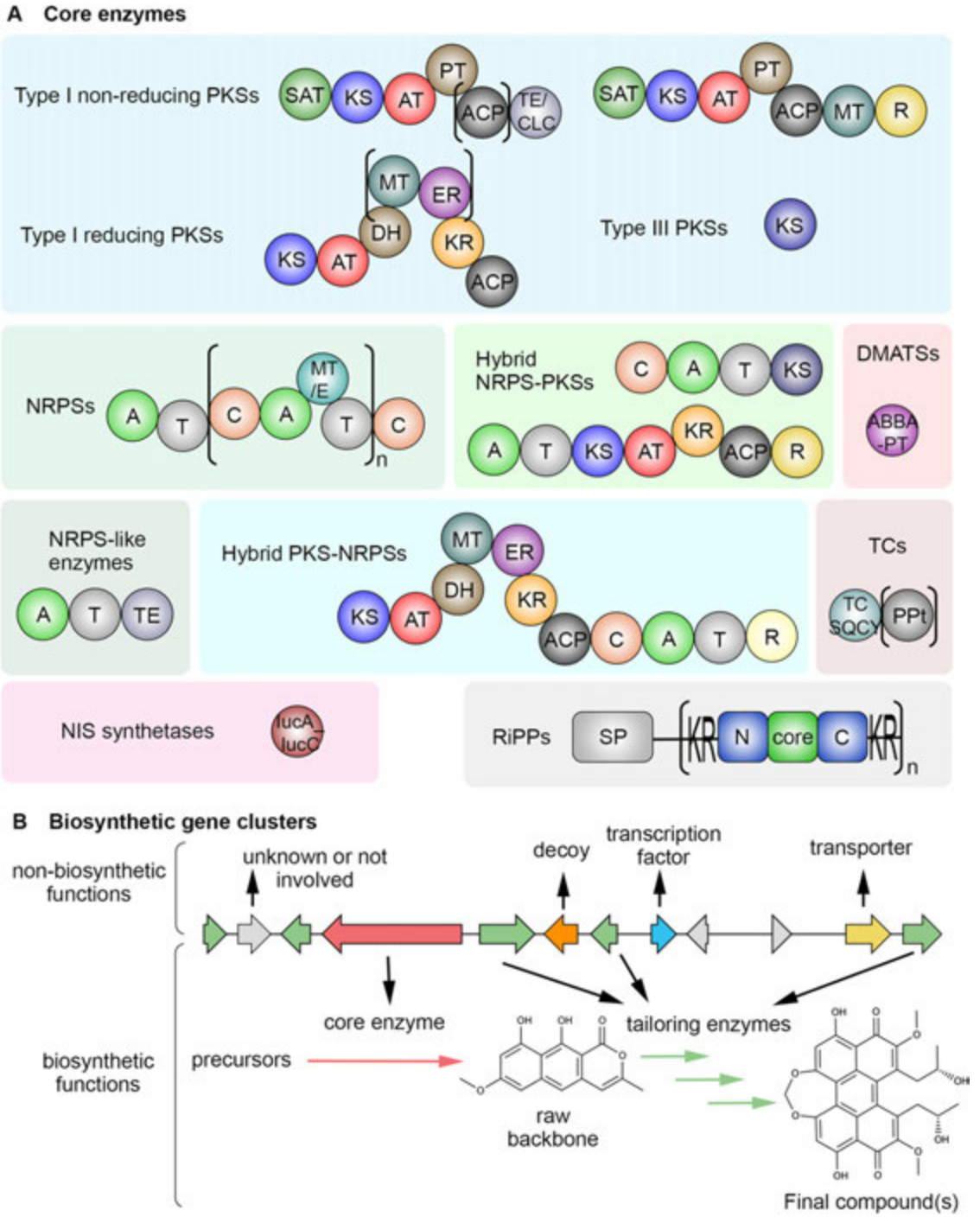

Fig. 3 Secondary metabolite (SM) biosynthetic pathways in fungal genomes. (A) Core genes and enzymes involved in the biosynthesis of the SM chemical backbone. The module organization of the core enzymes is shown with the arrangement of conserved domains. SAT: Starter Acyl-carrier protein Transacylase; KS: Keto-Synthase: AT: Acyl-Transferase; PT: Product Template; ACP: Acyl-Carrier Protein; TE: ThioEsterase; CLC: Claisen Cyclase; MT: Methyl Transferase; R: Reductase; DH: DeHydratase; ER; Enoyl Reductase; KR: Keto Reductase; A: Adenylation; T: Thiolation; C: Condensation; E: Epimerization; TC: Terpene Cyclase; SQCY: Squalene Cyclase; PPt: PolyPrenyl transferase; ABBA-PT: Aromatic Prenyl Transferase; SP: Signal Peptide. Two architectures are found for iterative type I non reducing polyketide synthases (PKSs), but both are characterized by the presence of the SAT and PT domains. The brackets indicate that the ACP domain is duplicated in certain enzymes. Type I reducing PKSs are characterized by the presence of at least one of the DH, ER and KR domain. The brackets indicate the domains that are not found in partially reducing PKSs. The most common structure of NRPS-like enzymes is represented, but diverse domains, including ferric reductase, polynucleotidyl transferase ribonuclease H and LPS-induced tumor necrosis alpha factor, linked to a single A domain are also found. Terpene Cyclases (TCs) are classified into different groups according to the conserved domain they harbor. Bifunctional TCs are characterized by the presence of a PPt domain. NRPS-independent siderophore (NIS) synthetases contain a single IucA_IucC domain. Ribosomally synthesized and post-translationally modified peptides (RiPPs) are encoded by genes. In many cases, the precursor peptide contains a SP followed by a repetitive sequence, which consists of the core peptide embedded between N- and C-terminal sequences and KR recognition motif for the kexin Golgi protease. (B) The biosynthetic gene cluster organization. Reproduced from Bushley, K.E., Turgeon, B.G., 2010. Phylogenomics reveals subfamilies of fungal nonribosomal peptide synthetases and their evolutionary relationships. BMC Evolutionary Biology 10 (1), 26. doi:10.1186/1471-2148-10-26.

amino acids are incorporated remains difficult. A recent study revealed that the activity of NRPS modules also depends on the presence of other surrounding modules (Degen *et al.*, 2019), making these predictions even more uncertain.

Hybrid enzymes between PKSs and NRPSs are also commonly found in fungi, either as PKS-NRPS or NRPS-PKS hybrid enzymes (**Fig. 3(A)**). The former consists of an rPKS fused to a single NRPS module and a final releasing domain, leading to the production of a polyketide which contains a single amino acid (Fisch, 2013). These enzymes are notably known for the production of cytochalasins, which are inhibitors of actin polymerization (Skellam, 2017). Statins are produced by hybrid PKS-NRPSs that are truncated and have lost the A and T domains, consistent with the absence of an amino acid in the statin chemical structure (Boettger *et al.*, 2012). Hybrid NRPS-PKSs are less widespread and thus less studied. The only two characterized fungal NRPS-PKSs either carry a single NRPS module upstream of a KS domain or an A-T module upstream of a prPKS, and they are respectively responsible for the production of tenuazonic acid in the rice pathogen *Pyricularia oryzae* (Yun *et al.*, 2015), and of swainsonine in the insect pathogen *Metarhizium robertsii* (Cook *et al.*, 2017).

Several other classes have been less studied in fungi (**Fig. 2** and **Fig. 3(A)**). NRPS-like enzymes consist of a single A-T module linked to various extra domains, including thioester reductase, ADH short chain dehydrogenase and ferric reductase domains (Bushley and Turgeon, 2010). Only a few compounds, such as atromentin and microperfuranone, have been identified as the product of NRPS-like enzymes (Schneider *et al.*, 2008; Yeh *et al.*, 2012). While most fungi produce siderophores, iron-binding compounds, through an NRPS pathway, it was reported that *Rhizopus delemar* produces the siderophore rhizoferrin using an NRPS-independent siderophore (NIS) synthetase that is characterized by an IucA/IucC conserved domain (Carroll *et al.*, 2017). Such synthetases have been characterized in bacteria (Carroll and Moore, 2018), but genome analyzes suggest that NISs could actually be produced by many fungi.

Certain SMs are not synthesized by conserved core enzymes and are therefore difficult to identify in fungal genomes. Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent a minor class of SMs in fungi (Vogt and Künzler, 2019). In contrast to the previous pathways, RiPPs are directly encoded by genes in the form of a precursor peptide (Yang and van der Donk, 2013). Remarkably, the precursors of the ustiloxin, phomopsin, asperipin-2a and epichloëcyclin RiPPs consist of a signal peptide followed by repeats of the core peptide in between KR signal sequences that are recognized by the kexin Golgi protease for cleavage (**Fig. 3(A)**) (Vogt and Künzler, 2019). Finally, SMs like kojic acid and psilocybin are also produced by unconventional biosynthetic pathways that do not include any core enzyme, but only enzymes for the modification of the respective precursors (Terabayashi *et al.*, 2010; Fricke *et al.*, 2017).

The Gene Cluster Organization

The core enzymes described above are involved in the production of the first stable intermediate. However, biosynthetic pathways are usually much more complex and can involve many additional modifications of the produced backbone. For example, the production of aflatoxin involves at least 15 tailoring steps after release of the norsorolinic acid precursor from the PKS (Bhatnagar *et al.*, 2003). These modifications are catalyzed by so-called tailoring enzymes with very diverse catalytic activities such as methylation, monooxygenation, decarboxylation, etc. (Walsh and Tang, 2017). The number and type of modifications steps after the release of the initial chemical backbone from the core enzymes expand the diversity of biosynthetic pathways and chemical structures (**Fig. 2**).

In fungi, the genes that encode core and tailoring enzymes in a given pathway often co-localize in the genome and are co-regulated, criteria that define a gene cluster organization (**Fig. 3(B)**) (Keller and Hohn, 1997). Additionally, these biosynthetic gene clusters (BGCs) can also comprise genes that encode MFS or ABC transporters for SM export or self-protection (Gardiner *et al.*, 2005; Wiemann *et al.*, 2009; Brown *et al.*, 2015; Dolan *et al.*, 2015), transcription factors for BGC regulation (Lyu *et al.*, 2019) and protein decoys for self-protection (Bushley *et al.*, 2013; Yeh *et al.*, 2016). Certain BGCS contain more than one core gene, leading to the production of hybrid molecules. For example, macrolide lactones are produced by a conserved pair of nrPKS and rPKS (Kim *et al.*, 2005), and meroterpenoids are the products of pathways involving PKSs and TCs (Itoh *et al.*, 2010). The diversity of core genes, tailoring genes and their combinations in BGCs makes it possible to produce a large diversity of bioactive compounds from a very limited set of precursors issued from primary metabolism.

How BGCs are formed in fungal genomes remains a matter of debate. A BGC organization has been suggested to facilitate the co-regulation of genes and interactions between enzymes from a given biosynthetic pathway (Hurst *et al.*, 2004; Santoni *et al.*, 2013; Rokas *et al.*, 2018). Another hypothesis suggests that the partial loss of genes from a given pathway would result in the production of toxic intermediates (McGary *et al.*, 2013). Finally, this organization could be the result of accumulated horizontal gene transfers (HGTs) (Rokas *et al.*, 2018). None of these hypotheses has been experimentally tested and thus requires further investigation.

Tight Regulation of Secondary Metabolite Production

Given that SMs often exhibit biological activities that could be detrimental to the producing organisms and that their production costs energy, SMs should be produced only when required. BGCs are tightly regulated to be expressed under very specific conditions.

The first level of regulation involves pathway-specific transcription factors, which are encoded by genes within the BGC they regulate (Lyu *et al.*, 2019) (**Fig. 4**). The best characterized example of such a local transcription factor is AflR that regulates the production of aflatoxins (Woloshuk *et al.*, 1994). AflR binds to the promoter of the other genes in the BGC and activate their transcription (Fernandes *et al.*, 1998; Ehrlich *et al.*, 1999).

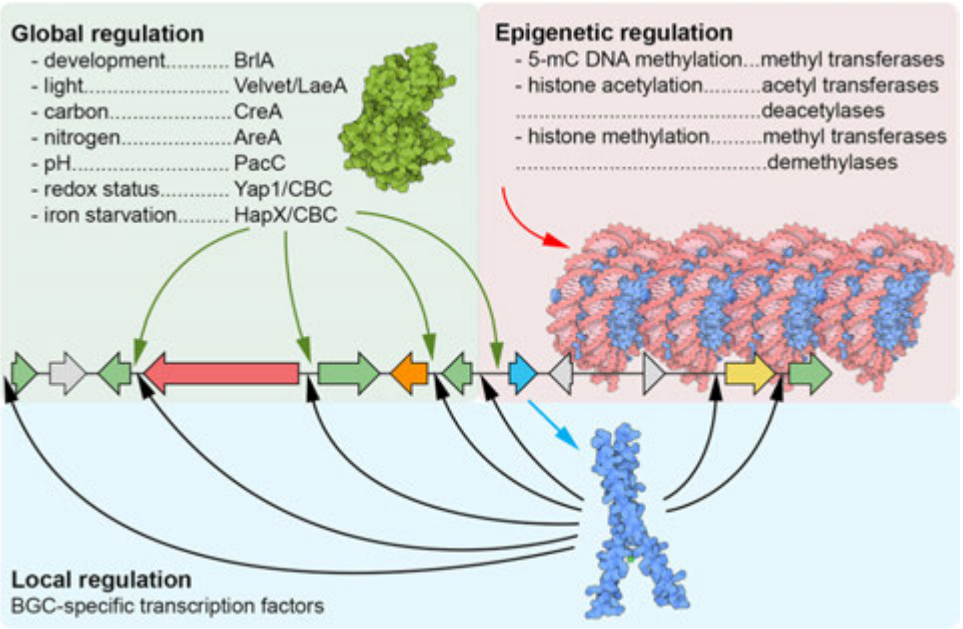

Fig. 4 Tight regulation of secondary metabolite production in fungi. The expression of biosynthetic gene clusters (BGCs) is controlled by several interconnected levels of regulation. BGCs can contain a gene that encode transcription factors that regulate the expression of the other genes in the BGC by binding to their promoters. Global regulators can also directly regulate the expression of fungal BGCs in response to environmental and developmental signals. Expression of fungal BGCs is also regulated through the modification of chromatin, the complex of DNA and histone proteins. Both DNA methylation and post-translational modifications of histones are epigenetic modifications that affect SM production in fungi. The protein structures are representations obtained with the Illustrate program. Reproduced from Goodsell, D.S., Autin, L., Olson, A.J., 2019. Illustrate: Software for biomolecular illustration. Structure 27 (11), 1716–1720. (Cell Press). doi:10.1016/J.STR.2019.08.011.

The second level of regulation involves global regulators that control the expression of a number of BGCs in response to developmental and environmental signals (**Fig. 4**). CreA, AreA and PacC are transcription factors that control BGC expression in response to the carbon source, nitrogen source, and pH, respectively (Lyu *et al.*, 2019). The Velvet and LaeA protein complex regulates BGC expression in response to light (Bayram *et al.*, 2008; Fischer, 2008), while Hap transcription factors control the response to redox status and iron starvation (Reverberi *et al.*, 2012; Wiemann and Keller, 2014; Hortschansky *et al.*, 2017). SM production is also interconnected with fungal development, often linked to conidiation and formation of survival structures like sclerotia (Calvo and Cary, 2015). This coordination involves specific signaling pathways, which are also activated in response to environmental signals (Lind *et al.*, 2018).

The third level of global regulation corresponds to epigenetic modifications (**Fig. 4**). The conformation of chromatin, the complex of DNA and histone proteins, determine the accessibility of DNA to regulatory proteins (Armeev *et al.*, 2019). Changes between euchromatin (open state) and heterochromatin (closed state) are especially determined by post-translational modifications of histones, mainly acetylation and methylation (Turner, 2007). As a result, BGCs located in euchromatin can be activated while BGCs located in heterochromatin remain silent (Strauss and Reyes-Dominguez, 2011). This research field has seen much progress in the last decade and a histone code for active and silent BGCs could be determined. BGC expression is mainly linked to acetylation of lysine residues in H3 and H4 histone proteins (Nützmann *et al.*, 2011; Gacek and Strauss, 2012; Soukup *et al.*, 2012; Nützmann *et al.*, 2013). In contrast, tri-methylation of lysine residues in H3 histones are a hallmark of BGC repression (Reyes-Dominguez *et al.*, 2010; Gacek and Strauss, 2012; Connolly *et al.*, 2013; Studt *et al.*, 2016). Several histone acetyltransferases and deacetylases, as well as histone methyltransferases and demethylases, have been characterized in fungi and were shown to impact the expression of several BGCs in a given fungus (Collemare and Seidl, 2019). However, BGC regulation by these enzymes is complex because altering the acetylation or methylation status of histones usually results in both activating and repressing different BGCs (Lee *et al.*, 2009; Connolly *et al.*, 2013; Rösler *et al.*, 2016). Although less studied because of its low abundance in fungi, DNA methylation was also found to modify SM production in several fungi, including *Aspergillus* and *Fusarium* species (Williams *et al.*, 2008; Fisch *et al.*, 2009; Yang *et al.*, 2016). More detailed information about each kind of regulation can be found on recent extensive reviews dedicated to this topic (Collemare and Seidl, 2019; Pfannenstiel and Keller, 2019).

Genomics of Fungal Secondary Metabolism

The study of fungal SMs had been focused on their diverse biological activities and functions until the advent of genomes. Since then, the genomics era has provided new research questions, especially regarding the origin and evolution of BGCs, and new tools to investigate fungal SMs. The number and diversity of available fungal genomes is increasing every day thanks to the development of Next

Generation Sequencing (NGS) technologies and the consequential drop in sequencing cost. The public repositories NCBI and Joint Genome Institute MycoCosm portal (Grigoriev *et al.*, 2014) currently host around 2000 and 1400 fungal genomes, respectively. This wealth of genomic information allows for the exploitation of a treasure trove in terms of biochemical reactions and biological activities.

Abundance of Biosynthetic Pathways in Fungal Genomes

The regain of interest in fungal SMs is certainly due to the availability of fungal genomes. For a given fungus, only a few SMs can be detected under standard laboratory conditions. However, genome analyzes have revealed that fungal species possess a much higher SM production capacity with many BGCs in their genomes remaining silent under laboratory conditions. These silent BGCs encode so-called cryptic biosynthetic pathways. For example, the genome of the well-studied model fungus *Aspergillus nidulans* contains 58 predicted BGCs, but we know the corresponding SM for only 26 of them (Romsdahl and Wang, 2019). The explanation for this discrepancy is the tight regulation of SM production under specific environmental conditions that are difficult to identify and reproduce in the laboratory.

Predicted numbers of core genes highlighted the very high SM production capacity of Ascomycota (**Fig. 5**). Apart from Saccharomycotina and Taphrinomycotina, the average number of core genes in Ascomycota genomes varies between 12 and 68 (**Fig. 5**). Basidiomycota fungi, which includes edible mushrooms and wood-decaying species, exhibit a more moderate production capacity with a predominance of NRPS-like and TC genes. With the exception of the Neocallimastigomycetes that are especially rich in NRPS genes, the other fungal lineages show a reduced SM production capacity (**Fig. 5**).

Even though more than 15,000 fungal SMs are reported in the Natural Product Atlas (van Santen *et al.*, 2019) (an open database of SM compounds curated by the scientific community), characterization of the BGCs behind their synthesis is still scarce. The largest collection of characterized BGCs is the Minimum Information about a Biosynthetic Gene cluster (MIBiG) (Medema *et al.*, 2015; Kautsar *et al.*, 2019), which currently contains around 270 fungal BGCs. This limited number of characterized BGCs and the observation that most of fungal BGCs predicted in fungal genomes are silent, highlight the task that is ahead. Especially, the correct identification and prioritization of silent BGCs using bioinformatic approaches are crucial before embarking on lengthy characterization efforts.

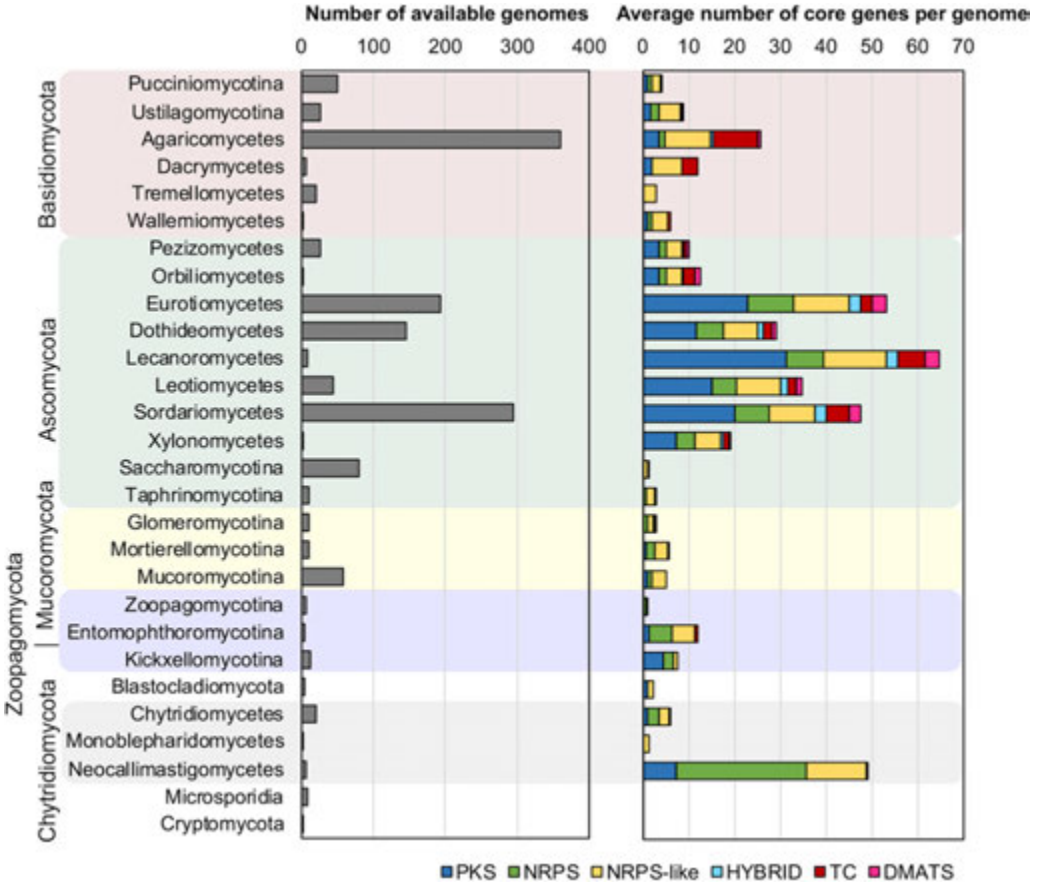

Fig. 5 Abundance of biosynthetic pathways in fungal genomes. Number of fungal genomes and predicted core genes in these genomes were retrieved from the Joint Genome Institute MycoCosm repository (November 7, 2019). Reproduced from Grigoriev, I.V., *et al.*, 2014. MycoCosm portal: Gearing up for 1000 fungal genomes. Nucleic Acids Research 42 (D1), D699–D704. doi:10.1093/nar/gkt1183.

Table 1 Software dedicated to the prediction and analysis of biosynthetic gene clusters (BGCs) in fungi

Name	*Main Function*	*Latest publication*	*Availability*	*Remarks*
SMURF	BGC prediction	(Khaldi *et al.*, 2010)	Webserver: http://smurf.jcvi.org	Motif-dependent prediction. Flexible BGC borders. Not updated anymore
fungiSMASH	BGC prediction	(Blin *et al.*, 2019)	Webserver: https://fungismash.secondarymetabolites.org Open source: https://docs.antismash.secondarymetabolites.org/install	Motif-dependent prediction. Fixed BGC borders. Active development
CASSIS	Fungal BGC border detection	(Wolf *et al.*, 2016)	Webserver: https://sbi.hki-jena.de/cassis/ Open source: https://sbi.hki-jena.de/smips/Download.php fungiSMASH module	Predict co-regulation by searching shared motifs in gene promoters Incorporated in fungiSMASH 4 (but currently not available in fungiSMASH webserver version)
MIDDAS-M	BGC prediction	(Umemura *et al.*, 2013)	Not available	Motif-independent prediction using gene co-expression
MIPS-CG	BGC prediction	(Takeda *et al.*, 2014)	Not available	Motif-independent prediction using non-syntenic blocks
FunGeneClusterS	BGC prediction	(Vesth *et al.*, 2016)	Webserver and open source: https://fungiminions.shinyapps.io/FunGeneClusterS/	BGC identification from gene co-expression
NaPDos	Phylogenetic analysis	(Ziemert *et al.*, 2012)	Webserver: http://napdos.ucsd.edu	Phylogeny of key biosynthetic domains
MultiGeneBlast	Comparative genomics	(Medema *et al.*, 2013)	Open source: http://multigeneblast.sourceforge.net	Used as a base for the KnownClusterBlast module in fungiSMASH
BiG-SCAPE	Comparative genomics	(Navarro-Muñoz *et al.*, 2019)	Open source: https://git.wur.nl/medema-group/BiG-SCAPE	Creates sequence similarity networks and defines BGC families
none	Comparative genomics	(Theobald *et al.*, 2018)	Open source: https://github.com/RoerdamAndersenLab/gene_cluster_networks_and_genetic_dereplication	Creates sequence similarity networks and defines BGC families

In Silico Prediction of Biosynthetic Gene Clusters

In silico prediction of BGCs usually starts with the detection of the core genes, which requires good quality genome assemblies and, more importantly, accurate gene prediction. While this can be accomplished with simple BLAST searches using reference data, several *in silico* BGC mining tools have been created to facilitate this process (Ziemert *et al.*, 2016), with most of them falling into two categories: motif-dependent and motif-independent detection. It is noteworthy to mention that many tools have been superseded or are not updated anymore, and in some cases, they only exist as a web tool with no open source code (**Table 1**).

Motif-dependent detection

This approach is based on the conserved sequences (domains) that characterize the SM core enzymes of the most commonly found biosynthetic classes. Protein conserved domains can be encoded as probabilistic mathematical models (hmm: hidden Markov model) that are built from multiple sequence alignments. The frequencies of every amino acid at each position in the alignment are recorded into a so-called profile and then used by software like HMMER (hmmer.org) to search and score similar sequences (Eddy, 1998). Nowadays, two tools that make use of hmm profiles (from the Pfam (El-Gebali *et al.*, 2019), TIGRFAM (Haft *et al.*, 2001) or custom-made databases) are predominantly employed to predict BGCs in fungal genomes with high confidence (Medema and Fischbach, 2015).

SMURF was the first tool specifically designed to detect fungal BGCs (Khaldi *et al.*, 2010). This tool uses domain detection not only to find NRPS, PKS, PKS/NRPS hybrid, NRPS-like, PKS-like and DMATS core enzymes in predicted proteomes, but also to find tailoring enzymes. These proteins are also commonly characterized by conserved domains and SMURF relies on a dataset of conserved domains found in 22 BGCs from *Aspergillus fumigatus*. Once a core gene is found, 20 genes upstream and downstream are scanned for the presence of tailoring SM domains and are either tagged as "SM domain positive" or "SM domain negative". The algorithm keeps scanning genes until (a) the 20-gene window is over; (b) a maximum intergenic distance to the next gene is found; or (c) a maximum number of "SM domain negative" genes are found. While this strategy is flexible to account for the different BGC configurations, its parameters are based on a very limited dataset *A. fumigatus* BGCs. Although SMURF is available online, it is not actively developed anymore.

Just after the release of SMURF, the first version of antiSMASH was published (Medema *et al.*, 2011), which follows the same principle of detecting core genes using profile hmms. antiSMASH was originally developed for mining bacterial BGCs, but it is nevertheless capable of finding all the usual fungal BGC types due to the fact that core enzymes are conserved in both kingdoms.

Then, a region defined by a simple extension of the up- and downstream sequences from the core gene(s) position(s) is reported as containing a BGC. Currently, version 5 of antiSMASH brought a complete revision of the code base, a new conceptual organization for the locus with biosynthetic genes (core, neighborhood, candidate gene cluster and region), and detection of fungal RiPP BGCs that resemble the ustiloxin BGC (Blin *et al.*, 2019). antiSMASH is actively maintained and can be used through the online web version, installed locally or used through Docker. It is worth noting that the fungal version of antiSMASH, named fungiSMASH, does not accept unannotated genomes, meaning that accurate gene prediction in the genome of interest has to be performed beforehand.

While detecting core genes is relatively straightforward, finding the borders of a given BGC is the major challenge in BGC prediction. Both SMURF and antiSMASH show low accuracy in predicting BGC borders. The key difference between fungiSMASH and the regular antiSMASH was the incorporation of the CASSIS algorithm to predict BGC borders (Wolf *et al.*, 2016; Blin *et al.*, 2017). In a given predicted BGC region, CASSIS reports genes that share a motif in their promoter region as this motif would indicate their potential co-regulation. A set of 38 characterized BGCs from *Aspergillus* spp. was used to determine CASSIS parameters.

Both SMURF and antiSMASH suffer from several limitations because they can only detect the major known core enzymes and they cannot report complete BGCs when they are dispersed at more than a single locus (e.g., cephalosporin (Singh *et al.*, 2019), melanin (Langfelder *et al.*, 2003), dothistromin (Schwelm and Bradshaw, 2010)) (Medema and Fischbach, 2015). In addition, the prediction of BGC borders is in most cases inaccurate and manual curation of the results is highly recommended, including validation of functional annotations. Many tools to predict substrate specificities and produced compounds are available either as standalone or integrated into antiSMASH. However, most of them are based on bacterial data and are most often inaccurate when applied to fungal sequences. Functional characterization of many more fungal BGCs is needed in order to develop accurate prediction tools.

Motif-independent detection

Considering the limitations of motif-dependent detection tools, other strategies have been employed to comprehensively identify BGCs in fungal genomes, including non-classical pathways (Umemura *et al.*, 2015). A first strategy is based on the observation that genes from the same biosynthetic pathway are co-regulated, even when they do not contain a classical conserved core gene. Such a strategy was developed in the MIDDAS-M algorithm which averages gene expression over a gene-window and normalizes expression ratios to yield significantly larger values for BGCs (Umemura *et al.*, 2013). This algorithm applied to several fungal species not only accurately reported characterized BGCs, but also identified a new BGC for the RiPP ustiloxin B in *Aspergillus flavus*, which was functionally validated through targeted gene deletion (Umemura *et al.*, 2013). Limitations of this approach are its dependency to experimental data and its ability to detect only the few BGCs that are expressed under laboratory conditions.

Comparative genomic analyzes revealed that fungal BGCs tend to be located in non-syntenic blocks (NSBs) (Machida *et al.*, 2005; Amselem *et al.*, 2011; Collemare *et al.*, 2014; Lind *et al.*, 2017). This observation led to the development of MIPS-CG, which attempted to detect novel BGC candidates by searching for conserved clustered genes between genomes in NSBs (Takeda *et al.*, 2014). This approach is independent of any prior knowledge on fungal BGCs, allowing the successful prediction of non-classical biosynthetic pathways as exemplified with the detection of the kojic acid BGC in *Aspergillus oryzae* (Takeda *et al.*, 2014).

Both MIDDAS-M and MIPS-CG tools are actually not available anymore, but the logic behind these tools can be easily implemented in tailor-made methods to study genomes of interest. The combination of motif-dependent and motif-independent strategies was also tested in *A. nidulans* by linking SMURF predictions to microarray expression data from diverse conditions (Andersen *et al.*, 2013). In this study, the expression data was used to calculate a Cluster Score as a measure for gene co-expression, which accurately predicted that two distant BGCs are responsible for the production of the hybrid peptide-terpene compound nidulanin A. FunGeneClusterS, a graphical online tool with an improved algorithm, was released in a follow up work (Vesth *et al.*, 2016).

Strategies for Prioritizing the Study of Biosynthetic Gene Clusters

Considering the large amount of predicted BGCs in fungal genomes, prioritization of BGCs for functional characterization is needed. Several strategies can be employed to select candidate BGCs that are likely to produce novel compounds or variations of known compounds (Bertrand and Sorensen, 2018; Kjærbølling *et al.*, 2019).

Phylogeny-informed prediction

General phylogenetic studies of core enzymes have been used to elucidate their evolutionary histories (nrPKS (Koczyk *et al.*, 2015)) and to define broad evolutionary clades that relate to enzymatic activities, often by focusing on a single domain (sesterterpenes (Narita *et al.*, 2017), nrPKS (Liu *et al.*, 2015; Throckmorton *et al.*, 2015), NRPS (Bushley and Turgeon, 2010)). Phylogenetic analyzes of core enzymes indicated that closely related core enzymes show functional homology as exemplified with nrPKSs involved in the production of emodin-like anthraquinones (Collemare *et al.*, 2014; Griffiths *et al.*, 2016) or siderophore synthetases (Bushley and Turgeon, 2010). This principle can be used to link BGCs with known metabolites (e.g., (Gibson *et al.*, 2014)) and to directly target novel BGCs whose core enzymes are divergent from characterized ones (Harvey *et al.*, 2018). NaPDos is a bioinformatic tool that was developed to perform functional predictions based on the phylogeny of key domains from characterized PKS (KS domain) and NRPS (C domain) enzymes (Ziemert *et al.*, 2012). While this phylogenetic approach is very useful, predictions of the final chemical structures is not possible because tailoring enzymes modify the backbone produced by the core enzymes. It is therefore crucial to employ also strategies that consider whole BGCs.

Comparative genomics and gene cluster families

Comparative genomics is based on pairwise comparisons between a query and a set of BGCs ("one-to-many"). One of the classical tools for pairwise BGC comparison is MultiGeneBlast (Medema *et al.*, 2013), which has been incorporated into antiSMASH in two analysis modules. A first module (ClusterBlast) compares each query BGC against predicted BGCs from antiSMASH DB (Blin *et al.*, 2018) (currently holding bacterial BGCs only), while the other module (KnownClusterBlast) compares the query against the set of characterized clusters from MIBiG (Medema *et al.*, 2015; Kautsar *et al.*, 2019). These tools remain of limited interest for fungal BGCs until a significant higher number of BGCs will become functionally characterized.

A more integrative approach makes use of sequence similarity networks (SSN) to provide a "many-to-many" overview, automatically defining gene cluster families (GCFs). GCFs corresponds to groups of BGCs that share a common origin and have diverged, resulting in the production of diverse, yet related, SMs. For example, BiG-SCAPE processes antiSMASH results, annotating protein domains in all regions containing a predicted BGC to define a distance matrix based on three components (domain content similarity, synteny and sequence identity) (Navarro-Muñoz *et al.*, 2019). Clustering into GCFs is performed using an affinity propagation algorithm on the resulting distance matrix. BiG-SCAPE accuracy was validated by comparing the GCF calling to metabolomics data for a bacterial case. In principle, BiG-SCAPE could also be used with fungal BGCs.

A similar approach was employed using BGC prediction with a modified version of SMURF in 32 *Aspergillus* spp. (Theobald *et al.*, 2018). The developed method aggregated percentages of identity of best bidirectional hits for each protein in the predicted BGCs, resulting in similarity scores that are translated into a BGC network. A random-walk algorithm is then applied to the network to define GCFs. In combination with expert knowledge of the malformin chemical structure, this method allowed to link malformin to a GCF in *Aspergillus* species, of which one BGC in *Aspergillus brasiliensis* was experimentally confirmed.

Function-guided prioritization

A different approach for BGC prioritization is not based on sequence comparisons but on researcher's interest in specific enzymatic functions. For example, new chlorinated polyketides from *Bipolaris sorokiniana* were discovered by selecting predicted BGCs which contain genes encoding halogenases (Han *et al.*, 2019).

The same strategy was successfully applied to search for BGCs that would produce SMs with specific biological activities. Several characterized BGCs contain a gene that encodes the enzymatic target of the SM, acting as a decoy and thus providing resistance to the toxic compound (Almabruk *et al.*, 2018). For example, the cyclosporine A BGC contains a gene encoding cyclophilin, the target of cyclosporine (Yang *et al.*, 2018). Such a strategy successfully identified the BGCs involved in the production of mycophenolic acid (MPA) in *Penicillium brevicompactum* (Regueira *et al.*, 2011) and aspterric acid in *Aspergillus terreus* (Yan *et al.*, 2018). The MPA BGC was identified in a cosmid library searching for a meroterpenoid BGC that would contain a copy of IMP dehydrogenase, the known target of MPA in B and T lymphocytes (Regueira *et al.*, 2011). The aspterric acid BGC was identified in the search for herbicides that target the enzyme dihydroxyacid dehydratase (DHAD) involved in the plant-essential branched chain amino acid pathway. Mining of fungal genomes for BGCs that contain a DHAD copy retrieved the aspterric acid BGC, which was functionally validated (Yan *et al.*, 2018).

Resistance gene-directed discovery has been implemented in ARTS (Alanjary *et al.*, 2017), a web tool for discovery of bacterial BGCs with different resistance mechanisms. Very recently, a similar pipeline called FRIGG has been designed for fungi (Kjærbølling *et al.*, 2019). This tool combines precomputed homolog protein families from different genomes with predicted BGCs. In this case, BGCs that contain genes homologous to genes present outside of the cluster may indicate they encode the target of the corresponding SM. Experimental validation of the FRIGG pipeline is still awaiting.

Linking Compounds to Biosynthetic Genes

Screenings for biological activities have resulted in the identification of many compounds from diverse fungi. In most cases, the BGCs behind these compounds is not known. When the genome of a given fungus becomes available, it is possible to implement the strategies mentioned above to select candidate BGCs to study. For this purpose, expert knowledge of the compound chemical structure is necessary to target specific biosynthetic classes using a "retro-biosynthetic" approach. The chemical structure will provide hints about the major chemical classes (polyketide, peptide, etc.) and the needed tailoring reactions (methylation, halogenation, dimerization, etc.). For example, the BGC involved in the production of aspirochlorine was identified thanks to the presence of an halogenase gene that correlate with the chlorinated residue found in this SMs (Chankhamjon *et al.*, 2014). Such a method can also apply to non-classical biosynthetic pathways. The genomes of psilocybin-producers *Psilocybe cubensis* and *Psilocybe cyanescens* were interrogated for the presence of clustered genes that would encode a methyltransferase, a hydroxylase, and a kinase, which are expected for psilocybin biosynthesis. This search retrieved a conserved locus between both species harboring all the expected genes and the predicted BGC was subsequently proven to be responsible for psilocybin production (Fricke *et al.*, 2017).

If the target compound is a chemical analog of a SM with a characterized BGC, it may be inferred that both compounds share the same biosynthetic origin. In this case, the characterized BGC may be used to guide a comparative genomics analysis. This approach have been applied in the *Aspergillus* genus and candidate BGCs could be identified for the production of novo-funigatonin, *ent*-cycloechinulin and *epi*-aszonalenin A and C in *A. fumigatus* and ochrindol in *Aspergillus steynii* (Kjærbølling *et al.*, 2018).

Activation of Silent Biosynthetic Pathways

Bioinformatics has revealed a large number of predicted BGCs in fungal genomes and has made it possible to explore effectively the diversity of naturally encoded pathways *in silico*. At the same time, only a very small fraction of these predicted BGCs are associated with characterized SMs and most pathways remain cryptic because they are not expressed under standard laboratory conditions. In the last 15 years, much effort has focused on methods to induce the expression of these silent BGCs and get access to the wealth of SMs they could produce. Several successful strategies have been developed, from the modification of culture conditions to genetic modifications, taking advantage of the accumulated knowledge about BGC regulation.

Modification of Culture Conditions

The tight regulation of fungal BGCs make them produce SMs under very specific conditions during their lifecycle. The laboratory conditions are usually adjusted for optimal growth and thus lack the specific signals for BGC activation. Considering that environmental signals are known to affect SM production (Takahashi *et al.*, 2013), one approach is to grow the fungus of interest under a variety of conditions (light, temperature, oxygenation, etc.) and substrates (different carbon, nitrogen sources, etc.), so that one of the conditions actually contains a signal required for the activation of a BGC. This approach is known as OSMAC (One Strain, Many Compounds) and has been successfully applied to many fungi. For example, cultivating *A. nidulans* under more than 40 different conditions resulted in the discovery of the compounds aspoquinolone (Scherlach and Hertweck, 2006) and aspernidine A and B (Scherlach *et al.*, 2010). Since the initial OSMAC study that discovered 15 SMs produced by *Aspergillus ochraceus* (Bode *et al.*, 2002), this approach has resulted in the discovery of many SMs as recently reviewed in (Romano *et al.*, 2018). Despite its success, the OSMAC approach can be quite tedious because there is no common signal that could always be used to activate the production of several SMs in many fungi. The multiplication of the conditions to test makes this approach difficult to employ on a large number of fungal species at once.

Nowadays, OSMAC approaches also include the use of chemicals to test their effect on SM production. In particular, chromatin remodeling agents are commonly employed because they provide a more consistent and broader effect on BGC activation due to their epigenetic regulation. Growing fungi in the presence of histone deacetylase inhibitors like suberoylanilide hydroxamic acid (SAHA) and of DNA methyltransferase inhibitors like 5-azacytidine resulted in successful activation of SM production in many studies (Henrikson *et al.*, 2009; Wang *et al.*, 2010; Vervoort *et al.*, 2011; Zutz *et al.*, 2013).

Co-Cultivation of Microorganisms

In contrast to competition-deprived laboratory conditions, fungi reside in natural habitats that are normally occupied by many other organisms, including bacterial and fungal competitors. The production of SMs is important to interact or fight with these other organisms. Co-cultivation of organisms (for example, bacteria with fungi or fungi with fungi) is used to mimic competition in natural habitats and induce SM biosynthesis. For example, co-culture of two extremophile *Penicillium* species from the Berkeley Pit Lake yielded eight new macrolide lactones (Stierle *et al.*, 2017). Similarly, co-culture of *A. nidulans* with the soil-dwelling actinomycete *Streptomyces rapamycinicus* demonstrated elevated synthesis of several polyketides, such as orsellinic acid, its derivative lecanoric acid, and two cathepsin K inhibitors, F-9775A and F-9775B (Schroeckh *et al.*, 2009). Co-cultivation of the human pathogen *A. fumigatus* with soil dwelling actinobacteria also induced the synthesis of the meroterpenoid fumicycline (König *et al.*, 2013). Physical contact with living bacteria seems necessary to trigger SM production because in both cases induction was not accomplished by supplementing fungal axenic culture with bacterial media, sterilized bacteria cultivation supernatant or co-cultivation with physical separation of organisms with dialysis tube.

The induction of SM production in co-cultures appears very specific because a single species out of 58 different *Streptomyces* bacteria induced SM production in *A. nidulans* (Schroeckh *et al.*, 2009). Such a specificity makes the outcome of the co-culture approach difficult to predict. The underlying molecular mechanism(s) has not been deeply investigated yet, but the co-culture of *A. nidulans* with *S. rapamycinicus* was showed to trigger modifications of fungal histone acetylation (Nützmann *et al.*, 2011). Higher acetylation at the locus of the transcription factor gene *basR* induced its expression, which in turn activated the expression of BGCs (Fischer *et al.*, 2018). Recently, it was shown that the bacterium *Pseudomonas piscium* suppressed SM production in *Fusarium graminearum* by secreting the histone acetyltransferase inhibitor phenazine-1-carboxamide (Chen *et al.*, 2018). Overall, it appears that the specific effect of co-cultures relies on the active secretion of SMs that trigger a general response in the fungus, either activation or suppression of SM production.

Genetic Modifications

Pathway-specific activation

Biosynthetic pathways can be regulated by BGC-specific transcription factors that are encoded within the corresponding BGC (**Fig. 4**). A strategy to activate a silent BGC which contains a transcription factor gene is thus to overexpress this regulatory gene. Such an overexpression can be achieved by introducing an extra copy of the transcription factor gene that is under the control of a constitutive or inducible promoter at another locus in the genome. This strategy successfully activated a silent PKS-NRPS BGC in

A. nidulans, leading to the discovery of aspyridone (Bergmann *et al.*, 2007). Instead of adding an extra copy of the transcription factor gene, an alternative strategy is to replace the native promoter of the regulatory gene. For example, replacement by the inducible *alcA* promoter of the promoter of the transcription factor gene found in a silent gene cluster with two PKS genes in *A. nidulans*, successfully induced the targeted BGC and yielded the new polyketide asperfuranone (Chiang *et al.*, 2009). However, in some cases, over-expression of the transcription factor gene does not result in BGC activation as shown for the fellutamide B BGC in *A. nidulans* (Yeh *et al.*, 2016). Activation of this silent BGC and discovery of fellutamide B was accomplished by serial promoter swap for each gene of this BGC.

Genetic manipulation of the global regulation

The study of BGC global regulation, involving both global regulators and epigenetic modifications (**Fig. 4**), through targeted gene deletion and over-expression resulted in the activation or repression of many BGCs at once. Thus, these functional studies were further developed as a strategy to activate silent BGCs. One of the most studied global regulators is the complex formed by Velvet proteins and LaeA. In *A. nidulans*, deletion of *LaeA* impairs the production of penicillin, sterigmatocystin and lovastatin, while over-expression increases penicillin and lovastatin production (Bok and Keller, 2004). Similarly, manipulation of other Velvet complex components leads to diverse effects on different BGCs. For example, deletion of *FfVel1* in *F. fujikuroi* resulted in the stimulation and down-regulation of bikaverin and fumonisin, respectively, while it did not influence fusarin C production (Wiemann *et al.*, 2010). Application of *LaeA* over-expression in the non-model fungus *Aspergillus fumisynnematus* successfully activated the production of a new compound in this fungus, which was identified as the known SM cyclopiazonic acid (Hong *et al.*, 2015).

Preferred targets for genetic modifications are genes involved in post-translational modifications of histones. Acetylation of histone proteins is commonly associated with active gene transcription, while their methylation is commonly associated with repression of gene expression (Strauss and Reyes-Dominguez, 2011). Consistently, the deletion of histone deacetylases (HDACs) and histone methyltransferases activates silent BGCs (Shwab *et al.*, 2007; Bok *et al.*, 2009). Similarly, over-expression of histone acetyltransferases activates SM production as shown in *A. nidulans* (Soukup *et al.*, 2012). In contrast, deletion of histone acetyltransferases and histone demethylases mostly results in reducing SM production (Gacek-Matthews *et al.*, 2015; Rösler *et al.*, 2016). Because the deletion of HDACs results in hyperacetylated chromatin and thus active gene transcription (Eberharter and Becker, 2002), these enzymes have been the preferred target to activate silent BGCs and SM production in a number of fungi (Lee *et al.*, 2009; Studt *et al.*, 2013; Maeda *et al.*, 2017; Pidroni *et al.*, 2018). A detailed overview of fungal chromatin-modifying enzymes that can be used as targets for deletion or overexpression has recently been published (Pfannenstiel and Keller, 2019).

Despite the success of this strategy to induce the expression of silent BGCs, their effect is more complex and cannot be predicted. For example, while deletion of the Gcn5 histone acetyltransferase in *F. fujikuroi* decreased the expression of most BGCs, the production of bikaverin was unexpectedly increased (Rösler *et al.*, 2016). In all examples mentioned above, both activation and repression of BGCs were actually found. In other fungi, manipulation of these global regulators only alters the production of already active BGCs and do not activate the production of any new SM (Griffiths *et al.*, 2015).

Heterologous Expression of Biosynthetic Gene Clusters

A major limitation to the genetic modifications mentioned above is that they can be efficiently implemented only in tractable fungal species. Thus, their use remains limited to mostly model fungi and does not allow exploiting the full SM production potential of fungi. An alternative that has been increasingly employed is to express BGCs in heterologous hosts (Skellam, 2019).

The cloning of large PKS and NRPS genes, as well as complete BGCs, is a challenge that was overcome, thanks to cloning technologies like Gateway (Reece-Hoyes and Walhout, 2018) and Golden Gate (Engler and Marillonnet, 2014), as well as the use of transformation-associated recombination (TAR), a method that relies on homologous recombination to assemble several overlapping DNA fragments in *Saccharomyces cerevisiae* (Kouprina and Larionov, 2016) and in *Escherichia coli* (Jacobus and Gross, 2015). Several cloning systems have been developed for heterologous expression of multiple genes in a fungal host, giving the possibility to express complete BGCs (Ishiuchi *et al.*, 2012; Pahirulzaman *et al.*, 2012; Nielsen *et al.*, 2013; Unkles *et al.*, 2014; Bok *et al.*, 2015; Gressler *et al.*, 2015; Geib and Brock, 2017).

Both yeast and filamentous fungi have been used as suitable heterologous hosts. *S. cerevisiae* benefits from decades of biotechnological development for protein and metabolite production. Engineering *S. cerevisiae* for successful SM production is required, especially for the production of polyketides and non-ribosomal peptides, which core enzymes need to be activated by a 4-phosphopantetheinyl transferase (Ishiuchi *et al.*, 2012; Billingsley *et al.*, 2016; Harvey *et al.*, 2018). In addition, incorrect splicing of foreign genes in yeast is commonly encountered, meaning that intron-less genes should be amplified for successful expression (Billingsley *et al.*, 2016; Harvey *et al.*, 2018).

Many different species of filamentous fungi have been used for heterologous expression, mostly from the *Aspergillus* genus (*A. nidulans*, *A. niger*, *A. oryzae*) (Pahirulzaman *et al.*, 2012; Chiang *et al.*, 2013; Richter *et al.*, 2014). *Penicillium* and *Fusarium* species have also been reported as suitable host for heterologous expression of fungal BGCs (Kindinger *et al.*, 2019; Nielsen *et al.*, 2019). So far, the most used host has been *A. oryzae* as it benefits from a long biotechnological development for fermentation processes, which makes this fungus suitable for safe production of enzymes and metabolites. Currently, the *A. oryzae* NSAR1 strain is becoming the main host for the expression of fungal BGCs, because its four auxotrophic markers make it suitable to transform with several plasmids harboring different

selection markers and safe to use as it cannot survive if unintentionally released to the environment (Jin *et al.*, 2004; Skellam, 2019). Intron splicing in filamentous fungal hosts is less of an issue than in yeast, but the foreign genes are not always accurately processed as exemplified by the attempts to express the *ACE1* gene from *P. oryzae* in *A. oryzae* (Song *et al.*, 2015).

Heterologous expression has mainly been applied to single targeted BGCs. A scaled-up heterologous expression system was designed, making use of fungal artificial chromosomes and metabolomic scoring (FAC-MS) (Clevenger *et al.*, 2017). A library of 156 FACs from *Aspergillus wentii*, *Aspergillus aculeatus* and *A. nidulans* was transformed into *A. nidulans*. Analysis of SMs produced by the transformants yielded 15 new SMs and their corresponding BGCs. Although successful, this method requires conserved signaling pathways and thus can only be applied in closely related fungal species.

Nowadays, we are entering a new era in which DNA synthesis is allowing large-scale targeted heterologous expression of silent BGCs. In a recent study, 41 fungal BGCs selected to cover a wide phylogenetic diversity were synthesized and expressed in yeast (Harvey *et al.*, 2018). The production of a SM was detected for 22 of these BGCs, of which 10 were new compounds (Harvey *et al.*, 2018). Incorrect gene structure prediction was suggested to be a major reason for failed SM production in this study. Indeed, manual inspection of intron prediction in the terpene cyclase TC5 identified a wrongly predicted intron and the corrected gene structure expressed in yeast successfully yielded new sesquiterpenoids (Harvey *et al.*, 2018). It is likely that DNA synthesis combined with heterologous expression will become the strategy of choice thanks to the development of new technologies that will lower its cost. The generation of high-quality genomic data and accurate gene prediction will be crucial for the success of large-scale heterologous expression.

Engineering Biosynthetic Pathways: Chimeric Enzymes, Pathway Refactoring, Combinatorial Biosynthesis, Artificial Pathways

The development of heterologous expression methods has been instrumental in elucidating biosynthetic pathways and activating silent BGCs. However, these methods are also opening new biotechnological opportunities towards the production of non-natural SMs.

Studies to understand the biosynthetic mechanisms of core enzymes, especially PKSs and NRPSs, made use of chimeric enzymes that combined conserved domains from different enzymes. For example, a chimeric PKS made of the SAT-KS-AT-PT domains from *Colletotrichum lagenarium* Pks1 and of the ACP-CLC domains from *A. nidulans* wA yielded the production of a novel hexaketide different from the precursor produced by Pks1 and wA (Watanabe and Ebizuka, 2002). Rational domain swapping between core enzymes often result in the production of new SMs which provide information about how these enzymes function, especially about chain length control in PKSs (Fisch *et al.*, 2011; Liu *et al.*, 2014).

Nearly a decade ago, the concept of plug-and-play synthetic biology was suggested as a method to engineer or optimize biosynthetic pathways (Medema *et al.*, 2011). In this concept, all core and tailoring genes are considered as pieces that can be rearranged and optimized in order to increase the production of a given SM or produce new SMs. Such a strategy was recently applied to the austinoid pathway. Austinol is a meroterpenoid compound with insecticide activity produced by *A. nidulans* under favorable fermentation conditions (Mattern *et al.*, 2017). *Aspergillus calidoustus* produces another derivative with higher insecticide activity, calidodehydroaustin, but only under conditions that are not favorable for industrial fermentation (Mattern *et al.*, 2017; Valiante *et al.*, 2017). The comparison of the austinoid BGCs in *A. nidulans*, *A. calidoustus* and *Penicillium brasilianum* identified four candidate genes for the conversion of austinol into calidodehydroaustin (Mattern *et al.*, 2017). Expression of the candidate *A. calidoustus* genes in *A. nidulans* under control of an inducible promoter resulted in successfully rewiring the austinol biosynthetic pathway in *A. nidulans* to produce calidodehydroaustin (Mattern *et al.*, 2017).

The plug-and-play concept allows envisaging combinatorial to produce new compounds. This possibility was exemplified with the study of BGCs involved in the production of macrolide lactones and which encode both an nrPKS and an rPKS. Different combinations of nrPKS and rPKS pairs from related BGCs in different fungal species resulted in the biosynthesis of diverse SMs, including unnatural lactone variants with altered biological activity (Xu *et al.*, 2014).

Theoretically, it is conceivable to create completely artificial pathways using genes from unrelated BGCs. While such an example with fungal sequences has not yet been published, an artificial biosynthetic pathway to produce carminic acid, a red food colorant of insect origin, in *A. nidulans* was recently reported. In this case, a type III PKS gene of plant origin was expressed in *A. nidulans* together with cyclase and aromatase genes of bacterial origin, resulting in the production of the expected carminic acid backbone (Frandsen *et al.*, 2018). This backbone was modified by endogenous monooxygenases, yielding the needed next precursor. The final step of carminic biosynthesis was completed with the expression of a C-glucosyltransferase from the insect *Dactylopius coccus*.

Fungal Secondary Metabolite Production: Future Perspectives

Our fundamental knowledge of fungal SM production has impressively improved in the first two decades of the 21st century, notably thanks to the development of genomics and biotechnological tools. Despite this progress, the study and exploitation of fungal SMs remains behind compared to bacterial SMs as clearly shown by the restricted number of characterized fungal BGCs. Further development of biotechnological tools to fully exploit the SM production capacity of fungi is still hampered from lack of fundamental knowledge.

Active development of bioinformatics tools dedicated to fungal genomes is needed to address several key challenges such as accurate BGC definition, integration of expression data, detection of BGCs split over different loci and evolution of biosynthetic

pathways in GCFs. With the increasing availability of both fungal genomes and characterized data, including transcriptomics and metabolomics data, there is now a timely opportunity to develop new fungal-specific approaches.

Although a better understanding of BGC regulation could provide new tools to activate silent BGCs in the future, biotechnological strategies relying on heterologous expression appear to be the most promising for the efficient production of new fungal SMs. Such strategies will allow obtaining bioactive compounds in industrial-scale quantities, developing semi-synthetic approaches to produce chemical structures that are otherwise difficult to obtain with synthetic chemistry (Asai *et al.*, 2015; Alberti *et al.*, 2017), and building artificial BGCs. These developments require the functional characterization of many more fungal core and tailoring enzymes, especially regarding substrate specificities and interactions between tailoring enzymes. The combination of functional characterizations, bioinformatics and biotechnological tools is promising to lead to the efficient and less labor-intensive production of diverse bioactive SMs of fungal origin.

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antiSMASH fungal version.
https://git.wur.nl/medema-group/BiG-SCAPE
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https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
Conserved Domain Search Service (CD Search).
https://fungiminions.shinyapps.io/FunGeneClusterS/
Fungal Gene Clustering.
http://hmmer.org
HMMER.
http://www.ebi.ac.uk/interpro/
InterPro
EMBL-EBI.
https://mibig.secondarymetabolites.org/
MIBiG: Minimum Information about a Biosynthetic Gene cluster.
http://multigeneblast.sourceforge.net/
MultiGeneBlast: Combined Blast search for multigene modules.
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TIGRFAMS I J. Craig Venter Institute.

Degradation of Homocyclic Aromatic Compounds by Fungi

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Introduction

Aromatic compounds are best known for having a distinctive smell (aroma) and are therefore used in many food and beverages for the aroma and flavor. The most known aromatic compounds are the aromatic amino acids phenylalanine, tryptophan and tyrosine. Well known aromatic compounds found in nature are the aromatic compounds vanillin and benzaldehyde, which are responsible for the distinct smell of vanilla and almonds, respectively. Aromatic compounds are chemical compounds which consist of a planar (flat) cyclic structure with alternating single and double bonds. This structure is known as an aromatic ring or benzene ring and the molecule is called benzene (C_6H_6) which was first isolated by Michael Faraday in 1825 and is the least complex aromatic compound (**Fig. 1**). In order to be an aromatic compound, it must follow the four criteria of the Hückel's rule of aromaticity which states that:

(1) The molecule must be planar (flat),
(2) The molecule must be cyclic,
(3) The ring is completely conjugated,
(4) It must contain an odd number of pairs of π electrons ($4n + 2$ π-electrons, in which $n =$ non-negative integer).

Homocyclic aromatic compounds contain only carbon atoms in the ring, while heterocyclic aromatic compounds contain non-carbon atoms such as oxygen, nitrogen or sulfur in their ring (**Fig. 1**). For example, pyridine and purine are both heterocyclic aromatic compounds. In addition, aromatic compounds can also consist of multiple benzene rings and are called polycyclic aromatic compounds. For example, naphthalene ($C_{10}H_8$) consists of two fused benzene rings and can be extracted from coal tar. This compound is used as the main ingredient of traditional mothballs.

Names of Aromatic Compounds

Aromatic compounds can have multiple atoms attached to its benzene ring and remain aromatic compounds as long as they follow the rules of aromaticity. Many aromatic compounds have several non-systematic names which are discouraged to be used by IUPAC. However, several non-systematic names have been generally accepted. For example, hydroxybenzene is commonly known as phenol, benzene carboxylic acid as benzoic acid, ethylbenzene as styrene and methylbenzene as toluene.

Some substituted benzenes are named using the *ortho* (*o*), *meta* (*m*) and *para* (*p*) nomenclature, *e.g.*, *p*-hydroxybenzoic acid (**Fig. 2**). However, more preferred is the numbering of the substitutions on the ring by choosing the point of attachment as position 1, while the second substituent has the lowest number as possible. Substituents in the name are written alphabetically, for example 4-hydroxy-3,5-dimethoxybenzoic acid. The carbons on the aliphatic chains are labeled with Greek letters starting with alpha (α), beta (β), gamma (γ), etc. (**Fig. 2**).

Due to the frequent use of non-systematic names, some are preferred over the IUPAC names since they are shorter and more reader friendly. For example, 4-hydroxy-3-methoxybenzaldehyde is the preferred IUPAC name, but this compound is better known through its non-systematic name vanillin.

Plant Derived Aromatic Compounds

In nature, many aromatic compounds exist and most organisms can synthesize aromatic compounds. However, plants and microorganisms are best known for their aromatic compound producing abilities. Many non-benzenoids and heterocyclic aromatic compounds exist, but these are not further discussed in this chapter. Therefore, only homocyclic aromatic compounds are discussed and were selected based on their occurrence in plants, global importance, such as pollution, or their applications in man made products.

Lignin

Plants are able to synthesize the largest polymeric aromatic molecule on Earth, called lignin. Lignin is synthesized from the aromatic amino acid phenylalanine and is converted to cinnamic acid which is one of the key building blocks for the lignin synthesis since it can be converted into the three essential monolignols (Humphreys and Chapple, 2002). Lignin mainly

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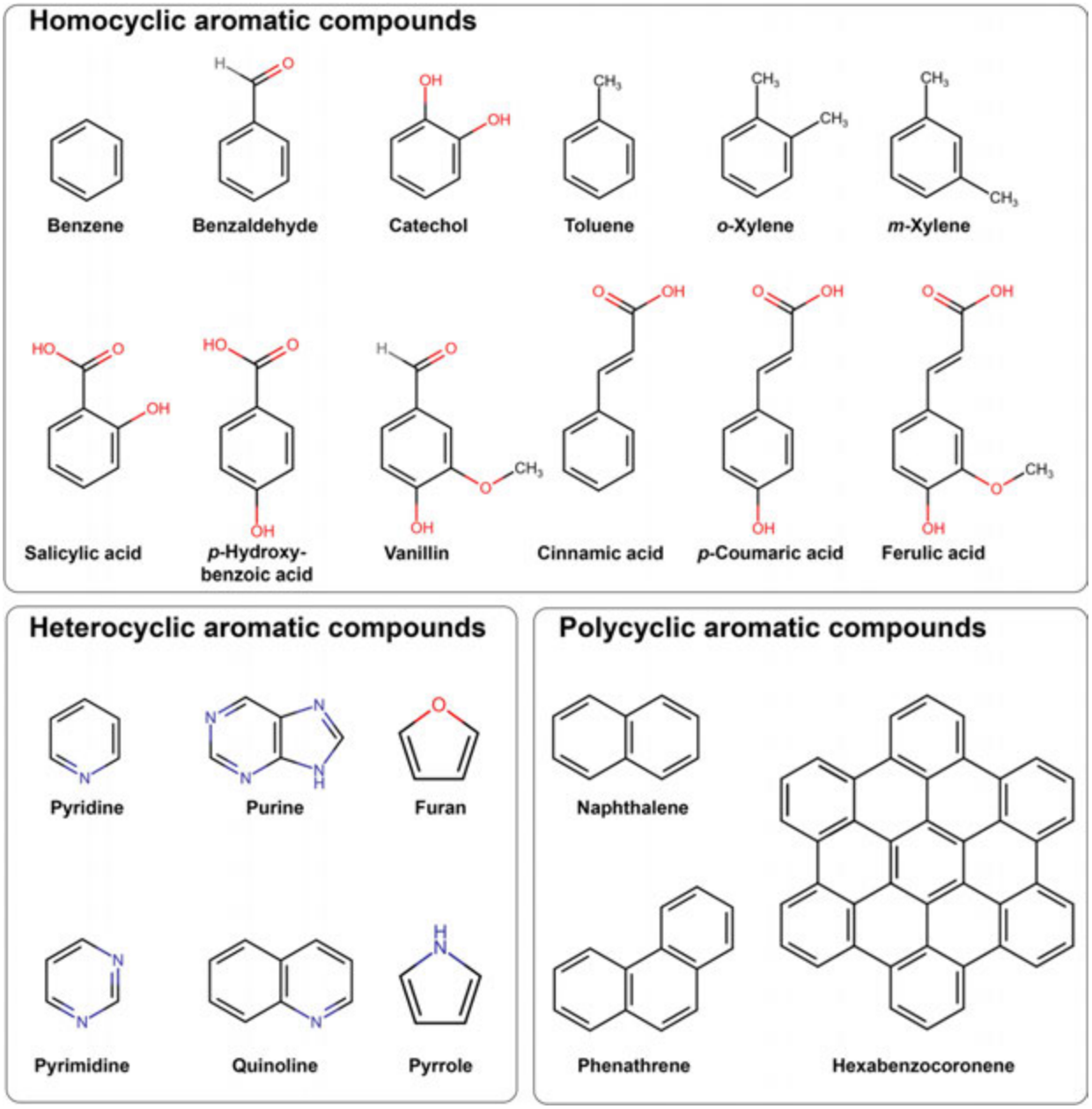

Fig. 1 Examples of homocyclic, heterocyclic and polycyclic aromatic compounds.

consists of the aromatic monolignols coniferyl alcohol, *p*-coumaryl alcohol and sinapyl alcohol that form the guaiacyl (G), *p*-hydroxyphenyl (H) and syringyl (S) units in lignin, respectively (Freudenberg, 1965; Humphreys and Chapple, 2002) (**Fig. 2**). Lignin provides for example stiffness and strength of the stem, and structural integrity of the cell wall (Davin and Lewis, 2005; Bhuiyan *et al.*, 2009; Ralph, 2010). These properties are derived by the crosslinking of lignin with other cell wall components and covalent binding to hemicellulose (Ralph, 2010; Mäkelä *et al.*, 2015; Dilokpimol *et al.*, 2017). Therefore, the development of lignin biosynthesis is considered as a crucial step in the colonization of plants from aquatic environments to land (Boerjan *et al.*, 2003; Weng and Chapple, 2010). The hydrophobic property of lignin is also essential for the plant to transport water and solutes through its vascular system. Next to that, lignin also plays a role as a barrier against pests and pathogens, and its metabolism is also involved in the response to abiotic stresses such as temperature, drought and salt stress (Bhuiyan *et al.*, 2009; Liu *et al.*, 2018). The synthesis of lignin in plants is a process that crosslinks monolignols through dimerization and lignification (Faulon and Hatcher, 1994; Boerjan *et al.*, 2003; Davin and Lewis, 2005). Interestingly, the amount and composition of lignin is highly variable among plant species, cell types and cell wall layers, and is even influenced by developmental and environmental cues (Boerjan *et al.*, 2003; Liu *et al.*, 2018). Angiosperm (hardwood) lignin consists mainly of S units, with low amounts of G units and traces of H units, while gymnosperm (softwood) lignin consists mainly of G units with a low amount of S and H

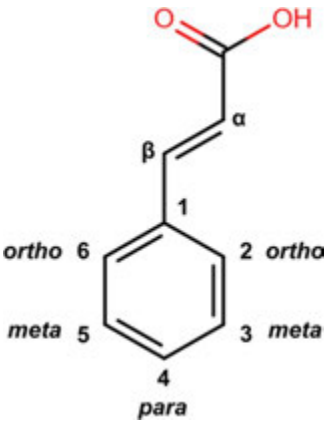

Fig. 2 Nomenclature of the aromatic ring and aliphatic chain.

units. Lignin from grasses (monocots) consists mainly of G and S units, but have a higher ratio of H units compared to angiosperms.

Plant Derived Monomeric Aromatic Compounds

In plants, aromatic compounds can serve as phytohormones, pesticides, anti-microbial agent, anti-nematocidal, repellents or as luring agents to attract insects, etc. Salicylic acid is known to be a phytohormone involved in plant defense signaling and plant development (Vernooij *et al.*, 1994; Shah, 2003; Zhang *et al.*, 2013; Lu *et al.*, 2016). Vanillin and cinnamaldehyde have strong anti-microbial activities and are found in *Vanilla planifolia* and *Cinnamomum verum* plants, respectively. Next to that, aromatic compounds can be part of cell wall components. For example, ferulic acid and *p*-coumaric acid are present in polysaccharides, such as xylan and pectin, and can form cross linkages between them and between polysaccharides and lignin through dimerization (Ralph, 2010; Mäkelä *et al.*, 2015; Dilokpimol *et al.*, 2017). Many aromatic compounds, such as caffeic acid, catechol, cinnamaldehyde, protocatechuic acid (3,4-dihydroxybenzoic acid), salicylic acid, syringic acid and vanillin, are found freely in plants (Link *et al.*, 1929; Link, 1933; Newby *et al.*, 1980; Cherney *et al.*, 1989).

Allelopathy

Aromatic compounds can also be found in high concentrations in soil when they are released during plant decay. Phenolic compounds that are released from plants (and other organisms) are known as allelochemicals, which results in the phenomenon called allelopathy. Allelopathy is the direct or indirect effect of a plant on other organisms, including other plants, through the release of biochemicals in the environment. These biochemicals can have beneficial or disadvantageous effect on the target or the community. For example, protocatechuic acid and catechol that diffuse from dead cell layers from the scales of pigmented onion, inhibit the spore germination and hyphal penetration of the onion fungal pathogen *Colletotrichum circinans* (Link *et al.*, 1929; Link, 1933). Other aromatic compounds, such as benzoic acid, caffeic acid, chlorogenic acid, cinnamic acid, 3,5-dinitrobenzoic acid, ferulic acid, gallic acid, gentisic acid, *p*-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid protocatechuic aldehyde (3,4-dihydroxybenzaldehyde), protocatechuic acid, vanillin and vanillic acid, are all suggested as allelochemicals (Chou and Lin, 1976; Whitehead *et al.*, 1982; Chou and Leu, 1992; Politycka, 1997; Sasikumar *et al.*, 2001, 2002).

Lignin Degradation

Fungi are considered to be the most efficient lignin degraders on Earth. Lignin degradation and modification by basidiomycete white-rot and brown-rot fungi has been studied for decades. However, ascomycete fungi are not known to degrade high molecular weight native lignin molecules (Mäkelä *et al.*, 2015, 2020), however exceptions have been observed (van Erven *et al.*, 2020). White-rot fungi have the ability to degrade and modify lignin, hemicellulose and cellulose. This often results in a white cellulose-enriched material, hence the name white-rot fungi. Brown-rot fungi can modify lignin to gain access to the plant cell wall polysaccharides. These lignin modifications result in a brown material of oxidized lignin, hence the name brown-rot fungi. In order to degrade or modify lignin, wood-decaying white-rot basidiomycetes release extracellular oxidative enzymes such as laccases and class II fungal heme peroxidases.

Less is known about the role of bacteria in lignin degradation, even though bacteria that are able to convert or degrade small lignin-derived aromatic compounds have been described previously (Bugg *et al.*, 2011b; Brown and Chang, 2014). Currently, much research is performed in the degradation of small lignin-derived aromatic compounds, especially with the bacterium *Sphingomonas paucimobilis* (Kamimura *et al.*, 2017).

Table 1 Examples of aromatic compounds released by the bacteria *Aneurinibacillus aneurinilyticus*, *Bacillus* sp., *Rhodococcus jostii* and *Sphingomonas paucimobilis* and the fungi *Ceriporiopsis subvermispora*, *Pleurotus eryngii*, *P. chrysosporium* and *Lentinula edodes* during growth on lignin containing substrates

Aromatic compound	*Systematic name*	*Lignin source*	*Species*
3,4,5-Trimethoxy benzaldehyde	–	Kraft lignin	*Bacillus* sp.
4-Methylguaiacol	–	Wheat straw	*C. subvermispora*, *P. eryngii, L. edodes*
Cinnamic acid	(2E)-3-Phenylprop-2-enoic acid	Kraft lignin	*Bacillus* sp.
Ferulic acid	4-Hydroxy-3-methoxycinnamic acid	Kraft lignin, lignocellulose	*A. aneurinilyticus*, *Bacillus* sp. *R. jostii*
Gallic acid	3,4,5-Trihydroxybenzoic acid	Kraft lignin	*A. aneurinilyticus*
Guaiacol	2-Methoxyphenol	Wheat straw	*C. subvermispora*, *P. eryngii, L. edodes*
p-Coumaric acid	4-Hydroxycinnamic acid	Kraft lignin	*Bacillus* sp.
Phenol	Hydroxybenzene	Wheat straw	*C. subvermispora*, *P. eryngii, L. edodes*
Phenylacetic acid	–	Kraft lignin	*A. aneurinilyticus*
p-Hydroxybenzoic acid	4-Hydroxybenzoic acid	Kraft lignin, spruce	*A. aneurinilyticus, P. chrysosporium*
Syringic aldehyde	4-Hydroxy-3,5-dimethoxybenzaldehyde	Lignocellulose	*S. paucimobilis*
Syringol	1,3-Dimethoxy-2-hydroxybenzene	Wheat straw	*C. subvermispora*, *P. eryngii, L. edodes*
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	Kraft lignin, spruce	*A. aneurinilyticus, P. chrysosporium*
Vanillin	4-Hydroxy-3-methoxybenzaldehyde	Wheat straw	*C. subvermispora*

Note: Raj, A., Krishna Reddy, M.M., Chandra, R., 2007. Identification of low molecular weight aromatic compounds by gas chromatography-mass spectrometry (GC–MS) from kraft lignin degradation by three Bacillus sp. International Biodeterioration and Biodegradation 59, 292–296. Bugg, T.D.H., Ahmad, M., Hardiman, E.M., Rahmanpour, R., 2011a. Pathways for degradation of lignin in bacteria and fungi. Natural Product Reports 28, 1883–1896. Chen, C., Chang, H., Kirk, T.K., 1982, Carboxylic acids produced through oxidative cleavage of aromatic rings during degradation of lignin in spruce wood by Phanerochaete chrysosporium. Journal of Wood Chemistry and Technology 36, 3–9. van Erven, G., Nayan, N., Sonnenberg, A.S.M., *et al.*, 2018. Mechanistic insight in the selective delignification of wheat straw by three white-rot fungal species through quantitative 13C-IS py-GC–MS and whole cell wall HSQC NMR. Biotechnology for Biofuels 11, 1–16.

Microbial degradation of lignin results in the release of aromatic compounds, which are toxic already at low concentrations for most microorganisms (Guiraud *et al.*, 1995; Friedman *et al.*, 2003; Adeboye *et al.*, 2014; Lima *et al.*, 2017). In order to survive this toxicity, the aromatic compounds need to be degraded or converted to non- or less toxic compounds by the microorganism. This is usually followed by the cleavage of the aromatic ring to eliminate toxicity, resulting in compounds that can be used as a carbon source. Many aromatic compounds have been observed to be released during bacterial and fungal lignin degradation (**Table 1**).

Degradation of Aromatic Compounds by Fungi

Most fungi are exposed to aromatic compounds in their environments and are able to degrade them. A common misconception is that aromatic compounds do not serve as carbon source for fungi and that they are only part of secondary metabolic pathways. However, several studies have shown that aromatic compounds can serve as carbon source (Guiraud *et al.*, 1992; Weber *et al.*, 1995; Martins *et al.*, 2015; Lubbers *et al.*, 2019b,a, 2020). The understanding of the microbial aromatic metabolic pathways can reveal, for example, how pathogens evade or suppress the plant defense system and cause diseases, or how some fungi can grow in hazardous environments while others cannot. More importantly, discovery of novel aromatic compound modifying enzymes are of great interest for the valorization of lignin and compounds derived from it. This will offer great opportunities for innovations to produce valuable compounds for many industries, e.g., food, plastic, cosmetic and pharmaceutical industries. To efficiently produce aromatic compounds, microorganisms have been genetically modified to produce desired compounds from glucose through the shikimate pathway or through lignin valorization (Wu *et al.*, 2017; Barton *et al.*, 2018). At this moment, only a few genes involved in the aromatic metabolism of fungi have been identified. Due to the many aromatic compounds present in nature, a selection of common and industrially relevant aromatic compounds will be discussed here.

Hazardous Aromatic Compounds

In this chapter aromatic compounds with hazardous properties are discussed. These aromatic compounds are released during industrial processes. The aromatic compounds benzene, toluene, ethyl benzene and xylenes, also collectively known as BTEX, are used in industrial synthesis and are components of gasoline and aviation fuels. However, BTEX are also contaminants commonly found in soil, sediments, water and the atmosphere, and are released in nature by improper waste disposal, leakages in underground storage tanks and pipelines, and accidental spillages (Nikolova and Nenov, 2005; García-Peña *et al.*, 2008).

Benzene

Benzene is a valuable chemical that is used as precursor for the synthesis of polymers, lubricants, rubbers, resins and synthetic fibers (Gentry, 2007; Nithyanandam *et al.*, 2018). Benzene is mainly converted to cumene, cyclohexane, nitrobenzene and ethylbenzene. More than half of the globally produced benzene is converted to ethylbenzene, of which 99% is converted to

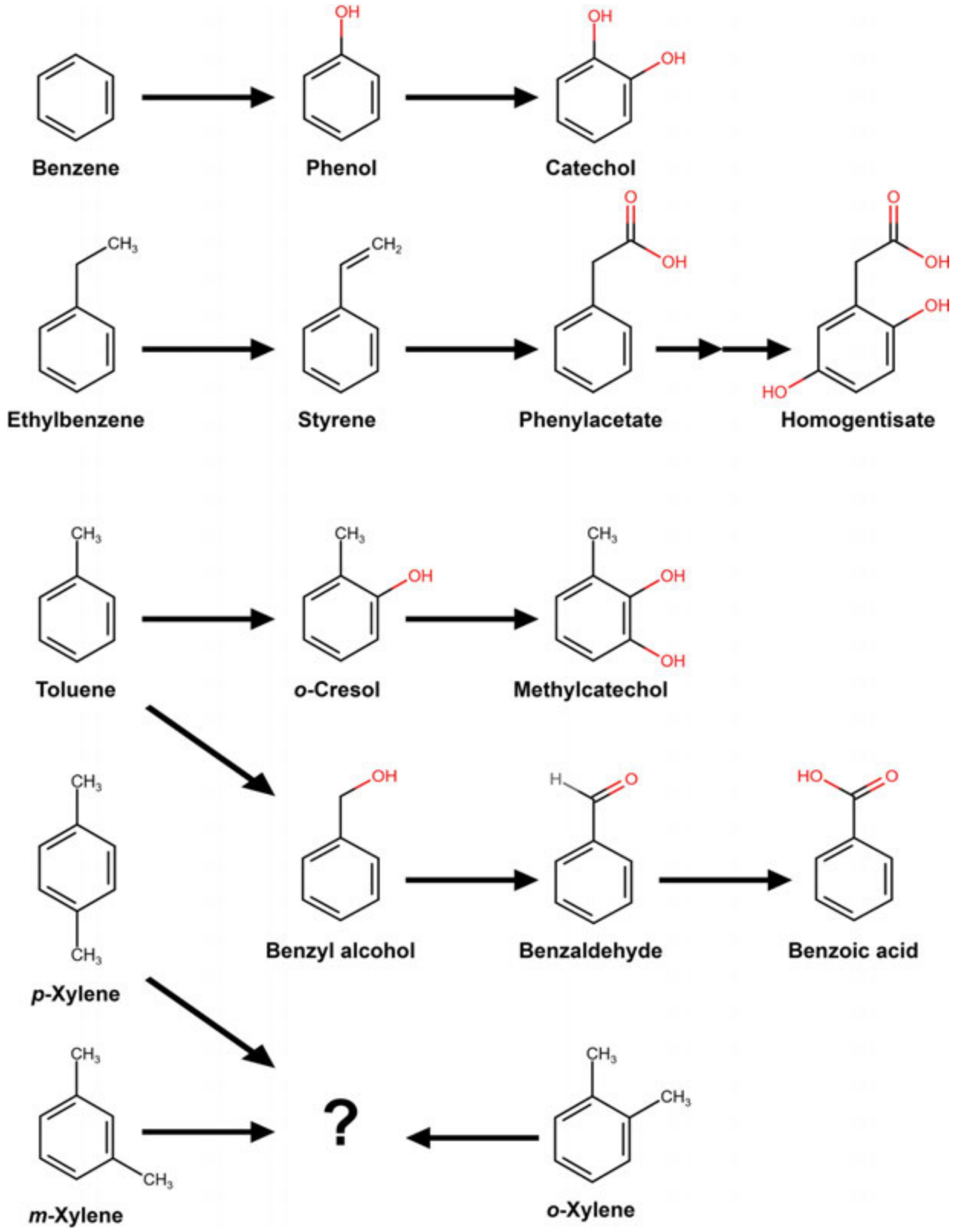

Fig. 3 BTEX metabolic pathways observed in fungi.

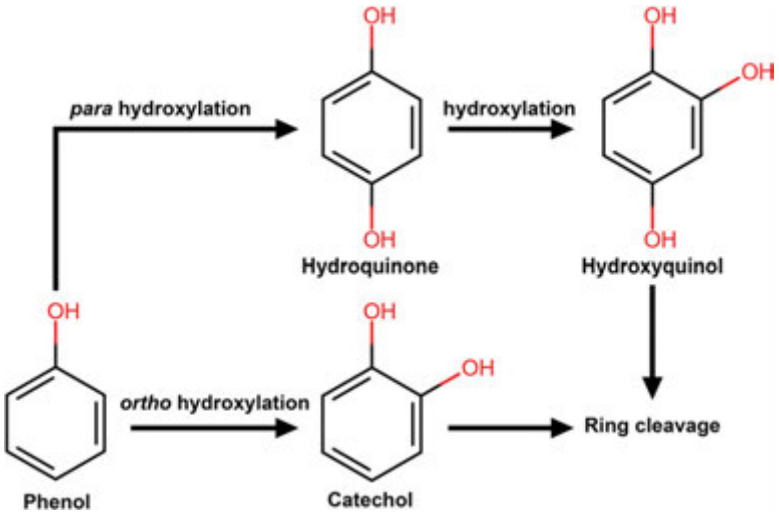

Fig. 4 Phenol metabolic pathway in fungi.

styrene. Styrene is used to produce synthetic rubbers and polymers such as polystyrene. Due to the many applications of benzene, the use of benzene is rapidly increasing and a global shortage is expected (Nithyanandam *et al.*, 2018). On the negative side, benzene is carcinogenic and its health risks have been studied for decades (Galbraith *et al.*, 2010; Gross and Paustenbach, 2018). Frequent exposure to high concentrations of benzene can cause non-Hodgkin lymphoma and leukemia, but also aplastic anemia, bone marrow abnormalities and cardiovascular disease (Vigliani and Saita, 1964; Velasco Lezama *et al.*, 2001; Galbraith *et al.*, 2010; Gross and Paustenbach, 2018). Exposure to low concentrations of benzene are primarily from petrol stations, gasoline combustion and tobacco smoke. Since benzene is highly volatile and can easily be inhaled, the exposure to benzene at workplaces is tightly regulated. In addition, the concentrations of benzene in various consumer products has been limited to less than 0.1% (Williams *et al.*, 2008).

Not much is known about the degradation of benzene by fungi. However, degradation of benzene has been observed in the fungi, such as the basidiomycete white-rot fungi *Phanerochaete chrysosporium* and *Trametes versicolor* (Yadav and Reddy, 1993; Aranda *et al.*, 2010). Currently, only one pathway has been described in *T. versicolor*. This fungus converts benzene through hydroxylation, which results in the formation of phenol that is further hydroxylated to catechol (1,2-dihydroxybenzene) (Aranda *et al.*, 2010) (**Fig. 3**). The metabolism of phenol and catechol is further discussed in Section "Phenol".

Ethylbenzene

Ethylbenzene naturally occurs in coal tar and petroleum. As mentioned before, ethylbenzene is mainly chemically synthetized from benzene through ethylation. Ethylbenzene is converted to styrene through catalytic dehydrogenation and is used for the production of polystyrene. Interestingly, the dimorphic yeast *Exophiala lecanii-corni* is also able to convert ethylbenzene to styrene (**Fig. 3**) (Gunsch *et al.*, 2005). It was suggested that styrene is converted to phenylacetate followed by two hydroxylation steps at the 2 and 5 position to homogentisate. Homogentisate is then cleaved to 4-maleylacetoacetate by homogentisate 1,2-dioxygenase. Similar styrene degradation pathways have been observed for the dimorphic yeast *Exophiala jeanselmei* and the white-rot fungus *Pleurotus ostreatus* (Cox *et al.*, 1996; Braun-Lüllemann *et al.*, 1997).

Toluene

The aromatic compound toluene (methylbenzene) can be extracted from crude oil, coal tar and petroleum. It is commonly used as a solvent in adhesives, coatings, paint and thinners but can also be used in fuels as an octane booster. Toluene is a hazardous aromatic compound, which has neurotoxic effects and can cause kidney and liver damage (Win-Shwe and Fujimaki, 2010).

The ascomycete fungus *Cladosporium sphaerospermum* was the first fungus shown to degrade toluene as sole carbon source (Weber *et al.*, 1995). This fungus was isolated from a compost biofilter used for the removal of toluene from waste gases. Degradation of toluene was also observed for two *Cladophialophora* strains and an *Exophiala* strain (Prenafeta-Boldú *et al.*, 2001). Currently, two toluene pathways have been described for fungi. In the first pathway, toluene is converted to benzyl alcohol by a toluene monooxygenase (Weber *et al.*, 1995; Luykx *et al.*, 2003) (**Fig. 3**). Benzyl alcohol is converted to benzaldehyde catalyzed by benzyl alcohol dehydrogenase. Benzaldehyde is further converted to benzoic acid by a benzaldehyde dehydrogenase. The metabolism of benzoic acid is further discussed below.

The second pathway was observed in the ascomycete fungi *Aspergillus ochraceous* and *Penicillium chrysogenum* (Smith and Rosazza, 1974). Toluene is hydroxylated at the *ortho* position resulting in the formation of *o*-cresol. This pathway was suggested to continue with an additional hydroxylation at *meta* position resulting in the formation of methylcatechol.

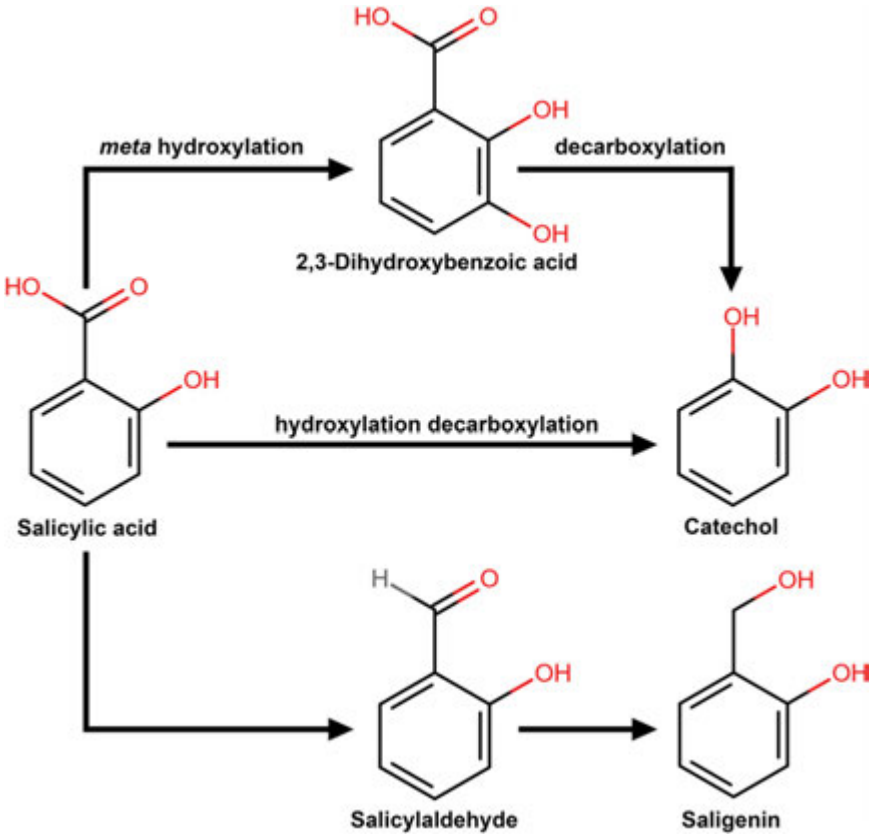

Fig. 5 Salicylic acid metabolic pathways in fungi.

Xylenes

Xylene (dimethylbenzene) has three isomers, *o*-xylene, *m*-xylene and *p*-xylene, which are widely used in the printing, rubber and leather industries, and in medical technology. In addition, *p*-xylene is a precursor for terephthalic acid and dimethyl terephthalate, which are used to produce the plastic polyethylene terephthalate (PET). However, xylene is toxic and has effects on the central nervous system, liver, kidney, reproductive system and respiratory track (Langman, 1994; Niaz *et al.*, 2015).

Currently, no metabolic pathways of xylene have been described in fungi. However, *Penicillium* sp. CHY-2, *P. chrysosporium* and *C. sphaerospermum* are able to degrade xylene (Jorio *et al.*, 2009; Chang *et al.*, 2016).

Phenol

The aromatic compound phenol (hydroxybenzene) is used in multiple applications and is mainly used as precursor for plastics and pharmaceuticals such as aspirin, but also for pesticides and herbicides. Phenol in combination with chloroform is also used in the molecular biology for the extraction of DNA and RNA. Phenol is a highly toxic, corrosive and mutagenic aromatic compound and is hazardous for humans, animals and plants at low concentrations (Bruce *et al.*, 1987). High concentrations of phenol can cause central nervous system disorders, myocardial depression and blindness, and harmful effects on the heart, liver and kidneys. Due to the toxicity and solubility in water, phenol is considered a highly hazardous pollutant. Phenol is also a byproduct of many industrial applications and can be found in high concentrations in the wastewater from coal refineries, tanneries, phenol manufacturing pharmaceuticals and industries of dyes, paints, resins, petrochemicals, textiles, pulp, paper and wood products (Senthilvelan *et al.*, 2014; Villegas *et al.*, 2016). In order to remove phenol from industrial wastewater, several methods are used, including adsorption, distillation, extraction, oxidation and membrane separation, but are costly and can yield hazardous byproducts (Mohammadi *et al.*, 2015). However, phenol can still be found in the environment, such as ground water and soil, due to disposal of untreated wastewater or leakages (Kahru *et al.*, 1994; Mohite *et al.*, 2011).

An alternative eco-friendly treatment of phenol containing wastewater is through bioremediation, in which microorganisms degrade phenol as a carbon source or convert it to a non-toxic compound. The biodegradation of phenol by bacteria is studied for decades, but is less studied in fungi. In fungi, two metabolic pathways have been observed (**Fig. 4**). The *ortho* hydroxylation of phenol results in the formation of catechol and is observed in the ascomycete fungi *Aspergillus fumigatus* (Jones *et al.*, 1993), *Aspergillus niger* (Supriya and Neehar, 2014), *Graphium* sp. FIB4 (Santos *et al.*, 2003; Santos and Linardi, 2004), *Fusarium oxysporum* (Park *et al.*, 2009), *Fusarium* sp. HJ01 (Cai *et al.*, 2007), *Paecillomyces variotii* (Wang *et al.*, 2010) and *Penicillium frequentans* (Hofrichter *et al.*, 1992), and the yeasts *Candida albicans* (Gérecová *et al.*, 2015), *Candida tropicalis* (Ahuatzi-Chacón *et al.*, 2004)

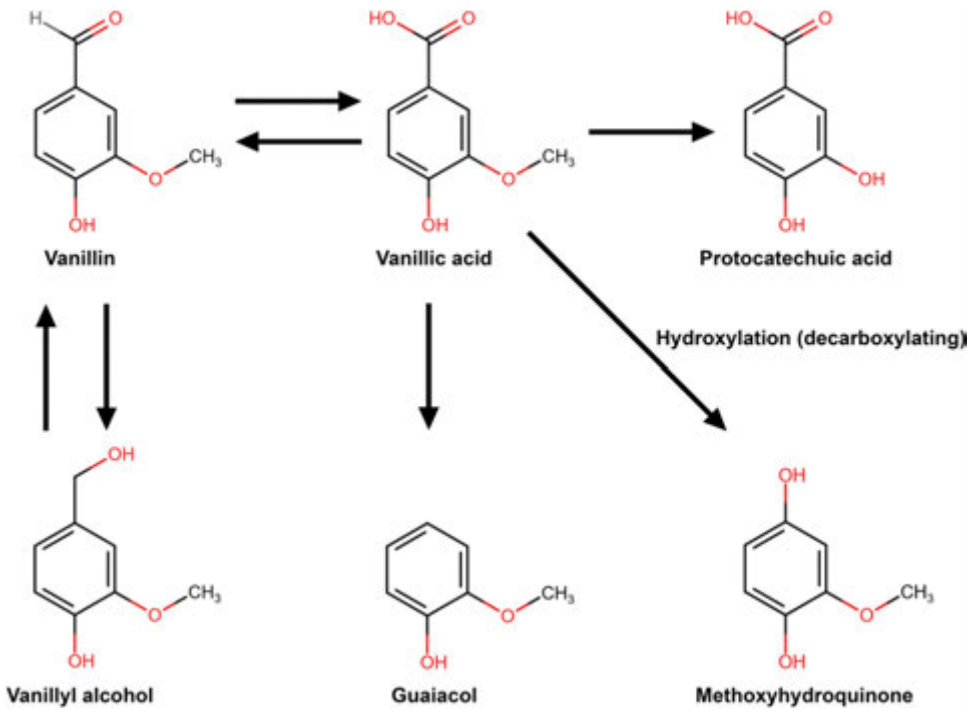

Fig. 6 Metabolism of vanillin and vanillic acid in fungi.

and *Trichosporon cutaneum* (Gaal and Neujahr, 1979). The aromatic ring of catechol is cleaved by an intradiol catechol 1,2-dioxygenase resulting in the formation of *cis,cis*-muconic acid. *Cis,cis*-muconic acid is further converted through the oxoadipate pathway to 3-oxoadipate and is then converted towards the tricarboxylic acid (TCA) cycle.

A second pathway in which phenol is hydroxylated at the *para* position, resulting in the formation of hydroquinone (1,4-dihydroxybenzene), was observed in *A. fumigatus* (Jones *et al.*, 1994), *P. chrysogenum* (Leitão *et al.*, 2007) and the yeast *C. tropicalis* (Gérecová *et al.*, 2015). Hydroquinone is further converted to hydroxyquinol (1,2,4-trihydroxybenzene) by a hydroxyquinol 1,2-dioxygenase to malylacetate and further to 3-oxoadipate.

Plant Derived and Food Related Aromatic Compounds

Salicylic acid

Salicylic acid (2-hydroxybenzoic acid) has many interesting properties and is mainly used as a peeling agent in skin care products (Arif, 2015). In addition, salicylic acid is also a precursor for aspirin and 4-aminosalicylic acid that is used to treat tuberculosis. Salicylic acid is mainly chemically synthesized from phenol through the Kolbe-Schmitt reaction (Kirimura *et al.*, 2010). In plants, the amino acid phenylalanine is converted to salicylic acid. As mentioned before, salicylic acid plays an important role as phytohormone involved in plant defense signaling, plant development, photosynthesis, transpiration and ion uptake and transportation (Vernooij *et al.*, 1994; Shah, 2003; Zhang *et al.*, 2013; Lu *et al.*, 2016). Salicylic acid degradation by fungi is considered to be an important factor for pathogenicity (Rabe *et al.*, 2013; Ambrose *et al.*, 2015; Hao *et al.*, 2019). Multiple salicylic acid degradation pathways have been described (**Fig. 5**).

The carboxyl group from salicylic acid is decarboxylative hydroxylated by a salicylate hydroxylase to a hydroxyl group resulting in the formation of catechol. This has been observed in the filamentous fungi *A. niger* (Lubbers *et al.*, 2021), *Aspergillus nidulans* (Martins *et al.*, 2015), *Aspergillus japonicus* (Milstein *et al.*, 1983), *Epichloë festucae* (Ambrose *et al.*, 2015), *Fusarium graminearum* (Hao *et al.*, 2019), *Sclerotinia sclerotiorum* (Penn and Daniel, 2013) and *Ustilago maydis* (Rabe *et al.*, 2013), and the yeast *Trichosporon cutaneum* (Sze and Dagley, 1984). As described before, the aromatic ring of catechol is cleaved resulting in the formation of *cis,cis*-muconic acid. In the second pathway, salicylic acid is first hydroxylated at the *meta* position resulting in the formation of 2,3-dihydroxybenzoic acid (Martins *et al.*, 2015). Thereafter, 2,3-dihydroxybenzoic acid is decarboxylated to catechol which is catalyzed by 2,3-dihydroxybenzoate decarboxylase (DhbA) (Santha *et al.*, 1995; Martins *et al.*, 2015). A third pathway was observed in *Neurospora crassa*, which is able to reduce salicylic acid to salicylaldehyde (2-hydroxybenzaldehyde) and saligenin (2-(hydroxymethyl)phenol) (Bachman *et al.*, 1960).

Vanillin

One of the best-known aromatic compounds is vanillin (4-hydroxy-3-methoxybenzoic acid) that is used for its sweet and appealing aroma. In addition, vanillin has antimicrobial activities and can be used as preservative (Fitzgerald *et al.*, 2003, 2004;

Ngarmsak *et al.*, 2006). Vanillin can be found naturally in the seed pods of vanilla orchids (*Vanilla planifolia*). Currently, vanillin is one of the most important flavor and fragrance compounds in food and cosmetics. Next to that, vanillin is also used as precursor in pharmaceuticals such as dopamine and papaverine. A promising industrial niche is the increasing consumer demand for "naturally" produced aromatic compounds. Only 0.2% of vanillin is derived from natural sources, while the rest is produced from guaiacol by chemical synthesis (Krings and Berger, 1998). Therefore, the production of vanillin by microorganisms is of great interest. Vanillin is also a flavoring agent of the traditional Japanese spirit, awamori. Ferulic acid (4-hydroxy-3-methoxycinnamic acid) that is present in rice koji (cooked rice) is converted to vanillin during the fermentation with *Aspergillus luchuensis*. The aromatic compounds ferulic acid, vanillin and vanillic acid were detected in rice koji and during fermentation (Maeda *et al.*, 2018; Taira *et al.*, 2018). Two metabolic pathways have been described, which result in the formation of vanillin. In the CoA-independent oxidative pathway, ferulic acid is decarboxylated to vinyl-guaiacol by the phenolic acid decarboxylase (Pad) and is further reduced to vanillin (Taira *et al.*, 2018). In the CoA-dependent non-oxidative pathway, ferulic acid is converted to feruloyl-SCoA by an feruloyl-CoA synthetase (Fcs). The next two steps are catalyzed by a multifunctional enzyme called enoyl-CoA hydratase/aldolase (Ech) which hydrates feruloyl-SCoA to HMPHP-SCoA and cleaves it to vanillin and acetyl-CoA. Currently, it remains unknown which metabolic pathway is used by *A. luchuensis*.

Fungi can degrade vanillin through multiple aromatic metabolic pathways (Lubbers *et al.*, 2019c). The foremost observed pathway is the dehydrogenation of vanillin to vanillic acid or reduction of vanillin to vanillyl alcohol (**Fig. 6**). Multiple vanillic acid metabolic pathways have been described that include conversion through:

(1) Hydroxylation of the carboxyl group to methoxyhydroquinone.
(2) Demethylation of the methoxy group to protocatechuic acid.
(3) Non-oxidative decarboxylation of the carboxyl group to guaiacol.
(4) Reduction of the carboxyl group to vanillin.

The hydroxylation of vanillic acid to methoxyhydroquinone has been described in multiple ascomycete and basidiomycete fungi and appears to be most common metabolic pathway in fungi (Yajima *et al.*, 1979; Busswell and Eriksson, 1988; Guiraud *et al.*, 1992). A vanillate hydroxylase was isolated from *Sporotrichum pulverulentum* (an anamorph of *P. chrysosporium*) that uses NADH or NADPH as cofactor. However, the encoding vanillate hydroxylase gene remains to be identified. The demethylation of vanillic acid to protocatechuic acid was observed in many fungi such as *A. fumigatus*, *Aspergillus ultus*, *Doratomyces microspores*, *Penicillium hirsutum*, *Penicillium italicum*, *Penicillium roqueforti* and *P. chrysosporium* (Guiraud *et al.*, 1992). The decarboxylation of vanillic acid to guaiacol has been observed in *Aspergillus oryzae*, *Aspergillus parasiticus* and *Paecilomyces lilacinus* (Guiraud *et al.*, 1992). The fungal vanillic acid decarboxylase remains to be identified. Several fungi, such as *Aspergillus carbonarius*, *Trichoderma harzianum* and *Trichoderma reesei*, can reduce vanillic acid to vanillin and further to vanillyl alcohol (Guiraud *et al.*, 1992). In *Penicillium simplicissimum*, vanillyl alcohol is converted to vanillin by vanillyl alcohol oxidase (Vao) (de Jong *et al.*, 1990).

Benzoic acid

Two other aromatic compounds added to foods, beverages and cosmetics are benzoic acid (benzenecarboxylic acid) and the salt sodium benzoate. Benzoic acid has strong antimicrobial properties and is therefore mainly used in the food industry as preservative. Benzoic acid can also be used as pH adjuster and fragrance ingredient.

As mentioned previously, toluene, benzyl alcohol and benzaldehyde are converted to benzoic acid. Currently, only one benzoic acid metabolic pathway has been observed in fungi and was reported in *A. niger*, *A. nidulans*, *Cochliobolus lunatus*, *P. chrysosporium* and *Rhodotorula minuta* (Boschloo *et al.*, 1990; Fukuda *et al.*, 1996; Matsuzaki and Wariishi, 2005; Korošec *et al.*, 2014; Martins *et al.*, 2015). Benzoic acid is converted to *p*-hydroxybenzoic acid by benzoate monooxygenase (BphA) together with a NADPH cytochrome P450 reductase (CrcA) (van Gorcom *et al.*, 1990). *p*-Hydroxybenzoic acid is further converted to protocatechuic acid (3,4-dihydroxybenzoic acid) which is catalyzed by *p*-hydroxybenzoate-*m*-hydroxylase (PhhA) (Lubbers *et al.*, 2019b). The aromatic ring of protocatechuic acid is then cleaved by the protocatechuate 3,4-dioxygenase (PrcA) to 3-carboxy-*cis*,*cis*-muconic acid.

Cinnamic acid

Cinnamic acid is commonly used as flavor compound in foods and drinks, and for its aroma in perfumes and cosmetics. Cinnamic acid is naturally found in the spice cinnamon, which is derived from the bark of trees from the genus *Cinnamomum*. The main aromatic compound responsible for the flavor of cinnamon is cinnamyl aldehyde. Cinnamic acid also plays a role as the building block for lignin units and is synthesized from phenylalanine (Humphreys and Chapple, 2002). As mentioned above, microorganisms can also be exposed to cinnamic acid through lignin degradation and allelopathy.

Currently, three cinnamic acid metabolic pathways have been described in fungi. The most observed metabolic pathway in fungi is the decarboxylation of cinnamic acid to styrene and has been observed in multiple Aspergilli and the yeast *Saccharomyces cerevisiae* (Plumridge *et al.*, 2008; Mukai *et al.*, 2010). This conversion is catalyzed by cinnamic acid decarboxylase (CdcA) and the co-enzyme flavin prenyltransferase (PadA) (Plumridge *et al.*, 2008, 2010; Payne *et al.*, 2015; Lubbers *et al.*, 2019a). The deletion of *cdcA* or *padA* results in abolished growth on cinnamic acid (Lubbers *et al.*, 2019a). In addition, both are also essential for the degradation of sorbic acid, which is a commonly used food preservative. In the *A. niger* genome, between *cdcA* and *padA*, lies a transcriptional regulator known as the sorbic acid decarboxylase regulator (*sdrA*) (Plumridge *et al.*, 2008). This has been shown to regulate *cdcA* and *padA*, and deletion of *sdrA* results in reduced growth on cinnamic acid and sorbic acid (Lubbers *et al.*, 2019a).

Homologs of *cdcA*, *padA* and *sdrA* are clustered on the genome in several *Aspergillus* species. In the genome of *S. cerevisiae*, ferulic acid decarboxylase (*fdc1*) and *pad1* are next to each other, but no transcriptional regulator is located in between or next to them.

Two other cinnamic acid metabolic pathways have been suggested. The reduction of cinnamic acid to cinnamyl aldehyde and cinnamyl alcohol and was described for *A. japonicus* and the basidiomycete *Schizophyllum commune* (Milstein *et al.*, 1983; Nimura *et al.*, 2010). In *A. japonicus* and *A. niger*, the conversion of cinnamic acid to benzoic acid was suggested (Milstein *et al.*, 1983; Lubbers *et al.*, 2019b). Deletion of *bphA*, *phhA* or *prcA* in *A. niger* resulted in reduced growth on cinnamic acid indicating that cinnamic acid is converted through the benzoic acid metabolic pathway (Lubbers *et al.*, 2019b). It remains unknown which metabolic pathway is used for the conversion of cinnamic acid to benzoic acid in *A. niger*.

Conclusions and Future Prospects

The metabolism of aromatic compounds by fungi is interesting for several industries, since fungi can convert toxic compounds into harmless or valuable compounds. The study of fungal aromatic metabolism is important for applications where fungi can play a valuable role. For example, fungi can be used in the bioremediation of polluted environments or the biotransformation of aromatic compounds to chemical building blocks. Despite the genomic era in fungal research, many genes involved in the fungal aromatic metabolic pathways remain to be identified and this is essential for the genetic modification of fungi to create efficient strains.

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Genetic Engineering for Strain Improvement in Filamentous Fungi

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Introduction

Filamentous fungi are a large group of eukaryotic organisms that have the ability to grow in many different environments, including biological wastes, soil, and plant and algal biomass. They also have the ability to grow on a wide variety of inexpensive substrates and produce a rich repertoire of interesting metabolites and enzymes, which has aroused considerable interest on the part of the scientific community for their exploitation as production organisms in biotechnology (Meyer, 2008). Nowadays, filamentous fungi are utilized as cell factories for the production of many industrially relevant compounds, such as organic acids, proteins, enzymes, exopolysaccharides, and secondary metabolites (Meyer, 2008; Nielsen and Nielsen, 2017) and fungi such as *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei* have become indispensable for enzyme production at industrial level (Frisvad *et al.*, 2018). Fungal enzymes currently make up more than half of the enzymes used in industrial applications (de Vries *et al.*, 2019), reflecting the importance of filamentous fungi as cell factories. In this context, one fundamental aspect for the future of fungal biotechnology is the improvement of production strains at the molecular level. Genetic engineering for strain improvement is a very powerful tool for increasing production levels, producing novel compounds and/or controlling/directing the synthesis of the desired products. Nevertheless, this will only be possible with the development of efficient methods to introduce (new) genes or control gene expression in filamentous fungi.

In this chapter, we summarize and discuss the currently available options for fungal transformation and genetic engineering of filamentous fungi.

Classical Genetic Engineering Approaches

Genetically Modified Strains (GMOs)

A genetically modified organism or strain (GMO) is defined as any (micro)organism whose genetic material has been modified through the application of genetic engineering methods. Genes can be transferred within the same species, across species, or even across kingdoms. Advances in the field of genetic engineering have allowed for precise control over the genetic changes introduced into an organism, overcoming the limitations of the naturally occurring genetic variation, thus optimizing the strain performance and/or improving the production of valuable substances. Despite gene transfer occur naturally between different species (horizontal gene transfer) (Gogarten and Townsend, 2005), there is a high social concern about the impact of GMOs in both human health and the environment. Unlike other methods for strain improvement, the application of genetic engineering technology is strictly regulated. Extensive legal framework on GMOs has been established in the European Union to protect human health and the environment (Directive, 2001/18/EC) and to ensure the free movement of safe and healthy genetically modified products in the market (Regulation (EC) No1829/2003). In the USA, GMO regulation is divided among three regulatory agencies: The Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the US Department of Agriculture (USDA). The USA approach to regulate GMOs is premised on the assumption that regulation should focus more on the nature of the final products, rather than the process in which they were produced, making regulation of GMOs in the USA relatively favorable for their development compared to other countries.

Genetic Engineering of Filamentous Fungi

In practice, genetic engineering of filamentous fungi meets with several difficulties, since different transformation methods are required for the great diversity of fungal species with their very different and complex structures (Li *et al.*, 2017). In order to genetically modify filamentous fungi, first the genetic material needs to be introduced into the recipient cells with an efficient transformation method. Secondly, the genetic modification of interest needs to be integrated into the genome of the recipient strain. Finally, transformant strains need to be selected under certain selection conditions, which can vary depending on each fungal strain (Goosen *et al.*, 1987; Punt *et al.*, 1987; Olmedo-Monfil *et al.*, 2004). To date, several transformation methods have been described for filamentous fungi, including the commonly applied protoplast-mediated transformation and the *Agrobacterium*-mediated transformation methods, as well as electroporation, biolistics, and shock wave-mediated transformation.

Protoplast-mediated transformation

The introduction of the desired genetic modification in our fungus of interest often represents a challenge, since the establishment of a suitable and efficient transformation method is not trivial for many fungal species. The application of the protoplast-mediated transformation (PMT) has been extended to many filamentous fungal strains since the first successful transformation of the yeast *Saccharomyces cerevisiae* (Hinnen *et al.*, 1978), becoming one of the most commonly and routinely used methods for the

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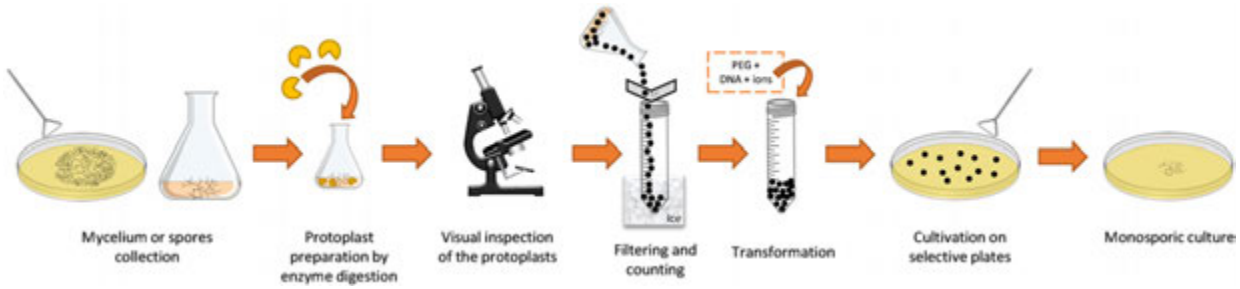

Fig. 1 Schematic representation of the basic steps for protoplast-mediated transformation.

transformation of filamentous fungi with high efficiency rates (Cantoral *et al.*, 1987; Dawe *et al.*, 2000; Meyer *et al.*, 2003). Protoplasts are cells from which the cell wall has been removed, usually by enzymatic digestion. The principle of PMT is to use commercially available cell wall-degrading enzymes to remove fungal cell wall components from either macroconidia, microconidia, or young mycelium to generate protoplasts, with the selection of a proper enzyme cocktail for the efficient protoplast formation (de Bekker *et al.*, 2009). Once formed, protoplasts are put into contact with the exogenous DNA of interest, which can be linearized or circular double-stranded DNA, and chemical reagents such as calcium ions and polyethylene glycol (PEG) are used to create small holes in the cell membrane and to promote the fusion of the exogenous DNA and the protoplasts, respectively (Li *et al.*, 2017). Finally, protoplasts are transferred to a selective medium (**Fig. 1**). Note that protoplasts are very sensitive to osmotic pressure, so it is necessary that a stable osmotic pressure is maintained to keep the protoplasts intact during the whole transformation process. Thus, osmotic stabilizers like sorbitol must be included in the buffers for protoplast generation to prevent cell damage. PEG also promotes cell agglomeration and together with the role described above, this makes PEG crucial for increasing transformation efficiency. Low transformation efficiencies can be in many cases improved by increasing the amount of PEG, and low molecular weight PEG (PEG3000, PEG4000 or PEG5000) is usually preferred over high molecular weight PEG (PEG8000) (Li *et al.*, 2017). Temperature also influences transformation efficiency. Normally protoplasts should be placed on ice, and the transformation process should be carried out at low temperature, although for some fungi such as *A. niger*, transformation at room temperature was demonstrated to also be very efficient (Kun *et al.*, 2020) . PMT has been successfully applied in many ascomycete species such as *T. reesei* (Penttilä *et al.*, 1987; Gruber *et al.*, 1990), *Neurospora crassa* (Case *et al.*, 1979), many *Aspergillus* sp. (Tilburn *et al.*, 1983; Yelton *et al.*, 1984; Hahm and Batt, 1988; Meyer *et al.*, 2003; Storms *et al.*, 2005) and *Penicillium* sp. (Sánchez *et al.*, 1987; Itoh *et al.*, 1994; Fierro, 2004; Villarino *et al.*, 2018), and basidiomycetes, such as *Laccaria bicolor* (Kropp and Fortin, 1986), *Coprinus cinereus* (Binninger *et al.*, 1987), *Pleurotus ostreatus* (Peng *et al.*, 1993), *Trametes versicolor* (Bartholomew *et al.*, 2001) and *Dichomitus squalens* (Daly *et al.*, 2017).

Theoretically, any fungal species would undergo protoplast generation. However, for some particular fungi, such as the basidiomycete *Ustilago violacea*, protoplasts cannot be generated in high amounts or their quality is not good enough to undergo efficient transformation (Bej and Perlin, 1989; Ruiz-Díez, 2002). In these cases, the transformation of intact fungal cells could be an alternative. In this context, it is possible to induce cell permeability by using high concentrations of lithium acetate in combination with PEG, dithiothreitol (DTT), and/or dimethyl sulfoxide (DMSO) (Olmedo-Monfil *et al.*, 2004; He *et al.*, 2017). This method was applied in a few fungal species such as *N. crassa*, *Coprinus cinereus* or *U. violacea* (Olmedo-Monfil *et al.*, 2004), although its application is limited and alternative transformation methods with higher efficiency rates are preferred instead.

Agrobacterium tumefaciens-*mediated transformation*

Agrobacterium tumefaciens is a Gram-negative soil bacterium that naturally infects plants throughout pre-existing injuries. It carries a tumor-inducing plasmid (Ti plasmid) which contains the transfer DNA (T-DNA) flanked by two directional repeats (known as right and left borders), and the necessary genes (called virulence genes) to introduce this T-DNA randomly into the plant genome, causing the crown gall disease (Gohlke and Deeken, 2014). *A. tumefaciens* has been extensively studied as a biotechnological tool for the introduction of foreign genes into plants and fungi. In fungi, *A. tumefaciens*-mediated transformation (ATMT) was first applied in *S. cerevisiae* (Bundock *et al.*, 1995). For ATMT of filamentous fungi, a binary vector is designed in which the target gene is inserted between the right and left borders of the T-DNA, and is then transformed into the bacterium. Finally, *A. tumefaciens* is used as a vehicle to integrate the gene of interest into the fungal genome (Li *et al.*, 2017). The basic steps for ATMT of filamentous fungi are shown in **Fig. 2**. ATMT consists of three main successive steps: (1) *A. tumefaciens* culture induction with an inducing agent such as acetosyringone, (2) co-inoculation of the bacterium and fungal cells in solid medium containing filters, and (3) selection of the positive transformants with the appropriate selection pressure (Michielse *et al.*, 2005). Acetosyringone is an important agent for the transformation process, since it induces the expression of the virulence genes necessary for the T-DNA transfer to the fungal cells. Other important factors affecting ATMT efficiency are the co-culturing conditions (e.g., time, temperature, pH, filters), and should be empirically determined and optimized for each fungal strain (Li *et al.*, 2017).

ATMT has many advantages, since this method can be applied for protoplast, spores, germlings, and/or hyphae transformation (Michielse *et al.*, 2005), and generates very stable transformants. In addition, ATMT leads to single-copy integration, in contrast to PMT, in which preferentially multi-copy integrations are observed (de Groot *et al.*, 1998; Meyer *et al.*, 2003). Currently ATMT is, together with

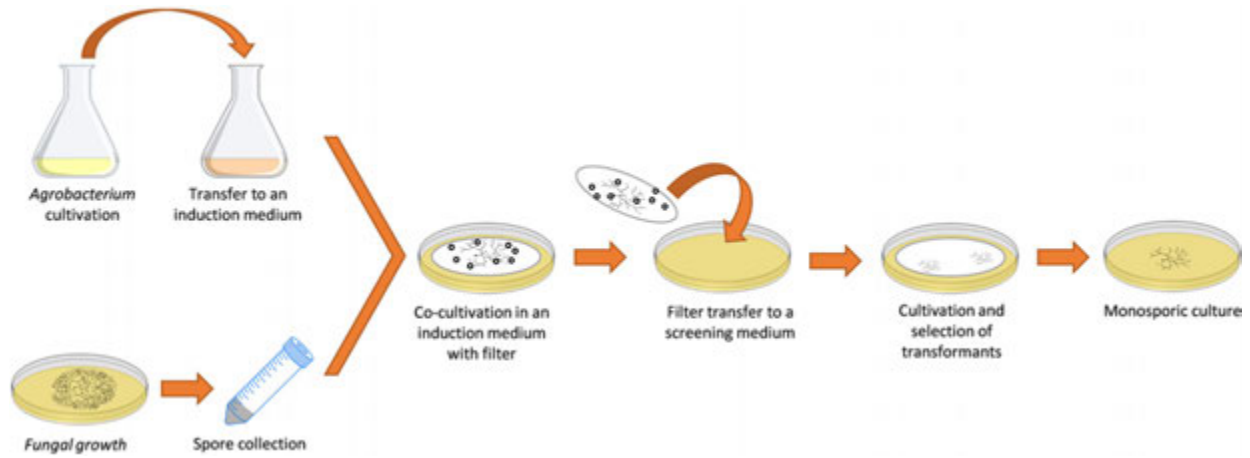

Fig. 2 Schematic representation of the basic steps for *Agrobacterium tumefaciens*-mediated transformation.

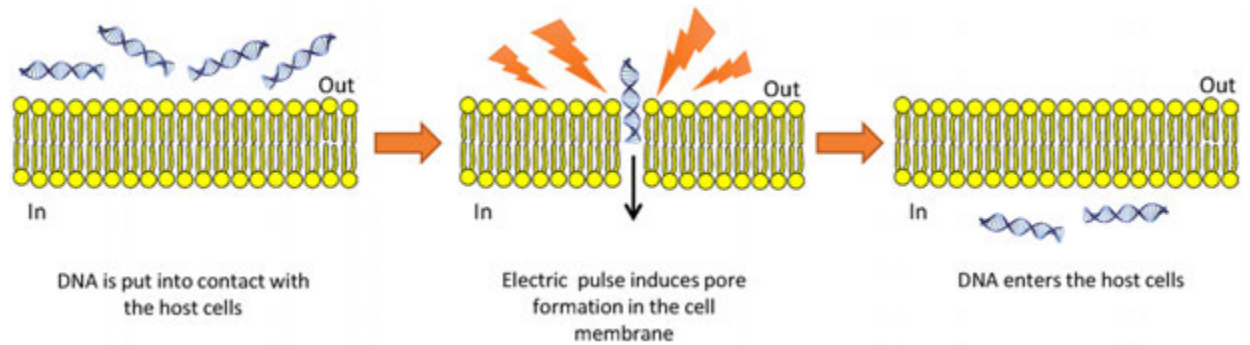

Fig. 3 Schematic representation of the electroporation-mediated transformation.

PMT, the method of choice for the transformation of many filamentous fungal species due to its high transformation rates and simplicity. ATMT has been successfully applied in a wide range of filamentous fungi, such as *Aspergillus giganteus* (Meyer *et al.*, 2003), *Botrytis cinerea* (Rolland *et al.*, 2003), *Fusarium oxysporum* (Mullins *et al.*, 2001), *Pyricularia* (*Magnaporthe*) *oryzae* (Rho *et al.*, 2001), *Aspergillus awamori*, *A. niger*, *N. crassa*, *T. reesei*, *Fusarium venenatum* (de Groot *et al.*, 1998), *Penicillium digitatum* (Wang and Li, 2008), *Penicillium expansum* (Zhang *et al.*, 2013), and in some basidiomycete and zygomycete species (Michielse *et al.*, 2005).

Electroporation-mediated transformation

Electroporation-mediated transformation (EMT) is a simple, fast, and efficient transformation method for both prokaryotic and eukaryotic cells (Haberl Meglič and Kotnik, 2017). This technology is based on the reversible capacity of cell membranes for permeabilization by short duration and high amplitude electric fields. After the application of the electric pulse, membrane permeabilization changes and pores can be formed, allowing the uptake of exogenous DNA into the cells (**Fig. 3**). After the electric shock, lipid and protein molecules in the biological membranes can restore their original structure, and DNA normally integrates into the genome, allowing the new traits to be inherited upon cell division. There are some factors that influence the efficiency of EMT, which include electric field intensity, variation in the electric charges (capacitance), pulse duration and frequency. However, the electric field intensity is the most important factor to control since high intensities correlate with higher rates of DNA uptake, but also with lower cell survival rates. For filamentous fungi, electric field magnitude ranges from 6 to 15 kV/cm and pulse width reaches the μs-ms scale (Li *et al.*, 2017). Due to the large amount of heat produced during EMT, low temperature (4°C) is recommended during the transformation process. Compared to PMT and ATMT, this technology is simpler and involves fewer transformation steps. It is also fast, reproducible, and multiple copies of DNA can integrate into the genome, providing the potential for increased yields of the desired products. However, the electroporation mechanism is still unclear, and the difficulty for its optimization together with instrumental costs hinder its applicability in filamentous fungi. Nonetheless, EMT has been successfully applied in some fungal species such as *Penicillium urticae*, *A. oryzae*, *N. crassa* (Chakraborty *et al.*, 1991), *A. niger* (Ozeki *et al.*, 1994), *Monascus purpureus* (Lakrod *et al.*, 2003), *T. reesei* (Benocci *et al.*, 2018) and *Pseudogymnoascus verrucosus* (Díaz *et al.*, 2019).

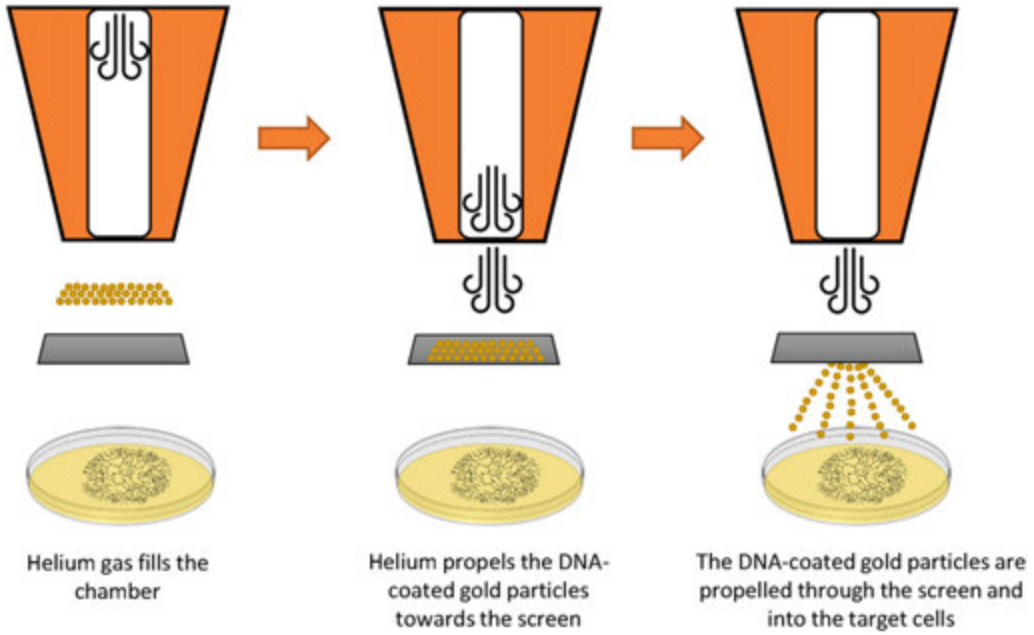

Fig. 4 Schematic representation of the biolistics transformation.

Biolistic transformation

Biolistic transformation, also known as particle bombardment or gene gun, was initially described as an alternative to ATMT for the transformation of plant cells (Takeuchi *et al.*, 1992). For fungal transformation, either gold or tungsten particles are coated with foreign DNA, accelerated to a high speed, and bombarded towards spores or hyphae (**Fig. 4**) (Su *et al.*, 2012; Li *et al.*, 2017). As a result, both stable and/or transient transformations can occur. Transiently transformed cells are able to express the foreign DNA without integrating it into their genome, while stable transformed cells integrate the foreign DNA as part of their own genomic sequence. To date, many fungal species have been successfully transformed by biolistics, such as *Aspergillus nidulans* (Barcellos *et al.*, 1998), *T. reesei*, (Te'o *et al.*, 2002), *Trichoderma harzianum* (Lorito *et al.*, 1993), and some mycorrhizal fungi (Harrier and Millam, 2001). Biolistics is one of the most powerful genetic transformation technologies, since it is not subject to the limitations described for the other transformation methods, such as protoplast generation, or fungal cell wall composition. However, this methodology requires high instrumental and consumable investment, thus it is only considered to be applied for recalcitrant strains when other transformation methods were proved to work unsuccessfully (Li *et al.*, 2017).

Shock wave-mediated transformation technology

Shock waves have been widely applied in medicine to treat several diseases (Thiel, 2001). Some studies have revealed that shock waves cause transient cell permeabilization through acoustic cavitation, allowing large molecules such as DNA to enter and become trapped inside the cells (Gómez-Lim *et al.*, 2015). The Shock wave-mediated transformation technology (SWMT) was initially applied for bacterial transformation (Loske *et al.*, 2011), and later to filamentous fungi (Magaña-Ortíz *et al.*, 2013). In principle, a solution of fungal conidia together with exogenous DNA is placed in heat-sealed polyethylene bags. Then, bags are located inside the water tank of the shock wave generator. Samples are subsequently exposed to 50–400 shock waves generated at 7.5 kV at a rate of 0.5 Hz. Finally, conidia suspension is transferred to agar plates with cellulose filters for conidia regeneration, and after 24 h filters are transferred to fresh agar plates with a selective agent (**Fig. 5**) (Gómez-Lim *et al.*, 2015). SWMT shows some advantages compared to other transformation methods reported above, since shock waves can act directly on spores, and only a few parameters need to be controlled: spore/DNA concentration, energy, and speed of the shock waves (Li *et al.*, 2017). SWMT is efficient in filamentous fungi, and in some cases efficiency is higher than ATMT (Magaña-Ortíz *et al.*, 2013). Nevertheless, some limitations are impeding SWMT to become a widely applied transformation technology in microbiology laboratories, since shock wave sources and instruments are very expensive, and during the transformation process a great proportion of DNA can be damaged, thus decreasing transformation rates. However, it has been reported that genetic transformation can be enhanced when a second shock wave is sent shortly before bubbles are formed after the cavitation start to collapse, which is also known as tandem shock waves (Gómez-Lim *et al.*, 2015) compensating for the loss of good quality DNA.

Non-GMO Genetic Engineering Approaches

Random mutagenesis is a classical and successful method that relies on a trial-and-error process and has therefore been widely used for fungal strain improvement (Ribeiro *et al.*, 2013; Chand *et al.*, 2005). It consists of the application of a physical or chemical agent

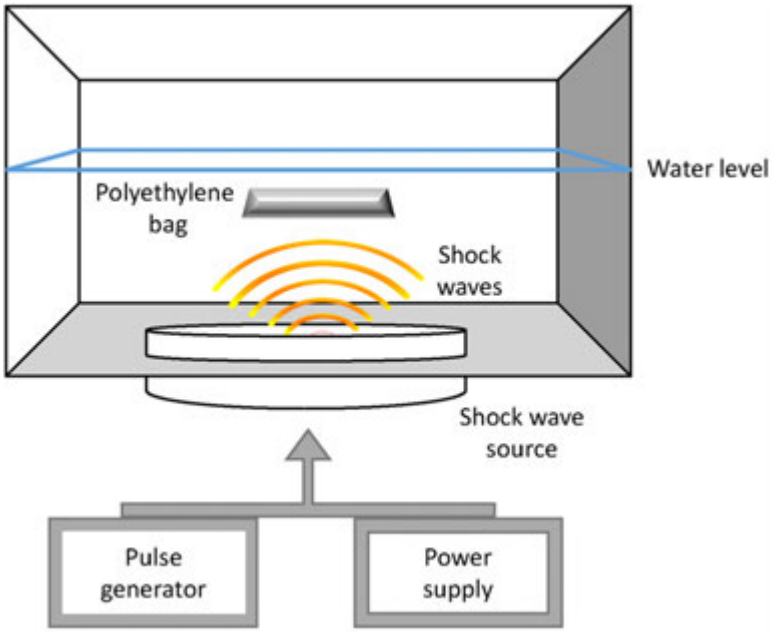

Fig. 5 Schematic representation of the shock wave-mediated transformation technology.

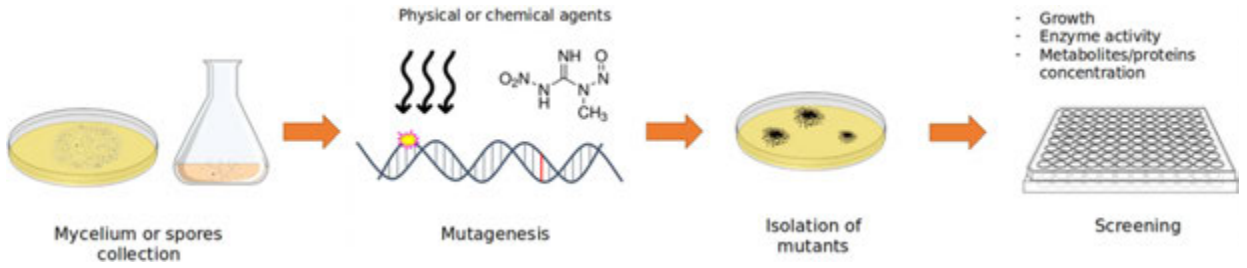

Fig. 6 In random mutagenesis physical or/and chemical agents are applied to the parental strain to obtain a diverse population of mutants. These mutants are isolated and screened by different methods to obtain strains with improved characteristics of interest.

to the fungus that reacts with the DNA, producing different genetic mutations. When it occurs in nature, these mutations can lead to a high death rate, but also to a genetically more diverse population, affecting natural evolution. In biotechnological strain improvement, the resulting population is screened after the mutagenesis step with a specific method to detect strains that present the trait of interest (e.g., overproduction of a protein, improved thermostability of an enzyme, increased conversion of a substrate) (**Fig. 6**).

Organisms obtained by means of these "traditional" techniques are not subjected to the GMO regulation (Directive 2009/41/EC) and, therefore, the use and commercialization of these strains are easier as they require less strict containment and isolation measures.

The use of ultraviolet irradiation (UV) or chemical mutagenesis has the advantage that although a laborious work on screening a large number of strains is needed, it is not necessary to know the underlying mechanism why the mutation is effective. At the technical level, random mutagenesis is simple and can be effective even in non-domesticated species that are recalcitrant to targeted genetic manipulation (Tapia *et al.*, 2012). Some drawbacks compared to more targeted methods, such as site directed mutagenesis, are that additional genes can be affected, e.g., resulting in poorly growing strains, and that the genetic stability of the strains can be impaired, leading to genotypic and phenotypic changes in the following generations (Chand *et al.*, 2005). However, recent novel genomic tools have enabled more in-depth analysis of these randomly modified strains, providing new insights on the molecular mechanisms of many pathways and a better control on the safety and stability of the industrial strains (Nikolaev *et al.*, 2013).

UV mutagenesis

Ultraviolet (UV) light is the most used physical mutagenic agent for strain improvement. This method consists on the use of UVC radiation (~254 nm), which is also used for microbial disinfection, at a sublethal dose to modify the DNA structure by a photochemical reaction. This radiation excites the DNA molecule resulting in the formation of pyrimidine dimers between adjacent pyrimidines, which usually causes point mutations that can be incorporated into the genome if they are not repaired. These mutations are mainly base substitutions of cytosine (C) to thymine (T), but the effects of UV light can be more complex

(Ikehata and Ono, 2011). UV radiation presents a good cost-benefit relation compared to other physical mutagenic agents such as X-rays, γ-rays, or heavy ion beams, since UVC lamps are widely available and easy to handle (Tapia *et al.*, 2012). Radiation can be applied to mycelium or spores, and the dose conditions (intensity, time) need to be optimized (Jafari *et al.*, 2017).

UV mutagenesis can be performed alone, although it is often combined with other physical and/or chemical agents to widen the mutation type range and improve the efficiency (Bai *et al.*, 2004). Other physical mutagenic agents that have been used in strain improvement of filamentous fungi are X-rays, γ-rays, heavy ion beams, and low energy ion implantation. They have been developed in specialized facilities and allow a high mutation rate and wide range of mutation types compared to UV, although they are not discussed here in detail (Jin *et al.*, 2017; Awan *et al.*, 2011; Hu *et al.*, 2014; Zhao *et al.*, 2012).

One of the most widely used strains of filamentous fungi for cellulase production, *T. reesei* RUT-C30, was obtained by three rounds of UV and chemical mutagenesis with a very thorough screening process focused on high production of cellulases and catabolite de-repression. This mutant produced approximately 20 mg of extracellular protein per ml, which represents a 15- to 20-fold increase in secreted protein compared to the original strain when grown in flasks (Peterson and Nevalainen, 2012). Mutagenesis by UV irradiation also resulted in a mutant with higher cellulase activity (71 units/ml) compared to the wild type strain (53.7 units/ml) in *A. niger* (Shafique *et al.*, 2009).

A classic example of UV mutagenesis application in *A. niger* is the isolation of protease deficient mutants that are valuable in protein production applications as they show a reduced level of extracellular protein degradation (Mattern *et al.*, 1992). In the case of metabolite production, a L(+)-lactic acid over-producing *Rhizopus oryzae* mutant was isolated by combination of UV, diethyl sulfate (DES) and ^{60}Co mutagenesis that produced 79.4 g/l L(+)-lactic acid, 52% more than the parent strain (Bai *et al.*, 2004).

Chemical mutagenesis

Intercalating molecules such as ethidium bromide interact with the DNA by inserting themselves between the base pairs and stretching the DNA strand. This can cause the DNA polymerase to insert an extra nucleotide in the opposite strand, resulting in a frameshift mutation when the modification is replicated (Ferguson and Denny, 2007).

Other common DNA reacting molecules that have been used as mutagenic agents for strain engineering are alkylating agents, such as N-methyl-N′-nitro-N-nitrosoguanidine (NTG), ethylmethane sulfonate (EMS), and 1-methyl-3-nitro-1-nitrosoguanidine (MNNG). These mutagens add an alkyl group to nucleotides which may result in a transition mutation where one pyrimidine of purine base is substituted by the other (Leitao, 2012). For instance, EMS was used for the improvement of an *Ashbya gossypii* strain for heterologous production of *T. reesei* endoglucanase I (EGI), and other cellulase-active native enzymes, achieving 2- to 3-fold improvements in the measured activities (Ribeiro *et al.*, 2013).

Combined use of mutagenesis approaches

As mentioned above, chemical agents are often used in combination with physical agents, such as UV irradiation, or other chemical agents for strain improvement. Mutagenesis by UV irradiation and NTG has been widely used due to its simplicity. The examples range from improving wild species for pectinase production in food industry (Huang *et al.*, 2019) to cellulases that are used in biomass treatment by various industries (Xu *et al.*, 2011). As an example, the combined use of UV radiation with NTG or ethidium bromide in *A. oryzae* achieved a mutant with 4-fold higher cellulase activity than the wild type strain (El-Ghonemy *et al.*, 2014).

Techniques with a high mutation rate like γ-rays can be used not only for improving the production of an enzyme of interest, but also to screen for an improved characteristic on this enzyme. For example, to improve thermal stability of α-amylases for maltose sirup production in *A. oryzae* (Aleem *et al.*, 2018). In this study, a 5-step screening process was needed to find the mutants that produced the thermoresistant enzyme in high amounts (Aleem *et al.*, 2018). A combined use of Co^{60} γ-rays, UV irradiation, and NTG has also been applied in other *Aspergillus* sp. (Vu *et al.*, 2011).

Combined mutagenesis has also been applied in pharmacology. As an example, *Aspergillus fumigatus* and *Alternaria tenuissima* that naturally produce the anticancer drug Paclitaxel were subjected to UV and γ-irradiation mutagenesis in order to improve their producing ability. In this study, two stable strains were identified, with Paclitaxel titers that were 1.22 and 1.24-fold higher compared to the parental strains (El-Sayed *et al.*, 2019). In the fungus *F. oxysporum*, two rounds of nitrosoguanidine mutagenesis together with optimization of the medium conditions helped to increase the antibiotic enniatin production 3-fold with respect to previous studies with submerged cultures (Madry *et al.*, 1983). Random mutagenesis is useful not only for improving the yield of a product, but also for improving the use of a specific substrate. In this context, the production of citric acid, an important biotechnological product for food and other industries, using black molasses as substrate was improved by UV followed by NTG mutagenesis from 31.1 g/l in the parental strain to 50.0 and 86.1 g/l in the most productive mutants (Ikram-Ul-Haq *et al.*, 2001).

Adaptive evolution

The main disadvantage of chemical and UV mutagenesis is the occurrence of random non-desired mutations that could have a negative effect on the performance of the strain in process conditions. In recent years an alternative, non-GMO, methodology has been developed, called adaptive evolution. This method is based on the principle that random mutations occur continuously in fungal genomes and that only those that are advantageous will be able to persist in a population. In fact, if the advantage of the mutation is significant, only individuals that contain this mutation will ultimately survive in a fungal culture. Therefore, a crucial step of this approach is the design of conditions under which strains with the desired mutation(s) have a competitive advantage to those lacking them, enabling them to outcompete the non-mutated strains.

Adaptive evolution was initially applied in the yeast *S. cerevisiae* to improve co-fermentation of glucose and xylose to ethanol (Sonderegger and Sauer, 2003). In filamentous fungi only few examples of adaptive evolution have been reported. Improvement of growth at 37°C was achieved through adaptive evolution in *Metarzhizium anisopliae* (de Crecy *et al.*, 2009). In *A. niger*, selection for improved growth on cellulose resulted in a strain that produced higher levels of enzymes degrading cellulose (Patyshakuliyeva *et al.*, 2016). Transcriptome analysis demonstrated that this was due to the loss of expression of a gene that was shown to encode a repressor of cellulolytic genes in *Podospora anserina* (Brun *et al.*, 2009). In the same species, growth on increasing levels of ferulic acid resulted in a strain with a higher tolerance to aromatic compounds, which was shown to be due to an increase in aromatic metabolism (Lubbers *et al.*, 2020). In *A. oryzae*, repeated inoculation on inulin containing plates resulted in a strain with increased exo-inulinase activity, but also increased activities of other plant biomass degrading enzymes (Culleton *et al.*, 2016). The molecular basis of this mutant has not yet been identified.

Despite the limited examples, the success of this method implies that it is suitable for a much broader set of applications. As only mutations that provide a benefit to the desired phenotype are enriched, this method results in less non-desired mutations than UV or chemical mutagenesis.

Novel Strain Engineering Approaches

The discovery of transformation procedures in the 1980s, the development of molecular tools for the modification of DNA in fungal genomes (e.g., homologous recombination), the current availability of a large number of fungal genomic sequences, and the development of novel genome editing methodologies have strongly expanded the toolkit for fungal strain engineering, enabling the generation of fungal strains with commercially valuable properties with high level of control of the strain modifications. In recent years, highly precise genome editing systems have revolutionized fungal biotechnology and broadened the range of modifications that can be performed at a target genetic locus, thus resulting in an endless number of possibilities of genomic modifications for strain improvement.

CRISPR/Cas Technology

The type II Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ CRISPR-associated protein (Cas) system originated from archaeal and bacterial adaptive immune systems (Jinek *et al.*, 2012), and has revolutionized high-throughput functional genomics. The CRISPR/Cas9 genome editing system contains two main components: the Cas9 endonuclease, and an easily customizable single guide RNA (sgRNA) formed by the fusion of the CRISPR RNA (crRNA) and trans-activating RNA (tracrRNA) (Brouns *et al.*, 2008; Deltcheva *et al.*, 2011). In order to induce genomic modifications, 20 nucleotides of the sgRNA target the genomic DNA via homology base pairing. Subsequently, Cas9 endonuclease binds to the target DNA through the interaction with the Protospacer Adjacent Motif (PAM) located immediately downstream of the target 20-bp genomic sequence. After binding, Cas9 will induce a double strand break (DSB) three nucleotides upstream of the PAM (Garneau *et al.*, 2010), which is detected as potentially lethal damage and needs to be repaired to ensure the survival of the organism (**Fig. 7**). In eukaryotic systems like filamentous fungi, there are two natural DNA repair mechanisms that allow DSB repair: the error-prone non-homologous end-joining (NHEJ) and high-fidelity homology-directed repair (HDR) pathways. With NHEJ, DNA breaks are directly re-ligated by the Ku70 and Ku80 heterodimeric complex, frequently leading to imprecise DNA repair and loss of genetic functionality (Davis and Chen, 2013). In contrast, HDR only occurs in the presence of a homologous duplex DNA template (referred as rescue template, RT) via homologous recombination (Liu *et al.*, 2018; Wright *et al.*, 2018), which can be used to precisely introduce the DNA sequence of interest in the target organism (**Fig. 7**).

Streptococcus pyogenes SpCas9 protein is the preferred Cas nuclease to date due to the abundance of its target PAM sequence (5′-NGG-3′) within genomes (Jinek *et al.*, 2012), which opens the door to an endless number of possibilities for genome editing, but also increases the probability of unintended mutations. Alternative CRISPR/Cas systems have been developed in order to overcome this limitation, including highly specific engineered Cas9 variants (e.g., eSp-Cas9, SpCas9-HF, or PAM-altered SpCas9), Cas9 homologs derived from other bacteria with different PAM specificities and sgRNA structures, and novel Cas proteins different from Cas9 (e.g., Cpf1, C2c2) (Nakade *et al.*, 2017).

In some cases, the introduction of a single genomic modification is not enough to improve a strain for industrial purposes, and editing of multiple traits is required to fine-tune metabolic networks of potential fungal cell factories. One possibility of introducing multiple genomic modifications with CRISPR/Cas is to perform iterative rounds of genome editing by recycling or using different selection markers. However, the multiplexing capabilities of this system for the introduction of multiple traits at the same time are currently being exploited with both Cas9 and more recently Cpf1 (also known as Cas12a), whose crRNA structure and processing activity increases the simplicity of multiplexing compared to Cas9 crRNA (Adiego-Pérez *et al.*, 2019).

The first application of the CRISPR/Cas9 technology in filamentous fungi was in the industrially relevant *T. reesei* (Liu *et al.*, 2015) and in six different *Aspergillus* species (Nødvig *et al.*, 2015). Nowadays, the CRISPR/Cas9 system enables the genetic improvement of a wide variety of filamentous fungal species, including *P. oryzae* (Arazoe *et al.*, 2015a), *N. crassa* (Matsu-Ura *et al.*, 2015), *A. oryzae* (Katayama *et al.*, 2016), *A. fumigatus* (Zhang *et al.*, 2016), *Penicillium chrysogenum* (Pohl *et al.*, 2016), *Alternaria alternata* (Wenderoth *et al.*, 2017), *Beauveria bassiana* (Chen *et al.*, 2017), *F. oxysporum* (Wang *et al.*, 2018b), *Fusarium solani*

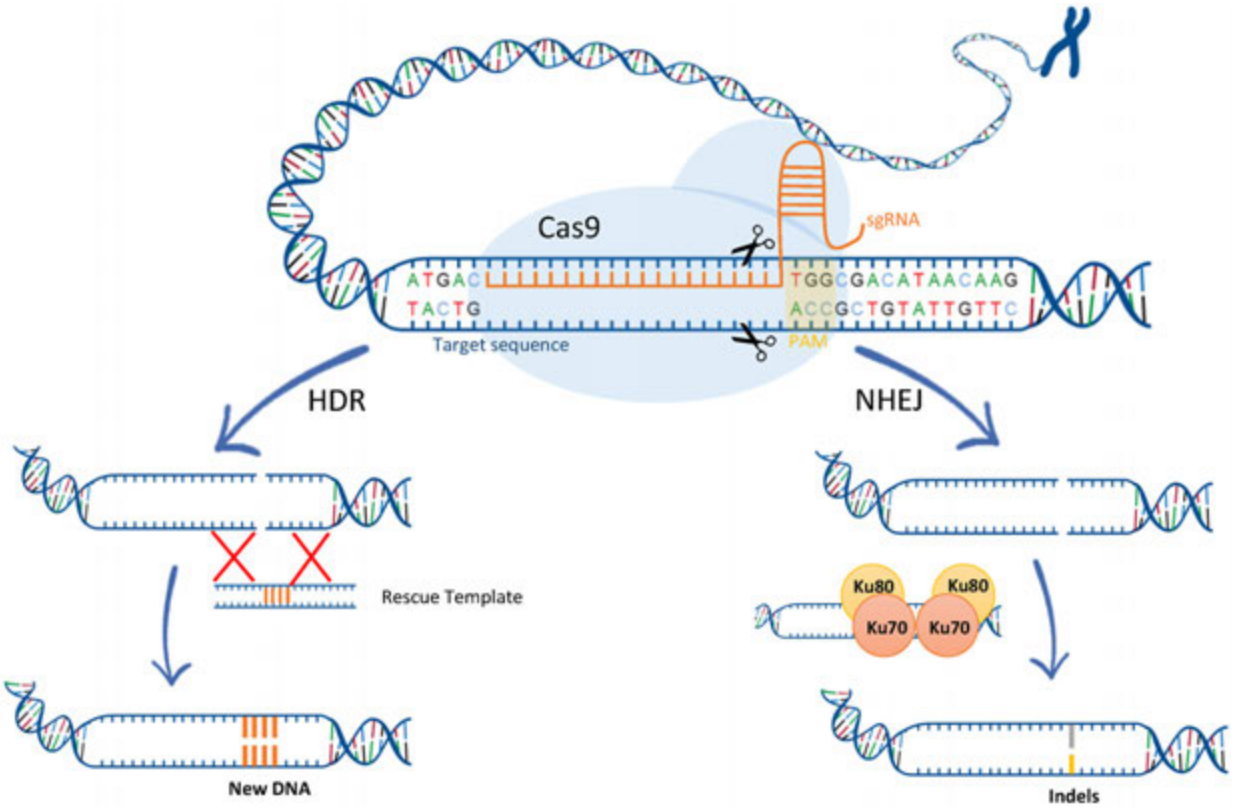

Fig. 7 Schematic representation of the CRISPR/Cas9 system. Double strand breaks (DSBs) induced by Cas9 can be repaired by two pathways. In the error-prone Non-Homologous End Joining NHEJ pathway, DSB are processed by endogenous DNA repair machinery, which may result in random mutations. Alternatively, a rescue template can be supplied to trigger the Homologous Directed Repair (HDR) pathway, which allows precise gene editing.

(Lightfoot and Fuller, 2019), *Fusarium fujikuroi* (Shi *et al.*, 2019) and *Penicillium subrubescens* (Salazar-Cerezo *et al.*, 2020) with different mutation efficiencies. Alternatively, CRISPR-Cpf1 has also been applied in some fungal species with high efficiency rates, such as *S. cerevisiae* (Li *et al.*, 2018) and the filamentous fungi *A. nidulans, A. niger* (Vanegas *et al.*, 2019), *Thermothelomyces thermophilus* (Kwon *et al.*, 2019) and *A. gossypii* (Jiménez *et al.*, 2019).

Other Novel Strain Engineering Approaches

Although CRISPR/Cas is becoming one of the most applied genome editing technologies for strain improvement, there are other valuable genome editing strategies that expand the toolbox for genetic engineering of filamentous fungi.

Zinc fingers (ZFs) are small proteins that have the ability to bind DNA through specific alpha helical protein motifs and have been adopted as gene-targeting tools. Naturally, each alpha helix of a ZF shows a unique amino acid sequence that interacts specifically with its corresponding DNA sequence. For strain engineering, ZFs are used as substrate for binding DNA, and are coupled with a restriction endonuclease (Zinc-finger nuclease, ZFN) to induce DSBs in the target DNA sequence (Carroll, 2011; Palpant and Dudzinski, 2013).

Transcription activator-like effector (TALE) nucleases (TALENs) also allow site-specific DNA DSBs, making them a valuable tool for strain improvement in many organisms, including filamentous fungi. TALE binding to DNA is mediated by a central region of these proteins that contains around 30 tandem repeats of 33–35 amino acids (Christian *et al.*, 2010). The amino acid sequence of each tandem repeat is invariant, except for the two adjacent amino acids (referred as repeat variable residue, RVD). Repeats with different RVDs recognize distinct DNA base pairs, allowing the development of this technology for directed mutagenesis when fusing customized TALE to endonucleases (e.g., Fok-I) that cut the target DNA, triggering the DSBs DNA repair pathways (Jiang *et al.*, 2013). TALENs has been successfully applied in several filamentous fungi such as *P. oryzae* (Arazoe *et al.*, 2015b), *A. oryzae* (Mizutani *et al.*, 2017), and *T. reesei* (Liu *et al.*, 2017b).

Even though these novel genome editing techniques allow the generation of specific genomic alterations more precisely than random mutagenesis techniques, in July 2018, the European Court of Justice ruled that (micro)organisms generated by directed mutagenesis techniques, such as CRISPR/Cas, ZFNs or TALENs require the same treatment as any other GMO in the European Union (Directive 2001/18/EC). In this context, meeting the obligations of the GMO Directive implies cost- and labor-intensive pre-market evaluations and long approval processes, which may decrease investments, and limit research and commercialization.

Applications of Strain Improvement

Filamentous fungi play a very important role in the production of biotechnologically interesting compounds, such as organic acids (Dörsam *et al.*, 2017), enzymes degrading complex plant biomass (de Vries, 2003), and drugs such as penicillin and other antibiotics (Brun *et al.*, 2009). Very often, wild-type strains do not produce these compounds at levels required in industry, or do not produce the desired proteins with the required properties or catalytic specificity. To overcome these problems, different genetic engineering approaches have been developed in order to improve the industrial potential of filamentous fungi for industrial applications.

Gene Overexpression

Gene overexpression is a procedure which leads to increased target gene expression for different purposes. In strain engineering of filamentous fungi, this approach is very useful for increasing the production of a protein or enzyme of interest. Overexpression of specific enzymes can increase the production of a metabolite of interest, or the conversion of a substrate. Moreover, the production of metabolites or proteins can be achieved indirectly by the overexpression of the regulators that control the expression of the involved genes or pathways. In the following sections, different gene overexpression strategies are described.

Replacing the promoter

Promoters drive the expression levels of genes and determine how this expression is affected by the activators and repressors of the regulatory system. There are constitutive promoters that are active independently of the environmental or internal signals, which typically drive the expression of housekeeping genes. Conversely, there are promoters that are controlled by transcription factors based on the environmental signals, for example, catabolic enzymes (Vogl and Glieder, 2013). To improve the production of a specific protein, the promoter of the corresponding gene can be switched for one with stronger expression, and, to improve the expression of a set of related genes of interest (e.g., biosynthetic or catabolic cluster), it may be possible to overexpress the transcription factor(s) controlling their expression (Gao *et al.*, 2017). Overexpression of genes can lead to overburdening of the cellular mechanisms or accumulation of toxic metabolites, which can be detrimental to the cell. As a solution to this, inducible promoters can be interesting tools to control gene expression in time. In the case of intermediate accumulation, this may happen because the enzymes in the pathway are expressed in different ratios than in the natural stoichiometry, and therefore some metabolites can accumulate due to limited enzymatic activity in the next step. This can be avoided by balancing the expression of the enzymes with specific promoters to mimic a more natural stoichiometry (Vogl *et al.*, 2018).

Regarding specific examples, the cellobiohydrolase I (*cbh1*) gene promoter, which is induced in the presence of cellulose, is often used in *T. reesei* and other fungi specially for heterologous protein production (Nevalainen and Peterson, 2014). Moreover, in *T. reesei*, this promoter has been optimized for improving the efficiency by replacing the negative regulator binding sites for positive regulator binding sites, demonstrating improved expression levels of heterologous genes (Liu *et al.*, 2008; Zou *et al.*, 2012).

In *Aspergillus* sp. there are several well-characterized promoters for high gene expression that can be used in the original organism or in different fungi. The most commonly used ones are the glucoamylase promoter (P*glaA*) from *A. niger*, the gycer-aldehyde-3-phosphate dehydrogenase promoter (P*gpdA*) from *A. nidulans* and the Taka-amylase A promoter (P*amyB*) from *A. oryzae* (Xie *et al.*, 2020). For instance, the P*adhA* promoter from *A. nidulans* was introduced in *Aspergillus terreus* to overexpress acetyl-CoA carboxylase in order to increase the production of the cholesterol lowering agent lovastatin (Hasan *et al.*, 2018). Some efforts have been made for the production of the organic acid L-malate directly from corn starch in order to avoid the saccharification step. To engineer this metabolic pathway in *A. oryzae*, the P*glaA* promoter and an engineered version of this one (PN5) were used to simultaneously overexpress three key enzymes, improving the L-malate titer from 55.5 to 78.5 g/l (Liu *et al.*, 2017a).

As an alternative to the use of known heterologous promoters, novel strong promoters of a specific strain can be detected by transcriptomic analysis, where the highly expressed genes may provide these kind promoters (Hu *et al.*, 2015; Wang *et al.*, 2018a).

Histone-encoding genes are highly expressed for the synthesis of DNA during the cell cycle. The activation of these promoters depends on the cell cycle stage, and the asynchronic growth of protein producing cultures may affect the promoter efficiency. At any specific time point, different cells are in different stages of their cell cycle, and therefore, while many of them will be highly expressing the gene, some may not, but the overall result is a constitutive and high expression of homologous and heterologous genes (Belshaw *et al.*, 2002). *Mortierella alpina* is an oil producing filamentous fungus of interest for nutrition and pharmacology industries as its biomass can consist of up to 50% triacylglycerol. The malic enzyme catalyzes the oxidative decarboxylation of L-malate to pyruvate and has been suggested to be important during fatty acid synthesis in oleaginous fungi as a NADPH-supplying enzyme. By introducing a construct of the malic enzyme encoding gene driven by the homologous histone H4.1 promoter, the content of arachidonic acid was increased 60%, without altering the total fatty acid content (Hao *et al.*, 2014).

Homologous recombination and gene editing tools can be applied to replace the endogenous promoter of the gene of interest by a stronger one, avoiding problems of random and multi-copy insertion of the construct. By using the CRISPR/Cas9 system in *N. crassa* it was possible to switch the endogenous cellulase regulator *clr-2* promoter for the *β-tubulin* constitutive promoter, achieving a higher cellulase production (Matsu-Ura *et al.*, 2015). These tools allow not only the replacement of the promoter but also the insertion of directed point mutations, where the characteristic modifications of hyper-producing mutants can be emulated. The implementation of this strategy in *A. niger* demonstrated that the mutation W361R in the transcription factor GaaR, found in a UV mutant, was responsible for constitutive pectinases production (Alazi *et al.*, 2018b).

As mentioned before, there are several examples of increasing the production of a set of proteins by overexpression of their regulator. The xylanolytic regulator Xyr1 of *T. reesei* induces the production of different cellulolytic and hemicellulolytic enzymes. Expression of the *xyr1* gene is induced in the presence of lactose and cellulose and controlled by carbon catabolite repression (Benocci *et al.*, 2017). By overexpressing this gene by switching the native promoter with a different promoter, either a constitutive (Wang *et al.*, 2013) or an inducible one (Lv *et al.*, 2015), cellulolytic enzyme production can be significantly increased independently of the induction conditions. Another example of overexpression of a regulator is the transcription factor encoding gene *gaaR* in *A. niger* that resulted in increased production of pectinases independently of the presence of the inducer D-galacturonic acid (Alazi *et al.*, 2018a).

An alternative kind of promoters are bidirectional promoters, which allow the expression of two genes at the same time, for instance, the gene of interest and the selection marker, two copies of the gene of interest, or different genes. Recently, natural bidirectional promoters have been found in filamentous fungi for various applications. The histone H4.1 and H3 promoters can act as natural bidirectional promoters that allow simultaneous expression of enzymes in the case of metabolic engineering in *Aspergillus* sp. (Rendsvig *et al.*, 2019). Interestingly, some inducible promoters such as *A. niger* D-galacturonic acid reductase promoter, show a bidirectional transcription, and can be used as bidirectional and inducible promoters (Kuivanen *et al.*, 2015). In this study, enzymes from the plant L-ascorbic acid pathway were introduced in *A. niger* for improving plant biomass D-galacturonic acid conversion to L-ascorbic acid, achieving titers up to 170 g/l.

Increasing the gene copy number

Increasing the copy number of a gene can increase significantly protein production, and this technique has been commonly used in fungal strain improvement (Archer *et al.*, 1994). This strategy has been used for example in the production of penicillin by *P. chrysogenum*, by inserting multiple copies of the penicillin biosynthesis cluster in the genome (van den Berg, 2010).

A high copy number can be achieved by (1) adding high amounts of DNA during transformation, using multiple constructs with different selection markers, (2) applying a strong antibiotic pressure, either by adding a high concentration or using a weak promoter for the selection marker, or (3) using bidirectional promoters, which would allow the transcription of two copies of the gene in the same construct. Increasing the number of copies can compromise the stability of the strain, which is especially undesired in industry where prolonged cultivation of strains is needed (Deckers *et al.*, 2020).

In some studies, depletion of transcription factors due to multicopy insertion of the gene of interest under the control of the same promoter has been detected. To avoid this, the possibility of inserting the gene under the control of different promoters that are induced under the same conditions has been explored (Miyauchi *et al.*, 2013).

Gene Downregulation

Gene downregulation can be defined as a genetic engineering approach in which one target gene of interest is made inoperative in an organism. Gene downregulation is a key approach to study gene function and to alter the characteristics of a fungal strain for different applications, such as improving enzyme production by the inactivation of repressor-encoding genes (Ichinose *et al.*, 2018), the inactivation of genes that results in increased gene recombination frequencies for subsequent transformations, or for the reduction of byproduct formation (Meyer, 2008). For this aims, genes can be either (partially) deleted, mutated, or silenced in filamentous fungi (Khan *et al.*, 2014).

Gene deletion

Gene deletion or gene knockout (represented with Δ) is a type of mutation that involves the total inactivation of a gene of interest through the loss of genetic material, and has been widely used to attribute physiological functions to a gene of interest by phenotype correlation after (partial) gene elimination.

Gene deletions can be applied for strain improvement in filamentous fungi. One of the main drawbacks for strain improvement of filamentous fungi is the low frequency of gene integrations into the genome, since DNA integration is mainly directed by NHEJ (Kuck and Hoff, 2010). To solve this problem, defective strains in NHEJ with improved site-specific recombination have been constructed in many fungal species by deletion of the Ku70 or Ku80-encoding genes, as for example *Neurospora* (Ninomiya *et al.*, 2004), *A. nidulans* (Szewczyk *et al.*, 2006), *A. oryzae* and *Aspergillus sojae* (Takahashi *et al.*, 2006), *Claviceps purpurea* (Haarmann *et al.*, 2008), *M. oryzae* (Villalba *et al.*, 2008), *Penicillium decumbens* (Li *et al.*, 2010), and *P. digitatum* (Gandía *et al.*, 2016).

Gene deletion approaches have also allowed the construction of gene knockout libraries in fungal species like *Aspergillus* and *Neurospora*, resulting in the identification of genes encoding novel transcription factors which are involved in the production of industrially relevant enzymes such as cellulases (Colot *et al.*, 2006; Ogawa *et al.*, 2013). Deletion of several transcription factor-encoding genes in some filamentous fungi can also result in an increased production of specific enzymes. For example, double deletion of the negative regulators CreA and CreB-encoding genes *creA* and *creB* in *A. oryzae* resulted in an increase of plant biomass degrading enzyme production such as α-amylases, xylanases and β-glucosidases (Ichinose *et al.*, 2018). In *T. reesei*, deletion of the cellulase and xylanase negative regulator encoding gene *ace1* increased gene expression in the presence of cellulose or the inducer sophorose (Aro *et al.*, 2003). In *P. anserina* and *A. niger*, the regulator NoxR was shown to negatively affect cellulase production, since Δ*noxR* mutants were able to degrade cellulose more efficiently than the parental strain (Brun *et al.*, 2009; Patyshakuliyeva *et al.*, 2016).

Gene knockout strategies have also enabled improved production of interesting metabolites and inhibition of toxic metabolites in filamentous fungi by genetic engineering of metabolic genes. For instance, deletion of L-galactonic acid dehydratase-encoding genes *gaaB* and *lgd1* in *A. niger* and *T. reesei*, respectively, increased extracellular accumulation of L-galactonic acid, with potential applications in the pharmaceutical, cosmetic, and many other industries (Kuivanen *et al.*, 2012). In *Myceliophthora thermophila*, multiple deletion of two fumarate reductase and a mitochondrial fumarase-encoding genes increased production of fumaric acid that is widely used in the food and pharmaceutical industries (Gu *et al.*, 2018). In *P. decumbens* and *Penicillium oxalicum*, deletion of *bgl2*, the major intracellular *β*-glucosidase encoding gene, resulted in significantly improved cellulase production (Chen *et al.*, 2013; Yao *et al.*, 2015). On the other hand, deletion of each of the fifteen genes involved in patulin biosynthetic pathway resulted in decreased (or abolished) ability of *P. expansum* to produce patulin, a mycotoxin that is often present as a contaminant in food, particularly in fruits and fruit-derived products (Li *et al.*, 2019).

Gene deletions can also be applied in many different plant and human pathogenic fungi in order to decrease their pathogenicity and/or virulence for different applications. For example, deletion of Mitogen-Activated Protein Kinases (MAPK)-encoding genes such as *slt2* or *kss1* in the phytopathogenic fungus *P. digitatum* resulted in strong reduction on virulence during infection on citrus fruits, which can be very interesting for the control of citrus postharvest diseases (Gandía *et al.*, 2019). Similarly, deletion of lipase-encoding genes *CpLIP1* and *CpLIP2* highly reduced virulence in the relevant human pathogen *Candida parapsilosis* (Gácser *et al.*, 2007), opening the door for the development of new antimycotic drugs.

Point mutations

Gene inactivation can also involve single missing DNA base pairs and single nucleotide changes. Point mutations can have a wide variety of consequences, ranging from no effect (synonymous or silent mutations) to amino acid changes (missense mutations or frameshifts) and the apparition of premature stop codons (nonsense mutations). In many cases, mutations can be harmful for the organisms or decrease their capability to be used as efficient cell factories. One example of a loss of gene functionality with negative consequences in filamentous fungi is the loss of sorbicillinoids production capability in *P. chrysogenum*, which are interesting secondary metabolites with antiviral, anti-inflammatory, and antimicrobial activities, due to a point mutation acquired in the *sorA* gene, which encodes for a polyketide synthase (Salo *et al.*, 2016). Another example is the non-citric acid production phenotype of a UV-derived *A. niger* mutant caused by a point mutation in *laeA* gene, encoding a putative methyltransferase-domain protein (Bok and Keller, 2004). However, certain mutations can make the organisms adapt better to the environment and improve their (biocatalytic) performance.

Point mutations for fungal strain improvement have been typically achieved by the application of physical and chemical mutagenesis (Parekh *et al.*, 2000). However, the new DNA-editing technologies such as CRISPR/Cas allow the precise (on-site) introduction of targeted point mutations in a more efficient and controlled way in filamentous fungi (Kun *et al.*, 2020). In the white-rot basidiomycete fungus *Pleurotus ostreatus*, a novel selection marker gene for transformation was developed by introducing a point mutation in a gene that encodes the iron-sulfur protein subunit of a succinate dehydrogenase (Honda *et al.*, 2000). Point mutations also resulted in improved cysteine biosynthesis in *Penicillium rubens* by the inactivation of enzymatic conversions that compete with the cysteine biosynthetic pathway, which plays a key role in penicillin production (Wu *et al.*, 2020). In addition, many *T. reesei* strains highly used at industrial level underwent point mutations leading to catabolite derepression, resulting in increased extracellular enzyme and protein levels compared to their wild type strain (Bischof *et al.*, 2016).

These examples demonstrate a wide range of possibilities of point mutations for gene inactivation and strain improvement in filamentous fungi. However, complete gene deletions by means of homologous recombination are still the preferred genetic engineering mechanisms to generate knockout strains throughout the scientific community.

RNA interference (RNAi)

The possibility to inactivate a given gene or metabolic pathway is not only restricted to DNA-based approaches. RNA interference (RNAi) is a mechanism for gene silencing/inactivation that represses gene expression by means of small non-coding RNAs (sRNAs) in eukaryotic organisms (Nicolás and Garre, 2017). In filamentous fungi, the gene silencing effects triggered by RNAi were firstly observed in *N. crassa*, a phenomenon that was initially called Quelling (Romano and Macino, 1992). The RNAi mechanism is highly conserved in the fungal kingdom, and is basically driven by two main components: a double-strand RNA endonuclease (Dicer), and a small RNA-binding protein (Argonaute). Dicer cleaves double strand RNAs (dsRNA) to generate sRNAs from 20 to 30 nucleotides that are subsequently carried by Argonaute and used to identify complementary sequences, triggering the degradation of the target sequences (Billmyre *et al.*, 2013).

RNAi is involved in a broad spectrum of biological functions, such as genome defense against virus or transposon invasion. However, this mechanism has been adapted as a potential biotechnological tool to improve fungal strains in different ways. For instance, RNAi was used to attenuate the expression of the *creA* gene in *P. chrysogenum*. Transformants expressing sRNA targeting *creA* showed increased penicillin production (Cepeda-García *et al.*, 2014). In *A. oryzae*, RNAi-based inactivation of three *α*-amylase-encoding genes improved heterologous protein production in the culture media (Nemoto *et al.*, 2009). Similarly, suppression of the expression of a cellobiohydrolase-encoding gene (*cbh1*) in *T. reesei* by RNAi increased gene expression of a heterologously expressed lipase-encoding gene from *A. niger* (Qin *et al.*, 2012).

Compared to conventional gene knockout strategies, such as gene deletion by homologous recombination, RNAi has potential advantages. One advantage is its applicability to inactivate gene expression regardless gene targeting efficiency, since the efficiency of homologous recombination varies among fungal species. In addition, RNAi is very convenient when multiple copies of a gene

of interest are present in the genome. Moreover, the function of essential genes in biological processes remains unknown since classical genetic approaches such as gene deletion are not available due to lethality. In this context, RNAi can be used for analyzes of lethal genes since it mostly induces knock-down of gene expression but not complete knockout (Nakayashiki, 2005). However, potential disadvantages of RNAi-based gene silencing are the incomplete reduction of the gene expression, and the instability of the transformants (Henry *et al.*, 2007).

Conclusions and Future Prospects

Genetic engineering of filamentous fungi has been an established approach in biotechnology, affecting processes in many industrial sectors. Non-GMO approaches have the widest applicability as they are not subject to GMO regulations that limit the use of GMO-based approaches in many applications. These regulations differ globally, with e.g., the USA allowing a broader application of GMO strains than the EU. The recent development of novel genome editing technologies, such as CRISPR/Cas9, that enable genetic engineering without the introduction of foreign DNA and without easy trackability has re-activated the discussion on GMO regulations and may open the door for a broader application of these technologies.

The global switch to a sustainable biobased economy provides many opportunities for fungi due to their high ability to convert plant biomass to a wide range of products. Fungal fermentations have the potential to replace many chemical processes that are based on fossil resources, but natural fungal isolates are unlikely to be efficient enough to result in economically sustainable processes. Genetic engineering will be needed to improve the productivity of fungal cell factories, reduce the production of side-products and increase the tolerance of the selected fungi to the process conditions. After the genomics era in fungal research, the coming decade(s) may well become the era of fungal cell factories.

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Strain Improvement and Genetic Engineering of *Trichoderma* for Industrial Applications

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Introduction

The filamentous fungi *Trichoderma* spp. (Hypocreales, Ascomycota) have been widely studied for their application in industry, agriculture, and other areas. For industrial applications, the well-known species *T. reesei* has been used almost exclusively for producing cellulolytic and hemicellulolytic enzymes and heterologous proteins. This is because enzymatic hydrolysis is considered a more commercially and environmentally beneficial procedure than chemical hydrolysis (Druzhinina and Kubicek, 2016; Payne *et al.*, 2015). In agriculture, highly mycoparasitic strains from several species such as *T. atroviride*, *T. virens*, *T. asperellum*, and *T. harzianum* have been used as bioeffectors in commercial formulations for the biological control of fungal pests (biocontrol). Some of these species can colonize root surfaces and elicit a plant defense response to different pathogenic fungi while simultaneously stimulating plant growth (Harman *et al.*, 2004; Druzhinina *et al.*, 2011). Moreover, *Trichoderma* spp. prolifically produce bioactive secondary metabolites; many of which have already been utilized as fungicides or plant growth-promoting agents, while others have the potential for application in the pharmaceutical industry (Contreras-Cornejo *et al.*, 2016; Keswani *et al.*, 2014; Zeilinger *et al.*, 2016).

Modifying particular genes in *Trichoderma* will enhance their abilities, such as those described above, for better application purposes. Several gene modification approaches have been used in filamentous fungi to obtain enzyme hyper-producers (Druzhinina and Kubicek, 2016; Mei *et al.*, 2019; Shi *et al.*, 2017). For instance, as reviewed by Druzhinina and Kubicek (2016), as well as others, almost all of the lignocellulolytic enzymes needed for the hydrolysis step can be produced by fermentation in *T. reesei*. However, the catalytic activity of some of the enzymes in the cellulase cocktail is still too low, and the composition of the secreted enzyme mixture may not be optimal due to some enzymes being present in limiting amounts. To overcome this bottleneck at the enzymatic hydrolysis step of lignocellulose, a significant amount of work has been directed towards understanding and improving the strain by improving the performance of the respective enzymes. These investigations have resulted in industry using *T. reesei* almost exclusively for cellulase production, despite the fact that other fungi also produce powerful cellulolytic mixtures (Druzhinina and Kubicek, 2016; Grujić *et al.*, 2019).

The genomic sequencing of many *Trichoderma* spp. has revealed that the number of secondary metabolite biosynthetic gene clusters (most of which are silent under laboratory conditions) far exceeds that of the identified compounds, suggesting great potential from a genetic perspective for drug or agent mining in these fungi (Kubicek *et al.*, 2019). Using genetic modification methods, many of the *Trichoderma* genes involved in interactions with other fungi have also been functionally characterized, and their activity can be further investigated for enhanced mycoparasitic abilities (Chenthamara and Druzhinina, 2016; Druzhinina *et al.*, 2011). Researches on these aspects in other filamentous fungi such as the genera *Aspergillus* and *Penicillium* (Eurotiales, Ascomycota) can be found in several reviews (Brakhage, 2013; Keller, 2019; Macheleidt *et al.*, 2016).

Improving the industrially or agriculturally relevant properties of *Trichoderma* spp. is closely related to the development of methods for genetic manipulation in these fungi. In this chapter, we will summarize and briefly describe the methods that have been developed for genetic modification of *Trichoderma* strains and illustrate some examples using these methods. We will then summarize some applications of the promising new technologies such as the CRISPR/Cas9 system, the currently most powerful gene-editing tool available, for further progress.

Untargeted Genetic Recombination by Classical Mutagenesis

The classical methods of mutagenesis, including forced random mutagenesis, sexual crossing, protoplast fusion, and parasexuality, have been developed to introduce mutations without previous knowledge of the gene or genome structure. These methods are applicable only when the desired genetic alteration has a readily identifiable phenotype.

Forced Random Mutagenesis Resulted in the Creation of the Industrial Hypercellulolytic Strains of *T. reesei*

The current most commonly used hypercellulolytic strain, *T. reesei* RUT C30, was obtained by multiple rounds of random mutagenesis of the parental wild-type strain QM 6a isolated from the Solomon Islands (see Peterson and Nevalainen, 2012 for the review), which massively increased its cellulase yields. The first attempt to obtain a high cellulase-producing strain by mutagenesis was conducted by Mandels *et al.* (1971), who introduced an irradiation method to mutate the wild-type *T. reesei* QM 6a strain. Conidia from QM 6a were suspended in distilled water and irradiated at 20°C with high energy electrons from a 24-million electron volt, 18-kw linear accelerator (**Fig. 1**). After applying the 0.05 megarad dose, a strain designated as QM 9123 was isolated

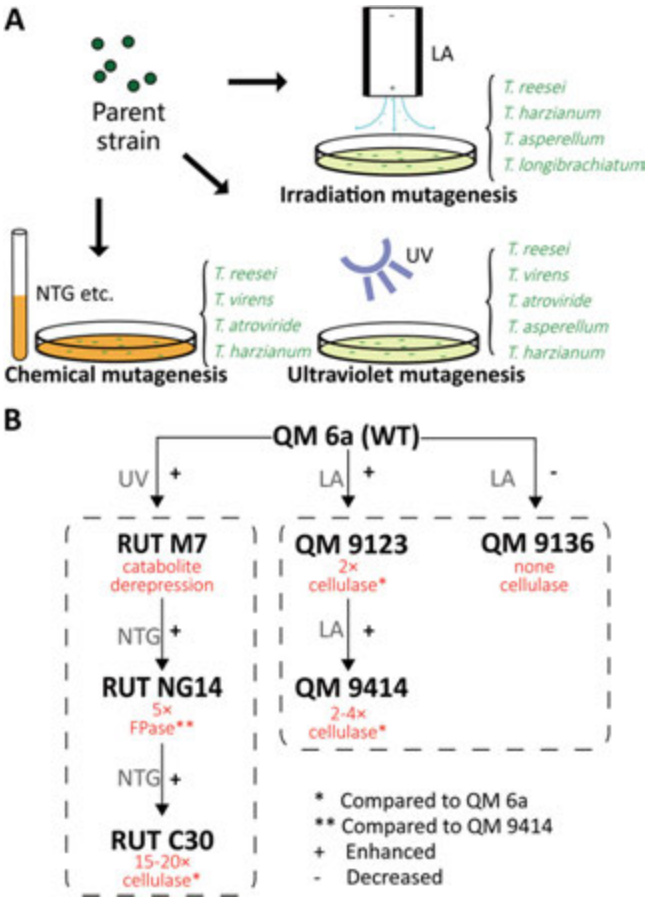

Fig. 1 The principles of three classical mutagenesis methods (A) and the *Trichoderma reesei* mutants derived via these methods (B). WT, wild-type *T. reesei* QM 6a; UV, ultraviolet; LA, linear accelerator; NTG, N-nitroguanidine.

because it produced twice as much cellulase as the parental strain. A second mutagenesis was performed using high-voltage electrons that led to the strain QM 9414. Compared to the wild type, this strain had a 2–4 fold increase in extracellular cellulase and protein production, but it was catabolite repressed (Montenecourt and Eveleigh, 1977b).

Another mutagenesis was performed on QM 6a via a three-step mutagenesis procedure that involved both ultraviolet (UV) irradiation and chemical mutagenesis. For the first step, QM 6a spores were exposed to UV irradiation under conditions to produce a 0.1% survival rate and plated on a catabolite-repression screening medium, which led to the isolation of strain M7. Chemical mutagenesis of this strain was then performed using N-nitroguanidine, leading to the partially carbon catabolite-derepressed strain NG14. This strain produced more extracellular protein with filter-paper activity than QM 9414 and had 2–5 times higher β-glucosidase activity. Another round of UV mutagenesis (as described above) was performed, which finally led to the hyper-cellulolytic (similar production and activity levels as NG14 in shaking flasks) and catabolite-derepressed strain RUT C30 (Montenecourt and Eveleigh,1977a,b, 1979). This strain is still one of the strongest cellulase-producing *T. reesei* strains available in the public domain. A genome-wide analysis revealed that the strain is missing an 85 kb genomic fragment that contains 29 genes, including primary metabolism enzymes, transport proteins, and transcription factors. Interestingly, this loss was already present in the strain NG14 (**Fig. 1**) and is not associated with catabolite-repression genes such as the *cre1* locus (Seidl *et al.*, 2008). However, the mutation of the *cre1* locus was shown to be specific to RUT C30.

In the last decade, to eliminate the glucose repression related to cellulase and hemicellulase gene expression, Ike *et al.* (2010) screened two *T.* cf. *reesei* ATCC 66589[1] (The accurate and precise molecular identification of this strain to the species level is not possible (Cai and Druzhinina, 2020). Based on the ITS1 and 2 (NCBI accession KU729058) and 28 S (NCBI accession KU729141) rRNA DNA barcode sequences, the strain can be attributed to a group of closely related species that includes *T. reesei*, *T. parareesei*, *T. gracile*, and *T. beinartii* (as for October 2020) (see "Relevant Website section"); the same hereafter) mutants that were able to produce cellulases when grown on a glucose-containing basal medium (Kawamori *et al.*, 1986). Sreenivasulu *et al.* (2014) improved the antagonism and carbendazim tolerance of the *T. reesei* strains TCT4 and TCT10 through ethyl methane sulfonate (EMS)-mediated mutagenesis.

Random mutagenesis has also been applied in other *Trichoderma* species. Abbasi *et al.* (2016) enhanced the antagonistic ability of *T.* cf. *harzianum* 65[1] against several plant-pathogenic fungi by mutating the wild type with a Gammacell irradiator at a dose of

250 Gray · s^{-1}. Wang *et al.* (2020a) further modified the mutant *T.* cf. *harzianum* EU2–77 (a mutant derived from *T. cf. harzianum* NP13a[1]) by UV irradiation, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, and EMS treatments, leading to a mutant (EUA20) with enhanced cellulase and reduced sugar production.

A schematic diagram of the random mutagenesis process using UV, irradiation and chemical mutagens is shown in **Fig. 1**. The best mutagens are those that can induce high mutation rates with little lethality. However, searching for the desired strains is laborious, unless a means of selecting the desired mutants is available. Choosing suitable mutagens and optimizing doses for the target strains can increase the mutagenesis efficiency.

In Vitro Sexual Crossing, the Dedicated Gift for *T. reesei*

Sexual reproduction is essential for the long-term population persistence of most eukaryotes, though sometimes asexual reproduction is a more efficient mechanism for dispersal. Sexual crossing, the crossing of the genetic information from two different parents, can make some progeny possess unique beneficial characteristics. However, this method can only be used to improve the strains of species with a sexual cycle (Seidl and Seiboth, 2010) completed *in vitro*. For a long time, this method was not available for any *Trichoderma* species. In 1996, Kuhls *et al.* (1996) found that the cellulase producing *T. reesei* was an asexual clonal line derived from a population of the tropical and sexually reproducing *T. reesei* (at that time named for its teleomorph *Hypocrea jecorina*, a name now abandoned). However, attempts to cross *T. reesei* QM 6a with sexually compatible strains isolated from the teleomorph repeatedly failed. Researches with the QM 6a strain discovered that it had a MAT1–2 mating-type locus, while a MAT1–1 mating-type locus was found in an isolate called CBS 999.97, suggesting these strains belong to a heterothallic species (Martinez *et al.*, 2008). The strain CBS 999.97 can be crossed with QM 6a and its mutants (Tisch *et al.*, 2017), but further analysis has shown that QM 6a and its derived strains can only act as the male mating partner. When the MAT1–2 locus in the QM 6a was replaced with the MAT1–1 locus, no fruiting bodies were observed during crosses between the transgenic strain and QM 6a, suggesting QM 6a was female sterile. However, after the mating-type locus was artificially inverted in the MAT1–1 isolates, the strains could be sexually crossed. A genomic analysis by Linke *et al.* (2015) revealed that two genes, encoding an unknown C2H2/ankyrin protein and the WD-protein HAM5, were essential for fruiting body formation when QM 6a was crossed with CBS 999.97. Importantly, the sexual cycle provides an invaluable tool for classical genetic analyzes and forms a basis that greatly facilitates genetic work and industrial strain improvement with this fungus. Unfortunately, thus far this is only possible with *T. reesei*, as no other *Trichoderma* species have been found that could mate under laboratory conditions (Seidl *et al.*, 2009; Chenthamara *et al.*, 2021).

Protoplast Fusion and Parasexuality, the Cloud of *Trichoderma* Strain Improvement

Fungal protoplasts, cells not protected by a cell wall, are formed by artificially removing the cell wall with lysing enzymes, and protoplast fusion is a recombination technique used to transfer cytosolic organelles from one protoplast to another (Muralidhar and Panda, 2000). The process generally consists of three steps: cell wall breakdown, protoplast fusion, and protoplast regeneration. The fungal cell wall is made up of chitin, glucans, mannans, and glycoproteins (Bowman and Free, 2006), and its breakdown requires activity of the relevant enzymes. Protoplast regeneration is then required to obtain viable progeny. The vital part of this process is protoplast fusion, which can be mediated by different agents such as a virus (Okada, 1988), a chemical or electric shock (Wang *et al.*, 2008). Protoplast fusion can be an appropriate tool for improving some fungal strains (Dillon *et al.*, 2008). To obtain fusants with desirable characteristics, the ideal approach should include intra-species, inter-species, and inter-genera fusions.

The first *Trichoderma* protoplast fusion was performed by Toyama *et al.* (1984) using immature conidia of the *T. reesei* mutant QM 9414 (they later also used the "smaller nuclei"; Toyama and Toyama, 1995). In that process, protoplasts derived from the two strains were mixed at an equal ratio, polyethylene glycol (PEG) 6000 and $CaCl_2$ were added to mediate the protoplast fusion process, and the protoplast suspension was plated on a regeneration medium to yield the fusion products. The conidia generated on the fusant colonies showed diverse phenotypes: DNA content was 1.2–2.0 times higher than that of the parents, colonies developed from a single conidia had a higher mycelia density, and their traits were stably inherited. In another experiment, Ogawa *et al.* (1987) used differentially pigmented mutants to conduct the protoplast fusion. The conidia (white and brown) of two mutants originating from *T. reesei* QM 9414 (green conidia) were used to generate the protoplasts, which were then treated with ethylene glycol and effectively fused. The heterokaryons had improved cellulase, xylanase, and *β*-glucosidase activities compared to the parental strain (QM 9414). Additionally, the use of pigmented mutants may also avoid a dependence on auxotrophic mutants, which can have decreased protein production compared to the parental strain.

Parasexual recombination, discovered by Pontecorvo and Roper (1952) in *Aspergillus nidulans*, is recombination outside of the sexual cycle in which neither meiosis nor fertilization occurs. Although the steps of the parasexual cycle are fundamentally different from those in the sexual cycle, they ultimately result in new genotypes. Classical genetic analyzes of asexual species depend on the parasexual cycle (Clutterbuck, 1996). Parasexual recombination related to reproduction can result in the recombination of genes from different individuals, but it does not involve the formation of a zygote by fertilization as in sexual reproduction. Parasexual crosses between strains with different beneficial mutations for the production of industrially valuable materials can result in the isolation of haploid progeny with the beneficial mutations from both parents combined through

chromosome reassortment (Bawa and Sandhu, 1994; Bodie *et al.*, 1994). Techniques for heterokaryosis and diploid formation have also been used to obtain higher yields of industrial metabolic products in many fungal strains. In addition, the use of markers such as different conidia colors allows one to follow the haploidization, diploid heterozygosis, and heterokaryosis processes during the cycle (Pontecorvo, 1956).

As previously mentioned, in *T. reesei* QM 9414, the heterokaryons from two differently colored mutants generated green conidia, suggesting that a parasexual cycle existed in the strain (Ogawa *et al.*, 1987). However, some steps of the parasexual process were putatively missing in *T. pseudokoningii*. Stable haploid and unstable hyperhaploid recombinants were recovered from heterokaryons without the isolation of a diploid (Bagagli *et al.*, 1995). The progeny strains from inter-strain crosses of *T. harzianum*, *T. hamatum*, *T. koningii*, and *T. viride* showed isozyme phenotypes identical to either one or the other of the parental strains, suggesting they did not originate from a parasexual mechanism, but rather that the nuclear form from one of the parents had degraded (Barcellos *et al.*, 2011; Sivan *et al.*, 1990; Stasz and Harman, 1990). Therefore, the parasexual cycle does not work in all the *Trichoderma* species thus far tested. Nevertheless, parasexual recombination has the potential to produce superior fungal strains for medical, industrial, and agricultural uses by recombining entire genomes to get numerous potentially useful properties in a single experiment, which may be better accepted by the public than are products derived via transgenesis (Strom and Bushley, 2016).

Targeted Genetic Recombination

Recombination Based on Selective Markers

Improving a strain by recombination with selective markers requires a potent transformation system that must meet at least three requirements: (1) unaltered phenotypes in mutants compared to the wild type; (2) a high rate of homologous recombination (gene replacement) events; (3) and the possibility for bidirectional positive selection (Gruber *et al.*, 2012; Steiger *et al.*, 2011). A selectable marker is a gene introduced into the genome that confers a trait suitable for artificial selection. Over the last three decades, more than ten species within the genus *Trichoderma* have been genetically investigated due to their industrial and/or agricultural application potential. Thus, efficient marker systems have been developed that can be divided into two main groups, the drug resistance genes and the auxotroph genes (**Table 1**).

For now, most *Trichoderma* mutants genetically engineered via recombination are produced based on the acquisition of resistance to certain xenobiotic compounds (**Table 1**) via introduction of the respective resistance genes into the strains. These marker genes give the recipient strains the ability to resist the antibiotic compounds. Thus far, most resistance marker genes are derived from bacteria. To be successfully expressed in fungi, the genes need to be placed into a cassette constructed with appropriate fungal promoters and terminators. The recipient cells are then plated on selective medium containing the antibiotic, and only the transformants harboring the marker cassette survive, and are thus identified.

Hygromycin B is an aminoglycoside antibiotic that inhibits protein synthesis in prokaryotes and eukaryotes by interfering with translocation and causing misreading (González *et al.*, 1978; Mach *et al.*, 1994). The *hph* gene from *Escherichia coli*, encoding a phosphotransferase that inactivates hygromycin B (Gritz and Davies, 1983), is the selectable marker gene used predominantly in *Trichoderma* spp. (**Table 1**). In 2012, (Gruber *et al.*, 2012) established the neomycin phosphotransferase II-encoding gene (*nptII* from *E. coli*, also termed *neo*) as a novel selectable marker for transformation into *T. atroviride* to confer geneticin (G418) resistance. This *nptII* marker cassette has since been efficiently transferred into other species such as *T. hypoxylon*, *T. reesei*, *T.* sp. aff. *guizhouense*, and *T. harzianum* (Liu *et al.*, 2018; Wu *et al.*, 2019; Chenthamara *et al.*, 2021). The bleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus* (Actinomycetales, Actinobacteria) has also been adapted for transformation into four *Trichoderma* spp., with phleomycin (bleomycin) used as the selection agent. The phosphinothricin resistance gene *bar* from *Streptomyces viridochromogenes* (Actinomycetales, Actinobacteria) has also been introduced into *T. reesei* for use as a selectable marker.

The drawbacks of using antibiotics, including fungicides, for selection is that they are expensive and can interfere with the regular function of the targeted gene (Calcáneo-Hernández *et al.*, 2020). An alternative to avoid the use of antibiotics is to generate auxotrophic transformation systems for genetic use. Auxotrophic selectable markers rely on the nutritional background of the recipient strains. The auxotrophic strains have either a mutation or a genetic form of a gene that makes them deficient in synthesizing an essential nutritional compound. By complementing the strains with a functional copy of the corresponding gene, the auxotrophic strains become prototrophic, obtaining the ability to synthesize the specific compound. For example, the *argB* gene from *Aspergillus nidulans* encodes ornithine transcarbamylase, which synthesizes arginine (Penttilä *et al.*, 1987). In the *T. reesei* autotrophs derived from UV-mutagenized conidia, this gene is functionally impaired, and thus they cannot grow without exogenous arginine. If a transformation in these strains is successful, the recipients obtain the ability to synthesize arginine and can grow without a direct arginine supply. Similarly, a selectable marker system employing D-mannitol as a selective carbon source and osmotic stabilizer has been developed based on the *hxk1* gene (encoding hexokinase) in the hexokinase-negative *T. reesei* strain TU-6H. Successful transformation with the *hxk1* gene allows the transformants to grow on D-mannitol as the sole carbon source (Zhang *et al.*, 2010).

For now, the most commonly used auxotrophic marker gene in *Trichoderma* spp. is *ura3*, encoding orotidine-5′-phosphate decarboxylase, the yeast *pyr4* homolog. The gene *ura5*, which encodes orotate phosphoribosyl transferase, can also be used as an auxotrophic marker, but it is less efficient compared to *ura3* (Bergès and Barreau, 1991; Steiger *et al.*, 2011). In this selection system, the pyrimidine analog 5-fluoro-orotic acid (5-FOA) is generally used for the positive selection of uracil (uridine)

Table 1 Selectable markers used for *Trichoderma* transformations

Marker origination	*Encoding gene*	*Marker gene function*	*Phenotype of transformants*	*Reported species*	*References*
Drug resistance	*hph*	Encoding hygromycin phosphotransferase	Hygromycin B resistance	*Trichoderma reesei*	(Mach *et al.*, 1994)
				T. harzianum	(Lorito *et al.*, 1993; Bae and Knudsen, 2000; Cardoza *et al.*, 2006; Cai *et al.*, 2020a)
				T. longibrachiatum	(Sánchez-Torres *et al.*, 1994; Cardoza *et al.*, 2006)
				T. atroviride[a]	(Herrera-estrella *et al.*, 1990; Zeilinger, 2004; Cardoza *et al.*, 2006)
				T. asperellum	(Cardoza *et al.*, 2006)
				T. hamatum	(Carpenter *et al.* 2008; Hohmann *et al.*, 2012)
				T. virens	(Catalano *et al.* 2011)
				T. viride	(Herrera-estrella *et al.*, 1990)
				T. arundinaceum	(Lindo *et al.*, 2018)
				T. hypoxylon	(Liu *et al.*, 2018)
				T. brevicompactum	(Tijerino *et al.*, 2011)
				T. guizhouense	(Zhang *et al.*, 2016; 2019; Meng *et al.*, 2019; Cai *et al.*, 2020a; Pang *et al.*, 2020)
				T. viride	(Zhu *et al.*, 2009)
	neo/nptII	Encoding neomycin phosphotransferase II	Geneticin (G418) resistance	*T. reesei*	(Wu *et al.*, 2019)
				T. atroviride	(Gruber *et al.*, 2012)
				T. hypoxylon	(Liu *et al.*, 2018)
				T. harzianum	(Cai *et al.*, 2020a)
				T. guizhouense	
	ble	Encoding bleomycin resistance protein	Phleomycin/ bleomycin resistance	*T. longibrachiatum*	(Cardoza *et al.*, 2006)
				T. harzianum	
				T. atroviride	
				T. asperellum	
Auxotroph	*amdS*	Encoding acetamidase	Acetamide prototrophy	*T. reesei*	(Penttilä *et al.*, 1987; Suominen *et al.*, 1993)
				T. virens	(Catalano *et al.*, 2011)
				T. harzianum	(Domínguez *et al.*, 2016)
	pyr4/pyrG (*ura3* and *ura5*)[b]	Encoding orotidine-5′-monophosphate (OMP) decarboxylase	Uridine prototrophy	*T. reesei*	(Gruber *et al.*, 1990; Bergès and Barreau, 1991; Hartl and Seiboth, 2005; Steiger *et al.*, 2011)
				T. hypoxylon	(Liu *et al.*, 2018)
				T. atroviride	(Calcáneo-Hernández *et al.*, 2020)
	arg2/argB	Encoding the small subunit of carbamoyl phosphate synthetase	Arginine prototrophy	*T. reesei*	(Penttilä *et al.*, 1987)
				T. virens	(Baek and Kenerley, 1998; Pozo *et al.*, 2004)
	hxk1	Encoding hexokinase	Mannitol prototrophy	*T. reesei*	(Zhang *et al.*, 2010)

[a]Identified as *T. harzianum* (IMI 206040) in the original publication of Herrera-Estrella *et al.*, 1990.

[b]Frequently used with 5-fluoroorotic acid (5-FOA) for bidirectional positive-selection system and also frequently used with *Cre/loxP* recombination system for reusable marker use.

auxotrophic mutants. 5-FOA is catalyzed by URA5 and URA3 to obtain 5-fluorouridine 5′-monophosphate and 5-fluorouracil (Boeke *et al.*, 1984). The latter product inhibits thymidylate synthase activity, which consequently results in thymine nucleotide depletion, affecting DNA synthesis (Boeke *et al.*, 1984; Calcáneo-Hernández *et al.*, 2020; Gruber *et al.*, 1990). Therefore, the toxicity of 5-FOA on those wild-type or complemented strains facilitates their selection, whereas mutants deficient in *ura3* or *ura5*, essential for uracil synthesis, can grow on 5-FOA-containing medium supplemented with uridine/uracil (Bergès and Barreau, 1991; Gruber *et al.*, 1990; Hartl and Seiboth, 2005; Steiger *et al.*, 2011). Another example is amidases, which are hydrolytic enzymes that catalyze the conversion of carboxylic amides to the corresponding carboxylic acid and ammonia. As amidases are present in only a few fungal species (Penttilä *et al.*, 1987), the *A. nidulans amdS* gene can be used as a selectable marker for successfully transforming *Trichoderma* spp. The transformants carrying the *amdS* gene can easily be selected using acetamide as the sole carbon or nitrogen source (Penttilä *et al.*, 1987; Suominen *et al.*, 1993).

However, the most commonly found *Trichoderma* species, such as *T. harzianum*, *T. guizhouense*, *T. virens*, *T. atroviride*, *T. asperellum*, *T. brevicompactum*, and *T. hamatum*, are prototrophic or sensitive to only one or two of the antibiotics mentioned, making it difficult to perform multiple gene transformations. One proposed strategy is to use the *Cre/loxP* recombination

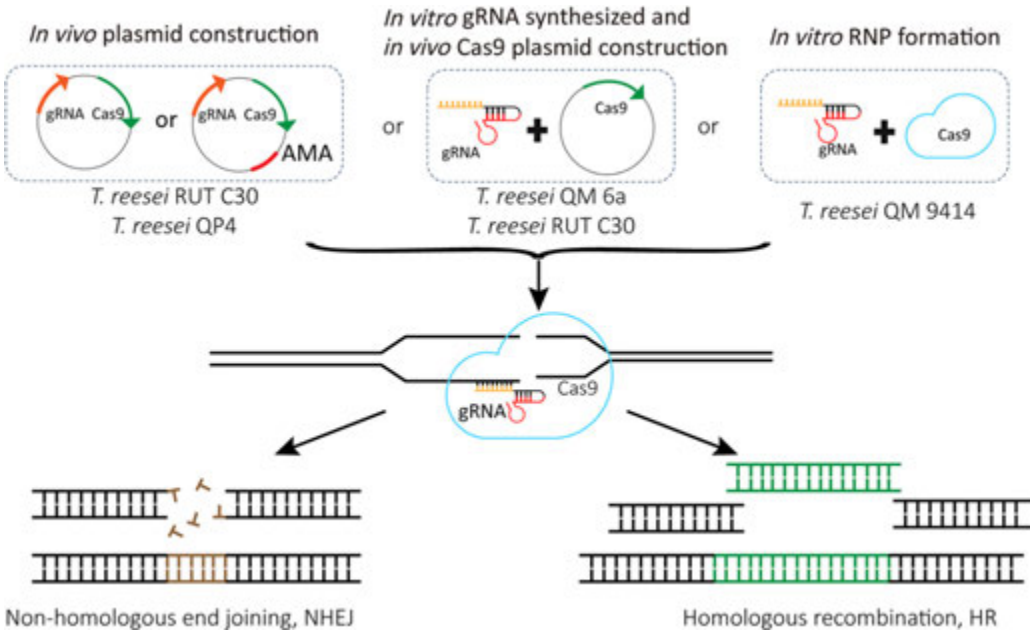

Fig. 2 Schematic diagram of CRISPR/Cas9-mediated gene manipulation. The Cas9 protein can be expressed *in vivo* in the host cell or added *in vitro*. The gRNA can either be synthesized *in vitro* or produced *in vivo* with an AMA-based autonomously replicating plasmid. Strains listed under each method are those that have already been established with the CRISPR/Cas9 system using that method.

system (also see below) adapted from bacteriophage P1 (Sternberg *et al.*, 1981), which involves the excision of DNA fragments (usually the marker gene cassette) flanked by *loxP* sites that catalyzed by the expression of CRE recombinase (Steiger *et al.*, 2011). In this circumstance, *Cre/loxP* offers the possibility of serially targeted gene deletions with absolute marker recycling in the fungus.

Recombination Based on Markerless Systems

Marker genes can be removed once a transformation has succeeded, and two methods have been reported for removing them by recombination (Bischof and Seiboth, 2014). The first method is homologous recombination-mediated excision. In previous research with *T. reesei* QM 6a, the auxotrophic *ura3* gene (termed *pyr4* in the original publication) was used as a selective marker gene (Hartl and Seiboth, 2005). The plasmid was constructed by inserting the *ura3* gene between two direct repeats of the *Sh ble* (zeocin resistance) gene. The up- and downstream regions of the targeting genes were then cloned into the plasmid to complete the final vector. After transformation, the targeted gene was deleted, and the *ura3* gene was inserted. Selection medium without 5-FOA and uridine (uracil) was used to screen for the positive transformants. After positive selection, the transformed mutants were plated on medium containing 5-FOA, and the *ura3* marker gene was excised via recombination among the direct repeats of the *Sh ble* fragments.

The other method for markerless recombination is the *Cre/loxP* system as briefly mentioned above, a site-specific recombination system mediated by heterologous site-specific recombinases (**Fig. 2**) that requires the introduction of a site-specific recombinase (CRE recombinase) by transformation. This method permits the unlimited reuse of the same marker and has been applied in *T. reesei* QM 6a (Steiger *et al.*, 2011). First, the human *LIG4*- (DNA ligase 4, which joins single-strand breaks in a double-stranded polydeoxynucleotide and is essential for DNA double-strand break repair via non-homologous end joining) homologous gene *tmus53* was deleted in QM 6a to create a non-homologous end joining-deficient strain, which can lead to significantly higher homologous recombination efficiency (Ninomiya *et al.*, 2004). For this purpose, a deletion cassette containing the phosphinothricin resistance (*bar*) marker was constructed and transformed to obtain a *tmus53* deficient strain (QM6aΔ*tmus53*). Another cassette containing two *loxP* sites flanking the *hph* and *amdS* cassettes (loxP-*amdS*-*hph*-loxP) was then used to replace the *bar* gene. At the same time, the *pyr4* gene was replaced by the *cre* gene (encoding CRE recombinase) under the control of the *xyn1* (encodes xylanase I) promoter. This step yielded the strain QM6aΔ*tmus53*Δ*pyr4*, which was resistant to hygromycin B and could utilize acetamide as a nitrogen source. The QM6aΔ*tmus53*Δ*pyr4* strain was then cultivated on xylan, resulting in excision of the marker genes *hph* and *amdS* by the *Cre/loxP* system, meaning these two marker genes can be reused repeatedly to delete any genes of interest. When the aim of the gene deletions was achieved, the *cre* gene was replaced by the *ura3* gene to obtain the marker free strain QM6aΔ*tmus53*. Nevertheless, this method always retains one *loxP* site in the genome after each deletion.

Methods for Introducing Recombinant DNA Into *Trichoderma*

Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens (syn. *Rhizobium radiobacter*, Rhizobiales, Proteobacteria) is a pathogenic Gram-negative alpha-proteobacterium that infects plants or fungi by inserting DNA into the nucleus of the host cell via a plasmid (the Ti-plasmid). In plants, it forces the abnormal growth of tissues (galls) that are used by the bacterium (Barton *et al.*, 2018; Gelvin, 2003). The Ti plasmid contains a virulence region with the *vir* genes that encode the virulence proteins involved in the formation, transport, and integration of the T-DNA. The concept of the *A. tumefaciens*-mediated transformation (AMT) method is based on the fact that all the T-DNA sequences can be replaced by other DNA sequences. Thus, an altered T-plasmid offers the opportunity for a DNA fragment to be placed between two 25 bp direct repeats (the left and right borders required to transfer the T-DNA) and thus be transformed into the host cell (Gelvin, 2003). Although frequently called "transformation", this process is more correctly described as "trans-conjugation" (Cai *et al.*, 2020b). The T-DNA transfer of *A. tumefaciens* requires inducers such as phenolic compounds, which are often used. After being induced by acetosyringone, the virulence genes begin to express, and the virulence proteins help generate single-stranded DNA copies of the T-DNA, which are then transferred into the recipient (Michielse *et al.*, 2005). If the T-DNA carries a homolog, homologous recombination will occur. Otherwise, it will integrate ectopically when the T-DNA does not homologously recombine. Many factors influence AMT efficiency in fungi, including the fungal starting materials (protoplasts, spores, mycelium, or fruiting body tissue), concentration of the inducer, and co-cultivation conditions (ratio between the *A. tumefaciens* and recipient, length of the co-cultivation period, temperature, and pH), making AMT a complicated procedure for fungal transformations.

T. reesei was the first species to be tested to check whether AMT can work for the fungus (de Groot *et al.*, 1998). The conidia of *T. reesei* were co-cultured with others from *A. tumefaciens*. T-DNA carrying the selectable marker gene was transformed into *T. reesei*, and the resulting conidia were then plated on selective medium to screen for positive transformants. The stability of the marker resistance was confirmed by repeatedly replating the transformants on fresh selective medium. Zhong *et al.* (2007) successfully applied the AMT method in *T. reesei* QM 9414 and established a procedure with a higher efficiency AMT. However, a characterization of the T-DNA insertion junctions suggested that T-DNA integration occurred at random positions in the *T. reesei* genome by a process of non-homologous recombination.

Another modified AMT method that can generate gene-deleting strains efficiently by homologous recombination was established and applied in *T. atroviride* P1 to delete two genes (Zeilinger, 2004). The respective gene deletion cassettes were constructed in a plasmid (pAN7–1) containing the *E. coli hph* gene cassette (see above). The whole disruption construct, flanked by the 5′ and 3′ non-coding sequences of the *tmk1* and *tga3* genes (the targeting genes), was inserted between the left and right border repeats of the binary vector pTAS5. The transformation was implemented by plating the co-cultivations onto sterile nitrocellulose membranes placed on an induction medium. After transformation and selection, the positive transformants showed highly efficient *tmk1* and *tga3* deletion. The AMT method has also been adopted for transforming different strains of *T. britannicum* (shown as *T. harzianum* in the publication of Cardoza *et al.*, 2006), *T.* cf. aff. *guizhouense* (Zhang *et al.*, 2016), *T. longibrachiatum* (Cardoza *et al.*, 2006), *T. asperellum* (Cardoza *et al.*, 2006), *T. brevicompactum* (Tijerino *et al.*, 2011), and *T. arundinaceum* (Lindo *et al.*, 2018). A detailed AMT procedure can be found in Cai *et al.* (2020b).

PEG-mediated protoplast transformation

Protoplast transformation is the most widely used method for introducing DNA into a fungal cell. The first successful attempt was based on a PEG/$CaCl_2$-mediated protoplast transformation in *T. reesei*. The *argB* gene from *A. nidulans* was used as an auxotrophic marker together with a corresponding *argB*-deficient mutant (Penttilä *et al.*, 1987). Protoplast transformations require the preparation of protoplasts from fungal cells using various cell-wall degrading enzymes. The starting cells can be germinating conidial spores or young mycelial fragments. The enzymes used to degrade the cell walls in *T. reesei* are usually from *T. harzianum*, while for other fungi, the lysis enzymes generally are from snail stomach and the bacterium *Arthrobacter luteus* (Actinomycetales, Actinobacteria). After hydrolyzation, the protoplasts are stabilized with sodium chloride, magnesium sulfate, mannitol, or most commonly, D-sorbitol or sucrose. They can then be frozen at −80°C for later use (see Bischof and Seiboth, 2014 for a review).

DNA uptake is mediated by the presence of calcium ions (Ca^{2+}) and a high PEG concentration. The calcium ions promote the salt precipitation of DNA on the protoplast surface, and PEG serves as a glue to keep the protoplasts in close contact with the DNA molecules, thereby facilitating DNA uptake. The protoplasts are then regenerated on selective media, where only the transformed cells can grow (Ruiz-Díez, 2002). In contrast to AMT, protoplast transformation often leads to a high copy number of DNA insertions. Recently, a well-established PEG-mediated protoplast transformation procedure has been adopted in species from the *Harzianum* clade (e.g., *T. harzianum* and *T.* cf. aff. *guizhouense*) that favors an outcome with genetically modified mutants for several different genes (Cai *et al.*, 2020a,b; Gao *et al.*, 2020; Meng *et al.*, 2020; Pang *et al.*, 2020; Zhang *et al.*, 2019). This procedure has been summarized by Cai *et al.* (2020b).

RNA Interference

RNA interference (RNAi) is a biological process in which gene expression is inhibited by targeting the specific mRNA (Bischof and Seiboth, 2014). The method is mediated by double-stranded RNA (dsRNA), and successful implementation requires two

components: RNA-induced silencing complex (RISC) and the enzyme Dicer. First, dsRNA with homology to the gene of interest is expressed in the fungal host strain, which is then cleaved into short interfering RNA (siRNA). The double-stranded siRNA is then unwound into two individual strands. One of the single-stranded RNAs, called the passenger strand, is degraded. The other, called the guide strand, is incorporated into RISC, pairs with its complementary mRNA, and induces cleavage, which results in the gene being silenced (Meyer, 2008).

RNAi was applied in *T. reesei* RUT C30 to silence the expression of the cellobiohydrolase II-encoding gene *cel6a* (Brody and Maiyuran, 2009), which was used as the DNA template to construct the silencing vector pMai148. The successful transformants had lower *cel6* expression levels, and an RT-qPCR evaluation showed that the level of *cel6a*-specific mRNA in the transformants was lower than that in wild type. Another improved RNAi system for *Trichoderma* was developed by He *et al.* (2015). This system has dual promoters for efficient RNA silencing in *T. reesei*. The *rp2* (encodes ribosomal protein P2; He *et al.*, 2013) promoter from *T. reesei* and the *trp*C (encodes a polypeptide involved in tryptophan biosynthesis; Hamer and Timberlake, 1987) promoter from *A. nidulans* were used to construct the pCAMBIA1300–1-dual plasmid. To construct a vector for gene silencing, the target gene is directly inserted into a specific site in pCAMBIA1300–1-dual. This modified system can also be used to co-silence more than one gene. Unfortunately, this RNAi system is uncontrollable in *T. reesei* or only works on certain substrates. Wang *et al.* (2018) developed a copper-controlled RNAi system in which the copper-responsive *tcu1* promoter was introduced into *T. reesei* QM 9414. This system was used to control the expression of *xyr1*, which encodes the transcriptional activator of the cellulase and hemicellulase genes (Stricker *et al.*, 2006), and *cel7*, including the exoglucanase-encoding gene *cel7a* and the endoglucanase-encoding gene *cel7b* (Kajisa *et al.*, 2009). The transcriptional levels of these two genes were both reduced when copper was absent in the medium, whereas they were restored when copper was added.

Promoter Engineering Approaches

Manipulating the promoter of genes is also an effective strategy to regulate their expression. Promoters are the regulatory regions upstream of the transcriptional start site, and they control gene transcription. Promoters also provide information and factors to RNA polymerase. In general, the promoters used for strain engineering can be classified into two types: constitutive promoters and tunable promoters.

Constitutive promoters are expressed independently and are not affected by environmentally induced factors. They regulate the expression of basal genes, assumed to be produced at a constant rate, and thus their activity is not flexible. Numerous constitutive promoters have been used such as *cdna1* (encodes a hypothetical protein with a high transcriptional level; Nakari-Setälä and Penttilä, 1995), *tef1* (encodes translation elongation factor 1 alpha; Nakari-Setälä and Penttilä, 1995), *eno1* (encodes enolase; Li *et al.*, 2012), *gpd1* (encodes glyceraldehyde-3-phosphate dehydrogenase; Li *et al.*, 2012), *pdc1* (encodes pyruvate decarboxylase; Li *et al.*, 2012), *pki1* (encodes pyruvate kinase; Kurzatkowski *et al.*, 1996), and *rp2* (see above). The *cdna1* and *tef1* promoters were isolated by screening cDNA libraries for genes highly expressed during growth on D-glucose-containing media, and they have been used for overexpressing different genes under these culture conditions (Nakari *et al.*, 1993). In *T. reesei* QM 9414, the *cbh1* (encodes cellobiohydrolase I) promoter is repressed by glucose (Penttilä *et al.*, 1993). Nakari-Setälä and Penttilä (1995) used the *cdna1* and *tef1* promoters to manipulate QM 9414, making the fungus secrete active cellobiohydrolase I and the endoglucanase I catalytic core when cultivated on medium containing glucose. The results showed that the *cdna1* promoter provided the highest yield, accounting for more than half of the total protein secreted by the mutant; the production levels obtained with the *tef1* promoter were much lower. The promoters for *eno1* and *pdc1* are also well-adapted for cultivation on D-glucose, as shown when their activities were compared by expressing xylanase in *T. reesei*. The recombinant strains obtained by Li *et al.* (2012) were cultivated in glucose-containing medium, which led to an extremely high yield of recombinant xylanase II (XYN II). Linger *et al.* (2015) utilized the *eno1* promoter to control the expression of the *cel7a* gene in a Δ*cel7a* strain, and the purified CEL7A produced from the mutant grown on glucose showed remarkably consistent activity. In contrast, purified CEL7A from the same strain grown on lactose demonstrated significantly higher variability in its activity.

Tunable promoters are inducible or repressible depending on the presence or absence of activating or repressing factors. An inducible promoter should have no basal expression, but have considerably enhanced expression on the addition of the inducer. Conversely, the expression of a repressible promoter should be significantly lowered by the addition of a repressing substance. For the mutant *T. reesei* D7, which was generated from *T. reesei* RUT C30, Li *et al.* (2017) used the *cbh1* promoter induced by cellulose to regulate the expression of *cbh2*, whose expression is usually lower than that of *cbh1*. The transformant, named *T. reesei* TH18, had the highest yield when induced by pre-treated corn stove. For *T. reesei* PC-3–7, the gene encoding XYN III showed its highest expression levels among the xylanase genes in response to cellulosic carbon. The *xyn3* promoter has also been found to be suitable for expressing specific genes encoding highly functional proteins. Hirasawa *et al.* (2018) has constructed various chimeric *xyn3* promoters by utilizing the *xyn1* cis-acting region. This efficient chimeric *xyn3* promoter was then used to control the high-performance BGL from *Aspergillus aculeatus* (AaBGLI) in the *T. reesei* PC-3–7 strain. The transformed strains had improved BGL activity, which also indicates a high saccharification ability with induction by cellulose.

Given that the non-directional mutations and DNA combinations achieved through forced random mutagenesis, sexual crossing, protoplast fusion, and parasexuality require time- and labor-intensive processes, the application of such classical mutagenesis techniques have been falling into disuse since the second sequencing revolution. Neither the recombinant methods based on a limited number of selectable marker genes nor the markerless systems allow for efficient multiple-gene editing. Even so,

by applying the methods described above in *Trichoderma* biology and biotechnology, great progress has been achieved in our understanding and utilization of this fungus. Given that the *Trichoderma* spp. genomes are largely unexplored, and the genetic bases of domestication traits of interest are poorly understood, robust genetic tools are still highly desired.

CRISPR/Cas9, the Next-Generation Genome Editing Revolution and Its Application in *Trichoderma*

In recent years, the type II clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) system has been applied for gene editing in various species of eukaryotes, including filamentous fungi. The CRISPR/Cas9 system is composed of a Cas9 nuclease and a single-guide RNA (sgRNA), which consists of a target-recognizing CRISPR RNA (crRNA) and an auxiliary noncoding trans-activating crRNA (tracrRNA) (Jinek *et al.*, 2012). The complex that the sgRNA binds to Cas9 catalyzes a double-strand break in the target DNA site (20 bp) that matches the sgRNA protospacer and a downstream protospacer adjacent motif (PAM) sequence (Mali *et al.*, 2013). The target sites must lie immediately at the 5′ end of the PAM sequence and match the canonical form 5′-NGG (N represents any possible nucleotide). The Cas9 nuclease can be directed to any DNA sequence of the form N_{20}-NGG simply by altering the first 20 bp of the gRNA corresponding to the target DNA sequence (Sander and Joung, 2014).

One of the first attempts to establish the CRISPR/Cas9 system in filamentous fungi was performed with *T. reesei* by Liu *et al.* (2015). In that study, a codon-optimized Cas9 with the SV40 nuclear localization signal (NLS) was integrated into the genomes of *T. reesei* strains QM 6a and RUT C30. Since knowledge of a confirmed RNA polymerase III-based promoter in *T. reesei* was lacking at that time, the authors introduced an *in vitro* synthetic sgRNA to edit the targeting genes by protoplast transformation. They first designed the sgRNA to target the *ura5* gene, which can undergo selection on minimal medium containing 5-FOA. To test the incidence of homologous recombination with the CRISPR/Cas9 system, they chose the gene *lae1* (encodes a putative methyltransferase; Seiboth *et al.*, 2012) as a target and used the exogenous gene *poura5* (encodes URA5 from *Penicillium oxalicum*) as the selectable marker. In this case, the designed sgRNA and the *lae1* donor DNA (dDNA) segment (containing the 5′ and 3′ regions of *lae1* and the *poura5* cassette) were co-transformed. The results indicated that the *lae1* gene was replaced by the selectable marker cassette in all transformants. A pair of 200 bp homology arms was sufficient to obtain homologous integration in the mutants, with homologous recombination frequencies of $\geq$93%. In another experiment, the two genes *vib1* (encodes a putative link between glucose signaling and carbon catabolite repression; Xiong *et al.*, 2014) and *clr2* (encodes a transcription factor for cellulase gene expression; Coradetti *et al.*, 2012) were chosen as targets to test the efficiency of disrupting multiple genes. The efficiencies for achieving the simultaneous homologous recombination of two (16%–45%, depending on the molar ratio of sgRNA to dDNA) or three (4.2%) genes were relatively low.

Liu *et al.* (2015) did not detect the off-target effect of the CRISPR/Cas9 system. Four years later, however, Hao and Su (2019) found an unexpected off-target gene disruption in *T. reesei* QM 9414 that was caused by intracellularly expressed Cas9. Hence, they utilized the *in vitro* assembled ribonucleoprotein (RNP) Cas9/gRNA complex to disrupt the *cbh1* gene in *T. reesei* TU-6 (the uridine auxotrophic mutant of QM 9414), which was shown to be successfully disrupted, with large DNA inserts in the edited *cbh1* locus. This transient *in vitro* method may prevent the side effects caused by the *in vivo* expression of Cas9, which may be due either to the enzyme itself or the transgenic procedures. This experiment supports using this alternative gene editing tool for those filamentous fungi in which it is difficult to manipulate genes as well as for those fungi lacking codon-usage databases (because the *in vivo* Cas9 system requires codon optimization for use in eukaryotic cells). Interestingly, Rantasalo *et al.* (2019) adapted the RNP-based method for multiple-gene deletions, achieving a 12% simultaneous triple deletion efficiency for the *cbh2* (encodes cellobiohydrolase II), *egl1* (encodes endoglucanase I), and *egl2* (encodes endoglucanase II) genes. More recently, Zou *et al.* (2020) improved the transformation efficiencies of both homologous recombination and gene disruption without the use of any foreign DNA, but rather via chemical reagents. Adding the surfactant Triton X-100 increased the number of RNP transformations by enhancing RNP penetration. Furthermore, the addition of inositol or benomyl increased the formation of monokaryotic protoplasts, which also improved the efficiency of homozygous transformations.

As two new U6 promoters (RNA polymerase III-based promoters) were recently confirmed by Wu *et al.* (2020), the simultaneous *in vivo* expression of both Cas9 and sgRNA in *T. reesei* has also now been successfully achieved. Expressing sgRNA in vivo can save time in the experimental procedure and guarantees the sgRNA concentration, thus increasing the efficiency of the CRISRPR/Cas9 system. Wang *et al.* (2020b) have also found that the native 5S rRNA promoter can be used to drive sgRNA expression in *T. reesei* (strain QP4), which was more efficient than using the heterologous promoter from *A. niger*. Fonseca *et al.* (2020) used this exogenous promoter to express an sgRNA *in vivo* to introduce six genetic modifications in *T. reesei* RUT C30, resulting in a remarkable increase in the protein secretion rates.

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Relevant Website

https://trichokey.com/index.php/trichoderma-taxonomy-2020
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Expression of Recombinant Fungal Proteins in *Pichia Pastoris*

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In studies of protein functions, the wild-type target protein can be used (Ayers *et al.*, 1978; Fägerstam and Pettersson, 1980), but endogenous expression levels are often low, and purification can be troublesome. Therefore, it is often more convenient to express recombinant proteins heterologously in another host. Various organisms, including bacteria, yeasts, insect cells, and animal cells, can be used as hosts. Each of them has characteristic advantages and disadvantages, and it is important to select an appropriate host for a particular purpose. In our research group, we often need at least several tens of milligrams of pure recombinant enzymes to study biomass-degrading systems derived from wood-decaying fungi. To obtain this level of recombinant protein production, we use *Pichia pastoris* as an expression host.

Introduction

In laboratory-scale production of recombinant and mutated proteins, *Escherichia coli* expression systems are most widely used (Rosano and Ceccarelli, 2014), and suitable vector systems are commercially available. However, protein synthesis in *E. coli* is intracellular, and the production amount of target protein per culture volume is limited. Moreover, since *E. coli* is a prokaryote, it is not suitable for the production of proteins derived from eukaryotes such as fungi, plantae and animalia.

We have used the yeast *P. pastoris* for more than 20 years as a host for production of recombinant fungal proteins and enzymes, as listed in **Table 1**. Most of the produced proteins are extracellular enzymes related to plant cell-wall metabolism, but we have also succeeded in synthesizing a membrane protein (anion exchanger) using this system (Tokuda *et al.*, 2011). Fewer host species and vector systems are available for *P. pastoris* compared to *E. coli* (Lin-Cereghino and Lin-Cereghino, 2007), but the yeast has the advantage that it can produce large amounts of the target protein due to the feasibility of high-density cultivation and the extracellular secretion of target proteins. Furthermore, *P. pastoris* secretes only low levels of endogenous proteins in culture (Cregg, 2007a), so purification of recombinant protein produced by *P. pastoris* is relatively easy. Levels of several grams per culture liter of recombinant fungal Carbohydrate-Active enZymes (CAZymes) have been obtained, and levels of several milligrams per culture liter are routinely obtained (Igarashi *et al.*, 2012; Mellitzer *et al.*, 2012; Nakamura *et al.*, 2013; Lombard *et al.*, 2014). High productivity of recombinant proteins is especially advantageous for studies such as enzymatic saccharification of biomass and preparation of crystals for neutron-beam structural analysis (Nakamura *et al.*, 2015). Moreover, since *P. pastoris* is an eukaryote, it can produce enzymes with post-translational modifications, such as glycan chain modification, or enzymes containing cofactors, such as flavin and heme (Yoshida *et al.*, 2001). Furthermore, *P. pastoris* is applicable to proteins that are generally difficult to express in recombinant form, such as G protein-coupled receptor (GPCR) and antibodies (Tokuda *et al.*, 2011; Shiroishi *et al.*, 2011; Ning *et al.*, 2003). Thus, *P. pastoris* has clear advantages over *E. coli* for producing eukaryotic proteins. **Fig. 1** shows a working scheme for recombinant protein production using *P. pastoris*. In this chapter, we will describe these procedures and focus on some key points for success.

Construction of Expression Strains

A variety of *P. pastoris* host strains and vectors for recombinant protein expression can be purchased through Invitrogen, bioGRAMMATICS and ATUM (Ahmad *et al.*, 2014). This section describes points to consider when using these resources to construct a recombinant protein expression system.

Vectors and Type of Induction System

The expression of recombinant protein in *P. pastoris* can be controlled by several promoter systems (Vogl and Glieder, 2013). Among them, the alcohol oxidase (AOX) gene promoter in pPIC vector and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene promoter in pGAP vector are commonly used for recombinant protein production in *P. pastoris*. The AOX promoter strongly induces the expression of recombinant proteins in the presence of methanol (Cregg *et al.*, 1993). Thus, the expression of a recombinant protein using pPIC can be controlled by the concentration of added methanol. In addition, protein production under this promoter can be performed in a higher-density culture environment than in the case of pGAP. Therefore, since a higher concentration of a recombinant protein solution is likely to be obtained under this promoter, the pPIC vector is typically the first choice. On the other hand, pGAP strongly induces the expression of recombinant protein in culture in the presence of glucose or glycerol as a substrate (Waterham *et al.*, 1997), so the target protein can be continuously and easily produced without changing to inductive culture. However, the density of cells tends to be limited, particularly in flask-scale culture, limiting the yield of the target protein.

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Table 1 Fungal-derived recombinant enzymes expressed in *P. pastoris* by our group

Family	*Protein*	*Source Organism*	*Reference*
Glycoside Hydrolase			
GH1	β-glucosidase (*Pc*Bgl1A)	*Phanerochaete chrysosporium*	Tsukada *et al.* (2006)
GH1	β-glucosidase (*Pc*Bgl1B)	*Phanerochaete chrysosporium*	Tsukada *et al.* (2006)
GH3	β-glucosidase (*Pc*Bgl3A)	*Phanerochaete chrysosporium*	Kawai *et al.* (2003)
GH6	Cellobiohydrolase II (*Pc*Cel6A)	*Phanerochaete chrysosporium*	Igarashi *et al.* (2012)
GH16	Laminarinase (*Pc*Lam16A)	*Phanerochaete chrysosporium*	Kawai *et al.* (2006b)
GH43	Exo-$\beta-1,3$-galactanase (*Pc*Gal43A)	*Phanerochaete chrysosporium*	Ichinose *et al.* (2005)
GH45	Endoglucanase V (*Pc*Cel45A)	*Phanerochaete chrysosporium*	Igarashi *et al.* (2008)
GH54	α-L-arabinofuranosidase (*Nc*Araf1)	*Neurospora crassa*	Takata *et al.* (2010)
GH54	Endo-β-(1$\rightarrow$6)-galactanase (*Nc*6GAL)	*Neurospora crassa*	Takata *et al.* (2010)
GH55	Glucan 1,3-β-glucosidase (*Pc*Lam55A)	*Phanerochaete chrysosporium*	Kawai *et al.* (2006a)
GH74	Xyloglucanase (*Pc*Xgh74B)	*Phanerochaete chrysosporium*	Ishida *et al.* (2007)
Carbohydrate Esterase			
CE1	Acetyl xylan esterase (*Al*AXEA)	*Aspergillus luchuensis*	Komiya *et al.* (2017)
CE1	Feruloyl esterase (*Ao*FaeB)	*Aspergillus oryzae*	Suzuki *et al.* (2014)
Polysaccharide Lyase			
PL20	Endo-$\beta-1,4$-glucuronan lyase A (*Tr*GL)	*Trichoderma reesei*	Konno *et al.* (2009)
Auxiliary Activities			
AA2	Manganese peroxidase (MnP5)	*Phanerochaete chrysosporium*	Lee *et al.* (2005)
AA3 + AA8	Cellobiose dehydrogenase (*Pc*CDH)	*Phanerochaete chrysosporium*	Yoshida *et al.* (2001)
AA8 + AA12	Sugar dehydrogenase (*Cc*SDH)	*Coprinopsis cinerea*	Matsumura *et al.* (2014)
AA8	Carbohydrate-binding cytochrome b562 (CBCyt. b562)	*Phanerochaete chrysosporium*	Yoshida *et al.* (2005)
AA9	Broad xyloglucan specific polysaccharide monooxygenase (*Gt*LPMO9A)	*Gloeophyllum trabeum*	Kojima *et al.* (2016)
AA9	Lytic cellulose monooxygenase (*Pc*LPMO9D)	*Phanerochaete chrysosporium*	Westereng *et al.* (2011)
Carbohydrate-Binding Module			
CBM1	Fluorescent protein-fused fungal cellulose-binding domains (RFP-CBDs)	*Phanerochaete chrysosporium and Trichoderma reesei*	Sugimoto *et al.* (2012)
Other			
AE1	Anion exchanger 1 transporter (*Pc*AEP)	*Phanerochaete chrysosporium*	Tokuda *et al.* (2011)

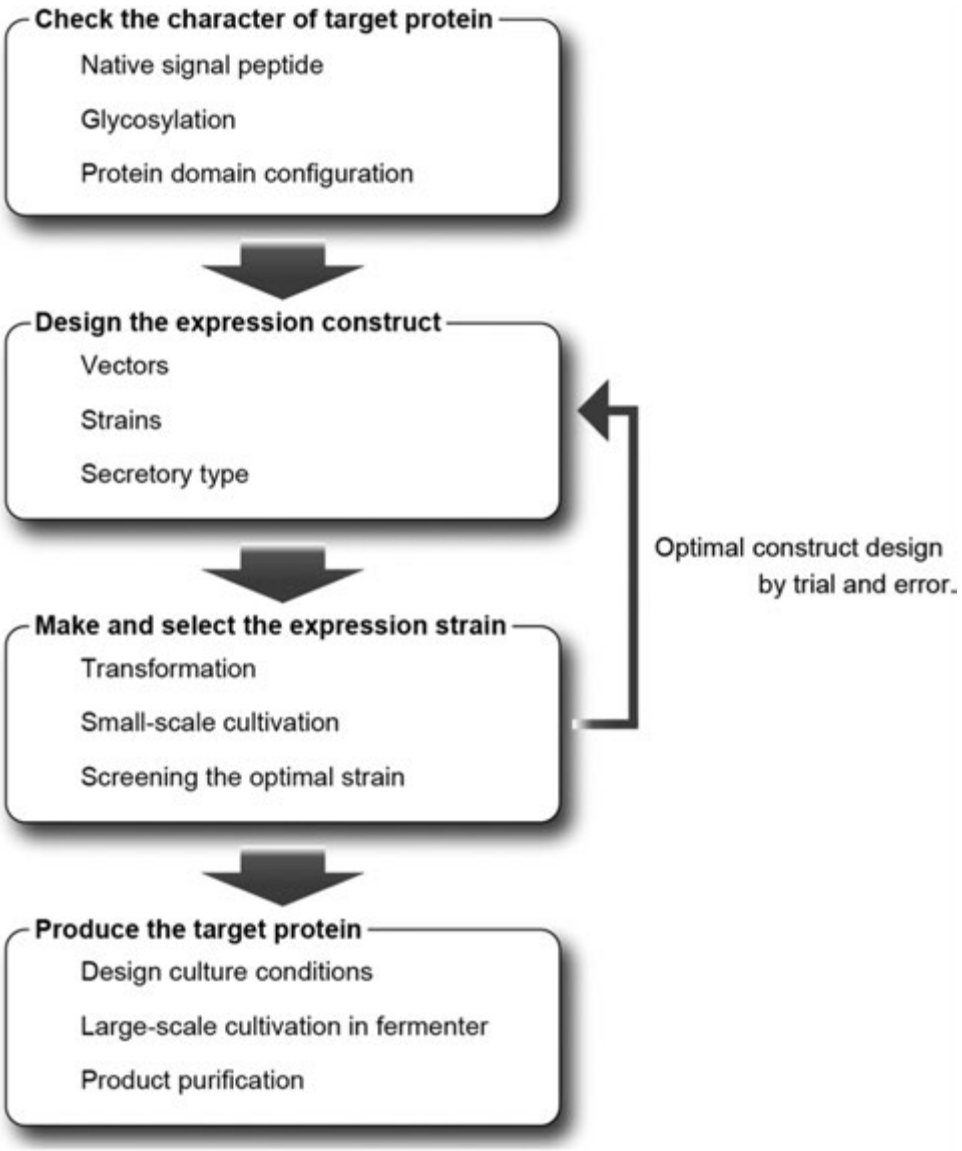

Fig. 1 Experimental scheme for the expression of recombinant proteins in *P. pastoris*.

Strains

Many types of *P. pastoris* hosts are available, differing mainly in the availability of selectable markers, methanol assimilation rate, and the presence or absence of protease genes (Lin-Cereghino and Lin-Cereghino, 2007; Ahmad *et al.*, 2014). The availability of a selection marker need not be considered when selection is done with antibiotics, but is relevant when performing auxotrophy screening using amino acids.

The rate of methanol assimilation greatly affects whether the final recombinant protein production can be performed on a large scale in a fermenter or on a flask scale, particularly in the case of the methanol-induction (pPIC) system. The rate of methanol assimilation in *P. pastoris* depends on the presence or absence of a mutation in the alcohol oxidase *aox1* gene, and this trait is expressed as a methanol utilization phenotype (Mut). Many *P. pastoris* strains, including wild strains, can grow well with methanol as a carbon source (the Mut^+ phenotype). Further, Mut^+ strains can continue producing recombinant proteins for a long period even when only methanol is available as a carbon source. Consequently, large amounts of recombinant protein per batch can be produced. However, since Mut^+ strains rapidly consume methanol in the culture solution, it is necessary to supply methanol continuously to achieve continuous induction. This is straightforward in cultivation using a jar fermenter, but in flask culture, methanol must be added at least every 12 h in order to maintain an inducing concentration, and this may be inconvenient. On the other hand, in the Mut^s phenotype, the AOX1 gene is partially deleted and replaced with the ARG4 gene of the yeast *Saccharomyces cerevisiae* (Cregg and Madden, 1987), and as a result, methanol consumption is slower than that of Mut^+ strains. Consequently, Mut^s strains can maintain induction for a long time with the initially added methanol; for example, in flask-scale culture,

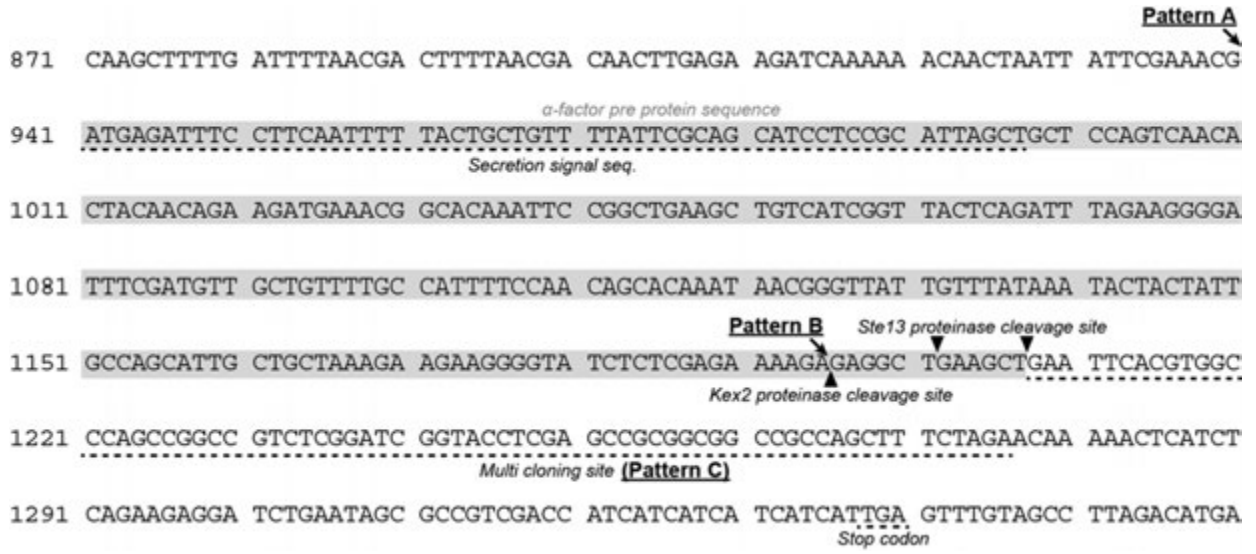

Fig. 2 Nucleotide sequences around the α-factor signal and the multi cloning site (MCS) of pPICZαA.

supplementation of methanol once every 24 h is sufficient. However, the growth phase of Mut^s is shorter than that of Mut^+ when only methanol is available as a carbon source. For the purposes of our research, we have usually found Mut^s strains more suitable.

Using Functional Host Strains

P. pastoris expression host strains with various functions are still being developed, including strains that can visually confirm the introduction of the recombinant gene (Thermo Fisher Scientific, PichiaPink™), that can mediate human-like glycosylation (Laukens *et al.*, 2015), that are easily adaptable for commercial purposes (ATUM, IP-Free® Cell Strains), etc. Among them, protease-deficient strains lacking one or several secretory protease genes are effective to protect recombinant products from proteolysis (Gleeson *et al.*, 1998). This is important, because degradation of recombinant proteins is a major problem in *P. pastoris* systems, especially when the production rate of the target protein is low. However, such gene-modified strains often have a lower productivity of recombinant protein than wild-type strain (Lin-Cereghino and Lin-Cereghino, 2007). Therefore, careful attention to the balance of production and degradation rates is required.

Vectors

Since the introduced plasmid vector is fused into the genome of *P. pastoris*, the recombinant strain is stable. We were able to produce recombinant proteins without addition of antibiotics even in a jar fermenter. This section describes the factors involved in the selection and construction of transformation vectors.

As described above, it is necessary to choose a suitable selection marker and promoter depending on the host species and induction system. For example, in selection with the antibiotic Geneticin 418 (G418) for the construction of multi-copy transformants, the initial selection based on histidine dependence requires a biosynthetic marker such as HIS4 in the pPIC9K vector (Clare *et al.*, 1991b). To use such selection system, it is necessary to select a host that supports multiple selections.

Secretory signals also play a very important role in the construction of recombinant strains. Various signal sequences have been investigated for secretory expression of heterologous proteins from *P. pastoris* (Raemaekers *et al.*, 1999; Kjeldsen *et al.*, 1999). In many commercial expression vectors, the sequence derived from α-mating factor of *S. cerevisiae* is adopted as a secretion signal. **Fig. 2** shows the gene sequences around the α-factor signal and the multiple cloning site (MCS) sequence of pPICZalpha (Invitrogen), which is often used in the construction of secretory expression strains. The α-mating factor protein is a secretory hormone of *S. cerevisiae*. The α-factor signal sequence in pPICZalpha is composed of a 19-amino-acid signal peptide, which translocates the target protein into the endoplasmic reticulum (ER), and a 66-amino-acid preprotein sequence that induces coat protein complex II (COPII)-mediated vesicle transportation (Otte and Barlowe, 2004; Dancourt and Barlowe, 2010). When the target gene is inserted into the MCS region, the target protein is connected to the C-terminal of the α-factor signal protein via a recognition site for proteases Kex2 and Ste2 (**Fig. 2** Pattern C). Recombinant protein synthesized in this way is transported to the ER by the action of the signal peptide sequence, then the preprotein is cleaved by Kex2 and Ste2 in the Golgi apparatus, and the target protein is secreted outside the cells (Julius *et al.*, 1983; Fuller *et al.*, 1988).

Introduction of the target gene into the MCS makes it possible to produce a secretory expression construct easily, regardless of the type of protein sequence. However, several additional amino acid residues derived from the vector and the remaining Ste2 protease recognition sequence are added to the N-terminus of the target protein when a gene is inserted into the MCS. Notably, the

number of amino acids derived from the Ste2 recognition sequence may be four residues (Glu-Ala-Glu-Ala-…), two residues (Glu-Ala-…) or none, due to the weak activity of Ste2 protease. Such amino acids that remain randomly at the N-terminal cannot be controlled during cultivation. Although such N-terminal heterogeneity does not affect the enzyme activity, the extra amino acids are problematic if a protein has an active site at the N-terminus (Koganesawa *et al.*, 2001; Daly and Hearn, 2005; Westereng *et al.*, 2011). Furthermore, if a recombinant protein is required for crystal structure analysis, this heterogeneity at N-terminus presents a serious problem, because crystallization may fail, or the crystal structure resolution may be reduced.

To avoid such problems, we routinely generate secretory constructs with no extra amino acids. For example, in the production of the Auxiliary Activities (AA) family 9 lytic polysaccharide monooxygenase (LPMO) from *Phanerochaete chrysosporium* in *P. pastoris*, the recombinant enzyme is active only when the natural N-terminal histidine of the enzyme is exposed (Westereng *et al.*, 2011). Subsequent structural analysis confirmed that this AA9 enzyme has an active site at the N-terminus, and that the enzyme activity requires coordination of a metal ion in the active site. We spent more than five years trying to identify the actual function of the enzyme just because of the effect of the extension generated from the MCS, indicating the importance of obtaining a recombinant enzyme as similar in structure as possible to the natural form, especially for a target protein whose function is not clear.

There are two ways to make a construct for secretory expression of a recombinant protein with the native N-terminal sequence, i.e., with a secretory signal sequence other than the α-factor signal (**Fig. 2** Pattern A) or without the Ste2 protease recognition site after the α-factor signal (**Fig. 2** Pattern B). In the case of pattern B, the target gene is introduced after the Lys-Arg sequence of the Kex2 protease recognition site located following the α-factor pre sequence. Since the Kex2 protease specifically cleaves the location immediately after the sequence, this construct can produce the target protein having a natural N-terminus. The secretory behavior is similar to that of the gene introduced using the MCS in this method, since the existing α-factor signal system can be used for transporting the translated protein to the ER. However, the type of N-terminal amino acid of the target protein affects the cleavage by Kex2 protease, because the substrate specificity of Kex2 depends on the amino acid at the recognition site (Manfredi *et al.*, 2016). In order to secrete a target protein incompatible with this restriction, it is necessary to use a secretory signal other than the α-factor signal.

In *P. pastoris*, it is possible to construct a secretory system using not only yeast-derived, but also non-yeast-derived enzymes, such as the yeast oligosaccharyl transferase subunit protein (OST1) signal, the yeast daughter cell-specific endo-1,3-*β*-glucanase (Dse4) signal, the plant lectin signal, the *Phaseolus vulgaris* agglutinin (PHA) signal, the *Rhizopus oryzae* alpha-amylase signal, and the human serum albumin signal (Forte *et al.*, 2011; Liang *et al.*, 2013; Raemaekers *et al.*, 1999; Li *et al.*, 2011; Xiong *et al.*, 2008). Predictive design of signal peptides is also underway with the aim of further improvement of productivity (Massahi and Çalık, 2016). Such a heterologous signal sequence is useful because it may have a secondary effect, such as increasing the expression level, though it is difficult to predict in advance what kind of signal sequence is likely to be effective for the expression of the target protein, and many combinations have to be tested. This uncertainty is an obstacle to applications of the *P. pastoris* system, because it is more laborious to construct a recombinant strain and to confirm target protein expression than in the case of *E. coli* systems. It is often necessary to use a trial-and-error approach.

Transformation

Most expression vectors for *P. pastoris* are designed to be introduced and retained at the target site in the genome, resulting in stable protein expression during cultivation. However, the transformation of *P. pastoris* requires several micrograms of plasmid DNA, whereas sufficient transformants can be obtained with a nanogram of plasmid when *E. coli* is used. This quantitative barrier has been an obstacle to the preparation of plasmids and the mutation of proteins especially for random mutagenesis. However, we recently developed a method to produce random mutant protein in *P. pastoris* using Phi29 polymerase, which at least partly overcomes this issue (Tachioka *et al.*, 2016).

Expression vectors designed to be introduced into the genome of *P. pastoris* are usually amplified in *E. coli*. When transforming such a plasmid, we have to linearize the plasmid at the promoter region or a selectable marker gene region before transformation (**Fig. 3**). In some vectors such as pPIC9K, it is possible to control the Mut phenotype, as described above, by changing the cleavage site in the plasmid. This makes it possible to evaluate the effect of the Mut phenotype on productivity of the target protein with one plasmid construct.

Both chemical (spheroplasts, LiCl) and electrical methods, as used for general yeast transformation, are suitable to introduce the plasmid (Cregg, 2007b). Electroporation is widely used because it is simple and sufficient transformants can easily be obtained. After gene introduction, *P. pastoris* cells are screened using an enriched medium such as Yeast extract/Peptone/Dextrose/Sorbitol (YPDS) with an antibiotic or a semi-synthetic medium such as Regeneration Dextrose (RD) medium in which the amino acid of interest is restricted. By adding sorbitol to the medium, it is possible to form colonies with slightly raised morphology instead of normal flat morphology. In the transformation of *P. pastoris*, one to several plasmids are inserted into the genome. In many cases, when more plasmids are inserted, the transcription amount of mRNA increases, causing an improvement of expression. Therefore, it is important to select colonies that are expected to contain more plasmids from the screened plates. For example, increasing the number of expression cassette integrations (copy number) from one to 14 for the tetanus toxin fragment C protein increased the level of protein expression by 6-fold (Clare *et al.*, 1991a; Daly and Hearn, 2005). In order to accurately evaluate the number of introduced plasmids in *P. pastoris* transformants, Southern blot analysis can be performed (Inan *et al.*, 2007). In our group, we first pick up larger and raised colonies from the screening plate and perform initial screening by small-scale cultivation. In our experience, large colonies tend to show higher expression of the target protein compared with smaller colonies.

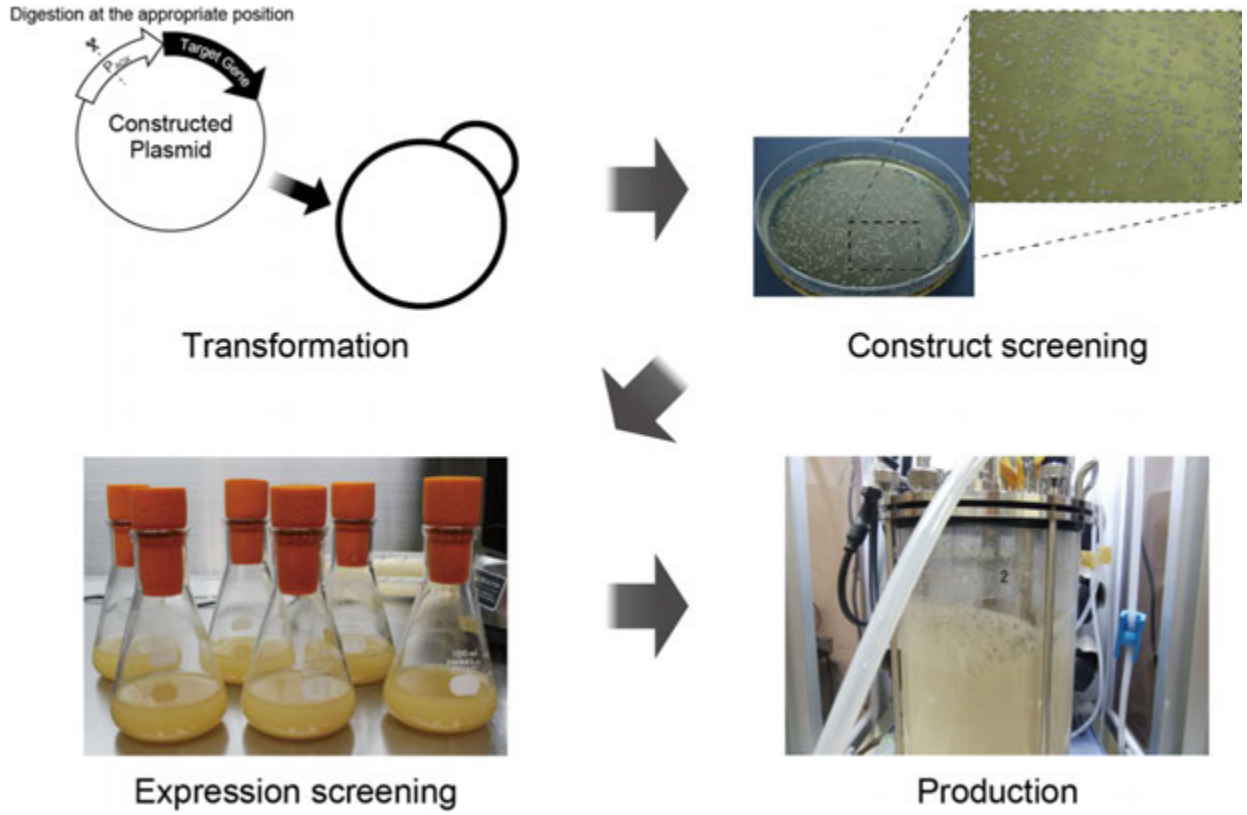

Fig. 3 Schematic flow chart of screening to optimize recombinant protein expression.

In order to select colonies with higher expression in plate screening, it is also effective to use a plate medium with an increased antibiotic concentration. In the case of the antibiotic zeocin, it is reported that increasing the concentration from 100 μg/mL to 2000 μg/mL increased the hepatitis B surface antigen (HBsAg) gene number from 1 to 4 copies (Vassileva *et al.*, 2001). When G418 is used as a selection marker, the number of target gene copies can reach 30 (Clare *et al.*, 1991b).

Cultivation

Medium and General Conditions

Recombinant proteins can be produced in *P. pastoris* in a wide variety of media, from semi-synthetic medium such as yeast extract/peptone/dextrose (YPD) medium to synthetic medium such as minimal salt medium (Darby *et al.*, 2012). These media are generally less expensive than the media used for Chinese hamster ovary (CHO) cells and baculovirus-insect cell expression systems, and this is one of the advantages of using *P. pastoris* expression systems (Lebozec *et al.*, 2018).

In general, protein production in *P. pastoris* is usually performed in the temperature range of 20–30°C and the efficiency depends upon the cultivation temperature, as is the case for many other recombinant protein expression hosts (Dragosits *et al.*, 2009). Shaking or stirring speed during cultivation is also an important factor, because sufficient aeration is necessary for cell growth (oxygen is required for methanol metabolism when the AOX promoter is used). Expression mediated by the GAP promoter involves one-step cultivation in both the cell-growth and protein-production phases. In contrast, two-step cultivation is required when the AOX promoter is used.

Buffered minimal glycerol complex (BMGY) medium and yeast extract/peptone/glycerol (YPG) medium are generally used for cell growth in flask-scale culture. In these media, glycerol is the carbon source. A medium containing glucose as the carbon source, such as YPD medium, can also be used. In order to perform methanol-induced protein production in a flask, grown cells have to be transferred to a medium containing methanol to remove residual glycerol and glucose, because the AOX promoter is repressed by glycerol and glucose (Cereghino and Cregg, 2000; Zhang *et al.*, 2010). On the other hand, in the case of constitutive expression with the GAP promoter, such differences of carbon sources have little influence on protein production, and continuous feeding of glycerol or glucose is not problematic.

Buffered minimal methanol complex (BMMY) or other similar media are generally used for methanol induction, but it is also possible to add methanol to a medium containing no carbon source, such as yeast extract/peptone (YP) medium. The concentration of methanol used is generally about 0.5%–1.0%. It is desirable to add the same amount of methanol approximately every 24 h for Mut^s phenotype strains and approximately every 12 h for Mut^+ phenotype strains. In the methanol-metabolizing phase, the cells are exposed to two kinds of stress: methanol metabolism and recombinant protein production (Vanz *et al.*, 2012). The optimum

methanol concentration depends on the type of target protein to be expressed, and addition of an excessive amount of methanol can sometimes kill the yeast strain. Moreover, the methanol-metabolizing system in yeast is not available immediately after switching the carbon source to methanol (Minning *et al.*, 2001). Therefore, excessive addition of methanol can be most damaging immediately after switching the culture phase. Under appropriate cultivation conditions, *P. pastoris* strains produce the target protein continuously for about seven days, though prolonged induction also causes cell lysis and leads to activation of proteases.

When *P. pastoris* grows well and consumes its carbon source effectively, the pH of the non-buffered culture becomes acidic, whereas the pH becomes alkaline under unfavorable cultivation conditions. There are multiple reasons for this increase of pH, including deficiency of nutrient carbon source and toxicity due to excessive addition of methanol. In some cases, synthesis of the target protein itself causes stress related to folding and secretion in the ER, which finally leads to cell lysis (Mattanovich *et al.*, 2004). This is often responsible for poor protein expression of the target. As an approach to solve this problem, co-expression with protein disulfide isomerase (PDI) and a molecular chaperone has been reported (Inan *et al.*, 2007). These co-expressed proteins support folding of the target protein, which is expected to improve the production.

Control of the pH of the medium also has an important influence on the degradation of the target protein by endogenous proteases. Wild-type *P. pastoris* has high levels of vacuolar proteases, protease A (*pep4*), protease B (*prb1*), and carboxypeptidase Y (*prc1*). In particular, protease B, which is a serine protease, exhibits relatively strong activity at neutral to alkaline pH (Nowak and Tsai, 1989; Bolumar *et al.*, 2005). In the case of production of glycoside hydrolase, in which two domains are connected by a hydrophilic linker peptide, the linker part is frequently degraded by this enzyme. To overcome this problem, protease-deficient strains such as SMD 1168 are useful (Gleeson *et al.*, 1998), but cultivation in an acidic environment, addition of protease inhibitors, addition of supplements, etc. are also useful to control the degradation. *P. pastoris* is capable of growing over a relatively broad pH range (3.0–7.0) (Macauley-Patrick *et al.*, 2005). Since the serine protease is most active in an alkaline environment, its activity can be suppressed by culturing under acidic conditions. In actual trials, good productivity was achieved under a strongly acid condition at pH 3, but in other cases neutral conditions were optimal, so a trial-and-error approach is necessary to find the optimum condition for each target protein (Brierley *et al.*, 1994; Clare *et al.*, 1991b). The use of a medium containing a buffer component such as BMMY is effective to maintain the pH at a desired level in flask culture. Although the use of protease inhibitor is expensive, it is an effective method. For example, phenylmethylsulfonyl fluoride (PMSF) is useful because it can irreversibly suppress the potent degradative activity of serine protease (Woo *et al.*, 2004). PMSF itself is extremely unstable in water, but this can be overcome by adding PMSF to a fed-batch methanol substrate in the case of methanol-induced culture. Supplements such as casamino acid and yeast extract can indirectly protect the target protein by serving as decoy substrates for proteases (Werten *et al.*, 1999; Clare *et al.*, 1991a). Furthermore, addition of these supplements can also prevent cell lysis due to lack of an nitrogen source.

Thus, optimal culture conditions for protein production by *P. pastoris* have to be assessed for each individual target protein. However, if the basal expression level of the target protein is high, detailed examination of culture conditions may not be necessary. On the other hand, it is essential when the expression level of the target protein is low.

Cultivation in a Jar Fermenter

Jar-fermenter cultivation (also called bioreactor cultivation) is a useful method to maximize the production of recombinant proteins from *P. pastoris*. Especially when methanol induction is used, a larger amount of crude enzyme solution can be obtained compared with culture in a shaken flask. In flask cultivation, the culture has to be concentrated before the methanol induction step to less than 1/5th of the initial amount, whereas in a fermenter, this step is not necessary. The expression levels in jar-fermenter cultures are typically up to 10 times higher than those in flasks (Daly and Hearn, 2005).

In jar-fermenter cultivation of *P. pastoris*, batch feeding can be used, and the cells can be cultivated to a higher density than in flask culture. This results in higher recombinant protein productivity in the fermenter (Stratton *et al.*, 1998; Zhang *et al.*, 2007). There are several methods to monitor the culture conditions, i.e., measurement of methanol concentration with a methanol sensor or enzymatic measurement of dissolved oxygen (DO) with an oxygen electrode. Fourier-transform mid-infrared spectroscopy (FT-MIRS) has also been applied to monitor the culture components (Guarna *et al.*, 1997; Crowley *et al.*, 2000; Hellwig *et al.*, 2001; Surribas *et al.*, 2003). The former two methods are often adopted based on low cost and easy maintenance.

In jar-fermenter culture, the use of a minimal salt medium is also useful because it allows sufficient cell growth to continue during batch feeding. Minimal salt medium such as basal salt medium (BSM) or FM22 medium does not contain contaminating proteins derived from medium components, which is a considerable advantage for easy purification of the target protein after production (Laroche *et al.*, 1994; Darby *et al.*, 2012). Aqueous ammonia is used for adjusting pH and as a nitrogen source. On the other hand, the presence of an excess of ammonia ions reduces production of the target protein, and it is important to identify the optimum concentration in order to optimize the target protein production (Xie *et al.*, 2003; Yang *et al.*, 2004).

Post Cultivation and Purification

Crude recombinant protein secreted and produced by *P. pastoris* is modified with N- and O-glycans. In particular, the N-glycosylation in *P. pastoris* is of high-mannose type, and alters the apparent molecular weight in SDS-PAGE (Bretthauer and Castellino, 1999). Moreover, hyper-glycosylation with a large amount of mannose sugar chains may occur, resulting in an apparent increase in

molecular weight of over 100 kDa (Scorer *et al.*, 1993). In addition, since the amount of the added sugar chains is not uniform, the obtained recombinant protein sometimes appears as a smear band on SDS-PAGE. In such cases, enzyme treatment to cleave the sugar chains is typically performed. Enzymes such as PNGase, EndoH, and α-mannosidase are used to remove N-glycans. Among them, PNGase cleaves the glycosidic bond between N-glycan and amino acid (asparagine), while EndoH cleaves the glycan between the initial and second N-acetyl glucosamine residues (Tarentino *et al.*, 1985; Trimble and Tarentino, 1991). However, the sugar chains are close to the protein backbone, and the treatment may be unsuccessful because the sugar chains are not readily accessible. In such cases, α-mannosidase can be used to trim sugar residues from the other end of the glycan (Li, 1966; Gnanesh Kumar *et al.*, 2014).

Generally, recombinant enzymes are purified from the crude enzyme culture solution by column chromatography. However, as described above, the crude enzyme solution still contains a large amount of protease, so care must be taken in controlling the temperature and pH. In our group, an initial purification step by hydrophobic chromatography is first carried out to roughly remove proteases and components of the medium, and then other purification procedures are applied (Igarashi *et al.*, 2012). Hydrophobic chromatography is employed because the pH can be freely selected, and the chromatography is easy to operate at a low pH to prevent protease-mediated cleavage of the recombinant protein.

Conclusion

The production of recombinant proteins by *P. pastoris* requires careful examination of constructs, selection of transformants, and optimization of culture conditions. However, after optimization, a large amount of the target enzyme can be obtained at moderate cost, which is especially advantageous for functional studies. In addition, the ability of *P. pastoris* to mediate post-translational modifications such as glycan attachment is important for the production of eukaryotic proteins. Of course, there are still some enzymes that cannot yet be produced in *P. pastoris*, but the technology is continually improving. We hope this chapter provides a helpful guide to the state of the art in recombinant protein production using *P. pastoris*.

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Transcriptional Regulation: How Saprobic Fungi Tune the Production of Plant Cell Wall Degrading Enzymes

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Introduction

Plant cell wall degrading enzymes (PCWDEs) produced by saprobic fungi have activity towards a wide array of polysaccharides such as cellulose, hemicelluloses and pectin. These enzymes have major applications in biotechnology, *e.g.*, for the saccharification of lignocellulose for second generation biofuel production (US-DOE, 2006) and clarification of fruit juices in the beverage industry (Kashyap *et al.*, 2001).

Fungi have evolved transcriptional regulatory mechanisms of enzyme production because different polysaccharides require particular sets of enzymes for their degradation and these enzymes are energetically costly for the fungus to produce. Transcription factors, the key regulatory proteins involved in these mechanisms, respond to environmental inputs and adjust expression of PCWDE-encoding genes to ensure optimal utilization of nutrients.

In this chapter, we discuss the main transcription factors that regulate production of PCWDEs in saprotrophic fungi, such as *Aspergillus* spp. and *Trichoderma reesei*. Fungi with a saprobic lifestyle degrade dead plant matter in nature and the saprobic lifestyle is beneficial for obtaining generally regarded as safe (GRAS) status for applications using these fungi and their enzymes. Furthermore, we outline methods used for transcription factors discovery and rational strategies for the engineering of transcription factors that result in strains with improved enzymatic capabilities. Finally, we provide insights into where the field might be moving to and highlight the areas that require more research in the future.

The Molecular Basis of PCWDE Production

There are five critical steps leading to a fungus producing a PCWDE. The first step is the presence of an environmental inducer which is most commonly a sugar released from plant biomass by constitutive or starvation-induced enzymes (van Munster *et al.*, 2014). In the second step, the reception of the inducer activates signaling cascades, leading to changes in the phosphorylation status (Horta *et al.*, 2019) and activation of transcription factors (for review of signaling cascades see Brown *et al.* (2014)). These transcription factors are the major final receivers of the signal, and in the third step, the DNA-binding domain of the transcription factor attaches to particular sequence motifs in a gene promoter and activates or represses transcription. Several hundred transcription factors are usually found in fungal genomes and can be categorized into approximately 80 DNA-binding domain families such as Zn_2Cys_6 zinc binuclear cluster superfamily (Shelest, 2017). Transcription factors in the fungal kingdom appear to have evolved substantially after the major phyla diverged (Todd *et al.*, 2014). Transcription factors are dynamic molecules that can move about inside the cell; *e.g.*, a study in *T. reesei* showed how the CRE1 transcription factor shuttles from the nucleus to the cytoplasm in response to external stimuli (Lichius *et al.*, 2014). A recent review summarized currently identified fungal transcription factors involved in plant biomass degradation (Benocci *et al.*, 2017). The fourth and fifth steps involve translation of the transcript and secretion of the enzyme, respectively.

Transcriptional regulation is especially important on/off control switch in the production of quickly secreted PCWDEs. Other regulatory mechanisms after translation of the transcript available for intracellular proteins (*e.g.*, proteolytic degradation) are not available to regulate secreted PCWDEs.

Transcriptional Regulation of PCWDEs in Fungi

Environmental Inducers and Their Corresponding Transcription Factors

Sugars or their metabolic conversion products are the major inducers of genes encoding plant biomass degrading enzymes

Complex carbon sources such as various types of crude plant biomass can induce a broad-spectrum of PCWDE-encoding genes in *Aspergillus niger* (Daly *et al.*, 2017; Khosravi *et al.*, 2019) and *T. reesei* (Häkkinen *et al.*, 2012; Bischof *et al.*, 2013; Novy *et al.*, 2019). Induction of these genes can largely be attributed to sugars released from the plant biomass as shown by analysis of expression changes in *A. niger* in response to a range of plant-derived monosaccharides (Gruben *et al.*, 2017). This sophisticated system enables the fungus to anticipate and tailor its response to the specific polymers present in the environment, and reduce the transcription of PCWDE-encoding genes as soon as the polymers are depleted. As with most biological phenomena, there are exceptions, such as how the disaccharide lactose functions as a major inducer of genes encoding hydrolytic enzymes in *T. reesei*

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Table 1 Summary of the major transcription factors from *A. niger* and *T. reesei* involved in the regulation of PCWDE-encoding genes. The major sugars (or sugar metabolites) that ultimately lead to induction of PCWDEs *via* these transcription factors are indicated

Species	*Transcription factor*	*Major sugars that signal through*	*Reference*
Aspergillus niger	XlnR	D-xylose	van Peij *et al.* (1998)
	AraR	L-arabinose	Battaglia *et al.* (2011)
	GaaR	2-keto – 3-deoxy-L-galactonate	Alazi *et al.* (2017)
	CreA	D-glucose, other favorable sugars *e.g.*, D-xylose in high concentrations	Ruijter *et al.* (1997), de Vries *et al.* (1999)
	RhaR	L-2-keto – 3-deoxyrhamnonate	Chroumpi *et al.* (2020)
Trichoderma reesei	XYR1	D-xylose, lactose	Stricker *et al.* (2006, 2007)
	ARA1	L-arabinose, D-galactose	Benocci *et al.* (2018)
	CRE1	D-glucose	Lichius *et al.* (2014)

(Bischof *et al.*, 2013) even though lactose is not a component of plant biomass. It is speculated that *T. reesei* responds to lactose because the disaccharide contains ß-D-galactoside linkages similar to those found in xyloglucan (Ivanova *et al.*, 2013). An alternative speculation is that lactose is structurally similar to another hexose disaccharide inducer called sophorose which may lead to the molecular components that recognize sophorose also recognizing lactose. Sophorose is a transglycosylation product of cellobiose and is another major inducer of PCWDE genes in *T. reesei* (Sternberg and Mandels, 1979).

The expression patterns of PCWDE-encoding genes are multifaceted and the expression on individual sugars cannot comprehensively explain the expression pattern on crude plant biomass as multiple sugars can induce the same genes (Gruben *et al.*, 2017; Kowalczyk *et al.*, 2017; Escalante-Chong *et al.*, 2015). Furthermore, the true inducer may be either the sugar released from the plant biomass or a metabolic conversion product of the sugar *e.g.*, the true inducer of the transcriptional activator of pectin-degrading genes RhaR is a catabolic conversion product of L-rhamnose that is released from pectin (Chroumpi *et al.*, 2020). This feature also has implications on whether transporters are required for induction, as the metabolic conversion product that acts as the inducer can only be generated after the sugar released from the plant biomass is imported inside the cell where metabolic conversions occurs.

There are a range of transcription factors that receive the signal from the inducing sugar (or a metabolic conversion product of the sugar) and subsequently activate the transcription of the PCWDE-encoding genes (**Table 1**). The most well-known broad-spectrum PCWDE-activating transcription factor is XlnR/XYR1. In *A. niger*, the XlnR transcription factor was identified through complementation of a mutant deficient in xylanase activity with a genomic library (van Peij *et al.*, 1998). As well as xylanases, XlnR in *A. niger* activates a broad-range of PCWDE genes including those encoding cellulases and pectinases (Gruben *et al.*, 2017; de Souza *et al.*, 2013). In *T. reesei*, XYR1 (Stricker *et al.*, 2006; Rauscher *et al.*, 2006), which was identified based on homology to XlnR, activates cellulase and xylanase gene expression (Benocci *et al.*, 2019; Ma *et al.*, 2016). However, it is difficult to generalize on the regulon (*i.e.* the genes regulated by a transcription factor) across different species that possess the same orthologous transcription factor; *e.g.*, large differences were found in the set of genes controlled by XlnR/Xyr1 in five ascomycete fungi from a proteomic analysis (Klaubauf *et al.*, 2014).

XlnR/XYR1 is not always the major transcription factor for activation of genes encoding enzymes involved in plant biomass degradation. In *Neurospora crassa*, CLR-1 & CLR-2 are the major transcription factors that activate cellulolytic gene expression (Coradetti *et al.*, 2012). In *A. niger* and *T. reesei*, there are also other transcription factors that regulate expression of PCWDE-encoding genes (**Table 1**). The *A. niger* transcription factor AraR, which likely originated from a duplication of XlnR (Battaglia *et al.*, 2011), activates genes involved in the degradation of hemicelluloses and pectin (de Souza *et al.*, 2013). In *T. reesei*, a corresponding function is performed by the ARA1 transcription factor (Benocci *et al.*, 2018). ARA1 is not a genetic ortholog of AraR, but performs a similar function, *i.e.*, is a functional ortholog that has arisen as a result of convergent evolution (Benocci *et al.*, 2018).

Other environmental inducers of genes encoding plant biomass degrading enzymes

While nutrient signaling is the major activator of PCWDE-encoding genes, other environmental variables such as pH and light can activate or contribute to the level of activation of gene expression (**Fig. 1**). Expression of genes encoding PCWDEs is responsive to pH whereby the fungus attempts to ensure that enzymes will be produced under pH conditions where they will have optimal activity. In *T. reesei* approximately 50 PCWDE-encoding genes were responsive to changes in pH (Häkkinen *et al.*, 2015). The transcription factor PacC/PAC1 is the key regulator responsive to changes in ambient pH; *e.g.*, in *Aspergillus nidulans*, during alkaline conditions PacC activates alkaline-expressed genes and represses acidic-expressed genes (Tilburn *et al.*, 1995). Additionally, expression of genes involved in plant biomass degradation is light-responsive as reviewed for *Trichoderma* by Schmoll (2018). In *T. reesei*, PCWDE-encoding gene expression is upregulated in light compared to dark conditions (Tisch *et al.*, 2011) and the blue light regulators 1 and 2 (BLR1 and 2) are key transcription factors/photoreceptors involved in the light response (Casas-Flores *et al.*, 2004).

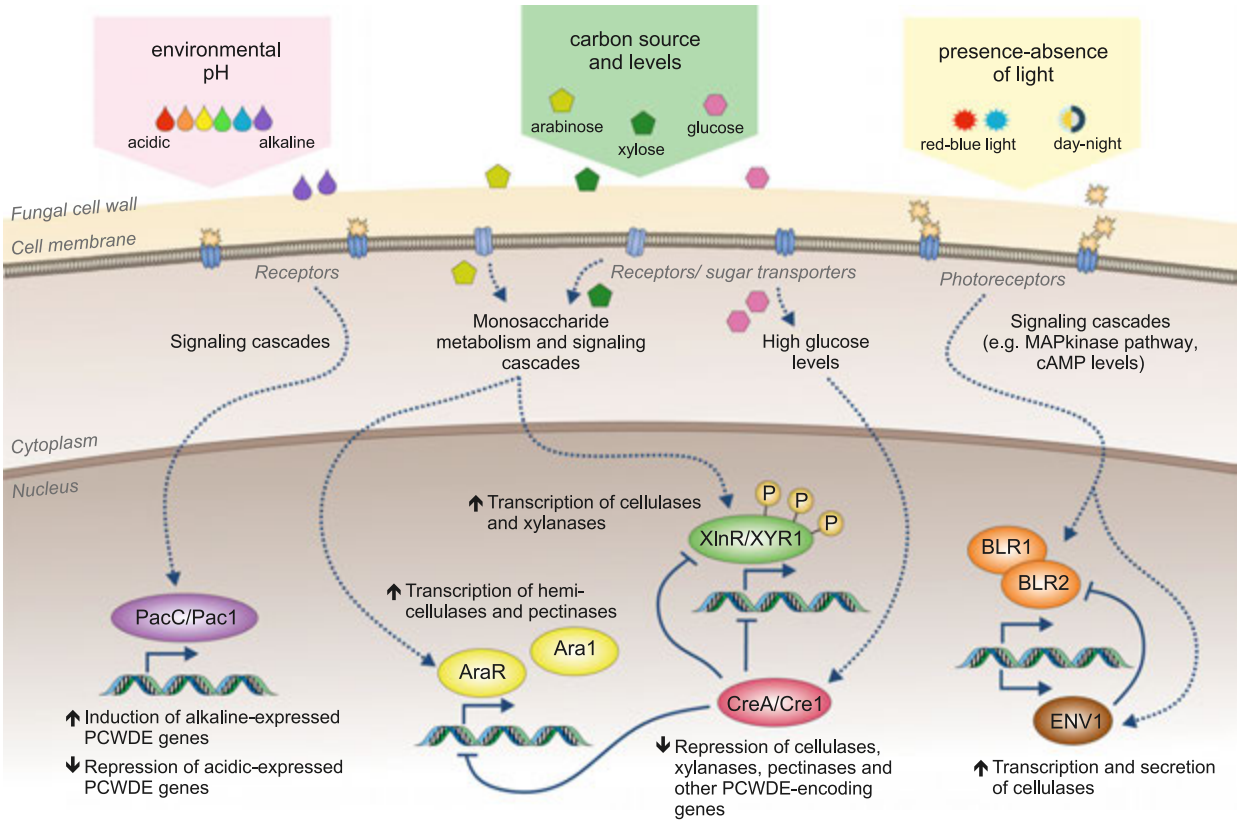

Fig. 1 Schematic overview of the environmental factors that contribute to the regulation of PCWD enzymes in saprobic fungi. Environmental signals (*e.g.*, presence of an inducing sugar) are sensed and/or transported into fungal cell, where they initiate signaling pathways leading to activation of responsive transcription factors. Transcription factors then enter the nucleus, bind to the promoter regions of their target genes and activate (or repress) transcription of PCWDE-encoding genes.

Environmental Repressors and Their Corresponding Transcription Factors

Factors in the environment also function as repressors of PCWDE gene expression, in particular those related to catabolite repression mechanisms. Carbon catabolite repression (CCR) occurs in the presence of sufficient concentrations of easily metabolizable carbon sources (*e.g.*, glucose), down-regulating the expression of PCWDE-encoding genes involved in the breakdown of complex carbon sources such as plant biomass as reviewed recently by Adnan *et al.* (2018). The wide-domain transcriptional repressor CreA/CRE1 is the key regulator of CCR in ascomycete fungi as reviewed by Benocci *et al.* (2017). CreA/CRE1 binds to the promotors of PCWDE-encoding genes when simple sugar concentrations are sufficiently high, thus preventing gene activation (**Fig. 1**). CCR can also occur independent of CreA such as by the exclusion of the inducing sugar as occurs in *A. nidulans* in the exclusion of L-rhamnose under glucose repressing conditions (Tamayo-Ramos *et al.*, 2012).

Regulation of Transcription Factor Activity

How the activity of transcription factors is regulated is multifaceted. Transcription factors can be autoregulated whereby the promoter contains binding motifs for the transcription factor whose expression it drives. For example, the expression of CreA/CRE1 in *T. reesei* is suggested to be autoregulated (Ilmén *et al.*, 1996). The expression of other transcription factors is also influenced by wide-domain regulators, *e.g.*, the wide-domain transcriptional repressor CreA/Cre1 can repress the expression of XlnR in *A. nidulans* (Tamayo *et al.*, 2008). Post-translationally, the phosphorylation status is a key regulator of transcription factor activity, influenced by kinases and phosphatases that add and remove phosphate groups changing the localization, interaction and stability properties of the transcription factor. In *Aspergillus oryzae*, the reversible phosphorylation of XlnR is triggered by xylose, with the active form of XlnR being highly phosphorylated (Noguchi *et al.*, 2011).

Methods for Discovery of Transcription Factors and Identification of Their Regulons

Discovery of transcription factors involved in plant biomass degradation in saprobic fungi began in the 1970s with several mutant selection screens (Bailey and Arst, 1975; Arst and Bailey, 1977) leading to identification of the CreA repressor in *A. nidulans* (Dowzer and Kelly, 1989, 1991). However, the real breakthrough in the field occurred in the post-genomic era, after many fungal

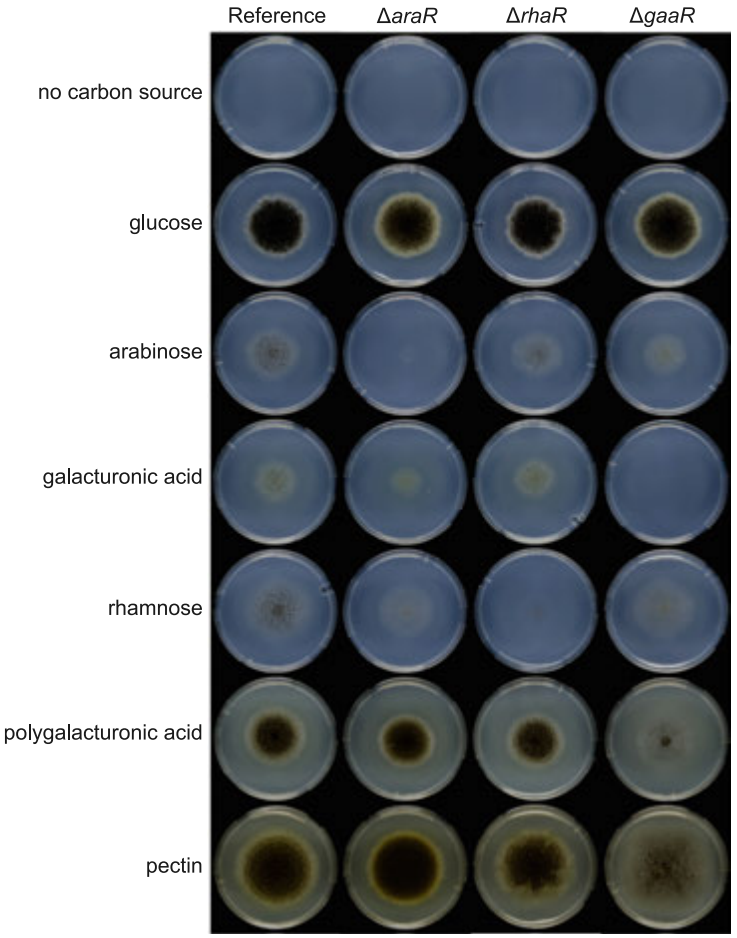

Fig. 2 Growth profiling of regulatory mutants on a range of plant biomass related carbon sources demonstrating the ability of this technique for phenotypic characterization. Reduced growth of regulatory mutants on monosaccharides (*e.g.*, rhamnose and galacturonic acid) indicates that a corresponding transcription factor regulates sugar uptake and/or metabolism, while reduced growth on polymeric carbon sources indicates that production of PCWDEs is reduced. Modified from Kowalczyk, J.E., Lubbers, R.J.M., Peng, M., *et al.*, 2017. Combinatorial control of gene expression in Aspergillus niger grown on sugar beet pectin. Scientific Reports 7, 12356.

genome sequences became available. The major approaches for discovery of transcription factors regulating PCWDE-encoding gene expression involve forward and reverse genetics. The former was a dominant method in the pre-genomics era before genome sequencing became commonplace and usually involved a random mutagenesis screen in an attempt to identify the genes responsible for the altered phenotype. Growth deficiency of mutants on particular carbon sources (**Fig. 2**) and reduced enzymatic activities are critical phenotypes that facilitated forward genetic approaches for identification of transcriptional regulators of genes involved in plant biomass utilization. The previous section described how XlnR in *A. niger* was identified by a forward genetic screen (van Peij *et al.*, 1998). Also, a forward genetic screen for constitutive expression of pectinases in *A. niger* discovered the transcriptional repressor GaaX (Niu *et al.*, 2017).

Reverse genetics uses bioinformatic methods to select candidates that are then functionally characterized using gene deletion or gene knockdown (*e.g.*, RNAi) approaches. Candidates can be selected using sequence homology; *e.g.*, in *A. niger* AraR was selected based on its close sequence similarity to XlnR from the same species (Battaglia *et al.*, 2011) and GaaR (Alazi *et al.*, 2016) was selected based on sequence similarity to GaaR from *Botrytis cinerea* (Zhang *et al.*, 2016). In *T. reesei*, XYR1 (Rauscher *et al.*, 2006) was selected based on sequence similarity to XlnR sequences from *Aspergillus* spp. Alternatively, co-regulation of expression with PCWDE-encoding genes can select candidate transcription factors; *e.g.*, RhaR in *A. niger* (Gruben *et al.*, 2014), ACE3 in *T. reesei* (Häkkinen *et al.*, 2014) and Ara1 in *Magnaporthe oryzae* (Klaubauf *et al.*, 2016). Genomic co-localization with other transcription factors was used to select GalX in *A. nidulans* (Christensen *et al.*, 2011) and co-localization with genes encoding related metabolic enzymes was used to select InuR in *A. niger* (Yuan *et al.*, 2008). Interestingly, high efficiency of gene deletion in certain fungal species led to the reverse genetic approach being used to generate and screen libraries of transcription factor deletion mutants, leading to identification of ManR in *A. oryzae* (Ogawa *et al.*, 2012) and CLR-1 and CLR-2 in *N. crassa* (Coradetti *et al.*, 2012), among others.

Identification of a transcriptional regulator is usually linked to identification of its regulon, *i.e.*, a group of genes that are regulated in a similar manner. Transcriptomic analysis using transcription factor deletion mutants is an essential technique to

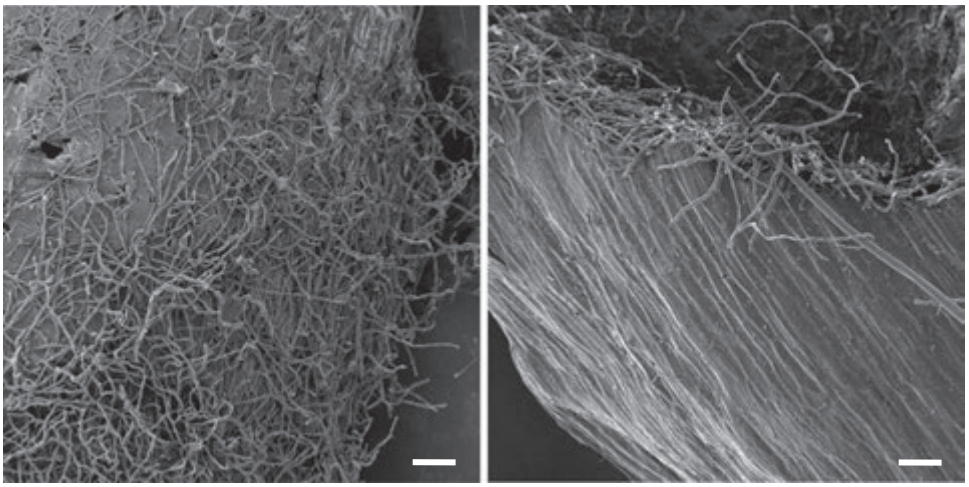

Fig. 3 Helium ion microscopy (HIM) images of the colonization of crude wheat bran flakes by an *A. niger* reference strain (left) and an Δ*xlnR* mutant (right). The impaired colonization ability of the regulatory mutant is a result of reduced production of PCWDE when compared to the reference strain. The scale bars represent 50 μm.

identifying the regulon of a particular transcription factor, as was shown *e.g.*, by analysis of the *xyr1* mutant of *T. reesei* on crude plant biomass (Benocci *et al.*, 2019). Additionally, to understand the relative importance of a transcription factor for overall growth and/or colonization abilities, regulatory mutants can be assayed using various visualization techniques such as growth profiling (**Fig. 2**) and high-resolution microscopy (**Fig. 3**).

It is worth mentioning that appropriate culturing conditions are critical to identifying primary effects (*i.e.*, genes that are likely bound by the transcription factor) from secondary effects (*e.g.*, a general stress response such as starvation). For example, analysis of the promoters of genes in a regulon for putative binding motifs, if known, can provide support for the gene being directly regulated by a transcription factor.

Manipulation of Transcriptional Regulation of PCWDEs in Biotechnology

In biotechnology, the manipulation of transcriptional regulation of PCWDEs has journeyed from random mutagenesis to rational engineering approaches. A major objective of commercial enzyme producers is to produce a desired enzyme cocktail without the need for the addition of potentially expensive inducers, *i.e.*, inducer independent production of PCWDEs. From random mutagenesis followed by screening for improved cellulase production and catabolite depression, the cellulase hyperproducer *T. reesei* strain RUT-C30 was generated as reviewed by Peterson and Nevalainen (2012). It was shown that altered transcriptional regulation was responsible for the catabolite depression due to truncation of the CRE1 transcriptional repressor in the RUT-C30 strain (Ilmén *et al.*, 1996). A recent study suggested that the truncation of CRE1 may have turned it into an activating regulator acting on the promoter of *xyr1* (Rassinger *et al.*, 2018).

Rational engineering of the regulatory system involves deletion or disruption of repressing transcription factors, overexpression of activating transcription factors and protein domain swapping or removal. Disruption of the CreA/CRE1 transcription factor is routinely used to improve enzyme production yields under catabolite repressing conditions (Yao *et al.*, 2015; Wang *et al.*, 2013). It is worth noting that disruption of the wide-domain regulator CreA/CRE1 shows phenotypes other than the desired elevated PCWDE production under repressing conditions such as smaller colonies (Nakari-Setälä *et al.*, 2009; Khosravi *et al.*, 2018). Overexpression of the activator GaaR in *A. niger* led to increased expression of pectinase genes in the absence of the D-galacturonic acid inducer of these genes (Alazi *et al.*, 2018). Modification of the domain structure of ManR (ClrB) from *A. niger* by fusing the DNA binding and transcriptional activation domain and exclusion of the middle domain resulted in the production of mannanases in a glucose medium (Gao *et al.*, 2019). Further strategies and examples can be found in a recent review of rational engineering strategies for PCWDE production (Alazi and Ram, 2018). Rational engineering approaches can also target the promoters of PCWDE-encoding genes for modification as reviewed by Fitz *et al.* (2018), such as the replacement of a native promoter with a constitutive promoter. Recently, the genome editing tool CRISPR-Cas9 has emerged as a promising tool for engineering strains (Nødvig *et al.*, 2015), such as a more efficient approach to edit transcription factors into a constitutively active form in *A. niger* (Kun *et al.*, 2020).

Research Technologies and Approaches for Future Research

There are many developing technologies and techniques in the fast-moving field of transcriptional research that have yet to be applied to the regulation of genes involved in plant biomass degradation in fungi. The accuracy and sensitivity of transcription

factor binding to DNA can be increased with methods such as SELEX-seq technology which can identify low affinity transcription factor binding sites (Rastogi *et al.*, 2018). Incorporating transcription factor shape-motif prediction alongside sequence motifs is a developing field in transcription factor research (Samee *et al.*, 2019). With the numbers of sequenced fungal genomes increasing rapidly over the past decade (Grigoriev *et al.*, 2014), high throughput gene deletion and functional characterization of transcription factors from these genomes is critical for future advances in the field. This will not only advance our understanding of a transcription factor function, but also allow for a far greater understanding of the diversity of transcriptional regulation of PCWDE-encoding genes across the fungal tree of life. Consequently, knowledge of functional diversity can inform rational strategies for the engineering of transcription factors and their binding motifs. In particular, the understanding of transcriptional regulation is still limited in wood rotting fungi and generally in other fungal species from the major fungal phylum of basidiomycetes (Mäkelä *et al.*, 2020).

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Bioinformatics Approaches for Fungal Biotechnology

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Introduction

Bioinformatics analysis plays a crucial role in today's biological research. Being an interdisciplinary branch of the life sciences, bioinformatics integrates computer science and mathematical methods to reveal the biological significance behind the continually increasing biological data. It does this by developing methodology and analysis tools to explore the large volumes of biological data, helping to query, extract, store, organize, systematize, annotate, visualize, mine, and interpret complex data. In this chapter, we aim to give a general overview on the basic operations and new developments of bioinformatics applications in fungal research, especially focusing on describing the bioinformatics tools and methods applied in fungal genomics, transcriptomics, proteomics and metabolomics studies, as well as the integrative analysis of multiple omics datasets. For each aspect, we highlight several examples to demonstrate how various bioinformatics methods have been successfully used to mine fungal omics data and improve our understanding of fungal physiology and evolution, specially emphasizing bioinformatics applications related to fungal plant biomass conversion.

Current Status of Fungal Genomic Analysis

The sequenced genome together with its annotation is the basis for understanding the biological process and evolutionary history of an organism. Due to the wide application of next generation sequencing (NGS) technology, biological research has entered the genome era. More than 1500 fungal genomes have been sequenced and still many more genomes are in progress since the first fungal genome sequence (*Saccharomyces cerevisiae*) was published in 1996 (Goffeau *et al.*, 1996). For instance, a broad range of reference fungal genomes have been sequenced, including the yeast *Schizosaccharomyces pombe* (Wood *et al.*, 2002), the filamentous saprobes *Neurospora crassa* (Galagan *et al.*, 2003), *Aspergillus nidulans* (Galagan *et al.*, 2005), *Aspergillus niger* (Pel *et al.*, 2007) and *Trichoderma reesei* (Martinez *et al.*, 2008), the filamentous cereal pathogens *Magnaporthe grisea* (Dean *et al.*, 2005), *Ustilago maydis* (Kamper *et al.*, 2006) and *Fusarium graminearum* (Cuomo *et al.*, 2007), and the white rot basidiomycete *Phanerochaete chrysosporium* (Martinez *et al.*, 2004). Moreover, the sequenced genomes together with their annotation information can be easily accessed from a list of the databases dedicated to fungal genomes (**Table 1**). The rapidly increasing amount of fungal genomics data not only provides us with a better understanding of the fundamental cellular processes, but also benefits a broad range of fungi related applications in human health, agriculture, enzyme biotechnology, bioenergy, and ecological diversity (Sharma, 2016).

Bioinformatics Workflow and Tools for Genomics Analysis

The advent of NGS technologies is accompanied with a rapid development of numerous bioinformatics methods and tools that were created to handle the vast amount and different types of data generated by different sequencing platforms. A specific set of bioinformatics tools are needed to process data in each different step of genomic analysis. Space and expertize constraints do not allow us to cover all bioinformatics tools deployed in the genomics field and here we mainly focus on tools that are frequently used in the community.

The past decades have witnessed dramatical changes in sequencing technologies. Early efforts of fungal genome sequencing were mainly made by the Sanger sequencing method, such as the genome sequencing of *S. cerevisiae* (Goffeau *et al.*, 1996), *T. reesei* (Martinez *et al.*, 2008), *P. chrysosporium* (Martinez *et al.*, 2004) and *Agaricus bisporus* (Morin *et al.*, 2012). However, due to the high cost and time-consuming use of the Sanger method, more recent genomes were mainly sequenced by alternative sequencing platforms, called "next generation sequencing (NGS)". Several NGS sequencing platforms are widely used by researchers, including Roche 454, Illumina,

Table 1 List of databases dedicated to fungal genomes

Database	*URL*
MycoCosm	https://mycocosm.jgi.doe.gov/mycocosm/home
Joint Genome Institute (JGI)	https://genome.jgi.doe.gov/portal/
FungiDB	https://fungidb.org/fungidb/
Ensembl Fungi	https://fungi.ensembl.org/index.html
Aspergillus Genome Database (AspGD)	http://www.aspgd.org/
Saccharomyces Genome Database (SGD)	https://www.yeastgenome.org/
Candida Genome Database (CGD)	http://www.candidagenome.org/

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Helicos, ABI SOLiD and newly developed Ion Proton, PacBio and Oxford Nanopore. These sequencing platforms differ substantially in terms of their sequencing mechanism, throughput, output (length of reads, number of sequences), accuracy and cost (Besser *et al.*, 2018). The decrease of sequencing cost compared to traditional Sanger sequencing has driven the rapid development of NGS and made it the primary sequencing method. In this chapter, we focus on the bioinformatics analysis of NGS data.

A typical workflow for genome analysis falls into the following steps: (1) quality control and preprocessing of raw reads; (2) alignment (especially for comparative assembly); (3) genome assembly; (4) genome annotation; (5) other advanced analyzes based on your research interest, such as phylogenetic analysis and comparative genomics analysis. Typically, the raw reads of NGS data are stored either as text in a Fasta file or with their qualities as a FastQ file. The latter is the most widely accepted format. Quality control (QC) of raw reads is the first step of the genomic data analysis and several tools are commonly used for this step. For instance, FastQC (See "Relevant Websites section") is a powerful QC tool, which can provide a range of useful QC statistics results, such as per-base quality, average read quality, GC content, N content, sequence length distribution, duplication levels, and overrepresented sequences. Read trimming is a preprocessing step which aims at removing unwanted information of sequence, including leftover adapter sequences, low-quality bases at reads ends, known contaminants (strings of As/Ts), reads containing too many N-bases along their length, and more. Popular trimming tools include the FASTA-Toolkit (See "Relevant Websites section"), Trimmomatic (Bolger *et al.*, 2014), Fastx_trimmer (See "Relevant Websites section") and cutadapt (Martin, 2011). In addition, NGS QC Toolkit (Patel and Jain, 2012) integrates various tools for performing multiple tasks, including quality control, trimming, format conversion, and generates corresponding statistics reports. After filtering the reads, the clean reads are aligned to the reference genome during a comparative assembly. Widely used aligners include Bowtie2 (Langmead and Salzberg, 2012), Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2010), STAR (Dobin *et al.*, 2013), HISAT2 (Kim *et al.*, 2015), and StringTie (Pertea *et al.*, 2015). The alignments results are usually stored as SAM files or its binary compressed implementation BAM files. Alignments can be viewed using user-friendly and freely available software, such as the Interactive Genome Viewer (IGV) (Thorvaldsdóttir *et al.*, 2013), the GALAXY server (Giardine *et al.*, 2005) or Genome Browse (See "Relevant Websites section").

Genome assembly refers to the process of taking a large number of short DNA sequences and putting them back together to create a representation of the original chromosomes from which the DNA originated. The genome assembly can be broadly categorized into two approaches: *de novo* assembly, for reconstructing genomes without available reference genomes, and comparative assembly, which uses the sequence of a closely related organism as a reference during assembly. Comparative approaches can only be applied to the genomes for which reference sequences are available. Even when a reference genome is available, the sequence can also be reconstructed through *de novo* assembly to improve the genome (Pop, 2009). In terms of different demands, there are several computational tools commonly used for genome assembly (**Table 2**).

Alongside the steady increasing performance of NGS technologies in genome sequencing, it still faces several persistent challenges to achieve a fully assembled genome. For example, errors in reads and large repeats in the genome complicate sequence alignment and genome reconstruction. Therefore, it is necessary to statistically assess the quality of an assembly. The quality of genome assembly can be assessed by several criteria, such as per-base error rates, insert size distributions, k-mer distributions and fragment (contig) length distributions. N50 is a widely used measure to assess the contiguity of a genome assembly, which is defined as the sequence length of the shortest contig for which longer and equal length contigs cover at least 50% of the genome. For quality evaluation of assembly results, several software can be applied, such as GAGE (Genome Assembly Gold-standard Evaluations) (Salzberg *et al.*, 2012), QUAST (Gurevich *et al.*, 2013), BUSCO (Simão *et al.*, 2015), SQUAT (Yang *et al.*, 2019c) and GenomeQC (Manchanda *et al.*, 2020). GAGE could evaluate repeat copy numbers, insertions, deletions, and assembly errors in the various assemblies, even the correctness of assemblies if the reference genomes are given. QUAST can evaluate assemblies with or without a reference genome, and usually presents comprehensive metrics, summary tables, reports and plots. BUSCO does a quantitative assessment of genome assembly and annotation completeness. SQUAT tool (See "Relevant Websites section") provides quality assessments for both pre-assembly and post-assembly, and examines the correctness of *de novo* assemblies and the input reads. GenomeQC characterizes both assembly and gene annotation quality, and provides a comprehensive assessment summary.

The next step after genome assembly is to provide functional annotation. The annotation is a bridge connecting the sequence with its possible biological function. Genome annotation includes structural annotation and functional annotation. The former

Table 2 Common assemblers for diverse sequencing data

Tools	*Description*	*Availability*	*Ref*
ABySS	Paired-end sequence assembler designed for large genome assembly of short reads	https://github.com/bcgsc/abyss	Simpson *et al.* (2009)
SOAPdenovo	Short read assembler	https://www.animalgenome.org/bioinfo/resources/manuals/SOAP.html	Li *et al.* (2010); Luo *et al.* (2012)
ALLPATHS-LG	Short read assembler	http://software.broadinstitute.org/allpaths-lg/blog/	Gnerre *et al.* (2011)
Canu	Long read assembler working on both third and fourth generation reads	https://github.com/marbl/canu	Koren *et al.* (2017)
Falcon	Long read assembler designed for diploid organisms.	https://github.com/PacificBiosciences/FALCON	Chin *et al.* (2016)
Velvet	*de novo* genome assembly and short read sequencing alignments	https://www.ebi.ac.uk/~zerbino/velvet/	Zerbino and Birney (2008)

refers to the identification features on a genome assembly such as protein-coding, non-coding RNAs (ncRNAs), repeats and transposable elements, and pseudogenes. There is a variety of programs available for ab initio gene predictions including GENSCAN (Burge and Karlin, 1997), GeneMark.hmm (Lukashin and Borodovsky, 1998), SNAP (Korf, 2004), GlimmerHMM (Majoros *et al.*, 2004), MAKER (Cantarel *et al.*, 2008) and AUGUSTUS (Stanke and Waack, 2003). To improve the accuracy of gene prediction and annotation, the NGS based transcriptome data is often integrated by many gene prediction tools. In addition, the RepeatMasker (See "Relevant Websites section") is routinely used to identify the repeat elements and mask them from the genome in preparation for a more focused gene finding exercise. The TransposonPSI (See "Relevant Websites section") is often used to assist in the discovery of transposable elements.

Functional annotation refers to the computational assignment of functions to the predicted genes. A common approach is using homology-based methods like BLASTp to infer biological function based on top hits in a well-curated and non-redundant database like the manually annotated SWISS-PROT provided at UniProt (See "Relevant Websites section") and the reference sequence (RefSeq) collection of the NCBI (See "Relevant Websites section") A widely used protein homology-based gene-modeling tool is GeneWise (See "Relevant Websites section"), which combines protein alignment and gene prediction into a single statistical model via a paired hidden Markov model (HMM) (Birney *et al.*, 2004). Additional functional characterization includes assigning enzyme activity (EC-number), gene ontology (GO), Pfam database (See "Relevant Websites section"), and KEGG-pathway. Several tools are commonly used for this analysis like InterProScan (See "Relevant Websites section"), Blast2GO software (See "Relevant Websites section"), the "hmmsearch" of the HMMER tool (See "Relevant Websites section"), and Funannotate (See "Relevant Websites section"). Furthermore, the secretion signal peptide and transmembrane characters can be predicted with the SignalP tool (See "Relevant Websites section") and TMHMM (See "Relevant Websites section"), respectively. Additionally, the comparative fungal genomics platform (CFGP, See "Relevant Websites section"), is a multifunctional fungal genome data warehouse, which enables online analysis of large set of fungal genomes by integrating 37 common bioinformatics tools and encompasses several specific repositories focusing on specific fungal protein groups such as secretome, transcription factor and cytochrome P450 proteins (Park *et al.*, 2008).

The annotation information of non-coding RNA is often derived from a reference genome. If this is not available, the common method is to map non-coding RNA sequences with known members from databases of non-coding RNA. Rfam (Griffiths-Jones *et al.*, 2003; Griffiths-Jones *et al.*, 2005) and INFERNAL (Eddy, 2002) serve useful resources for annotating transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and other short non-coding RNA with conserved sequences and secondary structures. NONCODE (See "Relevant Websites section") is an integrated knowledgebase dedicated to the complete collection and annotation of non-coding RNAs (ncRNA) (Liu *et al.*, 2005). lncRNAdb (See "Relevant Websites section") provides annotation of long non-coding RNAs (Amaral *et al.*, 2011).

Another method of functional annotation is the classification of orthologous genes. OrthoMCL (Li *et al.*, 2003) and OrthoFinder (Emms and Kelly, 2015) are commonly used to analyze orthologues. Due to their important biological function and industrial potential, some genes have been further annotated and classified into specific protein families, such as the carbohydrate-active enzyme annotation (CAZyme) and peptidases. CAZyme predictions are usually performed by running hmmscan from the HMMER tool (See "Relevant Websites section") by searching the HMM profiles derived from well-characterized CAZymes (Cantarel *et al.*, 2009; Zhang *et al.*, 2018). A similar approach could be used to identify the peptidases by using sequences provided on MEROPS website (See "Relevant Websites section") and HMMER tools. The resulting set of genes and functional annotation is typically less than perfect, and often need manual curation before its release to the public, which requires continuous refining efforts from the scientific community even after the release of the genome.

To make the best use of a genome, the newly assembled genome together with its annotation is uploaded to public databases, which can be accessed and shared by researchers worldwide. The most common databases dedicated to fungal genomes are shown in **Table 1**. In general, there are two types of databases. Several databases embrace a broader diversity of fungi, such as Mycocosm and FungiDB. Some other database focus on specific fungal species or genera. For example, the SGD (Sheppard *et al.*, 2016) and CGI (Skrzypek *et al.*, 2017) are specialized databases for *Saccharomycetes* and *Candida*, respectively. AspGD was designed for *Aspergillus* researchers, which provides visualization tools for genomic features and alignments as well as a comparative genomics analysis for the genus *Aspergillus* (Cerqueira *et al.*, 2014). Currently the AspGD database is no longer updated, and AspGD data has been integrated into FungiDB (See "Relevant Websites section"). Given their economic and scientific importance, several fungal genomes have received more attention and are selected to be deeply annotated to achieve gold-standard genome resources. Those flagship fungi include *A. niger* (Pel *et al.*, 2007), *A. nidulans* (Galagan *et al.*, 2005), *T. reesei* (Martinez *et al.*, 2008), and *P. chrysosporium* (Martinez *et al.*, 2004).

Examples of Fungal Genomic Studies

With abundant and diverse fungal genomes available, comparative genomics has emerged as a powerful approach to identify the link between genotype and phenotype via comparatively analyzing genomic features and their evolution across different species. In the past decade, numerous studies of comparative genomics have greatly enhanced our understanding of the genomic diversity and evolutionary mechanisms that are related to several important aspects of fungal biology, such as plant pathogenicity (Amselem *et al.*, 2011; de Wit *et al.*, 2012; Ohm *et al.*, 2012), plant biomass degradation (Floudas *et al.*, 2012; Peng *et al.*, 2017; Rytioja *et al.*, 2014; Berka *et al.*, 2011), as well as stress response (Acton *et al.*, 2017). The current rapid expansion of genomics data allows even large-scale comparison

of genomics at the genera or section level. For example, a recent comparative analysis of a large set of *Aspergillus* genomes has revealed remarkable genomic diversity and similarities within *Aspergillus* species and part of them could be associated with specific phenotypes (de Vries *et al.*, 2017). Another comparative genomics study of 23 *Aspergillus* species from section *Flavi* reassessed the phylogenetic relationships of the studied species, and highlighted the genetic and metabolic diversity within section *Flavi* by comparing evolution patterns of CAZymes, secondary metabolite gene clusters and fungal growth profiles (Kjærbølling *et al.*, 2020). A similar study of comparing inter- and intraspecies variation of 23 *Aspergillus* section *Nigri* also showed high genetic diversity and genome flexibility of section *Nigri* (Vesth *et al.*, 2018). Similar comparative studies have also been performed for 12 *Trichoderma* species (Kubicek *et al.*, 2019) and various sets of basidiomycete fungi. A comparative analysis of 31 basidiomycete genomes, including both white and brown rot basidiomycetes, reconstructed the evolution of lignin decay mechanisms and suggested that the evolution of ligninolytic capabilities was associated with the sharp decrease of carbon burial in geological time (Floudas *et al.*, 2012). A further comparison of 33 basidiomycete genomes revealed that the current dichotomous classification of white rot and brown rot fungi was not adequate to describe the whole spectrum of wood-decaying basidiomycetes (Riley *et al.*, 2014). For instance, *Botryobasidium botryosum* and *Jaapia argillacea* have lost ligninolytic class II peroxidases like brown rot fungi, but possess diverse enzymes acting on crystalline cellulose normally associated with white rot fungi, and thus represent so-called gray rot species. In summary, with the continuous expansion of fungal genomics, comparative genomics analysis have an enormous potential to help us better understand how fungi have adapted to their lifestyles and ecological niches, and to discover new biochemical mechanisms (Stajich, 2017).

Current States of Fungal Post-Genomics Analysis

In parallel with the increase in fungal genome sequences, the amount of fungal functional genomic data (transcriptomics, proteomics and metabolomics data) has also increased rapidly. Meanwhile, a broad range of bioinformatics tools have been developed as a response to the exponential growth in the amount, scale, and diversity of post-genomics data. In the post-genomic era, the focus of research will shift from revealing the genetic information of an organism to the functional biological research.

Transcriptomics

The transcriptome is the complete set of all RNA molecules in a cell, a population of cells or an organism. There are several major types of RNA inside a cell. The most studied one is the messenger RNA (mRNA) which is translated into proteins. Other types of RNAs serve other important functions, such as circular RNA (circRNA), microRNA (miRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small interfering RNA (siRNAs), and long non-coding RNA (lncRNA). Here we focus on mRNA related transcriptomics performed with RNA-Seq, which is the most commonly used experimental approach in current fungal studies. We focus on the bioinformatics analysis, and do not address the experimental details.

Bioinformatics Workflow and Tools for Transcriptomics Analysis

Generally speaking, the RNA-Seq downstream analysis can include four essential stages: quality control, alignment, qualification, and differential analysis. Several NGS sequencing platforms are used by researchers, including Roche 454, Illumina, Helicos, ABI SOLiD and PacBio (Chu and Corey, 2012). Among them Illumina is the most commonly used platform (Martin and Wang, 2011). Depending on the research requirement, single-end and/or paired-end reads can be generated. After obtaining a large volume of raw sequence reads, similar as for genomic analysis, quality analysis is needed to remove low quality reads by using tools such as FastQC (See "Relevant Websites section"), FaQCs (See "Relevant Websites section"), FASTX-Toolkit (See "Relevant Websites section"), Trimmomatic (Bolger *et al.*, 2014) and RSeQC (Wang *et al.*, 2012).

After the quality filtering, the clean reads are aligned to the reference genome or *de novo* assembly, depending on whether a reference genome is available. The former is referred to as "reference-based" transcript identification. The most widely used and efficient aligners for this purpose include Bowtie (Langmead *et al.*, 2009), TopHat2 (Kim *et al.*, 2013), STAR (Dobin *et al.*, 2013), GMAP (Wu and Watanabe, 2005), and HISAT2 (Kim *et al.*, 2015). If no reliable reference genome exists for the species, *de novo* transcript assembly can be used to identify the transcripts using tools such as Oases (Schulz *et al.*, 2012), rnaSPAdes (Bushmanova *et al.*, 2019), Cufflinks (Trapnell *et al.*, 2010), StringTie (Pertea *et al.*, 2015; Kovaka *et al.*, 2019), SOAPdenovo-trans (Xie *et al.*, 2014), CLC genomics workbench (Kumar and Blaxter, 2010) and Trinity (Grabherr *et al.*, 2011; Haas *et al.*, 2013). For species with a reference genome, both mentioned strategies can be used alone or in combination.

The next step is to estimate the expression abundance of genes or transcripts based on the alignment results. Gene (RNA) expression levels can be typically expressed as Reads Per Kilobase per Million (RPKM), Fragments Per Kilobase per Million (FPKM), Reads/Counts Per Million (RPM/CPM) and Transcripts Per Million (TPM). One of approaches for quantification is to aggregate raw counts of mapped reads using tools such as HTSeq-count (Anders *et al.*, 2015) and featureCounts (Liao *et al.*, 2014). The reads count tools rely on the alignment result file (SAM or BAM file) and gene feature file (GFF) as input and generate the count number of sequencing reads overlap for each of the gene features. In addition, transcriptome assemblers like Cufflinks and StringTie could be used to obtain the abundances of novel transcripts in addition to the known ones. Several other tools like RSEM

(Li and Dewey, 2011), Salmon (Patro *et al.*, 2017), and eXpress (Roberts and Pachter, 2013) are also commonly used to estimate transcript abundance as well as abundance of gene isoforms.

Once quantitative counts of each transcript are available, the following step is to identify differentially expressed genes (DEGs) across different samples and conditions. Multiple prevailing approaches are applied to accurately detect differentially expressed genes, which include count-based techniques like DESeq2 (Love *et al.*, 2014), limma (Ritchie *et al.*, 2015), and edgeR (Robinson *et al.*, 2010), assembly-based techniques like Cuffdiff (Trapnell *et al.*, 2013) and Ballgown (Frazee *et al.*, 2015), or sleuth (Pimentel *et al.*, 2017) which performs differential analysis on alignment-free quantifications. In addition, many genes involved in the same cellular pathways often share similar expression profiles and the detection of co-expression genes across different growth conditions has received broad attention. The weighted gene co-expression network analysis (WGCNA) is one of the most used tools to identify co-expressed groups of genes (Langfelder and Horvath, 2008).

In addition to the above quantitative analysis, RNA-Seq data can also be used to identify important genomic and transcriptomic variations. GATK (DePristo *et al.*, 2011) is the best practice workflow to call variants such as SNPs and indels from RNA-seq data. Furthermore, a useful tool is vcftools (Danecek *et al.*, 2011), which is a program package designed for working with VCF files and used to filter out specific variants or summarize variants etc. The widely used applications for functional annotation of variants include SnpEff (Cingolani *et al.*, 2012) and ANNOVAR (Wang *et al.*, 2010).

Transcriptome analysis is also commonly applied to identify target genes of a specific transcription factor (TF). This is often done by firstly identifying the DEGs through comparing transcriptomic profiles of a TF mutant and the corresponding reference strain. Next, the specific motif enriched in promoters of these DEGs could be used to infer the TF binding consensus sequence. Several tools are widely used for the motif analysis such as MEME (Bailey *et al.*, 2015) and RSAT (Medina-Rivera *et al.*, 2015).

The commonly used bioinformatics tools for each aspect of RNA-seq analysis are summarized in **Table 3**.

Examples of Fungal Transcriptomics Studies

RNA-seq analysis has been widely applied in a broad range of fungal studies and greatly advanced our understanding of fungal physiology and genetic regulation. For example, RNA-seq has been widely deployed to study fungal plant biomass degradation in diverse fungal species across different growth conditions. These studies range from the dynamic transcriptional responses of fungus to lignocellulose (Delmas *et al.*, 2012; Ries *et al.*, 2013; Daly *et al.*, 2017; Borin *et al.*, 2017) and lignocellulose-derived compounds (Casado López *et al.*, 2018; Lubbers *et al.*, 2019; Kowalczyk *et al.*, 2019), to complex transcriptional regulation network (Kowalczyk *et al.*, 2017; Li *et al.*, 2015; Craig *et al.*, 2015). In addition, sophisticated bioinformatics analyses of strand-specific RNA-seq data have indicated that antisense transcripts (Sibthorp *et al.*, 2013; Solomon *et al.*, 2018; Delmas *et al.*, 2012) and RNA editing (Wu *et al.*, 2018) may play a role during fungal grown on plant biomass. The combined analysis of large-scale transcriptome datasets across different fungal species and growth conditions have enabled us to detect conserved genes and pathways during fungal development or their adaption to natural biotopes (Krizsán *et al.*, 2019; Peng *et al.*, 2018).

Proteomics

The concept of proteome was first proposed in 1994 by Marc Wilkins (Wasinger *et al.*, 1995). The proteome is the entire set of proteins in a given cell, tissue or biological sample, at a precise developmental or cellular phase. Proteomics, the global analysis of proteins, aims for qualitative and quantitative measurements of the production, function and regulation of the entire set of proteins encoded by the genome at a specific condition. Mass spectrometry (MS) is the most common technique for proteomics analysis and has been widely applied in fungal research (Chalupová *et al.*, 2014; Patel, 2019; Studer *et al.*, 2016; Ball *et al.*, 2019; Kumar *et al.*, 2018; Mäkelä *et al.*, 2018). Due to the increasing throughput and accuracy of current MS instruments, the importance of bioinformatics analysis of vast amounts of proteomics data is becoming evident. Here we mainly focus on the proteomics related bioinformatics methods, while only give a very short summary of experimental methods. The experimental methods of proteomics can be found in other well-written reviews (Aslam *et al.*, 2017; Vidova and Spacil, 2017; de Oliveira and de Graaff, 2011).

The most widely-adopted proteomics methodology for large-scale proteomics analysis is the MS based bottom-up proteomics approach (Mayne *et al.*, 2016; Gillet *et al.*, 2016; Zhang *et al.*, 2013), while the top-down and middle-down proteomics strategy is still in the development stage (Catherman *et al.*, 2014; Donnelly *et al.*, 2019; Toby *et al.*, 2016). In bottom-up proteomics, we do not use MS to analyze the intact protein, but analyze a peptide mixture digested from proteins. Then MS detecting signals are used to infer peptide information, which can be further used to infer protein information. Owing to the recent advances in instrumentation, sample preparation and computational algorithms, proteomics researchers can routinely identify and quantify thousands of proteins in a single experiment. A generic proteomics workflow can be broadly categorized into three major steps, i.e., (1) sample preparation, (2) MS data acquisition and (3) data analysis or post-MS data acquisition. Proteins extracted from a biological sample are digested with a specific enzyme (typically trypsin). The resulting peptides are separated by liquid chromatography. Then the ionized peptides are introduced to mass spectrometry that detects the mass and ion intensity for peptides and their fragments. At the last step, via computational software, peptides and proteins are identified and quantified. The possible biological function can be further annotated via various bioinformatics analysis. Many strategies used in transcriptomic data analysis for biological function mining can be directly or slightly modified to be applied in proteomics data analysis.

Table 3 The widely used tools in transcriptomics analysis

Tools	*Quick links*
Quality control	
fastQC	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Trimmomatic	http://www.usadellab.org/cms/?page=trimmomatic
Fastx_trimmer	https://rdrr.io/github/abshah/RADseqR/man/fastx_trimmer.html
RSeQC	http://rseqc.sourceforge.net/
Alignment	
HISAT2	http://daehwankimlab.github.io/hisat2/
StringTie	https://ccb.jhu.edu/software/stringtie/
STAR	https://github.com/alexdobin/STAR
SOAPdenovo-trans	https://github.com/aquaskyline/SOAPdenovo-Trans
Cufflinks	http://cole-trapnell-lab.github.io/cufflinks/
Variant calling and genotyping	
GATK	https://gatk.broadinstitute.org/hc/en-us
Variant annotation	
SnpEff	http://snpeff.sourceforge.net/
ANNOVAR	https://doc-openbio.readthedocs.io/projects/annovar/en/latest/
Calculate reads count	
HTSeq-count	https://htseq.readthedocs.io/en/release_0.11.1/count.html
featureCounts	http://subread.sourceforge.net
Differential expression	
DEseq2	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
edgeR	https://bioconductor.org/packages/release/bioc/html/edgeR.html
Visualization	
IGV	https://software.broadinstitute.org/software/igv/
Gene annotation	
GO	https://go.princeton.edu/
KEGG	https://www.genome.jp/kegg/
GSEA	https://www.gsea-msigdb.org/gsea/index.jsp
BLAST2GO	https://www.blast2go.com/
Pfam	https://pfam.xfam.org/
WGCNA	http://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/
Utility tools	
Bedtools	https://bedtools.readthedocs.io/en/latest/
SAMtools	https://www.htslib.org/
Vcftools	http://vcftools.sourceforge.net/
Online platform	
Galaxy	https://usegalaxy.org/

Bioinformatics Workflow and Tools for Proteomics Analysis

Today, proteomics is a multi-disciplinary field, which has been extended from the initial large-scale protein identification, to quantification, and to the characterization of complex protein-protein interactions. In parallel, corresponding bioinformatics tools are also evolving so as to address problems arising from specific branches of proteomics research. In fact, intensive developments in these areas have led to the creation of a new field named "computational proteomics" dedicated completely to this pursuit (Kopczynski *et al.*, 2017; van den Toorn, 2017; Sarkar and Saha, 2016). In this section, we mainly focus on data analysis methods that are commonly used in most fungal proteomics studies including: (1) peptide and protein identification; (2) quantification of proteins; and (3) biological interpretation of proteomics results.

Peptide and Protein Identification

Normally, it takes two steps to infer protein identities from MS data. The first step is by matching a MS/MS spectrum to a peptide sequence, known as a peptide spectrum match (PSM). Subsequently, peptide sequences obtained from PSMs are used to infer proteins. As with any large-scale studies such as genomics and transcriptomics, statistical models play a key role in controlling the false discovery rate (FDR) for both peptide and protein identification. A number of computational algorithms and software

solutions have been developed to assign peptide sequences to fragment ion spectra. These tools can be classified into three categories according to reviews (Chen *et al.*, 2020; Hoopmann and Moritz, 2013; Kertész-Farkas *et al.*, 2012):

(1) Database searching: peptide sequences are identified by correlating an acquired MS^2 spectrum with a theoretical spectrum predicted from peptides obtained from in silico digestion of a protein sequence database (MacCoss *et al.*, 2002; Eng *et al.*, 1994). Alternatively, in the so-called spectral-library search approach a fragment ion spectrum can be correlated with a library of experimental MS/MS spectra identified in previous experiments (Dasari *et al.*, 2012; Tharakan *et al.*, 2010; Ning *et al.*, 2010).
(2) De novo sequencing: peptide sequences are directly inferred from fragment ion spectra without reference to any database information. Many newly developed tools designed for de novo peptide sequencing have introduced advanced machine learning algorithms and showed promising results, such as pNovo 3 (Yang *et al.*, 2019a), Novor (Ma, 2015), and DeepNovo (Tran *et al.*, 2017).
(3) Hybrid approaches that combine database search and de novo sequencing: based on the extraction of short "sequence tags" of 3–5 residues in length, followed by "error-tolerant" database searching (Zhang *et al.*, 2012; Kim *et al.*, 2009; Dasari *et al.*, 2010).

A list of tools commonly used for these different types of peptide spectrum matching is provided in **Table 4**. Currently, database searching strategies represent the most-used method for high-throughput peptide identification.

After peptide to spectrum matching, the next step is to assemble peptide sequences into proteins. However, mapping peptide sequences to proteins is not always straightforward, as some peptide sequences are shared among more than one protein, due to sequence homology or alternative splicing of genes. To solve this problem, a common strategy is simply reporting the parsimonious set of proteins to explain the observed peptides (Nesvizhskii *et al.*, 2007).

Table 4 Overview of software available for peptide and protein identification

Program	*Website*	*Ref*
Database search		
SEQUEST	http://fields.scripps.edu/sequest/	Eng *et al.* (1994)
MASCOT	http://www.matrixscience.com/	Perkins *et al.* (1999)
X!tandem	http://www.thegpm.org/tandem/	Craig and Beavis (2003)
Andromeda	http://www.coxdocs.org/doku.php?id=maxquant:andromeda	Cox *et al.* (2011)
Comet	http://comet-ms.sourceforge.net/	Eng *et al.* (2013)
MS Amanda	https://ms.imp.ac.at/?goto=msamanda	Dorfer *et al.* (2014)
MS-GF+	http://proteomics.ucsd.edu/software-tools/ms-gf/	Kim and Pevzner (2014)
SearchGUI	http://searchgui.googlecode.com	Vaudel *et al.* (2011)
ProLuCID	http://fields.scripps.edu/yates/wp/?page_id=821	Xu *et al.* (2015)
msFragger	https://github.com/Nesvilab/MSFragger	Kong *et al.* (2017)
De novo peptide sequencing		
pNovo 3	http://pfind.ict.ac.cn/software/pNovo/index.html	Yang *et al.* (2019a)
pNovoM	http://pfind.ict.ac.cn/software/pNovoM/	Yang *et al.* (2019b)
DeepNovo	https://github.com/nh2tran/DeepNovo	Tran *et al.* (2017)
DeNovoGUI	http://compomics.github.io/projects/denovogui	Muth *et al.* (2014)
Novor	https://www.rapidnovor.org/novor	Ma (2015)
PepNovo	http://proteomics.ucsd.edu/Software/PepNovo	Frank and Pevzner (2005)
PEAKs	https://www.bioinfor.com	Ma *et al.* (2003)
Hybrid approaches		
MS Dictionary	http://proteomics.ucsd.edu/Software/	Kim *et al.* (2009)
TagRecon	https://lab.vanderbilt.edu/msrc-bioinformatics/software/	Dasari *et al.* (2010)
PEAKS DB	https://www.bioinfor.com/peaksdb/	Zhang *et al.* (2012)
Statistical validation		
PeptideProphet	http://peptideprophet.sourceforge.net/	Keller *et al.* (2002)
ProteinProphet	http://proteinprophet.sourceforge.net/	Nesvizhskii *et al.* (2003)
Percolator	https://noble.gs.washington.edu/proj/percolator/	Käll *et al.* (2007)
Scaffold	http://www.proteomesoftware.com/	Searle (2010)
Proteomics data repositories		
Global Proteome Machine Database	http://gpmdb.thegpm.org/	Craig *et al.* (2004)
PeptideAtlas	http://www.peptideatlas.org/	Desiere *et al.* (2005)
jPOST	https://jpostdb.org/	Moriya *et al.* (2019)
ProteomeXchange Consortium	http://www.proteomexchange.org/	Vizcaíno *et al.* (2014)
Proteomics Identifications (PRIDE) Database	https://github.com/PRIDE-Toolsuite/pride-inspector	Perez-Riverol *et al.* (2016)
PASSEL	http://www.peptideatlas.org/passel/	Farrah *et al.* (2012)

As with other high-throughput "omics" technologies, proteomics is capable of generating massive amounts of data, albeit with a certain degree of noise, which affects peptide and protein identifications. As such, assessment of the quality of proteomics data is of utmost importance (Gauci *et al.*, 2009; Nesvizhskii *et al.*, 2007; Peng *et al.*, 2012; Käll *et al.*, 2007; Walzer *et al.*, 2014). Many probabilistic methods have been developed to provide a statistical measure of confidence and an estimate of error rates (Nesvizhskii *et al.*, 2007). For estimating the false-positive rate in peptide to spectrum matching, the most popular method makes use of a target-decoy analysis (Elias and Gygi, 2007). This method requires a decoy database, which is created by shuffling or reversing the protein sequences in the target protein database of the organism under study. It assumes that scores of spectra matched to a decoy database distribute similarly as those of incorrect matches to the original target database. The false discovery rate (FDR) of a certain score threshold can be determined by the number of PSMs in the decoy search divided by the PSM counts in the target search. Other approaches to separate the correct PSMs from incorrect ones are based on machine learning methods, such as PeptideProphet (Keller *et al.*, 2002) and Percolator (Käll *et al.*, 2007). For protein inference, several statistical models have been proposed to estimate the reliability of the assigned identified protein. For instance, ProteinProphet computes a probability that a protein is present in the sample by combining the probabilities of peptides assigned to this protein (Nesvizhskii *et al.*, 2003). In addition, several statistical methods have been specially developed to assess the confidence of the post-translation modification sites, such as Ascore (Beausoleil *et al.*, 2006), PTM score (Olsen *et al.*, 2006) and PhosphoRS (Taus *et al.*, 2011).

In addition, with continually increasing of proteomics application, sharing and reusing of data become essential for the research community. Currently, a number of proteomics tools, databases and repositories have sprung up which provide a highly valuable source for bioinformatics data mining (**Table 4**) (Martens and Vizcaíno, 2017; Perez-Riverol *et al.*, 2015).

Data Analysis of Quantitative Proteomics

MS based proteomics has not only been successfully used for large-scale protein identification, but is also widely applied for quantifying global protein production (Ong and Mann, 2005). Over the past decade, many quantification strategies for MS-based proteomics have been introduced (Chen *et al.*, 2020; Nesvizhskii *et al.*, 2007; Mirza, 2012; Lindemann *et al.*, 2017). In these methods, quantitative information from mass spectra at various levels can be extracted and used for estimating protein abundance. This information can either originate from a MS^1 feature (peaks in the MS^1 spectrum characterized by their intensity, *m/z* value, and the time of acquisition of the spectrum), or MS^2 features (fragment ion spectra) or even peptide features (the PSMs for each peptide or groups of isotopic mass peaks originating from the same peptide ion). In general, based on the particular sample preparation workflow, quantitative proteomics can be categorized in two flavors: (1) Based on stable isotope labeling or (2) Based on label free methods which mainly include spectral counting (PSM-based) and determination of precursor ion signal intensities (MS^1-based).

Quantitative proteomics analysis is not straightforward as the intensity detected by MS may be noisy, biased or contain missing values. Instead, the next step is to apply data analysis methods to clean up the data or to correct for these biases, followed by statistically determining and confirming differentially expressed proteins (Bantscheff *et al.*, 2012; Zielinska *et al.*, 2010; Nesvizhskii, 2012; Choi *et al.*, 2008). These statistical methods address key issues in data analysis ranging from data normalization (Karpievitch *et al.*, 2012; Callister *et al.*, 2006) to imputation of missing values and also significance analysis (Zhang *et al.*, 2006). As transcriptomics and proteomics data do bear some similarities, statistical methods successfully applied in quantitative proteomics are mostly adapted from transcriptome-based data analysis.

Biological Interpretation of Proteomics Results

Bioinformatics plays an important role in the research of functional protein annotation, and prediction of protein-protein interactions. For function prediction of an unknown protein, one simple and common method is to compare its sequence with the known proteins by using sequence alignment tools like ClustalW (Thompson *et al.*, 1994), Jalview (Clamp *et al.*, 2004), and MAFFT (Katoh and Standley, 2013), or using BLASTp to search the sequence against well curated databases such as NCBI (Camacho *et al.*, 2009) and Uniprot (UniProt, 2010). In addition, the general functional annotation of proteins, such as GO terms, KEGG, and PFAM are also useful to infer their function. Several tools can be used to achieve this goal such as Blast2GO (Gotz *et al.*, 2008), InterProScan (Jones *et al.*, 2014) and FungiFun2 (Priebe *et al.*, 2015). Specifically in fungi, the carbohydrate-active enzymes (CAZymes) which are responsible for the breakdown, biosynthesis or modification of glycoconjugates, oligo- and polysaccharides, could be assigned to a particular enzyme family in the CAZy database (Lombard *et al.*, 2014) to predict their function. Furthermore, the fungal secreted protein could be predicted with several common bioinformatics tools, such as SignalP, WolfPsort (Horton *et al.*, 2007) and Phobius (Käll *et al.*, 2007), or can be directly retrieved from the fungal secretome databases such as FunSecKB (See "Relevant Websites section") (Lum and Min, 2011) and FSD (See "Relevant Websites section") (Choi *et al.*, 2010). Enrichment analysis like GO enrichment, and gene set enrichment algorithms (GSEA) can be performed to further explore the function of a selected protein list (Subramanian *et al.*, 2005). Algorithms such as *motif-x* (Chou and Schwartz, 2011) or PhosphoMotif Finder (Amanchy *et al.*, 2007) analyze the sequence environment of phosphorylation modification sites in a proteomics dataset, reporting enrichment of certain amino acid motifs which can help to identify the kinases catalyzing the modification.

For protein-protein interaction, a widely used resource of interaction data is STRING (Franceschini *et al.*, 2013; Snel *et al.*, 2000), which is not only a widely used database for analyzing protein interaction data, but also providing various other resources for literature mining. Cytoscape (Shannon *et al.*, 2003) is a convenient tool to visualize the protein-protein interaction network.

With many plug-ins, Cytoscape enables further analysis of the protein network, such as the MCODE (Bader and Hogue, 2003) for topological analysis and BiNGO for Gene Ontology enrichment analysis (Maere *et al.*, 2005).

Examples of Fungal Proteomics Studies

The capability of high-throughput identification and quantification of proteins, the actual functional molecules in the cell, enables proteomics to provide complementary information to genomics and transcriptomics in reflecting the cellular state. In the past decade, numerous proteomics studies were carried out alone or together with transcriptomics to investigate fungal plant biomass utilization. For instance, MS based proteomics has been widely used to monitor the fungal secretome, which revealed dynamic changes of key lignocellulolytic enzymes in response to different carbon sources (Kolbusz *et al.*, 2014; Gaskell *et al.*, 2016). The comparative transcriptome and secretome analyzes of the brown rot fungus *Postia placenta* and the white rot fungus *P. chrysosporium* cultured in wood containing media elucidated the different mechanisms of lignocellulose degradation mechanisms employed by these model wood decay fungi (Vanden Wymelenberg *et al.*, 2010). Comparison of secretome from different fungi showed that the similar genomic content could generate a very diverse secretome, especially the CAZyme profile, in response to plant biomass degradation (Benoit *et al.*, 2015). In addition, several studies have indicated an important role of post-translational modification (such as phosphorylation) in regulating fungal plant biomass degradation through phospho-proteomics analysis (Xiong *et al.*, 2014; Horta *et al.*, 2019).

Metabolomics

Metabolomics originated from metabolic profiling. Fiehn proposed "metabolomics" in 2001 and defined it as "a comprehensive and quantitative analysis of all metabolites in a biological system" (Fiehn, 2001). Because metabolites and their concentrations directly reflect the underlying cellular biochemical activity, metabolomics is a useful and complementary approach to other omics techniques in deciphering the complexity of biological systems.

The general procedure for metabolomics analysis includes the separation, detection, identification and quantification of metabolites. Gas or liquid chromatography-mass spectrometry (GC-MS or LC-MS) and nuclear magnetic resonance (NMR) are the most frequently used tools for determining and quantifying as many metabolites as possible, including both known and unknown compounds. The analytical methodologies for metabolite profiling have been extensively reviewed in many papers and books (Villas-Boas *et al.*, 2005; Beale *et al.*, 2018; Ren *et al.*, 2018). The high separation efficiency, excellent reproducibility, and many well-established and available libraries for structural identification are key advantages of GC-MS analysis (Chen *et al.*, 2019). Typically, classes of metabolites routinely measured by GC-MS include sugars, amino acids, phosphorylated metabolites, organic acids, lipids, and amines. LC-MS is another technique commonly used in metabolomics. Sample preparation for LC-MS is simple and it caters well for compounds that are not well suited for GC-MS, such as macromolecular metabolites, thermally labile compounds, polar compounds, and compounds that do not volatilize easily. To date in fungi, LC-MS is a robust technique for examining and screening secondary metabolites (Gummer *et al.*, 2012). As for NMR, it has the advantage of simplicity of sample preparation, high precision as well as high resolution. However, it suffers from relatively low sensitivity, and concentration insensitivity on detecting metabolites with large concentration differences in biological systems (Markley *et al.*, 2017; Wishart, 2019), which hinders its application in fungal metabolomics (Chen *et al.*, 2019). Particularly for fungi, direct infusion mass spectrometry (diMS) (Smedsgaard and Nielsen, 2005) can probably be the best and most efficient approach used for the quantitative analysis of metabolites. In addition to relative easy-to-use and high resolution and accuracy, the major advantage of diMS is that each observed ion can be associated with a unique chemical formula so that the accurate mass of this ion can be determined. Therefore, diMS is applied to rapidly classify filamentous fungi. The classified species were typically identified based on their mass profile rather than a conventional phenotypic identification (Roessner and Bowne, 2009).

After the chromatographic separation of biological metabolites, a large amount of generated spectral and multivariate data needs to be analyzed by employing statistics and computational tools. Independent of the employed analytical technique, in broad terms, metabolomic profiling falls into two categories: targeted and untargeted analyzes. For the targeted approach, it is restricted to defined metabolites, for example, a group of particular metabolic enzymes, and can quantify and identify by monitoring a set of annotated known substances. By contrast, untargeted profiling methods try to detect all measurable compounds, including unknown or at least unidentified (Kluger *et al.*, 2015; Ribbenstedt *et al.*, 2018; Roberts *et al.*, 2012). Thus, untargeted approaches have the potential of probing the entire metabolic space (Schuhmacher *et al.*, 2013). GC-MS could be well applied to non-targeted metabolite profiling, especially for volatile and thermally stable polar and non-polar metabolites (Gummer *et al.*, 2012).

Bioinformatics Workflow and Tools for Metabolomic Analysis

Over the last decade, several software programs for automated processing of metabolomics data have been introduced to assist data mining in metabolomics. MZmine 2 (See "Relevant Websites section") is an open-source software for mass-spectrometry data processing, with the main focus on LC-MS data, which has been applied to both targeted and non-targeted metabolomic analyzes (Pluskal *et al.*, 2010). MetAlign has been successfully used in many metabolomics studies, which can process GC-MS and LC-MS

data with multiple threads for untargeted analysis (Lommen and Kools, 2012). OpenMS 2.0 (See "Relevant Websites section") provides many tools that are specifically designed for the metabolite quantification and metabolite identification in non-targeted metabolomics analysis (Röst *et al.*, 2016). XCMS (See "Relevant Websites section") also processes untargeted metabolomic data, and provides a complete workflow including feature detection, retention time correction, alignment, annotation, statistical analysis, and data visualization (Tautenhahn *et al.*, 2012). In addition, XCMS Online is integrated with METLIN (Smith *et al.*, 2005), a large metabolite database. Several metabolic pathways and biochemical databases are available for metabolomics analysis. Among them, BioCyc (See "Relevant Websites section"), MetaCyc (MetaCyc Encyclopedia of Metabolic Pathways, See "Relevant Websites section"), KEGG (See "Relevant Websites section") and YMDB (See "Relevant Websites section") are dominant databases for fungal research. However, a fungi-specific well-established metabolomics database is still lacking. Establishment of a microbial metabolomics database will accelerate the identification of compounds and species. Besides those mentioned databases, FUNGIpath (See "Relevant Websites section") appears to be a useful tool to assess fungal metabolic pathways predicted by orthology (Grossetête *et al.*, 2010). In addition, the metaP server (See "Relevant Websites section") can provide automated and standardized data analysis for quantitative metabolomics data, which is an easy-to-use and web-server-based platform covering series of analysis from data acquisition to biological interpretation (Kastenmüller *et al.*, 2011).

Previous studies have shown that genes involved in secondary metabolite biosynthesis are often co-regulated and physically linked into gene clusters on the chromosomes (Chavali and Rhee, 2018; Fedorova *et al.*, 2012). Those metabolic gene clusters are referred as biosynthetic gene clusters (BGCs). BGCs in fungi encode various unexploited high-value metabolites that are closely related to food, agriculture, and medical fields. In the past few years, various bioinformatics methods have been developed for detecting gene clusters in fungi, including antiSMASH (antibiotics & Secondary Metabolite Analysis Shell) (Fedorova *et al.*, 2012), BiG-SCAPE/CoRASoN (Navarro-Muñoz *et al.*, 2020), and SMURF (Secondary Metabolite Unknown Regions Finder) (Khaldi *et al.*, 2010), MIDDAS-M (Motif-Independent *De novo* Detection Algorithm for Secondary Metabolite gene clusters) (Umemura *et al.*, 2013), CASSIS-SMIPS (Cluster Assignment by Island of Sites and Secondary Metabolites by InterProScan) (Wolf *et al.*, 2016), C-Hunter (Yi *et al.*, 2007) and NRPSpredictor2 (Röttig *et al.*, 2011). Genome data and transcriptome data can also be integrated in the identification of gene clusters. For instance, a comparative genomic method was applied to analyze five *Aspergillus* species and ten other filamentous fungal species, which successfully identified secondary metabolite biosynthesis gene clusters by searching for a similar order of genes and their presence in non-syntenic blocks based on synteny analysis (Takeda *et al.*, 2014). A "multi-omics"-based method was performed on *Aspergillus nidulans*, which integrated metabolite profiling analysis and transcriptomics analysis to accurately determine fungal the secondary metabolites (SMs) gene cluster members (Andersen *et al.*, 2013). In addition, FunGeneClusterS software (Vesth *et al.*, 2016) was developed for accurate prediction of gene clusters from genome and transcriptome data, which can predict co-regulated clusters not limited to secondary metabolism. In response to an increasing interest in the research of secondary metabolites, several databases have been developed in recent year, including ClusterMine360 (See "Relevant Websites section") (Conway and Boddy, 2012), IMG-ABC (See "Relevant Websites section") (Hadjithomas *et al.*, 2015) and MIBiG (Minimum Information about a Biosynthetic Gene Cluster, See "Relevant Websites section") (Medema *et al.*, 2015). They provide useful information on gene clusters and related function annotation, which enable the exploitation of fungal metabolome.

Examples of Fungal Metabolomics Studies

Metabolomics has been widely used to investigate changes of fungal metabolites profile in response to various environmental stimuli or genetic modification (Peiris *et al.*, 2008; Bertrand *et al.*, 2013; Chatterjee *et al.*, 2016; Luo *et al.*, 2017). For instance, metabolomics was extensively used in studies of fungal pathogen-plant interactions to understand plant defense mechanisms, such as identifying fungi, determining infection mechanisms, and detecting interactions with the host (Chen *et al.*, 2019). Up to now, metabolomics analyzes of fungal pathogen-plant interactions are limited to several reference fungi and their host plants, such as *F. graminearum* (Bhandari *et al.*, 2018; Chen *et al.*, 2018), *Magnaporthe oryzae* (Jacob *et al.*, 2017), *U. maydis* (Doehlemann *et al.*, 2008), *Rhizoctonia solani* (Hayden *et al.*, 2019; Verwaaijen *et al.*, 2019), *Botrytis cinerea* (Hu *et al.*, 2019; Camañes *et al.*, 2015; Negri *et al.*, 2017), *Sclerotinia sclerotiorum* (Ghosh *et al.*, 2016), *Aspergillus oryzae* (Lee *et al.*, 2014), *Penicillium digitatum* (Tao *et al.*, 2019) and their hosts (Chen *et al.*, 2019). Metabolomics has also been commonly used to discover metabolic molecules related to fungi-fungi interaction. In addition, metabolomics has been increasingly used to help understanding fungal plant biomass degradation (Xiong *et al.*, 2017; Daly *et al.*, 2020).

Integrative Analysis of Multiple Omics

The single-level omics approaches (e.g., genomics, transcriptomics, proteomics, and metabolomics) have resulted in vast amounts of data and advanced our understanding of fungal biological processes. Combining multiple levels of omics information is an emerging approach, which helps uncover the hidden complex biological mechanism that may become evident only through integrative analysis of multiple molecular profiles (Huang *et al.*, 2017). The increase in different omics data available in public databases and newly developed bioinformatics tools accelerate the applications of integrative analysis. One of the straightforward applications of integrated multiple omics data analysis is using transcriptomics and proteomics for improving genome annotation and validating gene prediction. Other applications include directly correlating or sophisticatedly integrating different molecular profiles to discover new biological mechanisms. For instance, a comparative analysis of multiple transcriptome datasets from 10 different basidiomycetes

available in public databases showed that a large set of conserved genes, especially the CAZymes encoding genes, are highly expressed in plant biomass related substrates, suggesting that these genes are critical for fungal plant biomass conversion (Peng *et al.*, 2018). A large-scale co-expression analysis of *A. niger* transcriptome data was applied to improve the genome annotation and identify new function of several genes (Schäepe *et al.*, 2019). Several recent combined analyzes of transcriptome, secretome and even metabolome data have shown great potential for a comprehensive understanding of the process of fungal plant biomass degradation (Martinez *et al.*, 2009; Daly *et al.*, 2018; Daly *et al.*, 2020). In addition, more ambitious efforts have been performed to integrate different omics data, genetics and biochemical characterizations to reconstruct the networks of fungal plant biomass utilization, such as performed in *N. crassa* (Samal *et al.*, 2017) and *A. niger* (Brandl *et al.*, 2018).

To date, several tools have been developed to address the integration of different omics data based on biochemical pathway, ontology, network and correlation (see a recent review of (Wanichthanarak *et al.*, 2015)). For more advanced users with expertize in programming, a variety of tools are available for performing integrative analysis of multiple omics data, such as IntegrOmics (Lê Cao *et al.*, 2009), SteinerNet (Tuncbag *et al.*, 2012), Omics Integrator (Tuncbag *et al.*, 2016), MixOmics (Rohart *et al.*, 2017), SNF (Wang *et al.*, 2014), iCluster (Shen *et al.*, 2009), omicade4 package (Meng *et al.*, 2014), CIMLR (Ramazzotti *et al.*, 2018), and ShinyOmics (Surujon and van Opijnen, 2020). As most of these tools are originally designed for analyzing human and plant related data, some modifications are needed to enable them to be applied in fungal studies. Recently, Miyauchi *et al.* designed a bioinformatics pipeline for integrative analysis of transcriptomic and secretomic data based on self-organizing map algorithm and enrichment analysis. In the test case, it successfully identified condition-specific expressed genes and secreted enzymes that are synergistically involved in fungal plant biomass degradation (Miyauchi *et al.*, 2017).

Challenges and Perspectives for Future Bioinformatics Analysis

The progress of genomic and post-genomic technologies during the past decades has exponentially increased a variety of omics data and significantly enriched the list of potential genes and pathways related to fungal evolution, physiology and industrial applications. However, how to make best use of those large omics datasets remains a challenge. Firstly, the number of well-annotated genomes is severely lagging behind the total number of available draft genome sequences. Due to the remarkable genetic diversity that exists in the fungal kingdom, most gene function predictions based on sequence homology to well-annotated reference fungi cannot guarantee an accurate gene function assignment for many of the newly sequenced genomes. A large part of the genes in most fungal genomes are simply assigned as an unknown function based on computational prediction due to their low similarity with characterized genes. Since genome annotation is the starting point for analysis of genome content, the lack of well-annotated genomes could severely hinder proper interpretation of functional genomics data. For example, the accuracy of most functional enrichment analyzes relies heavily on genome annotation quality. To address this issue, a gold-standard genome program covering a wide range of fungi species is ongoing (Swift *et al.*, 2019; Aguilar-Pontes *et al.*, 2018). Transferring the annotation of gold-standard genomes to other closely related species could help to explore genome and gene function in a broader set of species, and ensure complete and consistent annotation sets. A modified gene annotation method, named RATT, can be applied using well-annotated genome and gene models as a reference to quickly transfer genome annotation (Otto *et al.*, 2011). Secondly, the integrative analysis of multiple omics datasets is still at an early stage. Most current integrative analyzes rely on manually collecting comparable datasets from different public databases or self-generated large-omics datasets, which is time consuming. Building a central database to connect all related biological data (including omics data, phenotypes, genetics and biochemical characterizations), could help to recycle existing datasets and discover new biological mechanisms via integrating analysis of multiple datasets. In addition, the tools and algorithms for integrative analysis of fungal omics lag behind, compared to the numerous tools designed for handling single omics data, and multiple integrative analysis tools developed for handling animal and plant related data. Thirdly, most current analyzes focus only on certain sets of genes depending on the specific research interest, but future bioinformatic studies need to conduct more network analysis, which could help to identify new genes associated with specific phenotypes. For instance, co-expression (Schäepe *et al.*, 2019) has been used to link unknown genes with known ones based on their similar expression patterns across a large set of transcriptomic data.

With many applications of various omics technologies and rapid expansion of omics data, bioinformatics has become an indispensable unit in today's biological research. In this chapter, we only selectively introduced a number of commonly used bioinformatics tools, which only represent a small part of total available tools. The Galaxy (See "Relevant Websites section") project, a web-based scientific analysis platform has currently collected more than 5500 tools that have been frequently used by tens of thousands of scientists across the world to analyze various omics data. In the future, we expect more advanced and user-friendly bioinformatics tools to be developed to deal with the increasingly large, complex and high-dimensional biological data and to help identify new biological mechanisms.

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Production of Biofuels From Biomass by Fungi

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The Demand for Biofuels

"The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust - almost anything. There is fuel in every bit of vegetable matter that can be fermented" - Henry Ford, "Ford Predicts Fuel from Vegetation", N.Y. TIMES, Sept 20, 1925 (Agarwal *et al.*, 2017).

Each discovery of a novel and robust way to harness and harvest energy has shaped civilization. This began with biofuels, fuel to be combusted for energy from plant biomass (Webb and Coates, 2012; Guo *et al.*, 2015). Before the 19th century, wood and plant oil was the prevailing fuel for cooking and lighting (Guo *et al.*, 2015). Turpentine tree resin was combined with alcohol to supply camphene, or lamp fuel (O'Connor, 2010); Ethanol powered stoves, heaters, and lamps and wood fed steam engines and turbines (Zerbe, 2006). Biofuels advanced with the development of cars. In 1826 Samuel Morey patented the first internal combustion engine to use a blend of ethanol, an alcohol fermented from plants, and turpentine (Songstad *et al.*, 2009). In 1860, Nicolaus Otto used an ethanol blend to fuel another internal combustion engine (Guo *et al.*, 2015; Songstad *et al.*, 2009). Later in the 19th century, German inventor Rudolf Diesel designed the first diesel engine to run on peanut oil (Songstad *et al.*, 2009). Henry Ford, a renewable energy titan, used ethanol as the main fuel source for his original 1908 Model T, his ideal car for the common American (Schubert, 2006; Meng, 2019). During World War II China developed "veg-gasoline" and "veg-diesel" from vegetable oil for motor fuels (Chang and Wan, 1947). Brazil prohibited the export of cottonseed oil so it could be refined into diesel for train fuel (Anonymous, 1943). Throughout history, transportation innovations have utilized plant based fuels.

However, biofuels remain marginalized with the commercialization of relatively inexpensive petroleum. Petroleum derivatives such as gasoline, a volatile C4-C12 hydrocarbon mixture (Guo *et al.*, 2015) and diesel remain low-cost sources of transportation fuel. Fossil fuels dominated the United States energy industry until the 1970s oil crisis, when petroleum reserves bottomed out due to the Organization of Arab Petroleum Exporting Countries oil embargo and the Iranian Revolution (Guo *et al.*, 2015; Webb and Coates, 2012; Lifset, 2014). History may repeat itself as petroleum reserves are expected to only last another 45 years (International Energy Agency, 2013; Guo *et al.*, 2015). Also sparking renewed interest in biofuels is an increased awareness of the negative environmental impacts of fossil fuels. Fossil fuels have played a obtrusive role in generating destructive climate change effects by elevating atmospheric CO_2 from pre-industrial level of 280 ppm to the present nearly 400 ppm (Guo *et al.*, 2015). Biofuels, on the other hand, offer four benefits: (1) decreased dependence on foreign oil, (2) the potential to be "carbon neutral" exploiting carbon currently "in circulation", or stored in growing plants (Meng, 2019), (3) biodegradability, reducing costs in recycling plant or biological waste streams and dangers associated with oil spills, and (4) ease of transition for already existing biomass production technology and infrastructure (Guo *et al.*, 2015).

In 1975, Brazil created the National Alcohol Program, or Proàlcool. Its purpose was to reduce oil imports by producing ethanol from sugarcane. The program also offered subsidies and tax rebates for sugar cane producers and distilleries to create an ethanol production chain. It established a requirement of mixing 10% anhydrous ethanol with petroleum (Mączyńska *et al.*, 2019). Most countries currently mandate ethanol blended fuel. These have "E" numbers, which indicate the percentage of ethanol fuel in the mixture by volume. The US, Mexico, Sweden, South Africa, and India required E10 "Gasohol", a mixture of 10% anhydrous ethanol blended with 90% gasoline (Meng, 2019). Brazil's current legal ethanol blend is E25, or about 27% ethanol (Meng, 2019; Mączyńska *et al.*, 2019). In 2003, Brazil introduced flex fuel vehicles, or cars that could run on either gasoline or biofuels (Mączyńska *et al.*, 2019). At present, 90% of Brazil's marketed automobiles are flex fuel, and they constitute 79% of the total fleet of light duty vehicles (Mączyńska *et al.*, 2019; Colares, 2007). Since 2003, these combined changes in the biofuel and automobile industry have decreased emissions of carbon dioxide in Brazil by more than 350 million tons (Mączyńska *et al.*, 2019). Although the Proàlcool program no longer exists, its impact on ethanol production and flex fuel vehicles has remained stable (Corteza *et al.*, 2016).

The U.S. Department of Energy promoted funding for biofuel research and development through the Energy Policy Act of 2005 and Energy Independence and Security Act of 2007 (Kraemer, 2006). President George W. Bush stated in his 2006 State of the Union address, "America is addicted to oil..(we must) move beyond a petroleum based economy" (Kraemer, 2006). In 2007, he mandated an increase in annual biofuel blending obligations for gasoline from 34 billion liters to 136 billion by 2022 with the U. S. Energy Independence and Security Act (Guo *et al.*, 2015). European ethanol production for automobile fuel rose from 47,500 tons in 1993–216,000 tons in 2001 (Van Thuijl *et al.*, 2003). Many Asian countries have sought biofuels that do not compete with food sources (Koizumi and Ohga, 2007). India formulated the National Mission on Biodiesel in 2003 to achieve 20% biodiesel blends by 2012 (Blanchard *et al.*, 2015). Worldwide ethanol production doubled between 2007 and 2016, from 13,123 million gallons, to 25,583 million gallons in 2016 (Dutta, 2018). **Table 1** tracks the steady increase in Ethanol production around the

Table 1 Ethanol production around the Globe (Mil. Gal)

Region	*2014*	*2015*	*2016*	*2017*	*2018*	*2019*
United States	14,313	14,807	15,413	15,936	16,061	15,800
Brazil	6760	7200	6760	6860	7920	8620
European Union	1445	1387	1377	1400	1430	1440
China	635	813	845	860	1050	900
Canada	510	436	436	470	480	500
India	85	195	275	210	400	530
Thailand	310	334	322	370	390	420
Argentina	160	211	264	290	290	290
Rest of World	865	391	490	414	549	600
Total	25,083	25,774	26,182	26,810	28,570	29,100

Note: Renewable Fuel Association, 2020. Available at: https://ethanolrfa.org/statistics/annual-ethanol-production/, accessed April 2021.

globe from 2014 to 2019. The sum of these changes invigorated biofuel research and industry. Scientists mobilized to perform research into conversion of plant material into biofuels. By 2050, bioenergy is predicted to provide 30% of the world's energy demands (Guo *et al.*, 2015). But this challenge is immense - biofuel production is a concert of enzymatic deconstruction of recalcitrant plant material and conversion of sugars or oils into acceptable compounds (Guo *et al.*, 2015). Fortunately, scientists utilize a group of organisms that have been harnessing energy from plant life for millions of years: fungi.

The Kingdom Fungi harbors a huge diversity of organisms that are eukaryotic, heterotrophic, and coenocytic or single cellular organisms (Stajich *et al.*, 2009; Kew, 2018; Walker and White, 2017). Their short generation times, large population sizes, and diverse life cycles enable them to evolve rapidly and occupy highly diverse habitats. Most fungi are primary decomposers. They secrete enzymes into the environment, digest the matter, and absorb it back through cell walls (Kew, 2018; Walker and White, 2017). These enzymes break down tough polymers in plant cell walls. Other fungi, like yeast, are able to ferment simple sugars into various compounds, like ethanol. We discuss here how plants store energy, the enzymes fungi use to degrade biomass, and how scientists have used these metabolic processes to improve biofuel production.

How do Plants Store Energy?

"Land, then, is not merely soil; it is a fountain of energy flowing through a circuit of soils, plants, and animals", Dr. Aldo Leopold (Leopold, 1939).

Algae, trees, and crops use sunlight and carbon dioxide to generate carbohydrates. Carbohydrates are composed of simple monosaccharide sugars such as glucose, fructose, arabinose and galactose. When monosaccharides are organized in linear or branched chains linked by glycosidic bonds they become sugar polymers called polysaccharides (Wang *et al.*, 2020). Polysaccharides can be classified as a homopolysaccharide, having one type of monosaccharide, or a heteropolysaccharide, having more than one type (Pontes, 2018). Polysaccharides form the basis of organic materials, or biomass. Plants utilize carbohydrate molecules to fulfill an array of biological and structural functions. They can be a defensive barrier, structural support, conduits for information, and a source of signaling molecules and developmental cues (Sørensen *et al.*, 2010). This section will detail the structures of common plant polysaccharides, and is summarized in **Table 2**. The next section will describe how fungi have evolved to digest each component for energy.

Plant polysaccharides fall into two categories: those used for storage and those that form the cell wall (Wang *et al.*, 2020). Storage polysaccharides are composed of starch and inulin (Wang *et al.*, 2020). Starch is a branched polysaccharide that comprises both amylose and amylopectin structures (Zeeman *et al.*, 2010). Amylose consists almost completely of linear α-1,4-D-glucose residues with a small amount of α-1,6 D-glucose as side chains (Wang *et al.*, 2020; Sanyang *et al.*, 2018; Pontes, 2018). Amylopectin is a larger branched structure with a majority of α-1,4-glycosidic linkages joining successive D-glucose residues (Pontes, 2018; Sanyang *et al.*, 2018; Varga and Samson, 2008). Inulin is a D-fructose based molecule and another storage carbohydrate. It is composed of β-2,1-D-fructosyl subgroups linked with α-1,2-D-glucose end groups (Varga and Samson, 2008; Pontes, 2018). Inulin is considered a dietary fiber and is abundant in chicory roots, Jerusalem artichokes, asparagus, leek, onions, and garlic (Shoaib *et al.*, 2016; Mensink *et al.*, 2015) **Fig. 2**.

The plant primary cell wall is synthesized during plant growth and is constructed of cellulose, hemicellulose, and pectin. Once matured, some plants add additional support by generating a secondary cell wall of polysaccharides and lignin (Pontes, 2018; Wang *et al.*, 2020). Harder materials such as wood have more abundant cellulose and hemicellulose compared to softer materials like straw. Cellulose constitutes approximately between 40.6% and 51.2% of all plant cell wall material (Pauly and Keegstra, 2008) and its primary purpose is to provide strength (Delmer and Amor, 1995). Cellulose chains are aggregates of β-1,4-linked glucan chains organized into microfibrils aligned to each other by hydrogen bonds. The chains interact to have both crystalline and amorphous regions depending on the degree of alignment (Pontes, 2018; López-Pérez *et al.*, 2015; López-Mondéjar *et al.*, 2019; Voiniciuc *et al.*, 2018).

Hemicellulose represents 28.5%–37.2% of plant cell walls (See **Table 3**) (Pauly and Keegstra, 2008) and is formulated from xyloglucan, xylan, and galacto(gluco)mannan. These are derived from the sugars D-xylose, D-galactose, D-glucose, and

Table 2 Components of plant biomass polymers

Polymer type	*Polymer*	*Main monomer*
Cellulose		D-glucose
Hemicellulose	Xylan	D-xylose
	Glucuronoxylan	D-glucuronic acid, D-xylose
	Arabinoglucuronoxylan	D-xylose, L-arabinose, D-glucuronic
	Arabinoxylan	D-xylose, L-arabinose
	Galacto(gluco)mannan	D-glucose, D-mannose, D-galactose
	Mannan/galactomannan	D-mannose, D-galactose
	Xyloglucan	D-glucose, D-xylose, D-fructose, D-galactose
Pectin	Homogalacturonan	D-galacturonic acid
	Xylogalacturonan	D-galacturonic acid, D-xylose
	Rhamnogalacturonan I	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, ferulic acid, D-glucuronic acid
	Rhamnogalacturonan II	D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose, L-fucose, D-glucose, D-manno-octulosonic acid (KDO), D-lyxo-heptulosaric acid (DhA), D-xylose, D-apiose, L-acetic acid
Inulin		D-fructose, D-glucose
Starch	Amylose	D-glucose
	Amylopectin	D-glucose
Lignin		Monolignols: p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol

Note: Renewable Fuel Association, 2020. Available at: https://ethanolrfa.org/statistics/annual-ethanol-production/, accessed April 2021.

Table 3 Comparison of biomass feedstock content

Feedstock	*Condition*	*% Cellulose*	*% Hemicellulose*	*% Lignin*
Corn stover	premilled	39.4	33.1	14.9
Wheat straw		34.9	22.5	21.3
Rice straw		41.6	31.5	12.5
Miscanthus		41.9	26.6	13.3
Sorghum	Whole sorghum pith and bark	15	12.3	5.8
Switchgrass	Late cut	46.1	32.2	12.3
Sugarcane		48.6	31.1	19.1
Hardwood	Beech	43.3	31.8	24.4
Softwood	Spruce	40.4	31.1	28

D-mannose (**Fig. 1**) (Wang *et al.*, 2020; Scheller and Ulvskov, 2010). Xyloglucan, the most abundant hemicellulose in the primary cell wall, has the same backbone as cellulose, but contains α-1,6-D-xylose residues and acetyl residues as sidechains. Attached to the D-xylose residues are D-fucose, α-1,2 L-arabinose, and α-and β-1,2-D-galactose residues (Dallabernardina *et al.*, 2016; Pauly and Keegstra, 2016; Scheller and Ulvskov, 2010). Xylan is a polymer with a β-1,4-D-xylose backbone with residues such as α-1,2-(4-O-methyl)-D-glucuronic acid (glucuronoxylan), α-1,2- or 1,3-L-arabinose (arabinoxylan), and α-1,4- and β-1,4-D-galactose (Pontes, 2018; Wang *et al.*, 2020; Scheller and Ulvskov, 2010). Galacto(gluco)mannan consists of a β-1,4-D-mannose residue backbone that can be interrupted by β-1,4-D-glucose residues (Varga and Samson, 2008). Side chains for Galacto(gluco)mannan include α-1,6-linked and β-1,2 linked D-galactose residues as well as acetyl groups (Timell, 1967; de Vries and Visser, 2001; Varga and Samson, 2008). All types of hemicellulose may be further crosslinked to cellulose via hydrogen bonds, creating a more rigid structure (Pontes, 2018; Varga and Samson, 2008; Scheller and Ulvskov, 2010).

Pectin has important roles in the strength and defensive properties of plant cell walls (Voragen *et al.*, 2009; Hahn *et al.*, 1981; Uluisik and Seymour, 2020). It is a complex heteropolysaccharide with an α-1,4-D-galacturonic acid backbone. It consists of different ratios of homogalacturonan, xylogalacturonan, or rhamnogalacturonan I or II (Pontes, 2018; Uluisik and Seymour, 2020). Homogalacturonan is the most abundant pectin domain (Yang *et al.*, 2018) and can be methyl-esterified (Pontes, 2018; López-Mondéjar *et al.*, 2019; Schmitz *et al.*, 2019). Xylogalacturonan polymers are decorated homogalacturonan, with D-xylose side chains that are β-1,2, β-1,3 β-1,4 (Nakamura *et al.*, 2002; Le Goff *et al.*, 2001; Zandleven *et al.*, 2006) linked to the main chain (Pontes, 2018; Schmitz *et al.*, 2019; Varga and Samson, 2008). Rhamnogalacturonan I has an alternating α-1,2-D-galacturonic acid and L-rhamnose backbone. The Rhamnogalacturonan I side chains include L-arabinose and/or D-galactose to the L-rhamnose residues (Pontes, 2018; Schmitz *et al.*, 2019). Rhamnogalacturonan II is an oligosaccharide with diverse side chains of over 30 different monomers (Pontes, 2018; Schmitz *et al.*, 2019; Varga and Samson, 2008) **Figs. 2** and **3**.

Since plant carbohydrates are high energy molecules, they are often targets for digestion by heterotrophs, such as fungi. Lignin functions as a chemical and physical barrier to prevent enzymatic degradation of nearby polysaccharides (Leong and Berka, 1991; Wang *et al.*, 2020). It consists of the monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (López-Mondéjar

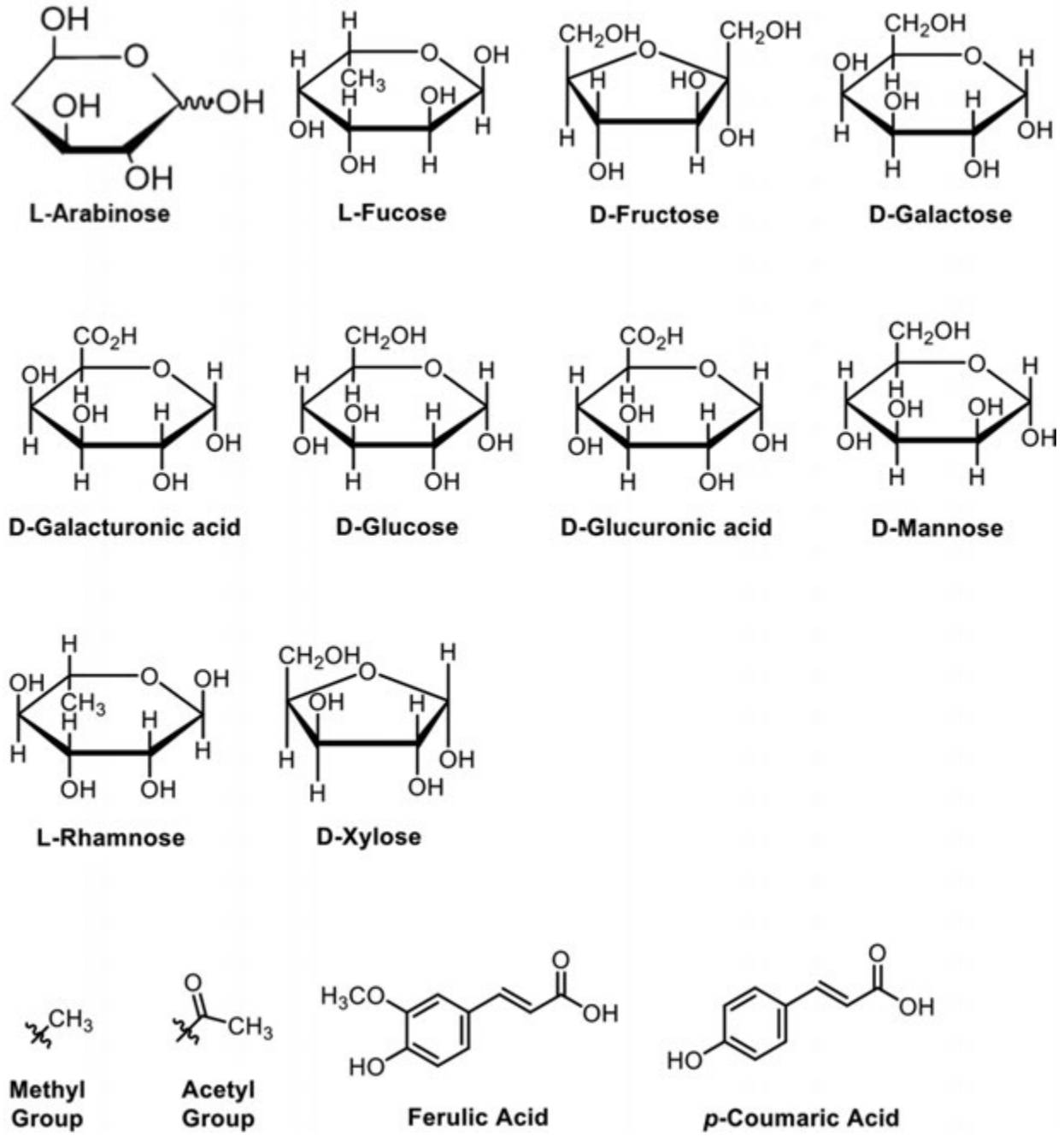

Fig. 1 Common monosaccharides.

et al., 2019). These monomers undergo enzymatic radical coupling polymerization to produce a heterogenous and amorphous matrix (Ganewatta *et al.*, 2019). As a result, lignin is thought to have no regularly organized linkage in the structure. Since there is no known single bond motif that a hydrolytic enzyme could break, it serves as an effective barrier to degradation (Pontes, 2018; Varga and Samson, 2008; Leong and Berka, 1991; Orth *et al.*, 1991).

Plants have evolved diverse ways to use and store carbohydrates. One of these specialized functions is to protect plant biomass from degradation, seen particularly in lignin. However, fungi have evolved many mechanisms to elude plant protections, in order to degrade and access the stored energy within plant carbohydrates. In fact, fungi can be found in almost every known biotope because they have become resilient to extreme conditions and can use a wide assortment of biomass substrates. The next section will review the diverse enzymatic pathways fungi use to degrade plant polysaccharide components.

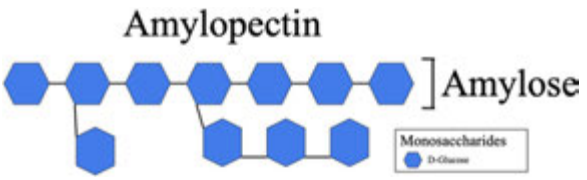

Fig. 2 Schematic structure of amylopectin and amylose.

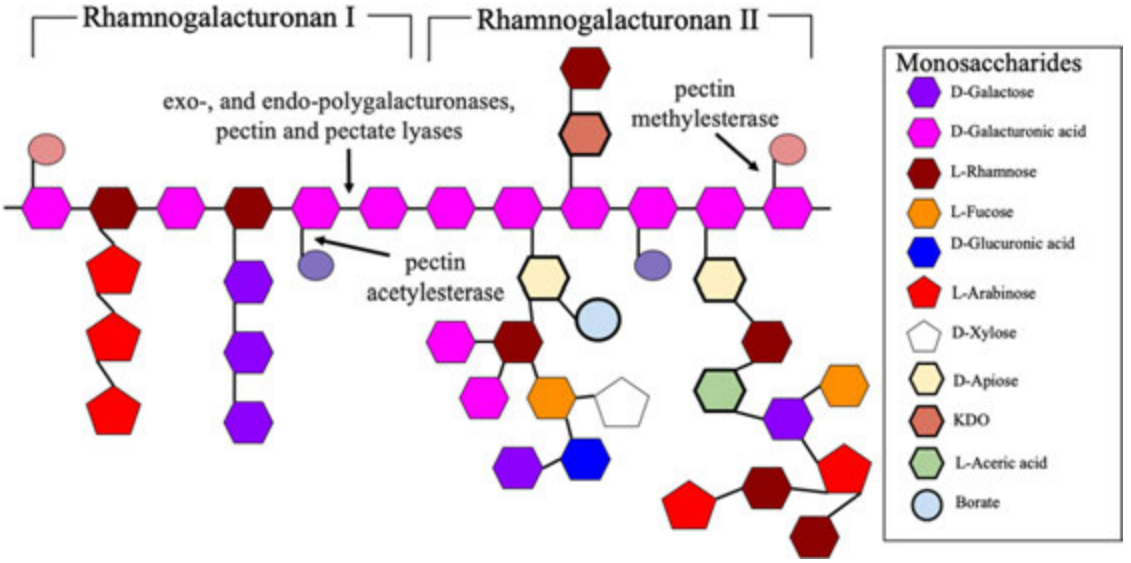

Fig. 3 Schematic structure of Rhamnogalacturonan I and II and modifications by several enzymes: Pectin methylesterase, Pectin acetylesterase, Exo- and endo- polygalacturonases, and Pectin and Pectate lyases.

How do Fungi Break Down Plant Polysaccharides?

"The world depends on fungi, because they are major players in the cycling of materials and energy around the world", Dr. E O Wilson (Watts *et al.*, 2018).

Fungi utilize extracellular proteins to break down plant polysaccharides into simpler monomers. These are called Carbohydrate Active Enzymes, or CAZymes (Garron and Henrissat, 2019). Each protein falls into one of six main families: glycoside hydrolases (GH), glycosyltransferases (Sørensen *et al.*, 2010), polysaccharide lyases (Laplace *et al.*, 1991), carbohydrate esterases (CE), auxiliary activities (AA) and carbohydrate binding modules (CBM) (Benocci, 2018; Garron and Henrissat, 2019). While there are many different CAZymes, only the main types for each polysaccharide group will be described. Furthermore, enzymes can be classified as hydrolytic or oxidative, with substrates that range from polysaccharides to lignin and open phenyl ring degradation (Andlar *et al.*, 2018). Different suites of enzymes determine the type of biodegradation. The biodegradation pattern then characterizes fungal decay as soft-rot, white-rot, or brown-rot (Andlar *et al.*, 2018; Daniel, 2014). A general overview follows describing how common fungal enzymes degrade cellulose, hemicellulose, lignin, pectin, and starch. Only the most common enzymes will be discussed, and **Table 4** represents a summary of relevant enzymes.

Much of the early study of cellulases was performed on a fungal strain of *Trichoderma reesei* discovered in southeast Asia on the Solomon Islands (Montenecourt, 1983; Reese, 1976; Reese *et al.*, 1950). *T. reesei* was collected as part of a project to understand the notorious ability of the fungus to destroy cotton clothes and tents in the warm and humid South Pacific during World War II (Leong and Berka, 1991; Bischof *et al.*, 2016; Wang *et al.*, 2020; Reese *et al.*, 1950). Subsequent isolation of strains of this fungus formed the basis for the Army Quartermaster fungal collection. In 1950, this mesophilic soft rot fungus was isolated and named *Trichoderma viride* by Dr. Elwyn T. Reese, Dr. Mary Mandels, and their coworkers of the US Army Natick Research Laboratory (Leong and Berka, 1991; Bischof *et al.*, 2016; Reese *et al.*, 1950). They screened 14,000 *T. viride* isolates, later changed to *T. reesei* after Dr. Reese, from the Quartermaster Collection and identified strain QM6a for its superior ability to degrade cellulose. *T. reesei* strain QM6a became a reference strain for cellulase discovery (Reese, 1956) and characterization, had its genome sequenced (Martinez *et al.*, 2008), and is the predecessor of many cellulase production mutants used today (Bischof *et al.*, 2016; Peterson and Nevalainen, 2012; Koike *et al.*, 2013; Le Crom *et al.*, 2009). They identified several cellulase enzymes required for the breakdown of cellulose to glucose (Leong and Berka, 1991; Montenecourt, 1983; Reese, 1956). When Reese retired in 1971, Dr. Mary Mandels

Table 4 Polymer degrading enzymes, EC number, and CAZy family

Polymer		*Enzyme*	*EC Number*	*CAZy Family*
Cellulose		cellulose 1,4-β-cellobiosidase	3.2.1.91	GH
		cellulase	3.2.1.4	GH
		β 1,4-glucosidase	3.2.1.21	GH
		cellobiose dehydrogenase	1.1.99.18	AA
		lytic cellulose monooxygenase	1.14.99.54,56	AA
Hemicellulose	Xyloglucan	endoglucanase	3.2.1.4	GH
		xyloglucanase	3.2.1.151	GH
		acetylxylan esterase	3.1.1.72	CE
		α-1,4 D-xylosidase	3.2.1.177	GH
		α-arabinofuranosidase	3.2.1.55	GH
		α- and β-galactosidase	3.2.1.22, 23	GH
	Xylan	endo β-1,4 xylanase	3.2.1.32,8	GH
		β-1,4 D-xylosidase	3.2.1.37	GH
		acetylxylan esterase	3.1.1.72	CE
	Galacto (gluco) mannan	β-1,4-mannanase	3.2.1.25	GH
		β-1,4-D mannosidase	3.2.1.25	GH
		β-1,4-D-glucosidase	3.2.1.3, 21	GH
		acetylxylan esterase	3.1.1.72	CE
		α-1,4-D-galactosidase	3.2.1.22	GH
Lignin		laccase	1.10.3.2	AA
		lignin peroxidase	1.11.1.14	AA
		versatile peroxidase	1.11.1.16	AA
		manganese peroxidase	1.11.1.13	AA
Pectin	Backbone	pectin lyase	4.2.2.10	PL
		pectate lyase	4.2.2.2,9	PL
		rhamnogalacturonan(endo/exo) lyase	4.2.2.23, 24	PL
	Homogalacturonan	exopolygalacturonase	3.2.1.67, 82	GH
		endopolygalacturonase	3.2.1.15	GH
	Xylogalacturonan	xylogalacturonan hydrolase	3.2.1.-	GH
Starch		α-amylase	3.2.1.1	GH
		α-1,4-glucosidase	3.2.1.20	GH
		glucoamylase	3.2.1.3	GH

took over conducting fundamental studies of the cellulase enzyme. She spearheaded the national bioconversion studies for four decades and characterized several cellulase genes (Allen *et al.*, 2009).

Hydrolytic cellulose degradation uses three main enzymes: endocellulases, exocellulases or cellobiohydrolases, and β-glucosidases. Endocellulases cleave within an intact cellulose chain (López-Mondéjar *et al.*, 2019). Exocellulases or cellobiohydrolases release cellobiose from the exposed ends the cellulose chain, and β-glucosidases convert cellobiose dimers to glucose monomers (López-Mondéjar *et al.*, 2019). Research has highlighted a unique partnership between two additional cellulolytic enzymes cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenases (LPMOs). The most accepted theory of CDH function is that it initiates a two-electron oxidation of cellodextrins from cellulose generating either reactive superoxides or hydrogen peroxide, which produce hydroxyl radicals through the Fenton reaction. The free radicals attack and break down localized biomass substrates (Ludwig *et al.*, 2010; Henriksson *et al.*, 2000; Mason *et al.*, 2003; Bissaro *et al.*, 2018). Recent evidence proposes CDH is a redox partner for AA9 LPMOs (Tan *et al.*, 2015; Courtade *et al.*, 2016; Bissaro *et al.*, 2018). LPMOs are metalloenzymes able to degrade cellulose, chitin, starch, and hemicellulose (López-Mondéjar *et al.*, 2019). They consist of four main families of auxiliary activity enzymes: AA9, AA10, AA11, and AA13 (Hemsworth *et al.*, 2014; Leggio *et al.*, 2015; Bomble *et al.*, 2017). LPMO oxidation uses a divalent metal ion, molecular oxygen, and electron donor to cleave the C1 or C4 carbon in the β-1,4 glycosidic bonds in cellulose (López-Mondéjar *et al.*, 2019; Müller *et al.*, 2015; Leggio *et al.*, 2015). Interestingly, fungal LPMOs use the plant defense mechanism of lignin to its advantage–cell wall lignin serves as an electron supply for long-range electron transfer, driving LPMO enzymatic activity on polysaccharides (Westereng *et al.*, 2015; Bischof *et al.*, 2016). They work in synergy with other carbon-active enzyme families. It has been suggested LPMOs fragment the surface of cellulose microfibrils to enhance GH activity (López-Mondéjar *et al.*, 2019; Bomble *et al.*, 2017). For example, when LPMOs from *Thermoascus aurantiacus* were added to commercial cellulase, cellulose solubilization increased by 60% (Müller *et al.*, 2015) **Fig. 4**.

Hemicellulose compounds like xyloglucan, xylan, and galacto(gluco)mannan are primarily degraded by enzymes from the CAZy glycoside hydrolase family. GH enzymes hydrolyze the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety (López-Mondéjar *et al.*, 2019). Xyloglucan is hydrolyzed by specialized endoglucanases, xyloglucanases, acetylxylan esterases, α-1,4-D-xylosidases, α-arabinofuranosidases, and α- and β-galactosidases (de Vries and Visser, 2001; Del Bem and Vincentz, 2010). Xylan degradation involves β-1,4-xylanases, β-1,4-D-xylosidases, and acetylxylan

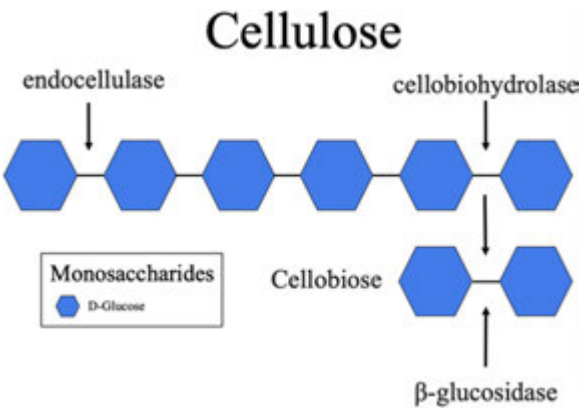

Fig. 4 Schematic structure of Cellulose and modifications by several enzymes: Endocellulase, Cellobiohydrolase, and β-glucosidase.

esterases. While these activities are best characterized, there are also new reports of xylan-specific LPMOs such as McAA9 which shows a preference for a xylan substrate (Hüttner *et al.*, 2019) and PcAA14B, which works synergistically with other xylanse enzymes (Zerva *et al.*, 2020). As an example of xylan degradation, *T. reesei* secretes two major inducible endo β-1,4-xylanases, which cleave the xylan backbone into shorter oligomers (Fukuda *et al.*, 2009). Acetylxylan esterase removes xylan side groups. β-1,4-Xylosidase hydrolyzes xylobiose into D-xylose. Lastly, galacto(gluco)mannan degradation requires combined action of main chain degrading enzymes like β-1,4-mannase, β-1,4-D-mannosidase, and β-1,4-D-glucosidase, and side chain degrading enzymes α-1,4-D-galactosidases (de Vries and Visser, 2001). β-1,4-Mannanase cleaves the β-1,4-D-mannose backbone to manno-oligosaccharides. β-1,4-D-Glucosidases further hydrolyze these released monomers. β-1,4-Mannosidase cleaves D-mannose from the terminal ends of mannan. β-1,4-D-Galactosidases remove side-chain substituents to create more sites for enzymatic hydrolysis (Varga and Samson, 2008; Moreira, 2008) **Figs. 5–7**.

Lignin forms a recalcitrant matrix with cellulose and hemicellulose to hinder enzymatic attack (Pontes, 2018). Further, it is a prime target for biofuel source material because it accounts for 25% of the energy that plants capture from the sun (Holzbaur *et al.*, 2017). Many fungi have evolved lignin degrading or modifying enzymes. For example, wood decaying white-rot fungi induce delignification by producing laccases, lignin peroxidases, versatile peroxidases, and manganese peroxidases (Leong and Berka, 1991; Orth *et al.*, 1991; Hatakka, 1994; López-Mondéjar *et al.*, 2019). Lignin peroxidase was first discovered in the model white-rot fungus *Phanerochaete chrysosporium* in 1983 (Gold *et al.*, 1983; Kirk and Tien, 1983). This enzyme oxidizes non-phenolic lignin substrates (Chan *et al.*, 2020; Hatakka, 1994; Hatakka and Hammel, 2011; Kirk and Farrell, 1987), and it's activity increases in the presence of the secondary metabolite veratryl alcohol (Schoemaker and Piontek, 1996; Vandana *et al.*, 2019). The "white-rot" classification arises when more extensive degradation of lignin and hemicellulose than cellulose occur, bringing a white appearance to rotting wood due to the remaining cellulose (Kirk and Farrell, 1987).

Brown-rot fungi, such as *Postia placenta*, evolved from white-rot fungi by genome contraction–loss of multiple gene families like peroxidases and oxidases. Brown-rot fungi selectively remove cellulose and hemicellulose and only slightly modify lignin (Kirk and Moore, 2007; Martinez *et al.*, 2009). They use endoglucanases and hemicellulases to open amorphous regions of cellulose microfibrils and break down the overall cellulose component (Mester *et al.*, 2004). However, the mechanism used to depolymerize cellulose is still unclear. Martinez *et al.* (2009) noted that the genome of the brown-rot fungus *P. placenta* lacks enzymes that are characteristic of all cellulolytic fungal aerobes. The decay resulting from brown-rot fungi causes wood to loose significant strength, shrink, and darken, hence their name (Green III and Highley, 1997).

Pectin degrading enzymes have been studied in phytopathogenic fungi as virulence factors. Common backbone-acting pectinases are endo-polygalacturonases (endo-PG), exo-polygalacturonases (exo-PG) (Li *et al.*, 2004; Chou *et al.*, 2015) and pectin- and pectate lyases (PLs) (Uluisik and Seymour, 2020). Exo-PGs remove D-galacturonic acid residues from the non-reducing ends of Homogalacturonan chains. Endo-PGs hydrolyze the α-1,4-glycosidic bonds of HG chains (Yang *et al.*, 2018). Pectin lyases prefer substrates with a high degree of methyl esterification while pectate lyases prefer a low degree of esterification (de Vries and Visser, 2001). Deletion of PG encoding genes can decrease virulence, such as targeted deletion of *Bcpg1* and *Bcpg2* in *Botrytis cinerea* (Kars *et al.*, 2005), *pg1* and *pgx6* in *Fusarium oxysporum* (Bravo Ruiz *et al.*, 2016), and *ccpg1* and *cppg2* in *Claviceps purpurea* (Oeser *et al.*, 2002), with some deletions rendering the fungus nearly non-pathogenic. PLs randomly cleave the β-1,4-linkages of homogalacturonan by β-elimination to generate oligogalacturonates (Uluisik and Seymour, 2020; Yang *et al.*, 2018). Genes that encode PLs have also been implicated as virulence factors, like *pelA* and *pelD* in *Fusarium solani* (Rogers *et al.*, 2000) and *pnl1* in *Penicillium digitatum* (López-Pérez *et al.*, 2015). Pectinases acting on the groups that decorate the pectin backbone include pectin methylesterases (PMEs) and endo-arabinanases (ABNs) (Schmitz *et al.*, 2019). PMEs remove methyl residues, catalyzing pectin de-esterification, and ABNs hydrolyze arabinan oligosaccharides in RGI side chains (Schmitz *et al.*, 2019) **Fig. 8**.

Starch is degraded by enzymes like α-amylase, α-1,4-glucosidase, and glucoamylase (Benocci, 2018). The α-amylase breaks down the 1,4-glycosidic linkages in starch into glucose, maltose, and other oligosaccharides (Wang *et al.*, 2020). Glucoamylase hydrolyzes the α-(1,4)- and α-(1,6)-glycosidic linkages from the nonreducing ends of starch and malto-oligosaccharides to free

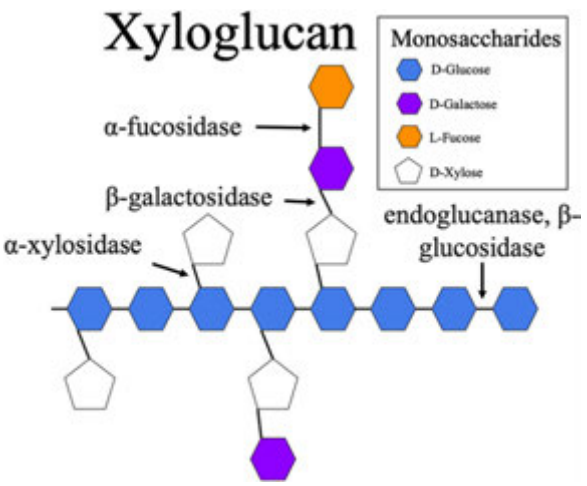

Fig. 5 Schematic structure of Xyloglucan and modifications by several enzymes: α-Fucosidase, β-Galactosidase, α-Xylosidase, Endoglucanase, β-Glucosidase.

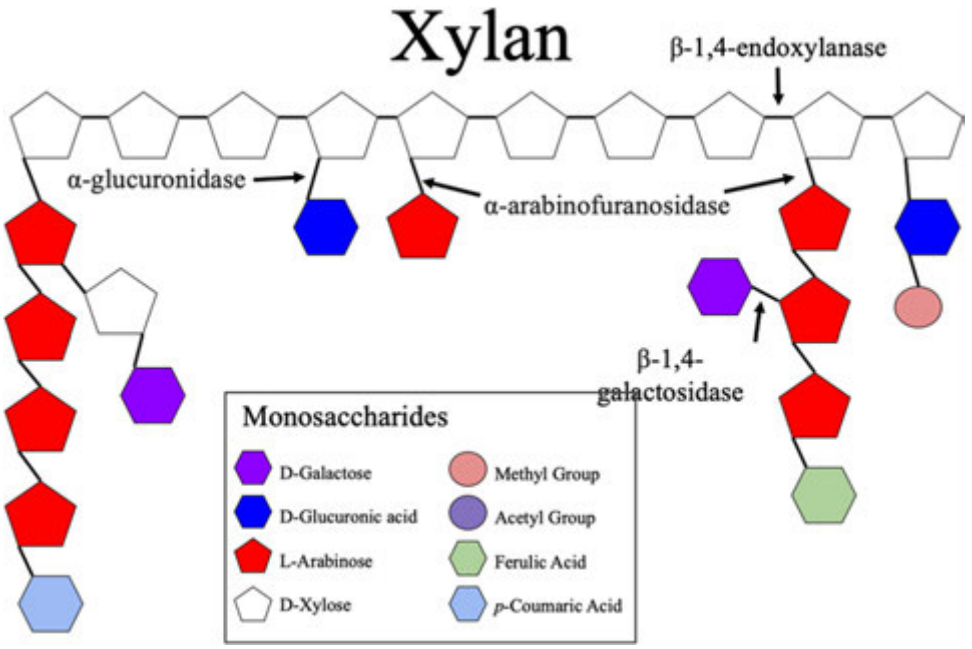

Fig. 6 Schematic structure of Xylan and modifications by several enzymes: β-1,4 endoxylanase, α-glucuronidase, α-arabinofuranosidase, and β-1,4 galactosidase.

glucose (Ashkari *et al.*, 1986). Most amylolytic enzymes were first identified in *Aspergillus oryzae* (Hata *et al.*, 1991; Wirsel *et al.*, 1989; Norihiro *et al.*, 1989) and more recent research has highlighted regulation of amylase genes. For example, the Zn_2Cys_6 *AmyR* transcriptional activator turns on expression of starch degrading enzyme encoding genes, like those encoding glucoamylase, α-amylase, and α-glucosidase (Varga and Samson, 2008). When two transcription factors were deleted in *A. oryzae*, α-amylase activity was 2-fold higher (Ichinose *et al.*, 2014). Additional transcription factors investigated for starch degradation include *devR* (Zhuang *et al.*, 2019a), *flbC* (Tanaka *et al.*, 2016) in *A. oryzae*, and *PoxNsdD* in *Penicillium oxalicum* (He *et al.*, 2018).

Production of fungal starch degrading enzymes has a long history in Koji production, reaching back 2500 years to enhance palatability of foods in China (Dunford, 2012; Machida *et al.*, 2008; Baker and Bennett, 2007). The application of Koji fungi like *Aspergillus*, *Rhizopus*, *Mucor*, *Monascus*, and *Penicillium* species for starch hydrolysis were used on solid fermentation substrates of different grains (Zhu and Tramper, 2013). In the late 19th century Dr. Jokichi Takamine adapted the koji process in the American distillery industry. He received a patent entitled "Process of making diastatic enzyme" for extracting an active amylase from *A. niger* growing on wheat bran (Zhu and Tramper, 2013; Takamine, 1894). Starch degrading enzymes were also an early US biotechnological development, seen in a patent for enzyme production by *A. niger* (NRRL 337) filed in 1948 (Le and Van Lanen,

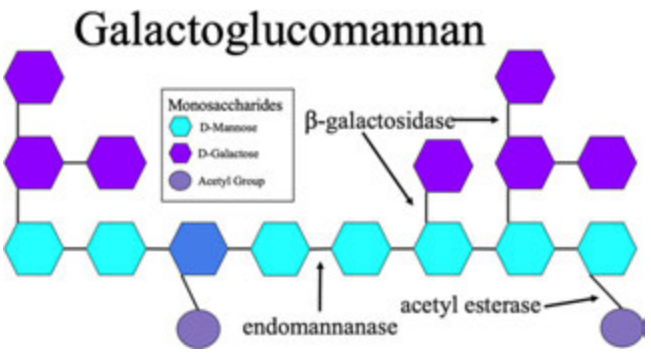

Fig. 7 Schematic structure of Galactoglucomannan and modifications by several enzymes: Galactosidase, Mannanase, and Acetyl esterase.

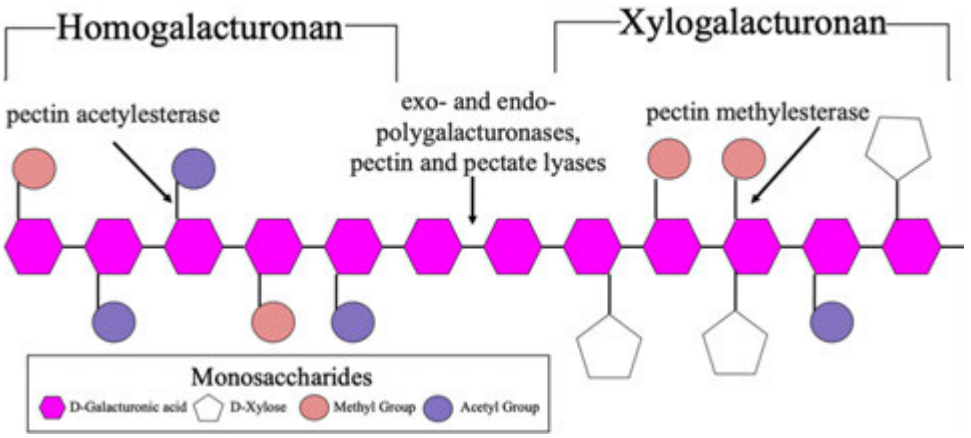

Fig. 8 Schematic structure of Homogalacturonan and Xylogalacturonan and modifications by several enzymes: Pectin acetylesterase, Pectin methylesterase, Exo- and endo- polygalacturonases, and Pectin and Pectate lyases.

1948). Fungal starch degrading enzymes have also been harnessed by animals in nature. Leafcutter ants cultivate *Leucoagaricus gongylophorus*, an efficient producer of starch degrading enzymes, in fungal gardens. They inoculate α-amylase-producing fungal mycelium on leaf pieces to break down plant starch into maltose. Ants then secrete maltase, an enzyme that converts maltose into glucose, which is used for food (Silva *et al.*, 2006; Bacci *et al.*, 2013).

Transcription Factors

Fungi have evolved an efficient set of transcriptional regulators to ensure the correct enzyme cocktail is produced at the right time (Benocci *et al.*, 2017). This enables a dynamic balance of transcriptional activation and repression to fit the needs of the environment and adapt to changing carbon sources by altering expression according to the necessities of the cell (Pontes, 2018; Varga and Samson, 2008; Benocci, 2018). Transcription factors are engineering targets to make fungi more efficient for biomass deconstruction and biofuel production. The availability of multi-omic analysis paired with classical genetics has identified important fungal transcriptional regulators controlling production of CAZymes in fungi. This section will review CAZyme-relevant transcription factors from the zinc cluster family (also called binuclear cluster), CreA, and some examples of how transcription factors have influenced the expression of lignocellulolytic enzymes.

The majority of DNA binding transcription factors belong to the zinc cluster family (Wang *et al.*, 2020), characterized by the presence of zinc fingers in their binding domains. Regulator families are further classified based on the overall shape of the protein backbone in the folded domain, which depends on cysteine and histidine residues. Most positive regulators belong to the Zn_2Cys_6 class, while most repressors belong to the Zn_2His_2 class (Keegstra, 2010). For example, a Zn_2Cys_6 gene called GalR in *Aspergillus nidulans* regulates genes encoding D-galactose catabolic enzymes, suggesting a role in pectin and hemicellulose degradation (Varga and Samson, 2008; Christensen *et al.*, 2011; Kowalczyk *et al.*, 2015). The zinc cluster positive regulator XlnR regulates xylanolytic and cellulolytic genes in *Aspergillus niger*, *T. reesei*, *Trichoderma. atroviride*, *Neurospora crassa*, *Penicillium canescens*, *Fusarium oxysporum*, *Magnaporthe oryzae*, and *Aspergillus* spp. (Wang *et al.*, 2020; Benocci, 2018). Exploiting the regulation of XlnR could enhance industrial processes for degrading cellulose and hemicellulose (Wang *et al.*, 2020).

Fungal enzyme production is controlled by environmental sensing and the repression of genes that enable metabolism of non-preferred carbon sources, known as carbon catabolite repression (CCR). This process is controlled by the transcription factor CreA,

a Zn_2His_6 DNA-binding repressor first discovered in *A. nidulans* (Wang *et al.*, 2020; de Assis *et al.*, 2018; Esperón *et al.*, 2014). CreA repression prevents wasting energy on the production of extracellular enzymes that are not needed, specifically when a preferred substrate is available (Wang *et al.*, 2020). De-repression of CreA leads to increased transcription of hemicellulolytic and cellulolytic enzymes (Pontes, 2018; Varga and Samson, 2008).

Transcriptional regulators are a target for strain optimization by both mutagenesis and synthetic design (Baker, 2018). For example, the Natick Army Research Laboratory and Rutgers University worked to improve the original *T. reesei* strain QM6a by mutagenesis (Bischof *et al.*, 2016). The team increased cellulase production up to 20 fold, creating the strain RUT-C30, where "Rut" stands for Rutgers (Bischof *et al.*, 2016; Montenecourt and Eveleigh, 1979). To further enhance cellulase production of *T. reesei*, Liu *et al.* (2019a,b,c) combined mutated genes of RUT-C30 and its sister strain SS-II (Le Crom *et al.*, 2009; Koike *et al.*, 2013), which contains unique mutations in 57 genes and a full length CCR gene *cre1*, the ortholog of CreA (Ilmén *et al.*, 1996; Rassinger *et al.*, 2018). The recombinant strain grew faster than RUT-C30 on various carbon sources and had more efficient cellulase production. First Liu *et al.* (2019a,b,c) tested if transforming/introducing the RUT-C30-characterized $cre1_{96}$ truncation into SS-II would improve cellulase production. Instead, it reduced growth rate in SS-II and decreased total cellulase production. However, several other SNP-containing genes in SS-II increased extracellular cellulase production over 70% when recapitulated in RUT-C30: tre108642, an unknown protein, and tre56839, annotated as a zinc-dependent alcohol dehydrogenase-like protein. The mutant tre108642 exhibited higher expression levels of transcription levels of five key cellulase genes and the transcriptional activator *xyr1* (Liu *et al.*, 2019b). Comparative genomic analysis promises additional gene-to-phenotype characteristics for improvement of fungal performance in industry.

Synthetic construction of CAZy regulators in the RutC30 background has also increased cellulase activity. For example, Zhang *et al.* (2018) enhanced cellulase and xylanase activity in RUT-C30 by replacing carbon-responsive regulators *ace2* and *cre1* with minimal transcriptional activators. These synthetic activators contain the respective DNA binding domain for *ace2* or *cre1* and a strong transcriptional activation domain VP16, but lack the central domain involved in repression. When tested for FPase (cellulase) activity, the *ace2* transformant (T-MTA139) was 26.5 fold higher than RUTC30. When T-MTA139 was tested on corn stover supplemented with β-glucosidase, glucose yields were comparable to those obtained with the commercial enzyme CTec2 (Zhang *et al.*, 2018). This illustrates that minimal transcription factor constructs can enhance cellulase production and offers a genetic engineering strategy for industrial production of fungal enzymes.

Many experiments modifying transcription factors find patterns in enzyme regulation by observing how deleting a gene effects enzyme production, for example with *fbx23* and *fibA*. Fbox protein Fbx23 was tied to loss of CreA repression and targeted CreA degradation in *A. nidulans*. The E3 Skip1-Cullin-Fbox (SCF) Ubiquitin Ligase Complex, which marks proteins for degradation, contains Fbx23. *fbx23* deletion strains ($^{\Delta}$*fbx23)* were grown under both CreA repressive (glucose) and de-repressive (xylan) growth conditions (de Assis *et al.*, 2018). They followed the expression of a known target of CCR, the endo-1,4-β-xylanase encoding gene *xlnA* by RT-qPCR. Expression of x*lnA* was unresponsive to xylan media in the $^{\Delta}$*fbx23* strains. Without Fbx23, the SCF ubiquitin ligase complex does not correctly target the CreA repressor complex for degradation, offering mechanistic insight into regulation of CreA itself.

There are multi-layered effects of transcription factors on enzyme production. The regulator FibA in *A. niger*, was investigated for its influence on enzyme secretion. FibA is implicated in many processes but is notable in sporulating regions of mycelial colonies, which have lowered enzyme secretion compared to nonsporulating regions. A $^{\Delta}$*fibA* mutant was grown on xylose medium and observed differential expression of 36 transcription factors (Aerts *et al.*, 2019). The most upregulated among these was *rpnR*. RpnR regulates constituents of the ribosome and is repressed when FibA is active. $^{\Delta}$*rpnR* mutants had reduced ribosomal subunit expression, which lowers protein secretion in sporulating regions. This finding suggests *rpnR* may be target for improving enzyme secretion of *A. niger* for industrial purposes.

In a similar engineering effort in *P. oxalicum*, Gao *et al.* (2019) modified ClrB, a Zn_2Cys_6 transcriptional activator of cellulolytic enzyme genes, constructing a fusion of the DNA binding domain, and the activator domain encoded at the protein C-terminus. Deletion of the central part of the *clrB* gene not encoding these two functions also seems to have ablated the post-translational requirement of cellulose as an inducer for ClrB-activation activity. The resulting derivative strain $clrB^{ID}$ allowed production of cellulase on normally cellulase-repressive carbon sources such as glucose and glycerol. Deletion of *creA* in the $clrB^{ID}$ fusion strain showed a further increase in cellulase expression, suggesting that removal of cellulase repression is independent of CreA function. A similar modification with a $clrB^{ID}$ analog in *A. niger* was made. The resulting strain produced increased cellulose degrading enzymes on glucose, like cellobiohydrolase and endoglucanase, and hemicellulose degrading enzymes, like mannanase (Gao *et al.*, 2019). Selective deletion of regulatory regions of a transcription factor can improve enzyme production. These insights on cellulase production on various substrates is important for engineering strains for industrial capabilities.

Silencing of transcriptional regulators has been used to improve thermal and chemical properties of cellulolytic enzymes. For example, RNA-seq profile analysis identified MHR1 as a repressor in the thermophilic fungus *Myceliophthora thermophila* (Wang *et al.*, 2018). This species is of particular interest due to its ability to produce thermostable cellulolytic enzymes, an attractive quality for high temperature industrial treatment and fermentation processes. Silenced *hmr1* expression increased transcription levels of cellulase degrading CAZymes encoding genes *cbh1*, *cbh2*, *egl3*, and the positive regulator *xyr1* (Wang *et al.*, 2018).

Transcription factors play key roles in regulating enzymes to deconstruct lignocellulolytic biomass. Deleting, silencing or truncating transcription factors can increase lignocellulolytic gene expression but may also cause unanticipated systemic effects on fungal growth and processes. Hyper-cellulolytic strains like RUT-C30 and SS-II exemplify how mutations in multiple transcription factors could hinder or improve growth and secretion. Additional research has investigated the transcription factors discussed, like *cre1* (Porciuncula *et al.*, 2013). More genes encoding transcription factors have been studied like *clr1*, *clr2*, *ace2*, and *ace3* (Benocci, 2018; Häkkinen *et al.*, 2014), *crz1*, *xyr1*, *ace1* (Chen *et al.*, 2016; Jiang *et al.*, 2016), *trpac1* (He *et al.*, 2014), *rce1* (Cao *et al.*, 2017),

AraR and *XlnR* (Ishikawa *et al.*, 2018), *pac1* (Häkkinen *et al.*, 2015), *FlbC* (Tanaka *et al.*, 2016), *xyr1* and *ace3* (Xue *et al.*, 2020), and *kap8* (Ghassemi *et al.*, 2015). Further insights on CAZymes and transcription factors will improve fungal engineering capabilities for industrial production of lignocellulolytic enzymes.

Fungi in the Biofuel Industry

"Better biofuels are a really big deal. That means we can precisely engineer the molecules in the fuel chain and optimize them along the way. So, if all goes well, they're going to have designer bugs in warm vats that are eating and digesting sugars to excrete better biofuels", John Doerr (Doerr, 2007).

Fungi achieved commercial viability in manufacturing, food, enzyme, and pharmaceutical industries for being able to secrete large amounts of valuable CAZymes (Dunford, 2012). Fungal enzymes account for 60% of the enzymes used in the food, cleaning product, textile and paper manufacturing industries (Kew, 2018). As we build the framework of the bioeconomy, fungal enzymes are especially valuable for plant biomass treatment in the creation of biofuels. For lignocellulosic feedstock inputs, the cost of enzymes represents up to 28% of the costs of biofuel production (Johnson, 2016; Aerts *et al.*, 2019). Scientists have engineered and bio-prospected fungi for desired characteristics, like withstanding higher temperature, specialized enzymes, all toward higher biofuel yields. The next few pages will describe enzymatic degradation by fungi, ethanol fermentation, and novel biofuels.

Ethanol as a Biofuel

Ethanol is currently used as a blending agent with gasoline to supplement octane and reduce carbon emissions (Gnansounou, 2008; Meng, 2019). A 2015 report from the U. S. Department of Agriculture revealed a high corn-based ethanol net energy yield of 2.1 (Meng, 2019; Gallagher *et al.*, 2016). The three predominant feedstocks for ethanol production are sugar crops, starch crops, and lignocellulosic biomass. Lignocellulosic feedstocks have become more popular as most do not compete with food crops, making them a second generation biofuel. However, they increase the cost of fuel production because the cellulosic material must be broken down into fermentable sugars while other feedstocks have sugars readily available.

With plant feedstocks as input, the production of ethanol is a complex and tailored process, but can be abridged to five steps. First, the plant biomass is compressed to become uniform. Then, the mass is exposed to extreme temperature and pressure (Merino and Cherry, 2007). This thermochemical pretreatment disrupts and hydrates the substrate to be accessible for enzymatic attack (Merino and Cherry, 2007). Methods of physicochemical pretreatment include gamma irradiation, strong acids like sulfuric acid, and steam explosion (Laxman and Lachke, 2009). The third step is enzymatic hydrolysis, or saccharification. Fungal enzymes are added to degrade the lignocellulosic biomass into simple sugars. Many pretreatments are directed to removing lignin, as it inhibits utilization of cellulose and hemicellulose (Wang *et al.*, 2020). Fermentation begins when conversion organisms, like a fermentative yeast, are added. Approximately 93% of the energy present in sugars is converted to ethanol during fermentation (Bhatia, 2014). In the last step, ethanol is removed and the residual organic waste is disposed of (Merino and Cherry, 2007).

First Generation Feedstocks

Sugar and starch crops are common feedstocks for first generation biofuels. Sugar crops are an ideal candidate because they store high concentrations of sucrose in the stalk (Eggleston *et al.*, 2010) and they reduce the field-to-fuel life-cycle GHG emissions relative to petroleum gasoline by 40%–62% (Wang *et al.*, 2012). The most common sugar crops are sugar cane, sugar beet, and sugar sorghum. As of 2009, sugar crops represented 50% of the world's fuel ethanol production (Eggleston *et al.*, 2010). During saccharification, enzymes catalyze the hydrolysis of sucrose into glucose and fructose. Sucrose hydrolysis is catalyzed primarily by the enzyme invertase, which is common to most yeasts (Eggleston *et al.*, 2010). Other enzymes convert the glucose and fructose into ethanol and CO_2. One ton of glucose or fructose theoretically supplies 511 kg of ethanol. However, practical production is 92% of this supply (Gnansounou, 2008). Byproducts of sugar refineries like molasses and bagasse are also viable feedstocks. Cane molasses contains 16%–52% w/w sucrose (Zagatto *et al.*, 1988). The refinery byproducts also contain more cellulose and hemicellulose, which must be converted to fermentable sugars (Liu and Cotta, 2008).

The most prevalent starch feedstocks are corn, composed of 60%–70% starch (Gnansounou, 2008), and potatoes. To convert starch to ethanol, manufacturers cook starchy biomass at a high temperature (140–180°C), then add enzymes for conversion to mono- or small oligo-saccharides, which are then fermented. Starch from corn must be treated enzymatically (using α-amylase and/or glycoamylase) to make sugars accessible (Alcantara *et al.*, 2019). Starch conversion enzymes are: (1) α-amylase, which cuts within the polysaccharide produced by pre-treatment, and (2) glucoamylase which chews the exposed ends of the sugar chains (Matsumoto *et al.*, 1982; Shigechi *et al.*, 2002).

Deconstruction of Lignocellulosic Material

Specific examples of lignocellulosic biomass includes sorghum, corn stover, grasses, industrial wastes, woody biomass and residues from starch and sugar crops, like molasses (Gnansounou, 2008). However, these feedstocks require a fungus with enzymes to more readily break down cellulose, hemicellulose, lignin, and starch. Mutagenesis has been deployed for many new organisms, as

was the case for *T. reesei* and cellulose degradation. In the case of lignin degradation, the white-rot fungus *Phanerochaete chrysosporium* has risen to prominence. Some scientists have sought ideal fungal degraders in unique places. Others have engineered fungi as whole cell biocatalysts. The importance of using enzymes suited to the target biofeedstock is a repeated theme in fungal bioconversion research. Inducing conditions for fungal enzyme production containing the wrong sugar proportions or types, or the wrong physical structure result in production of an incomplete set of enzymes to digest complex substrates. The next few pages will review a few cases of each method in the search to identify highly efficient lignocellulosic degrading fungi.

Breaking Down Cellulose

Before the development of engineered fungi, enzymatic hydrolysis of lignocellulose was too expensive to compete in the fuel market (Kim and Kim, 2014; NREL, 2010). The Cellulase Cost Reduction Project, a combined effort from the National Renewable Energy Laboratory, Novozymes, and Genencor, decreased enzyme production cost using the cellulase system from *T. reesei* (Merino and Cherry, 2007). The CCRP team improved enzyme activity by combining cellulase enzymes from *T. reesei* with β-glucosidase enzymes from *A. oryzae* or enzymes from *Thielavia terrestris* (Merino and Cherry, 2007). The CCRP achieved whole cell catalysis by expressing genes for β-galactosidase from *A. oryzae*, AA9 protein from *T. terrestris*, and a signal peptide from *Humicola insolens* Cel45A EG in *T. reesei* (Merino and Cherry, 2007). The recombinant strain significantly boosted the expression of target proteins and improved cellulase productivity. The project attained a ten-fold reduction in enzyme cost for the conversion of pretreated corn stover to ethanol (NREL, 2010; Kim and Kim, 2014).

Research on cellulase production in *T. reesei* has continued since then. For example, a mutation in the β-glucosidase II encoding gene *bgl2* in a strain of *T. reesei* had increased cellulase production than without the mutation(Shida *et al.*, 2015). *T. reesei* was tested on milled wheat starch, corn stover, and miscanthus biomass to measure the rate of hydrolysis and sugar yields on different substrates (Vintila *et al.*, 2017). Other organisms have been studied for their ability to break down cellulose. For example, cellulase induction of *Penicillium funiculosum*, *Fusarium verticillioides*, and *Cladosporium cladosporioides* was tested on *Moringa oleifera* softwood residues (Vázquez-Montoya *et al.*, 2020). Various *Talaromyces pinophilus* mutants were developed and tested for enhanced cellulase production on Solka Floc cellulose and corncob powder (Liu *et al.*, 2020). One mutant strain EMM displayed a 4.3 fold, 3.9 fold, and 8 fold increase in FPase activity, β-glucosidase activity, and cellulase production, respectively, compared to its parent strain (Liu *et al.*, 2020). The absence of a cell wall protein encoding gene *ncw-1* in *N. crassa* increased cellulase gene expression (Lin *et al.*, 2017). Advances in enzyme synergy and source organisms have improved enzyme systems related to biorefineries (Champreda *et al.*, 2019).

Many scientists have observed that co-cultivation and mixed fermentation methods improve enzymatic capabilities because of synergism amongst microbial consortia. Co-cultivation of *T. reesei* and *Monascus purpureus* on wheat straw exhibited significantly higher enzymatic activity than their respective individual cultures (Fatma *et al.*, 2020). Additional synergistic performance of microbial consortia on wheat (Kolasa *et al.*, 2014; Jiang *et al.*, 2016) and cereal straw (Ghosh *et al.*, 2017) has been studied. Mixed species fermentation conditions identified *T. reesei* and *A. oryzae* grown together to increase glucose release about 50% compared to individual cultivations on pretreated sugarcane bagasse (Maehara *et al.*, 2018).

Phanerochaete chrysosporium and Lignin Degradation

Novel feedstocks and organisms have diversified the potential of second-generation biofuels and bioproducts. There has been an increased interest in designing new methods to effectively break down crop straw, a lignocellulosic material abundantly available worldwide. However, straw degradation is difficult because it contains 21.3% lignin, nearly as much as hardwood (24% lignin) (see **Table 3**) (Pauly and Keegstra, 2008). *P. chrysosporium*, a white-rot fungus, has emerged as one of the most adept organisms for delignification (Laxman and Lachke, 2009). It was able to remove 42% and 17% of lignin from birch wood (Fan *et al.*, 1982) and grape cluster stems (Laxman and Lachke, 2009), respectively. Other white-rot fungi like *Polyporus adustus* can delignify up to 17% of birchwood (Laxman and Lachke, 2009). Genetic engineering has enhanced delignification. For example, *P. chrysosporium* only produces lignin peroxidase in limiting conditions. However, an engineered mutant, PSBL-1, could produce 4–10 fold more lignin and manganese peroxidase and glyoxal oxidases compared to the control in non-limiting conditions (Orth *et al.*, 1991). Additional research on *P. chrysosporium* has enabled exploitation of lignin degradation in multiple fields (Kiran *et al.*, 2019).

Recent advances on white-rot fungi highlight *Marasmiellus palmivorus*, *Pleurotus ostreatus*, and *Pycnoporus cinnabarinus*. *M. palmivorus* VE111 has become critical in lignin detoxification and the production of laccases for liberation of additional sugar and removal of microbial growth inhibitors from lignin of Eucalyptus wood (Schneider *et al.*, 2020). The secretome of *P. ostreatus* revealed over 170 proteins that were uniquely produced in the presence of wheat straw (Fernández-Fueyo *et al.*, 2016). *P. cinnabarinus* is able to produce high amounts of laccase and polysaccharide monooxygenases when grown on lavender straw (Lesage-Meessen *et al.*, 2018). Scientists are investigating delignifying fungi like *Pleurotus eryngii* (Salvachúa *et al.*, 2016), *P. ostreatus* (Li *et al.*, 2019; Wang *et al.*, 2019), *Trametes* species (Dong *et al.*, 2019; Liu *et al.*, 2019c; Rekik *et al.*, 2019; Jović *et al.*, 2018), *Flavodon ambrosius*, *Ceriporiopsis subvermispora* (Van Erven *et al.*, 2019), and more (Liu *et al.*, 2019a; Surendran *et al.*, 2018). Additional genetic engineering studies express heterologous delignifying enzymes from *Ganoderma lucidium* (Gopalakrishnan *et al.*, 2020). However, lignin degradation releases inhibitor compounds for fermenting organisms; this will be discussed in the fermentation section.

Starch Degradation

Early research into starch substrates has grown into exploration of enzymes from a broader range of environments, such as from cold-active microorganisms. Cold-active enzymes improve industrial processes by decreasing energy inputs of fermentation and broadening the range of process conditions. For example, glucoamylases are often limited to optimal activity at temperatures between 45 and 60°C. However, the cold-adapted yeast *Tetracladium* secretes a glucoamylase Amy1T with optimal activity at 30°C. Protein modeling analysis suggests that Amy1T is suitable for industrial processes, like biofuel production (Carrasco *et al.*, 2017). A screen of enzymes from cold tolerant yeast *Rohodotorula gracilis* showed elevated amylase and cellulase activity at 10–22°C and 22–37°C, respectively (Carrasco *et al.*, 2016). *Mrakia blollopis* showed cellulase activity as low as 4°C (Carrasco *et al.*, 2016). Recent research on temperature conditions for starch degrading enzymes has been investigated fungi like *Fomitopsis palustris* (Tanaka *et al.*, 2020), *M. thermophila* (Xu *et al.*, 2018), *Thamnidium elegans* and *Cordyceps farinose* (Roth *et al.*, 2019). Improvements in our library of available enzymes will decrease limitations of pH or temperature for large scale biomass conversion processes.

Lignocellulose Degrading Specialists

Researchers have found cellulose busting fungi in unique places. Ruminant animals like cows, sheep and buffalo have cultivated anaerobic microbes in their digestive tracts to break down plant matter. For example, the fungus *Neocallimastix patriciarum* was isolated from buffalo rumen (Chen *et al.*, 2012). When Chen et al expressed the *N. patriciarum* β-glucosidase gene in *S. cerevisiae* or *Kluyveromyces marxianus*, both yeast species illustrated more cell growth, glucose production, and ethanol yield on cellulosic media than growth media treated with the commercial enzyme Novozyme 188, a cocktail of cellulase enzymes from *T. reesei* and β-glucosidases from *A. niger* (Rosgaard *et al.*, 2007; Chen *et al.*, 2012). A fungal specimen isolated from a donkey in Indiana, *Piromyces* strain UH3–1, shows efficient hydrolysis of biomass in the presence of high syringyl-lignin, a growth inhibitor for other fungal species (Hooker *et al.*, 2018). Xylose utilizing fungi have been isolated from within the guts of either wood eating termites in China (Ali *et al.*, 2017) or elephants in India (Kuyper *et al.*, 2003).

Another location for novel species of fungi is inside plant tissues as endophytes. A study of four endophytic species identified a range of CAZymes and terpene synthases capable of deconstructing plant lignocellulose, as well as direct production of hydrocarbon fuel molecule candidates (Wu *et al.*, 2017). Of 14 strains of endophytic fungi tested on various substrates and in different conditions, *Botryosphaeria* sp. and *Saccharicola* sp. were the most versatile and promising in the production of cellulases and xylanases (Marques *et al.*, 2018). Additional endophytic fungi have been investigated like *Persea indica* (Tomsheck *et al.*, 2010), *Gliocladium roseum* (Stadler and Schulz, 2009) and *Homonema* sp. (Fillat *et al.*, 2017).

Fermentation Processes

Metabolism of plant carbohydrates to biofuels may require different conditions for fermentation, varying across the sugars present and the acting organisms. A common fermentation organism, *S. cerevisiae*, can ferment in submerged (SmF), low oxygen environments. Traditional fermentation methods involve SmF, because it has low processing costs and high productivity (Ng *et al.*, 2020). Some fermentation processes use very high cell densities, like in Brazil where more than 90% of yeast cells are often reused between batch fermentations (Basso *et al.*, 2008). Other fermentation processes like solid state fermentation (SSF), aerobic or anaerobic gas conditions, batch or continuous processes, whole slurry fermentation, separate simultaneous saccharification, and mixed, sequential, or successive feedstock fermentation, have been investigated (Dutta *et al.*, 2010; Florencio *et al.*, 2016).

Yeast strains must endure high temperatures, osmotic stress, acidity, bacterial contamination and high ethanol concentration conditions (Basso *et al.*, 2008). The model fermentation organism is *S. cerevisiae*, because it has been extensively studied and well characterized, is genetically tractable, has GRAS (Generally Recognized as Safe) status by the FDA and a high ethanol and pH tolerance (Tesfaw and Assefa, 2014; Albergaria and Arneborg, 2016). However, *S. cerevisiae* can only convert hexose sugars, especially glucose, to ethanol. It cannot utilize pentose sugars, like ribose, xylose, arabinose, or aldopentose (Hahn-HäGerdal *et al.*, 1991; Laluce *et al.*, 2012). To resolve *S. cerevisiae*'s narrow substrate range, scientists have genetically engineered *S. cerevisiae*, or performed co-fermentation to utilize the metabolic abilities of other organisms. For example, earlier work with yeasts identified that *Pichia stipitis*, *Pachysolen tannophilus*, or *Candida shehatae* can ferment xylose directly to ethanol or convert it via glucose isomerase (EC 5.3.1.5) for *S. cerevisiae* fermentation subsequently. However, xylose fermenting yeasts have a low ethanol tolerance, which limits fermentation of high sugar concentrations (Laplace *et al.*, 1991; Madhavan *et al.*, 2017a). Additional fermenting fungi have been investigated like mucoralean fungi, which are advantageous for their resistance to inhibitors, valuable byproducts, and flexible morphology (Satari and Karimi, 2018).

Recent research has investigated the optimization and changes in enzyme production between SmF and SSF often finding differences in the transcriptomes (Téllez-Téllez and Diaz-Godinez, 2019) and enzyme types. SSF is a method that uses solid growth medium to culture fungi. As the fungi grow, they have access to oxygen, which has shown higher production of enzymes or products at the surface. Several defining characteristics of SSF are low water activity and non-uniformity of fungal growth. It has the additional benefit of no wastewater discharge, no power input for stirred fermentations, and maximizes nutrient utilization (Ng *et al.*, 2020). The hydrolysis performance of four lignocellulolytic complexes was tested on wheat bran using SmF and SSF, and higher glucose concentrations were observed with SSF after lignocellulose hydrolysis for 72 h (Prévot *et al.*, 2013; Ng *et al.*, 2020).

In another study, cellulolytic and xylanolytic activities of *T. reesei* on wheat chaff were enhanced by 27% and 13% in SmF, and 23% and 43% in SSF (Jovanović *et al.*, 2019). Additional research has expanded and optimized SmF and SSF to increase enzyme production of *A. niger*, *T. reesei*, or *P. oxalicum* on corn stover or wheat bran (Gong *et al.*, 2015; Zhao *et al.*, 2019), *T. reesei* on switchgrass, sawdust, peanut hull, corn cob, molasses, glucose, and xylose (Nanjundaswamy and Okeke, 2020), and *Mucor indicus* on rice straw (Molaverdi *et al.*, 2019). A combination of organisms and fermentation characteristics can be used to optimize biofuel production, such as in *S. cerevisiae* and *Zymomonas mobilis* co-fermenting hexoses and pentoses into ethanol using liquid and solid state fermentations (Dutta *et al.*, 2010).

Fermentation conditions such as low oxygenation and inhibitor compounds from the biomass material form technical barriers for efficient use, prompting bioprospecting for other fungi. Biomass derived degradation compounds like vanillin (Delgenès *et al.*, 1996), furan derivatives like furfural (Feldman *et al.*, 2019) and 5-hydroxymethyl-furfural (Yee *et al.*, 2018), and acetic acid (Kim *et al.*, 2017), inhibit the growth of organisms that utilize biomass. Current research targets engineering recombinant genes to degrade these compounds. For example, furfural can be degraded by recombinant manganese peroxidase (rMnP) produced by *Pichia pastoris*. When *S. cerevisiae* was treated with rMnP then the effect of furfural toxicity was reduced (Yee *et al.*, 2018; Bronikowski *et al.*, 2018). Additional research has been performed, like new fungal isolates able to hydrolyze inhibitory compounds (Hooker *et al.*, 2018), genetic engineering (Kim *et al.*, 2017), and screening inhibitory responsive genes (Feldman *et al.*, 2019).

First Generation Ethanol Fermentation

Brazil's primary production of renewable energy comes from sugar cane products. In 2013, sugar cane fueled 29% of the country's energy supply (Espinasa *et al.*, 2016). In 2008, Basso *et al.* (2008) collected monthly yeast population samples from 70 major distilleries. They sought strains that both dominated and persisted/prevailed in the population over a 200-day season. Those selected were identified by karyotyping, then isolated and screened for desirable fermentation traits like high ethanol yield, recyclability, and low foam formation (Basso *et al.*, 2008). Fourteen promising strains were reintroduced in industrial processes, with some persisting more than 200 days of recycling. The two most popular *S. cerevisiae* strains, CAT-1 and PE-2, were used in about 150 distilleries during 2007–2008, becoming responsible for 60% of the country's total ethanol production (Basso *et al.*, 2008; Basso *et al.*, 2011).

Expansion of Brazilian ethanol production into other raw materials, or "flex mills" is a current area of research. Use of the *S. cerevisiae* CAT-1 strain has spawned fundamental research into sugar preferences, as well as tailored assays into performance on secondary biofuel substrate. CAT-1 showed a favorable performance on a mixture of 50% corn derived broth and 50% molasses broth; the mixture serves to dilute the inhibitory phenolic compounds in molasses and increase cell viability, and budding rate compared to 100% of either broth (Alcantara *et al.*, 2019). Others have approached the issue of phenolic inhibitors with engineering inhibitor resistance into yeast (Brandt *et al.*, 2019). Just as Basso (Basso *et al.*, 2008) sought new organisms and strains, exploration of yeast strains from other fermentation environments have also been investigated for their bioethanol potential. Cachaca distilleries produce Brazilian white rum from sugar cane. One strain isolated from the distillery has fermentation characteristics similar to PE-2 (Araújo *et al.*, 2018).

Heterologous Xylose Degradation in *S. cerevisiae*

The ability to ferment D-xylose, the major hemicellulosic-derived sugar, has guided discovery and manipulation of xylolytic genes (Delgenès *et al.*, 1996). However, since *S. cerevisiae* cannot ferment pentose sugars, novel fungi are being investigated for xylose utilization. Recently isolated fungi are being investigated for their capacity to ferment xylose, like a new clade of yeasts *Spathaspora hagerdaliae* (Cortivo *et al.*, 2020), *Spathaspora passalidarum* (Rodrussamee *et al.*, 2018), *Candida tropicalis* (Martins *et al.*, 2018), *Meyerozyma caribbica* (Sukpipat *et al.*, 2017) and *Rhodosporidium toruloides* (Zhuang *et al.*, 2019b). In some cases, the native xylose pathway is altered to improve fermentation capability. For example, xylitol yield increased five-fold when xylose reductase was overexpressed and xylitol dehydrogenase was simultaneously knocked out in *Meyerozyma guilliermondii* (Atzmüller *et al.*, 2020). In other cases, xylose utilization is introduced into unconventional fungi. For example, The xylose utilization genes were introduced into *Issatchenkia orientalis*, a multi-stress tolerant yeast, using novel genetic tools based on the autonomously replicating sequence (ARS) from *S. cerevisiae* (Cao *et al.*, 2020). The xylose utilization pathway was functional in the resulting strain, representing a step in the efficient construction of foreign biochemical pathways in nonconventional organisms.

More recent research has engineered the ability to ferment xylose directly into *S. cerevisiae* and other nonconventional yeasts, expanding the range of organisms for xylose fermentation (Xiao *et al.*, 2019; Cunha *et al.*, 2019). However, one of the biggest challenges to this is glucose repression. In *S. cerevisiae*, the presence of glucose inhibits pentose utilization, resulting in delayed fermentation and reduced ethanol productivity (Li *et al.*, 2010). Scientists began to work around this obstacle by testing intracellular glucose utilization. Genes for intracellular β-glucosidases and the cellodextrin transport system from *N. crassa* were expressed in *S. cerevisiae*. The resulting strain fermented cellobiose with an ethanol productivity comparable to industrial yields (Galazka *et al.*, 2010). Similar genetic engineering was performed using *S. cerevisiae* expressing xylose utilizing genes from *Pichia stipitis* (Li *et al.*, 2010; Ha *et al.*, 2011). The resulting strains utilized intracellular glucose, but did not inhibit xylose uptake showing minimized glucose repression. When the recombinant yeast were tested on a mixture of cellobiose and xylose, the ethanol productivity increased by 2.2 fold (Li *et al.*, 2010) and 2.4 fold (Ha *et al.*, 2011) compared to the control. These findings

demonstrate that mixed sugars can be simultaneously consumed in *S. cerevisiae* to produce ethanol at high yields. These insights will facilitate the future manipulation of xylose metabolism in microbial cell factories.

Heterologous Starch Degradation in *S. cerevisiae*

To decrease the cost of adding enzymes, scientists have genetically engineered yeast to co-display starch degrading enzymes. For example, two recombinant *S. cerevisiae* strains increased ethanol productivity on soluble potato starch when expressing either glucoamylase (pGA11) from *Rhizopus oryzae* (Kondo *et al.*, 2002) or a combination of glucoamylase from *R. oryzae* and α-amylase from *Bacillus stearothermophilus* (Shigechi *et al.*, 2002). These strain were as effective at a lower temperature (80°C), reducing costs associated with high temperatures (Shigechi *et al.*, 2004a). When the same glucoamylase from *R. oryzae* and a more robust α-amylase from *Streptococcus bovis* was tested on raw corn starch, the recombinant strain was even more efficient, producing 61.8 g of ethanol/liter in a 72-hour fermentation (Shigechi *et al.*, 2004b). Recent research has tested amylolytic *S. cerevisiae* strains on other feedstocks, like broken rice (Myburgh *et al.*, 2019).

Starch degradation by purified enzyme addition is costly for industrial scale processes. However, recombinant production of starch-specific enzymes has effectively engineered whole cell biocatalysts. This synthetic strategy can consolidate multiple enzymatic pathways into a single expression host (Madhavan *et al.*, 2017b). We may also look to our food production processes, and fungi such as *A. oryzae*, the koji fungus, which is capable of producing large amounts of amylase (Machida *et al.*, 2005; Kitamoto, 2015). Additional research has investigated the secretome, proteome, and metabolome of *A. oryzae* (Nemoto *et al.*, 2012; Watanabe *et al.*, 2011; Tanaka *et al.*, 2016; Zhuang *et al.*, 2019a; Hiramoto *et al.*, 2015). The breakdown of starch-containing biomass can benefit from long-cultivated and newly engineered fungi (Wang *et al.*, 2020).

Biodiesel

Biodiesel is a biofuel made by catalytically combining vegetable oil or animal fat with alcohol to produce methyl esters. It can be derived from a wide range of oils like sunflower, peanut, cotton, castor bean, palm, coconut oil, and even spent coffee grounds (Soccol *et al.*, 2005; Park *et al.*, 2018). Biodiesel yields 93% more energy than the energy invested in its production (Hill *et al.*, 2006). The triglycerides in the fats and oils undergo transesterification by a lipase catalyst, or the displacement of alcohol from an ester by another alcohol, such as ethanol or methanol. The results are methyl esters, the biodiesel, and glycerol, a byproduct (Vasudevan and Briggs, 2008).

Adding purified lipase is expensive because it requires a complicated recovery, purification and immobilization to prevent enzyme loss (Ban *et al.*, 2001). To circumvent these issues, researchers use lipase producing fungi with high stability and biosynthetic ability, like *Candida cylindracea*, *Candida antarctica*, *Geotrichum candidum*, and *Yarrowia lipolytica* (Vakhlu, 2006). Lipase-producing yeasts were isolated from contaminated palm oil samples from factories in southern Thailand (Srimhan *et al.*, 2011). Among the 102 samples, the *Rhodotorula mucilaginosa* isolate had a 51.26% fatty acid methyl esters yield. When methanol was added to support catalysis, P11189 increased its maximum FAME yield to 83.29% over 72 h. Another model yeast with an impressive host of lipases is GRAS verified *Y. lipolytica*. It is an oleaginous yeast able to accumulate enough lipids to represent more than 80% of its cell dry weight (Darvishi *et al.*, 2017). The lipids it produces can be used as feedstock for biodiesel. Further, it can consume a wide variety of oils, fats, and hydrocarbons, including from waste streams, making it an attractive fungus for bioremediation of oil contaminated areas. *Y. lipolytica* has also been engineered to increase native lipase production. For example, when a strong promoter was inserted to control the *LIP2* gene, a lipase precursor, the result increased lipase production thirty-fold (Pignède *et al.*, 2000). *Y. lipolytica* grown on sugarcane bagasse produces lipids that could be converted into biodiesel (Tsigie *et al.*, 2011). *Y. lipolytica* represents a multi-functional cell factory, able to simultaneously degrade feedstocks, accumulate lipids, and potentially convert them to biodiesel. Other organisms that have been investigated for fatty acid derived biofuels production include *Aspergillus wentii* (Shoaib *et al.*, 2018), and *S. cerevisiae* (Runguphan and Keasling, 2014; Buijs *et al.*, 2013).

Researchers have scaled up fungi-driven biodiesel production with two other techniques: yeast immobilization, a packed bed reactor system and, more recently, SSF. Immobilizing *Rhizopus chinensis* on polyurethane foam biomass support particles (BSPs) enhanced intracellular lipase production (Nakashima *et al.*, 1990), increasing intracellular inter-esterification lipase activity several fold compared to freely suspended cells. When this experiment was repeated on other *Rhizopus* species, like *R. javanicus, R. oryzae, and R. niveus*, they observed a similar enhancement (Nakashima *et al.*, 1990). *R. oryzae* was tested in a packed bed reactor system, which emulsifies reactants by ultrasonication prior to each reaction cycle, and increases the contact between the reaction mixture and the immobilized cells (Fukuda *et al.*, 2009; Hama *et al.*, 2007). Researchers were able to achieve a FAME yield of 90% in the first cycle, followed by a high value of 80% after 22 reaction cycles (Fukuda *et al.*, 2009; Hama *et al.*, 2007). SSF can also be applied to palm biomass waste, which is rich in oil. *Aspergillus tubingensis* could grow and product oils with good biodiesel properties using non-sterile SSF, reducing potassium content by 90%–a combustion inhibitor when palm biomass is burned for fuel. Fermentation also increased cellulose content to >57%. This growth was repeatable with 90% batch replacement of total palm biomass waste, presenting an zero-waste treatment process (Intasit *et al.*, 2020).

Ethanol and biodiesel are not the only sustainable fuels currently being investigated. Biogas production uses a variety of feedstocks like wheat straw (Gaworski *et al.*, 2017). Endophytic fungi have demonstrated the ability to produce volatile organic compounds, called myco-diesel (Wu *et al.*, 2017; Stadler and Schulz, 2009; Maxwell *et al.*, 2018). Higher alcohols like butanol and

isobutanol are viable gasoline substitutes that have been produced by *S. cerevisiae* (Buijs *et al.*, 2013). Terpenes have been investigated as a valuable fuel source because of their low viscosities, high energy densities, and high volumetric net heats of combustion (Mewalal *et al.*, 2017; Zhuang *et al.*, 2019b). For example, *S. cerevisiae* was engineered to produce bisabolene, sesquiterpene and alternative to diesel (Peralta-Yahya *et al.*, 2011). Terpenes have been explored as jet fuel substitutes (Buijs *et al.*, 2013; Wu *et al.*, 2018; Meylemans *et al.*, 2013), diesel or gasoline fuels (Mewalal *et al.*, 2017; Hellier *et al.*, 2013) and more (Meylemans *et al.*, 2012). Various fungi have been investigated for cineole and terpene synthases, like endophytic fungi (Wu *et al.*, 2017; Tomsheck *et al.*, 2010) and the yeast *Rhodosporidium toruloides* (Zhuang *et al.*, 2019b). Traditionally petroleum derived molecules can also be produced, such as isobutylene from the wood-rot fungus *Fomitopsis palustris* (Kim *et al.*, 2019), and itaconic acid produced by *Aspergillus terreus* and *Ustilago maydis* (Zhao *et al.*, 2018).

Conclusion

Overall, fungal enzymes are critical to increase the efficiency of biofuel production from plant matter. Model fungi like *T. reesei*, *R. oryzae*, and *P. chrysosporium* uniquely break down complex plant polysaccharides like cellulose, hemicellulose, and pectin into simple sugars like glucose and xylose. These sugars are then fermented by yeast like *S. cerevisiae*. Scientists have improved these processes by combining various mixtures of microbes, discovering new species with unique metabolic processes, increasing enzymatic activity with genetic engineering, and adjusting industrial methods. For example, altering transcription factors can increase enzyme production and secretion, resulting in decreased costs. Further, saccharification and fermentation have been consolidated with whole cell catalysis, engineering ethanologenic organisms to become cellulolytic or vice versa. Certain species thrive on specific substrates or fermentation conditions. Additional biofuels have been investigated like biodiesel, biogas, and terpenes. Improving fungal efficiency will lower the cost of biofuels and enable them to be a competitive alternative to fossil fuels.

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Oleaginous Fungi in Biorefineries

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Introduction

Many unicellular microorganisms including yeasts, fungi, microalgae, and to a lesser extent bacteria (**Fig. 1**), can produce intracellular edible oils under normal and specialized conditions (Papanikolaou and Aggelis, 2011). Most of these organisms can accumulate microbial lipids, or single-cell oils (SCOs), to 20%–90% (w/w) of their dry cell biomass. For example, fungi in the genus *Mortierella* and *Umbelopsis* can accumulate lipids at concentrations that exceed 86% of their dry weight (Meng *et al.*, 2009). Microbial lipids can be produced using low-priced organic materials, including waste-streams from the food industry, as growth substrate. The fatty acid of microbial lipids is very similar to the conventional vegetable oils in type and composition (Madani *et al.*, 2017). Furthermore, microbial lipids have many potential applications, including human food additives, nutraceuticals, pharmaceuticals, cosmetics, biopolymers and feed ingredients for aquaculture, and as an alternative feedstock for the production of biofuel (Lewis *et al.*, 2000). Due to the reduced availability of cultivable land and increasing human population growth, producing lipids via traditional methods will not satisfy the rapidly growing global demand. Therefore, microbial lipids accumulated from microorganisms, especially oleaginous fungi, are considered as a vital and renewable oil resource and have been regarded as an alternative to animal and plant lipids in recent years, given their unique characteristics and functions in energy, chemical, and food industries (Huang *et al.*, 2017). The production of microbial lipids is particularly attractive when low or negative cost substrates are used as the feedstock.

Oleaginous Fungi and Their Advantages for SCO Production

Fungi consist of a large group of diverse species and represent their own Kingdom of life. Fungi have conquered almost all habitats on the planet, from glacial deep seawater to hot and dry desert, and associate with single prokaryotes, eukaryotic mammals and plants (Gupta *et al.*, 2013). Some fungi are biotrophic and cause negative impacts on native organisms, such as the rice blast pathogen *Pyricularia oryzae* (previously known as *Magnaporthe oryzae*) (Zhang *et al.*, 2015). However, many fungi are beneficial to their hosts, such as mycorrhizas that provide their plant hosts with mineral nutrition. Some filamentous fungi produce high amounts of lipids, and are called oleaginous fungi. Examples of oleaginous filamentous fungi (**Fig. 1**) include *Mortierella alpina* (Wang *et al.*, 2011), *Umbelopsis isabellina* (previously known as *Mortierella isabellina*) (Harde *et al.*, 2016), *Mucor circinelloides* (Song *et al.*, 2001), and *Cunninghamella echinulate* (Fakas *et al.*, 2009b). These species have attracted much attention for their high yield of long-chain polyunsaturated fatty acids (PUFAs). With the advancement of fermentation technology, microbial lipids (primarily from fungi and yeast) can be produced in quantities equivalent to acres of agricultural land (Alvarez and Steinbüchel, 2002), and SCO produced by oleaginous fungi could contribute to meeting human demands for industrial oils.

Biorefineries are facilities that produce multiple products including chemicals, biofuels, and are specialized at making valuable bioproducts from waste residues. Applications of oleaginous fungi in biorefineries have become more attractive given their renewable, resource-efficient, and environment-friendly features (Peng and Chen, 2008), as well as their versatility and high efficiency in utilizing complex and diverse substrates (Ferreira *et al.*, 2012). Overall, the main advantages of oleaginous fungi are shown as follows: (1) High yield of lipid content (up to 90%) and high-value compounds such as PUFAs. For example, *U. isabellina* was able to produce large amounts of cellular lipids amounting to over 80% of their dry cell biomass (Chatzifragkou *et al.*, 2010; Gao *et al.*, 2013). (2) Outstanding lipid profiles for high-quality biodiesel. Not all biological lipids are suitable for biodiesel production. In fact, only saponifiable lipids and free fatty acids can be turned into fatty acid methyl esters (FAMEs), the main content of biodiesel. Most fungus-derived microbial lipids are suitable for high-quality biodiesel production. For example, 98% of the lipids produced by *M. circinelloides* were saponifiable lipids and free fatty acids, which could directly be acid-catalyzed into biodiesel (Vicente *et al.*, 2010). Microbial lipids produced by the filamentous fungus *Aspergillus oryzae* had appropriate fatty acid profiles for biodiesel production: 11.6% of palmitic acid, 15.6% of palmitoleic acid, 19.3% of stearic acid, 30.3% of oleic acid, 5.5% of linolenic acid, and 6.5% of linoleic acid (Muniraj *et al.*, 2013). (3) Easy to form pellets under submerged fermentation that can reduce the viscosity of the fermentation culture and it is easy and cheap to harvest (Koike *et al.*, 2001; Reis *et al.*, 2014; Xia *et al.*, 2014; Xia *et al.*, 2011); (4) Flexibility of various carbon sources for lipid production including *e.g.*, glycerol, wheat straw, tomato waste hydrolysate, potato processing wastewater, corn stover, brewery waste, and glucose (André *et al.*, 2010; Fakas *et al.*, 2007; Fang *et al.*, 2016; Gema *et al.*, 2002; Muniraj *et al.*, 2013).

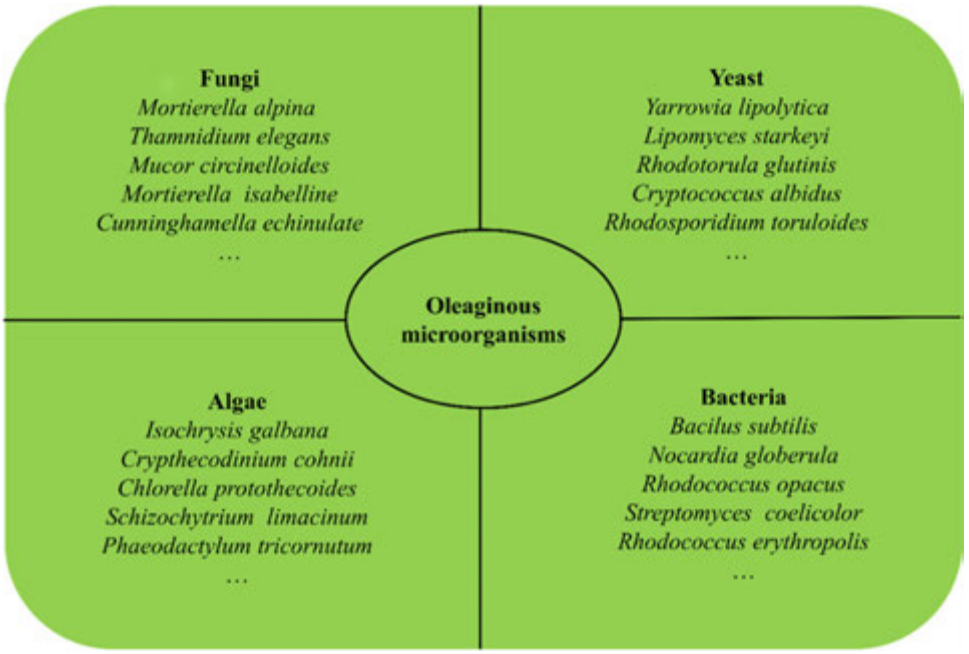

Fig. 1 Common oleaginous microorganisms.

Limiting Factors of Large-Scale SCO Production

Although microbial lipids from oleaginous fungi have promising potential for many applications, their high production cost compared to plant and animal-derived lipids still limit the broader use of microbial lipids (Zhao *et al.*, 2011). There are three major costs associated with producing microbial lipids: carbon and nutrient source, fermentation, and post-processing operations associated with separating lipids from oleaginous organisms. According to the previous study, the cost of feedstocks or carbon sources can account for up to 75% of the total costs for producing microbial lipids (Subramaniam *et al.*, 2010). Thus, the production cost could be dramatically reduced when cheaper feedstocks or waste residues are used for microbial lipid production. During recent years, a series of new microbial lipid processing techniques have been developed and used to cultivate oleaginous fungi using agricultural residues as the carbon source to lower costs and increase sustainability (Carota *et al.*, 2018; Economou *et al.*, 2011; Fang *et al.*, 2016; Zhang and Hu, 2012; Zheng *et al.*, 2012). For example, the fungus *Aspergillus niger* accumulated 41%–57% of lipid when it was cultured on biodiesel-derived waste glycerol (André *et al.*, 2010).

Many studies have been carried out to reduce downstream processing costs, one of the major obstacles that need to be solved to improve the economic viability of producing microbial-derived lipids, especially for large-scale commercialization (Ochsenreither *et al.*, 2016). It has been reported that the filamentous fungus *U. isabellina* formed pellets with an average diameter of 0.11 mm when cultured on non-detoxified liquid hydrolysate (Zheng *et al.*, 2012), which makes the downstream harvesting process easier. Some feasible ways have been reported to induce fungal pelletization by adding $CaCO_3$ or adjusting the pH (Liao *et al.*, 2007; Xia *et al.*, 2011), which offered a feasible direction for low-cost harvesting, a major step during downstream processing.

Lipid Synthesis Mechanism of Oleaginous Fungi

Biochemistry of Lipid Accumulation

Insights on the lipid biochemistry and physiology in oleaginous microorganisms can promote both basic research and industrial applications. A better understanding of the lipid synthesis mechanism in oleaginous microorganisms will help us improve lipid yield and acquire the desired fatty acid profiles. Previous studies have elucidated the biochemical mechanisms of lipid accumulation in various microorganisms, including key enzymes, regulation of lipid accumulation, and key intermediates in lipid biosynthesis (Alvarez and Steinbüchel, 2002; Burton *et al.*, 2005; Hao *et al.*, 2014; Ratledge, 2014; Shuib *et al.*, 2018; Tang *et al.*, 2016; Zhao *et al.*, 2016). There are similarities in the lipid accumulation mechanisms among different oleaginous organisms. Regarding the oleaginous microorganisms, lipid accumulation often happens during the starvation of nutrients including nitrogen (N), phosphorus (P), sulfur (S), vitamins, and metals such as zinc (Zn), iron (Fe), and magnesium (Mg) (Laoteng *et al.*, 2011; Madani *et al.*, 2017). Significant lipid accumulation rarely occurs under enriched nutrient conditions. However, the cells react to the limits of a key nutrient, *i.e.*, N, P, K, Mg, S and Fe that are required for cell proliferation, and they will enter into lipid storage

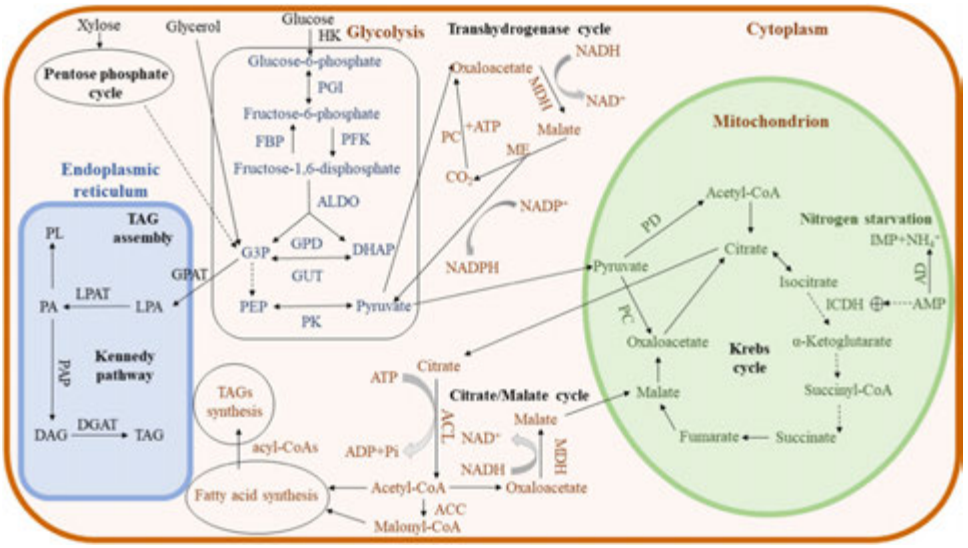

Fig. 2 Outline of the main biochemical process causing lipid accumulation in oleaginous fungi. AD, adenosine deaminase; ALDO, aldolase; DHAP, dihydroxyacetone phosphate; FAS, fatty acid synthase; FBP, fructose-1,6-biphosphatase; G3P, glycerol-3-phosphate; GPD and GUT, isoforms of G3P dehydrogenase; HK, hexokinase; ICDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PEP, phosphoenolpyruvate; PFK, phosphofructokinase; PGI, glucose-6-phosphate isomerase; PK, pyruvate kinase.

phase, in which excess carbon is converted to lipid reserves by unique lipid biosynthetic pathways. The limited supplement of nutrients inhibits cell proliferation and the synthesized lipid can be stored in the oil bodies of undivided cells (Alvarez and Steinbüchel, 2002; Beopoulos *et al.*, 2009a,b; Dourou *et al.*, 2018; Ratledge and Wynn, 2002; Shields-Menard *et al.*, 2018).

Among those key nutrients, N-limitation is the most important factor for lipid accumulation. The fatty acids for microbial lipids from oleaginous fungi are typically synthesized from acetyl-CoA with a reversed β-oxidation in the cytoplasm. As shown in **Fig. 2**, when the N supply is limited, the activity of adenosine monophosphate (AMP) deaminase will be upregulated, which breaks down AMP, an essential cofactor of isocitrate dehydrogenase (ICDH), and releases ammonia to N-starved cells. ICDH is a key enzyme in the citric acid cycle. It will become inactive in the absence of N and unable to convert isocitrate to α-ketoglutarate. As a consequence, the tricarboxylic acid cycle (TCA) is blocked at isocitrate due to the declined concentration of cofactor AMP along with the increase in AMP deaminase activities. Subsequently, the accumulated citrate is transferred from the TCA cycle and diverted into the cytoplasm from mitochondria via citrate-malate translocase. In the cytosol, the citrate is catalyzed and decomposed into acetyl-CoA and oxaloacetate by ATP citrate lyase (ACL). The latter is then converted into malate by malate dehydrogenase. The malate could be translocated to the mitochondria by citrate-malate translocase or converted into pyruvate via the malic enzyme (ME) with the production of NADPH and CO_2 during the cytosolic transhydrogenase cycle. Resulting from N limitation, accumulated acetyl-CoA and NADPH enter into fatty acid and triacylglycerol (TAG) synthesis (Jin *et al.*, 2015; Ratledge and Wynn, 2002; Ratledge and Wynn, 2020). In contrast, when the N availability increases, the cell will convert storage lipids into cellular materials (Daum *et al.*, 2007; Ratledge, 2002).

In addition, the ratio of carbon to nitrogen (C/N) in the culture is a crucial factor for lipid accumulation in oleaginous microorganisms. It has been shown that microbial lipid accumulation could be improved by optimizing the process parameters and culture conditions including the C/N ratio, N source content, and oxygen concentration through new process configurations such as solid-state or semi-solid-state fermentation methods (Kosa and Ragauskas, 2011). Increasing the ratio of C/N in culture media significantly improves the lipid yields from oleaginous fungi (Fakas *et al.*, 2007). According to current studies, the optimal C/N ratio depends on the organism, but exceeds 65 and may exceed 100 (Ageitos *et al.*, 2011; Jin *et al.*, 2015). Increasing the ratio of C/N was observed to promote lipid accumulation with longer growing periods in oleaginous fungi (Papanikolaou *et al.*, 2004; Ruan *et al.*, 2012), and there is usually an optimum carbon concentration for each oleaginous fungal species at which lipid accumulation is maximized (Murphy, 1990). Under C/N ratios of 35, 44, and 57, *U. isabellina* ATHUM 2935 accumulated about 36%, 51.2% and 64.3% of lipids, respectively, using rice hull hydrolysate (Economou *et al.*, 2011). *Cunninghamella echinulata* ATHUM 4411 was able to produce ~25% of lipids at a C/N ratio of 78 in an N-limited medium supplemented with xylose. Its lipid content reached a maximum of 57.7% at a higher C/N ratio of 285 (Fakas *et al.*, 2009b). When cultivated in xylose media, *U. isabellina* ATHUM 2935 behaved similarly to *C. echinulata* ATHUM 4411 with a higher lipid yield of 65.5% compared to 57.7% in *C. echinulata* ATHUM 4411 (Fakas *et al.*, 2009b).

Two types of lipid synthesis pathways have been described in oleaginous microorganisms, designated "*de novo*" and "*ex novo*" lipid accumulation (Papanikolaou and Aggelis, 2011). The *de novo* lipid accumulation process is often fermented on hydrophilic materials (sugars and related substrates) with excessive exhaustion in the medium of an essential nutrient (typically N). In contrast, the *ex novo* lipid accumulation pathway is carried out on the hydrophobic materials (oils, alkane, *etc.*) along with the

growth process and does not require N-limiting culture conditions (Huang *et al.*, 2013; Papanikolaou *et al.*, 2001, 2002). The main difference between the two pathways is that the *ex novo* lipid accumulation occurs simultaneously with cell growth and is independent with the nutrient-limiting culture conditions. Each pathway has unique advantages: *de novo* lipid accumulation provides great lipid yield, while *ex novo* lipid accumulation allows modification on the ratio between intracellular and extracellular lipid composition to meet the requirement for different applications (Huang *et al.*, 2013, 2017; Papanikolaou and Aggelis, 2011). The two pathways could be engineered simultaneously. For example, Huang *et al.* (2017) combined "*de novo*" and "*ex novo*" lipid fermentation using corncob acid hydrolysate with soybean oil as a mixed-medium to cultivating oleaginous yeast *Trichosporon dermatis*, which could simultaneously and efficiently utilize both hydrophilic and hydrophobic substrates in the medium.

Lipid Profiles in Oleaginous Fungi

The growth of fungi is usually accompanied by relative changes in the number of lipid fractions and unique types of lipids, as well as in fatty acid composition. Oleaginous fungi accumulate lipids predominantly in the form of TAG. Other accumulated lipids include monoacylglycerols, diacylglycerols, free fatty acids, sterols, sterol esters, and polar lipids (glyco-, sphingo-, and phospholipids) (Khot *et al.*, 2018). TAGs are neutral, insoluble fatty acid tri-esters of glycerol, which possess a higher caloric value than carbohydrates and proteins, and thus provide an abundant reserve with much higher energy via β-oxidization (Alvarez and Steinbüchel, 2002). TAGs represent the predominant storage form of carbon and energy in oleaginous microorganisms. Following phospholipid synthesis during the exponential growth phase, TAGs usually accumulate in the stationary phase when cellular growth is impaired under excess carbon and limited N conditions (Ratledge and Wynn, 2002). Fatty acids in microbial lipids range from lauric acid (C12:0) to docosahexaenoic acid (DHA, C22:6). Palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are the most common fatty acids in fungi. Among those fatty acids, $\sim$50% are unsaturated fatty acids (Subramaniam *et al.*, 2010).

It has been reported that the fatty acid profiles in microbial lipids are mainly dependent on the species (Xu *et al.*, 2012). For example, in *U. isabellina*, the lipids consist of 49%–54% oleic acid, 24%–35% palmitic acid, 2%–11% linoleic acid, 3.5%–8% stearic acid, 1%–2% palmitoleic acid, and 0.4%–2% γ-linolenic acid (Economou *et al.*, 2010), which is similar to *Microsphaeropsis* sp.: 46.1% oleic acid, 27.3% palmitic acid, 20.0% linoleic acid, 3.5% palmitoleic acid, and 3.0% stearic acid (Peng and Chen, 2008). In contrast, *Aspergillus tubingensis* TSIP9 accumulated 42.7% saturated and 55.3% unsaturated fatty acids including 35.8% oleic acid, 21.5% palmitic acid, 18.8% linoleic acid, and 9.6% stearic acid (Cheirsilp and Kitcha, 2015). *U. isabellina* and *C. echinulate* have the same types of fatty acids, and the composition was independent of the carbon sources. At the beginning of the growth period, linoleic acid (C18:2) and γ-linolenic acid (C18:3) were the most abundant fatty acids. However, oleic (C18:1) and palmitic (C16:0) acids became the key fatty acids after the lipid accumulation over 20% (Fakas *et al.*, 2009a,b).

The content of microbial lipids in fungi also depends on hereditary factors and culture conditions like agitation, pH, temperature, light, and nutrient content (Subramaniam *et al.*, 2010). Meng *et al.* (2009) reported that the oil content and composition in oleaginous fungi were approximate 7%–23% of C16:0, 1%–6% of C16:1, 2%–6% of C18:0, 19%–81% of C18:1, 8%–40% of C18:2, and 4%–42% of C18:3 according to the fungal strains and growth conditions such as pH, temperature, and culture phase. The optimal culture temperature for oleaginous fungi ranges from 20 to 28°C and pH 4–7 is optimal for their growth (Jin *et al.*, 2015). To some extent, metal ions affect the oil content of oleaginous microorganisms. Several studies have been attempted to improve the PUFAs of oleaginous fungi via optimizing different kinds of metal ions in the culture medium. For example, Cu and Zn have a positive effect on the γ-linolenic acid (GLA, C18:3, ω-6) production of *Umbelopsis ramanniana* (Hansson and Dostálek, 1988). In *Cunninghamella* sp., Mg, Fe, and Zn have significant effects on lipid accumulation because the supplement of each ion resulted in a 64%, 43%, and 33% increase in lipid content compared to the cultures deprived with each of these ions, respectively (Muhid *et al.*, 2008). Among the three ions, Zn has the most significant effect on fatty acid content and contributed to a 74% increase in GLA (Muhid *et al.*, 2008). In addition, lipid accumulation and fatty acid compositions could be affected by the carbon sources (*e.g.*, glucose and xylose) in various zygomycete fungi (Dyal *et al.*, 2005; Hansson and Dostálek, 1988; Sajbidor *et al.*, 1988; Somashekar *et al.*, 2003).

Different Substrates for Microbial Lipids Production

Conventional Carbon Substrates

As shown in **Table 1**, various kinds of carbohydrates, such as sucrose, molasses from sugar beet and sugarcane, glucose and dextrins from hydrolyzed starch, whey (a by-product of cheese production), fructose produced from inulin, and some natural hydrolysates like rice straw hydrolysate, tomato waste hydrolysate, wheat straw hydrolysate, sugarcane bagasse hydrolysates, and potato processing wastewater, are raw materials derived from agro-industrial processes, which could be used as substrates for microbial lipids production (Chatzifragkou *et al.*, 2010; Fakas *et al.*, 2008a,b; Muniraj *et al.*, 2013). Several monosaccharides, disaccharides, and carboxymethyl-cellulose (CMC) were tested as carbon sources for microbial lipid production by the filamentous fungus *U. isabellina* (Zeng *et al.*, 2013). Cultivated with C5 (xylose) and C6 (fructose and glucose) monosaccharides, *U. isabellina* was able to produce over 60% cellular lipids of total biomass, compared to the 17% and 41% of lipid using disaccharide sucrose and cellobiose, respectively. Due to the absence of cellulase enzymes, CMC did not stand out as a good substrate for *U. isabellina* with only 5% of lipid produced (Zeng *et al.*, 2013). Glycerol is a common by-product of biodiesel production and it could be used as

Table 1 Lipid production of oleaginous fungi using various carbon sources.

Oleaginous fungi	*Lipid content (%, w w^{-1} CDW)*	*Biomass (g L^{-1})*	*Lipid yield (g L^{-1})*	*Substrate*	*Cultivation time (h)*	*References*
Alternaria sp. DM09	55.10	13.80	7.60	Glucose	240	(Dey *et al.*, 2011)
Aspergillus oryzae	40.00	8.75[a]	3.50	Potato processing wastewater	120	(Muniraj *et al.*, 2013)
A. oryzae A-4	18.15	4.31	39.08[b]	Cellulose	144	(Lin *et al.*, 2010)
Aspergillus tubingensis TSIP9	NA	NA	31.10[b]	Palm pressed fiber	120	(Kitcha and Cheirsilp, 2014)
Colletotrichum sp. DM06	46.08	16.20	7.30	Glucose	240	(Dey *et al.*, 2011)
Cunninghamella echinulata ATHUM 4411	25.00	31.2[a]	7.80	Tomato waste hydrolysate + glucose	300	(Fakas *et al.*, 2007)
	57.70	7.80	4.50	Xylose	192	(Fakas *et al.*, 2009b)
	46.00	15.00	6.90	Glucose	360	(Fakas *et al.*, 2009b)
	25.60	7.80	2.00	Glycerol	340	(Fakas *et al.*, 2009b)
	49.00	8.90	4.40	Glucose	480	(Gema *et al.*, 2002)
	36.30	4.30	1.56	Raw glycerol	135	(Chatzifragkou *et al.*, 2011)
	30.00	12.90	3.90	Commercial glucose	309	(Chatzifragkou *et al.*, 2010)
	32.00	12.10	3.80	Molasses	356	(Chatzifragkou *et al.*, 2010)
Mortierella ramanniana MUCL 9235	37.10	7.30	2.71	Raw glycerol	216	(Chatzifragkou *et al.*, 2011)
Mucor sp. LGAM 365	18.10	5.30	0.96	Raw glycerol	237	(Chatzifragkou *et al.*, 2011)
Thamnidium elegans CCF1465	42.60	6.80	2.90	Raw glycerol	271	(Chatzifragkou *et al.*, 2011)
Trichosporon dermatis CH007	41.50	23.60	15.10	Corncob acid hydrolysate with soybean oil	168	(Huang *et al.*, 2017)
Umbelopsis isabellina	57.34	44.94	78.66[a,b]	Corn stover	222	(Fang *et al.*, 2016)
U. isabellina ATCC 42613	30.00	16.80	5.10	Acid- and alkali-pretreated corn stover hydrolysate	93	(Ruan *et al.*, 2014)
U. isabellina ATHUM 2935	65.50	8.70	5.70	Xylose	216	(Fakas *et al.*, 2009b)
	44.60	27.00	12.04[a]	Glucose	360	(Fakas *et al.*, 2009b)
	53.20	6.20	3.30	Glycerol	264	(Fakas *et al.*, 2009b)
	74.00	13.20	9.90	Commercial glucose	237	(Chatzifragkou *et al.*, 2010)
	61.00	12.10	7.40	Commercial fructose	405	(Chatzifragkou *et al.*, 2010)
	54.00	9.50	5.10	Molasses	150	(Chatzifragkou *et al.*, 2010)
U. isabellina IFO7884	NA	NA	47.9[b]	Soybean hull	8[c]	(Zhang and Hu, 2012)
U. isabellina MUCL 15102	33.20	5.60	1.86	Raw glycerol	168	(Chatzifragkou *et al.*, 2011)
U. isabellina NRRL 1757	66.68	5.98	3.99	Xylose	144	(Zeng *et al.*, 2013)
	48.21	5.84	2.82	Arabinose	144	(Zeng *et al.*, 2013)
	30.23	3.37	1.02	Ribose	144	(Zeng *et al.*, 2013)
	51.16	9.37	4.80	Mannose	144	(Zeng *et al.*, 2013)
	49.14	8.17	4.01	galactose	144	(Zeng *et al.*, 2013)
	62.50	6.09	3.82	Fructose	144	(Zeng *et al.*, 2013)
	66.50	8.69	5.77	Glucose	144	(Zeng *et al.*, 2013)
	40.87	5.79	2.36	Cellobiose	144	(Zeng *et al.*, 2013)
Zygorhynchus moelleri MUCL 1430	42.40	3.70	1.57	Raw glycerol	192	(Chatzifragkou *et al.*, 2011)

[a]Calculated from the available data.
[b]Measured in 1 mg lipid produced per 1 g dry substrate.
[c]Measured in week.
Abbreviations: CDW, cell dry weight; NA, not available.

the carbon source for oleaginous fungi. Chatzifragkou *et al.* cultivated 15 strains of fungi and yeast with glycerol and the tested stains accumulated 18%–43% (w/w) cellular lipids (Chatzifragkou *et al.*, 2011).

Lignocellulosic Biomass

Lignocellulosic biomass including wood, grass, forestry waste, and agricultural residues, is considered as the most abundant and renewable organic materials (Palmqvist and Hahn-Hägerdal, 2000). Plant-derived lignocelluloses consist of cellulose (35%–50%, dry weight), hemicelluloses (20%–35%) and lignin (10%–25%) (Huber and Corma, 2007; Sun and Cheng, 2003), which have been tested as a suitable feedstock for microbial lipids and biodiesel production because of their abundance and low cost.

Lignocellulosic polymers need to be pretreated and hydrolyzed into readily fermentable sugars by a series of lignocellulolytic enzymes, such as cellulases and hemicellulases (Dyk and Pletschke, 2012). After that, sugars could be utilized by various oleaginous microorganisms (Kitcha and Cheirsilp, 2014). The current challenge for using lignocellulosic biomass includes the high expense of enzymes, and the rate of hydrolysis that may be inhibited by the substrate (sugar), and productivity of microorganisms.

To date, many oleaginous fungi have been demonstrated to utilize lignocellulosic biomass. *U. isabellina* yields 16.8 g L^{-1} of biomass with 5.1 g L^{-1} of lipids (30%, w/w), when cultured on acid- and alkali-pretreated corn stover hydrolysate (Ruan *et al.*, 2014). Two endophytic fungal isolates, *Colletotrichum* sp. (DM06) and *Alternaria* sp. (DM09), produced 68.2 mg gds^{-1} (per gram dry substrate) and 60.32 mg gds^{-1} lipid using rice straw and wheat bran under solid cultivation (Dey *et al.*, 2011). Filamentous fungi are considered as strong cellulolytic enzymes secreting strains in solid-state fermentation conditions (Singhania *et al.*, 2009). For example, *A. tubingensis* TSIP9 could convert palm pressed fiber and palm empty fruit bunches into lipid with the highest production of 31.1 ± 1.7 or 37.5 ± 2.2 mg gds^{-1}, respectively. This species produces diverse cellulolytic enzymes with high activity, yet the productivity of cellulase and xylanase can be improved by alkaline pretreatments (Kitcha and Cheirsilp, 2014). In *A. oryzae*, starchy waste substrates were used as the carbon source for microbial lipid production without the need for external amylase. This is because the fungus could secrete amylase and accumulate lipids simultaneously (Muniraj *et al.*, 2013). Direct bioconversions by fungal isolates efficiently reduces time, energy, and costs associated with the enzyme digestion and hydrolysis steps, which are promising for microbial lipid production using lignocellulosic biomass.

The Main Applications of Microbial Lipids From Oleaginous Fungi

The potential applications of oleaginous fungi in biorefineries have been intensively studied in recent years (**Fig. 3**).

Biodiesel

Over the past few decades, fossil fuels, such as petroleum, natural gas, and coal, have been major and essential energy sources consumed globally. However, gas emissions from fossil fuels are harmful to the environment, particularly due to their greenhouse gas contribution to the atmosphere that is the main driver of climate change. Moreover, fossil fuels are inherently finite and they are non-renewable resources in a diminishing process (Wang and Wan, 2009). In fact, many studies have suggested that petroleum reserves will be extracted within 50 years, natural gas within 65 years, and coal within 200 years at the present rate of consumption (Soetaert and Vandamme, 2009). The instability of crude oil supplies and the volatility of prices have further sparked widespread interest in alternative energy sources. With the rapidly increasing global population and the decline of fossil fuel availability,

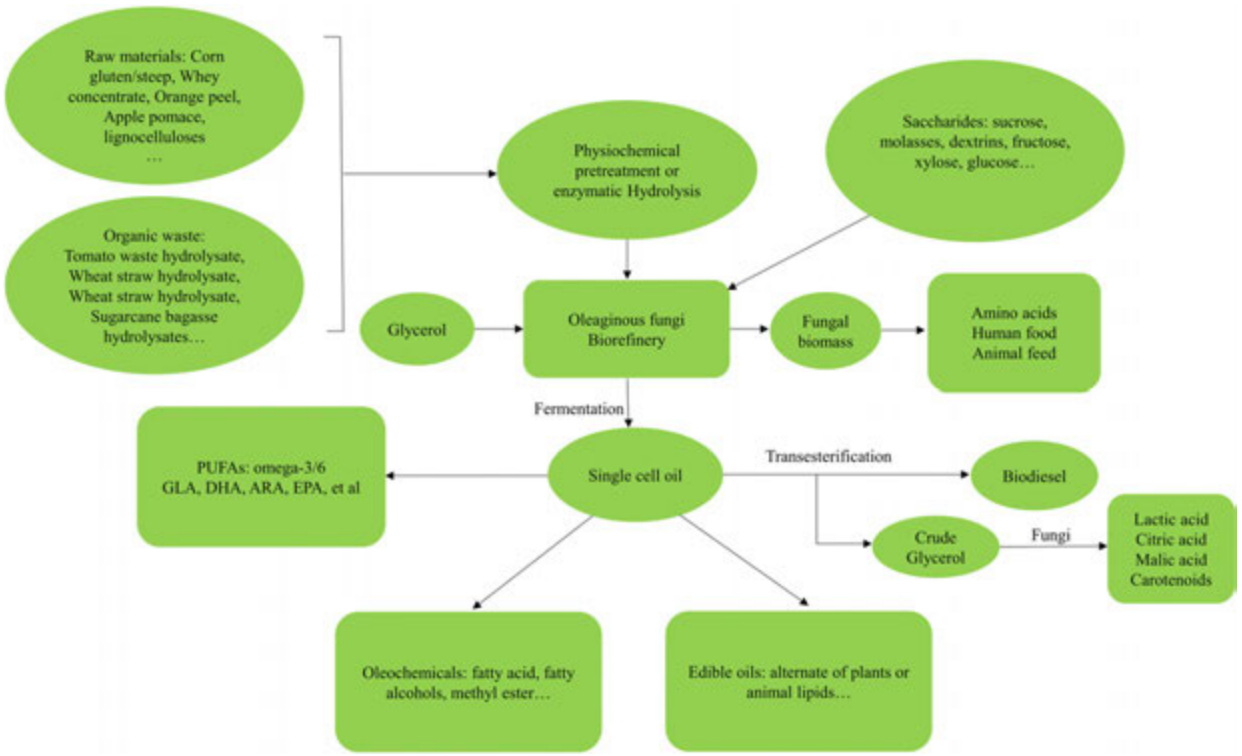

Fig. 3 Schematic diagrams of oleaginous fungi in biorefineries.

traditional methods of producing fuels will not satisfy increasing demands. Thus, it is imperative to develop and utilize sustainable and renewable energy sources. At present, biofuels such as bioethanol and biodiesel are the primary alternative fuels. Compared to bioethanol, biodiesel has several significant advantages: (1) Higher energy density than carbohydrates-based fuel. Biodiesel has an energy density of at least 25% higher than that of ethanol (Durrett *et al.*, 2008). (2) Higher net positive energy balance. For example, soy oil yields 93% more energy than the total energy invested for the production, while the number of corn starch, a major feedstock of bioethanol is 25% (Hill *et al.*, 2006). (3) Lower greenhouse gas emissions. Compared to fossil fuels replaced by biofuels, biodiesel reduced 41% of greenhouse gas emissions and bioethanol reduced them by 12% (Hill *et al.*, 2006). (4) Biodiesel can be used directly for efficient diesel engines and the storage and transport are less expensive compared to ethanol (CharlesDismukes *et al.*, 2008; Durrett *et al.*, 2008). Thus, biodiesel has become a focus of renewable energy research (Demirbas, 2007; Du and Benning, 2016).

Biodiesel is made from monoalkyl esters of long-chain FAMEs produced by transesterification of plant, microbial and algal oils or animal fats. Technically, biodiesel has many advantages including its renewable and non-toxic features, ease of production, good flashpoint, and biodegradability (Meng *et al.*, 2009). More than 95% of the conventional feedstocks for biodiesel production are edible oils including plant oils or animal fats such as palm oils, soybean oils, rapeseed oils, and castor oils, uses which compete with the food industry (Alptekin *et al.*, 2014; Felizardo *et al.*, 2006; Navas *et al.*, 2018; Predojević, 2008; Zabaruddin *et al.*, 2019). Moreover, these conventional feedstocks and raw materials are expensive to produce, which has become the main economical challenge for biodiesel (Srinivasan, 2009). Therefore, it is necessary to find alternative cheap non-edible oil sources and waste feedstock for biodiesel production. In order to lower costs and reduce the environmental impacts of oil-based raw materials, increasing effort has been made on the research of microbial oils. The long-chain fatty acids from microorganisms including oleaginous microalgae, bacteria, and fungi are enriched with energy that makes them ideal precursors for biodiesel (Zhang *et al.*, 2011; Li *et al.*, 2008). Although photosynthetic microalgae have exclusive advantages by utilizing sunlight and carbon dioxide for lipid production, algal biodiesel is facing problems such as high cost of photobioreactors and limitation of large-scale outdoor cultivation (Khot *et al.*, 2018). In contrast, single-cell oils of oleaginous fungi are not affected by light, seasons nor climate, and are promising sustainable feedstocks for biodiesel production (Meeuwse *et al.*, 2013). Further, the content of fatty acid influences the saponification number and iodine value of some specific lipids, and has an impact on the quality of biodiesel, such as cetane number, oxidative stability, the heat of combustion, cloud point, and lubricity (Knothe, 2009). As mentioned above, fatty acid profiles of oleaginous fungi are dominated by myristic, palmitic, stearic, oleic, linoleic, and linolenic acid, all of which could be turned into biodiesel by transesterification reaction (Ruan *et al.*, 2012). The heterotrophic cultivation of oleaginous fungi has many excellent characteristics for industrial biofuel production compared to other oleaginous microorganisms, although there are still many difficulties associated with the industrial production of fungal oils.

The goal of fungal fuels is to develop high quality, low cost, and environmentally-friendly products, which can replace at least a portion of the conventional fuels. Oleaginous fungi, especially yeasts, are efficient in lipid synthesis and have been considered by the biodiesel industry to replace the traditional fossil fuel in the future. Nonetheless, production costs of microbial lipids are still a major problem that restricts their large-scale industrial application. Cheap carbon sources should be developed for the cultivation of those oleaginous fungi. In fact, many alternative economical and sustainable substrates like nongrain plants, agricultural and forest residues, lignocellulosic biomass, food waste, and other waste materials, were used for biodiesel production. For example, using corncob waste liquor as the carbon substrate, a filamentous species of *Aspergillus* was able to produce a high amount of lipids (22.1%; 2 g dry biomass; 48 h), which was ideal for biodiesel production due to its high fraction of saturated fatty acids and other appropriate fuel properties (acid number: 0.40 mg KOH per g of acid; iodine value: 11 g I_2 per 100 g oil; density: 0.8342 g cm^{-3}) (Subhash and Mohan, 2011). Bagy *et al.* (2014) combined fermentative oleaginous fungi with hydrogen-producing bacterium *Clostridium acetobutylicum* ATCC 824 as an integrated system to maximize the biodiesel yield from sugarcane molasses, which increased the economic feasibility of the biodiesel production in molasses.

Identifying novel high-yield oleaginous fungal species that produce hydrolytic enzymes is a strategy for reducing the cost of biodiesel production. For example, several strains of *Aspergillus terreus*, an oleaginous fungus that secrets high amounts of lignocellulolytic enzymes, can utilize low-price renewable carbon sources for economical biodiesel production (Khot *et al.*, 2012).

Functional Oils

Many oleaginous microorganisms could produce microbial lipids that are rich in ω-3 and -6 PUFAs such as GLA (C18:3, ω-6), arachidonic acid (ARA, C20:4, ω-6), eicosapentaenoic acid (EPA, C20:5, ω-3), and DHA (C22:6, ω-3). These essential fatty acids (EFAs) that humans cannot synthesize, are now widely accepted by the public for their health benefits (Ward and Singh, 2005). The synthesis pathway of fatty acids in most oleaginous microorganisms is shown in **Fig. 4**. Fatty acids are synthesized from acetyl-CoA and malonyl-CoA by the FAS complex of enzymes. Subsequently, the saturated fatty acid, stearic acid (SA, C18:0) is desaturated and elongated by a battery of reactions and turned into various ω-3/6 PUFAs according to the position of the first double bond from the methyl terminal group (**Fig. 4**) (Ratledge, 2004). Traditionally, the vast majority of EFAs were extracted from cold-water fish and seeds of plants such as evening primrose, borage, and black currant that offer relatively low yields at high costs (Dyal *et al.*, 2005). Also, some concerns remain about using fish oils as nutritional supplements due to the presence of environmental pollutants including dioxins, polychlorinated biphenyl (PCB), and heavy metals that bioaccumulate in fish and become concentrated in the liver and other organs (Ratledge, 2004). Therefore, finding new alternate economical sources and

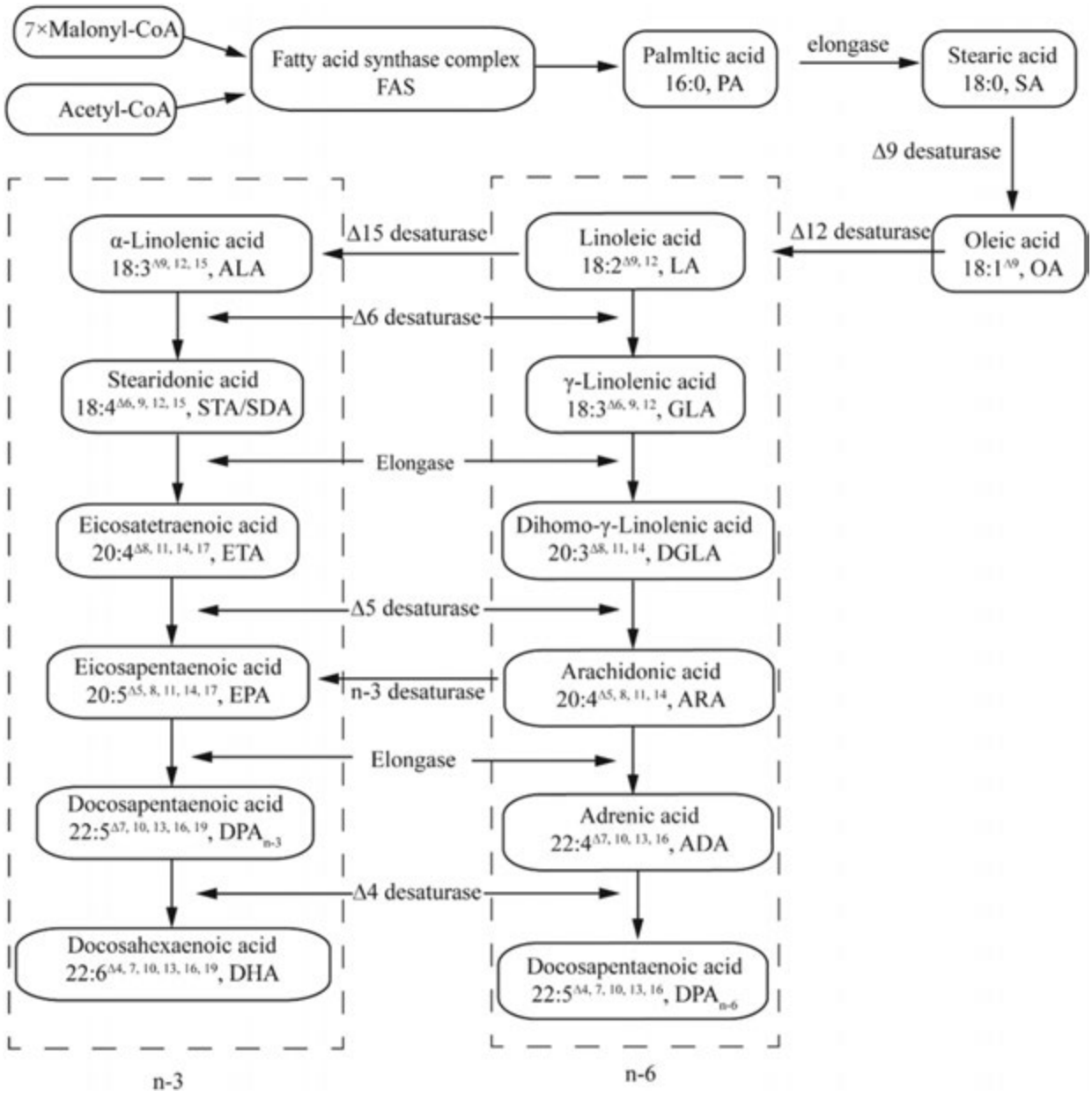

Fig. 4 Biosynthesis of PUFAs in oleaginous microorganisms.

novel dietary products to supplement EFAs has become a hot topic in both academia and industry. With in-depth research on oleaginous fungi, scientists found that fungal species possess unrivaled advantages compared to traditional EFA sources, which make them attractive alternatives for EFAs. Compared to tedious genetic manipulation and strenuous breeding work on plants for EFAs, fungal strains are able to produce a substantial amount of EFAs by controlling growth conditions. Moreover, oleaginous fungi do not require agricultural land for their growth, and can accumulate PUFAs at a level of over 70% of the total cellular lipids. Thus, oleaginous fungi that produce desirable PUFAs (as well as TAGs) have become crucial sources of EFAs (Bellou *et al.*, 2016; Passoth, 2017; Ratledge, 2004). High-value fungal oils will be an important and promising way to provide human diet supplement in the future.

γ -linolenic acid

Among PUFAs, the production of γ -linolenic acid (GLA) by different oleaginous microorganisms, especially fungi, was investigated due to its selective anticancer characteristics and negligible systemic toxicity (Kenny *et al.*, 2000). GLA belongs to the ω-6 PUFAs produced from linoleic acid (LA, C18:2, ω-6) by a Δ-6 desaturase enzyme and it can be further elongated and desaturated to synthesize other long-chain PUFAs including dihomogamma linolenic acid (DGLA, C20:3, ω-6) and ARA. In

industry, GLA could be synthesized and accumulated in different oleaginous fungi such as *U. isabellina* (Papanikolaou *et al.*, 2004), *Cunninghamella echinulate* (Fakas *et al.*, 2007), *U. ramanniana* (Dyal *et al.*, 2005), *M. alpina* (Jang *et al.*, 2005), and *Mucor rouxii* (Mamatha *et al.*, 2008). The filamentous fungus *C. echinulate* produced over 35% of cellular storage lipids and over 11% of GLA under a high C/N ratio of over 100. Following the cellular lipid accumulation, GLA concentration gradually increased in *C. echinulate*, while the highest GLA yield (720 mg L^{-1}, 80 mg g^{-1} of dry biomass) was observed when the C/N ratio reached 163 (Gema *et al.*, 2002). Different nutrient sources for *C. echinulate* ATHUM 4411 varied in GLA yield with 8.7 g L^{-1} of lipids and 1018 mg L^{-1} of GLA from tomato waste hydrolysate and 3.8 g L^{-1} of lipids and 540 mg L^{-1} of GLA from potato starch (Fakas *et al.*, 2008b; Papanikolaou *et al.*, 2007). This suggests the significance of optimal culture conditions for both lipid quality and productivity. *C. echinulate* CCRC 31840 exhibited a higher GLA yield of 964 mg L^{-1} using starch as a substrate, which could be further increased to 1349 mg L^{-1} by optimizing the inoculum (Chen and Liu, 1997; Chen and Chang, 1996).

Many Mucorales species, including *M. circinelloides* and *M. rouxii*, are famous for producing high levels of lipids and GLA (Mamatha *et al.*, 2008). For example, *M. rouxii* CFR-G15 produced a maximum GLA level (18.55%, w/w) using central composite rotatable design (CCRD) along with response surface methodology (RSM) to select optimal medium (Mamatha *et al.*, 2008). *Umbelopsis ramanniana* var. *ramanniana* was reported to produce 13.3% of GLA in the total lipids using 5% dextrose and 1% yeast extract as substrates, which is higher than the GLA yield from evening primrose (Dyal *et al.*, 2005). Similar increases of GLA were observed in *U. isabellina* when grown in a culture broth including 2% octadecanol and 1% yeast extract (Xian *et al.*, 2002), suggesting the enormous potential of oleaginous fungi for producing PUFAs by establishing individual optimum culture conditions.

Arachidonic acid

Arachidonic acid (ARA, C20:4, ω-6) is an important precursor of several key eicosanoid hormones with pharmacologically active metabolites and it has wide applications in medicine, cosmetics, pharmacology, food industry, agriculture, and many other fields (Eroshin *et al.*, 2000). Strains of *Mortierella* species belonging to the subphylum Mortierellomycotina have shown an outstanding capability to produce ARA (Fernandes *et al.*, 2017; Jacobs *et al.*, 2010; Kikukawa *et al.*, 2018). A fast-growing *Mortierella alliacea* YN-15 was shown to accumulate ARA in TAGs: 46.1 g L^{-1} of total dry cell biomass, 19.5 g L^{-1} of total fatty acid with 7.1 g L^{-1} of ARA in 7 d cultivation (Aki *et al.*, 2001). *M. alpina* is well documented as a suitable source for ARA production. *M. alpina* LPM 301 could produce lipids to 16.4% or 18.8% of their dry biomass with 46.0% or 60.4% of ARA content under batch cultivation in glucose-containing media with urea or potassium nitrate as N sources, respectively (Eroshin *et al.*, 2000). *M. alpina* M6 was reported to accumulate 72.3% of ARA in lipids and the yield of ARA could reach 4.82 g L^{-1} (Zhu *et al.*, 2004).

Eicosapentaenoic acid

Eicosapentaenoic acid (EPA, C20:5) is an ω-3 PUFA and has medically therapeutic capabilities against cardiovascular diseases, cancer, schizophrenia, and Alzheimer's disease (Liang *et al.*, 2012; Ursin, 2003). Many fungal species are known to produce a substantial amount of EPA. For example, at low temperature (12°C), *Mortierella* species were able to make EPA comprised 5%–20% of the total extractable fatty acids, including *Mortierella elongata* CBS 121.71 (5%), *M. elongata* IS-5 AKU 3999 (7.9%), *Mortierella hygrophila* IFO 5941 (10.4%), *Mortierella parvispora* 2S-13 AKU 3994 (10.9%), *M. alpina* 2O-17 (17.1%), and *M. alpina* 1–83 (19.8%) (Shimiziu *et al.*, 1988). No obvious EPA was detected in the same strains grown at high temperature (28°C), which produced 18%–48% ARA instead of EPA. Expressing the gene encoding *Saprolegnia diclina* Δ17 desaturase in *M. alpina* ST1358 allowed the transformant to produce EPA at both 12 and 28°C, which had 26.4% EPA of total fatty acid and reached 1.8 g L^{-1} at 28°C (Okuda *et al.*, 2015). The oomycete *Pythium irregulare*, a fungal-like protist that was formerly classified as a fungus, shows great potential in producing EPA. It was reported that *P. irregulare* has the capability to utilize various substrates for its growth and EPA production, including crude soybean oil, soymeal waste, sweet whey permeates, and sucrose waste stream (Cheng *et al.*, 1999; O'Brien *et al.*, 1993). It also has been reported that the oleaginous yeast *Yarrowia lipolytica* was able to produce EPA at 15% of dry cell weight by metabolic engineering approaches (Xie *et al.*, 2015).

Docosahexaenoic acid

Docosahexaenoic acid (DHA, C22:6, ω-3) is a primary structural component of human organs such as the brain and eye. It's essential for the growth and functional development of infant brains and for maintaining normal brain function in adults (Horrocks and Yeo, 1999). The marine fungus *Thraustochytrium aureum* ATCC 34304 has been considered as a promising producer for DHA, which was observed to contain approximately 50% of total fatty acids as DHA, depending on the growth conditions, and produce over 500 mg L^{-1} of DHA under optimized conditions for 6 days (Bajpai *et al.*, 1991). Other *Thraustochytrium* species were reported to offer high amounts of DHA, such as *Thraustochytrium* sp. ATCC 20892 (67.6 mg L^{-1}, 5 days) (Singh *et al.*, 1996) and *Thraustochytrium roseum* ATCC 28210 (1011 mg L^{-1}, 5 days) (Singh and Ward, 1996).

Future Strategies for Productive Oleaginous Fungi

In the past two decades, microbial lipids have been used for food production, and as nutritional supplements, detergent, lubricants, and biodiesel. There are two major barriers to microbial lipid production: the high cost of the glucose substrate and the

low productivity of the oleaginous microorganisms (Lin *et al.*, 2010). At present, many studies have focused on reducing these costs. For large-scale production of SCO, finding suitable feedstock is one key issue for its industrialization (Huang *et al.*, 2017). Various low cost, hydrophilic or hydrophobic substrates were used for SCO production (Huang *et al.*, 2013), and screening of optimal oleaginous fungi has become a vital mission to utilize the low-price substrates. Scientists have been isolating and screening oleaginous strains from field environments, where these fungi could be grown on a large scale. For example, approximately 30 strains of the endophytic fungi were isolated from the oily wood stems of seven oleaginous plants. Fungi belonging to five genera including *Microsphaeropsis*, *Phomopsis*, *Cephalosporium*, *Sclerocystis*, and *Nigrospora*, were able to secrete cellulases and accumulate oils simultaneously, ranging from 21.3% to 35% of dry weight in straw-based solid-state medium (Peng and Chen, 2007).

Aside from screening natural isolates, genetic and metabolic engineering approaches have been carried out to enhance lipid productivity and quality in oleaginous fungi. For example, overexpression of a gene encoding native malic enzyme, a major provider of NADPH for lipid production, led to a 2.5-fold increase in lipid content in the oleaginous fungus *M. circinelloides* (Zhang *et al.*, 2007). In *M. alpina* 1S-4, different approaches such as chemical mutagenesis, RNA interference (RNAi) and overproduction of key enzymes, such as desaturases, were used to engineer fatty acid compositions: the JT-180 mutant lacking Δ12-desaturase had increased Δ5- and Δ6-desaturase activities, resulting in higher levels of Mead acid (MA, C20:3), whereas overexpression of an endogenous Δ12-desaturase encoding gene in the JT-180 strain increased ARA (2.0 g L^{-1}), but reduced MA production (Sakuradani *et al.*, 2002). Disruption of Δ5-desaturase led to substantial production of dihomo-γ-linolenic acid (DGLA, C20:3, ω-6) (Kikukawa *et al.*, 2016), while ARA content could be significantly increased by overexpressing the genes encoding GLELO (Takeno *et al.*, 2005; Wynn and Ratledge, 2000) and multiple desaturases (Δ5, 6, and 12) (Kikukawa *et al.*, 2018). Newer gene-editing technologies such as CRISPR-Cas9 have been used in oleaginous fungi and yeast for genome editing and modification of transcriptional regulation, facilitating more efficient engineering of microbial lipid production (Otoupal *et al.*, 2019; Schwartz and Wheeldon, 2018; Shi *et al.*, 2017).

Conclusions and Perspectives

After decades of research and development, oleaginous fungi have become one of the best performing producers of microbial lipids due to a number of great advantages. Therefore, efforts towards the applications of microbial oils often involve the development of oleaginous fungi. Various biotechnology methodologies, such as mutagenesis, cell fusion, and directed evolution, have been used to engineer fungal strains to improve the productivity of oils and high-value compounds. Additional approaches including genomic, transcriptomic, proteomics, metabolomics, and lipidomic techniques could help to build more impeccable and desirable lipid production, as well as convenient application systems to explore oleaginous fungi. Fungal lipid products could be expanded in the future with better strains and fermentation technologies, which will eventually contribute to meet the demand for energy, food, and essential dietary nutrition of human beings.

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Role of Fungi in Fermented Foods

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Introduction

Cultures and civilizations across the globe have known to embrace the process of fermentation for food production. Fermented foods often have numerous advantages over the raw materials from which they are made. In this regard fungi are used to improve the digestibility of raw materials (e.g., soybeans), to make food safe (e.g., alcoholic beverages) and to produce much valued aroma compounds (e.g., beer, wine, cheese, soy sauce). The process of fermentation can be seen as the chemical transformation of organic substances into simpler compounds by the action of single enzymes or complex organic enzymatic cascades that are produced by microorganisms such as fungi or bacteria. Spoilage and growth of pathogenic organisms is inhibited during food fermentation due to the generation of secondary metabolites by the fermenting microorganisms. For instance, strains of the filamentous fungal genus *Penicillium* that are used for cheese production act as protective cultures preventing the settlement of other microorganisms (Nielsen *et al.*, 1998). Besides preservation, fermentation also imparts a variety of organoleptic properties, such as characteristic aroma, flavor and texture, to food. Fermentation also helps to improve the nutritional profile of food substances by removing toxins and synthesizing vitamins (Terefe, 2016). Tempeh is a classic example for this case, where fungal fermentation of soybean leads to a reduction of phytic acid and trypsin inhibitors and results in the hydrolysis of complex soy proteins into more bioavailable peptides and amino acids (Chen *et al.*, 2013; Soni and Dey, 2014; Terefe, 2016). Fungi possess superior capabilities in breaking down complex molecules into their smaller and more absorbable forms. Frequently used fungal species in food fermentation are yeasts like *Candida* spp., *Kluyveromyces* spp., *Phaffia* spp., *Pichia* spp. and *Saccharomyces* spp. as well as filamentous fungi like *Aspergillus* spp., *Mucor* spp., *Penicillium* spp. and *Rhizopus* spp. (Khachatourians, 2004). This review will mainly focus on the usage of filamentous fungi in food fermentation.

Filamentous fungi and yeasts belong to the eukaryotic kingdom of fungi. Like species of the animal kingdom, fungi are heterotrophs and, therefore, require organic material for growth. The two major phyla in the fungal kingdom are the Ascomycota and the Basidiomycota, sometimes referred to as higher fungi. Commonly known as sac fungi, many filamentous fungi such as *Fusarium*, *Penicillium*, *Aspergillus*, *Morchella* (true morels), *Tuber* (truffles), and most unicellular yeasts belong to the phylum Ascomycota. On the other side, the species of the phylum Basidiomycota, also known as club fungi, represent the group to which almost all mushroom forming fungi belong. Common representatives of the basidiomycete fungi are the button mushroom (*Agaricus bisporus*), the shiitake (*Lentinula edodes*) and the oyster mushroom (*Pleurotus* spp.). The filamentous character of most of the higher fungi enables a colonization of solid substrates, while simultaneously digesting it.

Many fungal fermented foods have long established traditions in certain countries. Asian countries are famous for their fermented foods, such as soy sauce and tempeh. Many of the European countries are famous for their bread, beer, wine or cheese. Lately, some new products can be found in the market. Quorn is a fungal meat substitute, whose main ingredient is a mycoprotein. This mycoprotein is made up of the mycelium of *Fusarium venenatum* and has become one of the world's best-selling meat alternative (Wilson, 2001). In the further course of this review, a depiction on the various new developments along with traditional filamentous fungal fermented foods will be presented (see **Table 1** for an overview).

Foods Fermented With Filamentous Fungi

Koji

Koji is not considered a food, but a pre-culture used for food fermentation processes in Asian countries. Traditionally, it consists of the fungus *Aspergillus oryzae* (current name: *A. flavus* var. *oryzae*) and a solid substrate, such as soybeans, rice or wheat. It is used to produce sake, soy sauce or miso. Besides the traditional "koji mold" *A. oryzae*, other filamentous fungi like *Aspergillus sojae*, *Aspergillus niger*, *Rhizopus* spp. or yeasts like *Hansenula anomala* (current name: *Wickerhamomyces anomala*) are also used (Nout and Aidoo, 2002). The principal function of koji fermentation is the induction and the supply of hydrolytic enzymes by fungi, such as amylases, peptidases and lipases, which break down macromolecules present in the grains into their constituent parts. Starch is hydrolyzed into maltose and glucose by amylases, proteins into peptides and amino acids by peptidases, and fats into the corresponding fatty acid chains by lipases (Chen *et al.*, 2009). The fermentation process itself can be considered as solid substrate fermentation (SSF), in which filamentous fungi colonize heat-sterilized grains or soybeans under controlled conditions. In the traditional process of koji making, a starter culture known as tane-koji (asexual spores of *Aspergillus* spp.) is mixed with steamed rice or grains and put into an aeratable container (e.g., wooden tray) (Steinkraus, 1996). Nowadays, fully controlled SSF bioreactors are available that provide better control over aeration and humidity during the koji process (Zhu and Tramper, 2013).

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Table 1 Role of fungi in the production of fermented food and beverages

Fermented food/Beverage	*Main fungi involved*	*Function/Role of fungi*
Koji	*Aspergillus flavus* var. *oryzae*, *Aspergillus sojae*	Supply enzymes (amylases, peptidases, lipases) required to breakdown macromolecules to their respective counterparts
Soy sauce	solid state: *A. oryzae, A. sojae* liquid state: *Zygosaccharomyces rouxii*, *Candida versatilis*	Secretion of enzymes that break down plant polymers present in the grains and soybeans osmotolerant microorganisms produce the characteristic flavor of the soy sauce
Miso	*A. oryzae*, *A. sojae*, *Z. rouxii*	Hydrolysis of soybean components and production of the characteristic flavor
Tempeh	*Rhizopus microsporus* var. *oligosporus*	Alteration of the nutritional parameters of soybeans; increase of water soluble nutrients; degradation of antinutritive components; generation of a food matrix
Angkak	*Monascus purpureus*, *M. ruber*	Natural food color production; development of beneficial metabolites including alcohols, antibiotics and antihypertensives
Oncom	red oncom: *Neurospora sitophila*, *N. intermedia* black oncom: *R. microsporus* var. *oligosporus*	Increase economic efficiency of food production by fermenting side-streams to human quality food products
Sufu	*Actinomucor* spp., *Mucor* spp., *Rhizopus* spp.	Increase digestibility; increased contents of calcium, free amino acids, soybean derived peptides and aromatic compounds
Cheese	internally ripened: *Penicillium roqueforti* surface ripened: *Penicillium camemberti*	Flavor enhancement; production of characteristic texture and color; ripening assistance; production of lipolytic and peptidolytic enzymes; reduction in fat rancidity
Meat	*Debaryomyces hansenii*	Production of consistent flavor, taste, drying rate, and appearance; inhibition of growth of undesirable microorganisms
Quorn	*Fusarium venenatum*	Provision of mycoprotein as raw material, which is processed into meat alternatives
Other fungal proteins	*Pleurotus sapidus*, *A. oryzae, N. intermedia*	Alternative protein source; upcycling of low-quality agricultural side streams; production of vegan sausages
Injera	*Candida humilis*, *Rhodotorula mucilaginosa* var. *mucilaginosa*, *Kluyveromyces marxianus*, *Debaryomyces hansenii*	Improve bioavailability of minerals and phytochemicals from cereals; increase glycemic index
Bread	Sourdough: *Kazachstania exigua, Candida humilis* Currently used in industries and bakeries: *S. cerevisiae*	Generation of CO_2 responsible for dough leavening; production of secondary metabolites including organic acids, glycerol, and aroma compounds; improvement of flavor and texture
Cacao	*Kluyveromyces marxianus, Hanseniaspora guilliermondii, H. opuntiae*	Reduction in astringency of cacao seeds; exert pectinolytic activity through the production of pectinases
Wine	*Saccharomyces* spp.	Production of ethanol; increased production of higher alcohols, esters, volatile/aromatic compounds, fatty acids, terpenols; inhibition of undesirable organisms
Sake	*A. oryzae*, *S. cerevisiae*	Saccharification and production of ethanol and various flavor compounds; suppression of invasion by wild microorganisms
Beer	top fermenting (ales): *S. cerevisiae* bottom fermenting (lager): *S. pastorianus* var. *pastorianus*	Production of ethanol, CO_2 and various aroma compounds, such as esters and higher alcohols
Basidiodrinks	various fungi of the phylum Basidiomycota	Production of flavor or degradation of undesirable aroma compounds

Soy Sauce

Soy sauce is a brownish salty and meat like tasting liquid used to refine various dishes. Traditionally, soy sauce derived from China is made of soybeans, whereas in Japan (Japanese: shōyu) and nowadays also in Western countries roasted wheat or rice is added (Zhu and Tramper, 2013). The multiple-stage fermentation process of soy sauce can be divided into two main steps. During the first step, the koji production, mainly *A. oryzea* or *A. sojae* colonize pre-treated grains and soybeans. This step requires guaranteed aeration while the temperature and relative humidity should be controlled at around 25–35°C and 90% or higher, respectively. In this first step, enzymes are secreted by the fungus to degrade the plant polymers. In a second step, also known as the moromi stage, the koji is soaked in brine containing around 20% NaCl. Hence, the SSF is replaced by a submerged fermentation, where fungal enzymes produced during the koji stage break down the plant polymers and proteins present in the grain and soybeans, e.g., peptidases hydrolyze proteins into oligopeptides and amino acids and polysaccharides are hydrolyzed by means of different enzymes, including amylases, cellulases and invertases. During the submerged fermentation (moromi), a combination of yeast(s) (*Zygosaccharomyces rouxii, Candida versatilis*) and lactic acid bacteria (*Pediococcus halophilus, Pediococcus soyae)* are added to produce the characteristic flavor of soy sauce (Nout and Aidoo, 2002; Steinkraus, 1996). The moromi stage may last up to 12 months (Nout and Aidoo, 2002). After filtration or decantation, the raw soy sauce is pasteurized. Due to its low pH and high salt content, soy sauce can be stored at room temperatures and does not require cooling (Krämer, 2007).

Fig. 1 Fresh tempeh: soybeans coated with the mycelium of *Rhizopus oligosporus*.

Miso

A traditional Japanese seasoning, Miso is a fermented soybean paste produced from soybeans, salt and the fermentation starter koji. The koji is prepared by inoculating mainly polished, steamed, and cooled rice with *A. oryzae* based tane-koji (Steinkraus, 1996). In contrast to the soy sauce production where brine is blended with the koji, the koji for miso production is mixed with salt and pre-treated soybeans resulting in a paste. In addition to the koji derived filamentous fungi (*A. oryzae*, *A. sojae*), yeasts (*Z. rouxii*) and lactic acid bacteria (*Pediococcus* spp. and *Streptococcus* spp.) are added to the soybean paste to start the fermentation, which results in the hydrolysis of the soybean components as well as starch and proteins derived from rice or barley, if added. The characteristic flavor of miso results not only from the fermentation process, but also from several reactions that occur during the ripening, which can last several months, and during the preparation of miso based meals due to the Maillard reaction which takes place at high temperatures during cooking (Inoue *et al.*, 2016). Miso is classified into several types according to the type of koji used, the usage of cereals, the color, the salt concentration and the period of ripening (Steinkraus, 1996).

Tempeh

Tempeh (or tempe) originates from Indonesia and is commonly made by fermentation of soybeans (soybean tempeh, tempeh kedele, **Fig. 1**). Besides, coconut press cake (tempeh bongkrek), peanut press cake, fava beans and cowpeas can also be used for tempeh (Krämer, 2007; Nout and Aidoo, 2002). Briefly, the single-stage fermentation process starts with de-hulling of raw soybeans. After soaking for 3–20 h at 30°C in water, the beans are drained, washed with fresh water and boiled. The pre-treated beans are inoculated with *Rhizopus* species such as *R. oligosporus* (current name: *R. microsporus* var. *oligosporus)* and *R. oryzea* (current name: *R. arrhizus*). Other fungal species detected in tempeh are *Mucor* spp. and the yeast *Trichosporon beigelii* (Nout and Aidoo, 2002). Comparable to the koji fermentation, the tempeh fermentation can be considered as SSF during which enzymatic activities of the microorganisms lead to an alteration of the nutritional parameters of the soybeans. Anti-nutritive compounds present in soybeans are partly degraded by the *Rhizopus* species during fermentation. These include protein based trypsin inhibitors or potential allergens, which are hydrolyzed by peptidases, glycosylated isoflavones that are converted into their aglycones, and the oligosaccharides raffinose and stachyose, which lead to flatulence in humans, are hydrolyzed by fungal α-galactosidases (Cao *et al.*, 2007). Besides the significant increase in water-soluble nutrients by fungi, enhanced vitamin biosynthesis leading to a remarkable vitamin B12 presence in tempeh can be achieved by the addition of *Propionibacterium freudenreichii* (Wolkers – Rooijackers *et al.*, 2018). The tempeh fermentation takes place in perforated plastic beds or bags, or even more traditionally in banana leaves to assure sufficient aeration for one to two days at 25–37°C. After fermentation, the mycelium covers the beans completely and binds them together into a solid. Thus, the fungus not only increases the nutritional value of the soybeans, but also generates a specific food matrix. This white and solid fresh tempeh is cooked or fried prior to consumption. Tempeh is a very popular meat replacer or side dish and can also be used as a snack. Fresh tempeh is not stable for a longer time due to its comparably high water activity and, therefore, has to be dried or preserved in a different way to guarantee a longer shelf life (Krämer, 2007).

Angkak

Angkak, also known as traditional Chinese red rice, was originally produced from fermentation of cooked non-glutinous rice (*Oryza sativa* L. *Gramineae*) by filamentous fungi like *Monascus purpureus and Monascus ruber*, (Pattanagul *et al.*, 2007). It has been used extensively in Asia as a natural food colorant in fish, Chinese cheese, red wine, sausages, and sufu (soybean cheese) due to its intense purple red color. Though angkak is traditionally produced by SSF of polished, steamed and cooled rice with spores of *Monascus* for one week at 25–30°C, production of angkak via submerged fermentation leads to higher pigment yields (Nout and Aidoo, 2002). The produced pigments have been well investigated (Nout and Aidoo, 2002; Pastrana *et al.*, 1995): orange, purple and yellow pigments such as monascorubin, monascorubramine, ankaflavin or monascin have been reported to be present in angkak. They are heat-stable, which makes angkak an increasingly favorable source of pigment in the food industry. Apart from

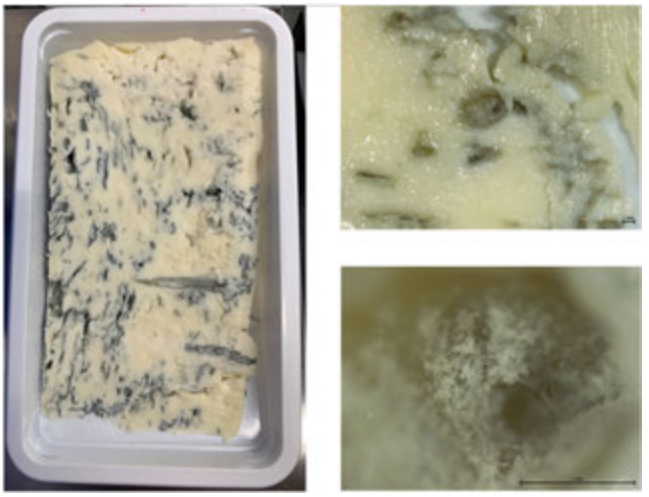

Fig. 2 Blue cheese of the type Gorgonzola with mycelial hyphae of *Penicillium roqueforti*.

pigments, the fungal fermentation of starchy rice leads to the development of several metabolites such as alcohols, antibiotics, antihypertensives, enzymes, fatty acids, flavor compounds, flocculants, ketones, organic acids, pigments and vitamins (Pattanagul *et al.*, 2007). Today, angkak is still used as traditional medicine and food colorant in Asia and in Chinese communities in North America. Due to its antibacterial activity, considerable interest has been devoted to the application of angkak as a nitrite/nitrate substitute for the preservation of meats (Pattanagul *et al.*, 2007).

Oncom

Oncom is a traditional Indonesian fermented food that has been produced and consumed mainly in West Java (Surono, 2016). The raw materials of oncom are mainly the waste of agricultural products and, consequently, there are many kinds of oncom derived from the combination of several filamentous fungi and raw materials. Generally, two types of oncom can be differentiated: red oncom and black oncom. Red oncom is produced by the ascomycete *Neurospora sitophila* or *Neurospora intermedia*. It is the only human food produced by *Neurospora* species. Meanwhile, black oncom is produced by fermenting fungi like *R. microsporus* var. *oligosporus* and *Mucor* species. Generally, red oncom is made from tofu waste, while black oncom is made from peanut dregs mixed with cassava dregs or cassava powder (Surono, 2016). Comparable with the production of tempeh and koji, the fungal fermentation process is a SSF that takes 36–48 h at room temperature, guaranteeing a relative humidity of about 90% (Steinkraus, 1996). Since oncom production uses by-products to make food, it increases the economic efficiency of food production. In the production of oncom, sanitation and hygiene are important to prevent bacterial or fungal contaminations, such as *Aspergillus flavus*, which can produce the mycotoxin aflatoxin (Surono, 2016).

Sufu

Originating from China, sufu is a traditional fermented soybean curd, which has a long history of being consumed as an appetizer (Cheng *et al.*, 2011). It is a soft cheese-like product with a spreadable creamy consistency and is produced by SSF of soybean curd (Cheng *et al.*, 2009). Many different methods are available for sufu production, but the fungal-fermented sufu is the most consumed type. This sufu is prepared in four major steps: (1) Preparing tofu (soybean curd), (2) Preparing pehtze (pizi) by fungal SSF of tofu using starter cultures containing *Actinomucor* spp., *Mucor* spp. or *Rhizopus* spp. (3) salting of pehtze, and (4) ripening in a dressing mixture (Wang and Hesseltine, 1970; Han *et al.*, 2004). The composition of the dressing mixture eventually determines the type of sufu (red or white) that is obtained (Han *et al.*, 2004).

Cheese

Filamentous fungi play important roles in the production of a variety of cheeses. The most well-known are the blue mold and the white mold cheeses. Fungal ripened cheeses can be classified into two categories depending on the type of ripening process they undergo – internally ripened cheeses and surface ripened cheeses. The semi-soft, blue mold cheese, such as Roquefort or Gorgonzola, produced in different countries under different names, is the most famous internally ripened cheese that makes use of *Penicillium roqueforti* as the principal fungus. This fungus creates blue veins in the cheese as it grows (**Fig. 2**). Besides, the applied *Penicillium* species produce the mycotoxin roquefortine C. Due to the relative low toxicity of roquefortine C and its low concentrations, blue cheese is non-hazardous for human consumption (Finoli *et al.*, 2001). A lesser known variety of an internally

ripened cheese is Gamalost. It is a traditional Norwegian cheese that makes use of *Mucor mucedo* and *Mucor racemosus* for its production. These filamentous fungi give Gamalost its characteristic flavor, texture and the yellow brown color.

Among the surface ripened cheeses, the soft, white mold cheese, such as Camembert or Brie are the most well-known. *Penicillium camemberti* is used as the main ripening agent to produce this cheese, wherein the fungus grows over the entire surface of the cheese and produces a white coat. Various other surface ripened cheeses include the semi-hard Saint Nectaire cheese, which is ripened by fungi such as *Mucor* spp., *Trichothecium roseum*, and *Bisifusarium domesticum*, Cantal, Salers and Rodez cheeses, which utilize *Sporendonema casei* for their production, and various French and Austrian cheeses which are produced by the *Scopulariopsis* species. These fungi are either inoculated on the cheese as a starter culture or stand out in mixed cultures during spontaneous fermentation (Metin, 2018). During ripening, proteins and fat are hydrolyzed by peptidases and lipases secreted by the fungi. Aroma compounds such as C_6-, C_8-and C_{10}-fatty acids and methyl-ketones are produced, resulting in the special characteristics of these aforementioned cheeses (Krämer, 2007).

Meat

In many countries all over the world, fermented meat products often represent elements of culinary heritage (Macedo *et al.*, 2017). In Europe, especially Italy, Germany and Spain, a wide variety of fermented meat products account for 20%–40% of total processed meat. Fermented sausages are obtained through fermentation of a meat batter composed of lean and fat meat, curing agents (nitrate and nitrite), spices, sodium chloride, sugars (to favor lactic acid fermentation) and starter cultures stuffed in natural or synthetic casings, and then subjected to a ripening process under controlled temperature and relative humidity (Coloretti *et al.*, 2014). Starter cultures are formed by mixing different types of microorganisms, each of which has a specific function. The most common microorganisms used as starter cultures in meat fermentations are lactic acid bacteria and coagulase-negative cocci (Macedo *et al.*, 2017). Besides, yeasts and filamentous fungi are also used as starter cultures. Yeasts are usually found on the surface of the product and contribute to the development of the characteristic flavor and texture of cured meat. The most prominent yeast species isolated from dry-cured meat products is *Debaryomyces hansenii*, although minority species of the genera *Rhodotorula* and *Trichosporon* have also been isolated (Aquilanti *et al.*, 2007). Filamentous fungi are used to process meat in Southern European regions mainly on dry sausages. Inoculation of sausages with fungi was traditionally done with native flora from plants, the so-called "house flora", which was mainly composed of species from the genera *Penicillium*, *Aspergillus* or *Scopulariopsis*. This kind of uncontrolled, spontaneous fermentation can lead to sausages covered with potential pathogenic fungi. *Penicillium nalgiovense* was the first non-hazardous and technologically suitable fungus used as starter culture for fermentation of meat, and later made commercially available as "Edelschimmel Kulmbach" (Sunesen and Stahnke, 2003). The usage of fungi on processed meat has two aims. Firstly, *P. nalgiovense* or other non-toxic fungi act as protective cultures reducing the risk of mal-fermentation and inhabitation of mycotoxin producing fungi. Secondly, sausages inoculated with a suitable starter culture achieve a more consistent flavor, taste, drying rate, and uniform appearance (Sunesen and Stahnke, 2003).

Fungi as Alternative Protein Sources

Alternative protein sources are urgently required because of the constantly growing world population and changes in living standards. Fungi in the form of edible fruiting bodies are consumed worldwide due to their taste and nutritional value. They are being artificially cultivated for hundreds of years by means of SSF on different agricultural and forestry side streams (Rühl and Kües, 2007). Although the fermentation of lignocellulose containing substrates, such as wood or straw, is necessary for mushroom production, the derived fruiting bodies are strictly spoken of as not fermented food and thus are not discussed here. Nevertheless, several reviews focus on the fermentation of recalcitrant substrates for mushroom production (Sánchez, 2004; Rühl and Kües, 2007). In contrast to the fruiting bodies, the fungal biomass from filamentous fungi grown in submerged cultures that directly results from a fermentation is rich in proteins, fibers and vitamins, thereby making it highly suitable for human consumption (Ahlborn *et al.*, 2019).

Quorn

Although one would think that fungi of the phylum Basidiomycota, to which almost all mushroom forming fungi belong to, would be the direct way towards fungal based food; an ascomycete was found in 1960, within a target-oriented screening for a microorganism that was able to grow fast on an inexpensive media to generate valuable protein. Since 1985, this filamentous fungus has been deliberately cultured for use as a primary source of protein for human consumption. Produced by Marlow Foods, this is high protein rich biomass from the ascomycete *Fusarium venenatum* A3/5 (ATCC PTA-2684). The fungal biomass of the ascomycete is produced in a continuous flow culture by submerged fermentation and is sold under the trade name Quorn. It makes for a meat alternative, has sustainable functional ingredients and forms the basis of all Quorn brand products (Wiebe, 2004). After being produced, the fungal biomass is subjected to a heat treatment for the reduction of ribonucleic acid content (Edelman *et al.*, 1983). The mycelial suspension is further heated to 90°C and concentrated by centrifugation to give a paste with a bio dry mass of more than 20% (w/v) (Wiebe, 2002). The mycelial paste generated is called mycoprotein and may further be combined with a binding

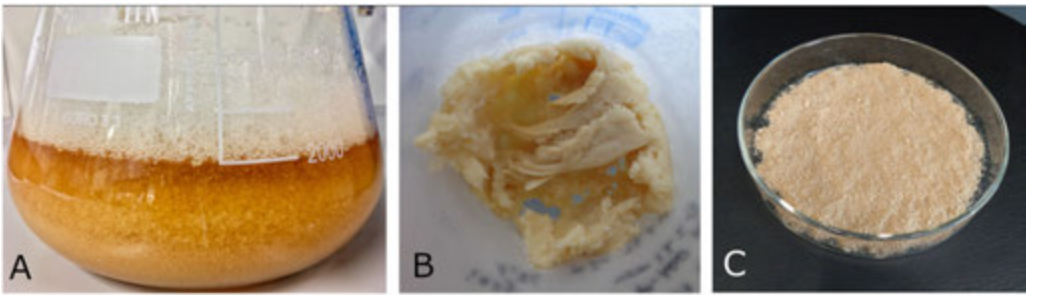

Fig. 3 Fungal mycelium produced by means of liquid fermentation (A) is separated from the supernatant (B) and dried via lyophilization (C).

agent such as egg albumin and various spices or flavorings. Standard food processing technology is used to shape the final products, which include unflavored chunks, mince, sausages, burgers, fillets, and steaks (Wiebe, 2004).

Other Types of Mycoprotein

Adapted from work of Justus Liebig in the 19th century on the usage of spent yeast (*S. cerevisiae*) for human consumption (Liebig, 1846), a yeast based condiment, known as Marmite™, was established around 100 years ago (Wadlow, 2011). At the same time - during the First World War, Germany had replaced half of the imported fodder protein by yeast. Since the spent brewer's yeast was not sufficient, yeast biomass was produced using a semi-defined medium containing ammonium salts as the nitrogen source (Ugalde and Castrillo, 2002). Two decades later, spent brewer's yeast and fermented yeast were used to supplement human and animal diets (Ugalde and Castrillo, 2002). Although Baker's yeast is currently not being used as a primary food, it is still produced using molasses as a carbon source and ammonium salts as nitrogen source and sold in supermarkets as a leavening agent. In recent years, research has again focused on the use of vegetative fungal biomass as a protein source, especially due to the possibility of higher fungi to grow on recalcitrant side-streams. Recently, Ahlborn *et al.* screened seven mushroom-forming fungi on their ability to grow on various industrial side streams, such as different pomaces (apple, pomegranate and aronia), leaf spinach and beet molasses for the production of fungal biomass (Ahlborn *et al.*, 2019, **Fig. 3**). Fungal biomass of the oyster mushroom *Pleurotus sapidus* cultivated on apple pomace or isomaltulose-molasses has been used to produce vegan sausages (Stephan *et al.*, 2017). Mahboubi *et al.* used different dairy wastes and its by-products as substrates for cultivation of the filamentous ascomycetes *A. oryzae* (see Koji) and *N. intermedia* (see Oncom) (Mahboubi *et al.*, 2017).

Yeasts in Fermented Foods

Injera

Injera together with "wot" (sauce) is the major staple food eaten by Ethiopians and Eritreans, as well as people from some areas of Somalia and Sudan (Adem and Ambie, 2017). It is generally prepared from cereals like sorghum, tef, corn, finger millet and barley, although tef (*Eragrostis tef*) is the main cereal ingredient of the Ethiopian injera (Leykun *et al.*, 2020). To make injera, the grains are de-hulled and milled into flour. The flour is mixed with water to form a dough, the starter culture (ersho) is added and the dough is fermented for two or three days. Ersho is a fluid obtained from previously fermented doughs containing mainly yeasts, such as *Candida humilis*, *Rhodotorula mucilaginosa* var. *mucilaginosa*, *Kluyveromyces marxianus*, *Ogataea naganishii* and *Debaryomyces hansenii*, some filamentous fungi, including species of the genera *Pullaria*, *Aspergillus*, *Penicillium*, and a number of lactic acid bacteria (Ashenafi, 1994; Blandino *et al.*, 2003). Due to the activity of lactic acid bacteria, the pH of the dough decreases to about 3.5 (Ashenafi, 1994) and the lactic acid concentration rises, which gives Injera a sour flavor. After fermentation, the sourdough is thinned down to a thick batter and baked or eventually steamed. The major quality attribute of a good injera is its slightly sour flavor.

Bread

Among plant-fermented food, bread is of great importance, both from a cultural and a nutritive point of view. Leavened bread was traditionally made with flour, water and a fermenting agent, which was either a fermenting beverage or a fermenting dough, termed sourdough (Venturini Copetti, 2019). This sourdough was generally initiated from a mixture of flour and water, naturally colonized by lactic acid bacteria and yeasts (Gobbetti *et al.*, 2016). Yeast cells ferment the carbohydrates present in the dough and generate carbon dioxide that are responsible for dough leavening during the fermentation phase and the oven rise (Struyf *et al.*, 2017). Sourdough was either maintained from one bread making process to the other or initiated again (Decock and Cappelle, 2005). The typical sourdough yeasts are *Saccharomyces exiguus* (current name: *Kazachstania exigua*) and *Candida humilis* (Iacumin

et al., 2009). Sourdough ecosystems are also inhabited by fungal species like *Pichia anomala (*current name: *Wickerhamomyces anomalus)*, *Torulaspora delbrueckii*, *Debaryomyces hansenii*, and *Pichia membranifaciens* (Corsetti and Settanni, 2007). In the 19th century, yeast starters made of *S. cerevisiae*, also referred to as baker's yeast, were proposed as an alternative to sourdough and are more commonly used nowadays by local bakeries and industries (Struyf *et al.*, 2017). According to research, it has been shown that baker's yeast serves other functions as well. It produces metabolites such as organic acids, glycerol and aroma compounds. These compounds have an important impact on the bread making process and final bread quality (Cho and Peterson, 2010; Jayaram *et al.*, 2013). Dough fermentation can further be affected by different factors like dough ingredients, dough fermentation conditions, yeast pre-growth conditions, and the genetic makeup of the yeast strain (Randez-Gil *et al.*, 1999; Rezaei *et al.*, 2015).

Cacao

The starting material for cocoa and chocolate is the seed of *Theobroma cacao* which are embedded in the pulp of the tree pods (Money, 2016). To manufacture chocolate, the pods are cut open, the pulp-bean mass is arranged in heaps, boxes, or baskets and allowed to ferment in the open air for 4–7 days. The spontaneous fermentation of sugars and citric acid in the mucilaginous pulp kills the cacao embryos and reduces the astringency of the seeds. Generally, cacao fermentation takes place spontaneously at the farmer's site and, thus, involves a complex community of yeasts and bacteria. During fermentation, the pulp becomes contaminated with diverse microorganisms from the environment. At the start of fermentation, the low pH and high sugar in the surrounding pulp favors anaerobic fermentation by yeasts and the growth of lactic acid bacteria. The ethanol produced represents a substrate for the acetic acid bacteria, which predominate when the sugars are exhausted (Money, 2016). Pectinolytic activity is supplied by *Kluyveromyces marxianus, Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae*, but also other yeasts have been found like *Saccharomyces*, *Torulopsis*, *Pichia* and *Candida* that play significant roles (Money, 2016; Bamforth and Cook, 2019). The pectinolysis leads to the draining of the pulp off the beans as "sweatings". This allows air into the spaces between the beans and so, late in fermentation, aerophiles develop, including *Bacillus*, as well as filamentous fungi (Bamforth and Cook, 2019). The succession of microorganisms reflects changes in the physical and chemical environment within the pulp-bean mass. At the end of the fermentation, the seeds are dried, packed and used for chocolate production.

Beverages

Wine and Fruit-Based Alcoholic Fermented Beverages

As a traditional fermented alcoholic beverage with substantial territorial and socio-cultural significance, wine has a long history that is patterned by a tradition of innovation (Pretorius, 2020). It is made by alcoholic fermentation of grape or grape juice by *Saccharomyces* spp. mainly, with a subsequent aging process. Wine fermentation is carried out in barrels, open vats, or industrial fermenters. Following the first phase of the fermentation, the wine is transferred to wooden casks and other containers for maturation and storage. The wine is filtered before bottling to eliminate bacteria that might spoil the wine by converting the ethanol to acetic acid and carrying out other reactions (Money, 2016). The microflora used for fermentation of wine may also include various non-*Saccharomyces* yeasts, such as *Candida colliculosa*, *Candida stellata* (current name: *Starmerella stellata*), *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii*, and *Kloeckera apiculata*. These species dominate the early stage of wine-making process and may also persist during other fermentative stages (Sun *et al.*, 2014). Furthermore, they have been reported to exhibit low production of undesirable components and probably promote the release of many different compounds (Comitini *et al.*, 2011). *H. uvarum* was found to increase the quantity of some desirable compounds, such as higher alcohols and esters (Medina *et al.*, 2013). *M. pulcherrima* has been shown to have a synergistic effect in co-culture with *S. cerevisiae* and promote the production of many aromatic compounds, including fatty acids, esters and terpenols (Sun *et al.*, 2014; Comitini *et al.*, 2011).

Unless otherwise specified, the term "wine" is generally applied to the product made by the alcoholic fermentation of grape or grape juice by yeasts. Like the wines from grapes, there are different fruit-based alcoholic beverages, termed as "fruit wines" as they employ the same production process as wine. One of the most widely produced non-grape fruit wine all over Great Britain and Europe is cider, or apple wine, which is fermented apple juice. Boukha is wine made from figs, while Perry is a wine prepared from pear juice. Asians have their own unique versions of fruit wines made from plums. When dealing with fruits other than grapes, sugar may be added to accelerate the fermentation process, if the fruit does not contain enough fermentable natural sugar. While the entire gamut of winemaking involves *S. cerevisiae*, natural fruit fermentations for wine production have been dominated by fungi like *Aspergillus flavus*, *A. oryzae*, *Mucor* spp., *Rhizopus* spp., and bacteria like *Pediococcus*, *Leuconostoc*, and *Pseudomonas* (Joshi *et al.*, 2017).

Sake

The global popularity of sake, a traditional Japanese alcoholic drink, is on a continuous rise. Sake production is conducted by fermenting steamed rice and malted rice (*kome-koji*; see Koji) with water. *Kome-koji* is solid-fermented steamed rice prepared using *A. oryzae* as a saccharifier to degrade rice starch to glucose and oligomers. *Kome-koji* is then used for preparing a starter brew, called *moto*, by manually mixing it with steamed rice and water in an open tank. The sugars produced by the koji enzymes are further

fermented into ethanol by *S. cerevisiae* that is applied as *moto* (Winans *et al.*, 2020). *Moto* significantly influences the flavor and richness of sake. In the traditional starter, *yamahai-moto*, the growth of naturally occurring lactic acid bacteria represses putrefactive microorganisms, whereas in the modern starter, *sokujo-moto*, this is achieved by addition of lactic acid (Koyanagi *et al.*, 2016). *Sokujo-moto* and *yamahai-moto* take approximately 14 and 30 days, respectively, to ensure sufficient viability for sake fermentation (Akaike *et al.*, 2019). Finally, a main mash called *moromi* is prepared. The process involves several steamed rice, water and koji additions to the final *moto*, at temperatures between 12–20°C. This stepwise addition applies a high osmotic pressure on the yeast due to the different rice mash ratios (Winans *et al.*, 2020). It also plays an important role in suppressing the invasion of wild microorganisms together with lowering the mashing temperature in each addition. Temperature control is extremely important to balance saccharification and fermentation, both of which occur simultaneously in *moromi* (Kanauchi, 2013). Fermented *moromi* is mushy, dense, and difficult to filter because little water is used for its preparation (Yoshizawa, 1999). It is separated into sake filtrate and solids using a special filter press. Slightly turbid fresh sake is clarified, some of which is bottled directly for sales. Fresh sake is stored after it is pasteurized to kill yeasts and inactivate enzymes. During storage, sake matures, its color deepens, and a smooth taste develops. It is then blended and diluted with water to the appropriate alcohol content. Finally, sake is pasteurized, bottled, and shipped (Yoshizawa, 1999). To produce red sake, *Monascus* ssp. are used to produce the purple red color pigments (Ray and Bhunia, 2008).

Beer

Beer is a beverage whose history can be traced back for 6000–8000 years, and the process, whilst increasingly regulated and well-controlled because of tremendous strides in the understanding of it, has remained unchanged for hundreds of years (Bamforth and Cook, 2019). Brewing technologies are generally based on a recipe that includes water, malted barley, hops, and yeasts. The Bavarian Beer Purity Law of 1516, known as *Reinheitsgebot*, listed barley malt, hops and water as the only approved materials for brewing with the exception of some other malted grains like wheat and rye. The conventional brewing process consists of a series of batch processes, which can be compared to the sake/soy sauce production (Zhu and Tramper, 2013). The malting of barley is the first step, which is needed to produce mainly amylases that are able to degrade starch during mashing, and thus has the same aim as the koji by providing hydrolytic enzymes. The malt-based enzymes are extracted out of the grinded malt by water and act on the starch and proteins present in the malt during mashing, which takes place at different temperatures. Afterwards, the spent grains are separated from the liquid that is known as wort. The wort is boiled and hops are added. After cooling to the fermentation temperature, yeast is added. Yeasts used for beer production belong to the *Saccharomyces* genus. In the past, before the role of yeasts was understood, fermentation involved microorganisms naturally present in the air. Today, fermentation is generally carried out by the addition of standard yeast cultures (Baiano *et al.*, 2012). Depending on physiological characteristics of the yeast, and on the quality of other ingredients, two main types of beers are produced, i.e., ale or lager-style beers. Ales are brewed at relatively high temperatures (15–26°C) with top-fermenting *S. cerevisiae* strains, which rise to the surface of the fermenting wort during the flocculation process. Lager beers are brewed at lower temperatures (5–14°C) with bottom-fermenting yeasts (initially classified as *S. carlsbergensis*, now known as *S. pastorianus* var. *pastorianus*) that have a tendency to sediment after flocculation and sink to the bottom of the fermenters (Baiano and Petruzzi, 2017). The concentration of ethanol that can be achieved in the beer depends on the initial carbohydrate concentration in the wort, the amount of yeast biomass inoculated, the fermentation temperature, and the yeast attenuation capacity (Telini *et al.*, 2020). Besides the different yeast strains used, the temperature during fermentation has a high impact on the beer aroma. At higher temperatures, fruity and flowery aroma are produced by *S. cerevisiae*, e.g., the ester isoamyl acetate which has a strong banana odor. In contrast, at lower temperatures less or no fruity odors are produced and a "cleaner" aroma is demanded, which can be guaranteed by *S. pastorianus* var. *pastorianus*.

Kombucha

Traceable to as early as 220 BC in China, kombucha is a fermented product produced by symbiotic associations of several yeast strains and acetic acid bacteria (Hartmann *et al.*, 2000). The dominant bacterium is *Acetobacter xylinum*, while the yeasts belong to the genera *Zygosaccharomyces*, *Schizosaccharomyces*, *Saccharomyces*, *Saccharomycodes*, *Candida*, *Pichia*, *Brettanomyces* and *Torulopsis* (Teoh *et al.*, 2004). Under aerobic conditions, kombucha symbiosis is capable of converting sucrose and black or green tea, over a period of 7–10 days, into a slightly carbonated, mildly sour and refreshing beverage. The yeasts ferment the sugar in the tea medium to ethanol, which is then oxidized by the acetic acid bacteria to produce acetic acid (Teoh *et al.*, 2004). Analysis of this beverage shows the presence of sugars, gluconic, glucuronic, L-lactic, acetic, malic, tartaric, malonic, citric, and oxalic acid, ethanol, different free amino acids and water soluble vitamins and minerals (Bauer-Petrovska and Petrushevska-Tozi, 2000; Chen and Liu, 2000; Malbaša *et al.*, 2011). It has been reported that the kombucha beverage can act as a digestive, gives relief from arthritis, acts as a laxative, prevents microbial infections and even help in combating stress (Malbaša *et al.*, 2011).

Beverages Produced Using Basidiomycetes

While lactic acid bacteria, acetic acid bacteria, yeasts, and other ascomycete fungi are the most commonly used microorganisms for the preparation of traditional fermented beverages such as beer, wine, kvass and kombucha, a few studies that utilize

basidiomycetes for beverage production, have also been published (Zhang *et al.*, 2014). Lin *et al.* developed a novel beverage by *Saccharomyces* that utilized shiitake stipe extract as a nitrogen source and cane sugar as the carbon source (Lin *et al.*, 2010). Hou *et al.* prepared a novel beverage by mixing polysaccharides extracted from wild edible BaChu mushroom produced in Xinjiang Province, China, with hawthorn juice and apple juice (Hou *et al.*, 2008). Okamura *et al.* reported the production of beer-like fermented drinks using *Pleurotus ostreatus*, *Tricholoma matsutake*, or *Agaricus subrufescens* instead of *Saccharomyces cerevisiae* (Okamura *et al.*, 2001; Okamura-Matsui *et al.*, 2003). Further, Zhang *et al.* screened 31 different filamentous fungi of the phylum Basidiomycota by cultivating them in submerged cultures using wort as the sole carbon source. The most promising fermented refreshments with pleasant flavors were those that were produced by *Lentinula edodes* (shiitake) and *Trametes versicolor* (turkey tail) (Zhang *et al.*, 2014, 2015a). Wort fermented by L. *edodes* produced a very pleasant flavor (fresh, fruity, plum-like, sweet and slightly sour) within 48 h (Zhang *et al.*, 2015b). Fermentation of wort with *T. versicolor* resulted in a fresh, fruity, sweet and slightly floral aroma (Zhang *et al.*, 2015a). The aroma profile of both wort-based beverages was completely different to the profile of the original wort (Zhang *et al.*, 2015a,b).

Conclusion

Humans have employed fungi for thousands of years. For most of our history this remained unacknowledged like in cases of dough fermentation and brewing. Mushroom cultivation on decaying wood is an example of early biotechnology that is now utilized widely in Asian medicine and adds flavors in cuisines all around the world. The use of fungi in the pharmaceutical sector, especially for antibiotic production, is a more recent advancement and genetic manipulation of fungal strains has revolutionized biotechnology. It is important to remember that fermentation processes involving fungi are not limited to alcohol production. It also provides indispensable foods, beverages, and ingredients for various cuisines. While most fungal fermentation processes are spontaneous, methods for controlling these fermentations have developed and further deeper understanding of these processes is needed to preserve and to improve technologies and traditions.

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The Application of Fungal Biomass as Feed

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Introduction

World population is steadily increasing and based on predictions, it will exceed 9 billion people by 2050. Moreover, population growth has been followed by improvements in the standard of living. This reality has its implications, such as boosting present concerns regarding food security and energy supply, and an increasing environmental footprint, due to rising waste production (Taherzadeh, 2019). Reasonably, adding pressure to the production of food concomitantly increases the demand of high-quality compound feed, in which protein sources are the main components (Henchion *et al.*, 2017). The problem is exacerbated by the use of food-grade substrates, such as fishmeal and soybean meal, as the main protein sources for feed, influencing feed price and the sustainability of the whole sector (Henchion *et al.*, 2017). As a result, a great deal of research attention has been given to the search for alternative protein sources for feed applications. Feed recipes can be of variable complexity depending on the application, and can additionally include e.g., fat, minerals, amino acids, and vitamin sources (Fihurska and Thorenko, 2019; Rezaei *et al.*, 2019). Therefore, alternative protein sources that provide additional needed nutrients can potentially ease its inclusion in presently standard feed diets.

Several organisms have been proposed as potential alternative protein sources, including insects, algae, floating aquatic plants (van der Spiegel *et al.*, 2013), earthworms (Sharma and Garg, 2019), bacteria, and unicellular and filamentous fungi (Souza Filho *et al.*, 2019). Particularly, filamentous fungi are well-known microorganisms due to their long use for commercial production of e.g., food products, enzymes, and organic acids (Gibbs, 2000). Additionally, they have largely been investigated in a context of circular bioeconomy, towards valorization of side-streams, residues, and wastes (Ferreira *et al.*, 2016; Ferreira *et al.*, 2013). Filamentous fungal biomass has been proposed for feed applications in view of its nutritious composition rich in e.g., protein, fat, minerals, vitamins, and pigments (Karimi *et al.*, 2018). Protein and fat profiles are characteristically rich in essential amino acids and polyunsaturated fatty acids, respectively, while the cell wall is a potential source of immunostimulating compounds (Karimi *et al.*, 2018). Therefore, filamentous fungal biomass offers nutritional quantity and variability, and can contribute to the sustainability of the feed sector, if the biomass originates from growth on low-value substrates.

This chapter aims to provide an overview of the nutritional composition of filamentous fungal biomass, potential growth substrates and needed cultivations conditions, and insights gathered on the effect of using filamentous fungal biomass in feeding trials. The chapter also provides a clear view on the main bottlenecks and knowledge gaps within the area, and consequently proposes future research and development avenues.

Feed Market Prospects

The world compound feed market can be regarded by animal type, namely ruminants, poultry, swine, aquatic animals, and others; by ingredients, namely cereal, cakes and meals, by-products, and supplements; by supplements, namely vitamins, amino acids, enzymes, acidifiers, probiotics and prebiotics, and other supplements; and by geography, namely North America, Europe, Asia-Pacific, South America, and Africa (Mordor Intelligence, 2019). The world compound feed production was just over 1 billion tons in 2018 (IFIF, 2020), and a compound annual growth rate (CAGR) of 4.8% is projected during 2020–2025 (Mordor Intelligence, 2019). The growth is driven by the meat and fish sectors, that in turn are related to changing diet patterns, urbanization, population, and economic growth, mainly in developing countries (Mordor Intelligence, 2019). In a long-term perspective, the Food and Agriculture Organization of the United Nations (FAO) stated that an increase of 60% in the demand of food proteins must be met by 2050, indicating that a yearly 1.7% expansion of the sector must occur from 2010 to 2050. In turn, this demand represents a needed increase of 70%, 90%, and 55% in the production of meat, aquaculture, and dairy products, respectively (FAO, 2020).

Compound Feed: Composition and Requirements

Farmed animals usually do not feed by natural prey or forages, and nutrients must be provided in the form of compound feed. Moreover, animals are not capable to synthesize an array of nutrients de novo; therefore, they must be supplied in the diet to meet their requirements. Feed ingredients can vary from simple compounds such as salt (NaCl) to more complex compounds such as animal/plant meals, vitamins, oils, etc., and are typically classified into three major groups, namely protein, fat, and carbohydrate ingredients (Jobling, 2001). A number of supplements such as vitamins, minerals, pigments, and antioxidant agents, are also normally added to the feed (McDonald *et al.*, 2011). The type and proportion of compounds in feed recipes are variable and depend on animal species, life stage, animal age, local availability, final farming product, etc. (Halver and Hardy, 2002). The

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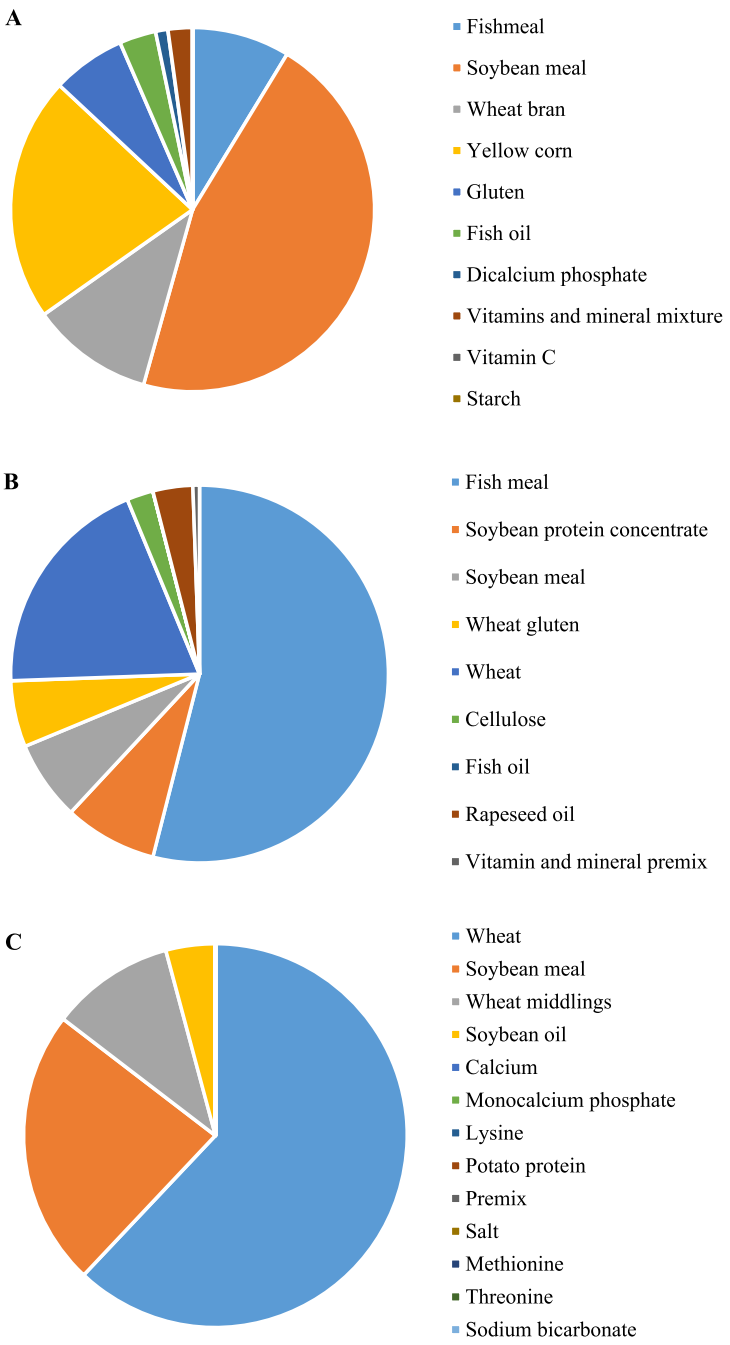

Fig. 1 Examples of feed recipes used for the growth of (A) Nile tilapia (*Oreochromis niloticus*), (B) Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*), and (C) broiler chickens. Reproduced from (A) Dawood, M.A.O., Eweedah, N.M., Moustafa, E.M., Farahat, E.M., 2020. Probiotic effects of *Aspergillus oryzae* on the oxidative status, heat shock protein, and immune related gene expression of Nile tilapia (Oreochromis niloticus) under hypoxia challenge. Aquaculture 520, 734669. (B) Langeland, M., Vidakovic, A., Vielma, J., *et al.*, 2016. Digestibility of microbial and mussel meal for Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*). Aquaculture Nutrition 22, 485–495. (C) Rezaei, M., Wall, H., Tarshan, M., Ivarsson, E., 2019. Evaluation of broiler chickens' digestibility of Neurospora intermedia biomass. Poultry Science 98, 5017–5022.

compound feed composition must be formulated according to the target animal species to balance a range of essential and non-essential nutrients. Examples of feed recipes for fish and poultry are presented in **Fig. 1**.

Feed and feeding practices represent the most expensive part of animal farming industries, comprising around half of final production costs. Furthermore, product quality is directly related to the supplied feed quality. In view of its key functions in any organism's body and consequent amount needed, protein sources represent the highest cost among feed compounds (FEFAC, 2019). Commonly-used protein sources in animal feed are presented in **Table 1**. The type and number of protein sources used vary

Table 1 Commonly-used protein sources in animal feed recipes

Ingredient	*Dry matter (%)*	*Crude protein (%)*	*Crude fiber (%)*	*Ash (%)*	*Energy (MJ/Kg)*
Maize	86.4	8.1	2.2	1.2	16.2
Wheat gluten	87.9	14.5	6.1	4.1	16.7
Corn gluten	89.5	60.6	1.1	1.8	20.6
Cottonseed	90.6	21.2	23.4	3.9	21.8
Linseed, full fat	90.3	22.6	9.2	4.3	24.4
Rapeseed	92.2	19.1	8.2	4.0	26.4
Soybean, full fat	88.1	34.8	5.2	5.2	20.4
Molasses	73.7	4.0	0.0	10.3	11.0
Potato protein concentrate	92.3	77.6	0.8	2.6	20.5
Alfa Alfa	84.8	20.9	18.9	11.6	16.1
Milk powder	96.4	23.4	–	6.0	22.7
Fishmeal	91.7	65.3	–	16.2	19.0
Blood meal	94.3	89.7	–	2.8	22.9
Feather meal	92.1	79.0	–	0.3	21.8
Meat and bone meal	96.3	51.3	–	30.5	16.6

Note: Sauvant, D., Perez, J. -M., Tran, G., 2004. Tables of Composition and Nutritional Value of Feed Materials: Pigs, Poultry, Cattle, Sheep, Goats, Rabbits, Horses and Fish, second ed. Amstelveen: Wageningen Academic Publishers & INRA.

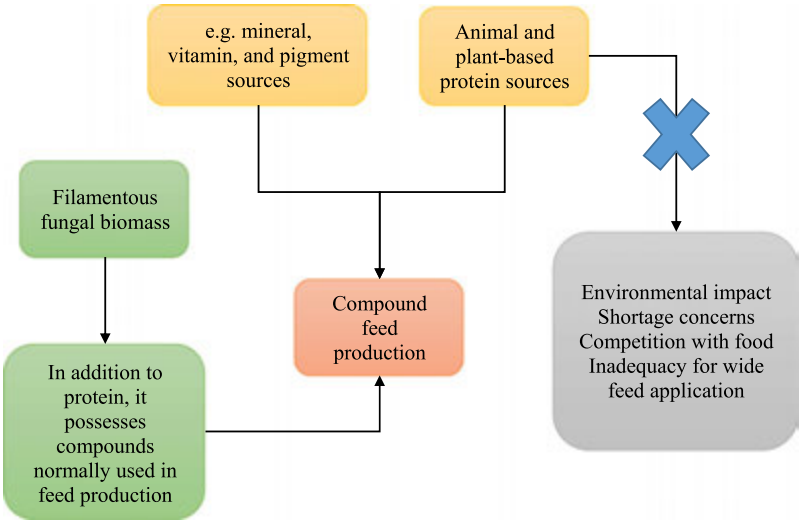

Fig. 2 Potential of filamentous fungal biomass to replace protein, mineral, vitamin, and pigment sources in compound feed production.

according to the type of animal being farmed. For instance, fishmeal and soybean meal are preferred for aquaculture practices, whereas corn meal, sorghum, or alfa alfa are preferred for ruminants, and soybean meal is the most common protein source in poultry feed (FEFAC, 2019). Nonetheless, a mixture of protein sources such as corn meal, cereal grains, sunflower meal, etc. can also be used in animal feed.

However, different constraints are related to different protein sources. Low availability and high price have limited animal-based protein sources usage in animal feed production; for example, fishmeal and fish oil production has been stagnant since the 1990s at its maximum sustainable yield (MSY) from natural resources (Pietzsch *et al.*, 2020). Other protein sources such as blood meal and feather meal have low digestibility in most animals, while inclusion of plant-based protein sources in feed is debatable since it rises human food competition concerns (FEFAC, 2019). Therefore, to cover the high demand for feed ingredients, new alternative ingredients that can circumvent these constraints are highly demanded.

Filamentous fungi are microorganisms that can grow on a wide range of substrates. The fungal biomass is rich in protein and fat, which are rich in essential amino acids and polyunsaturated fatty acids, respectively, in addition to cell wall components with immunostimulant activities, vitamins, minerals, and pigments (Karimi *et al.*, 2018). Filamentous fungi can grow on low-value substrates resulting from human activities (Ferreira *et al.*, 2013, 2016). Therefore, the process of fungal biomass production can be sustainable and readily available, while providing compounds commonly used for production of compound feed (**Fig. 2**).

Filamentous Fungi

The kingdom Fungi is a large and diverse group of organisms, estimated to comprise between 1.5 and 7.1 million species (Dornburg *et al.*, 2017). The group ranges from unicellular yeasts to multicellular filamentous fungi that also include macroscopic fungi that form large fruiting bodies (Boland *et al.*, 2012).

Multicellular filamentous fungal biomass originates through germination of microscopic spores into highly intertwined hyphae, ultimately leading to a 3D macroscopic structure. These fungi are used in the commercial production of various products under submerged fermentation (high water activity) and solid-state fermentation (low water activity), normally using high-quality growth medium recipes. For instance, members of ascomycetes are used for production of antibiotics (e.g., *Penicillium chrysogenum*), enzymes (*Aspergillus* spp. and *Trichoderma* spp.), and organic acids (*Aspergillus* spp.) through submerged fermentation. Additionally, both submerged fermentation and solid-state fermentation have been used for production of human foods (Ferreira *et al.*, 2016). The products under the trademark Quorn™ are produced through submerged fermentation of the ascomycete *Fusarium venenatum* (Wiebe, 2002), while solid-state fermentation is used for production of e.g., tempe (Wikandari *et al.*, 2012), oncom (Sastraatmadja *et al.*, 2002), and red rice (Patakova, 2013) using zygomycetes *Rhizopus* spp., and ascomycetes *Neurospora* spp., and *Monascus* spp., respectively.

This successful transfer of filamentous fungi from nature to the industry demonstrated their metabolic diversity regarding both the range of valuable products produced and the range of enzymes existing and enabling their growth in a wide range of substrates. This laid down the foundations for further investigations using filamentous fungi in a context of circular bioeconomy. Here, low-value substrates are converted in a wide range of valuable products, similarly to their role in natural nutrient cycles (Taherzadeh, 2019). Similarly, the wide and long use of several species of filamentous fungi in the production of human foods laid the foundations for the consideration of their biomass for feed applications (Rasmussen *et al.*, 2014; Ferreira *et al.*, 2014).

In addition to the environmental impact, the use of low-value substrates has also an influence in the production costs of a given valuable product. While defined (synthetic) media are popular among biologists, as they allow the accurate control of the media composition and establishment of cause-effect relationships, they are costly (Sezonov *et al.*, 2007). Substrates used for fungal cultivation should be easily biodegradable, cheap, readily available, and contain sufficient required micro- and macronutrients (Demirbas, 2009). Organic-rich industrial by-products and waste streams, such as those from bioethanol, dairy, and starch production industries, may meet these requirements for feasible fungal cultivation (Ferreira *et al.*, 2013, 2016, 2018).

Fungal Cultivation Process

The successful design of a fungal cultivation process lies on the close control of a range of parameters. These can impact fungal growth rate, morphology (mycelial suspension evenly distributed through the medium, pellets, or clumps), range and concentration of products, and biomass composition (Gibbs, 2000; Zhang *et al.*, 2007; Papagianni, 2004).

The choice of a certain fungal strain affects the growth rate, morphology, biomass composition, the range of nutrients needed, and physicochemical parameters such as pH, temperature, and oxygen supply rate. Strains within fungal groups differ genotypically, resulting in dissimilar enzyme expression both qualitatively and quantitatively (Papagianni, 2004). This, in turn, will influence the hydrolysis and assimilation rate of nutrients, and biomass composition regarding profiles of amino acids, polyunsaturated fatty acids, and cell wall components, in addition to the proportion of protein, fat, and cell wall contents on a dry weight basis (Ferreira *et al.*, 2012; Karimi *et al.*, 2019). Filamentous fungi can be grouped according to their cell wall composition, where varying amounts of chitosan, mixed chitin/chitosan, glucans, and mannans, can be found (Free, 2013). Chitosan and chitin are distinguished by the acetylation level of the building block glucosamine into N-acetyl-glucosamine. Chitosan has variable but relatively low acetylation degrees, whereas chitin is fully acetylated.

In addition to the fungal strain, cultivations conditions and cultivation time can alter the cell wall content in filamentous fungi, and the share among glucosamine and N-acetyl-glucosamine in zygomycetes (Ferreira *et al.*, 2012). For instance, longer cultivations times are normally associated with a higher acetylation level of the cell walls of zygomycetes (Zamani *et al.*, 2008). Altogether, considering that immunostimulant activities have been attributed to cell wall components, the choice of the filamentous fungus and cultivations conditions can steer the potential positive effect when the fungal biomass is used in feed.

The medium composition in the cultivation determines fungal growth rate, the need of additional process steps, and biomass amount and composition. For instance, the use of lignocellulosic materials normally entails a pretreatment step in order to make the nutrients available to filamentous fungi, and enhance growth rates and nutrient conversion (Nair *et al.*, 2018, 2015). Biomass amount and composition can be related to the C/N ratio, where higher ratios motivate production of metabolites over biomass and lower protein contents, or to the presence of suspended solids (Rasmussen *et al.*, 2014; Nitayavardhana, 2012). The product obtained via solid-state fermentation is normally a mixture of fungal filaments and remaining substrate, whereas in submerged fermentation a purer filamentous fungal biomass can be obtained through tailored cultivations conditions and content of suspended solids (Souza Filho *et al.*, 2019). High content of the latter normally leads to entanglement with fungal filaments; hence influencing the final composition of the fungal biomass (Rasmussen *et al.*, 2014). This can have both positive and negative impacts depending on the final applications and composition of the suspended solids. For instance, the suspended solids in stillage, the left-overs from industrial bioethanol production and normally used for feed applications, possess a protein content of ca. 16% that can be increased through fungal cultivation, due entanglement with hyphae (Bátori *et al.*, 2015).

Table 2 Functions of amino acids in organisms

Amino acid	*Functions*
Essential amino acids	
Arginine (Arg)	Protein synthesis and anabolic processes, urea production, metabolism of several amino acids such as proline and glutamic acid, precursor for creatine and nitric oxide synthesis, stimulant of insulin and growth hormone, effect on immunological functions in mammals, modulate innate immune mechanisms, regulating endocrine and reproductive functions, extra-endocrine signaling, waste detoxification, wound healing, bone repair.
Histidine (His)	Involved in the one-carbon metabolism and DNA and protein synthesis, energy fuel during starvation, induces desirable taste (e.g., sweetness), improves sensory attributes (e.g., flavor).
Isoleucine (Ile)	Anabolic activity, amino acid synthesis, energy source.
Leucine (Leu)	Critical roles in protein structure and composes relatively high proportion of most proteins, stimulates muscle protein synthesis, inhibits proteolysis, stimulates muscle protein synthesis.
Lysine (Lys)	Regulation of carnitine synthesis, fatty acid metabolism, osmoregulation in aquatic animals, maintaining the acid-base balance, nitrogen retention in the tissue, increasing the muscle growth, increasing weight gain.
Methionine (Met)	Precursor for other amino acids, innate immunity modulator, lipid metabolism, antioxidant activity.
Phenylalanine (Phe)	Precursor for neurotransmitter production such as cholecystokinin (CCK) and norepinephrine (NE), induces saturation feeling after eating, needed for thyroid gland, amino acid synthesis, melanin synthesis.
Threonine (Thr)	Affect immunity responses, component of mucin in the small intestine, maintaining the intestinal barrier integrity and function, immunological responses.
Valine (Val)	Deposited in skeletal muscles, key roles in determination of 3D shapes of structural proteins, synthesis of myelin, anabolic activity.
Tryptophan (Trp)	Serotonin (5-HT) synthesis, pain sensitivity reduction, reduces anxiety and tension, sleep induction.
Non-essential amino acids	
Alanine (Ala)	Gluconeogenesis precursor, energy supplier, nitrogen carrier for amino acid metabolism, stimulatory effects on feeding rate.
Asparagine (Asp)	Roles in stress tolerance, weight gain, increase feed intake.
Cysteine (Cys)	Antioxidant and detoxifying activity in particular in case of heavy metal removal from the body, DNA repair.
Glutamine (Glu)	Purine and pyrimidine nucleotide synthesis, acid-base balance regulation, protein synthesis stimulation, energy substrate for enterocytes, enhances weight gain, feed intake, intestinal development and digestive enzymes activity, essential for fish immunity response. Regulatory effects on hormones secretion such as norepinephrine and glucan-like peptide 1, glutathione production, cell regeneration, brain nutrient.
Glycine (Gly)	Hepatic thyroxine monodeiodinase activity increase, enhances the efficiency of nutrient absorption and anabolic events, protein synthesis.
Ornithine (Orn)	Synthesis of proline and polyamines, role in the synthesis of arginine in the urea cycle, growth promotor, immune enhancer, detoxifier.
Proline (Pro)	Metabolic precursor for glutamine, osmoregulation, roles in protein structure formation, collagen synthesis.
Serine (Ser)	Participates in gluconeogenesis, sulfur amino acid metabolism, fat digestion, stimulates feed intake.
Tyrosine (Tyr)	Precursor for CCK and NE, regeneration of red and white blood cells, improve memory, mental alertness.

Note: NRC, 2011. Nutrient Requirements of Fish and Shrimp. Washington DC: The National Academies Press. Wu, G., 2010. Functional amino acids in growth, reproduction, and health. Advances in Nutrition 1, 31–37. Holeček, M., 2018. Branched-chain amino acids in health and disease: Metabolism, alterations in blood plasma, and as supplements. Nutrition & Metabolism 15, 33.

Processes that aim for the production of fungal biomass are aerobic and therefore they need the correct reactor design for proper cultivation conditions and sustained scale-up. Stirred-tank bioreactors have been frequently used in industry. However, they are more suitable for the growth of unicellular microorganisms since high entanglement of filamentous fungi hyphae with internal spare parts takes place, leading to suboptimal mass transfer rates (of e.g., nutrients and oxygen) (Träger *et al.*, 1989). Although the fungal morphology can smooth the effect of reactor design, the cultivation of filamentous fungi is normally preferred to be carried out in airlift bioreactors. This reactor design, simply based on two concentric cylindrical tubes, decreases the entanglement of fungal hyphae, and improved mass transfer rates are attained (Träger *et al.*, 1989). Lastly, the air supply rate will influence fungal growth rate, morphology, and product type and concentration. For instance, if a filamentous fungus capable of producing ethanol is used, higher aeration rates are needed in order to reduce the amount of nutrients being converted into ethanol and enhance biomass production (Ferreira *et al.*, 2015).

Filamentous Fungal Biomass Composition

Protein and Amino Acid Profile

The importance of protein in feed recipes is given by the functions that its composing amino acids play when absorbed by animals in the intestine, following digestion (**Table 2**). The major roles of amino acids include participation in protein synthesis, use as a substrate for necessary metabolite synthesis, e.g., peptides (glutathione, carnosine, etc.) and neurotransmitters, and use as energy source through amino acid oxidation (Deutz *et al.*, 2014). Supplementing feed with the appropriate amount of amino acids can

Table 3 Profile of different groups of amino acids in different animals, namely in mammals, poultry, and fish

Mammals			*Poultry*			*Fish*		
EAA	*NEAA*	*CEAA*	*EAA*	*NEAA*	*CEAA*	*EAA*	*NEAA*	*CEAA*
Arg	Ala	Gln	Arg	Ala	Gln	Arg	Ala	Gln
Cys	Asn	Glu	Cys	Asn	Glu	Cys	Asn	Glu
His	Asp	Gly	Gly	Asp	Tau	His	Asp	Gly
Ile	Ser	Pro	His	Ser		Ile	Ser	Tau
Leu		Tau	Ile			Leu		
Lys			Leu			Lys		
Met			Lys			Met		
Phe			Met			Phe		
Thr			Phe			Pro		
Trp			Pro			Thr		
Tyr			Thr			Trp		
Val			Trp			Tyr		
			Tyr			Val		
			Val					

Note: Wu, G., 2010. Functional amino acids in growth, reproduction, and health. Advances in Nutrition 1, 31–37. Halver, J., Hardy, R., 2002. Fish Nutrition, third ed. New York: Academic Press.

Table 4 Protein content present in the biomass originated from cultivation of filamentous fungi in various substrates

Fungi	*Substrate*	*Protein content (%w/w dry weight)*	*References*
Aspergillus oryzae	Palm oil waste	39	Barker *et al.* (1981)
Rhizopus oligosporus	Starch processing wastewater	46	Jin *et al.* (1999)
Rhizopus sp.	Spent sulfite liquor	50–60	Ferreira *et al.* (2012)
Mucor circinelloides	Corn ethanol stillage	30.4	Mitra *et al.* (2012)
Pythium irregulare	Corn ethanol stillage	28	Liang *et al.* (2012)
Rhizopus, Mucor, Rhizomucor	Tempeh	47–63	Wikandari *et al.* (2012)
Mucor indicus Rhizopus sp.	Spent sulfite liquor	30–50	Lennartsson (2012)
Rhizopus oryzae	Vinasse	49.7	Nitayavardhana *et al.* (2013)
Neurospora intermedia	Wheat ethanol stillage	56	Ferreira *et al.* (2014)
Aspergillus oryzae		48	
Rhizopus sp.		55	
Rhizopus oligosporus	Corn ethanol stillage	43	Rasmussen *et al.* (2014)
Aspergillus oryzae	Wheat ethanol stillage	43	Bátori *et al.* (2015)
Neurospora intermedia			
Rhizopus oligosporus	Wheat bran	40	Yunus *et al.* (2015)
Neurospora intermedia	Dairy waste	40	Mahboubi *et al.* (2017)
Aspergillus oryzae			
Neurospora intermedia	Wheat lignocellulosic residues	50	Nair (2017)

reduce the risk of cardiovascular disease, prevent cholestasis, liver problems, and have neuro-modulation effects, antioxidant activity, and intestinal health and immunity (Dwyer, 2003; Holeček, 2018; Wu, 2016).

The 22 amino acids identified among living organisms have been grouped into essential amino acids (EAAs), conditionally essential amino acids (CEAAs), and non-essential amino acids (NEAAs). EAAs represent those that animals are unable to synthesize in contrast to NEAAs, while CEAAs represent those amino acids which carbon skeleton is not synthesized de novo in animals (Wu, 2013). The synthesis of CEAAs may be influenced by the life stage and physiological conditions of the body when its consumption rate surpasses synthesis (Reeds, 2000). Several factors can affect amino acid synthesis in the animal body including availability of substrate, animal species, animal life stage, physiological circumstances, composition of intestinal microbiota, environmental condition, and animal health status (NRC, 2011). Although the essentiality of each single amino acid is dependent on animal species, most of EAAs are common among animals (**Table 3**).

With a few exceptions, the protein content (%w/w dry weight) of the filamentous fungal biomass obtained following growth in various substrates had been within 40%–60% (**Table 4**), which points out the potential of fungal biomass for feed applications, when compared with feed recipes and main sources of protein for animal feed (**Table 1**). Furthermore, the profile of EAAs of filamentous fungal biomass show a high level of compatibility with that from fishmeal (**Fig. 2**). Although lower levels of lysine and methionine are available in fungal biomass in comparison to those in fishmeal, their concentrations are still higher than in

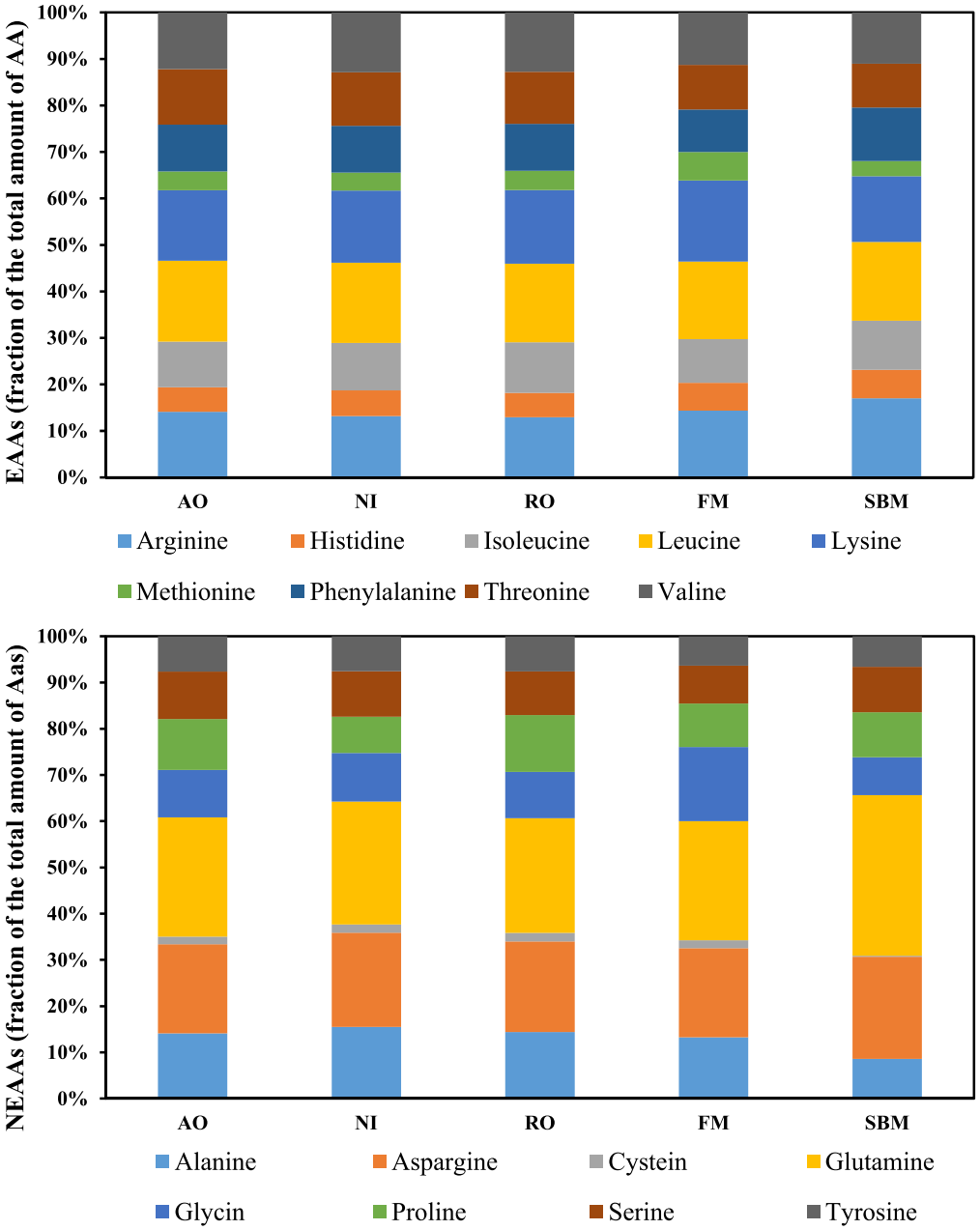

Fig. 3 Comparison of profiles of EAAs and NEAAs found in filamentous fungi (*Aspergillus oryzae* (AO), *Neurospora intermedia* (NI), *Rhizopus oryzae* (RO)), fishmeal (FM), and soybean meal (SBM). Reproduced from Karimi, S., Mahboobi Soofiani, N., Lundh, T., *et al.*, 2019. Evaluation of filamentous fungal biomass cultivated on vinasse as an alternative nutrient source of fish feed: Protein, lipid, and mineral composition. Fermentation 5, 99.

soybean meal that currently is the preferred alternative protein source of fishmeal (**Fig. 3**). Filamentous fungal biomass contains high concentrations of valine, threonine, and phenylalanine in comparison to those in fishmeal that can stimulate growth, elevate immune responses, and lead to efficient physiological and hormonal responses, respectively (NRC, 2011). With the exception of glycine, present at higher concentrations in fishmeal, the remaining NEAAs are present at higher levels in fungal biomass than in fishmeal. Although NEAAs can be synthesized in the body, their supplementation in the diet may have an energy sparing effect for the organism. Furthermore, in some life stages such as the reproduction period or a disease outbreak, the body may not provide sufficient levels of required NEAAs (Wu, 2013).

In addition to the amino acid composition, amino acid balance is another important aspect while considering potential protein sources for feed applications. An imbalanced amino acid profile may induce amino acid antagonism and toxicity, which in the long run, can decrease feed intake, induce aggression and abnormal activity, and a suboptimal growth rate (Dwyer, 2003).

Fat and Fatty Acid Content

Lipids are primarily an energy source for the cells. However, as components of the cell membrane and of all other membranes in the cell, they also play critical roles in cell integrity and transport (FAO, 2010). In addition, lipids participate in various key processes in living organisms, which affect growth, health, and activity. They can also make complexes with other cell structural components and form different molecules such as lipoproteins and glycolipids, which are very important for cell functionality (Burlingame *et al.*, 2009).

Similar to proteins, the importance of fat in feed is linked to its profile of fatty acids. Long-chain polyunsaturated fatty acids (LC-PUFAs), composed of ≥ 18 carbons, are especially relevant due to proven beneficial health properties (Blondeau *et al.*, 2015). Plants and some other organisms cannot synthetize LC-PUFAs, and those that are able to carry out their synthesis might not achieve adequate levels, pointing out the importance of addition of sources of LC-PUFAs in feed (Bhardwaj *et al.*, 2011). In addition to the profile of fatty acids, the PUFA/saturated fatty acids (SFA) ratio is also important to consider during feed applications, where a ratio of ≥ 0.45 is considered to be healthy (Gomez Candela *et al.*, 2011).

Due to their phylogenetic diversity, the fat content of filamentous fungi can vary widely among species in addition to the variation induced by cultivation conditions (Sibirny, 2017). For instance, the fat content of *Aspergillus oryzae*, *Neurospora intermedia*, and *Rhizopus oryzae* has been reported to be 3.5%–7% (Karimi *et al.*, 2019), while in the so-called oleaginous filamentous fungi it can account for up to 80%, on a cell dry weight basis (Sibirny, 2017). Considering the profile of fatty acids, filamentous fungal biomass is clearly a potential alternative to soybean meal and fishmeal (**Fig. 4**). Although the profile varies greatly among different fungal strains (**Fig. 4**), the ratio of PUFAs/SFA was higher than 1 pointing out its healthy status for feed applications (Karimi *et al.*, 2019).

Cell Wall Components

Filamentous fungi, similarly to plants, some algae and bacteria, consist of a functional rigid cell wall. Although the general functions that are attributed to the cell wall of filamentous fungi are highly similar to those described for the cell wall of other organisms, its composition may differ. For instance, bacterial cell walls are made of peptidoglycan, those of algae are based mainly on cellulose, while the cell walls of filamentous fungi can differ regarding the presence and amount of a number of polysaccharides including chitosan, chitin, glucans, and mannans. In spite of the prominence of polysaccharides, comparatively lower amounts of proteins and lipids can also be found in fungal cell walls (Bowman and Free, 2006). To emphasize on the importance of cell wall of filamentous fungi, it can account to up to 30% of the cell dry weight, and roughly 20% of their total genetic material is involved in the regulation of cell wall composition and structure (Bowman and Free, 2006).

One approved application of filamentous fungal cell wall polysaccharides is their use as immunostimulants. Cell wall biopolymers such as chitin, chitosan, glucans, and mannans can improve the immunity status of animals, including humans (Karimi *et al.*, 2018; Hernandez Chavez *et al.*, 2017; Sakai, 1999). Application of immunostimulants as diet therapy have been approved to elevate the immune status, stress related responses, and resistance to diseases (Ringo *et al.*, 2010).

Disease outbreak has become a critical issue in human health and animal farming industries. Several kinds of antibiotics and other medicines have been utilized to prevent bacterial, viral, and parasitic disease incidence, while promoting growth. However, the use of such medicines has led to the generation of resistant pathogenic strains, which promoted the development and administration of vaccines, an expensive and laborious approach (Peek and Landman, 2011). Hence, providing compounds with immunostimulant activity in feed has become very important, especially in Europe where the use of antibiotics is restricted.

Other Compounds

Filamentous fungal biomass is also a potential source of minerals, pigments, and vitamins. Minerals include calcium, potassium, sodium, phosphorus, and sulfur. Minerals are essential components of the skeleton and other hard tissues. They are involved in several key functions such as electron transfer, regulation of acid-base balance, and in processes influencing cell wall functionality (Fairweather-Tait and Cashman, 2015). A comparison of mineral profiles among filamentous fungi and plant-based sources e.g., soybean meal, reinforces the potential of filamentous fungal biomass for incorporation in animal feed (Karimi *et al.*, 2019).

Various genera of filamentous fungi such as *Neurospora*, *Monascus*, *Fusarium*, and *Penicillium* can synthesize an array of pigments including carotenoids, melanins, flavins, phenazines, quinones, and sometimes monascins, violacein, or indigo. Some of these pigments are presently available in the market. Pigments are important additives in animal feed recipes, improving the animal health status via improvement of its antioxidant capacity (Stahl and Sies, 2003). Furthermore, pigments are usually added to feed to enhance meat quality and satisfy consumer's preferences (Teles *et al.*, 2011). One example includes the use of pigments during salmon farming (Lim *et al.*, 2018).

Vitamins are organic compounds playing key roles in living organisms mostly as cofactors of enzymes. Ascorbic acid (vitamin C), riboflavin (vitamin B_2), pyridoxine (vitamin B_6), and nicotinic acid are among the vitamins found in filamentous fungal biomass (Archer *et al.*, 2008).

Feed Applications

Filamentous fungal biomass has been added to animal feed recipes functioning as supplements, namely as probiotics, or as feed ingredients (**Table 5**), and its impact on e.g., growth performance, digestibility, immunological responses, and antioxidant

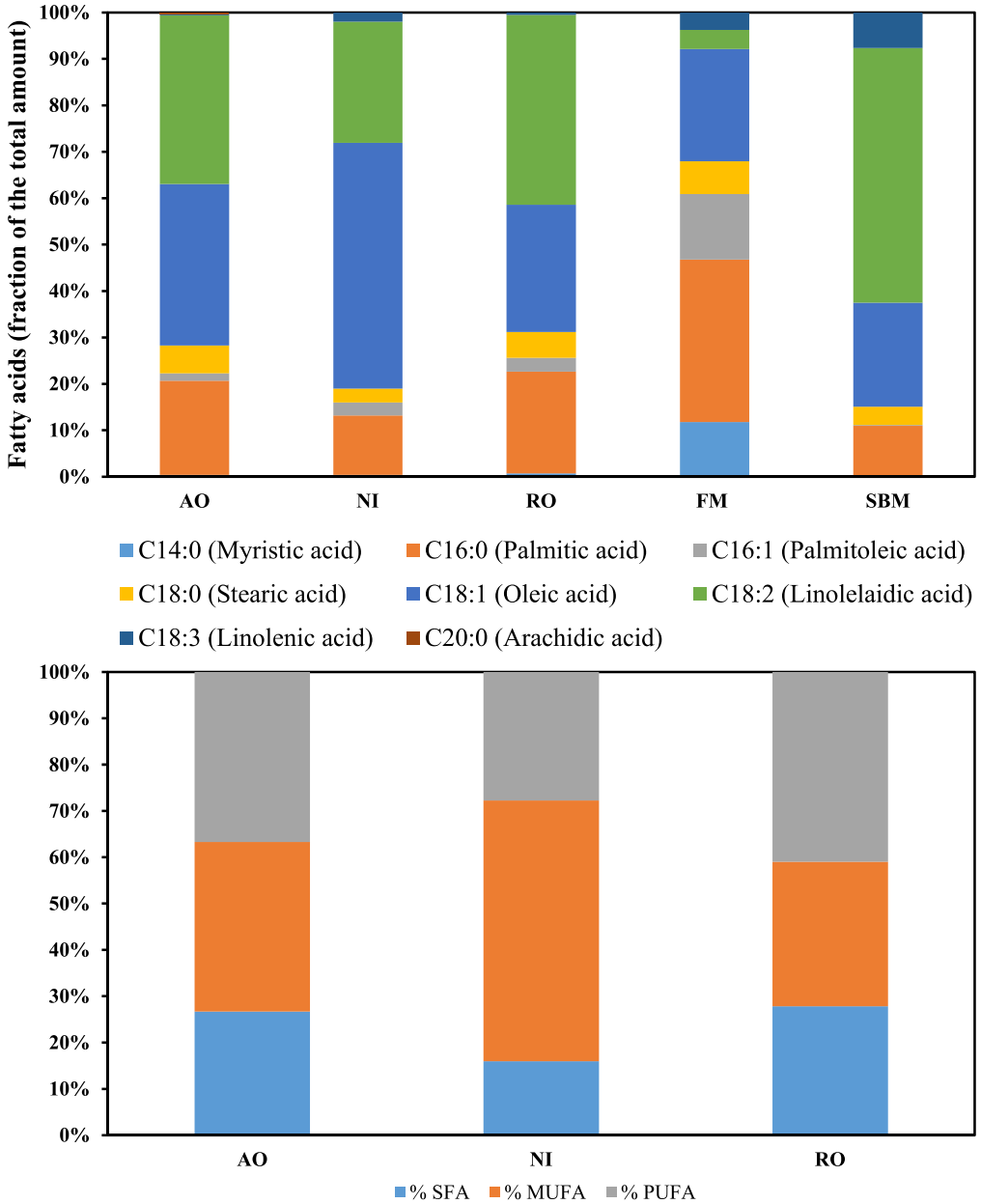

Fig. 4 Comparison of the profile of fatty acids found in filamentous fungi (*Aspergillus oryzae* (AO), *Neurospora intermedia* (NI), *Rhizopus oryzae* (RO)), fishmeal (FM), and soybean meal (SBM). The fraction of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) is presented in the lower Figure. Reproduced from Karimi, S., Mahboobi Soofiani, N., Lundh, T., *et al.*, 2019. Evaluation of filamentous fungal biomass cultivated on vinasse as an alternative nutrient source of fish feed: Protein, lipid, and mineral composition. Fermentation 5, 99.

capacity, has been studied (**Table 5**). In general, the use of filamentous fungi in feed has been related either to the absence of negative effects in comparison to a standard diet, or to improved feeding outputs.

Probiotics are living microorganisms that when ingested can settle in the host gut and enhance e.g., intestinal immunity and digestibility. Accordingly, probiotics have attracted high interest among animal nutritionists as feed supplements, as an alternative to antibiotics (Sugiharto *et al.*, 2017). Improved growth has been reported in most of the studies using different filamentous fungal species as probiotics (Sugiharto *et al.*, 2017; Sugiharto, 2019; Saleh *et al.*, 2019; Yamamoto *et al.*, 2007).

The use of filamentous fungus *Neurospora intermedia* biomass as alternative protein source in broiler chickens feeding has primarily been motivated by a suitable profile of amino acids, particularly lysine, and low level of fibers (Rezaei *et al.*, 2019). The latter are mostly related to the fungal cell wall polysaccharides, with proven positive effects on immune system, gut microbiota, and growth rate (Bowman and Free, 2006). During fish feeding studies, minor mortality was observed when arctic char was fed for 14 weeks with a recipe containing 40% zygomycete biomass (Wagner *et al.*, 2019). This result was likely due to increased levels of

Table 5 A summary of the studies related to animal feed applications of filamentous fungi

Fungal species	*Tested animal*	*Effect*	*References*
As probiotic			
A. awamori	Broilers	Higher weight gain, better nutrient digestibility, and increased carcass weight	Yamamoto *et al.* (2007)
A. niger and *A. oryzae*	Broilers	Improved growth performance	Saleh *et al.* (2019)
		Enhancement in nutrient digestibility and higher level of intestinal health	KyungWoo *et al.* (2006)
		Decreased saturated fatty acids and increased unsaturated fatty acids in the muscle fat	Sugiharto (2019)
Chrysonilia crassa	Broilers	Increased antioxidant capacity, more resistant when exposed to the different sources of stressors such as heat stress, better growth rate and immune responses, modified intestinal microbiota community	Sugiharto *et al.* (2017)
Filamentous fungi	Poultry	Enhancement in feed utilization, higher growth rate	Saleh *et al.* (2014), Sugiharto (2019)
Fungal biomass as feed ingredient			
Rhizopus oryzae	Arctic charr and Eurasian perch	Normal feed intake, no significant differences in digestibility for dry matter, crude protein, and gross energy	Langeland *et al.* (2016)
Zygomycetes	Atlantic salmon	No negative effect on fish performance	Pan (2013)
Neurospora intermedia	broiler	No significant difference in feed intake and final body weight, positive effects in immune responses, gut microbiota modification, and growth improvement	Rezaei *et al.* (2019)
Zygomycetes	Arctic charr	Minor mortality, improved muscle fatty acid profile, higher level of mono unsaturated fatty acids (MUFAs) and PUFAs	Wagner *et al.* (2019)

o-phosphocholine, which influences the initiation of recognition and phagocytic immune responses. The use of zygomycete biomass in feed also improved the fatty acid profile, likely through induction of higher gene expression of $\Delta - 6$ fatty acid desaturase, which plays a role in the synthesis of PUFAs. A higher level of MUFAs and PUFAs, including $n - 6$ and $n - 3$, were obtained in the fish fed with zygomycete biomass. An increased amount of 54% and 24% in docosahexaenoic acid and phospholipid content, respectively, and a 42% decrease in triacylglycerol content were observed in the muscle of fish fed with zygomycete biomass.

Conclusions and Perspectives

By steering the filamentous fungal species and cultivation conditions, a fungal biomass containing several compounds needed in compound feed production can be obtained. In addition, the studies available on the use of filamentous fungal biomass in feed point out the potential for replacement of common animal and plant-based sources. Nonetheless, the research field could benefit from a wider screening of animal species regarding the potential of using filamentous fungal biomass as replacement in standard feed diets. This would strengthen the use of filamentous fungal biomass as feed source and evaluate its application versatility. Furthermore, studies on the application of filamentous fungal biomass for animal feeding should be concomitantly supported by techno-economic and life-cycle assessment studies. This will shed light on the impact that filamentous fungi can have on the valorization of low-value substrates and on the whole compound feed sector, due to the replacement of animal and plant-based feed sources.

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Applications of Fungal Polysaccharides

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Introduction

Fungal polysaccharides are a subject of research in many fields of science due to their numerous applications. They are a large group of biopolymers constituting a component of the cell wall. Polysaccharides are the main component (90%) of the fungal cell wall, and their structure depends on the fungal species. The central core of the cell wall is a usually composed of β-1,3 glucan with β-1,6- branches, which is linked to chitin via a β-1,4 linkage. It is known that polysaccharides are synthesized by many species of fungi with different ecological positions such as saprotrophic and endophytic, pathogenic or symbiotic-mycorrhizae fungi. Therefore, these polymers can play different biological functions, for example in the protection against environmental stress factors and in interactions with other organisms. Polysaccharides may be also accumulated inside cells as a reserve of energy (Giavasis, 2013; Osińska-Jaroszuk *et al.*, 2015).

In terms of chemical structure, polysaccharides are a diverse group of compounds occurring most often in the form of large polymers – oligosaccharides comprising monosaccharide sub-units connected by glycosidic linkages. Combinations of monosaccharides may be present in many different positions of the molecule, which means that polysaccharide polymers may have a linear or branched structure (**Table 1**). In terms of their location in the cell, polysaccharides are divided into exopolysaccharides (EPS), intracellular polysaccharides (IPS), and fungal cell wall polysaccharides (FCW) (Jaroszuk-Ściseł and Kurek, 2012; Yan *et al.*, 2014).

Fungal polysaccharides exhibit a wide spectrum of bioactive properties, e.g., antioxidant, antimicrobial, immunomodulatory, antitumor, hypolipidemic, hypoglycemic, or hepatoprotective activities (Kozarski *et al.*, 2011; Miranda-Nantes *et al.*, 2011; Usui, 1983). A sulfated mannogalactan isolated from the fungus *Agaricus brasiliensis* has been described as an anticoagulation agent (Yu *et al.*, 2018).

The bioactive properties of polysaccharides depend on their spatial structure, molecular weight, presence of other monosaccharides in the molecule, and the type of glycosidic bonds. Great importance is also ascribed to the source of isolation thereof (type of organism, strain), conditions of the microbial culture, and extraction and isolation methods. Due to the large variety of fungal polysaccharides and their physicochemical properties, these macromolecules have a broad spectrum of activity in various fields of biotechnology, medicine, cosmetics or food and pharmacy industries (**Table 2**). A schematic illustration of the bioactivity properties of fungal polysaccharides and their use in industry is presented in **Fig. 1**. With their natural properties, i.e., capability of heavy metal sorption, ability to support solubilization of mineral compounds in fungi, and induction of plant immunity, polysaccharides have been widely used in agriculture for bio-fertilization of plants, bioremediation of water, and biological plant protection (Seneviratne *et al.*, 2008; Welch *et al.*, 1999).

Medicine and Pharmacy

Medical Properties of Fungal Polysaccharides

Antibacterial and antiviral effect

Fungal polysaccharides have recently become an object of research conducted by numerous scientific teams in connection with the growing problem of antibiotic resistance of bacterial strains and emergence of new varieties of pathogenic viruses. One of the proposals is the polysaccharide-protein complex isolated from *Phellinus linteus* acting as an anti-inflammatory agent (Patel and Goyal, 2012). Polysaccharides EP-AV1 and EP-AV2 obtained from *Phellinus pini* by hot-water extraction are described as promising agents against HSV-1 infections (Lee *et al.*, 2010). Strong antibacterial activity against *Escherichia coli* was detected for polysaccharides isolated from spent mushroom substrate after three harvesting cycles of a *Lentinus edodes* strain. It was detected that two fractions included in the preparation are mainly composed of glucose, rhamnose, and mannose and their FTIR spectra were very similar to those of lentinan (Zhu *et al.*, 2012). The 1,3- and 1,6-glucans isolated from the L. *edodes* can inhibit the growth of the following bacterial cells: *Bacillus megaterium* (NCIB 2602), *Enterococcus pheniculicola* (JLB-1T) – minimum inhibitory concentration (MIC): 150 mg/sample, and *Klebsiella pneumoniae* (K4) – MIC: 80 mg/sample (Rasmy *et al.*, 2010). Water-soluble sulfated derivatives of β-glucans from *Pleurotus tuber-regium* can be effective in the control of herpes simplex virus type 1 and 2 by preventing the processes of binding of virus particles to the host cells (Zhang *et al.*, 2004, 2003). EPS from *Schizophyllum commune* and *Schizophyllum gluconicum* have been found to increase resistance of the yellowtail fish to *Streptococcus* sp. infection by triggering a non-specific immune response (Zhu *et al.*, 2015). β-Glucan produced by *A. brasiliensis* is described in the literature as an antiviral agent, which is able to inhibit the replication of bovine herpes virus 1 (BoHV-1) in HepG-2 cells. The probable mechanism of this effect is connected with early events of the viral infection (Minari *et al.*, 2011; Zhu *et al.*, 2015).

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Table 1 The most common polysaccharide structures used in biotechnology and industry

Fungal sources	*Polysaccharides (common names)*	*Structure (main chain)*	*References*
Aureobasidium pullulans	Pullulan	(1→6)-α-polymer/maltotriose	Prajapati *et al.* (2013)
Grifola frondosa	Grifola	(1→3)-β-D-glucan	Zhuang *et al.* (1994)
	Grifolan	with 1→6 branches	Mizuno and Zhuang (1995), Zhuang *et al.* (1994)
		Mannoxyloglucan/glucan	
		Fucomannogalactan/galactose	Zhang *et al.* (2007)
		Mannogalactofucan/fucan	Mizuno and Zhuang (1995), Zhuang *et al.* (1994)
Lentinus edodes	Lentinan	(1→3)-β-D-glucan with 1→6 branches	Zhang *et al.* (2007)
Pleurotus spp.	Pleuran	Mannogalactan/galactan	Wasser (2002)
		Xyloglucan/glucan	Wang *et al.* (2005)
Pleurotus pulmonarius *Pleurotus citrinopileatus*		Arabinogalactan/galactan	
Schizophyllum commune	Schizophyllan	(1→3)-β-D-glucan with 1→6 branches	Zhang *et al.* (2013)
Sclerotium glucanicum	Scleroglucan	(1→3)-β-D-glucan with 1→6 branches	Survase *et al.* (2007)
Saccharomyces cerevisiae	Glucan	(1→6)-β-D-glucan	Klis *et al.* (2006)
		(1→3)-β-D-glucan	Kwiatkowski and Kwiatkowski (2012)
		(1→4)-α-(1→3)-β-D-glucan	
		(1→3)-α-(1→6)-β-D-glucan	
		(1→2)(1→3)-α-(1→6)-α-D-mannan/protein complexes	

Anticancer effect

Many polysaccharides of fungal origin are described as potential anticancer agents. One of them is β-(1-3)-linked glycan from *Pleurotus linteus* belonging to the Hymenochaetaceae family, which has been found to inhibit cancer cell growth and metastasis (Baker *et al.*, 2008). A polysaccharide-protein complex isolated from this organism is also able to induce apoptosis in SW480 human colon cancer cells (Li *et al.*, 2004). It has been demonstrated that a polysaccharide extract obtained from *Pleurotus ostreatus* biomass can inhibit the proliferation of HT-29 colon cancer cells and induce apoptotic changes therein (Lavi *et al.*, 2006). The POPS-1 preparation of water-soluble polysaccharides from this strain showed substantially higher cytotoxicity towards HeLa cancer cells, in comparison to human embryonic kidney 293T cells, than the commonly used anticancer drug 5-fluorouracil (Tong *et al.*, 2009).

Water-soluble polysaccharides from *P. tuber-regium* exhibited cytotoxicity as well as antiproliferative and proapoptotic effects towards promyelocytic leukemia cells (HL-60) (Wong *et al.*, 2007). Lentinan (β-glucan) obtained from the L. *edodes* is often described as a well-known anticancer preparation with high effectiveness against proliferation of leukemia cells (Patel and Goyal, 2012). Lentinan injections are used as an adjuvant in the therapy for gastric, pancreatic, colorectal cancer and hepatocellular carcinoma (Bisen *et al.*, 2010; Hazama *et al.*, 2009; Yu *et al.*, 2018). Another popular polysaccharide Krestin isolated from the white rot fungus *Trametes versicolor* can be potentially used in the prevention of breast cancer development and inhibition of the proliferation of human hepatoma cells (QGY) e.g., by induction of the pro-apoptotic pathways (Cai *et al.*, 2010). The polysaccharide-K from *T. versicolor* in combination with fluoropyrimidines evidently increases the survival of patients with colorectal and gastric cancer (Sakai *et al.*, 2008). An interesting effect was exerted by a chemically sulfated polysaccharide (S-GAP-P) obtained from *Grifola frondosa* mycelium. This preparation alone and in combination with 5-fluorouracil significantly inhibited the growth of human gastric carcinoma cells (SGC- 7901) and promoted apoptosis processes in these cells (Shi *et al.*, 2007). The MD fraction isolated from *G. frondosa* also induced apoptosis of cancer cells through stimulation of the BAK-1 gene (Soares *et al.*, 2011). Another example of a polysaccharide with anti-cancer properties is curdlan. It can stimulate the maturation of dendritic cells, which are very important elements of the tumor environment and their dysfunction can hinder the development of effective immunotherapy of cancer (Kim *et al.*, 2010). A fraction of polysaccharides isolated from *Lactarius flavidulus* mycelial cultures inhibited the proliferation of Sarcoma 180 cells (Patel and Goyal, 2012). It was also observed that polysaccharides isolated from *Fomes fomentarius* mycelium showed an anti-proliferative effect towards human gastric cancer cells (SGC-7901 and MKN-45), in contrast to the normal gastric cells (GES-1). A similar effect was detected in the case of exopolysaccharide with addition of doxorubicin (Dox) (Chen *et al.*, 2008). Polysaccharides extracted from fresh and dried fruit bodies of *Lentinus polychrous* at a concentration of 1 mg/ml induced cytotoxicity against human breast adenocarcinoma (MCF-7) cells at the level of 38% and 45%, respectively (Thetsrimuang *et al.*, 2011). The fungus *Ganoderma lucidum* is a source of a polysaccharide inhibiting human breast malignant carcinoma MT-1 and MDA-MB-231 cells (Xie *et al.*, 2006; Zhao *et al.*, 2010). Some researchers have reported anti-cancer

Table 2 Applications and bioactivity of fungal polysaccharides

Fungal sources	*Polysaccharides (common names)*	*Applications and bioactivity*	*References*
Aureobasidium pullulans	Pullulan	Food and feed	
		food coating or packaging material, oxygen barrier films for food and nonfood applications, dietary fiber, diabetic food, stabilizer, low viscosity filler, spice and flavoring, prebiotic, sugar syrup, intensifier, adhesive, binder, starch replacement, preservative	Cheng *et al.*, (2011), Oğuzhan and Yangılar (2013), Singh *et al.* (2008, 2010, 2019), Singh and Saini (2012)
		Medicine and pharmaceutical	Childers *et al.* (1991), Hijiya and Shiosaka (1975), Izutsu *et al.* (1987), Miyamoto *et al.* (1986), Yatmaz and Turhan (2012)
		capsules for drug delivery, dental care, pharmaceuticals, tablets, pills, granules (preventing brownish color change of the composition with lapse of time)	
		Cosmetics	Hyde *et al.* (2010), Leung *et al.* (2006)
		ingredient of cosmetic lotions, cosmetic powders, cosmetics around eyes, facial packs, shampoos, specific hair dressings	
		Industrial	Hijiya and Shiosaka (1975), Hirohara *et al.* (1981), Kawahara *et al.* (1984), Sasago *et al.* (1988), Tsukada *et al.* (1978), Vermeersch *et al.* (1995)
		printing, textile, photography, plywood, plasma extender, adhesives or pastes, lithographic and electronic applications, chromatography standards, cross-linked pullulan beads in gel permeation chromatography.	
		Agriculture	Chang *et al.* (2016)
		soil binders	
Agaricus blazei Murill.	–	*Medicine and pharmaceutical*	Angeli *et al.* (2009), Machado *et al.* (2005), Menoli *et al.* (2001), Mizuno *et al.* (1990), Wu (2015)
		Antitumor, anti-mutagenic, anticlastogenic, anti-genotoxic, immunomodulating	
Auricularia auricula-judae	AAG	*Food and feed*	Fan *et al.* (2007)
		Wheat flour replacement	
Flammulina velutipes	Flammulin	*Medicine and pharmaceutical*	Chou *et al.* (2013)
		Prebiotic	
Ganoderma lucidum	GI-1, GLP-2	*Medicine and pharmaceutical*	Jin *et al.* (2017), Wu (2015)
		Prebiotic, immunomodulating	
		Agriculture	Zhang *et al.* (2019)
		Prevention and control of plant diseases	
		Promote growth in cotton	
Grifola frondosa	Grofolan	*Medicine and pharmaceutical*	Patel and Goyal (2012), Shi *et al.* (2007), Wasser (2002), Yu *et al.* (2018)
		Antitumor, immunomodulating	
Trametes versicolor	Krestin	*Medicine and pharmaceutical*	Barros *et al.* (2016), Cai *et al.* (2010), Cui and Chisti (2003), Patel and Goyal (2012), Sakai *et al.* (2008), Wasser (2002), Yu *et al.* (2018)
		Anticancer (chemotherapy to treat cancers), immunomodulating, prebiotics, neutraceutical polysaccharopeptide preparations in the form of capsules, ground biomass tablets, syrups, food additives, and teas	
Lentinula edodes	Lentinan	Food and feed	
		wheat flour replacement (low calorie, fiber-rich functional baked food, noodles), yogurts (the addition of hydrogels of β-glucans)	Giavasis (2013), Hozova *et al.* (2004), Kim *et al.* (2008), Kim *et al.* (2011)
		Medicine and pharmaceutical	Chou *et al.* (2013), Hyde *et al.* (2010), Patel and Goyal (2012), Rasmy *et al.* (2010), Wasser (2002), Yu *et al.* (2018), Zhu *et al.* (2012)
		anticancer (chemotherapy to treat cancers), antibacterial, immunomodulating, prebiotic	
		Cosmetics	Wu (2015)
		teeth whitening	
		Agriculture	Aguiar *et al.* (2018)
		antibacterial (protective) effects on tomato cultivars	
Pleurotus spp.	Pleuran	*Medicine and pharmaceutical*	Li *et al.* (2007), Liu *et al.* (2010), Patel and Goyal (2012), Synytsya *et al.* (2009), Wang *et al.* (2013), Wong *et al.* (2007), Zhang *et al.* (2003, 2004)
		Antibacterial, anticancer, antioxidative, immunomodulating, prebiotic	
		Agriculture	Aguiar *et al.* (2018)
		Antibacterial (protective) effects on tomato cultivars	

(*Continued*)

Table 2 Continued

Fungal sources	*Polysaccharides (common names)*	*Applications and bioactivity*	*References*
Schizophyllum commune	Schizophyllan	*Food and feed*	Okamura-Matsui *et al.* (2001)
		Functional cheese-like food	Wasser (2002), Yu *et al.* (2018), Zhu *et al.* (2015)
		Medicine and pharmaceutical	Kumari *et al.* (2008), Wu (2015)
		Antibacterial, immunomodulating	www.macrocare
		Cosmetics	
		Moisture emulsion, skin protection (decreases inflammation, irritation and other damage due to UV and toxic environment exposure of the skin)	
Saccharomyces sp.	Yeast glucan	*Food and feed*	Kwiatkowski and Kwiatkowski (2012), Worrasinchai *et al.* (2006), Zechner-Krpan *et al.* (2009)
		Emulsifiers and fat replacers, dietary fibers, fat replacer in mayonnaise	
Saccharomyces cerevisiae	Zymosan	*Food and feed*	Hayen and Pollmann (2001), Kwiatkowski and Kwiatkowski (2012), Zechner-Krpan *et al.* (2009)
		Gelling thickeners for noncaloric functional food products, pet's food	Jamas *et al.* (2000)
	Yeast glucan	*Medicine and pharmaceutical*	Aguiar *et al.* (2018)
		Compositions useful in the treatment of dietary disorders, and as dietary additives to provide a source of fiber, of short chain fatty acids and as bulking agents in humans and animals	
		Agriculture	
		Antibacterial (protective) effects on tomato cultivars	
Sclerotium glucanicum	Scleroglucan	*Food and feed*	Coviello *et al.* (1999), Giavasis (2013), Giavasis (2014), Farina *et al.* (2001), Singh *et al.* (2010), Survase *et al.* (2007)
Sclerotium rolfsii		Thickener, gelling and stabilizing agent; stabilizer-texturizer, edible films, low calorie foods, tablets for neutraceuticals	Matricardi *et al.* (2006)
		Medicine and pharmaceutical	Coviello *et al.* (2005)
		Ophthalmic solutions, tablet coating, paints	Brigand (1993), Coviello *et al.* (2005), Holzwarth (1984)
		Cosmetics	Brigand (1993)
		Hair control compositions, skin care preparations, creams and protective lotions	
		Industrial	
		Oil industry for thickening, drilling muds and for enhanced oil recovery, preparation of adhesives, water colors, printing inks and liquid animal feed composition, stabilization of bitumen emulsions, ceramics	
		Environmental and agricultural	
		Antisettling agent for phytosanitary products used in agriculture; it facilitates the preparation of spraying mixtures, and in particular, it improves contact of the droplets sprayed onto the leave	
Sclerotinia sclerotiorum	Scleroglucan	*Medicine and pharmaceutical*	Matricardi *et al.* (2006)
		Antitumor, antiviral, antimicrobial	Halleck (1970, 1972)
		Cosmetics	
		Creams and protective lotions	
Alcaligenes faecalis	Curdlan	*Medicine and pharmaceutical*	Kanke *et al.* (1995a,b), Kim *et al.* (2010), Patel and Goyal (2012)
		Dry tablet encapsulation of theophylline, gel encapsulation of salbutamol sulfate, prednisolone, and indomethacin in the form of gel suppositories, anticancer	
Phellinus linteus	–	*Medicine and pharmaceutical*	Baker *et al.* (2008), Patel and Goyal (2012)
		Anti-inflammatory, anticancer	
Fomes fomentarius	–	*Medicine and pharmaceutical*	Chen *et al.* (2008), Patel and Goyal (2012)
		Anticancer	
Ganoderma applanatum	–	*Medicine and pharmaceutical*	Osińska-Jaroszuk *et al.* (2014)
		Antibacterial, anticancer, immunomodulating	
Pestalotiopsis sp.	Pestan	*Agriculture*	Moon *et al.* (2006)
		Biosorption properties of Pb (II) and Zn (II)	
Aspergillus fumigatus	–	*Agriculture*	Yin *et al.* (2011)
		Biosorption properties of Cd (II), Pb (II) and Cu (II)	

properties of an endopolysaccharide fraction isolated from *Inonotus obliquus* mycelial biomass (Kim *et al.*, 2006; Wang *et al.*, 2017b). An exopolysaccharide obtained from the mycelial culture of *Ganoderma applanatum* (GpEPS) demonstrated selective activity against SiHA tumor cells (Osińska-Jaroszuk *et al.*, 2014).

Antioxidant potential of polysaccharides

The antioxidant activity of fungal polysaccharides is very often described. Both intra- and extracellular compounds with such characteristics have been isolated from many different species of fungi. An exopolysaccharide obtained from the mycelial culture of *Cordyceps sinensis* contained a β-D-glucan backbone and exhibited antioxidant activity measured as a Trolox equivalent at the level of 35–40 μM of Trolox/g (Leung *et al.*, 2009). It has also been detected that a polysaccharide from *Cordyceps* can protect neuronal cells against free radical-induced destruction (Li *et al.*, 2003; Wang *et al.*, 2011). A polysaccharide-peptide complex isolated from the fungus *Pleurotus abalones* markedly increased the activities of enzymatic antioxidants SOD, CAT, and GSH-Px in the kidney, liver, and brain of senescence-accelerated mice (Li *et al.*, 2007). Polysaccharides produced by *Pleurotus nebrodensis*, *Pleurotus eryngii*, and *Pleurotus cornucopiae* were described as effective free radical scavengers (Liu *et al.*, 2010). Very interesting results were obtained for a water-soluble polysaccharide isolated from the fruiting bodies of somatic hybrid PCH9FB, which was a result of intergeneric protoplast fusion of *Pleurotus florida* and *Calocybe indica* var. (Maity *et al.*, 2011). Besides the activation of macrophages, splenocytes, and thymocytes, this substance had antioxidant potential. A very good radical scavenging effect has been demonstrated in the case of polysaccharides isolated from *G. lucidum* and *P. linteus* strains, with the evidently low EC_{50} values <0.1 mg/ml (Kozarski *et al.*, 2011). An analysis of polysaccharide extracted from *Ganoderma tsugae* representing Ganodermataceae showed its very interesting antioxidative potential with EC_{50} values <0.1 mg/ml (Tseng *et al.*, 2008). Three different preparations of a polysaccharide (GLP-H, GLP-V, and GLP-F) produced by the white rot fungus *G. lucidum* were very effective radical scavengers in in vitro conditions (Fan *et al.*, 2012). Another fungal polysaccharide isolated from *Morchella* sp. was able to modulate the activity of antioxidant enzymes glutathione peroxidase and superoxide dismutase (Meng *et al.*, 2010). Antioxidant properties were also detected in a polysaccharide isolated from *I. obliquus* (Xiang *et al.*, 2012). The scavenging abilities were also reported for endopolysaccharides obtained from mycelial biomass of the white rot fungus *Cerrena unicolor* (Jaszek *et al.*, 2013).

Anti-diabetes polysaccharides

Hepatoprotective effect of polysaccharides

According to the current research data, a combination of two well-known fungal polysaccharides isolated from *C. sinensis* and *Ganoderma atrum* has been found to protect the liver cells of cyclophosphamide treated mice. A decrease in the liver toxicity markers, e.g., alanine aminotransferase, aspartate transaminase, and alkaline phosphatase, was observed (Fan *et al.*, 2018).

Immunomodulating activity

β-(1-3) linked glycan isolated from the fungus *P. linteus* demonstrated immunomodulating activity (Baker *et al.*, 2008). Schizophyllan obtained from *S. commune* is known as a water-soluble homopolysaccharide effective in immunomodulation of the human immune system (Kumari *et al.*, 2008). The well-known lentinan and schizophyllan alone or in combination with chemical drugs are used as immune system protectors in clinical therapies (Yu *et al.*, 2018). Polysaccharides isolated from *Pleurotus citrinopileatus* fermentation broth evidently increased the number of macrophages as well as CD4 +, CD8 +, and T cells in mice (Gregori *et al.*, 2007). Extracts containing polysaccharides isolated from *G. lucidum*, *Agaricus bisporus*, *A. brasiliensis*, and *P. linteus* exhibited immunomodulatory activity towards activated human PBMCs. In the case of *G. lucidum*, *A. bisporus*, and *A. brasiliensis*, the polysaccharides induced IFN-γ synthesis (probable proinflammatory effect), whereas samples obtained from *P. linteus* decreased IFN-γ synthesis (probable immunosuppressive action) (Kozarski *et al.*, 2011). It has been described that β-glucans obtained from *G. lucidum* can effectively induce immune response of the host organism for prevention and reduction of malignant tumors (Wang *et al.*, 2014). Another organism from this group, i.e., *G. atrum*, was found to produce polysaccharides that were able to enhance phagocytosis performed by murine macrophages RAW264.7 (Yu *et al.*, 2013). A preparation of lentinan from *L. edodes*, D-fraction isolated from *G. frondosa*, schizophyllan from *S. commune*, and polysaccharide-K (PSK) from *T. versicolor* are currently used in anticancer immunotherapy (Wasser, 2002, 2011; Yu *et al.*, 2018). As demonstrated by Chen *et al.* (2006) the mannose component included in the EPS produced by *Tremella mesenterica* can induce the stimulation of the nitric oxide (NO) and cytokine production by RAW264.7 macrophages. The endopolysaccharide PTP from *Polyporus albicans* (Imaz.) Teng has been reported to stimulate the proliferation of murine lymphocytes induced by lipopolysaccharide or concanavaline A (Sun *et al.*, 2008). An endopolysaccharide fraction (c-EPL) that was able to induce the secretion of TNF-α and IL-6 by THP-1-derived macrophages was isolated from the mycelium biomass of *C. unicolor* (Mizerska-Dudka *et al.*, 2015). An exopolysaccharide isolated from the culture fluid of *G. applanatum* (GpEPS) induced TNF-α production by THP-derived macrophages (Osińska-Jaroszuk *et al.*, 2014).

Pharmaceutical Application

Due to their structural properties and numerous bioactive properties, polysaccharides have found application as drug delivery systems. Kanke *et al.* (1995a) studied theophylline-containing curdlan tablets and showed that addition of curdlan to theophylline improved the pharmacokinetics of this drug. This polysaccharide was also used in the form of gel suppositories containing salbutamol sulfate, prednisolone, and indomethacin (Kanke *et al.*, 1995b). Pullulan is another widely used polysaccharide in the

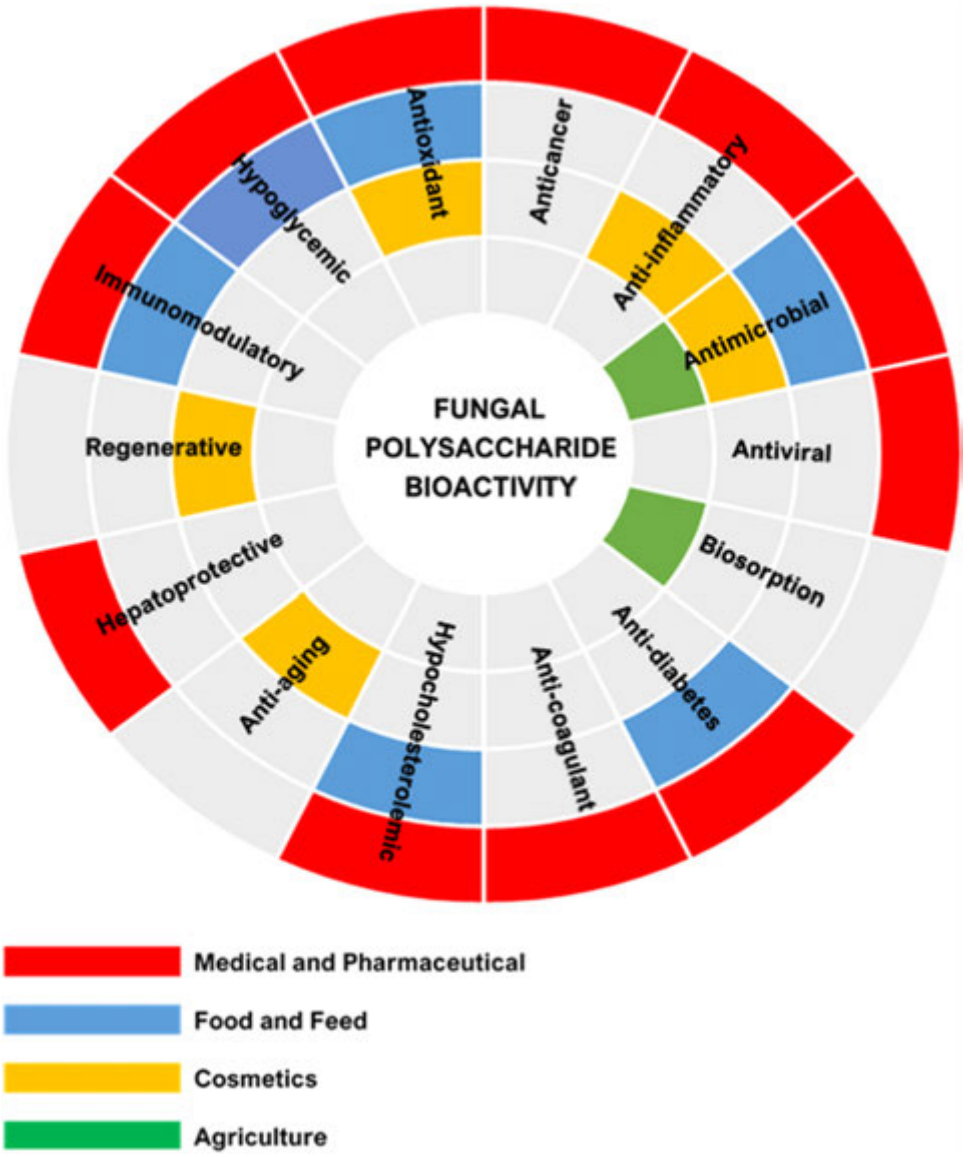

Fig. 1 The bioactivities of fungal polysaccharides in different industrial sectors.

drug delivery system due to its unique properties such as water solubility, fiber formation, adhesion, and water absorption. It can be used in the form of nanogels, nanoparticles, and microspheres; thus, it is easier to target the drug to the cancer cell and reduce the toxicity of the drug to normal cells (Singh *et al.*, 2017). Pullulan is also used in the pharmaceutical industry to create sugar-coated compositions such as tablets, pills, and granules to prevent browning of such preparations (Childers *et al.*, 1991; Izutsu *et al.*, 1987) and as a denture adhesive (Hijiya and Shiosaka, 1975). Despite the successful use of fungal polysaccharides in anti-cancer therapies in Asia, there are not enough clinical studies of these compounds in Western countries. Clinical studies were conducted on a polysaccharide isolated from *G. frondosa*, which was tested in phase I/II clinical trials in postmenopausal breast cancer patients. The extract of this polysaccharide exerted immunomodulatory effects but, measurable immunological effects after oral administration are unknown (Deng *et al.*, 2009). Lentinian from *L. edodes* has been investigated clinically for its anticancer activity in conjunction with chemotherapeutic agents such as paclitaxel, tegafur, epirubicin and hydroxycamptothecin, and javanica oil emulsion (Ina *et al.*, 2013; Wang *et al.*, 2017a). It has been used in cancer immunotherapy in Japan and China for the treatment of advanced lung, gastric (esophagus, colorectal), and cervical cancers. Standis *et al.* (2008) showed a potential use of immune therapy based on *T. versicolor* polysaccharide constituents in treatment of breast cancer. Other potential medical applications of fungal polysaccharides include gene delivery, tissue engineering, vaccination, or insulinotropic activity (Singh *et al.*, 2017).

Fungal Polysaccharides in Food Industry

Polysaccharides extracted from various fungi have been extensively studied in many fields of food and feed industry as food additives, edible and/or biodegradable films, prebiotics, or dietary food supplements for application in nutraceutical industry (**Table 2**). Given their biological activities, such as hypocholesterolemic, hypoglycemic immunomodulatory, antitumor, antioxidant, and anti-inflammatory effects, this large group of biopolymers (mainly β-glucans or heteropolysaccharides) are acknowledged as functional and bioactive food ingredients (Du *et al.*, 2019).

Food Additives

Fungal polysaccharides, including those from mushrooms and yeast, are widely used as thickening, stabilizing, texturizing, or gelling agents, conferring good sensory properties, extended shelf life, and easier processing to the products (Jindal and Khattar, 2018). One of the most common and well-studied fungal polysaccharides in the context of applications in the food industry is pullulan. This biopolymer is an extracellular and neutral microbial polysaccharide produced by the yeast-like fungus *Aureobasidium pullulans* (Singh *et al.*, 2008). Pullulan is a non-toxic, non-mutagenic, non-carcinogenic, odorless, tasteless, and edible polymer that has been certified harmless for usage in food products by food safety regulations in many countries (Kimoto *et al.*, 1997; Prajapati *et al.*, 2013; Singh *et al.*, 2019). Pullulan, scleroglucan, and yeast glucan may be used as thickeners, binders, and gelling and stabilizing agents. Many applications of these biopolymers have been granted patent protection, e.g., the Japanese patents (San-Ei Chemical Industries, Ltd.) for scleroglucan describe improvement of the quality of frozen or heat-treated edibles, such as Japanese cakes, steamed foods, rice crackers, and bakery products (Coviello *et al.*, 2005). The use of pullulan to stabilize fatty emulsions, the quality and texture of mayonnaise (Yamaguchi and Sunamoto, 1991) and as a binder and stabilizer of food pastes (Hijiya and Shiosaka, 1975), anti-sticking substances, cookies, and tobaccos has also been protected by patents (Cheng *et al.*, 2011; Miyaka, 1979).

Yeast β-glucans improve food rheological, gelling, water-holding, and oil-holding properties, without influencing its taste or odor (Petravić-Tominac *et al.*, 2011). Food-grade yeast β-glucans are used as ingredients in baked foods such as soft dough and panning dough, a mixture for cake filling, beverages such as fruit juices, yogurt and other milk products, and chocolate. They are also used as food thickeners in salad dressings, frozen desserts, mayonnaise, sauces, and cheese. Given its ability to retain water, β-glucan can be also used in the production of sausages and other meat products (Thammakiti *et al.*, 2004). The majority of these applications have been patented (Thammakiti *et al.*, 2004; Zechner-Krpan *et al.*, 2009) and critical reviews of 300 patented applications are available (Kwiatkowski and Kwiatkowski, 2012; Laroche and Michaud, 2007). Due to the low viscosity of pullulan, which is not affected by addition of sodium chloride, heating, and changes in pH, its solutions can be used to impart viscosity and gloss to food products with a high salt concentration, e.g., barbecue sauce, soya sauce, pickled fruits, and vegetables (Singh *et al.*, 2019). A solution of this biopolymer can be sprayed on dried powdered food products for easy granulation and to enhance the solubility of the products in water (Tsujisaka and Mitsuhashi, 1993). It can be used to bind nuts on cookies or sesame seeds on the rice cracker surface and for preparation of snack foods like fish sticks and beef or pork sheets (Chaen, 2009). Hozova *et al.* (2004) established that the addition of hydrogels of β-glucans extracted from fresh *P. ostreatus* fruiting bodies (pleuran) and dried *L. edodes* fruiting bodies (lentinan) to yogurt had no negative influence on the sensory acceptability of the product, but improved its health-promoting properties (Hozova *et al.*, 2004). When incorporated into products, such preparation can provide additional prophylactic and functional properties, such as immunostimulant, hypoglycemic, anti-radical, antitumor, anti-inflammatory, and other activities (Khan *et al.*, 2017).

Edible and/or Biodegradable Films

The viscosity, gelling, and film forming properties of fungal polysaccharides have been exploited for specific applications in the food and allied industries, e.g., production of edible and "active" packaging. In addition to these properties, pullulan has a considerable mechanical strength and adhesiveness; hence, it can be used as a packaging material for various food items e.g., fruits, vegetables, poultry, meat, dried fish, cashew nuts, peanuts, noodles, and confectionaries (Chaen, 2009; Singh *et al.*, 2019). Due to their oxygen resistance, pullulan films are suitable for protection of readily oxidized fats and vitamins in food (Oğuzhan and Yangılar, 2013). With its biodegradability, impenetrability to oxygen, and resistance to the digestive enzymes of the human gut, pullulan can be used as a carrier for oral delivery of drugs (Prajapati *et al.*, 2013). However, one of the most important applications of pullulan in food packaging can be its use as an edible coating, i.e., a relatively thin layer of material applied and formed directly on the surface of the food product, which can be eaten along with the product (Farris *et al.*, 2014; Pavlath and Orts, 2009). Additionally, pullulan improves the shelf life of food, as it is not a readily assimilable carbon source for bacteria and fungi responsible for spoilage (Singh *et al.*, 2008). This exopolysaccharide has been shown to be an inherently antimicrobial biopolymer (Yuen, 1974; Chlebowska-Śmigiel and Gniewosz, 2009). The antimicrobial properties of pullulan-based films and its "active" features allow these coatings to be classified as "functional" packaging (Farris *et al.*, 2014). Another polysaccharide isolated from the yeast cell wall for film formation was described by Novák *et al.* (2012). These authors produced biodegradable films with β-(1,3)-glucans isolated from commercial baker's yeast (*Saccharomyces cerevisiae*) (Peltzer *et al.*, 2018). Their adhesive and biological properties can be also utilized in the production of coatings for surgical

instruments (Klein, 2003) and in the manufacture of packaging for the food industry (Cope, 1987; Kwiatkowski and Kwiatkowski, 2012). Scleroglucan can also be utilized in the formation of edible films and tablets for neutraceuticals, owing to its chemical stability, biocompatibility, and biodegradability (Coviello *et al.*, 1999).

Functional Foods and Nutraceuticals

Fungal polysaccharides can be incorporated into foods and feed, creating novel "functional foods" with many health-promoting benefits strengthening and stimulating the immune system and reducing the risk of chronic diseases, in addition to the nutritive value of both. Nowadays, commercial fungal polysaccharides, especially β-glucans, are found in the form of capsules or tablets as food supplements and to a lesser extent as ingredients in food products, and this market is still developing (Eleftherios *et al.*, 2014). Besides its role as a food additive and packaging material, pullulan can be appropriately used in the production of dietary foods as a partial replacement for starch in pastas or baked goods (Cheng *et al.*, 2011). β-Glucan from *L. edodes* (lentinan) is another polysaccharide used as a replacement for a portion of wheat flour in baked foods in an attempt to produce a novel functional food with low calorie and high fiber content (Giavasis, 2013; Kim *et al.*, 2011). In similar studies, this biopolymer was added to noodles as a partial wheat flour replacement and resulted in a fiber-rich functional food with antioxidant and hypocholesterolemic effects and improved quality characteristics (Kim *et al.*, 2008). *Auricularia auricula* polysaccharide (AAP) flour blended bread was developed. The results showed that the incorporation of AAP in bread markedly increased the antioxidant property of the bread. It was also indicated that the concentration of the AAP flour below 9% could be used without altering the sensory acceptance of the blended bread (Fan *et al.*, 2007). Other examples of application of fungal polysaccharides in functional food are the *P. ostreatus* and *L. edodes* β-glucans used as additives to low-fat yogurt (Hozova *et al.*, 2004) and extruded snack products with a low glycemic index (Brennan *et al.*, 2013). Similarly, chicken burgers were enriched with *Pleurotus sajor-caju* β-glucan (Wan Rosli *et al.*, 2011), and *P. ostreatus* was incorporated into sausages in an effort to lower their fat content (Chockchaisawasdee *et al.*, 2010; Eleftherios *et al.*, 2014).

The application of fungal polysaccharides as a functional food ingredient is connected with easy manipulation of the composition of colonic microbiota in the human gut by inhibition of exogenous pathogens (Rycroft *et al.*, 2001), and thus with improvement of host health (Roberfroid, 2002; Singdevsachan *et al.*, 2016). For this reason, such sugar biopolymers as β-glucans, homoglucans, and heteroglucans with β $(1\rightarrow3)$, β $(1\rightarrow4)$, and β $(1\rightarrow6)$ glycosidic linkages are considered as prebiotics. Many research studies have indicated that polysaccharides from *Pleurotus* spp. (Synytsya *et al.*, 2009), *L. edodes*, *Tremella fuciformis* (Guo *et al.*, 2004), *G. lucidum* (Delzenne and Bindels, 2015), *A. bisporus* (Giannenas *et al.*, 2011), and *A. pullulans* (Cheng *et al.*, 2011; Masakazu *et al.*, 1990) have prebiotic activity. They are regarded as dietary fiber promoting the growth of beneficial bifidobacteria in the human intestine (Chou *et al.*, 2013). The most widely studied polysaccharides schizophyllan and lentinan are already available and marketed as nutraceuticals (pharmaceutical formulation); however, the addition of their purified form to food has not been commercialized worldwide yet (Giavasis, 2013).

Fungal polysaccharides can also be used as a source of sugar syrups that are commonly applied in many areas of the food industry. An example of such a biopolymer is pullulan used as a substrate for the production of maltotriose syrup via enzymatic hydrolysis with the participation of pullulanase. This syrup possesses many excellent properties such as low freezing point depression, mild sweetness, moisture retention, and prevention of retrogradation of starch in foodstuffs. These properties are useful in food industry for the manufacture of desserts, baking, and brewing, and in the pharmaceutical industry for replacement of glucose in intravenous feeding (Mishra *et al.*, 2016; Singh *et al.*, 2010).

Animal Feed

In the feed industry, there are many commercial products available based on fungal polysaccharides. They are used as feed supplements to stimulate the animal's immune system and thus avoid excessive use of antibiotics in the feed industry. Such immunostimulation has been noticed in the case of β-glucans from *P. ostreatus* fed to chicken (Muthusamy *et al.*, 2013). Based on the current knowledge of the use of edible mushrooms as a feed additive with dietary and health-promoting activities in the nutrition of broiler chickens and laying hens, it can be concluded that many other mushroom species e.g., *L. edodes*, *A. bisporus*, *Agaricus blazei*, *Hericium caput-medusae*, *P. eryngii*, *Fomitella fraxinea*, *Flammulina velutipes*, *G. lucidum*, *Cordyceps sinensis*, and *Cordyceps militaris* can also be a source of active substances with a positive effect on poultry performance and health status (Bederska-Łojewska *et al.*, 2017). Additionally, β-glucan from *L. edodes* has been applied to fish feed for immunomodulation in the rainbow trout (*Oncorhynchus mykiss*) (Djordjevic *et al.*, 2009), from *P. florida* in the major carp (*Catla catta*) (Kamilya *et al.*, 2008), and from *P. ostreatus* in the common carp (*Cyprinus caprio L.*) (Dobšíková *et al.*, 2013; Eleftherios *et al.*, 2014). In turn, yeast β-glucan with bioactive properties including anti-cancer, anti-inflammatory, and immune-modulating properties is an interesting natural supplement used in combination with milk hydrolysate influencing the growth performance and gut health in newly weaned pigs (Mukhopadhya *et al.*, 2019). It has been proved that fungal polysaccharides not only influence the immune system, but may also reduce cholesterol levels. Lim *et al.* (2015) applied glucans from the yeast-like fungus *A. pullulans* in an experimental animal model (hamster) of hyperlipidemia induced by a high-fat diet. Studies comparing the effects of several different β-glucans showed that yeast-derived β-glucan 300 markedly lowered total cholesterol levels in mice with experimentally induced hypercholesterolemia. Supplementation with three other β-glucans, i.e., Krestin, ImmunoFiber, and Now glucan, induced similar, albeit less pronounced effects (Sima *et al.*, 2018; Vetvicka and Vetvickova, 2007).

Cosmetics

Due to their many bioactivities such as anti-aging, anti-cancer, and anti-oxidant effects, fungal polysaccharides can be used in supportive human health-care products, especially functional cosmetics or cosmeceuticals. They are mainly ingredients of skin care cosmetics and hair products. The main applications of fungal polysaccharides are presented in **Table 2**. Given their significant antioxidant bioactivities and the ability to stimulate the proliferation of human dermal fibroblasts and the biosynthesis of collagen, *G. frondosa* exopolysaccharides can be useful in skin-care cosmetics (Kim *et al.*, 2007). Bais *et al.* (2005) demonstrated that scleroglucan produced by *Sclerotium filamentum* was used for preparation of cosmetic emulsions. Scleroglucan can also be used to produce various skin-care cosmetics such as creams and protective lotions (Halleck, 1970, 1972). In turn, due to its immunomodulatory properties and ability to fight diseases and infections, lentinan isolated from *L. edodes* can be applied as an ingredient in creams and is used in Aveeno Positively Ageless Night Cream (Bisen *et al.*, 2010). Commercial products containing fungal polysaccharides are shown in **Table 3**. With its non-toxic, non-immunogenic, non-carcinogenic, and non-mutagenic properties, pullulan produced by the yeast-like fungus *A. pullulans* is widely used in the production of many commercial preparations. In addition, this polysaccharide exhibits excellent moisture absorptivity, good water solubility, and viscosity, which make it an ideal ingredient in many cosmetics. Pullulan has become an important ingredient of cosmetic products, e.g., cosmetic lotions, cosmetic powders, eye area cosmetics, facial packs, shampoos, specific hair dressings, and oral care products (tooth powders) (Leung *et al.*, 2006; Singh *et al.*, 2008). Schizophyllan containing β-1,6-branched β-1,3-glucan isolated from *S. commune* soothes the skin and decreases inflammation, irritation, and other UV-induced damage (Kumari *et al.*, 2008). It is used in the commercial Murad's Sleep Reform Serum preparation (Hyde *et al.*, 2010). The fungus *Aspergillus niger* is a source of a chitin–glucan polymer which consists of two types of polysaccharides, namely chitin poly (N-acetyl-D-glucosamine) and β $(1 \rightarrow 3)$ glucan. This polymer is known under the commercial name KiOsmetine-CG™ and can be used as a cream ingredient due to its good moisturizing and anti-aging properties (Gautier *et al.*, 2008). Polysaccharides obtained from various species of *Tremella* possess many bioactivities, which include improvement of immunity, reduction of blood sugar, lowering blood fat, anti-aging, anti-ulcer, and anti-thrombosis effects, and antimutagenicity (Wu *et al.*, 2019). In addition, their special emulsion stabilizing, film forming, and skin conditioning properties are used in pharmaceutical and cosmetic industries. Commercial preparations containing polysaccharides isolated from *T. fuciformis* include Curerex-Celipid, Vegeluron® Gel, and Tremoist™ TP. *Tremella* polysaccharides are also cosmetics ingredients of formulations improving eye area skin and moisturizing gels (Surkran Grape Seed Lift Eye Mask U. S., BeautyDiy Aqua Circulation, Hydrating Gel and Sulwhasoo Hydroaid) (**Table 3**).

Environment and Agriculture

Exopolysaccharides are main component of exopolymers of fungal origin commonly found in various natural environments (soils, waters, and air) (Donot *et al.*, 2012; Osińska-Jaroszuk *et al.*, 2015). The main role of EPS is the capacity to aggregate soil particles, which is highly important for soil structure, health, and fertility (Costa *et al.*, 2018; Nouha *et al.*, 2018). The EPS have a slimy texture and ionic charges and can hold solid particles together (create aggregate of particles). EPS affect the development of microorganisms in the environment and the course of microorganism-microorganism and microorganism-plant interactions. As the main (50%–70%) component of microbiological biofilms formed on the surface of roots and on soil colloids in the root zone, EPS determine the intensity of rhizosphere colonization by microorganisms (Bashan *et al.*, 2004). EPS can be a reservoir of elements that are necessary for microorganisms. In addition to biogenic elements (carbon, nitrogen, oxygen, hydrogen, and nitrogen), EPS contain a number of physiological elements such as sodium, potassium, magnesium, and calcium and binds metals, including heavy metals. In the soil rhizosphere, EPS in combination with microbial organic acids contribute to the release of soil minerals (sparingly soluble phosphates and aluminosilicates) inaccessible to plant elements, primarily phosphorus and potassium. They provide environmental adaptations protecting against various stresses, including water stress, by strengthening water retention or regulating the diffusion of organic carbon sources (Chenu, 1993; Chenu and Roberson, 1996). These natural abilities of fungal polysaccharides can be used in agriculture in processes increasing soil aggregation. Research confirmed that the best results were obtained for filamentous fungi such as *Absidia*, *Mucor*, *Rhizopus*, *Chaetomium*, *Fusarium*, and *Aspergillus* (Swaby, 1949). Fungal polysaccharides have also found application as soil binders in construction and geotechnical engineering (Chang *et al.*, 2016). A commercial β-1,3/1,6-glucan (Polycan™) from *A. pullulans* was used to improve the strength of Korean residual soil (strength of soil increased by more than 200%) (Chang and Cho, 2012).

EPS afford self-protection for cells from desiccation, predation, the effects of antibiotics, antimicrobial substances, antibodies, bacteriophages and adherence to other microorganisms, animal and plant tissues in different stress conditions such as biotic stress, competition, and abiotic stresses, including temperature, light intensity, or pH (Donot *et al.*, 2012; Staudt *et al.*, 2012). Additionally, EPS supply bacterial aggregation, surface attachment, and plant-microbe symbiosis; hence, it is a crucial property for wastewater treatment and soil aggregation. Furthermore, microbial pathogenicity is related to the production of capsular EPS and depends on the rate and amount of EPS synthesis (Kumar and Mody, 2009). Polysaccharides isolated from *G. lucidum* can be used for the prevention and control of plant diseases. It has been described that these polysaccharides can promote growth in cotton (Zhang *et al.*, 2019). In turn, polysaccharides from fungal wastes extracted from spent mushroom substrate of *P. ostreatus*, residual brewery yeast (*S. cerevisiae*), and basidiocarps discarded from L. *edodes* production showed protective effects against bacterial spot caused by *Xanthomonas gardneri* on tomato cultivars (Aguiar *et al.*, 2018). Scleroglucan is used as an antisettling agent for

Table 3 Commercial products containing fungal polysaccharides

Commercial name of the product	*Product function*	*Fungal sources/ polysaccharides*	*References*
Lentinan™ for injection; Immune-Assist™	Therapeutic	Lentinian	Wu (2015)
Aveeno Positively Ageless Night Cream	Cosmetics	Lentinian	www.aveeno.com
Krestin™	Pharmaceutical	Krestin (PSK)	Wu (2015)
Murad's Sleep Reform Serum	Cosmetics	Schizophyllan	Hyde *et al.* (2010)
Cuskin™	Moisture emulsion	Schizophyllan	Wu (2015)
KiOsmetine-CG™	Cosmetics	KitoZyme's chitin-glucan	Gautier *et al.* (2008)
Bel-mélo	Cosmetics, dietary supplements	β-glucans	Hyde *et al.* (2010)
Surkran Grape Seed Lift Eye Mask U.S.	Cosmetics (improves skin around eyes)	*Tremella* polysaccharide	Wu *et al.* (2019)
BeautyDiy Aqua Circulation Hydrating Gel Sulwhasoo Hydroaid	Moisturizing gel	*Tremella* polysaccharide	pinkybeauty.com.au
Curerex-Celipid Vegeluron® Gel Tremoist™ TP	• Emulsion stabilizing • Film forming • Skin conditioning	*Tremella fuciformis*	cosmetics.specialchem.com
EcoActiva	Dietary supplements (a feed supplement, on non-specific immune parameters and the growth rate of snapper (*Pagrus auratus*))	*Saccharomyces cerevisiae*	Cook *et al.* (2003)
Glucan #300	Therapeutic/pharmaceutical (immunostimulating activity, stimulation of both humoral and cellular immunity)	Yeast	Vetvicka and Vetvickova (2018)
Immunowall®	Prebiotic, feed additive (prevents colonization of the intestinal tract by pathogens, stimulates the immune activity of the phagocytic cells, enhances the action of beneficial bacteria such as *Lactobacillus* and *Bifidobacteria*)	*Saccharomyces cerevisiae*	Abu-Elala *et al.* (2018)
MacroGard®	Commercial feed supplement (immune modulator with protective effect against tumor cells, viruses, bacteria, fungi, parasites, and carcinogen)	*Saccharomyces cerevisiae*	Bagni *et al.* (2005), Miest *et al.* (2016)
Zymosan A	Chemical reagent (used to induce sterile inflammation, including proinflammatory cytokines, arachidonate mobilization protein phosphorylation and inositol phosphate formation)	*Saccharomyces cerevisiae*	Cui *et al.* (1998), Miyasato *et al.* (1994)
Nutricafe-Organic Performance Coffee	Therapeutic (increases physical endurance and helps to remove toxins from the body)	*Cordyceps* & *Ganoderma*	Rathore *et al.* (2017) www.alohamedicinals.com
Yestimun®	Therapeutic (immunomodulatory effect)	*Saccharomyces cerevisiae*	Stier *et al.* (2014)
Polycan™	Soil-treatment material (used to improve the strength of the Korean residual soil)	*Aureobasidium pullulans*	Chang and Cho (2012)

phytosanitary products. This substance facilitates preparation of a spraying mixture and improves the contact of droplets sprayed onto leaves. In agriculture, scleroglucan is also used in pesticides, defoliant sprays, and seed coatings (Brigand, 1993; Farina *et al.*, 2001). Biosorption of cations by EPS occurs via mechanical, physical, ion exchange, chemical sorption, and complexation processes. Various functional groups (hydroxyl, carboxyl, amino), which are ligands forming coordination bonds with appropriate metals, are responsible for the complexation of elements by polymers. EPS are a component of preparations used in agriculture: bio-fertilizing, biostimulating, and biological plant protection preparations, among others as inducers (elicitors) of systemic plant resistance (SAR or ISR) (Jaroszuk-Ściseł and Kurek, 2012; Li *et al.*, 2011). EPS are used in the bioremediation of an environment contaminated with heavy metals, e.g., Cd or Pb (da Silva *et al.*, 2014) or petroleum derivatives (Raghukumar *et al.*, 2006). One of them is EPS isolated from *Zoogloea* spp. and *A. niger*, which helps in degradation of pyrene in contaminated soils (Jia *et al.*, 2011). A polysaccharide isolated from *Aspergillus fumigatus* also exhibited sorption capacity for two heavy metals: lead and copper (Yin

et al., 2011). In turn, pestan produced by *Pestalotiopsis* sp. KCTC 8637 has been used as a biosorbent of lead and zinc in wastewater treatment (Moon *et al.*, 2006).

Other Industrial Applications

Fungal polysaccharides have also been widely applied in other branches of industry. Bancerz *et al.* (2018) demonstrated a possible application of fungal polysaccharides isolated from *G. applanatum* (GaEPS), *Abortiporus biennis* (AbEPS), *Piptoporus betulinus* (PbPS), and *Laetiporus sulfurous* (LsPS) for enhancement of esterification (butyl caprylate and butyl oleate synthesis) based on *Rhizomucor variabilis* lipase (Bancerz *et al.*, 2018). Two structurally similar polysaccharides, scleroglucan synthesized by *Sclerotium glucanicum* and *Sclerotium rolfsii*, and schizophyllan produced by *S. commune*, are used in oil recovery. Scleroglucan may be an alternative to xanthan due to its better efficiency and stability. In oil recovery, this polysaccharide is used to extract oil or for lubrication of the drill and control of the backpressures created during drilling (Hamed and Belhadri, 2009; Holzwarth, 1984; Survase *et al.*, 2007). Besides, scleroglucan can be used in preparation of water-based paints, printing inks, and ceramic binders (Survase *et al.*, 2007). Curdlan and schizophyllan are attractive for nanoscience applications, where they have been used to encapsulate single-walled carbon nanotubes (SWCNT) (Lehtovaara and Gu, 2011). Pullulan is known from different applications in industry (Singh *et al.*, 2008). It can be used in the paper industry as a novel paper-coating material improving paper printing gloss, adhesive strength, and viscosity stability during storage (Nakashio *et al.*, 1976; Nomura, 1976). This polysaccharide can be also used as an addition to paints in order to improve their quality (Nakashio *et al.*, 1975) as well as in photographic, lithographic, and electronic applications (Sasago *et al.*, 1988; Tsukada *et al.*, 1978; Vermeersch *et al.*, 1995). In biotechnology and science, pullulan gels have been used as matrices for enzyme immobilization and as cross-linked pullulan beads in gel permeation chromatography (Hirohara *et al.*, 1981). Kawahara *et al.* (1984) demonstrated the possibility of commercial use of this polysaccharide to produce chromatography standards. Pullulan can also be used in the textile industry to produce fibers resembling nylon or rayon by wet or dry spinning (Hijiya and Shiosaka, 1974).

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Development of Mycoherbicides

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Introduction

Undesirable vegetation (primarily, weeds and invasive plants) has become a common component of anthropogenic ecosystems. To avoid their seed bank accumulation in the soil, there is a continuous need for weed control using various approaches. Average yield losses caused by weed infestation are about 10% but depend on the crop, fertilization/manuring and weather conditions resulting sometimes in over 50% losses. Moreover, at high infestation density, many agricultural methods, such as use of highly productive crop varieties, fertilizing and application of plant growth promoters, do not compensate the losses. That is why the volume of chemical herbicides usually considerably exceeds the number of other agrochemicals applied for plant protection (Aktar *et al.*, 2009; Cantrell *et al.*, 2012).

Despite the rich assortment of active ingredients for chemical herbicides, there is a number of problematic undesirable plants that are difficult to control with standard approaches. For instance, several mechanical soil cultivations (tillage, harrowing) along with application of high doses of chemical herbicides are necessary for eradication of perennial weeds. Control of parasitic weeds is more complicated, which should additionally include the use of resistant crop varieties. On the other hand, many weeds, especially invasive species, grow on ruderal or non-arable land near human habitation that makes their control highly problematic.

Intensive use of chemical herbicides may cause very serious consequences, such as water and soil pollution, and accumulation of their residues in crop yield (Aktar *et al.*, 2009). For instance, pollution monitoring in France revealed mainly herbicides among 15 pesticides detected in lake and ground water (Cordeau *et al.*, 2016). In addition, there is some evidence that Alzheimer's disease is the most common in the areas with high use of chemical pesticides (Yan *et al.*, 2016). Many studies have indicated genotoxicity and carcinogenicity of glyphosate, the most sold herbicide in the world (Bakry *et al.*, 2015).

Another serious problem is weed resistance to convenient herbicides. Considerable number of resistant weed populations is found in the countries where the herbicides are used heavily. Moreover, chemical companies do not promise new herbicidal compounds with novel mode of action to strike resistance problem in the nearest future. That is why new solutions are being considered to be integrated in weed control systems (Shaner and Beckie, 2014).

Connected with aforesaid, the wish of both farmers and consumers to improve herbicide efficacy as well as to use less effective but safer plant protection means is understood. Following this trend, the development of ecologically safer means of pest control is carried out in the entire world. Such alternatives are biological control (natural enemies of weeds) and biorational herbicides (herbicidal formulations based on natural products).

The idea to use phytopathogenic microorganisms against weeds was evidently based on natural observations: weeds as well as cultivated plants may strongly suffer from some infectious diseases. Fungal plant diseases are common to cause damage for plants followed by decreasing yield and product appearance/attractiveness. In nature, the most noticeable diseases are caused by rust, smut, mildew and downy mildew fungi, especially when systemic infection take place.

The rust fungus, *Puccinia punctifromis* (syn. *P. suaveolens*), causing systemic infection in Canada thistle, attracted attention of scientists about 100 years ago. Attempts for the rust multiplication and application were performed in USA and the Soviet Union. First results, which demonstrated the weed reduction level about 30%, were not very promising as supposed by the pioneers (Potapov, 1925; Hasan, 1983). One of first trials to develop a commercial biological herbicide, which was based on *Alternaria cuscutaceae*, against parasitic dodder (*Cuscuta* spp.) weeds was made in the Soviet Union in the early 1960 s. However, the project was not successful (Rudakov, 1961; Wapshere, 1982).

There were only sporadic reports of microbial weed control studies before 1970. In the following 25 years, the problem received increased attention to establish theoretical and practical basis for weed control with fungi and their metabolites. As a result, two main approaches were proposed: classical biocontrol for suppression of invasive species with introduction of phytopathogens from countries of their origin, and bioherbicidal approach employing mainly endemic fungi for mass production and following application similar to chemical herbicides (TeBeest *et al.*, 1992).

The first success of classical biocontrol was the introduction of *Puccinia chondrillina* to Australia (1972) and USA (1975) for suppression of rush skeletonweed (*Chondrilla juncea*) that originated from South Europe. In 1981, the first mycoherbicide Collego® was registered in USA based on *Colletotrichum gloeosporioides* f. sp. *aeschynomene* for biocontrol of northern jointvetch (*Aeschynomene virginica*) infesting rice and soybean fields. These achievements stimulated several hundreds of weed biocontrol projects over the world (Hasan, 1983). Interestingly, the earlier studies were also continued. For instance, soil application of teliospores of *P. punctifromis* in autumn gave systemic infection of 70% aerial shoots of Canada thistle in New Zealand (Berner *et al.*, 2013). In 2005, a bioherbicide Smolder® was registered in USA for dodder biocontrol with *Alternaria destruens* (Bailey, 2014).

Table 1 Mycoherbicides that have been registered worldwide

Product name	*Registration*		*Active ingredient*	*Target weed (s)*
	Year	*Country*		
Di-Bak® Parkinsonia	2018	Australia	*Lasiodiplodia pseudotheobromae, Neoscytalidium novaehollandiae, Macrophomina phaseolina*	*Parkinsonia aculeata*
Bio-Phoma™	2016	Canada	*Phoma macrostoma*	Numerous broad-leaved weeds
Phoma™	2012	USA		
Sarritor®	2009	Canada	*Sclerotinia minor*	*Taraxacum officinale* and other broad-leaved weeds
Chontrol™	2004	Canada, USA	*Chondrostereum purpureum*	*Populus* and *Alnus* spp.
Collego™	1982	USA	*Colletotrichum gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*
LockDown™	2006			
DeVine™	1981, 2006	USA	*Phytophthora palmivora*	*Morrenia odorata*

Note: Morin, L., 2020. Progress in biological control of weeds with plant pathogens. Annual Reviews of Phytopathology 58, 201–223.

What is Mycoherbicide

Bioherbicides are formulations consisting of living microorganisms and ancillary components (surfactants, adjuvants, preserving agents, water retaining additives, inert fillers etc.) for weed control. More specifically, weed control formulations based on fungi are called mycoherbicides (Bailey, 2014). Plant or microbial extracts with phytotoxic properties, purified or semi-purified natural phytotoxins are sometimes also referred as bioherbicides (Bordin *et al.*, 2018). However, this group of pesticides is better to assign to biorational (natural or biochemical) herbicides (Duke *et al.*, 2019), which will be discussed below.

Mycoherbicides aim to cause local epidemics in the populations of undesirable plants. In contrast to that, classical biocontrol leads to uncontrolled dispersal of the disease and following epidemics in populations of target host-plants. In both cases, the disease is expected to weaken susceptible weeds and to decrease their competitive ability. Some necrotrophic fungi (e.g., belonging to genera *Alternaria*, *Bipolaris*, *Drechslera*, *Fusarium*, *Myrothecium* and *Phoma*-like fungi) release phytotoxins during the infection process that directly kill plants acting similarly to biorational herbicides (TeBeest *et al.*, 1992).

Why do endemic species of phytopathogenic fungi not cause the destructive epidemics of weeds similar to some fungal diseases on cultivated plants? The main obstacles to the epidemics in the weed population are: (1) uneven distribution of host plants in the field and their different susceptibility to phytopathogens; (2) the use of fungicides, crop rotations and soil cultivation, which destroy the trophic relations of pathogens with host plants; (3) insufficient aggressiveness; (4) climatic conditions unfavorable for epidemics. Therefore, phytopathogenic microorganisms are artificially multiplied on host-plants or in bioreactors and are applied to weeds at high concentrations in a period of time favorable for infection in the same way as chemical herbicides. Mycoherbicides should be used regularly, although it is not excluded that their effect will be stable for several seasons after establishing successful infection and epidemics (TeBeest *et al.*, 1992).

Mycoherbicides are (or are considered to be) selective biologics that suppress one or several species of weeds. The selectivity of potential mycoherbicides is carefully studied before commercialization. Without questions, narrow host-range is a strict requirement for classical biocontrol agents. However, from commercial point view, it is commonly a significant disadvantage of mycoherbicides when compared with chemical herbicides. In addition, the effectiveness of mycoherbicides is lower comparing with chemical herbicides; the area of treatments cannot be large, and their shelf-life period is limited. However, development of mycoherbicides is much cheaper. They can be used in organic farming or as a component of the integrated management of troublesome weeds (Hershenhorn *et al.*, 2016).

The host range of the fungus and selectivity of its formulations should be proven both theoretically and experimentally. Some mycoherbicides are based on wide-spectrum pathogens recorded both on target weeds and crops. Such pathogens can be used at particular situations when susceptible crops are not included in a crop rotation in the region where weed biocontrol is performed. If the conditions for the use of mycoherbicides are violated, damage to the protected crops is possible (Watson, 2019). To date, just seven of 15 registered mycoherbicides could be available, but none of them are broadly used (**Table 1**). This is partially because most of them are selective biologicals that have been developed to suppress specific problematic weed species.

Biocontrol agents *a priory* look environmentally safe. However, allergenicity and toxicity, persistence in soil, effect on useful organisms should also be evaluated. Little is known about these properties of phytopathogens, except for some *Alternaria* spp. and wide range of *Fusarium* fungi (Żukiewicz-Sobczak, 2013; Chou *et al.*, 2014).

Table 2 Requirements for novel chemical and biological herbicides

Properties	*Herbicides*	*Mycoherbicides*
Performance	• Fast action and high efficacy over a relatively wide temperature range • Low application rates	• Reasonable dosage of inoculum • Short incubation period • Strong negative effect on the fitness and reproducibility of target plants • Action in a wide range of temperatures and relative humidity of air or soil • Application with standard equipment (sprayers, granule spreaders, etc.)
Mode of action	• A unique/novel mechanism of action for controlling resistant species	• High aggressiveness to target weeds/low resistance levels in populations of target weeds • Virulence and fitness determinants (toxins, enzymes, compatible solutes etc.) should be characterized
Selectivity	• Broad spectrum to selective action	• Selective to narrow action: the biologicals affect a target weed or a group of weeds without injury of crops or ornamentals in the accepted crop rotation or typical homestead plots
Safety	• Low toxicity for non-target organisms • Rapid decomposition in nature with non-toxic metabolites	• Low toxicity and allergenicity • No residues of fungal bioactive metabolites in water, soil, plants and foodstuff
Manufacturability	• Low-cost production • Stability in the formulation	• Large quantities of inoculum (conidia, mycelium or its modifications) are obtained on cheap media using standard fermentation technologies • Inoculum is easy to stabilize, it is stored for at least a year without refrigerating

Important Stages of Mycoherbicide Development

General Considerations

In nature, phytopathogenic fungi control plant populations, often leading to succession, maintaining biological diversity, and taking part in the initial decomposition of plant biomass (Zadoks, 1987). However, plant pathogens cause diseases of crops and ornamental plants, are producers of mycotoxins and allergens, and, unfortunately, are potential biological weapons. Therefore, the use of fungi for weed control is justified only if the safety of non-target plants, humans and environment is guaranteed.

Despite biologicals cannot kill weeds so quickly and effectively as chemicals, chemical herbicides are often used as an etalon at determining the effectiveness of mycoherbicides. Obviously, there are a number of requirements for development of chemical herbicides (Cobb and Reade, 2010) that could be compared to those for mycoherbicides (**Table 2**). It is seen that the requirements for fungal bioherbicides are just as high. Simply put, a mycoherbicide is "an ideal phytopathogen in an ideal formulation" while a chemical herbicide is "a perfect phytotoxin in a perfect formulation".

Although a typical algorithm for development of a mycoherbicide is presented on **Fig. 1**, it is not a standard and straight way because any fungal species has its own biological and ecological properties.

Selection of Appropriate Biocontrol Agent

At this early stage, the well-planned work requires mycological research, which includes the study of mycological databases and herbaria on pathogens of the target and phylogenetically related plant species, field surveys, establishing a collection of pure cultures, accurate species identification using classical, chemotaxonomic and molecular methods, and analysis of literature related to selected candidates for further evaluation. The most common and visually noticeable plant diseases are caused by parasites – biotrophic pathogens (some rusts, smuts, downy mildews and other fungi), but their use is limited due to the difficulties of inoculum production and the need to determine their race composition. Those rust fungi that do not have intermediate host plants seem to have a certain mycoherbicidal potential. For example, two *Puccinia* species were registered as mycoherbicides: *P. canaliculata* (Dr. BioSedge™) for the control of yellow nutsedge (*Cyperus esculentus*) and *P. thlaspeos* (Woad Warrior™) for the control of dyer's woad (*Isatis tinctoria*).

From a technological point of view, facultative parasites and facultative saprotrophs (necrotrophic and hemibiotrophic fungi) are preferable for the development of mycoherbicides because these phytopathogens can be cultured on artificial nutrient media. Many potential mycoherbicides are based on species from the genera *Alternaria*, *Ascochyta*, *Bipolaris*, *Cercospora*, *Colletotrichum*, *Drechslera*, *Fusarium*, *Microsphaeropsis*, *Phoma*, *Phomopsis*, *Phytophthora*, *Sclerotinia*, *Septoria* and *Stagonospora* (Harding and Raizada, 2015). Some examples with fungi of the genera *Alternaria* and *Colletotrichum* will be discussed below. Useful information on *Phoma*-like fungi, which are often considered as weed biocontrol agents, can be found in the review of Hynes (2018).

In nature, few types of plant killers occur. These are pathogens causing systemic infections (some rusts, smuts, downy mildews, wilts) and death of seedlings (root and stem rots). The wilts and rots are caused by *Fusarium*, *Phytophthora*, *Pythium*, *Sclerotinia*, *Sclerotium* and *Verticillium* species. However, there are risks in their use due to high persistence of these fungi in the

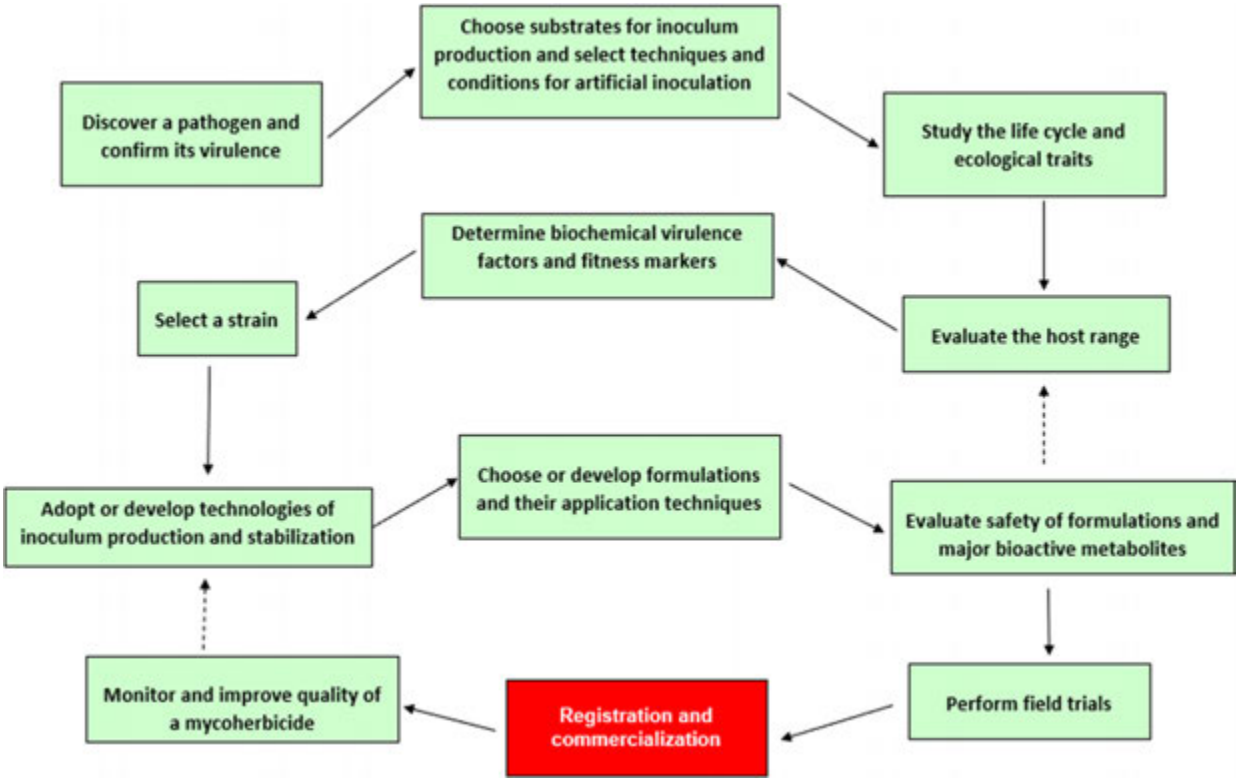

Fig. 1 A roadmap for development of a mycoherbicide. Fig.1

soil and subsequent possible damage to cultivated plants. On the other hand, crop pathogens are successfully tested against weeds, for example *Alternaria helianthi* (sunflower pathogen) against common cocklebur (*Xanthium strumarium*) (Sanyal *et al.*, 2008), *Colletotrichum coccodes* (potato and tomato pathogen) against velvetweed (*Abutilon theophrasti*) (Wymore *et al.*, 1988) and non–specialized pathogens, such as *Sclerotinia sclerotiorum* (Saharan and Mehta, 2008) and *Sclerotium rolfsii*, against various dicot weeds (Gibson *et al.*, 2014). Two mycoherbicides (Bio-Phoma ™ and Sarritor®) selective to dicots and based on the pathogens *Phoma macrostoma* and *Sclerotinia minor*, respectively, were registered in Canada for biocontrol of the weeds on grass lawns (Morin, 2020).

Several mycoherbicides were registered to control unwanted woody vegetation. A strain of *Chondrostereum purpureum* originated from apple trees was commercialized in USA and Canada as mycoherbicides (Chontrol™, Myco-Tech™) for control weedy trees as birch, aspen, alder, and blackthorn (Evans, 2013). Di-Bak® Parkinsonia was registered in Australia to control Parkinsonia spikenard (*Parkinsonia aculeata*). This mycoherbicide consists of three unspecialized phytopathogens (**Table 1**), which are safely formulated as capsules for injection into the trunk of trees with a special device (Morin, 2020).

When isolating phytopathogens from both healthy and diseased plant organs, saprotrophic, endophytic and epiphytic fungi are usually detected. The most common species are *Alternaria alternata*, *A. tenuissima*, *Aureobasidium pullulans*, *Boeremia exigua*, *Colletotrichum dematium*, *Cladosporium herbarum*, *Curvularia lunata*, *Fusarium* spp., *Aspergillus* spp., *Penicillium* spp., *Mucor* spp. and *Phoma herbarum*. These fungi grow well and sporulate abundantly on many substrates. Under a long period of relative high air humidity and high inoculum concentration, they can affect plants both in the laboratory and field. For these fungi, the production of phytotoxic compounds and lytic enzymes is very characteristic. Many novice researchers are tempted to test these fungi as mycoherbicides and they may sometimes work under favorable conditions of high humidity and optimal temperature in the laboratory. However, it should also be taken into account saprotrophs are non-host selective, dangerous as allergens and mycotoxin producers. However, laboratory samples of bioherbicide based on the mild toxigenic fungus, *Myrothecium verrucaria*, are being tested in the United States to control kudzu. It has been shown to be particularly effective in combination with standard herbicides, destroying up to 90% of this weed and freeing up space for local flora (Weaver *et al.*, 2016). Compared with chemical herbicides, the *Sclerotium rolfsii* SC64-based bioherbicide significantly improved the weed community structure and dramatically increased the biodiversity in habitats invaded by *Solidago canadensis* in several Chinese provinces (Zhang *et al.*, 2019).

Unfortunately, it is not always possible to find a suitable (or perfect) pathogen of the target plant as some of them are non-selective and harmful for crops, weakly pathogenic after production in artificial conditions or toxigenic. The effectiveness of mycoherbicides may be improved using various biotechnological approaches. However, if this does not work, it would be better to switch to biorational herbicides or phytophagous insects, which may be more effective than phytopathogens.

Biology and Ecology of Weed Pathogens

This complex and comprehensive phase of research includes: (1) selection of solid substrates or liquid media, and conditions (e.g., temperature, light, pH, agitation) for production of fungal propagules (spores, mycelium or its modifications), (2) choice of plant inoculation techniques and study of conditions for weed infection by the phytopathogen (effects of leaf wetness period, inoculum concentration, growth stage of the weed, temperature, relative humidity etc.), (3) evaluation of the host range of the fungus, and (4) studying dispersal and persistence (overwintering, survival in the soil and on plant debris) in the nature. In general, this is almost the same set of studies to develop a forecast of a particular crop disease.

Choice of the appropriate propagules is defined by their virulence, the potential for relatively long shelf time (up to two years at 5°C–25°C), and stress (desiccation, thermal and UV) tolerance. Generally, fungal conidia that fit best the environment can be produced by solid-state fermentation (SSF) using cheap natural substrates. However, this method is inferior to liquid submerged fermentation (LSF), in which the cultivation control (aeration, agitation, dissolved oxygen (dO) level, pH and temperature) is easier. The morphological, physiological, biochemical and physical features of submerged conidia or blastospores are notably different from those properties of the aerial conidia produced in solid cultures being usually more hydrophilic and less stress tolerant. If the conidia production is technologically problematic (e.g., due to near-UV-irradiation demands for sporulation, low spore production or low pathogenicity of this inoculum type), the mycelium or its modifications (chlamydospores and microsclerotia) can be used as alternative infection material. Application of vegetative mycelium has been more effective than conidia in many "weed–fungus" pathosystems, for instance in those of sicklepod (*Cassia obtusifolia*) – *Alternaria cassiae*, black cherry (*Prunus serotina*) – *Chondrostereum purpureum*, dandelion (*Taraxacum officinale*) – *Phoma herbarum* and Canada thistle – *Stagonospora cirsii* (Sokornova and Berestetskiy, 2018).

The dew requirement for germination of fungal propagules is one of the main constraints for artificial inoculation of plants. If the infection of target plants requires a dew period of more than 24 h, the successful use of such a fungus as a mycoherbicide is unlikely, since optimal humidity conditions are infrequent in the field. For instance, infection and significant suppression of spurred anoda (*Anoda cristata*) by *Alternaria macrospora* was achieved with inoculation of seedlings in the phase of cotyledon leaves or one true leaf by conidial suspension at a concentration of about 0.25–1 million spores mL^{-1} at 20–30°C with and leaf wetness period of 24h (Walker, 1981).

For the host range studies of potential mycoherbicides, representatives of local flora and main crops and ornamentals are included. For instance, *P. macrostoma* 94–44B (Bio-Phoma™) was evaluated against 94 plant species in 34 botanical families of economically important agricultural, horticultural and ornamental species, as well as target and nontarget weeds. Fifty-seven species from 28 families were found to be resistant to *P. macrostoma*, while 38 species from 12 families, six of which also contained resistant species, were found to be susceptible. *P. macrostoma* was pathogenic to many dicotyledonous plant species, but non-pathogenic to monocots showing its prospects for weed management in turfgrass (Bailey *et al.*, 2011). Berner (2010) published an approach for a host-range study of weed biocontrol agents.

The fungus *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (Collego™) does not survive well in soil and on plant debris, and has a limited distribution due to the uneven density of the host weed. Therefore, the mycoherbicide should be added to the fields annually. On the contrary, spores of the fungus *Phytophthora palmivora* (DeVine™) were well preserved in the soil; after one application, the pathogen suppressed plants and seedlings of strangler vine on citrus plantations for several years (TeBeest and Templeton, 1985). Fungi of the genera *Fusarium*, *Verticillium* and *Sclerotinia* are well-known to persist in the soil as chlamydospores, microsclerotia and sclerotia, respectively, so their use should be carefully studied to avoid crop infection the next year after their application.

Production, Formulation and Application

A commercial mycoherbicide product is the result of close collaboration between scientists and companies involved in production, formulation, and application. At this stage of the development of mycoherbicides, methods for mass production of fungal inoculum are optimized and scaled up, and techniques for its stabilization and storage as well as the composition of solid and liquid formulations, and spray solutions are selected and improved (Daigle and Connick, 2002).

Liquid state fermentation (LSF) is the most commonly used technology in microbial biotechnology. The first mycoherbicides, Collego™ and DeVine™, have been manufactured this way. The full process control and relatively short duration (several days) is an undoubted advantage of LSF over solid state fermentation (SSF). However, the culture medium composition takes up 30%–40% of the production costs by LSF, while cheap or waste natural substrates are appropriate for SSF. For instance, a commercial LSF medium for *C. truncatum* conidia production includes glucose (20 g L^{-1}), yeast extract (2.5 g L^{-1}), cottonseed flour (7.5 g L^{-1}) and various salts (Silman and Nelsen, 1993). In the case of SSF, the fungi can "simply" be grown on cereal grain to be applied as granular formulations like *P. macrostoma* and *Sclerotinia minor* based mycoherbicides (Bailey *et al.*, 2009).

To obtain a high yield of viable and stress tolerant infectious material, the composition of the liquid nutrient medium requires optimization. This process usually includes three main steps: (1) selection of the basal medium with a set of vitamins and trace elements, on which the fungus grows and/or sporulate well; (2) selection of carbon and nitrogen sources and their optimal concentration and ratio, and (3) replacement of artificial carbon and nitrogen sources by cheap natural ones (Jackson, 1997). Both LSF and SSF process parameters may need optimization as well, in particular duration of fermentation, aeration level, agitation speed and incubation temperature. Statistics approaches, such as factorial design experiments and response surface methodology,

were successfully used to optimize the growth parameters required for large scale conidia and microsclerotia production of potential mycoherbicides based on *Gloecercospora sorghi*, *Septoria polygonorum*, and *Plectosporium alismatis* (Mitchell, 2003; Mitchell *et al.*, 2003; Dedjell and Cliquet, 2019).

Biomaterial produced by fermentation should be separated from a substrate, stabilized and stored. In the ideal situation, the modern biopesticides should stand 0.5–2 years depending on temperature and relative humidity. However, some mycoherbicides have to be used within several weeks after fermentation was finished like DeVine™ (Wraight *et al.*, 2001; Auld *et al.*, 2003). There are no universal recipes for stabilization of fungal spores and mycelium. Generally, fungal germination and growth depend on temperature, free water availability (water activity), relative humidity, and pH and oxygen concentration. As a good example, see the protocol for production and stabilization of fungal microsclerotia (Jackson and Payne, 2016).

There are two general types of herbicide formulations: liquid and dry. In the case of mycoherbicides, no solvent is used in the liquid formulations, but concentrated water (e.g., DeVine™) and oil-based suspensions of fungal inoculum are prepared with various adjuvants and preservatives. Wettable powders (e.g., Collego™) and granules (e.g., Sarritor®) are those types of dry formulations that are usually known for preparation of mycoherbicides. Alginate and wheat gluten ("Pesta") granules (Walker, 1981; Connick and Boyette, 1991) as well as invert emulsions (Auld *et al.*, 2003) still seem to be model formulations due to the high cost of food components in their composition. Trends relating to the type of biopesticide formulations would probably go the way from wettable powders and suspension concentrates to water dispersible granules, for safety reasons, and from single component to multi-component formulations to increase their efficacy (Gašić and Tanović, 2013).

The concentrates and wettable powders are dissolved in water to prepare the spray solution, while granules (colonized grain, alginate or "Pesta" granules) are applied on soil surface. Generally, the application rate of liquid formulations of herbicides is about 100–400 L ha^{-1} while the optimal spray volume for mycoherbicides varies from 400 to 2000 L ha^{-1} because fungi need high relative humidity for successful infection (Boss *et al.*, 2007c). The rate of application of bioherbicidal granules is relatively high, about 50–100 g m^{-2} as for turf weed mycoherbicides based on *Sclerotinia minor* (Abu-Dieyeh and Watson, 2009) and *P. macrostoma* (Smith *et al.*, 2015), while application rate of chemical herbicides is five to 10 times lower (Metzger *et al.*, 2019). Solid application of selective mycoherbicides may not be feasible because weed density on large fields is not uniform. Hershenhorn *et al.* (2016) suggested and listed several innovative site-specific application methods such as the use of drip irrigation systems or the combination of optical sensors able to detect the presence of the weed in the field, with automated spraying systems. Such approaches may allow to decrease costs of application of mycoherbicides in the future.

Safety and Registration

The use of plant pathogens as biocontrol agents is not without risks, and these need to be accurately and thoroughly assessed prior serious research input and field experiments. The safety evaluation includes the above discussed data on the host range of the candidate fungus and its persistence in nature (on plant debris and in soil), toxicity and allergenicity of biocontrol agents and their metabolites, and environmental fate of those that were proven to be toxic. The method of inoculum production, as well as its formulation should be considered, because both largely determine the safety of mycoherbicides as selectivity, toxigenicity, and allergenicity. Thus, toxicological studies should be done with the microorganism, its formulations and bioactive metabolites. For quality control and safety reasons, reliable molecular markers should be developed for strain-specific identification of biocontrol agents and their tracking post release into the environment (Dauch *et al.*, 2002; Boss *et al.*, 2007a; Pan *et al.*, 2010; Zimmermann *et al.*, 2015).

The combination of publication surveys, observations in nature and experimental evaluation of the host-range of candidate fungal strains makes it possible to predict the safety of a potential mycoherbicide to crops. However, some formulations, especially oil-based ones, influence the host range of mycoherbicides. The effects of an invert emulsion (IE) on the host range and weed control efficacy of the fungus *Colletotrichum coccodes* (NRRL strain 15547) for biocontrol of Eastern black nightshade (*Solanum ptycanthum*; EBN) were examined. Greenhouse tests demonstrated that several other solanaceous weeds were also infected and killed, and field tests revealed $>$ 90% EBN control and dry weight reduction in plants treated with the fungus-IE formulation. These results demonstrate that this IE formulation can promote the efficacy of this bioherbicidal pathogen (Boyette *et al.*, 2018). IE abolished the selectivity of other potential mycoherbicides i.e., *Alternaria cassiae* and *A. crassa* (Amsellem *et al.*, 1991).

Most necrotrophic and hemibiotrophic phytopathogenic fungi produce a variety of biologically active compounds, primarily phytotoxins (substances that are toxic to plants) and mycotoxins (substances that are toxic to humans and animals). It is important to note that only some of these compounds have a narrow spectrum of action, but most of them have a wide range of biological activity. The production of mycotoxins by any biocontrol agent can become a serious problem, making the work of operators dangerous. On the other hand, the accumulation of mycotoxins, which are produced by toxigenic fungi such as *Alternaria* and *Fusarium* spp., in food and feed, water and soil poses a serious problem for agriculture. Thus, for the vast majority of mycoherbicide producers, it is necessary to study their production of their bioactive metabolites both in vitro and in vivo. However, it is still under question which microbial metabolites should be evaluated for safety reasons (Scheepmaker *et al.*, 2019). Two examples are discussed below.

Stagonospora convolvuli strains that are promising for biocontrol of field bindweed (*Convolvulus arvensis*) produce three main toxins, leptosphaerone, cercosporin, and elsinochrome A, which have a wide range of biological activity. The production of elsinochrome A (ELA) in vitro correlated with the pathogenicity of fungal strains (Ahonsi *et al.*, 2005). ELA has shown toxicity to

various organisms, from bacteria and fungi to water organisms such as *Artemia salina* and *Daphnia magna*. However, neither ELA nor other toxins of *S. convolvuli* were detected in the aboveground shoots of field bindweed and in the fruits of the protected crop-strawberries treated with mycoherbicide. ELA is stable for a long time in the dark, but quickly degraded when exposed to sunlight (the half-decomposition period is about 14 h). It was concluded that *S. convolvuli* does not pose a threat to the environment and consumers of agricultural products (Ahonsi *et al.*, 2006; Boss *et al.*, 2007b).

Various strains of *Myrothecium verrucaria* are promising for the biocontrol of certain weeds (Anderson and Hallett, 2004; Boyette *et al.*, 2014; Hoagland *et al.*, 2020). This fungus is known to produce a number of mycotoxins, e.g., roridin E and H, epi-isoridin E, and verrucarin A and J, during growth on solid and liquid media (Abbas *et al.*, 2002). The mycotoxins were found in the conidia of the fungus, but they were not detected in the mycelium obtained on a liquid medium based on soy flour and corn sirup (Boyette *et al.*, 2008). However, even when using conidial inoculum, the fungal toxins were not detected in infected target weed (*Pueraria montana*) (Abbas *et al.*, 2001). Thus, a set of studies has shown the relative safety of using the fungus as a mycoherbicide. Therefore, for producers of bioherbicides and toxic metabolites it is important (1) to develop protocols for determining the trace amounts of toxins in fungal cultures, infected plants, soil and water, and (2) to select fungal strains or/and culture conditions minimizing toxin production. Moreover, biocontrol candidates should be checked for safety further, taking into account that some phytopathogenic fungi (at least *Alternaria*, *Cladosporium*, *Colletotrichum*, *Curvularia* and *Fusarium* spp.) are able to cause human diseases or allergy (De Lucca, 2007; Kazerooni *et al.*, 2020).

Currently, there are protocols of toxicological studies in vitro using model organisms including some bacteria, cell lines, protozoa cells and brine shrimp. Their results can be approximated for preliminary safety assessment of biocontrol fungi producing toxic metabolites (Altomare *et al.*, 2012). For more details, one may read a review of Evans and Seier (2012) on the safety and regulation of microbial control of weeds. A good road map for collecting scientific and practical data necessary for registration of microbial herbicides and their herbicidal metabolites is "Guidelines for the registration of microbial, botanical and semi-ochemical pest control agents for plant protection and public health uses" published by Food and Agriculture Organization of the United Nations and World Health Organization in 2017. For instance, extensive research was conducted on possible harmful effects of Collego™ on humans and animals (TeBeest and Templeton, 1985).

Alternaria or Colletotrichum?

The species of the genera *Alternaria* and *Colletotrichum* have been most often evaluated as potential mycoherbicides (Triolet *et al.*, 2020). For example, two mycoherbicides were registered in the United States (CASST™ and Smolder™) and Myco-herb® based on *A. chlamidosporiformans* (*Lewia chlamidosporiformans*) was pre-registered in Brazil. However, these products have not been implemented in practice. The reasons for this may include unstable or unsatisfactory efficacy in the field or problems with obtaining large volumes of conidia using solid state fermentation, since *Alternaria* fungi do not sporulate in liquid culture (Sokornova and Berestetskiy, 2018; Watson, 2019). CASST™ based on *A. cassiae* conidia for the control of *Senna obtusifolia* and *Crotalaria spectabilis* was registered in the 1980 s (Bannon, 1988a). The bioherbicidal composition consisted of a non-phytotoxic vegetable oil, an adhesive, and conidia to be used with standard spray equipment (Bannon, 1988b). The bioherbicide can be combined with some chemical herbicides such as glyphosate, 2,4-dichlorophenoxyacetic acid, and lactofen. These synergistic compositions allowed to reduce the application rates and to expand the range of natural conditions for successful infection of the target weeds with *A. cassiae* (Caulder and Stowell, 1987). Efficacy of *A. destruens* against the dodder was increased by the combined use of the fungus (18 billion conidia L^{-1}), a vegetable oil (7.5%), phytotoxic adhesive ammonium sulfate (0.125%) and herbicide glyphosate (20 g of active substance L^{-1}). This mixture was effective in the field, causing the death of the parasitic plant five weeks after treatment and not affecting lemon trees (Cook *et al.*, 2009).

A. cirsinoxia was found to cause leaf lesions on *Cirsium arvense* in Canada and the Kyrgyz Republic. The main advantages of this pathogen are good survival of conidia and mycelium of the fungus under adverse conditions, as well as the ability to cause disease over a wide temperature range at the absence of a long period of dew. The disadvantages are that the pathogen can infect other plants of the *Asteraceae* family, including sunflower, safflower, and chicory, the lower leaves of the weed are mainly susceptible to the pathogen and large conidia clogs the holes of standard sprayers at inoculum concentrations above 10^5 conidia mL^{-1} (Green and Bailey, 2000; Green *et al.*, 2001; Gannibal and Berestetskii, 2008).

Some *Alternaria* spp. can infect plants by submerged mycelium or its modifications that is important when the sporulation on solid substrates is poor. For this reason, a liquid nutrient medium (30 g of sucrose, 20 g of yeast extract and 4 mL of Tween 80) and the culture conditions (pH 6.9–7.0, incubation temperature 25–30°C, agitation speed 150 rpm) were selected for obtaining chlamydospores of *A. chlamidosporiformans*, a potential mycoherbicide against *Euphorbium heterophylla* (Vieira and Barreto, 2010).

Due the toxigenic and allergenic properties of *Alternaria* fungi there are obvious risks of their use as bioherbicides. For instance, *A. alternata* is known to accumulate mycotoxin alternariol in conidia (Häggblom, 1987) and a number of allergenic proteins (Alt A family) were found on conidial surface (Kustrzeba-Wójcicka *et al.*, 2014). Information about the toxigenic and allergenic properties of other *Alternaria* pathogens of weeds is still minimal. It should be noted, that *A. alternata* has been a research object in the past (Singh *et al.*, 2010; Motlagh, 2012; Aneja *et al.*, 2014) and at the present time (Abdessemed *et al.*, 2020).

Several *Colletotrichum*-based mycoherbicides were registered in China, the United States and Canada. LuBao™ reduced soya infestation with dodder species and prevented 30%–80% of crop losses (Templeton, 1991; Barreto *et al.*, 2012). Collego™ contains dried conidia (15%) and inert ingredients (85%) (Bowers, 1986). The development of anthracnose of *Aeschynomene virginica* begins 48 h after treatment with this mycoherbicide, and after 7–10 days, multiple necroses appear on the leaves (Bailey, 2014). BioMal™ based on *C. coccodes* was developed for biocontrol of roundleaf mallow (*Malva pusilla*) in wheat, lentil and flax (Mortensen, 1988).

Velgo™ is another mycoherbicide based on *C. coccodes* to combat *Abutilon theophrasti* in corn and soy crops. It has been used in some areas of the United States, where corn was cultivated, as well as in southern Ontario. When applying at the stage of two to three true leaves, the mycoherbicide killed 40% of the target weeds (Mortensen, 1988; Dauch *et al.*, 2002). Hakatak™ is manufactured as a granular formulation consisting of dried mycelium of *C. acutatum*, wheat gluten and soy flour. This mycoherbicide was targeted for biocontrol of *Hakea gummosis* and *H. sericea* in South Africa, but its production was halted due to low demand (Morris *et al.*, 1999).

Many other *Colletotrichum* species are also known as potential mycoherbicides. One of them, *C. truncatum* is considered as a biocontrol agent of *Sesbania exaltata*. This fungus has become a model in studies on optimization of liquid and solid substrates for the production of both conidia and microsclerotia (Schisler *et al.*, 1991). The isolate *C. truncatum* UFU 280 was proposed for development of a mycoherbicide against beggartick (*Bidens pilosa* and *B. subalternans*), one of the major weeds of Brazilian agriculture (Vieira *et al.*, 2018). *C. graminicola* has been evaluated for the control of *Echinochloa crus-galli* and *Alisma plantago-aquatica* (Yang *et al.*, 2000; Motlagh *et al.*, 2011) and *C. typhae* against *Typha domingensis* (Maia *et al.*, 2020).

Some of the main problems with the use of mycoherbicides are their insufficient field efficacy, too high selectivity and, consequently, a limited market. Studies with some *Colletotrichum* species became model for strain improvement and the development of formulations and application of mycoherbicides. The effect of various surfactants, which are typical components of spray suspensions, on the conidial germination and growth of ten *Colletotrichum* species was evaluated to show the inclusion of Tween® − 40 and Tween® − 80 in the solution at a concentration of 1% to stimulate the germination of conidia of the studied fungi, while Tween® − 20 had an inhibitory effect (Zhang *et al.*, 2003). The use of *Colletotrichum*-based mycoherbicides in the invert emulsion reduces their selectivity and increases their efficacy in the field. The oil phase of the emulsion may include paraffin oil, a monoglyceride-based emulsifier, and paraffin wax (Boyette, 2006; Boyette *et al.*, 2018). The combination of a conidial suspension of *C. truncatum* with some herbicides (2,4-D ether, clopyralid and metribuzin) had a synergistic or additive effect to significantly increase the control of *Matricaria perforata* and *Sesbania exaltata* (Graham *et al.*, 2006).

Strains of *C. gloeosporioides* and *C. coccodes* became models for the development of more effective (hypervirulent) transgenic mycoherbicides. Robinson and Sharon (1999) developed a simple method for obtaining *C. gloeosporioides* f. sp. *aeschynomene* transformants by electroporation of germinating conidia. They embedded the marker genes *gfp* (green fluorescent protein) and *hyg* (resistance to hygromycin) in the genome of this mycoherbicide producer to track the pathogen in plants. A biocontrol strain of *C. gloeosporioides* f. sp. *aeschynomene* genetically modified with the apoptosis *Bcl-2* showed to increase the yield of fungal biomass and tolerance to external stresses (Sharon and Goldstein-Barhoom, 2004). A mycoherbicidal strain of *C. coccodes* was transformed by a number of genes responsible for the biosynthesis of various fungal virulence factors including phytohormones (indolylacetic acid), enzymes (pectinase), effectors (NEP1, ceratoplatanin) and inhibitors of phytoalexin synthesis (oxalic acid). In most cases, the transformants were more pathogenic than the wild-type strains. However, the achieved level of pathogenicity was not sufficient for the commercialization of mycoherbicide (Gressel *et al.*, 2007).

Potential Use of Fungal Secondary Metabolites

Various plant pathogenic fungi (mainly *Ascomycota*), including weed biocontrol agents, produce numerous bioactive metabolites, for instance phytotoxins, mycotoxins, antibiotics, phytohormones, pigments and antioxidants. Many of these metabolites may have potential for agricultural and industrial applications. For instance, fungal phytotoxic metabolites can be used as biorational (natural, biochemical) herbicides or as selection and quality markers for mycoherbicidal agents (Dayan and Duke, 2014; Cimmino *et al.*, 2015). Other metabolites have been evaluated as pro-drugs (Chen *et al.*, 2020), indicating that production of mycoherbicides has possibilities to obtain useful by-products.

Biorational herbicides are chemicals and therefore their activity is less depended on abiotic and biotic factors than mycoherbicides. Moreover, for pure chemical compounds, toxicology and mode of action issues are clearer than for living organisms or crude extracts obtained from them. For instance, phytotoxins have been isolated from cultures of many potential mycoherbicides produced by the fungal species from order *Pleosporales*, especially *Alternaria* spp. From the culture fluid of *Alternaria cirsinoxia* (a pathogen of Canada thistle) and *A. cichorii* (isolated from Russian knapweed) phytotoxic zinniol was obtained (Stierle *et al.*, 1993; Berestetskii *et al.*, 2010). Radicinin (a phytotoxin produced by a number of *Alternaria* and *Cochliobolus* spp.) has been evaluated as an herbicide for the control of Buffalo grass (*Cenchrus ciliaris*), an invasive weed plant in North America (Masi *et al.*, 2019). Tenuazonic acid (actually, being a mycotoxin) produced by some *Alternaria* spp., was patented as a natural herbicide with an original mechanism of action and was proven to control some troublesome weeds under the field conditions (Zhou *et al.*, 2019).

Some phytotoxic compounds may obviously serve as markers for selection of fungal strains with stronger herbicidal activity as well as quality markers for such strains. However, this approach seems not to be universal. For instance, no correlation was found with production of a selective phytotoxin ascosonchine and pathogenicity of *Ascochyta sonchi* (*Boeremia exigua* var. *exigua*) strains to perennial sowthistle (Evidente *et al.*, 2006). As well, overproduction of phytotoxic fusaric acids, which inhibits the germination of the angiosperm parasite *Orobanche ramosa*, by *Fusarium oxysporum* strains was not correlated with their pathogenicity to the weed (Abouzeid *et al.*, 2004; Bouizgarne *et al.*, 2006). In the case of *Stagonospora convolvuli* and *Cercospora piaropi* (a pathogen of water hyacinth), high aggressiveness of the fungal strains was related to elsinochrome A and cercosporin production level, respectively (Ahonsi *et al.*, 2005; Tessmann *et al.*, 2008). Phytotoxic macrocidins were proven to be active components of the *P. macrostoma*-based mycoherbicide and their granular formulation was standardized by "macrocidin units", a proprietary measure of active ingredient content (Wolfe *et al.*, 2016).

The yield of target phytotoxins can be increased using biotechnological approaches. For instance, strain and media selection, temperature of incubation and duration of cultivation allowed to optimize the production of mevalocidin, a phytotoxin of *Coniolariella* sp. demonstrating broad spectrum post-emergence herbicidal activity (Gerwick *et al.*, 2013; Sica *et al.*, 2016). Zonno *et al.* (2008) found optimal medium and cultivation length for production of potential natural herbicide, phyllostictine A produced by *Phyllosticta cirsii*. To combat the parasitic *Striga* spp., a mycoherbicidal strain of *Fusarium oxysporum* was selected for overproduction of tyrosine, an amino acid that inhibits the development of the weed (Nzioki *et al.*, 2016).

The development of commercial biorational herbicides based on fungal phytotoxins is delayed because little is still known about their selectivity, stability and general toxicity. Moreover, most natural phytotoxins seem to be unable to penetrate the plant cuticle. Some adjuvants (for-instance, oil-based adjuvants like Hasten™) were demonstrated to increase herbicidal efficacy of fungal phytotoxins (Poluektova *et al.*, 2018; Dubovik *et al.*, 2020). Nanoformulations (nanoemulsions, nanodispersions, polymer-based granules) can potentially improve efficacy, reduce effective doses, and increase shelf-life and persistence of natural herbicides (Kremer, 2019; Vurro *et al.*, 2019). Some toxicity effects of pesticides including natural herbicides can be lowered by encapsulation in biodegradable polymers with tuning of the release bioactive compounds (Serino *et al.*, 2020).

The natural origin of a substance is not equal to its environmental safety. AAL-toxin isolated from the fungus *Alternaria alternata*, which showed potent phytotoxicity and selectivity to some nightshade weeds, was patented as a natural herbicide. However, when its chemical structure was established, it turned out that it is very similar to the structure of fumonisins – a group of dangerous mycotoxins produced by some *Fusarium* fungi. Toxicological studies confirmed carcinogenic properties of AAL-toxin (Abbas *et al.*, 1995; Wang *et al.*, 1996). Toxicological studies of phytotoxins of the fungus *Ascochyta caulina*, a mixture of which is considered a pro-herbicide for the control of fat hen (*Chenopodium album*), have shown that they are highly toxic to *Daphnia*, but showed low toxicity for other tested organisms, i.e., planktonic crustaceans, zebrafish and earthworms (Fumagalli *et al.*, 2013).

Inocula of mycoherbicidal fungi (spores and mycelium) are produced by liquid- and solid-state fermentation, and the spent substrate may be used for isolation of valuable by-products. The culture liquid of *Stagonospora cirsii*, a potential mycoherbicide for biocontrol of perennial thistles, may be used for isolation of phytotoxic stagonolide A and herbarumin I (Dalinova *et al.*, 2019). The spent solid substrate for conidial production of *Alternaria sonchi* was found to be a rich source of a number of bioactive metabolites from xanthone and chromone groups with various bioactivities (Dalinova *et al.*, 2020). Ophiobolin A and terpestacin produced by some weed biocontrol fungi has been evaluated as very promising anticancer drugs (Park and Choi, 2013; Morrison *et al.*, 2017; Masi *et al.*, 2018).

Future Prospects

Biological and biorational herbicides based on fungi and their metabolites are already being proposed and developed in various countries as a supplement or alternative to chemical herbicides. However, they have not become a common part of pest management systems. The main well-known drawbacks of mycoherbicides are the delayed action, and insufficient and unstable biological effectiveness compared to chemical herbicides. It should also be noted that the biologicals have limited shelf life. Moreover, there are not enough criteria for evaluating their quality. There are some obvious problems in their registration in many countries: having a lower payback and efficiency, bioherbicides should pass the stages of this expensive process, as do any chemical pesticide. Since 2007, the publication activity in the development of mycoherbicides has slowed down (Triolet *et al.*, 2020). Therefore, it is necessary to develop novel approaches that can improve the effectiveness and manufacturability of potential mycoherbicides These approaches should be based on a deep understanding of the ecology, biochemistry and physiology of mycoherbicidal fungi in relation to their pathogenic properties, tolerance to various stresses (mainly, sub-optimal temperature and relative humidity). As well, the modern biotechnology should be employed for (1) enhancement of strain performance in the field, (2) increased inoculum yield, (3) improved formulations, (4) adequate shelf life of mycoherbicides, and (5) utilization of waste culture substrates.

Some attempts to reanimate a mycoherbicidal approach of weed control have been done. For instance, methods for obtaining virulent and stress-tolerant inoculum based on microsclerotia and mycelium of mycoherbicide-producing fungi are being developed (Sokornova and Berestetskiy, 2018). Original methods of applying mycoherbicides are being investigated, for example, using various feeding and irrigation systems (Boari *et al.*, 2008). Biochemical quality control markers, such as lipid content of the fungal mycelium or phytotoxins in the formulation, have been proposed (Bailey *et al.*, 2011; Frolova *et al.*, 2019). Finally, there is a lot of experimental data showing that some well-chosen combinations of mycoherbicides and chemicals can act synergistically (Gressel, 2010), which suggests supplementation of chemical herbicides with selective biologicals to target particular herbicide-resistance weeds. However, much more needs to be done and systemized to ensure that biological and biorational herbicides become full-fledged products on the market.

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Biofungicides: An Eco-Friendly Approach for Plant Disease Management

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Introduction

In a scenario of further population growth, the control of fungal plant diseases has become necessary to meet the demand for food supply (Ram *et al.*, 2018). Phytopathogenic fungi consist of a highly diverse group of economically devastating threats to both food supply and security. The extensive use of synthetic fungicides for managing fungal crop diseases over the years has led to the increase of antimicrobial resistance and damage to the environment and animal health (Bondori *et al.*, 2019; Leiter *et al.*, 2017). Therefore, many researchers have focused their efforts on developing alternative methods for managing fungal plant diseases. Among these methods, the use of biofungicides with potential to control fungal plant pathogens has been increasingly recognized as a promising alternative because it is practicable, environmentally safe, cost-wisely affordable, and field persistent when compared with other conventional non-sustainable control methods (Abbey *et al.*, 2019a). Biofungicides is the general name given to formulations derived from animals, plants, microorganisms, or their products that control plant pathogenic fungi in an environmentally friendly manner (Anuagasi *et al.*, 2007). Disease control by microbial-based biofungicides is the result of interactions between the biological control agent, the local microbial community, the target plant pathogen, and the host plant. These multitrophic interactions are directly influenced by the physical-chemical properties of the environment in which the biological control agent is introduced. It is desirable that the microbial-based biofungicide agents act as antagonist of plant pathogens, are well adapted to the treated plants and environmental conditions of the local agroecosystem, and cause no damage or side-effects to both plants and environment.

Biofungicide Agents

Thousands of microorganisms with particular niche association properties have been identified as antagonists of fungal pathogens. For instance, endophytic microorganisms can establish inside the host plant tissues and produce bioactive compounds that are elicitors of the plant resistance response against diseases and plant growth promoters (Segaran and Sathiavelu, 2019). Several endophytic filamentous fungal species with relevance for the biocontrol of phytopathogenic fungi have been described, such as species from the genera *Acremonium*, *Chaetomium*, *Cladosporium*, *Gliocladium*, *Paraconiothyrium*, *Sarocladium* and *Trichoderma* (De Silva *et al.*, 2019; Gama *et al.*, 2020; Madbouly and Abdel-Wareth, 2020; Maia *et al.*, 2018). Species of *Aureobasidium*, *Candida*, *Pichia*, *Rhodotorula* and *Saccharomyces* are examples of endophytic yeasts used in biocontrol (Madbouly and Abdel-Wareth, 2020). Bacterial endophytes have also been used in microbial biofungicide formulations, including *Bacillus amyloliquefaciens*, *B. subtilis*, *Bacillus velezensis*, *Burkholderia*, *Mesorhizobium*, *Pantoea*, *Pseudomonas fluorescens*, *Rhizobium and Streptomyces* (Bolívar-Anillo *et al.*, 2020; Cheffi Azabou *et al.*, 2020; Hassan *et al.*, 2018; Nagpal *et al.*, 2020).

Another type of niche association, includes the phylloplane-resident microorganisms, which are known for their advantageous adaptation to stand harsh conditions, such as direct solar irradiation, resulting in their usefulness for the management of foliar plant diseases (Wang *et al.*, 2020). The phylloplane fungus *Cladosporium cladosporioides* is an example of a biocontrol agent with activity against the rice blast disease (*Pyricularia oryzae*), by inhibiting the pathogen's pre-infection conidial germination and appressorium formation, and post-infection mycelial growth, besides secreting lytic enzymes (Chaibub *et al.*, 2020). Phylloplane bacteria (e.g., *Bacillus*, *Pseudomonas*) and yeasts (e.g., *Candida*, *Pichia*) were described as biocontrol agents for postharvest fruit diseases caused by *Botrytis*, *Colletotrichum*, *Monilinia* and *Penicillium*. Their antagonism included the production of lytic enzymes, antibiotics and volatile compounds, induction of host resistance and alleviation of fruits oxidative damage (Dukare *et al.*, 2019).

Rhizoplane-resident microorganisms are the third category of niche associations described for the biocontrol agents. These microorganisms inhabit the plants root surface or soil particles adhered to the root system and their competition abilities are associated with the capability of colonizing very complex soil niches, producing a great diversity of antimicrobial secondary metabolites (Srivastava and Singh, 2020). Species of *Trichoderma* are the main rhizoplane-resident microorganisms exploited as fungal biofungicides, exerting their antagonism against several fungal plant pathogens by parasitism, antibiosis, competition, resistance induced-resistance and plant growth promotion (Adnan *et al.*, 2019; Zhang *et al.*, 2016). Strains of *Trichoderma asperellum*, *T. atroviride*, *T. harzianum* and *T. virens* are among the most common biocontrol agents in products labeled for the management of plant diseases, either for seed or soil treatments (Woo *et al.*, 2014).

The last category of niche associations includes the biofungicides based on fungus-infecting viruses (mycoviruses). There are only few examples of such biofungicides for the management of air-borne and soil-borne plant pathogens (e.g., *Alternaria alternata* and *Sclerotinia sclerotiorum*, respectively) (Hu *et al.*, 2020).

Screening for Novel Biofungicides

The development of novel biofungicides requires the screening of numerous antagonists or candidate bioactive antimicrobial compounds according to their niche association and resulting ecological interactions, modes of action, field efficacy, formulation stability, manufacturing costs, environmental risk and impacts, and regulatory laws (Köhl *et al.*, 2011; Raymaekers *et al.*, 2020).

A successful biofungicide should have four crucial features as an effective, long-lasting, affordable, cropping system-compatible, and environmentally safe choice for the management of fungal plant disease: (1) genetic stability of the microbial antagonistic properties; (2) an efficient and low-cost manufacturing process; (3) full compatibility with other control strategies, especially with the regular fungicide sprays and the cultural practices required by the cropping system; (4) non-pathogenic to humans. More specifically, it is desirable for the biocontrol agent that the microorganism in the biofungicide formulation acts as antagonist by more than one mechanism, including antibiosis, resistance induction, competition, parasitism, predation, and/or growth-promotion. For formulations of biofungicides based on microbial natural compounds, their mode of action may be the direct inhibition of a plant pathogen, or indirect, by inducing resistance. Besides the desired efficacy against plant disease, microbial-natural-compounds-based biofungicides should have a low-cost efficient production process, no phytotoxicity to the target plant crop, no toxicity to humans, and no environmental risk.

The criteria for screening biological control agents and natural compounds as potential biofungicides are described as follows.

First, as a criterion of high environmental fitness, the antagonist candidates should be preferentially prospected from the same pathosystem and pathogen's niche as an assurance for a potential high performance as a formulated biofungicide. To attend this criterion, one option is to conduct the bio-prospection by exploring rich microbial diversity sources, such as tropical soils, disease-suppressive soils, and phylloplane and rhizoplane of native plants from existing natural ecosystems, using appropriate isolation methods (Maia *et al.*, 2018; Mwajita *et al.*, 2013; Srivastava and Singh, 2020). Publicly shared libraries of microorganisms or natural compounds from cataloged collections in reference research institutes or companies could be reliable sources of biocontrol agents, if a perfect match between the origins of the antagonists and the target pathosystem/niche could be made.

Once the biofungicide candidates have been selected, the process continues with the adoption of key exclusionary assays. An initial selection of potential biofungicide candidates may begin with an exclusion step based on biosecurity and ecological adaption features (Köhl *et al.*, 2011). For instance, to avoid potential human pathogens among the candidates, all prospective microbial species and lineages capable of growing at 37°C should be eliminated. Subsequently, drought tolerance could be evaluated if the microbial candidate may face water constrains and osmotic stress conditions in the field. This test may be conducted using culture media osmotically modified by addition of ionic solutes (NaCl or KCl) and non-ionic solutes (glycerol and glucose) (Sartori *et al.*, 2020). The sensitivity to UV irradiance is another critical aspect in this exclusionary selection process, especially if the microbial biofungicide is meant for delivery at the plant leaves and will be exposed to the solar radiation. The microbial biofungicide candidates are then exposed, several times during incubation, to UV-A and UV-B irradiation at relevant sunlight intensity (Köhl *et al.*, 2011; Sartori *et al.*, 2020). Fungal species candidates can be also evaluated for compatibility with the fungicides commonly labeled for controlling the target plant pathogen, because fungicide sprays will indistinctly affect both the fungal pathogen and the biological control agent simultaneously. Species inherent reduced sensitivity to fungicides would be an important phenotype of fungal-based biofungicides.

After eliminating a large part of the microbial biofungicides candidates through the exclusionary steps described above, several types of screening are run for determining their direct or indirect biocontrol activity (Raymaekers *et al.*, 2020).

One of the most common screening for direct antagonism detection is the in vitro "dual culture assay" since it is very simple to execute. The in vitro co-culture of biofungicide agent and fungal plant pathogen is checked for the formation of inhibition zones of the pathogen's growth (Bedine Boat *et al.*, 2020). Other screening tests based on co-cultures in partitioned Petri dishes are used to identify the role of volatiles in the antagonism. Co-cultures in microtiter plates containing liquid medium can be used for simultaneously determining the production of natural compounds in culture filtrates (e.g., essential oils) combined with measurements of the pathogen growth using absorbance readers or its spore germination percentage using light microscope. This screening in microtiter plates can also be used for determining the effective concentration of the biofungicide to inhibit mycelial growth or spore germination in 50% (EC_{50}) or for determining the minimum inhibitory concentration (MIC) (Perina *et al.*, 2019). An alternative to growth measurements, we can determine the biofungicide effect on the respiration activity of the pathogen using the resazurin assay (Jethva *et al.*, 2020).

The methods for assessing the antagonism indirectly are phenotypic- or physiological-based assays (Raymaekers *et al.*, 2020). Phenotypic-based assays take as a parameter the ability of the biocontrol agent to decrease disease severity, lesion development and other disease-associated symptoms such as rotting and wilting. On the other hand, the physiological-based assays are based on the quantitative analyses of specific plant response, such as the production of reactive oxygen species (ROS), coumarin derivatives and occurrence of cell death. ROS production and cellular death can be screened using microplate assays based on specific fluorescent dyes like dichlorofluorescin diacetate (DCFDA) or Evans-blue, respectively (Noutoshi *et al.*, 2012).

The last step in the development of a biofungicide is its formulation. Formulations include adjuvants to the microbial bioactive (i.e., produced in vivo) ingredients in order to ensure its survival (shelf life) and to stabilize the microbial activity at its maximum even at harsh environmental conditions. Therefore, the general industrial manufacturing processes for biofungicides production should include microbial culturing optimization, scale-up fermentation, optimized formulation, appropriate packing and storage allowing the biofungicide to stand long distance transport and deployment. Recent studies demonstrate improvement of the biofungicides efficacy by the incorporation of nanotechnologies in the production steps, such as encapsulation of the microbial bioactive with alginate-silica nanoparticles and carbon nanotubes (Hersanti *et al.*, 2020; Pour *et al.*, 2019).

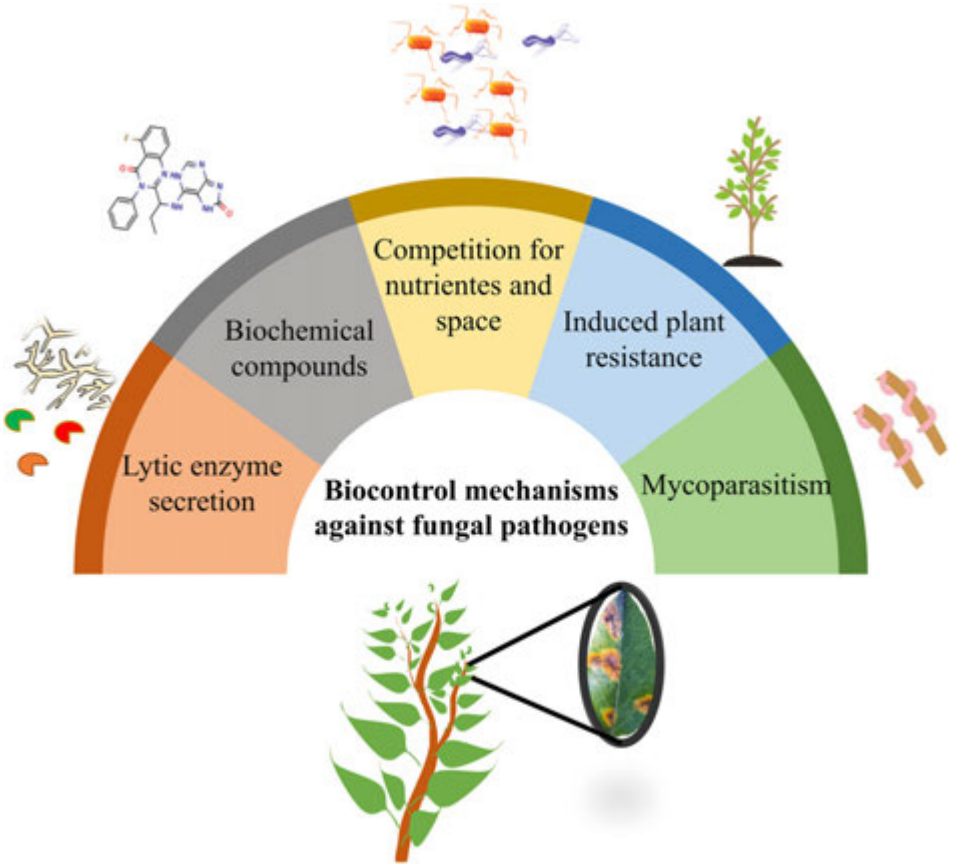

Fig. 1 A summary of the biocontrol mechanisms of action against fungal pathogens.

Biocontrol Mechanisms Against Fungal Plant Pathogens

A biocontrol mechanism of action may be described as the strategy used by a biocontrol microorganism against a plant pathogen (Spadaro *et al.*, 2002).

Several biocontrol mechanisms against fungal phytopathogens have been described (**Fig. 1**), including direct and indirect antagonism between antagonistic and plant pathogen species. Direct interactions may occur through (1) parasitism (mycoparasitism) – parasites invading and killing fungal pathogen's hyphae; (2) antibiosis, by which microbial secreted molecules present an inhibitory effect on pathogens' growth; (3) production and secretion of cell wall and membrane degrading enzymes, and other antimicrobial secondary metabolites such as enzyme inhibitors, antifungal proteins and peptides (Köhl *et al.*, 2019). Indirect interactions may occur via competition for nutrients and space, and by microbial induced enhancement of plant defense responses.

Mycoparasitism is characterized by the growth of the biocontrol fungus towards the pathogen, secretion of cell wall and membrane degrading enzymes to invade and destroy the target pathogen (Crowder and Harwood, 2014). Mycoparasitism is a typical mechanism used by *Trichoderma harzianum* against *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*; *Aspergillus niger* against *Macrophomina phaseolina*; and *Coniothyrium minitans* against *Sclerotinia* sp. (Ram *et al.*, 2018).

Antagonistic activities have also been described by yeasts, including *Metschnikowia pulcherrima*, effective against several fungal species from the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Monilinia* and *Penicillium* (Gore-Lloyd *et al.*, 2019; Oro *et al.*, 2014), *Pichia guilliermondii*, against *Botrytis cinerea* (Freimoser *et al.*, 2019; Wilson *et al.*, 1991; Zhang *et al.*, 2011) *Saccharomycopsis schoenii* against *Candida auris* (Abbey *et al.*, 2019b), besides others species of *Saccharomyces*, *Candida*, *Pichia* and *Aureobasidium* (Freimoser *et al.*, 2019).

There are many examples of antimicrobial secondary metabolites secreted by antagonistic agents (Köhl *et al.*, 2019). These molecules can target, for instance, the fungal pathogen cell wall and membrane or protein synthesis, or inhibit key metabolic enzymes (Ram *et al.*, 2018). Some examples of secondary metabolites with antifungal activity are the Bacillomycin D produced by *Bacillus amyloliquefaciens* strain FZB42 and *B. subtilis* AU195, active against *Fusarium oxysporum*, *Fusarium graminearum* and *Aspergillus flavus*; the Gliotoxin from *T. virens* against *R. solani*; the Iturin A from *B. subtilis* QST713 against *Botrytis* sp. and *R. solani* (Hilber-Bodmer *et al.*, 2017; Oro *et al.*, 2014; Saravanakumar *et al.*, 2008; Spadaro *et al.*, 2002).

A number of small molecular weight proteins with antifungal activities have also been described. Their biochemical characteristics of high content of basic amino acids favor their interaction with the negatively charged cell membrane and may cause membrane permeabilization, induction of ROS and apoptosis (Leiter *et al.*, 2017). Because (1) their primary structure

includes cysteine molecules interacting by disulfide bonds and favoring both thermal and pH stability; (2) their intrinsic inability to select for resistance in fungal plant pathogens, and (3) their lack of cytotoxic effect to plants and animals, these molecules are considered very promising as natural biofungicides (Hegedus and Marx, 2013; Leiter *et al.*, 2017). Examples of these molecules are the antifungal proteins PAF secreted by *Penicillium chrysogenum*, and AFP secreted from *Aspergillus giganteus*. PAF has been described as an effective molecule against filamentous fungi including *B. cinerea*, *Fusarium* species, *Cochliobolus carbonum*, *Trichoderma koningii*, *A. flavus*, *Puccinia graminis*, *Puccinia recondita* (Marx *et al.*, 2008; Leiter *et al.*, 2017; Tóth *et al.*, 2020), and AFP against *Fusarium* species, *B. cinerea*, *Erysiphe graminis*, *Mycosphaerella grisea*, *A. alternata*, and *P. recondita* (Lacadena *et al.*, 1995; Leiter *et al.*, 2017; Oldach *et al.*, 2001; Vila *et al.*, 2001). Competition can be described as active demand between two or more microbial populations for the same limited environmental resource (Wilson *et al.*, 1991). The rhizosphere is an example niche of high nutrient competition between biocontrol microorganisms and plant pathogenic fungi (Mwajita *et al.*, 2013).

The mechanism of enhancing the plant defense against pathogens by microbial biocontrol agents is an important strategy to prevent crops diseases. This microbial inducible plant defense usually occurs in direct response to infection by necrotizing pathogens, resulting in a local or systemic acquired resistance response, or when plants are exposed to contact with beneficial biocontrol microorganisms (Ram *et al.*, 2018; Zhao *et al.*, 2020). Cell components from fungal biocontrol agents, including proteins, chitin and β-1,3 glucan, are known as microbe-associated molecular patterns (MAMPs), which trigger plant resistance mechanisms against phytopathogens. These cell components are recognized by specific plant receptors and can stimulate local or systemic responses in the plant (Köhl *et al.*, 2019).

Another alternative for fungal biocontrol is the application of enzymes to lyse cell wall and membrane. This strategy is supported by the possibility of hydrolysis of the cell structures of the pathogen with reduced damage to the host and the environment. Chitin filaments, β-1,3 glucan and proteins support the fungal cell wall that offers e.g., osmotic resistance to the cell. These components are targets for the attack of enzymes – the hydrolysis of these polymers results in the weakening of the cell wall, making the protoplast a more susceptible target to osmotic pressure.

The main hydrolases of interest in biocontrol of fungi are chitinolytic enzymes or chitinases which can hydrolyze the β-1,4 bonds of N-acetyl glucosamine residues in chitin (Senol *et al.*, 2014); peptidases that are capable to cleave peptide bonds in protein or peptides (Da Silva *et al.*, 2017), and β-1,3 glucanases, which are glycosidases capable of cleaving β-1,3 bonds in glucans (Zhou *et al.*, 2017). In general, these enzymes are important elements in antagonism, as an essential part of mycoparasitism, however, in addition to the interaction between living organisms, these cell-wall degrading enzymes can also be applied as cell free biofungicides.

Chitinases, β-1,3 glucanase and peptidases can cause damage to the fungal cell wall and prevent sporulation (Silva *et al.*, 2019). Furthermore, chitinases are important enzymes for fungal morphogenesis since they lead to hyphal fragmentation and elongation (Langner *et al.*, 2015). Thus, chitinase inhibitors, such as allosamidin and argadin, can compromise the mycelial fragmentation, budding and consequently reduce the fungal growth (Hamid *et al.*, 2013). Cell wall lysis by chitinases has been reported for the control of fungi, such as *B. cinerea* (Fernandez-Caballero *et al.*, 2009), *Fusarium oxysporum*, *Fusarium solani*, *A. alternata*, *R. solani*, *Cladosporium* sp. (Anitha and Rabeeth, 2010; Sharma *et al.*, 2020), *Ceratocystis fimbriata*, *Verticillium dahlia* and *Ustilaginoidea virens* (Liu *et al.*, 2020).

Plant Extracts as Potential Biofungicides

Plants produce a wide range of natural substances such as essential oils and other compounds with antifungal properties that are able to delay and/or inhibit the growth of pathogens (Tyagi and Malik, 2011; Tripathi and Shukla, 2010). Overall, plant extracts are considered non-phytotoxic and potentially effective for the control of phytopathogenic fungi being a sustainable and economically viable alternative for use in agriculture (Bajpai *et al.*, 2008).

The process for obtaining plant extracts in pure form, consists of the maceration of plant material with different solvents, followed by various organic fractionation methods for purification until isolation of the metabolites (Zaynab *et al.*, 2018). Many plant extracts include more than one antifungal compound, which has a great advantage if these compounds have different mechanisms of activity, and therefore, these extracts can lead to a decrease in the development of pathogens resistance (Shuping and Eloff, 2017).

There are many reports demonstrating antifungal activity of plants extracts. Aqueous extracts of *Vachellia nilotica* (previously *Acacia nilotica*) showed antifungal activity against eight species of fungi from the genus *Aspergillus* (Satish *et al.*, 2006). Plants extracts obtained from parts of leaves, flowers, root and fruits from *Trachystemon orientalis*, *Smilax excelsa*, *Rhododendron ponticum*, *Phytolacca americana* and *Prunus laurocerasus* significantly inhibited mycelial growth of three pathogens that cause economically important diseases, i.e., *Alternaria solani*, *B. cinerea* and *Rhizoctonia solani* (Onaran *et al.*, 2016).

Essential oils are highly volatile compounds, naturally produced by aromatic plants and reproduced by steam distillation techniques. The term *essential oil* refers not only to complex oils isolated from plants, but also to their constituent components, which are lipophilic molecules that volatilize at low temperatures and give the characteristic odor (Floudas *et al.*, 2012; Raveau *et al.*, 2020).

The oils are usually complex mixtures of several low molecular weight components with varying concentrations. The constituent compounds of essential oils may be responsible for the antimicrobial characteristics, which can be attributed to the presence of various terpenoid and phenolic compounds (Bakkali *et al.*, 2008).

Secondary metabolites are bioactive compounds that are toxic to the pathogens (Amiri *et al.*, 2017; Tripathi and Shukla, 2010). Based on biosynthetic pathways, they can be classified into three main groups: phenolic, terpenes and nitrogen compounds. Phenolic compounds are among the secondary metabolites produced by plants, which have important functions in growth, pigmentation, reproduction and resistance to pathogens (Lattanzio *et al.*, 2006). During the pathogen attack in the host tissues, some antibiotic phenolics that are stored in plant cells as inactive bound forms, are readily converted into biologically active antibiotics by plant hydrolyzing enzymes (glycosidases). Many studies have demonstrated phenolic compounds with antifungal properties against various pathogens, such as *B. cinerea* and *P. oryzae* (Lattanzio *et al.*, 2006; Beckman, 2000).

Terpenes represent the largest class of secondary metabolites and are synthetized in the cytoplasm of plant cells through the mevalonic acid pathway. In general, they are classified by the number of isoprene units used to build them, i.e., monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids (Singh and Sharma, 2015).

Gossypol is a sesquiterpenoid produced naturally by cotton *Gossypium hirsutum*, which has antifungal properties that demonstrated strong growth inhibition activity against many economically important fungi, such as *R. solani* and *F. oxysporum* (Mellon *et al.*, 2014).

Finally, alkaloids represent a diverse group of nitrogen-containing basic natural compounds that are found in many vascular plants (Coley and Barone, 2001). Many alkaloids are synthesized from common amino acids such as lysine, aspartate, tyrosine and tryptophan (Freeman and Beattie, 2008). Caffeine is a purine alkaloid found in coffee (*Coffea arabica*), tea (*Camellia sinensis*) and cocoa (*Theobroma cacao*). It has a common toxic effect in a range of pathogenic fungi as *Botryosphaeria dothidea* and *Aspergillus nidulans* (Yuvamoto and Said, 2007).

Biofungicides in the Post-Genomic Era

Through the last decades, most of the findings and observations on biofungicides research have been based on traditional microbiological and biochemical techniques (Massart *et al.*, 2015). Although these approaches have extensively contributed to this field, the development of high-throughput technologies has rapidly emerged in new tools towards improving the efficiency of biological control agents against fungal phytopathogens. A number of recent combinatorial approach of genomics, transcriptomics, proteomics, metabolomics, and strain engineering have significantly advanced our understanding of the molecular mechanisms involved in this process (**Fig. 2**).

Genome-level information on relevant biocontrol species is a fundamental starting point for many unanswered and novel aspects of their potential and overall biology. *Trichoderma*-based biofungicides have been widely used in agriculture and successfully commercialized due to their ability to induce a combination of antagonistic responses against plant pathogens. In 2008, the complete genome of *Trichoderma reesei* was released (Martinez *et al.*, 2008), which provided a deeper understanding about its potential for plant biomass degradation. Some years later, the genome sequencing of the two biocontrol species *T. atroviride* and *T. virens* were analyzed and revealed new insights into the complex repertoire of genes involved in mycoparasitism, which explained their abilities to antagonize fungal plant pathogens through the production of plant biomass degrading enzymes and antibiotics. More recently, the genome of *Trichoderma atrobrunneum* (*T. harzianum* species complex) ITEM 908 reported a wide range of carbohydrate active enzymes (CAZymes) encoding genes, proteins involved in secondary metabolites production and siderophores potentially involved in parasitism, saprotrophic degradation as well as in biocontrol and antagonistic activities (Fanelli *et al.*, 2018).

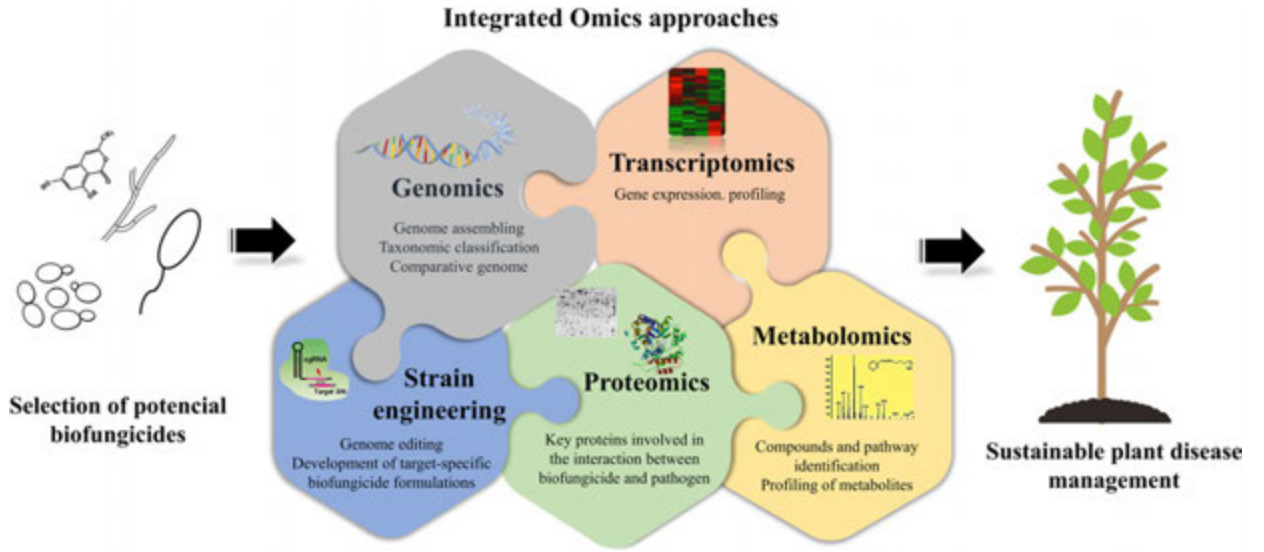

Fig. 2 The puzzle of high-throughput omics analyses towards a sustainable plant disease control.

Research on biofungicide agents and plant defense mechanisms is changing in the advent of proteomics technologies. Proteomics refers to the qualitative and quantitative analysis of all proteins produced by a genome (Massart *et al.*, 2015). In 2004, the proteome of the biocontrol agent *T. harzianum* was the first to become available. *T. harzianum* secretes a range of cell wall-degrading enzymes (CWDEs) that break down the cell wall of phytopathogenic fungi (Grinyer *et al.*, 2004).

Metabolomics greatly improved our understanding of global metabolic characteristics, elucidation of metabolic mechanisms and identification of metabolic biomarkers (Peng *et al.*, 2015). Once the composition of the metabolites is known through different techniques, it is possible to predict the activation of certain genes and understand the molecular mechanisms.

Comparative transcriptome profiling has become affordable and easy to describe the metabolic processes and contributing to understand adaptation mechanisms and lifestyle of biofungicides agents to an unfamiliar environment (Hasin *et al.*, 2017).

B. cinerea is an airborne plant pathogen responsible for one of the most destructive fungal disease of fresh fruits and vegetables (Wilson *et al.*, 1991). Genomic information of *B. cinerea* has become an important model system to elucidate the molecular basis of other fungal pathogens (Choquer *et al.*, 2007). To avoid the potential damages of several chemical fungicides, the application of beneficial and antagonist microorganisms has emerged as an ecofriendly alternative against *B. cinerea*. Chitosan has a broad-spectrum antimicrobial activity and has been successfully used to protect grapes against *B. cinerea* (Hua *et al.*, 2019; Vega *et al.*, 2020). Transcriptomic and metabolomic analyzes revealed that chitosan significantly improves the resistance of grapefruit to *B. cinerea* by activating grape disease resistance-related signals and plant hormone signals (Zhang *et al.*, 2020).

The soilborne fungus *Helminthosporium solani* is the causal agent of silver scurf, a disease with significantly economic consequences, which affect the quality of potato. The biofungicide agent *Clonostachys rosea* strongly reduced the severity of silver scurf symptoms. Transcriptomics analysis was performed during the interaction between the biofungicide and the pathogen. The gene expression of *H. solani* was significantly reduced when inoculated with *C. rosea*, whereas gene expression of *C. rosea* was upregulated. Transcriptomic analysis demonstrated that the mode of action of *C. rosea* as biofungicide agent is a combination of mechanisms, including mycoparasitism, microbial competition for nutrients and antibiosis (Lysøe *et al.*, 2017).

Transcriptomic analysis has also identified gene clusters involved in the synthesis of antifungal secondary metabolites of *C. rosea*. *C. rosea* strain ACM941 is a patented biofungicide against *Fusarium* head blight disease, caused by several species of the genus *Fusarium*. A greater level of understanding in the behavior of the mycoparasite *C. rosea* could provide the improvement of environmentally friendly biofungicide agents (Demissie *et al.*, 2018).

Another prominent biofungicide widely used in agriculture is the nucleoside antibiotic wuyiencin, which is a secondary metabolite produced by the bacterium *Streptomyces ahygroscopicus* var. *wuyiensis* (Liu *et al.*, 2014). A study based on transcriptomics data analysis reported the pathway and regulatory mechanisms of wuyiencin involved in the growth and differentiation of *B. cinerea*. The expression of genes involved in DNA replication, cell cycle and stress response were significantly downregulated. Furthermore, this nucleoside antibiotic has a remarkable effect on primary metabolism in *B. cinerea* by activating the expression of genes involved in amino acid metabolism, such as valine, leucine, and isoleucine degradation (Shi *et al.*, 2020).

A revolutionary method for the modification of genes based on knowledge of the functional genome has allowed advances in the alteration of specific genes. Nowadays, the clustered regularly interspaced short palindromic repeats (CRISPR) is becoming the most popular genome-editing technology (Kun *et al.*, 2019). The system consists of an endonuclease, such as multidomain Cas9 with a single enzymatic activity guided by a single guide RNA (sgRNA) that match with the target DNA region followed by a short Protospacer Adjacent Motif (PAM) and a downstream chimeric scaffold sequence (gRNA scaffold) (Borrelli *et al.*, 2018). Recently, CRISPR/Cas9 was established to edit the genome of the entomopathogenic fungus *Beauveria bassiana* via blastospore transformation, thus showing that this technology allows significant advances in functional genome studies (Chen *et al.*, 2017).

In conclusion, molecular biology technologies have an exciting potential for applicability in biological control. While strain engineering of biofungicides agents is still in its infancy, we are learning with the support of omics technologies research how to introduce these biofungicides in the market to overcome the challenges of agriculture. It requires appropriate use of synergistic combinations, as well as multidisciplinary studies aimed at a comprehensive assessment of the impact on ecological systems and human health. Resistance, environmental impact, economic debates, technology efficiency, risk analysis, regulation and policies are prominent issues to be analyzed for the future use of these innovations.

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Degradation of Plastics by Fungi

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Introduction

Plastic is a term describing a wide range of high molecular weight polymers formulated by polyaddition or polymer condensation reactions (Frank and Biederbick, 1984). According to their melting properties, two types of polymers can be distinguished: thermoplastics and thermosets. Thermoplastics can be reversibly melted by heating and hardened again upon cooling allowing the material to be reshaped repeatedly. Ubiquitous types and uses are polypropylene (PP; pipes, containers); polyethylene (PE; bottles, grocery bags); polyvinylchloride (PVC; pipes, packaging); polystyrene (PS; insulation material, food containers, disposable cups); polyethylene terephthalate (PET; beverage bottles, textile fibers), polyamides (PA; fibers, construction), polycaprolactone (PCL; production of PU, biomedical applications). (**Fig. 1**). In contrast, thermoset polymers cannot be reshaped once heated and their chains are highly cross-linked. A common example are polyurethanes (PU; insulating foams, mattresses, coatings, adhesives). Several hundred million tons of synthetic polymers are produced worldwide annually. It has been estimated that by 2050 about 12,000 million tons of plastic waste will be accumulating in the oceans and in landfills if current waste management strategies will persist (Geyer *et al.*, 2017). The plastic waste found in the environment is mainly composed of synthetic polymers made from fossil feedstocks. Common characteristics are their recalcitrance against biological degradation due to their high molecular weight, high crystallinity, and hydrophobicity which renders them resistant to attack by microorganisms and their enzymes. So-called biodegradable plastics can be mineralized in the environment by microorganisms, including fungi, and used for cell growth. Examples are the aliphatic polyesters poly(3-hydroxybutyrate) (PHB), polycaprolactone (PCL), and polylactic acid (PLA) used to manufacture, i.e., bottles, containers, and cups (Tokiwa *et al.*, 2009). The rate and degree of their biodegradation in the environment is however depending inter alia on their molecular weight, their degree of crystallinity, the molecular orientation of the polymer chains, and their melting temperature.

While polyesters, especially aliphatic polyesters like PCL, PLA, and PHA are more amenable to microbial degradation, non-hydrolysable synthetic polymers such as PE, PP, PVC, and PS are considerably more resistant to biodegradation.

Plastics in the Environment

The global production of plastics has continuously increased over the last 50 years reaching 360 million tons in 2018 (PlasticsEurope, 2019). This ever-increasing production has resulted in staggering amounts of plastic waste accumulating in landfills and in the oceans due to littering, industrial discharges, and waste mismanagement. Once in the environment, plastic materials will disintegrate over time due to both biotic and abiotic factors such as UV radiation and mechanical disruption to produce debris of various sizes. Together with discarded plastic particles in the form of granulates, microbeads and pellets, they are ultimately forming microplastics with a diameter of <5 mm and nanoplastics with a diameter of <1 μm (Sivan, 2011). The potential threat of micro- and nanoplastics, especially in the

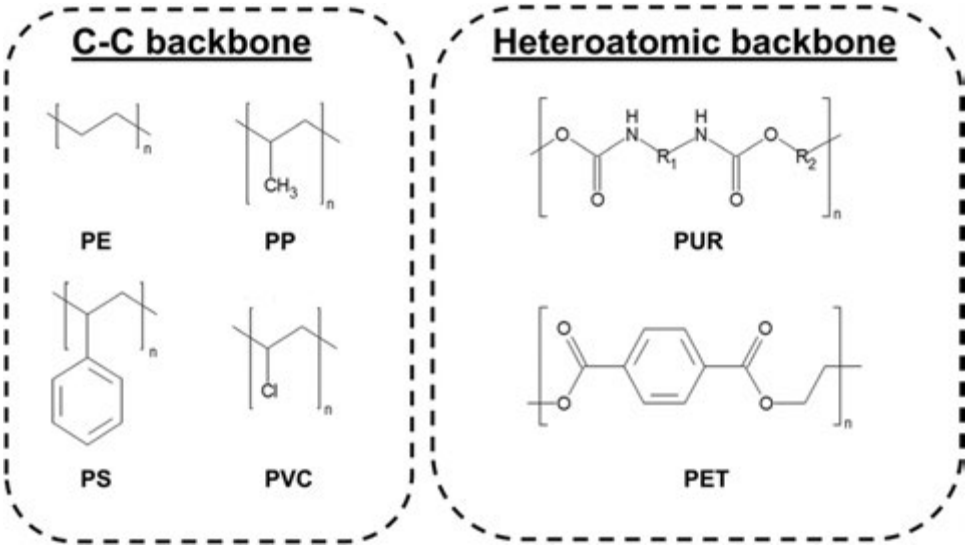

Fig. 1 Backbone structural formula of widely used petroleum-based plastics. Reproduced from Wei, R., Zimmermann, W., 2017. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: How far are we? Microbial Biotechnology 10 (6), 1308–1322.

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marine environment, to human and animal health by entering the food web is of growing concern (Ivar do Sul and Costa, 2014; Kong and Koelmans, 2019; Eerkes-Medrano *et al.*, 2015; Smith *et al.*, 2018; de Souza Machado *et al.*, 2018).

While many publications have reported effects of fungi and their enzymes on plastic materials, such as morphological changes, weight reduction of the materials, changes in molecular size of the polymers, etc., a complete breakdown of the polymers to CO_2 or CH_4 and biomass unequivocally confirmed, i.e., by respirometry analyses has been rarely demonstrated. While some fungi that have been found attached to plastic in the environment may be able to attack the high molecular weight polymers, others may only utilize additives or oligomers generated by abiotic factors or by biofilm consortium members. However, several fungi and their hydrolytic enzymes have shown high activity against high molecular weight polyester-type plastics, specifically aliphatic polyesters. Polyesters containing aromatic moieties such as PET are however considerably more resistant to biodegradation (Müller *et al.*, 2001).

Plastic materials are often degraded in a natural environment by the synergistic action of both prokaryotic and eukaryotic microorganisms (Nishida and Tokiwa, 1993; Reisser *et al.*, 2014). For example, efficient degradation of PE by a mixed culture of the fungus *Aspergillus niger* and the bacterium *Lysinibacillus xylanilyticus* and synergistic effects on the weight reduction of a modified PE caused by a biofilm composed of a fungal and bacterial, i.e., *Penicillium* and *Bacillus*, species have been described (Esmaeili *et al.*, 2013; Seneviratne *et al.*, 2006). Strong synergistic effects of a mixed culture of *A. niger* and a bacterial species *Pseudomonas aeruginosa* on the degradation of PU have also been reported (Fernandes *et al.*, 2016).

The formation of a biofilm by a consortium of microorganisms, including fungi, on the surface of plastic materials is considered a major first step for a substantial biodegradation to occur in the environment (Mathur *et al.*, 2011; Ghosh *et al.*, 2019; de Tender *et al.*, 2017). In this process, hydrophobins, i.e., hydrophobic proteins produced by many filamentous fungi, may render them more capable of attacking plastic materials compared to bacteria. The hydrophobic surface layers formed by hydrophobins are enabling the attachment of fungal hyphae to the hydrophobic surfaces of plastics (Kershaw and Talbot, 1998). In addition, filamentous fungi can penetrate and disrupt plastic substrates by their invasive hyphal growth, contributing to the disintegration of the material in the course of the biodegradation process (Money, 2007).

In aquatic environments, the detection of microorganisms, including fungi, in biofilms has suggested the presence of plastic-specific species (Kirstein *et al.*, 2019). Information on the biodiversity of fungal communities on plastic debris in the aquatic environment is however still limited (Amaral-Zettler *et al.*, 2020). An analysis of fungi attached to PE and PS in aquatic environments has identified 81 fungal taxa, among them mainly members of Chytridiomycota, Cryptomycota, and Ascomycota (Kettner *et al.*, 2017). However, it has been discussed that many microorganisms growing on microplastics belong in fact to opportunistic colonists, which do not display plastic-degrading activities (Lobelle and Cunliffe, 2011; Oberbeckmann and Labrenz, 2019; Debroas *et al.*, 2017).

Reported indications of fungal attacks on plastic substrates are the appearance of cracks, pits, and holes, surface erosions and weight reduction of the materials as well as changes of the molecular weight of the polymer and the formation of new functional groups. Once a high molecular weight plastic polymer is reduced in size by abiotic factors or by the action of extracellular enzymes produced by microorganisms attached to its surface, the oligomers and monomers can be taken up by the cells and further utilized by microorganisms with appropriate metabolic pathways (Hakkarainen, 2002; **Fig. 2**).

Fungal Degradation of Polymers

Polymers Containing Ester Bonds

Polyethylene terephthalate (PET)

PET is a synthetic linear thermoplastic composed of ethylene glycol and terephthalic acid. Depending on its processing and thermal treatment, PET contains amorphous and crystalline regions. While the crystalline regions are resistant to microbial attack, several fungal enzymes hydrolyzing the amorphous part of PET have been identified (Wei and Zimmermann, 2017).

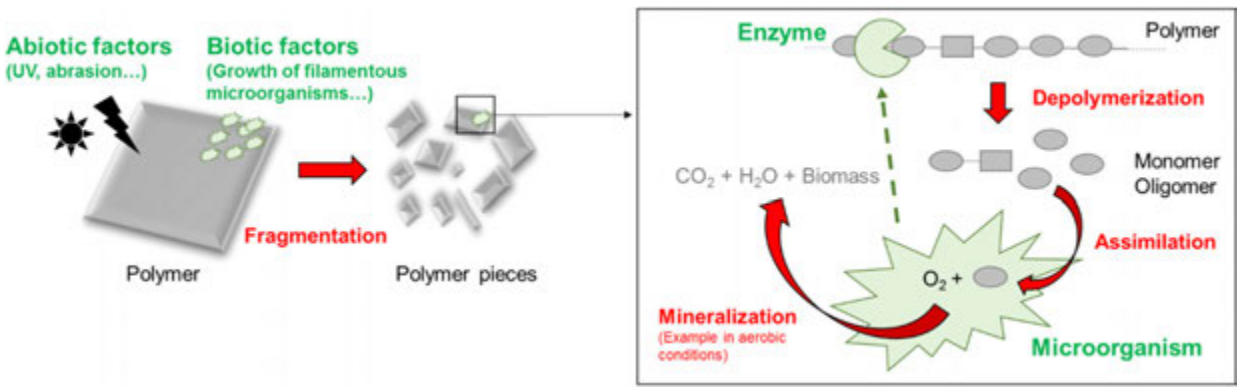

Fig. 2 Polymer biodegradation process. Reproduced with permission from Magnin, A., Pollet, E., Phalip V., Avérous, L., 2020. Evaluation of biological degradation of polyurethanes. Biotechnology Advances 39, 107457. doi:10.1016/j.biotechadv.2019.107457.

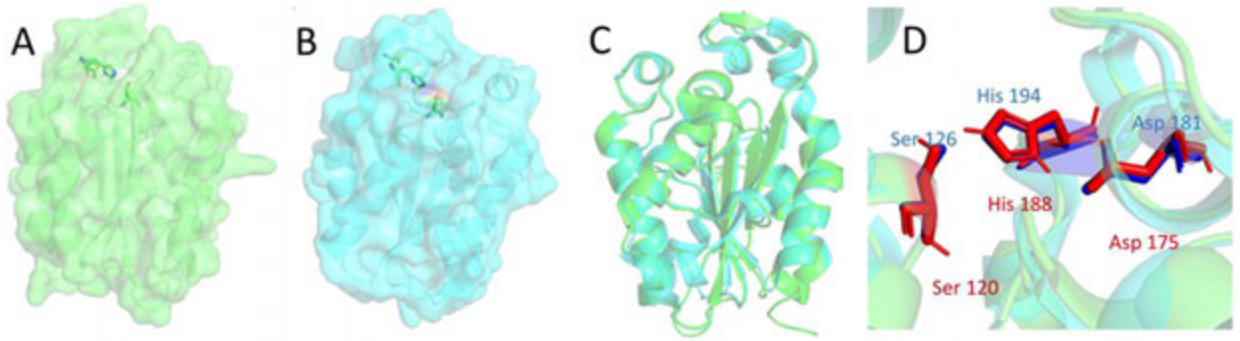

Fig. 3 Three-dimensional structures of cutinases from *Fusarium solani* (PDB code 1CUS) (A) and *A. oryzae* (PDB code 3GBS) (B). Superposition of *A. oryzae* (cyan) and *F. solani* (green) cutinases revealing nearly identical structural similarity (C). Superposition of catalytic triad of *A. oryzae* (blue) and *F. solani* (red) cutinases (D). Reproduced with permission from Urbanek, A.K., Mirończuk, A.M., García-Martín, A., *et al.*, 2020. Biochemical properties and biotechnological applications of microbial enzymes involved in the degradation of polyester-type plastics. Biochimica et Biophysica Acta – Proteins and Proteomics 1868 (2), 140315.

Phytopathogenic fungi such as *Fusarium solani* produce cutinases (EC 3.1.1.74) to degrade the cuticula in higher plant cell walls in the course of the infection process (Purdy and Kolattukudy, 1975; **Fig. 3**). Cutinases are esterases and members of the family of α/β-hydrolases (Longhi and Cambillau, 1999). In contrast to typical lipases, cutinases show an exposed and flexible active site accessible to polymeric substrates allowing activity against synthetic polyesters such as PET. The cutinase of *F. solani* has been studied in detail (Zimmermann and Billig, 2010; Martinez *et al.*, 1992; Longhi *et al.*, 1997; Egmond, 2000). The enzyme has a molecular mass of 22 kDa and consists of 197 amino acids with four cysteines forming two disulfide bridges. Besides PET, the enzyme hydrolyzes a wide range of esters, including PCL. A strain of *Fusarium oxysporum* isolated from plant samples in the tropics produced a cutinase able to release terephthalic acid from highly crystalline PET fibers with a higher efficiency compared to the enzyme from *F. solani* (Nimchua *et al.*, 2007). The enzyme from *F. oxysporum* has been further characterized. While its crystal structure showed only minor differences from the *F. solani* cutinase, distinct surface properties observed could explain its better performance in the hydrolysis of PET (Dimarogona *et al.*, 2015). A cutinase from *Glomerella cingulata*, another fungal phytopathogen, hydrolyzed PET model compounds and PCL. Analysis of its structure indicated that the enzyme undergoes a conformational rearrangement during its catalytic cycle (Nyon *et al.*, 2009).

The thermophilic soft-rot fungus *Thermomyces insolens* produced a thermostable cutinase able to hydrolyze both amorphous and biaxially oriented PET films of high crystallinity close to its glass transition temperature resulting in high degradation rates. Hydrolysis of amorphous PET films at 70°C resulted in an almost 100% weight reduction after 96 h of reaction with the enzyme, corresponding to a reduction of film thickness of 30 μm per day (Ronkvist *et al.*, 2009). A similar PET- and PCL-degrading cutinase from *Thermomyces lanuginosus* has also been characterized (Fernandez-Lafuente, 2010). A PET-degrading cutinase from another thermophilic fungus, *Thielavia terrestris*, was stable up to 65°C (Yang *et al.*, 2012). *Penicillium* species have also been reported to be involved in PET degradation (Sepperumal *et al.*, 2013). A *Penicillium citrinum* strain produced a cutinase that partially hydrolyzed PET fibers (Liebminger *et al.*, 2007).

Since synthetic polyesters are not the natural substrates for cutinases, efforts have been made to improve their activity by genetic engineering strategies. The cutinase from *F. solani* was successfully modified by enlarging the active site of the enzyme to better accommodate PET polymer chains (Araújo *et al.*, 2007).

Polyurethanes (PU)

PU are heteropolymers synthesized from a wide range of polyols and isocyanates and can be thermoplastics or thermosets with different macromolecular architecture and susceptibility to fungal attack. As observed with other types of plastics, crystalline regions in the polymer restrict the accessibility of the PU chains to biological degradation while the amorphous regions are more amenable to biodegradation. PU can contain ether or ester, and urethane bonds. The latter are susceptible to microbial degradation and many soil- and compost-inhabiting fungi, among them strains of *Aspergillus, Alternaria, Gliocladium, Fusarium, Candida, Monascus,* and *Cladosporium* have been described to be involved in the hydrolysis of polyester-PU (Darby and Kaplan, 1968; Pommer and Lorenz, 1985; Barratt *et al.*, 2003; Mathur and Prasad, 2012; Khan *et al.*, 2017; Osman *et al.*, 2017; Shuttleworth and Seal, 1986; Zafar *et al.*, 2013; El-Morsy, 2017).

A large number of fungi, among them genera of *Aspergillus, Trichoderma, Paecilomyces, Penicillium, Alternaria, Cladosporium,* and *Fusarium* were able to grow on PU as a sole carbon and nitrogen source (Filip, 1979; Loredo-Treviño *et al.* 2011; Lugauskas *et al.*, 2003). An *Aspergillus flavus* isolate utilizing PU as sole carbon source caused a 60% reduction in weight of a PU film within 30 days of incubation in liquid cultures (Mathur and Prasad, 2012). *Geomyces pannorum* and a strain of *Phoma* were the dominant species detected colonizing PU buried in garden soils in the UK. Both strains degraded a polyester-PU suspension (Cosgrove *et al.*, 2007). An *Alternaria solani* strain isolated from a soil sample in Jordan caused more than 60% weight reduction of polyester-PU after three weeks of incubation (Ibrahim *et al.*, 2009). *Cladosporium* spp., *Aspergillus fumigatus, Aspergillus tubingensis* and *Penicillium chrysogenum* strains also

degraded polyester-PU suspensions and solid PU foams (Khan *et al.*, 2017; Álvarez-Barragán *et al.*, 2016). One of the isolates, *Cladosporium pseudocladosporioides*, degraded almost 90% of a polyester-PU suspension after 14 days of incubation. Another strain of *Penicillium* of the section Lanata-Divaricata was found to be an efficient degrader of a PCL-based thermoplastic PU (Magnin *et al.*, 2018).

Several endophytic fungi have also been reported to efficiently degrade polyester-PU. Among them, *Pestalotiopsis* isolates from the Amazonian rainforest and from Malaysia could grow on PU as the sole source of carbon at both aerobic and anaerobic conditions (Russell *et al.*, 2011; Bong *et al.*, 2017). Isolated from Lake Zurich, Switzerland, *Cladosporium cladosporioides*, *Xepiculopsis graminea*, *Penicillium griseofulvum* and *Leptosphaeria* sp. likewise degraded polyester-PU suspensions (Brunner *et al.*, 2018).

Most of the fungal enzymes described to be involved in PU degradation are hydrolases active against the ester and urethane bonds in polyester-PU. Esterases from *Rhizopus delemar* (Tokiwa *et al.*, 1988), *Curvularia senegalensis*, *Aureobasidium pullulans*, *F. solani*, *Cladosporium* sp. (Crabbe *et al.*, 1994) and a serine hydrolase from *Pestalotiopsis microspora* have been described (Russell *et al.*, 2011). Lipases (EC 3.1.1.3) from a number of yeasts, among them *Candida rugosa* (Gautam *et al.*, 2007), *Candida antarctica* (Takamoto *et al.*, 2001), and *Candida cylindracea* (Kim and Kim, 1998) were also able to hydrolyze polyester-PU samples. Specific hydrolysis of the urethane bonds in PU has been less frequently reported. Polyether-PU displays a high resistance to microbial degradation (Darby and Kaplan, 1968). A strain of *Alternaria* has been shown to secrete enzymes degrading the urethane bonds in polyether-PU (Matsumiya *et al.*, 2010). Furthermore, an *Alternaria tenuissima* strain was isolated which caused structural changes in polyether-PU chain-extended with pyridine derivatives (Oprea *et al.*, 2017) and an esterase and urease (EC 3.51.5) producing strain of *C. pseudocladosporioides* caused up to 65% weight reduction from a polyether-PU foam (Álvarez-Barragán *et al.*, 2016).

Polylactid acid (PLA)

PLA is a thermoplastic aliphatic polyester with monomers derived from plant biomass.

The polymer shows a higher resistance against microbial attack compared to other polyesters and only few reports on its biodegradation in the environment are available. Fungi with activity against PLA and PLA oligomers have been detected in the genera *Tritirachium*, *Fusarium*, *Rhizopus*, and *Penicillium* (Jarerat and Tokiwa, 2001). The PLA-hydrolyzing enzyme from *Tritirachium album* was identified as a serine protease (Williams, 1981; Torres *et al.*, 1996). Likewise, a cutinase from *Aspergillus oryzae* and from a *Cryptococcus* yeast strain also hydrolyzed PLA (Maeda *et al.*, 2005; Masaki *et al.*, 2005).

Polycaprolactone (PCL)

PCL, poly(hexano-6-lactone) is a synthetic partially crystalline linear polyester mainly used for the production of PU. Several filamentous fungi have been reported to degrade PCL to water-soluble products (Kim and Rhee, 2003). The degradability of PCL by fungi depends on its molecular weight and degree of crystallinity. Similar to other polyesters, a PCL of high molecular weight and a high degree of crystallinity is generally more difficult to degrade (Cook *et al.*, 1981). For example, *Pullularia pullulans* only degraded low molecular weight PCL (Mw = 2.000) in three weeks at 30°C (Fields *et al.*, 1974). *Aspergillus*, *Penicillium*, *Chaetomium*, and *Fusarium* species were able to also degrade PCL of higher molecular weight up to 35,000 (Tokiwa *et al.*, 1976; Benedict *et al.*, 1983; Vivi *et al.*, 2018). A thermotolerant *Aspergillus fumigatus* strain and a thermophilic *Thermoascus aurantiacus* strain degraded high molecular weight PCL (Mw = 67,300) at 50°C (Sanchez *et al.*, 2000). A strain of *Clonostachys (Gliocladium) rosea* isolated from Arctic soil degraded more than 30% of PCL films during an incubation for 30 days even at a low temperature of 21°C (Urbanek *et al.*, 2017).

PCL-hydrolyzing fungal enzymes such as lipases and esterases have been identified in *Rhizopus* spp. (Tokiwa and Suzuki, 1977).

Several phytopathogenic fungi such as *Alternaria alternate*, *Colletotrichum lagenarium*, *Fusarium culmorum*, and *Penicillium oxalicum* formed clear zones on agar plates containing PCL suggesting the secretion of PCL-degrading enzymes (Tokiwa and Calabia, 2007; Li *et al.*, 2012). At least one of the enzymes detected in *Fusarium* spp. was identical with a cutinase, while a lipase has also been described to be involved in PCL hydrolysis by this strain (Murphy *et al.*, 1996, 1998).

Other fungal cutinases are also able to hydrolyze PCL. Examples are the enzymes from *Thielavia terrestris*, *Alternaria brassicicola*, *A. fumigatus*, *A. oryzae*, *Humicola insolens*, and the yeasts *Cryptococcus* and *Arxula adeninivorans* (Masaki *et al.*, 2005; Yang *et al.*, 2012; Baker *et al.*, 2011; Liu *et al.*, 2009; Bischoff *et al.*, 2015; Ping *et al.*, 2017).

Polyhydroxyalkanoates (PHA)

PHA are polyesters produced by a wide range of bacteria as energy storage compounds. Poly-3-hydroxybutyrate (PHB) has been extensively studied as a biodegradable non-toxic alternative to conventional synthetic polymers (Lichtenthaler, 2010). Many filamentous fungi and yeasts produce enzymes able to degrade PHB and its co-polyesters with 3-hydroxyvalerate to non-toxic water-soluble oligomers and monomers (Matavulj and Molitoris, 1992; Kim and Rhee, 2003; Tokiwa and Calabia, 2004). While *Penicillium* and *Aspergillus* were among the prevalent genera, *Fusarium*, *Trichoderma*, and *Alternaria* have also been identified as degraders of PHA in soils and compost (Mergaert *et al.*, 1992; Lee *et al.*, 2005; Aburas, 2016). A thermotolerant strain of *Aspergillus* degraded more than 90% of PHB films within 3–6 days of incubation at 50°C (Sanchez *et al.*, 2000).

PHB-depolymerizing enzymes hydrolyze the polymer to water-soluble products. A number of extracellular PHB hydrolases, mainly from *Penicillium* as well as from *Aspergillus*, *Emericellopsis*, *Paecilomyces*, *Fusarium*, and *Cryptococcus* species have been characterized (Kim and Rhee, 2003; Shivakumar *et al.*, 2011; Panagiotidou *et al.*, 2014; Oda *et al.*, 1995; Bhatt *et al.*, 2010; Scherer *et al.*, 1999; Iyer *et al.*, 2000; Jung *et al.*, 2018; Oda *et al.*, 1997; Sang *et al.*, 2006; Han *et al.*, 1998; Han and Kim, 2002; Liu *et al.*, 2006; Su-Qin *et al.*, 2006; Kim *et al.*, 2002; Gowda and Shivakumar, 2015; Shivakumar, 2013; Masaki *et al.*, 2005; Li *et al.*, 2019; Zhou *et al.*, 2008). The PHB hydrolase from *Penicillium funiculosum* was identified as a serine esterase (Brucato and Wong, 1991). It

showed a high hydrolytic activity also against 3-hydroxybutyrate oligomers (Miyazaki *et al.*, 2000). The elucidation of the structure of this enzyme indicated the presence of a number of subsites for binding the PHB substrate. The PHB polymer chain was supposed to bind to a cleft at the surface of the enzyme by hydrophobic interaction with several hydrophobic residues located around the cleft (Hisano *et al.*, 2006).

Polyamides (PA)

Synthetic PA such as nylons are linear polymers containing amide bonds. PA are considered to be very resistant to biological attack due to the high symmetry of their polymer structure.

The white-rot fungi *Phanerochaete chrysosporium*, *Trametes versicolor* and the fungal isolate IZU-154 degraded membranes made of nylon-66 (poly[imino(1,6-dioxohexamethylene) iminohexamethylene]) (Deguchi *et al.*, 1997). *P. chrysosporium and T. versicolor* also slowly degraded fibers of nylon-6 (poly[hexano-6-lactam]), a semicrystalline PA (Klun *et al.*, 2003). The enzyme degrading nylon-66 produced by the fungal isolate IZU-154 was further characterized and identified as a manganese peroxidase (EC 1.11.1.13), which is also a part of the lignin-degrading enzyme system of white-rot fungi. Lignin, an important structural component of plant cell walls, is a heterogeneous aromatic polymer composed of phenyl propane units crosslinked by various ether and C-C bonds. The enzyme degraded nylon apparently by an oxidative process, and not by a hydrolytic process, to soluble oligomers with a different reaction mechanism as described for the degradation of lignin by this enzyme (Deguchi *et al.*, 1998). It was assumed that methylene groups adjacent to the nitrogen atoms in the polymer were attacked by the enzyme and that the degradation of the PA subsequently proceeded via an auto-oxidative mechanism (Nomura *et al.*, 2001). Nylon-66 membranes were also degraded by a laccase isolated from *T. versicolor* in the presence of 1-hydroxybenzotriazole. The laccase caused a disintegration of the membranes and reduced the molecular weight and polydispersity of the PA (Fujisawa *et al.*, 2001). A manganese peroxidase was also produced by *Bjerkandera adusta* incubated with nylon-6 (poly[hexano-6-lactam]) in a liquid medium. The white-rot fungus disintegrated the nylon fibers and formed soluble degradation products (Friedrich *et al.*, 2007). The degradation of nylon-4 (polybutyrolactam) and its use as a carbon source by a *Fusarium* isolate has been reported (Tachibana *et al.*, 2010). Nylon-4 is however more susceptible to biological degradation compared to other synthetic PA (Hashimoto *et al.*, 1994).

Non-Hydrolysable Synthetic Polymers

Polyolefins (polyethylene and polypropylene)

The synthetic polymers PE and PP made from petrochemical feedstocks are polyolefins produced by polymerizing ethylene or propylene, respectively. Polyolefins are the most widely produced and commercialized synthetic polymers with a concomitant important contribution to the global plastic waste problem. Composed of saturated high molecular weight hydrocarbons, a partial crystallinity and chemical resistance against acids and bases, polyolefins show a high resistance against microbial and enzymatic attacks. Depending on the extent of branching and the molecular weight, various types of PE and PP with different mechanical properties can be produced, such as high-density PE/PP (HDPE/HDPP) and low-density PE/PP (LDPE/LDPP), among others. The slow degradation of PE and PP observed in the environment appears to be mainly the result of a combination of UV, thermal, mechanical, and biotic processes (Hakkarainen and Albertsson, 2004). The abiotic factors result in a decrease of the molecular weight of the polyolefins and the introduction of new functional groups in the polymer chain rendering them more amenable to a subsequent biological degradation.

A large number of fungi colonizing and attacking PE have been described. The establishment of a biofilm on the highly hydrophobic surface of polyolfins is facilitated by the formation of hydrophobins produced by many fungi. Fungal strains have been isolated from a variety of often tropical habitats such as soils, composts, and aquatic sources. Among them, *Aspergillus* and *Penicillium* species have been frequently detected. For example, *P. oxalicum* and *P. chrysogenum* isolates degraded more than 50% of HDPE and LDPE sheets after 90 days of incubation. The surface of the degraded sheets showed cracks and grooves as evidenced by atomic force microscopic (AFM) and scanning electron microscopic (SEM) analysis (Ojha *et al.*, 2017; **Fig. 4**). An *A. flavus* strain isolated from the guts of the wax moth *Galleria mellonella* degraded HDPE microplastic as indicated by a reduction of the molecular weight and the formation of carbonyl and ether groups in the PE evidenced by Fourier transform – infrared (FTIR) spectroscopy (Zhang *et al.*, 2020). *Trichoderma*, *Cladosporium*, *Chaetomium*, *Fusarium*, *Gliocladium*, *Mucor*, *Phanerochaete*, and *Lasiodiplodia* are examples of other genera detected which caused structural and surface changes, weight reductions or changes in the molecular weight distribution of PE samples. Mostly LDPE has been studied as substrate, some of which was pretreated chemically, thermally or by UV/ gamma irradiation (Restrepo-Flórez *et al.*, 2014; Yamada-Onodera *et al.*, 2001; Sowmya *et al.*, 2014a; Bonhomme *et al.*, 2003; Arutchelvi *et al.*, 2008). To which extent the fungi reported have been able to attack high molecular weight PE is not clear in all observed cases. A mixed culture of the white-rot fungi *Fomes annosus*, *Peniophora gigantea* and *Odontia bicolor*, as well as a culture of *Fusarium redolens* liberated only 0.5% $^{14}CO_2$ from ^{14}C-labeled HDPE after 24 months of incubation (Albertsson, 1978; Albertsson *et al.*, 1978).

A number of marine fungal species with activity against PE has also been reported. *Aspergillus* and *Penicillium* strains isolated from the Red Sea reduced the weight of LDPE films and powder by more than 40% (Alshehrei, 2017). Species of *Candida*, *Acremonium*, *Alternaria*, *Emericella*, *Eurotium*, *Exophiala*, *Geosmithia*, *Paecilomyces*, *Pichia*, *Zalerion*, *Phialophora*, *Cladosporium*, and *Aspergillus* have also been identified (Pramila, 2011; Paço *et al.*, 2017; Ameen *et al.*, 2015; Sangeetha Devi *et al.*, 2015; Sindujaa *et al.*, 2011). Kathiresan (2003) reported weight reductions of PE bags of almost 30% per month incubated with two strains of Aspergilli isolated from mangrove soil. In contrast, from a freshwater source, among more than 100 fungal strains, including

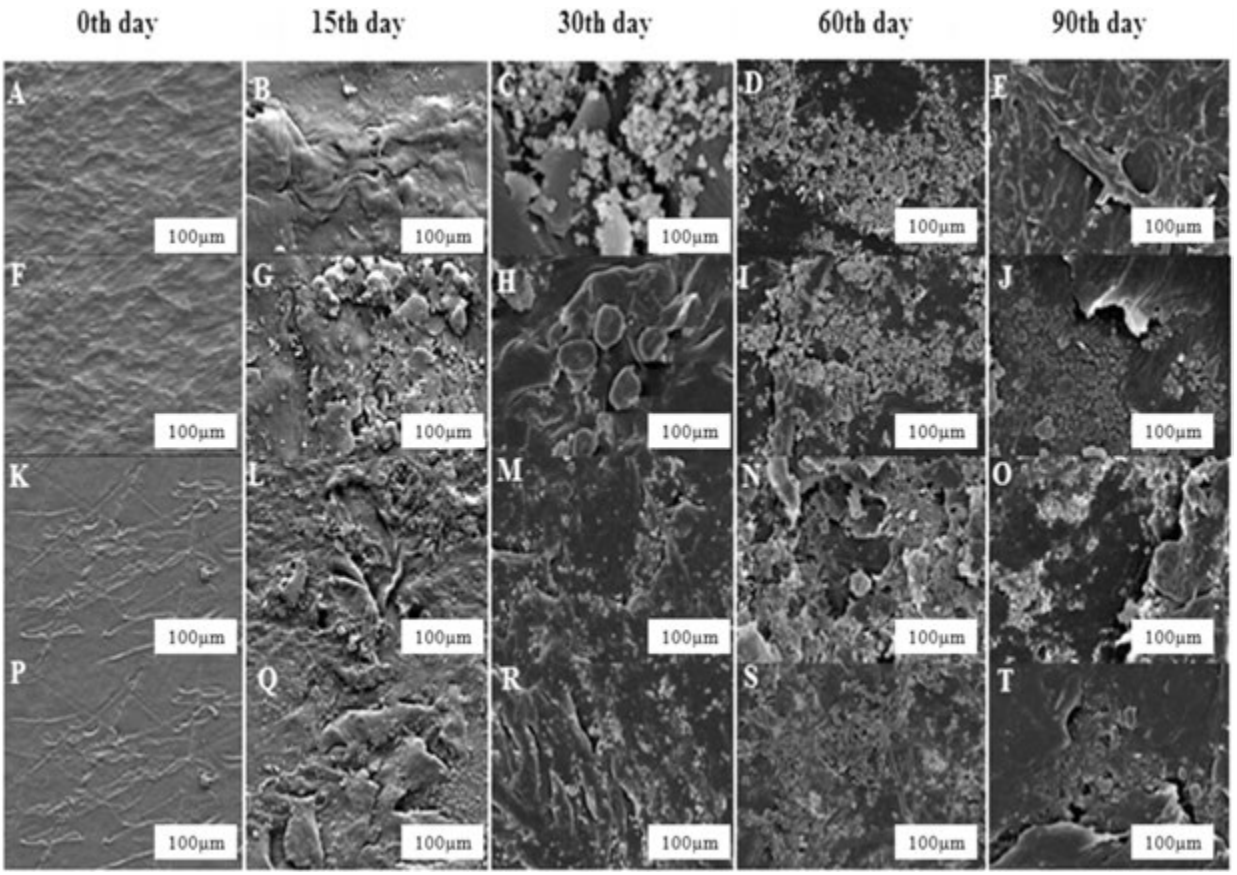

Fig. 4 Field emission scanning electron microscopic view of HDPE and LDPE sheets degraded by strains of *P. oxalicum* and *P. chrysogenum.* (A) HDPE sheet (control). HDPE sheets after (B) 15 days, (C) 30 days, (D) 60 days and (E) 90 days of incubation with a *P. oxalicum* strain. (F) HDPE sheet (control). HDPE sheets after (G) 15 days, (H) 30 days, (I) 60 days and (J) 90 days of incubation with a *P. chrysogenum* strain. (K) LDPE sheet (control). LDPE sheets after (L) 15 days, (M) 30 days, (N) 60 days and (O) 90 days of incubation with a *P. oxalicum* strain. (P) LDPE sheet (control). LDPE sheets after (Q) 15 days, (R) 30 days, (S) 60 days and (T) 90 days of incubation with a *P. chrysogenum* strain. Reproduced from Ojha, N., Pradhan, N., Singh, S., *et al.*, 2017. Evaluation of HDPE and LDPE degradation by fungus, implemented by statistical optimization. Scientific Reports 7, 39515.

saprotrophic fungi and plant pathogens isolated from plastic particles floating at the shoreline of Lake Zurich, Switzerland, none was able to degrade PE (Brunner *et al.*, 2018).

Many fungal isolates collected from landfills and garbage dumping sites containing plastic waste in India and Africa showed activity against PE (Pramila and Vijaya Ramesh, 2011; Deepika and Jaya Madhuri, 2015; Aderiye *et al.*, 2019; Singh and Gupta, 2014; Balasubramanian *et al.*, 2014; Das and Kumar, 2014). Incubation of LDPE with *P. chrysogenum, Rhizopus nigricans, Chaetomium murorum, Memnoniella echinata, Aspergillus fumigatus, A. niger, Aspergillus flavus, Stachybotrys chartarum, Chaetomium globosum,* and *Fusarium oxysporum* isolated from waste dumping sites caused between 2%–9% weight reduction of LDPE after incubation for 30 days (Saxena and Pareek, 2017). *Aspergillus* isolates appeared as prominent PE degraders. For example, an *A. niger* strain isolated from a plastic waste dumpsite caused a 3.4% weight reduction and a 61% reduction in tensile strength of thermally oxidized HDPE after 1 month of incubation (Mathur *et al.*, 2011). SEM, AFM, and FTIR analyses also indicated the degradation of LDPE incubated for 90 days in a liquid medium with an *Aspergillus clavatus* strain (Gajendiran *et al.*, 2016). *A. flavus* and *Aspergillus terreus* strains caused a weight reduction of PE of 30% and 11%, respectively, after incubation in soil for 9 months (Verma and Gupta, 2019) and an *A. fumigatus* strain isolated from a landfill degraded UV-irradiated LDPE in a solid waste medium (Zahra *et al.*, 2010). An *Aspergillus terreus* and an *Aspergillus sydowii* strain isolated from the mangrove rhizosphere at a dumping site were identified as the most efficient degraders of PE based on the reduction of tensile strength and FTIR analysis (Sangale *et al.*, 2019). An *A. oryzae* strain isolated from a dumpsite in Kenya caused weight reductions of LDPE sheets and the formation of new functional groups evidenced by FTIR analysis (Muhonja *et al.*, 2018).

Synergism between PE-degrading fungi in the degradation of PE has also been observed. Incubation of PE with a consortium composed of *Curvularia lunata, Alternaria alternata, Penicillium simplicissimum* and *Fusarium* species isolated from a landfill site showed a higher weight reduction of PE after three months of incubation compared to treatments with the single strains (Sowmya *et al.*, 2014b, 2015).

An improved biodegradability of PE and PP was observed following their modification by UV/gamma irradiation, heat treatment or by the addition of starch or metals to act as pro-oxidants (Fontanella *et al.*, 2010). These modified polyolefins have therefore often been probed as potential substrates for fungal growth and biodegradation (Volke-Sepúlveda *et al.*, 2001; Karlsson *et al.*, 1988; da Luz *et al.*, 2013). It has been shown that some of the fungi preferentially degraded the amorphous parts or oligomeric fractions of the PE liberated by a pretreatment of the samples (Manzur *et al.*, 2004; Raghavan and Torma, 1992). An *A. oryzae* strain caused a decrease in tensile strength of a LDPE sample pretreated with the pro-oxidant manganese stearate and UV-irradiation (Konduri *et al.*, 2011). Likewise, the weight and mechanical properties of films of disposable bags made of a PE blend containing starch to render it more amenable to biodegradation were changed following incubation with cultures of *Mucor rouxii* NRRL 1835 and *A. flavus* (El-Shafei *et al.*, 1998). *Rhizopus oryzae* could grow on thermally pre-treated LDPE causing weight and tensile strength reduction of the PE after one month of incubation. The fungal hyphae penetrated the film and analysis by AFM showed an increased surface roughness (Awasthi *et al.*, 2017). A mixed culture *of A. niger*, *P. funiculosum*, *Paecilomyces variotii* and *Gliocladium virens* also caused surface erosions in thermally pretreated PE of low molecular weight (Weiland *et al.*, 1995). In contrast, Lee *et al.* (1991) detected no degradation of a modified PE containing a pro-oxidant and starch following incubation with *P. chrysosporium*.

Due to its mechanical properties and higher thermal stability compared to PE, PP is even more difficult to attack by microorganisms, and few reports are available on fungi involved in its biodegradation. *P. chrysosporium* and *Engyodontium album* caused a weight reduction of UV-pretreated and pro-oxidant-containing PP after incubation for 12 months (Jeyakumar *et al.*, 2013). Alariqi *et al.* (2006) found that γ-irradiation improved the susceptibility of PP for a colonization with *A. niger*.

Fungal enzymes involved in lignin degradation, such as laccases (EC 1.10.3.2), manganese peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14), have shown activity against PE. However, their mode of action in the depolymerization of PE has not yet been explored in detail. PE membranes were degraded by treatment with a laccase, a copper-containing enzyme from *Trametes versicolor*, in the presence of 1-hydroxybenzotriazol as a mediator (Fujisawa *et al.*, 2001). The plant pathogenic ascomycete *Lasiodiplodia theobromae* also produced a laccase when incubated with γ-irradiated LDPE (Sheik *et al.*, 2015). The upregulated expression of two laccase-like multicopper oxidases from an *A. flavus* strain degrading PE microplastic suggested their involvement in the PE degradation process (Zhang *et al.*, 2020). Manganese peroxidases were shown to be involved in the degradation of PE by the lignin-decomposing white-rot fungus *P. chrysosporium* ME-446 and a white-rot fungus isolate IZU-154 (Iiyoshi *et al.*, 1998). Another strain of *P. chrysosporium* degraded 70% of chemically oxidized PE bags in a liquid medium and produced a lignin peroxidase and a manganese peroxidase. It was assumed that the pretreatment resulted in the formation of low molecular weight PE which was further degraded by the enzymes (Mukherjee and Kundu, 2014). Laccase, lignin peroxidase and manganese peroxidase activities were also detected during the degradation of plasma-pretreated LDPE by the white-rot fungus *Pleurotus ostreatus* (Gómez-Méndez *et al.*, 2018). A *Penicillium simplicissimum* strain isolated from a landfill site degraded 38% of UV-pretreated PE after three months of incubation. However, crude enzyme preparations from this strain did only cause PE weight reductions of less than 1% (Sowmya *et al.*, 2014b).

Polystyrene (PS)

PS is a synthetic aromatic polymer made from the monomer styrene. This hydrophobic thermoplastic of high molecular weight is extremely persistent in the environment and only a few observations on the degradation of PS by fungi have been reported (Otake *et al.*, 1995). Hyphae of the ascomycete *Corvularia* were able to adhere and penetrate oxidized PS within nine weeks of incubation (Motta *et al.*, 2009). Kaplan *et al.* (1979) found very limited degradation of ^{14}C-labeled PS by strains of *Coriolus hirsutus*, *Gloeophyllum trabeum*, *Coriolus versicolor*, *Bjerkandera adusta*, *Daedalea quercina*, *Phellinus pini*, *Aureobasidium pullulans*, *Fomes annosus*, *Peniophora gigantea*, *Fomes everhartii*, *Poria xantha*, *Aspergillus fumigatus*, *Paecilomyces varioti*, *Trichoderma koningii*, and *A. niger* (Kaplan *et al.*, 1979). One of the strains, the brown-rot fungus *Gloeophyllum trabeum*, caused a 50% reduction in molecular mass of water-soluble PS-sulfonate after 20 days of incubation (Krueger *et al.*, 2015).

Polyvinyl chloride (PVC)

PVC is a synthetic polymer composed of vinyl chloride monomers and represents after PE and PP the most widely produced plastic worldwide. While a small number of fungi have shown activity against PVC, a degradation of the high molecular weight polymer has not been reported. Morphological and structural changes of low molecular weight PVC were observed after incubation with a laccase-producing *Cochliobolus* strain isolated from a landfill site (Sumathi *et al.*, 2016). A *P. chrysosporium* strain grew on PVC and starch-blended PVC films causing structural changes and a decrease in the molecular weight of the PVC films (Ali *et al.*, 2013a,b). *Aureobasidium pullulans, Aspergillus fischeri* and *Paecilomyces* strains colonized plasticized PVC which is more susceptible to biodegradation compared to PVC without the addition of plasticizers such as dioctyl phthalate and dioctyl adipate. The *A. pullulans* strains could utilize components of the plasticized PVC as a source of carbon causing a reduction of weight which was probably only due to the degradation of the plasticizers. The polymer itself was apparently resistant to the fungal attack (Webb *et al.*, 2000; Roberts and Davidson, 1986).

Conclusions

Plastics have been generally considered as resistant to microbial attack. However, several types of plastics, especially those containing ester bonds in the polymer backbone are modified or even fully degraded by fungi and their enzymes. In contrast, the biodegradation

of non-hydrolysable plastics by fungi is typically a slow process supported by abiotic factors, such as chemical and physical aging. While numerous reports have identified a number of fungi which colonize and modify plastic substrates, further research on their biodiversity, enzymatic degradation mechanisms and metabolic pathways will be necessary to evaluate their role in plastic biodegradation in the environment and their possible application in bioremediation and polymer modification processes.

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Treatment of Industrial Wastewaters and Liquid Waste by Fungi

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Introduction

Water is the essence of life. It is a basic and a necessary resource for every natural being and its depletion and/or unsuitability in terms of the required quality will cause unimaginable damage to the environment. According to an UN report, global water demand is still increasing and by 2050 this is expected to be 30% more than the current level of water use (WWAP, 2019). This is mostly due to rising water consumption in different sectors, distributed among agriculture (69%), industry and energy production (19%) and households (12%). It is estimated that the two latter sectors will contribute to this growth most strongly (Burek *et al.*, 2016). What also needs to be highlighted, is that climate change, which is nowadays the most urgent global issue, will significantly affect the availability of water resources. Facing these facts, any kind of action taken to protect existing water resources through the development of novel wastewater treatment (WWT) technologies, minimizing the negative impact of the industrial sector on the aquatic ecosystem, and spreading the knowledge about the sustainable water management becomes the greatest challenge, not only for researchers and politicians, but for entire mankind of the 21st century.

Apart from life, water is an integral part of almost every industrial technology and process. Depending on the sector, it is used for heating or cooling, diluting, fabricating, washing, processing and finally transporting. The list of unit operations is probably much longer and not limited to the above-mentioned examples. It is hard to clearly indicate the sector or type of industry responsible for the greatest damage to the aquatic environment. The negative impact is a result of water consumption, the characteristics, properties and quantity of pollutants introduced into water used for processing, as well as technologies used to limit their emissions to the external environment. To show the complexity of the problem, its scale and global significance, two examples are presented. First, the pharmaceutical industry, which requires consistent, high-quality, ultra-pure water on the one hand, but does not consume a large volume of water. Despite this, the presence of pharmaceuticals in ground- and surface water is today considered to be the most harmful environmental pollution (Gadipelly *et al.*, 2014; Ebele *et al.*, 2017). On the other hand, the highest water consumption is assigned to other sectors, such as energy, paper and textile production, which – in most cases – are oriented towards sustainable water management and prevent spreading water pollution through investments in novel technologies for WWT. In summary, an effective treatment of industrial effluents is necessary and applies to all areas of global industry, regardless of the size, specialization and location of the company.

Efficient and cost-effective industrial WWT has been an area of an intense research and development for decades. Conventional technologies have been upgraded or replaced with novel ones, following the innovations that have been introduced to industrial production. Slowly but consequently, a change in wastewater management began to appear, and versatile, universal wastewater treatment plants (WWTP) were found to be insufficient for removal of newer and more recalcitrant chemical compounds, and therefore technologies specialized and dedicated to the individual sectors conquered the markets. This switch did not only affect advanced chemical and physio-chemical installations, but at the same time classical biological WWT procedures were also verified and soon revealed to be a great area for innovation and technological breakthroughs. The most important one referred to the type of microorganisms harnessed to decompose the rapidly increasing and persistent water pollutants. Whereas the time of widespread application of bacteria seems to be coming to an end, the role of fungi in biological WWT begins to gain momentum and seems to become a long-awaited solution for industrial effluents, waste and wastewater issues.

Fungi, as well as bacteria, form a group of the most widespread organisms in the world. Their ability to survive in various habitats and at different, often harsh conditions, is widely documented (Espinosa-Ortiz *et al.*, 2016; Bano *et al.*, 2018). Due to their nutrition system (chemoheterotrophs), as well as their enzymatic repertoire, fungi play a very important role in different ecosystems by decomposing organic matter and cycling of organics and nutrients. However, their utility is not only limited to the natural environment, since fungi are also commonly applied in many industrial sectors and processes, e.g., food production, brewing, medicine or biotechnology (Hyde *et al.*, 2019). Already for decades, fungi and fungal biomass have been used as a source of elements and compounds, such as proteins and enzymes. Therefore, their potential significance in environmental engineering still increases and in the very near future fungi may become major subjects of extensive research.

Although biological treatment of industrial wastewater is currently dominated by bacterial systems, existing reports clearly indicate that the situation may change in a short-term perspective. This chapter aims to provide understanding of the growing global research attention for fungal WWT systems and provides information about the uniqueness of the fungal kingdom and identified features which enable fungi to outcompete bacteria in degradation of contaminants. In addition, examples of the mechanisms involved in fungal WWT are presented and discussed. The technical aspects in reactors' operation together with their advantages and drawbacks are discussed based on selected examples. The conclusions and a brief summary of the future perspectives and challenges related to fungal wastewater systems provide knowledge about the needs and potential research gaps that have to be explored for successful technology implementation.

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The Role of Fungi in Industrial Wastewater and Liquid Waste Treatment

A wide group of industrial WWT technologies (physical, chemical, biological) is currently applied for the decomposition and/or removal of various pollutants introduced to process water and further, to the environment. Among those methods, the biological ones are commonly used worldwide due to their well-known operating conditions, low operating costs and relative technical simplicity (i.e., design, construction and operation). The general idea of biological WWT is to involve specific groups of microorganisms in the water purification process. Those microorganisms need to show great capability to decompose a wide spectrum of different organic compounds present in water. To date, in most of the existing biological WWTP, multiple consortia of bacteria are applied, which are supported by simple eukaryotic organisms such as protozoa and algae. However, the latest studies on fungal cultures and their role in organic matter cycling, and thus in WWT, evidently shows their high biodegradation potential for recalcitrant waste (e.g., plastic, petroleum, agro-chemical) and wastewater containing persistent compounds, such as pharmaceuticals and personal care products, polyaromatic hydrocarbons, textile dyes, and pesticides.

The complexity of wastewater and liquid waste composition, as well as the diversity of their sources is another reason for continuous development of treatment technologies. New substances with harmful effects to the environment are being identified every year. The largest challenges of WWT, i.e., removal of recalcitrant organic compounds (pharmaceutically active compounds (PhACs), endocrine disruptive compounds (EDCs), polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) etc.) and heavy metals, are not yet solved. Those substances are transferred constantly to industrial wastewater, being responsible for its toxicity and confirmed resistance to bacterial degradation (Ventura-Camargo and Marin-Morales, 2013; Wang and Chu, 2016; Ebele *et al.*, 2017; Lellis *et al.*, 2019).

Utilization of fungal biomass in WWT has gained interest after World War II and is a promising solution for the removal of recalcitrant organic pollutants (ROP). The removal of ROPs by fungi has sprung as a result of the discovery of lignocellulosic biomass degrading enzymes (Bennet *et al.*, 2002). Fungi are a diverse group of organisms, including single-cell organisms (yeasts) and multicellular organisms (filamentous fungi), belonging to kingdom Eumycota. Many fungal species have already been studied for their application in WWT, nevertheless the potential of the fungal kingdom has not been fully addressed as there are between 2.2 and 3.8 million of fungal species (Hawksworth and Lücking, 2016).

In the environment, white-rot fungi (WRF) degrade lignin to monomeric compounds, mainly phenols (Rabinovich *et al.*, 2004), and even mineralize lignin. The ability of fungi to decompose many of the recalcitrant organic pollutants results from their molecular structure resemblance to lignin. Dyes, pharmaceuticals, personal care products, etc. all contain phenolic rings in their structure.

The use of fungi provides many advantages in comparison with other organisms used for wastewater and liquid waste treatment:

(1) ability to grow in diverse habitats, due to simple nutrient requirements and adaptation to stress conditions,
(2) higher growth ability, providing higher biomass by simple fermentation techniques,
(3) ability to produce various extra- and intracellular enzymes able to degrade high-molecular-weight compounds, e.g., PAHs,
(4) higher resistance to high concentrations of pollutants,
(5) a high percentage of the biomass consists of cell walls, build from chitins and proteins, glucans and other polymers and copolymers (e.g., chitosan, chitin-glucan polymers, mannans, chemicellulosic and lignin polymers), lipids, polysaccharides and pigments (e.g., melanin) with functional groups present at the cell surface that are responsible for the high heavy metals binding ability (Gadd, 2009; Álvarez *et al.*, 2017; Manna *et al.*, 2018).

Various approaches are being studied worldwide towards the use of the observed benefits of fungal enzymes.

Most commonly, living fungi are being used for ROP removal, however there is a trend towards the use of extracellular enzymes produced by fungi (Bilal *et al.*, 2019). The latter approach has many disadvantages, the main one being loss of enzymes. In order to counteract that problem, enzymes are being immobilized on solid carriers. This allows for recovery and reuse of enzymes. Another interesting solution is the utilization of fungal consortia (Sharma and Malaviya, 2016) (**Fig. 1**), which benefits from synergistic interactions between fungal species. Heavy metals can be successfully removed from soils, waste, waste liquids and wastewater using living fungi as well as other microbial taxa, including archaea, bacteria, cyanobacteria, and algae (Gadd, 2009; Das *et al.*, 2019). Additionally, dead fungal cells can be used as a sorption material for heavy metal removal.

Different mechanisms are involved in the removal of organic pollutants and heavy metals; hence those issues will be addressed separately. A selection of the published research on the removal of both groups of these pollutants is presented in **Table 1**.

Removal of Recalcitrant Organic Pollutants

Advantages of fungal-based wastewater and liquid waste treatment result from extracellular and intracellular enzymes produced by fungi and their cell wall adsorbing properties. These enzymes help fungi to digest substrates in nature, but due to their nonspecific action, they can break down a wide range of organic pollutants. These include recalcitrant pollutants of high molecular weight like PAHs: pollutants that are persistent in the conventional activated sludge process, i.e., highly resistant to bacterial degradation (Rabinovich *et al.*, 2004; Naghdi *et al.*, 2018). For instance, Lee *et al.* (2014) confirmed that the basidiomycete fungi *Pheniophora incarnata* and *Phlebia brevispora* can degrade four PAHs, i.e., phenanthrene, anthracene, fluoranthene and pyrene.

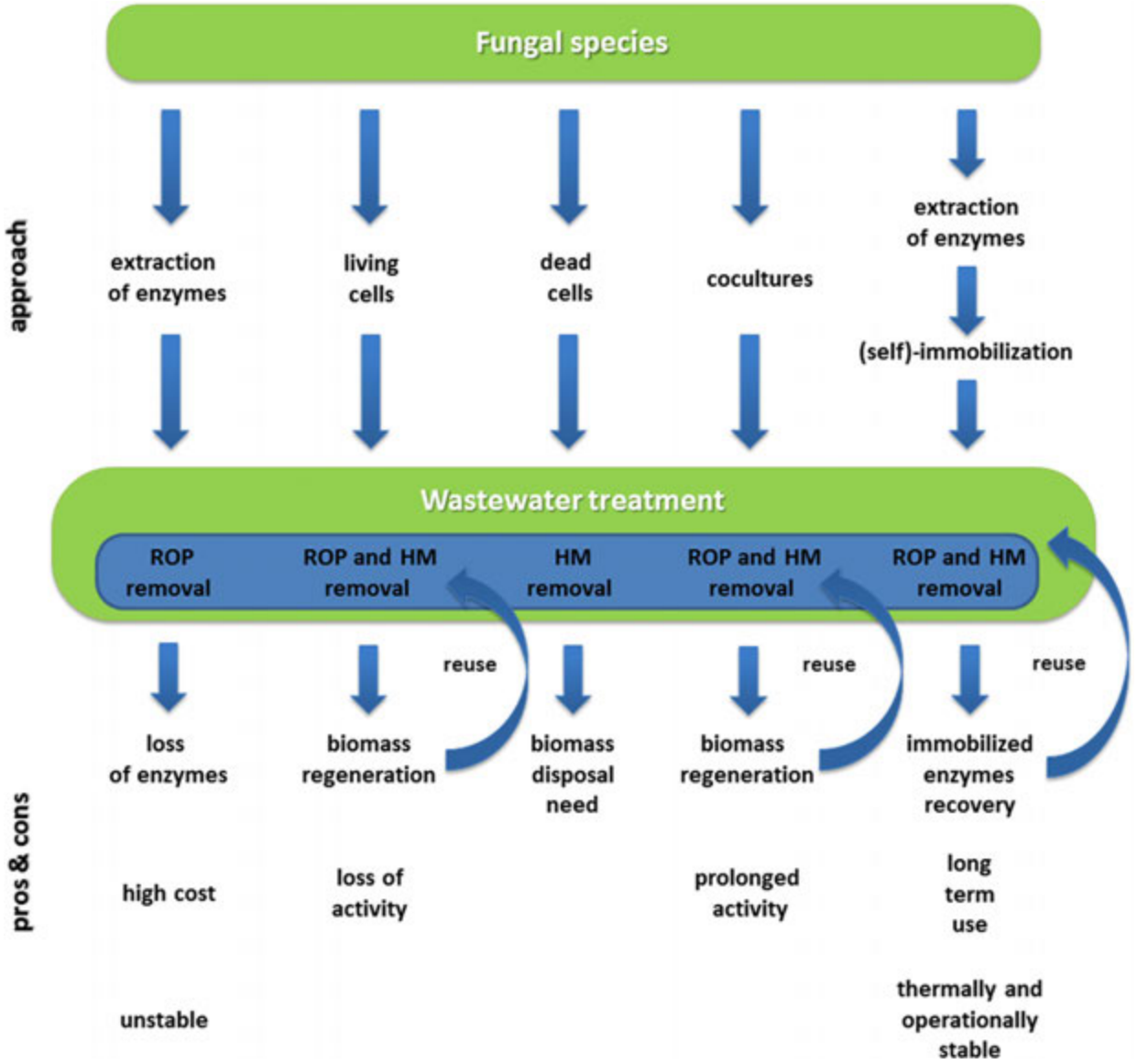

Fig. 1 Summary of the use of fungi for WWT (ROP – recalcitrant organic pollutants, HM - heavy metals).

The potential of many fungal species to degrade pollutants has been studied, from Basidiomycota (e.g., *T. versicolor*) (Lucas *et al.*, 2018; Perna *et al.*, 2019); *Pleurotus ostreatus* (de Araujo *et al.*, 2017; Chefetz *et al.*, 2019) to Ascomycota (e.g., *Aspergillus* sp.) (Almeida and Corso, 2014; Isik *et al.*, 2019); *Beauverias bassina* (Gola *et al.*, 2019), and Mucoromycota (e.g., *Mucor hiemalis*) (Esterhuizen-Londt *et al.*, 2016). Apart from the use of fungal biomass in the degradation of recalcitrant organic pollutants, also crude enzymes extracted from fungi are being used in wastewater purification technologies as green catalysts (**Fig. 1**) (Ramírez-Cavazos *et al.*, 2014; Bilal *et al.*, 2019). For example, 100 U L^{-1} of thermally stable LCCs extracted from *Pycnoporus sanguineus* can remove EDCs with 95% efficiency in 8 h of treatment (Ramírez-Cavazos *et al.*, 2014), which is faster than with the use of fungal biomass. Even though the use of crude enzymes provides clear advantage when used for the removal of pollutants, a major disadvantage links with high operational and investment costs arising from the need for enzyme downstream processing (separation and purification) (Blatkiewicz *et al.*, 2017), which restricts their use for industrial wastewater and liquid waste treatment. One of the possibilities to lower the operational cost of crude enzyme utilization is their immobilization on solid carriers (**Fig. 1**), which allows for increased reusability and storage stability of enzymes (Bilal *et al.*, 2019).

Mechanisms responsible for removal of recalcitrant organic pollutants

Fungi generally produce a wide variety of enzymes. The most promising enzymes for decomposition of contaminants are produced by wood–decaying fungi. Among them, WRF produce significant amounts of extracellular enzymes that help to decompose pollutants, including laccase (LCC), lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP) and dye-decolorizing

Table 1 Pollutant degradation by fungi in wastewater and liquid waste treatment. The selection has been organized based on the group of the treated pollutant

Type of pollutant removed	*Fungal species*	*Concentration of pollutant*	*Efficiency*	*Comments*
Dyes				
Textile wastewater	*Aspergillus terreus*[a]	NA	80% color removal within 48 h	NA
Azo dye Procion Red MX-5B	*Aspergillus niger* (CCT1435)[b] *A. terreus* (CCT 2679)[b]	200 μg mL^{-1}	98% decolorization	100 mL Erlenmeyer flasks, 25 mL of dye solution, pH = 4.0
Textile wastewater	*Aspergillus carbonarius* M333[c]	COD: 1.372 $\pm$ 16.6 mg L^{-1} Color: 240 $\pm$ 8.9 Pt/Co	91% decolorization, 73% COD removal	Nanofiltration bioactive membrane
Remozal Brillant Blue R (RBBR)	*A. carbonarius* M333[c]	10 mg L^{-1}	95% decolorization	Nanofiltration bioactive membrane
Basic Red 18 (BR18)	*A. carbonarius* M333[c]	10 mg L^{-1}	96% decolorization	Nanofiltration bioactive membrane
Textile dye water	*Ganoderma lucidum*[d] MTCC- 1039	No data	81.4% decolorization 90.3% COD removal	Batch reactor, optimized conditions T = 26.5°C, pH = 6.6, 200 rpm
Poly R-478 (polyvinylamine sulfonated backbone with anthrapyridon chromophore)	*Trametes versicolor (Coriolus versicolor)* (NBRC 9791)[e]	50 mg L^{-1}	55.06% BOC removal, 67.47% COD removal	Batch reactor - 300 mL flask containing 200 mL of the culture medium at aerobic conditions and 28°C
Poly S-119 (polyvinylamine backbone with azo chromophore)	*T. versicolor* (NBRC 30388)[e]		53.06% BOC removal, 67.47% COD removal	
Other types of wastewater				
Olive mill wastewater (OMWW) (polyphenols)	*Phanerochaete chrysosporium*[f]	OMWW concentrations (25, 50, 75 and 100%)	60.1% total phenols removal	125 mL Erlenmeyer flask containing 25 mL of diluted OMWW
Pulp and paper wastewater (lignin)	*Bjerkandera adusta*[g] *P. chrysosporium*[g]	5 g L^{-1}	97% degradation 74% degradation	1 L Erlenmeyer flask containing 0.5 L of wastewater and 50 mL fungal culture incubated for 10 days at 24°C with 60 rpm
Polycyclic aromatic hydrocarbons (PAHs)				
Phenanthrene	*Pheniophoraincarnata* KUC8836[h] *Phlebia brevispora* KUC9033[h]	25 mg L^{-1} 25 mg L^{-1}	95.3% degradation ~70% degradation	Erlenmeyer flasks (250 mL) containing 100 mL of 2% malt extract (ME) liquid medium. Rotary shaker at 150 rpm (27°C)
	Pleurotus ostreatus[i] *Agaricus bisporus*[i]	0.05 mg mL^{-1} 0.05 mg mL^{-1}	60% after 3 days 95% after 7 days 80% PHEN after 7 days, 100% after 14 days (remaining metabolites – quinone)	24–26°C Basidiomycetes rich medium
Anthracene	*P. incarnata* KUC8836[h] *P. brevispora* KUC9033[h]	25 mg L^{-1} 25 mg L^{-1}	50% degradation 80.4% degradation	Erlenmeyer flasks (250 mL) containing 100 mL of 2% ME liquid medium. Rotary shaker at 150 rpm (27°C)
	P. ostreatus[i] *A. bisporus*[i]	0.05 mg mL^{-1} 0.05 mg mL^{-1}	50% after 3 days 85% after 21 days 80% PHEN after 7 days 100% after 14 days (remaining metabolites – quinone)	24-26°C Basidiomycetes rich medium
Fluoranthene	*P. incarnata* KUC8836[h] *P. brevispora* KUC9033[h]	25 mg L^{-1} 25 mg L^{-1}	95.0% degradation ~65% degradation	Erlenmeyer flasks (250 mL) containing 100 mL of 2% ME liquid medium. Rotary shaker at 150 rpm (27°C)
Pyrene	*P. incarnata* KUC8836[h] *P. brevispora* KUC9033[h]	25 mg L^{-1} 25 mg L^{-1}	97.7% degradation ~60% degradation	Erlenmeyer flasks (250 mL) containing 100 mL of 2% ME liquid medium. Rotary shaker at 150 rpm (27°C)

(*Continued*)

Table 1 Continued

Type of pollutant removed	*Fungal species*	*Concentration of pollutant*	*Efficiency*	*Comments*
Endocrine disrupting chemicals (EDCs)				
Polychlorinated biphenyls (PCBs), i.e., PCB 28, 52, 101, 118, 138, 153, and 180	*Thermothelomyces thermophila*[j]	400 μl of a PCB mix 3 μg mL^{-1} of each congener	93%–94% degradation	Erlenmeyer flask containing 20 mL of medium, 25°C, 120 rpm for 48 h
	Thermothelomyces heterothallica[j]		80%–87% degradation	
	Thermoascus crustaceus[j]		95% degradation	
	Fusarium solani[j]		85%–88% degradation	Erlenmeyer flask containing 20 mL of medium, 37°C, 120 rpm for 48 h
	Schizopyllumcommune[j]		81%–86% degradation	
Mix of seven polychlorinated biphenyls (PCBs), i.e., PCB 28, 52, 101, 118, 138, 153, and 180	*Myceliophthora thermophila* S2-1[k]	3 μg mL^{-1} of each congener	74.96 ± 1.10% degradation	Erlenmeyer flasks, 25°C or 37°C, 180 rpm for 7 days
	Thermoascus crustaceus S2-7[k]		78.41 ± 0.74% degradation	
	Doratomyces nanus S2-12[k]		73.81 ± 2.78% degradation	
	Phoma eupyrena S2-26[k]		81.59 ± 1.81% degradation	
	Doratomyces purpureofuscus S2-32[k]		82.01 ± 3.97% degradation	
	Doratomyces verrucisporus S2-33[k]		70.02 ± 7.08% degradation	
Pharmaceuticaly Active Compounds (PhACs)				
Lamotrigine (anticonvulsant medication)	*Pleurotus osteratus* PC9[l]	1 – 10 μg L^{-1}	96% after 20 days	Batch 250 mL, 250 rpm, 28°C for 10 days
Acetaminophen (paracetamol)	*Mucor hiemalis* EH5 (DSM 14200)[m]	5, 10, 50 and 100 ng mL^{-1}	33, 29, 7 and 5% internalization	50 mL sterile Sabouraud dextrose broth SAB together with ten pellets
Trimethoprim (TMP) Sulfamethoxazole (SMX)	*P. ostreatus*[n]	50 mg L^{-1} of each antibiotic	70% removal of agents in combination	Erlenmeyer flasks (125 mL), three mycelium plugs (ø 15 mm), static conditions, 28°C, for 35 days
	Pleurotus pulmonarius[n]		62% removal of agents in combination	
	Trametes sp.[n]		40% removal of agents in combination	
Heavy metals				
Cu	*Beauveria bassiana*[o]	30 mg L^{-1} of each heavy metal	74.13% removal	1 mL *B. bassiana* spore suspension (10^6 spores mL^{-1}) in 100 mL of composite media
Zn			75% removal	
Cd			63.4% removal	
Cr			67.8% removal	
Ni			61.13% removal	
Multimetal mixture		total heavy metals concentration 30 mg L^{-1}	84.54% removal	
Multimetal mixture (Pb, Cu, Zn, Cd, Cr, Ni)	*B. bassiana* MTCC4580[p]	5 mg L^{-1} of each metal (total 30 mg L^{-1})	93% initial removal 55.4% after 12 months of storage	Mycogranules
			93% initial removal 39.9% after 12 months of storage	Mycocapsules
			83.5% initial removal 35% after 12 months of storage	Mycotabletes
Cd	*Alternaria alternate*[q]	5 mg L^{-1}	99.99%	2% homogenous spore suspension, 30°C, 120 rpm
		500 mg L^{-1}	43.72%	
Pb			97.39%	
			13.9%	
Cd	*Trametes versicolor*[r]	1 mg g^{-1}	Absorbed up to 0.300 mg g^{-1} after 7 days 90.3% removal	Conical flasks at 28°C at 150 rpm for 7 days

Cr (VI)	*A. niger*[s]	50 mg L^{-1}	100% after 30 min	28°C, 1 g of dry fungal biomass, 100 rpm, 24 h
Zn		100 mg L^{-1}	100% after 165 min	
Hg		100 mg L^{-1}	83.2% removal	
Co		100 mg L^{-1}	71.4% removal	
As (V)	*A. niger* coated with iron oxide[s]	1.0 mg L^{-1}	69% removal	
As (III)		1.0 mg L^{-1}	66% removal	
Pb	*A. niger*[s]	100 mg L^{-1}	59.0% removal	
Cd		5.0 mg L^{-1}	57.0% removal	
Ag		100 mg L^{-1}	48.0% removal	
Cu		100 mg L^{-1}	37.0% removal	

[a]Gomaa *et al.* (2010).
[b]Almeida and Corso, 2014.
[c]Isik *et al.*, 2019.
[d]Selvakumar *et al.* (2013).
[e]Hossain *et al.* (2016).
[f]Salman *et al.* (2014).
[g]Costa *et al.* (2017).
[h]Lee *et al.* (2014).
[i]Pozdnyakova *et al.*, 2018.
[j]Périgon *et al.* (2019).
[k]Mouhamadou *et al.* (2013).
[l]Chefetz *et al.*, 2019.
[m]Esterhuizen-Londt *et al.*, 2016.
[n]de Araujo *et al.*, 2017.
[o]Gola *et al.*, 2016.
[p]Gola *et al.*, 2019.
[q]Das *et al.*, 2019.
[r]Manna *et al.*, 2018.
[s]Acosta-Rodríguez *et al.*, 2018.

Table 2 Examples of fungal enzyme applications for WWT

Enzyme	*Species of fungi*	*Properties*
Laccase	*Trametes versicolor*	ability to decolorize Remazol Brilliant Blue R dye (RBBR) in agar medium by production of ligninolytic enzymes[a]
	Phanerochaete chrysosporium	ability to decolorize sewage dye (mainly nitrogen compounds)[b]
	Bjerkandera adusta	ability to decolorize synthetic dyes (anthraquinone, azo, phthalocyanine, polyaromatic dye)[c] ability to degrade polycyclic aromatic hydrocarbons[d]
	Pleurotus ostreatus	ability to remove phenols from wastewater derived from pressing of olive oil[e] ability to purify polluted environment from bisphenol A[e]
	Irpex lacteus	decolorization capacity of dyes in a liquid medium[f] ability to degrade resistive organic substances (PAHs) found in soil[g] ability to decolorize synthetic dyes (anthraquinone, azo, phthalocyanine, polyaromatic dye)[g] capacity to decolor colorful, liquid matter[h] ability to degrade lignin[i]
	Cerrena unicolor	decolorization capacity of textile dyes without the use of mediators[j]
Manganese peroxidase	*T. versicolor*	ability to decolorize RBBR in agar medium by production of ligninolytic enzymes[a]
	P. chrysosporium	ability to degrade contaminations from phenol derivatives; ability to degrade herbicides[k] ability to decolorize sewage dye (mainly nitrogen compounds)[b]
	B. adusta	decomposition xenobiotic compounds[l] ability to decolorize synthetic dyes (anthraquinone, azo, phthalocyanine, polyaromatic dye)[m] degradability of synthetic polymers[n] ability to degrade polycyclic aromatic hydrocarbons[o]
	I. lacteus	decolorization of dyes in a liquid medium[f] degradation of resistive organic substances (PAHs) found in soil[g] ability to decolorize synthetic dyes (anthraquinone, azo, phthalocyanine, polyaromatic dye)[g] decolorization capacity[h]
Lignin peroxidase	*T. versicolor*	ability to decolorize RBBR in agar medium by production of ligninolytic enzymes[a]
	P. chrysosporium	ability to decolorize sewage dye (mainly nitrogen compounds)[p]
	B. adusta	ability to degrade polycyclic aromatic hydrocarbons[d]
	I. lacteus	ability to degrade resistive organic substances (PAHs) found in soil[g] ability to decolorize synthetic dyes (anthraquinone, azo, phthalocyanine, polyaromatic dye)[g]

[a]Sari *et al.* (2015).
[b]Kiran *et al.* (2019).
[c]Eichlerová *et al.* (2007).
[d]Andriani and Tachibana (2016).
[e]Fountoulakis *et al.* (2002).
[f]Novotný *et al.* (2004).
[g]Novotný *et al.* (2009).
[h]Šíma *et al.* (2017).
[i]Kim *et al.* (2002).
[j]Moilanen *et al.* (2010).
[k]Vágvölgyi *et al.* (2014).
[l]Heinfling *et al.* (1998).
[m]Eichlerová *et al.* (2007).
[n]Mohorčič *et al.* (2009).
[o]Andriani and Tachibana (2016).
[p]Khamrai *et al.* (2018).

peroxidase (DyP) (**Table 2**). While LCCs use O_2 as electron acceptor, for peroxidases this role is performed by H_2O_2 (**Fig. 2**) (Rodríguez-Rodríguez *et al.*, 2013). Other enzymes that help in decomposition of pollutants are intracellular enzymes represented mainly by the cytochrome P450 system and many other accessory enzymes such as glyoxal oxidase, aryl alcohol oxidase and oxalate degrading oxalate decarboxylase.

The accepted mechanism for pollutant removal by WRF can be divided into three steps: (a) sorption of the pollutant onto the cells, (b) degradation by extracellular enzymes or mycelium-bound enzymes, and (c) degradation in the fungal cell by intercellular enzymes (Naghdi *et al.*, 2018) (**Fig. 2**).

Sorption is important for both organic substance and heavy metal removal by fungi. The main component of fungi responsible for their high sorption properties is their cell wall polymer chitin (Gadd, 2009; Almeida and Corso, 2014). Sorption is involved in removal of ROP, especially when treating dye-containing wastewaters, but the degradation by enzymes remains as the main process responsible for their removal (Almeida and Corso, 2014).

Not all WRF species produce the same set of enzymes. The set of enzymes released outside the fungal cell also varies depending of the fungal species (Sen *et al.*, 2016; Asif *et al.*, 2017a). Single or a combination of enzymes can be involved in the pollutant

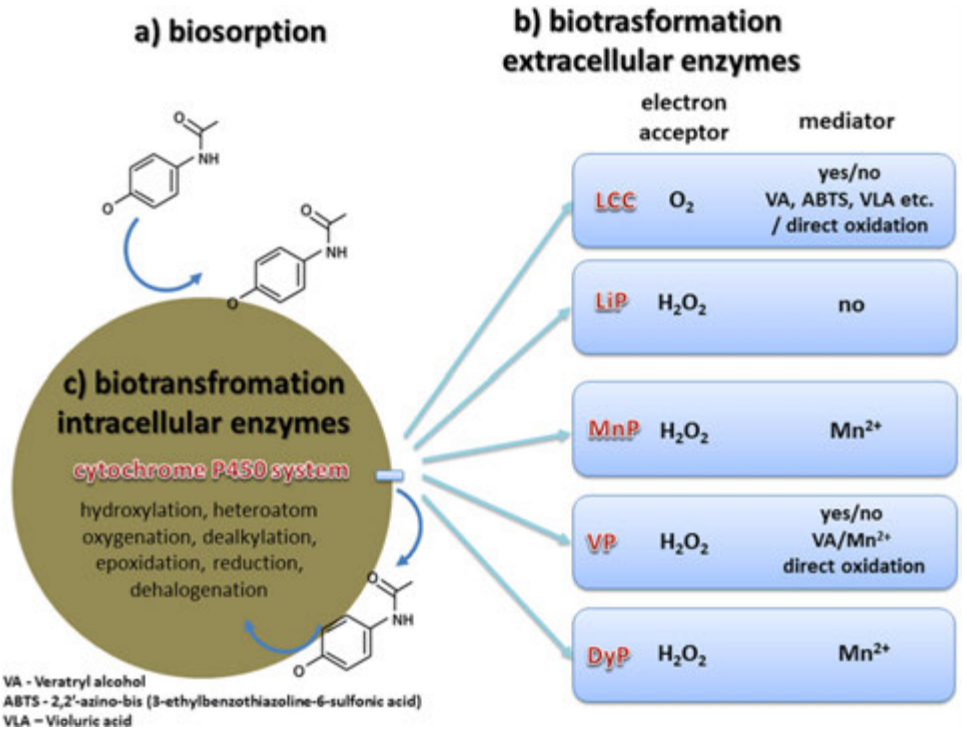

Fig. 2 Mechanisms involved in recalcitrant organic pollutant removal by fungi.

degradation, hence the mechanism of this process can vary significantly. Together with the variety of recalcitrant pollutants, this makes the degradation a complex process and an interesting field of research.

Fungal LCCs' redox potential is reported to be between 780 and 800 mV vs Normal Hydrogen Electrode (NHE) (Christopher *et al.*, 2014; Munk *et al.*, 2015; Muszyńska *et al.*, 2017). Hence, their application is limited to phenolic compounds that have low oxidation potential (Madhavi and Lele, 2009). However, these enzymes display a greater oxidation capacity in the presence of natural and artificial low-molecular mediator compounds, e.g., veratryl alcohol, ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), and violuric acid, that act as an electron carrier between LCC and the substrate, and therefore can be also successfully utilized in numerous biotechnological applications, including the oxidation of non-phenolic lignin compounds (Cañas and Camarero, 2010; Munk *et al.*, 2015; Hilgers *et al.*, 2018).

Lignin peroxidase (LiP) is an enzyme that catalyzes H_2O_2-dependent direct oxidative degradation of lignin (Daniel, 2016; Falade *et al.*, 2017). For non-phenolic compounds, LiP oxidation involves electron oxidation and cationic radical formation, which leads to side chain cleavage, demethylation, and intramolecular addition and rearrangement (Falade *et al.*, 2017). In addition, LiP has an ability to oxidize various phenolic compounds, such as amines, aromatic ethers and polycyclic aromatic hydrocarbons whose redox potential exceeds 1.4 V (versus NHE) (Kersten and Cullen, 2007; Wong, 2009).

Among WRF, manganese peroxidase (MnP) is a more common extracellular enzyme than LiP. The MnP catalytic cycle is similar to the two-electron oxidation of LiP, but MnP is capable of oxidizing Mn^{2+} ions, resulting in the formation of diffusive oxidants (Mn^{3+}), which can penetrate the plant cell wall and oxidize phenolic substrates (Hofrichter, 2002; Wong, 2009).

Versatile peroxidase (VP) can be considered a hybrid between MnP and LiP. VP can oxidize substrates of low and high redox potential, e.g., phenolic and non-phenolic lignin dimers, substituted phenolic compounds and various types of dyes (Mäkelä *et al.*, 2015).

Other enzymes with potential to decompose polluting compounds are dye-decolorizing peroxidases (DyPs). They form a relatively new family of peroxidases that lacks homology to other peroxidases, but requires H_2O_2. It can be distinguished by e.g., wide substrate specificity and activity under low pH (Sugano *et al.*, 2007). DyP is named after its ability to degrade anthraquinone dyes (Yoshida and Sugano, 2015) and hence, it is a promising enzyme for textile WWT.

The role of intracellular enzymes (e.g., cytochrome P450 and epoxide hydrolase) is also large. Cytochrome P450 is a large group of enzymes present in all living organisms. In fungi those enzymes deal with compounds formed during wood decomposition, which can be potentially toxic towards fungi (Morel *et al.*, 2013). They can catalyze a wide array of reactions (**Fig. 2**), hence they may play important role in removal of organic pollutants from wastewater. They are involved in the initial oxidation of phenanthroline as suggested by Pozdnyakova *et al.* (2018). The role of cytochrome P450 in the decontamination of organic pollutants was further confirmed during lamotrigine decomposition by *P. ostreatus* (Chefetz *et al.*, 2019). The intracellular enzymes do not lead to complete mineralization of the recalcitrant pollutants, but they are involved in ring cleavage and mineralization of the pollutants to CO_2 (Pozdnyakova *et al.*, 2018).

The most reported and characterized LCCs originate from WRF such as *Trametes* (*Coriolus*) *versicolor*, *Trametes hirsuta*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Phlebia radiata*, *Coriolopsis polyzona*, *Lentinus edodes*, *P. ostreatus*, *Pycnoporus cinnabarinus* and *Coprinopsis cinerea* (Madhavi and Lele, 2009; Upadhyay *et al.*, 2016). LiP isoenzymes have been identified in *Phanerochaete chrysosporium*, *T. versicolor*, *P. radiata* and *Phanerochaete sordida*, but many WRF do not produce LiPs (Falade *et al.*, 2017). Dye-decolorizing peroxidases have for instance been identified in *Auricularia auricula-judae* and *Exidia glandulosa* (Liers *et al.*, 2013).

A problem directly connected to the various mechanisms involved in decontamination of pollutants is the formation of potentially harmful byproducts (Olicón-Hernández *et al.*, 2017). Therefore, monitoring of degradation byproducts for their toxicity is an extremely important factor with all new WWT technologies, especially advanced oxidation processes. It is not uncommon for the degradation byproducts to be more toxic then the primary pollutant, and this is also true in the case of biodegradation of PhACs (Naghdi *et al.*, 2018). Nevertheless, many studies still focus mainly on the pollutant degradation efficiency, ignoring the problem of potential toxicity of degradation byproducts (de Araujo *et al.*, 2017).

Removal of Heavy Metals by Fungi

As mentioned above, removal of heavy metals can take place in various microbial taxa. While some heavy metals are essential for fungal metabolism at small concentrations, i.e., Cu, Fe, Mn, Mo, Zn, Ni (Baldrian, 2003), even these essential heavy metals will be toxic if present in excess. Many fungal species have been studied for their resistance to heavy metals and their ability for heavy metal removal (**Table 1**). The response to the presence of specific heavy metals can differ between fungal species (Baldrian, 2003; Oladipo *et al.*, 2018). For example, *Aspergillus niger* is highly resistant to some heavy metals, e.g., Cr, which is one of the most hazardous metals (Álvarez *et al.*, 2017; Acosta-Rodríguez *et al.*, 2018). Most commonly, the tolerance and ability of heavy metals removal by fungi is tested in single-metal systems (Xu *et al.*, 2012; Gola *et al.*, 2016; Wang *et al.*, 2019). As industrial wastewaters and liquid waste never contain just one heavy metal, research conducted on mixed heavy metals removal is needed. Das *et al.* (2019) showed that a manglicolous fungus *Alternaria alternate* isolated from Indian Sunderban has a good ability to remove both Pb and Cd as single metals ($>80\%$), but that the efficiency drops significantly in the mixed systems. However, lately Gola *et al.* (2016, 2019) demonstrated that effective removal of mixed heavy metals is achievable using *Beauveria bassiana*. This ability has been additionally confirmed with the use of the WRF *P. ostreatus* for real coal washery effluent containing numerous heavy metals, e.g., Mn, Zn, Ni, Cu, Co, Cr, Fe and Pb (Vaseem *et al.*, 2017). The lowest efficiency was observed for Mn and Pb in both raw coal washery effluent as well as 50% and 25% diluted effluents.

Heavy metal removal by fungi is affected by many factors. First of all, fungal species show different tolerance to heavy metals, while also the initial concentration of heavy metal influences the process efficiency (Vaseem *et al.*, 2017; Das *et al.*, 2019). It has been shown that the efficiency of heavy metal removal depends on temperature, pH, ionic strength, biomass dosage, contact time, agitation speed, salinity, and heavy metal concertation and its properties (Chanda *et al.*, 2016; Das *et al.*, 2019; Saad *et al.*, 2019).

Heavy metal removal due to biosorption is especially interesting, since the process can be reversed, and hence heavy metals, platinum group metals as well as rare earth elements, can be recovered from different waste streams (Zhuang *et al.*, 2015; Kanamarlapudi *et al.*, 2018). Lately, municipal and industrial wastewaters and other liquid wastes have been considered as a resource for biomining (bioleaching) metal recovery (Zhuang *et al.*, 2015). This process allows not only the recovery of heavy metals from biomass, but also ensures that the biomass can be regenerated and reused (Kanamarlapudi *et al.*, 2018). Das *et al.* (2019) proved that alkaline regeneration enabled reuse of biomass from *A. alternate* even after five cycles.

Real wastewaters and liquid wastes usually contain a variety of pollutants, including both organic compounds and heavy metals. This unfortunately generates problems, since presence of heavy metals affects efficiency of the enzymatic process (Baldrian, 2003). Nevertheless, Mishra and Malik (2014) have shown that efficient removal of pollutants can be achieved by using fungal consortia.

Mechanisms of heavy metals removal

The process involved in heavy metal removal is broadly called biosorption – a physicochemical process that takes place on biological material (dead or alive) (Shakya *et al.*, 2018). The term bioaccumulation is used when metal is being removed via a growing cell.

Fungal species and strains show different sensitivity towards heavy metals. The ability for heavy metal removal from the environment by fungi results from their defense mechanisms against the toxicity of heavy metals. It is based on metal chelation by extracellular and intracellular compounds (Baldrian, 2003). As biosorption is a physio-chemical process, it is not very different from sorption processes occurring in solids. Biosorption is a complex process resulting from various mechanism that can be divided into three groups: cell surface sorption, intracellular accumulation and extracellular precipitation/accumulation (Dhankhar and Hooda, 2011; Álvarez *et al.*, 2017). The specific mechanism is shown in **Fig. 3**. Heavy metal removal firstly and mainly takes place at the cell wall surface, and up to 50% of heavy metal is bound on the cell wall (Baldrian, 2003). Functional groups of the fibrillary and matrix components of the fungal cell wall, i.e., chitin, cellulose, β-glucan and α-glucan, chitosan, polyuronide, glycoproteins, lipids, inorganic salts and pigments, bind and remove heavy metals through adsorption, ion exchange, surface complexation and precipitation (**Fig. 2**) (Gadd, 2009; Dhankhar and Hooda, 2011). Metal binding sites are mainly amino, carboxyl, hydroxyl and carbonyl groups (Gadd, 2009; Álvarez *et al.*, 2017), which have been confirmed with Fourier Transform Infrared spectroscopy (FTIR) analysis (Gola *et al.*, 2016; Álvarez *et al.*, 2017; Manna *et al.*, 2018). The remaining 50% percent is divided between the cytoplasm and vacuoles (Baldrian, 2003). Heavy metals are transported into the fungal cell by transporters that import metals that are essential for

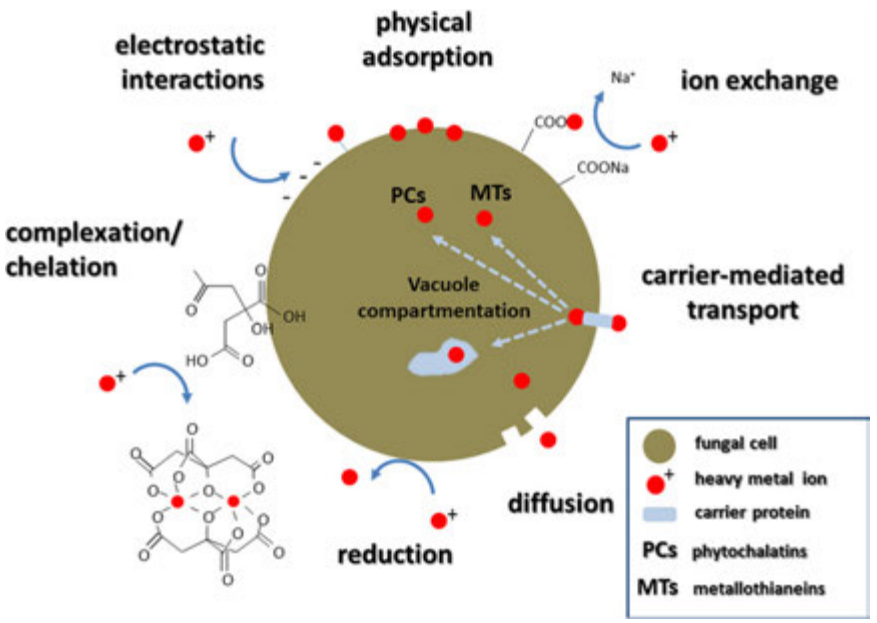

Fig. 3 Mechanisms involved in heavy metal removal by fungi.

fungi, due to their low specificity (Baldrian, 2003). Heavy metals are then intracellularly chelated by phytochalatins or metallothioneins (Álvarez *et al.*, 2017; Vaseem *et al.*, 2017). Extracellular chelation proceeds with the chelation agent, such as oxalic acid produced by WRF and brown-rot fungi (Baldrian, 2003), leading to formation of oxalates of heavy metals.

Benefits of Fungal Consortia

The future of fungal WWT seems to be the use of co-cultures or microbial consortia (Lade *et al.*, 2012; Sharma and Malaviya, 2016; Kasonga *et al.*, 2019; Kurade *et al.*, 2019). Most industrial effluents are multicomponent matrices, where more than one type of contaminant is present. For instance, in industries like pulp and paper, and tannery and textile, process effluents are rich in metals and dyes (Mishra and Malik, 2014). Hence, the processes used to treat those waste streams would benefit from the use of fungal consortia. Mishra and Malik (2014) have shown that fungal consortia of *Aspergillus lentulus*, *Aspergillus terreus* and *Rhizopus oryzae* are successful in removing both heavy metals and dyes. Cr^{6+}, Cu^{2+}, Acid Blue 161 and Pigment Orange 34 were removed with 100%, 83%, 98% and 100% efficiency, respectively, showing better performance than any of these fungal strains separately.

Recently, de Oliveira Rodrigues *et al.* (2020) showed that solid-state fermentation of fungal co-cultures increases production of lignocellulose-degrading enzymes when compared to monocultures. Furthermore, alteration of the fungal consortia has the highest influence on which enzymes are produced and at which levels (de Oliveira Rodrigues *et al.*, 2020). Consortia of *T. versicolor* 561, *Ganoderma lucidum* 601 and *P. ostreatus* PL06 showed the highest production of LCC, whereas co-cultures of *A. niger* SCBM1, *Aspergillus fumigatus* SCBM6 and *P. ostreatus* PL06, *A. niger* SCBM1, *T. versicolor* 561 and *P. ostreatus* PL06, and *A. niger* SCBM1, *G. lucidum* 601 and *P. ostreatus* PL06 were the most efficient in producing cellulases and hemicellulases (de Oliveira Rodrigues *et al.*, 2020). Kasonga *et al.* (2019, 2020) studied a consortia of South African indigenous fungal strains (*A. niger*, *Mucor circinelloide*, *Trichoderma longibrachiatum*, *Trametes polyzona*, *Rhizopus microspores*) in two types of reactors, i.e., sequential batch reactor and a non-sterile stirred fluidized bioreactor for their ability to remove pharmaceuticals ibuprofen, carbamazepine and diclofenac, also proving the benefit of using consortia of various fungi. The main gain from the use of multiple species in the biological treatment of various pollutants is the fact that species can specialize for degradation or uptake of particular contaminant. In addition, strains capable of heavy metal accumulation can protect the rest of the community, facilitating efficient removal of organic pollutants (Mishra and Malik, 2014).

Emerging Trends in Fungal Wastewater Treatment Research

Utilization prospects for fungal WWT are wide and ever expanding, owning to the increasing number of fungal species used, range of targeted pollutants, isolated enzymes, etc. Below, we outline some of the emerging and interesting research directions in the field. The process called advanced bio-oxidation is very interesting (Vasiliadou *et al.*, 2019). It is based on an extracellular quinone redox mechanism, where H_2O_2 formed in that process is decomposed in a Fenton-like reaction in the presence of chelated iron ions, which allows for hydroxyl radical formation. Vasiliadou *et al.* (2019) have shown that both *T. versicolor* and *G. lucidum* are able to remove mixtures of 13 PhACs (e.g., ibuprofen, sulfamethoxazole, carbamazepine, progesterone and ranitidine) in the presence of gallic acid or dimethyl-benzoquinone as mediators and Fe(III)-oxalate (metal precursor). The best results were

obtained for *T. versicolor* when gallic acid was added, as more than 60% of all PhACs studied were removed. The latest research also focuses on genetically modified organisms with the ability to degrade polluting compounds. Most commonly this refers to the use of recombinant enzyme in a new biocarrier (e.g., bacteria or yeast). For example studies consisted of the use of a recombinant MnP enzyme, produced in the yeast *Pichia pastoris*, in dye discoloration and denim bleaching (Zhang *et al.*, 2020). Numerous approaches are available for fungal strain engineering, e.g., classical mutagenesis, genetic engineering and genome editing (CRISPR/Cas9) (Kun *et al.*, 2019). Direct genome modification of fungi, aimed at improved pollutants degradation ability, is not as often studied as in other applications of fungi. The reason might be that basidiomycete fungi that are able to produce lignocellulose-degrading enzymes are less amenable to genetic manipulation (Rodgers *et al.*, 2010).

Another promising direction of research is extraction of heavy metals by metal chelation, as exemplified by *Penicillium tricolor* that produces citric and oxalic acids (Zhuang *et al.*, 2015). Furthermore, it has been shown that due to the precipitation process by fungal-produced organic acids, metal nanoparticles formation is possible. Joseph *et al.* (2012) proved that silver nanoparticles are formed when *B. bassiana* grows on silver containing compounds.

Technical Aspects of Fungal Wastewater Treatment

Principles of Microbial Wastewater Treatment

Biological WWT involves the natural processes occurring every day in our environment allowing for matter cycling and keeping the balance in nature. Organic matter (here: pollutants) that is introduced to water is systematically and continuously converted into neutral (inert) product by means of microorganisms and their metabolic pathways. From the technical point of view the most important requirements for the efficient biological WWT systems are to:

(1) Select the proper microorganism belonging to chemoheterotrophs;
(2) Ensure the proper conditions for their growth and reproduction.

The mineralization (decomposition) of organic compounds in WWT takes place as a result of microbial oxidative or fermentative catabolism. Due to the fact, that the greatest number of existing WWTPs is based on aerobic processes, the oxidative catabolism will be considered of the real importance within this section. At these conditions, the main routes for pollutants oxidation can be described with the following reactions (1 and 2):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + Energy \tag{1}$$

$$C_xH_yO_z + \frac{1}{4}(4x + y - 2z)O_2 \rightarrow xCO_2 + \frac{y}{2}H_2O \tag{2}$$

where $C_xH_yO_z$ represents the simplified molecular formula for a single organic compound. To understand the role of microorganisms in biological WWT it is necessary to identify and formulate some basic principles and indicators to describe the progress and efficiency of the microbial degradation. Therefore, the characterization of carbonaceous matter will be given below, followed by the definitions of the two key variables representing the substrate.

Organic matter present in wastewater is usually divided into biodegradable and non-biodegradable (inert) fractions. Both can occur in two forms: soluble and particulate. Importantly, whereas the form of the inert fraction does not change within the biological system, the biodegradable fraction undergoes transformation and leaves the system with the lower concentration, depending on its susceptibility to microbial (enzymatic) action. Soluble, rapidly biodegradable carbonaceous matter is the fraction directly consumed by heterotrophic microorganisms. For the degradation of slowly biodegradable matter (mostly particulate), an additional step (i.e., conversion into soluble fraction) involving the action of extracellular enzymes, is required. Finally, the non-biodegradable fraction is removed from wastewater with the residual biomass (due to the sorption on the cells' surface) or remains in the effluent at the same level. The above-mentioned characteristics allow for defining two major variables of WWT, describing the quality and quantity of substrates (pollutants), namely: chemical oxygen demand (COD) and biological oxygen demand (BOD). For the whole group of the WWT processes COD is defined as an amount of oxygen that is consumed for the decomposition (chemical oxidation) of organic matter present in wastewater. For biological systems, BOD is more commonly applied and it expresses the amount of oxygen consumed by microorganisms during the decomposition of organic matter. In other words, BOD is the measure of the concentration of biodegradable substrates, whereas COD reflects the concentration of the total organic (and partially inorganic) matter in wastewater. Those simple variables and their mutual ratios indicate the applicability of the microbial system for wastewater purification.

To ensure the stable growth of the microorganisms involved in biological WWT, accurately specified conditions must be provided. They refer not only to temperature, carbon and moisture content, but also to the pH of the reaction mixture, concentration of the dissolved oxygen and availability of nutrients (mostly expressed as N:P or C:N:P ratio). Depending on the type of microorganism employed in wastewater purification these conditions will differ. In the case of the well-known bacterial systems, i.e., conventional activated sludge process (CASP), these conditions are already defined. However, for fungal systems, which are scarcely considered for big-scale application, their establishing requires long-term and tedious laboratory studies conducted on real, non-sterile wastewater, focused on the cultivation procedures and screening for the best species to withstand severe conditions. Therefore, so far only basic knowledge referring to the optimal conditions for fungal WWT is available. Similarly to bacteria, the WRF typically grow in aerobic conditions with an optimum humidity of about 70%–80%. Municipal or industrial wastewater

usually contains a mixture of many impurities present in low concentrations (Verlicchi *et al.*, 2010; Wang and Ren, 2014), including organic carbon and nitrogen. For growth of WRF in wastewater, an additional, easily absorbable source of carbon might be needed, since fungi may not be able to assimilate those elements from the compounds originated from wastewater. Research has highlighted the necessity of glucose and ammonium tartrate or ammonium chloride addition to maintain WRF activity in sewage (Hadibarata and Kristanti, 2012; Cruz-Morató *et al.*, 2013a; Badia-Fabregat *et al.*, 2015). Moreover, the pH of the municipal wastewater is usually around 7, while effective treatment by WRF requires a pH around 4.5 (Verlicchi *et al.*, 2010; Mir-Tutusaus *et al.*, 2018), which can pose another technical issue for fungal WWT systems. Another difference (and drawback) of fungal WWT in comparison to the CASP is the requirement of a longer hydraulic retention time (HRT) to remove organic matter (Mir-Tutusaus *et al.*, 2018). This parameter, defined as the ratio of volume of liquid in the system to volume of liquid removed per unit time, determines the volume of the bioreactor to be operated in the system. When longer HRT is required for efficient WWT, bigger systems must be constructed. Another strategy is to increase the solid retention time (SRT), defined as the ratio of mass of solids in the system to mass of solids removed per unit time. In biological systems SRT is therefore the measure of the cells in the unit, and can be increased by internal recirculation of the sludge within the system (Mir-Tutusaus *et al.*, 2018).

Possible Technical Solutions

Fungal WWT can be realized through several technical solutions. The final choice of the concept will depend on the general aim of the treatment, i.e., whereas it will be the removal of all pollutants present in wastewater, decomposition of residual COD or preliminary biodegradation of the compounds extremely harmful for the following stages of biological WWTP. Schematically three of the most explicit configurations for fungal-based WWTP are given on **Fig. 4**, followed by the brief description of the examples for their possible applications.

The first concept (**Fig. 4(A)**) is the easiest configuration for fast implementation nowadays and reinforces the idea of using fungal operations as on-site treatments in specific contaminated streams. Typically, it consists of the first stage of treatment (mechanical, physical, chemical, e.g., coagulation/flocculation), followed by the fungal bioreactor, where the complex wastewater decomposition takes place. At the end of the system an additional unit may be considered for tertiary treatment, especially if there is a necessity for the disinfection of the effluent. Due to the fact that this system does not provide for the use of bacteria, both the volume of the reactor (HRT) and the process conditions (C:N, temperature, pH) can be precisely optimized solely in agreement with the requirements of fungal growth. Such a solution can be applied for wastewaters containing compounds which are not easily degradable by bacteria, i.e., hospital wastewaters, pharmaceutical wastewaters, in pulp and paper industry etc., or with lower pH (acidic, 4–5), and can be located and operated on-site, prior to discharge to municipal WWTP.

Both the second (**Fig. 4(B)**) and the third (**Fig. 4(C)**) concepts include bacterial action on WWT, and as such can be considered as a complement to the existing biological WWTPs. The difference between them is only the location of the fungal bioreactor: it can be placed upstream or downstream of the bacterial bioreactor. The first option allows to avoid the problem of bacterial contamination and/or competition, which will be described in the next section. It should be applied when the concentration of compounds toxic to bacteria is too high. Fungi can not only withstand such harmful conditions, but also are capable to degrade those compounds to some extent. The latter option (**Fig. 4(C)**) can be considered wherever the efficiency of CASP does not meet the restrictions in terms of

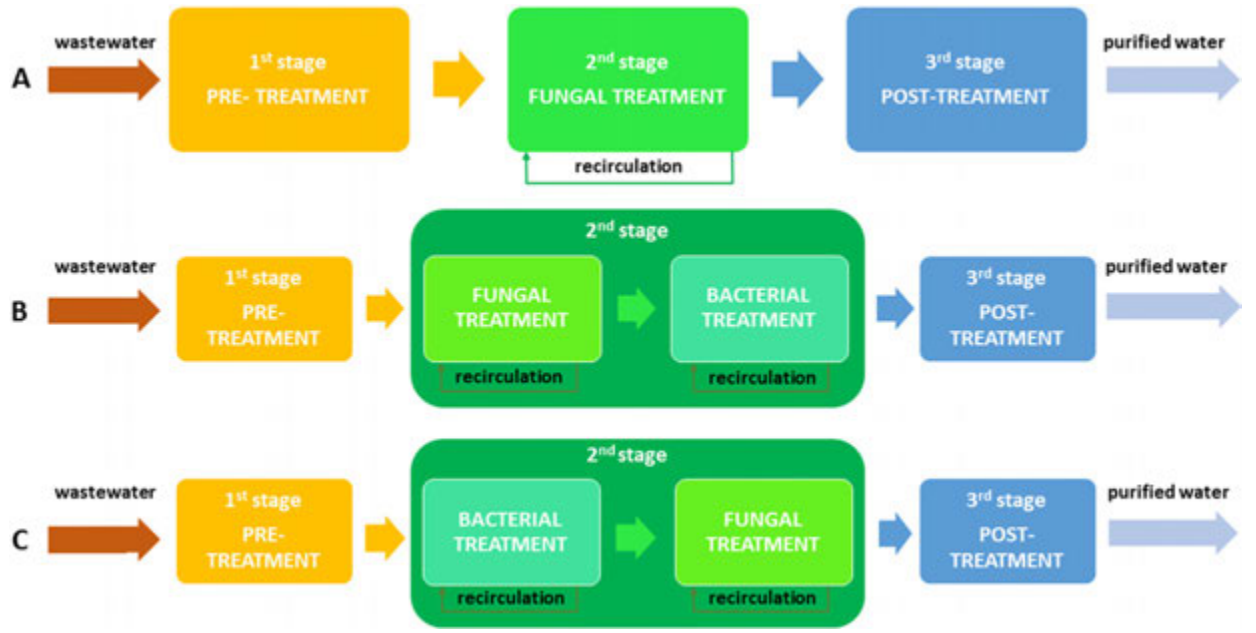

Fig. 4 Possible configurations of fungal WWTP.

the released concentration of specific compounds, such as pharmaceuticals and personal care products. In such systems, fungal bioreactor plays the role of tertiary treatment and its operation might be negatively affected by bacterial inflow or insufficient nutrients concentration. Nevertheless, from a technical point of view, this option still seems to be more attractive than the previous one, since ensuring the required pH or HRT is easier to obtain for the last stage of installation than for the first one.

Bioreactors

Biological, including fungal, WWT takes place in the unit called bioreactor. Bioreactors are apparatus of a different shapes (e.g., cylindrical, cuboid) and geometry, commonly made of stainless steel and equipped with supporting devices, such as baffles, oxygen distributors and agitators. Depending on the operating regime they can be classified as batch or continuous flow bioreactors.

Typically, the biological stage of each WWTP is equipped with the specified number of bioreactors followed by the clarifier (**Fig. 5**). While the biological (enzymatic) processes in bioreactor ensure the decomposition of biodegradable pollutants, in the second unit the separation of purified wastewater from the solids is constantly conducted. In most cases, the separated solids are partially recycled to the bioreactor, to maintain the desired SRT and partial biomass renovation stabilizing the age of fungal biomass.

In order to facilitate the use of WRF and other fungi in chemical processes, different reactor concepts have been proposed and tested over time, mostly on a laboratory scale. Different process aspects and process parameters need to be considered to achieve the desired effect, which is the mineralization of organic species. The main parameters are the mobility of the culture, aeration, agitation, HRT and product separation. Early research on the use of WRF was conducted in the 1980s and since then many studies discussing various reactor concepts was published. The main interest in the fungi-driven processes for WWT arose in the last two decades, with the number of publications increasing fifteen times in 2019 (1845) compared to 1999 (120) (Elsevier, 2019). This section presents a concise overview of the literature related to the different reactors and reactor configurations.

For optimal performance, the fungal colony needs to be present in the reactor in a way that offers a maximum contact with the influent, while minimizing the mechanical stress on the colony. One of the first key concepts was the use of stirred tank reactors that are the largest group of reactors used for the cultivation of fungi in WWT applications (Espinosa-Ortiz *et al.*, 2016). It was, however, shown that application of a too severe mixing or bad impeller design can damage (tear apart) the pellets causing a reduction of treatment efficiency (Moreira *et al.*, 2003). To overcome this problem, alternative reactor designs have been proposed, which eliminate the need for mechanical stirring: the airlift reactor (ALR), the bubble column reactor (BCR) and the fluidized bed reactor (FBR). In the first two types, the mixing is induced by the air fed to the bottom of the reactor (**Fig. 6**). In the ALR, an internal circulation is established by the density difference between the liquid rich in gas bubbles and the degassed liquid; an internal baffle (draft tube, riser) helps to guide the circulating fluids. In the BCR, no internal flow separator is used and the bubbles will be uniformly dispersed rising through the liquid. In the FBR, a fine bulk solid (e.g., biomass, sand) is brought into a fluid-like state by another medium (liquid or gas) that flows through the initially stationary packed bed of the solid in an upward direction. A very good mixing and high mass and energy transfer rates between the fluidization medium and the solids can be achieved in an FBR, which are two of its main advantages.

For the use of fungal processes, multiple researchers reported the use of an FBR. However, in most cases the distinction between a true FBR and a BCR is not clear, because also in the BCR the fungal colonies float in the entire volume of the reactor, lifted by the gas bubbles. A comparison between the experiments performed in a fixed-bed reactor and in an FBR are reported in the literature (Jaganathan *et al.*,

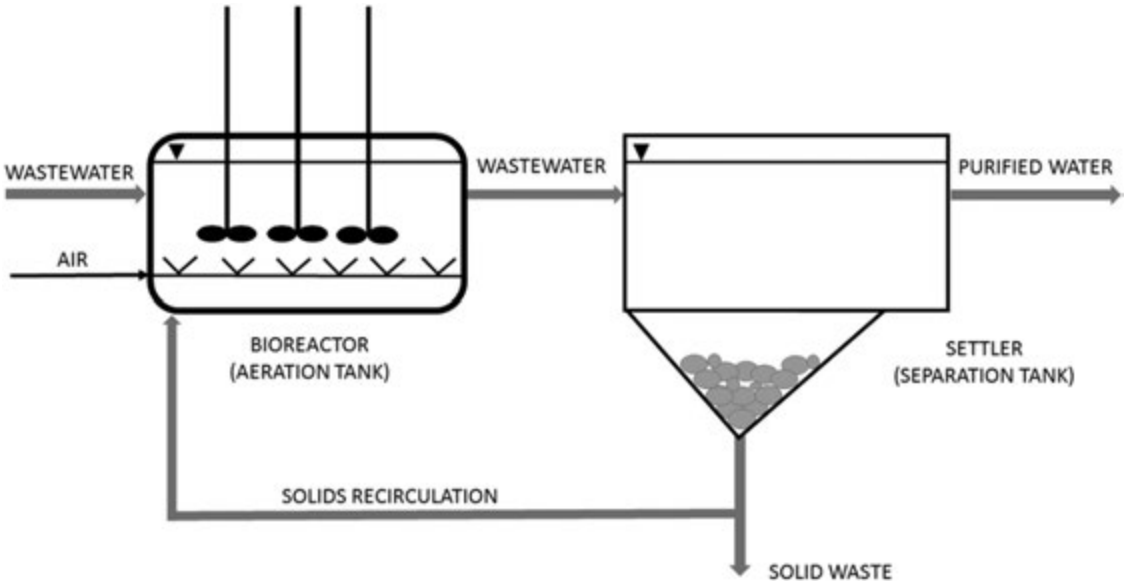

Fig. 5 A scheme of biological stage of WWTP.

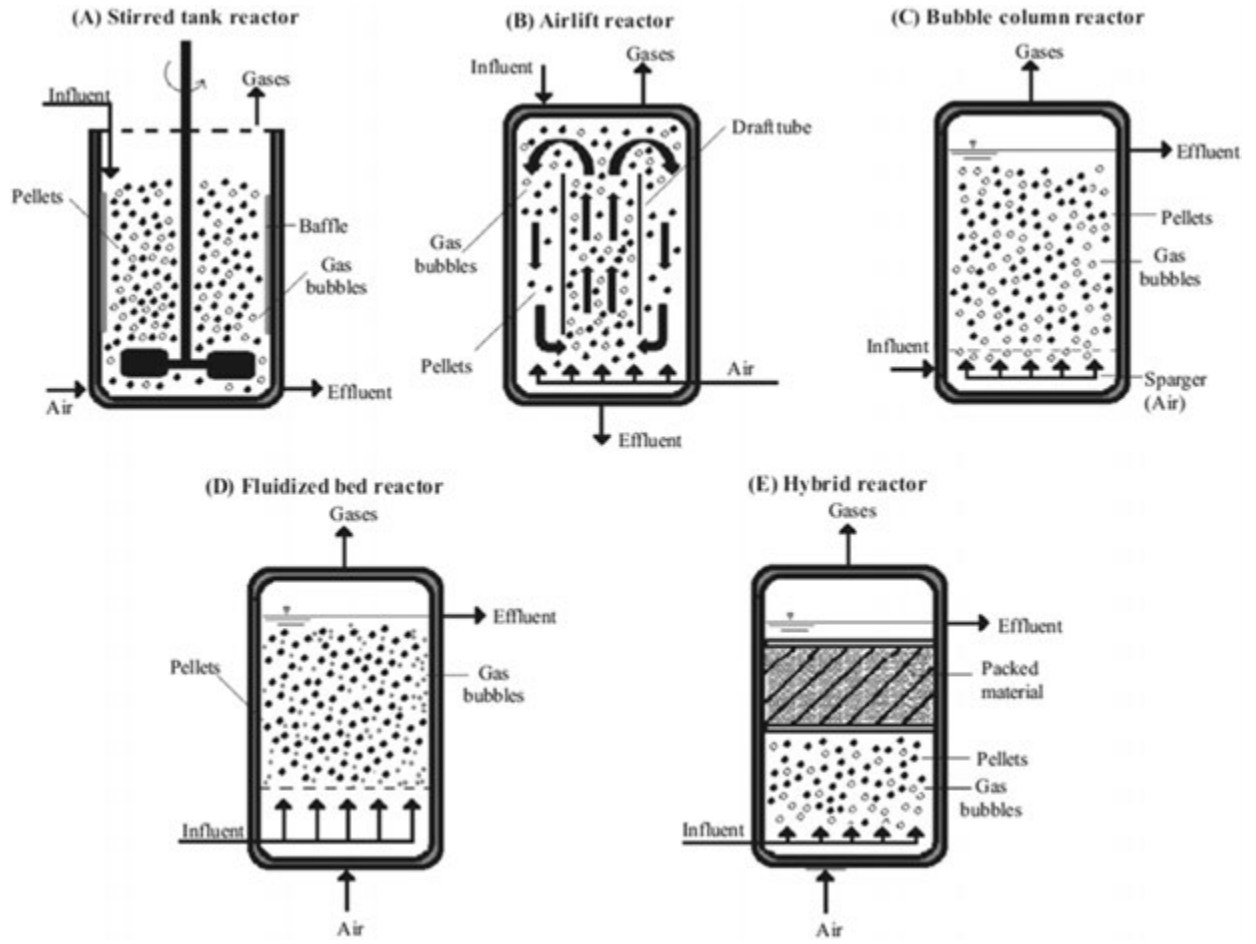

Fig. 6 Different types of fungal bioreactors. Reprinted from Espinosa-Ortiz, E.J., Rene, E.R., Pakshirajan, K., van Hullebusch, E.D., Lens, P.N.L., 2016. Fungal pelleted reactors in wastewater treatment: Applications and perspectives. Chemical Engineering Journal 283, 553–571, with permission from Elsevier

2009), however only with limited details about the reactor geometry. The motion of the fungal colonies in the reactor does not need to be beneficial under all circumstances. A comparison of the use of a laboratory batch fermenter stirred at 400 rpm to a packed bed reactor led to the conclusions that the degradation of the tested model compound (2-chlorophenol) was more efficient when the fungus was immobilized, either on porous silica or on sodium alginate beads (Lewandowski *et al.*, 1990). Here the morphology of the fungal colony comes into the picture, as "fungal pellets can be efficiently used as immobilized cell systems due to their morphological characteristics" (Espinosa-Ortiz *et al.*, 2016). This statement suggests the preferred use of fungal pellets over a filamentous form "in which the mycelium consists of a tangled mass of hyphae homogenously dispersed throughout the medium" (Pirt, 1966).

Looking for an industrial solution to treat wastewater using fungi, laboratory scale bioreactors of 0.5 and 1.5 L were scaled up to 10 L volume, where the biomass fluidization and biomass phase homogenization was achieved by supplying air pulses at the bottom of the reactor (Blánquez *et al.*, 2008). This system, referred to as a "fluidized bed bioreactor", was used to investigate the treatment of hospital wastewater, containing pharmaceuticals and endocrine disrupting compounds (Cruz-Morató *et al.*, 2013a,b, 2014). The results showed at least partial or even complete removal of 48 out of 52 detected compounds. The hydraulic retention time played an important role, which will be discussed below. Another important aspect was the noted need for pH neutralization after the treatment, due to organic acid secretion by the fungi. As an alternative reactor concept, a drum-type reactor was used to investigate the reduction of the amount of sulfur in lignite coal using *Alternaria* sp. Cf1 (Şener *et al.*, 2018).

The employed bioreactor needs to provide sufficient HRT to achieve the required conversion, as was indicated above. Depending on the type of contaminant and probably also the matrix of other compounds present in the solution (wastewater), the required HRT can vary from hours to several days. This obviously will influence the up-scaling of the process for industrial applications, as the treatment of industrial amounts of wastewater will require an installation of considerable size. Therefore, it is crucial to select only the waste streams that cannot be treated efficiently using different methods.

Another group of reactors used to investigate the removal of impurities from wastewater using fungi are Membrane BioReactors (MBR). The main advantages of the fungal MBR reported in the literature are "suspended solids and macro-colloidal material free permeate, retention of high biomass concentration requiring a small footprint and allowing the process to be operated at a low F/M ratio (i.e., food to mass or food to microorganisms), hence, yielding reduced excess sludge production" (Hai *et al.*, 2006, 2009). A fungal

Table 3 Basic characteristics of fungal bioreactors

Reactor type	*Fungi location*	*Mixing*	*Growth control*
Fixed Bed[a,b]	immobilized	none	none
Stirred Tank[c]	floating	very good	good
Bubble Column[d,e]	floating	good	weak
Air Lift[f,g]	floating	good	weak
Fluidized Bed[h,i]	floating[j]	very good	very good

[a]Jaganathan *et al.*, 2009.
[b]Lewandowski *et al.*, 1990.
[c]Moreira *et al.*, 2003.
[d]Cerrone *et al.* (2011).
[e]Kantarci *et al.* (2005).
[f]Espinosa-Ortiz *et al.*, 2016.
[g]Merchuk and Gluz (2002).
[h]Blánquez *et al.*, 2008.
[i]Cruz-Morató *et al.*, 2013a,b.
[j]can be locally immobilized on a substrate material, which can be beneficial to restrain the growth of bacteria (Gao *et al.*, 2008).

MBR was also used in series with a photocatalytic membrane reactor employing TiO_2 and ZnO catalysts and UVA lamps (Deveci *et al.*, 2016). In this reactor, high rates of removal of organic substances were observed in the fungal reactor, while the decolorization occurred mainly in the photocatalytic reactor. A reduction of the COD of ca. 60% was achieved after a five-day treatment in the MBR using *P. chrysosporium*. It must be emphasized that all studies cited above used (micro)filtration membranes, which acted purely as a physical barrier. Such a reactor concept has to be distinguished from MBR used for bacterial processes, where the membrane functions as an electrochemical (ion) barrier. An extensive recent review of this type of MBRs is given in Giwa *et al.* (2019) "**Table 3** presents a brief summary of the main characteristics of fungal bioreactors discussed above".

Key Monitoring Parameters

Production of fungal enzymes is influenced by many factors, mainly process temperature, oxygenation, pH, type and concentration of the substrate and/or inducer (Rodríguez Couto and Sanromán, 2005; Rouches *et al.*, 2016). Hence, regardless of the type of the reactor employed for the processes involving fungi and aqueous solutions, numerous process parameters need to be monitored. A possible configuration of the process control equipment is shown in **Fig. 7**. The key parameter is the measurement of the enzymatic activity, e.g., of LCC. The measurement is usually done off-line using substrates like ABTS and spectrophotometry. For a more accurate determination of the activity of different LCCs, a method using FTIR was proposed (Perna *et al.*, 2019). As the fungal degradation processes are slow, as indicated above, a manual/off-line quantification method can be considered sufficient, although for optimal process monitoring a continuous and automatic method would be preferred. Once the relationships between the parameters like LCC activity and the degree of impurity removal from wastewater are identified, such a parameter can be used to control the process, including the governing of the HRT, nutrient feeding (e.g., glucose, ammonium tartrate) and ultimately the refreshment of the entire fungal culture. Temperature and O_2 concentration can be controlled using common process control equipment. For the control of pH, algorithms have been proposed (e.g., Tyagi and Davison, 1993). The aspect of fungal growth to avoid excessive agglomeration, maintaining its flotation and the avoidance of sticking to the equipment or to the reactor walls is important for long-term operation. For the laboratory tests, visual inspection and periodic sampling is the current practice. Absolute and differential pressure measurements can be used as an indicator for the changes in the reactor inventory (fungal growth, agglomeration or disruption). High-frequency pressure measurements have been applied for the monitoring of agglomeration in gas-solid fluidized beds (Bartels *et al.*, 2010) and perhaps this technique could also be used for monitoring the fungal bioreactors to check if the fungal pellets remain suspended in the reactor and if any changes in the morphology of the fungal biomass occur. Control of both parameters is necessary for the optimal progress of the biological processes.

The description of the technical aspects of fungal bioreactors given above obviously does not exhaust the topic and is only a brief comment to the on-going research and findings in this wide area. Although great progress has already been achieved in this field, several technical challenges still exist and pose a key barrier for commercialization of fungal WWT systems. The most important limitations for up-scaling and long-term operation of fungal bioreactors will be presented in the next section, together with other restrictions that must be fulfilled for successful and industrial-scale fungal technology.

Challenges and Opportunities

As presented in the previous sections, the use of filamentous fungi to treat highly contaminated, industrial wastewater seems to be an attractive and promising option, but their application and operation on a larger scale is still limited. The major reason for this is insufficient

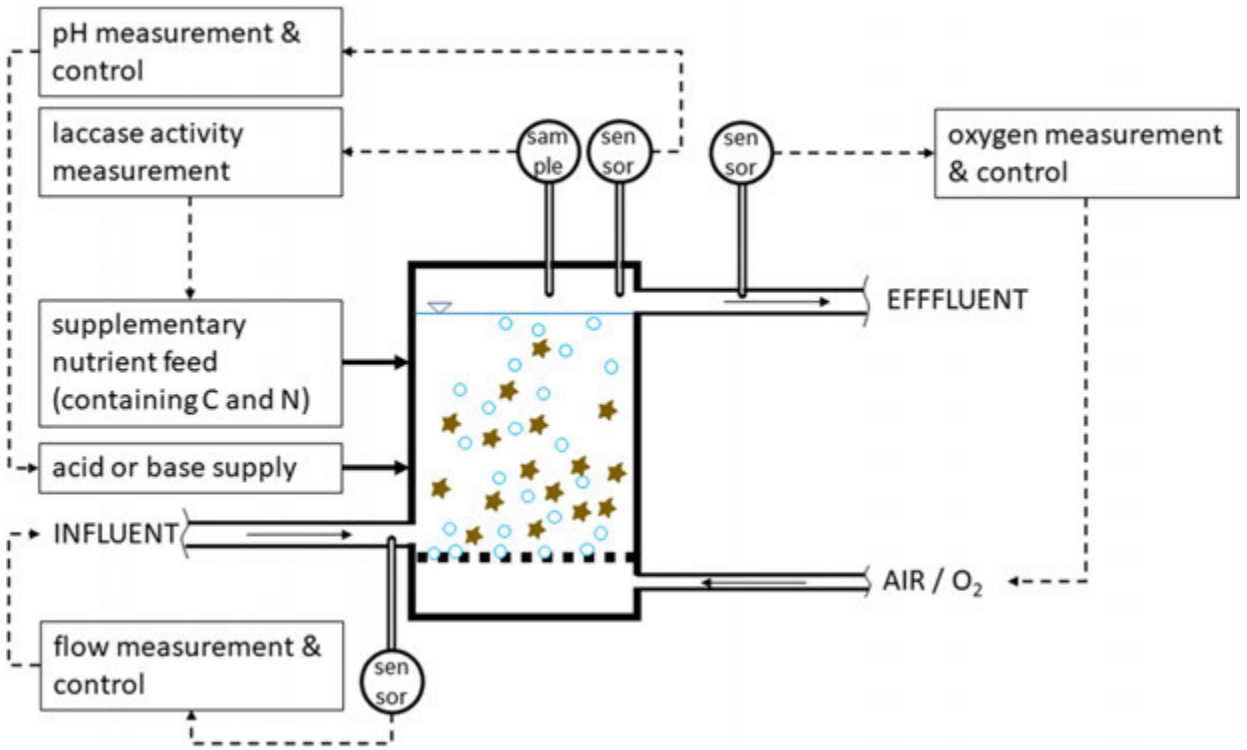

Fig. 7 A possible configuration of key process control equipment in a fungal reactor.

knowledge related to fungal growth kinetics and cultivation requirements. Therefore, to gain general and widespread acceptance for fungal WWT technology and promote its implementation, it is necessary to establish a better understanding of fungal systems.

The description given within the article clearly indicates that the fungal kingdom is still not fully explored, and the number of fungal species being discovered increases rapidly. That requires the development of fast and reliable methods for fungal characterization at the genomic, transcriptomic and proteomic level. Fungal omics should therefore be widely applied to support and stimulate further developments and improvements in their future industrial applications (Xiao *et al.*, 2017). Omics-based strategies in WWT by fungi are expected to dominate the upcoming shift in the current research approach (Silva, 2016). So far more than 2000 fungal genomes have been sequenced, which means that the major part of the fungal kingdom remains undescribed at the genomic level. An abundance of such kind of data facilitates the discovery of fungal proteins and enzymes, and following development of the advanced models for degradation pathways and kinetics, which play important role in designing industrial systems for WWT (Edwards *et al.*, 2017; Téllez-Téllez and Díaz-Godínez, 2019; Wilken *et al.*, 2019). Bioinformatics will be used intensively in mining the data to assist in selection of species with a high potential to produce the desired enzyme activities. Hand in hand with such studies also further extended lab scale tests should be done, with the focus on characterization of enzymes and other key substances such as chelators and organic acids excreted from fungal cells. It is worth to mention that a part of the degradation pathways goes through nonspecific oxidation reactions. Therefore, delivery of this kind of information should be useful for determining fungal potential for mineralization and degradation of organic pollutants. On the other hand, broad and common application of adaptive evolution for fungal cells will allow for their in-depth characterization and determination of the key parameters crucial for the efficient, large-scale cultivation of the selected species. Such an approach will ensure their utilization in more severe conditions resulting from the presence of recalcitrant and often toxic compounds in industrial wastewaters (Zhang and Xu, 2018).

The largest challenge for the successful and widespread implementation of fungal WWT technologies seems to be the development of effective strategies to prevent bacterial contamination, which typically occurs in processes conducted under non-sterile conditions. Although this issue is recognized as crucial, no solution has been offered and confirmed as sufficient so far, despite years of in-depth research and efforts made (Yang *et al.*, 2013a; Mir-Tutusaus *et al.*, 2016; Asif *et al.*, 2017a; Vasina *et al.*, 2017). Entering bacteria into the bioreactors adversely affects the efficiency of fungal WWT for many reasons, among which the most important are: interference with fungal growth, decreasing fungal activity, causing damage in mycelia and competing for substrate (Yang *et al.*, 2013b; Asif *et al.*, 2017a). To date only a few strategies are proposed for dealing with bacterial contamination, i.e., disinfection of the influents by means of ozone, equipping the reactors with micro-screens for washing-out bacteria cells, system operation under lower, acidic pH or applying a coagulation-flocculation treatment prior to the treatment in fungal bioreactor (Sankaran *et al.*, 2010; Jureczko, 2018). However, more research and new approaches are needed to be developed and carefully evaluated to take full advantage of fungal bioreactors. This, in turn, requires more intense studies performed under non-sterile environments combined with further testing and operation of fungal WWT on large scale to verify the obtained results.

The future success of fungal WWT relies also on additional improvements related to costs optimization. Cost reduction and industrial scale application can be boosted by increasing the lifecycle of enzymes, maintaining their activity at a constant level and avoiding their loss by washing out of the system. Several strategies are being tested, mostly at lab scale, to ensure the long-term stability of enzymatic operations. The most promising are enzyme immobilization and application of whole cell fungi instead of pure enzymes (Cabana *et al.*, 2007; Rodríguez-Rodríguez *et al.*, 2010; Asif *et al.*, 2017a). Oxidative enzymes can be immobilized on various synthetic and natural supporting materials, including nano-materials, which significantly improve their stability and prevent outflow with the reactor effluent (Corvini and Shahgaldian, 2010; Datta *et al.*, 2013). Furthermore, it allows for continuous operation of the system, when internal recirculation is considered, and an easier separation of the biocatalyst from the product (Rodríguez Couto, 2009; Gao *et al.*, 2010; Datta *et al.*, 2013; Guzik *et al.*, 2014). The application of whole fungal cells simplifies WWT and reduces the time required for its completion compared to the systems with isolated enzymes. Replacing pure enzymes with the whole cell system may enhance removal of a broader spectrum of pollutants due to the synergistic action of different enzymes (availability of extracellular, intercellular, and/or mycelium bound enzymes), supported by the sorption of pollutants onto fungal biomass (Yang *et al.*, 2013a; Nguyen *et al.*, 2014). Whole cell cultures could be used to enrich microbial populations in existing WWT technologies, or can be applied solely as a tertiary treatment step to wastewater polishing (Shreve *et al.*, 2016). From an economic point of view, also scaling up the process with crude fungal biomass to the level of industrial implementation will always be much cheaper and easier than for pure enzymes.

A key factor influencing successful technology implementation directly concerns bioreactors configuration and further up-scaling to the industrially desired scale. To date only a few studies reported good results in scaling up of fungal WWT systems (Borràs *et al.*, 2008; Cruz-Morató *et al.*, 2013a; Mir-Tutusaus *et al.*, 2017). Therefore, there is still an unquestionable research gap and global need for large-scale studies verifying bench scale experiments. However, it is extremely important to perform this kind of investigations using real industrial effluents. Lab-scale experiments often involve model streams, which are not necessarily representative of real WWTPs, as due to many reasons it is impossible to replicate the latter ones in a defined medium. Also, process operation under non-sterile conditions gives more insight and information about the problems related to biochemistry, toxicology and efficiency of the process, which in turn will help to make any improvements necessary to avoid these process- and technology-related issues. Aiming at an industrial or commercial application of fungal bioreactors different process aspects still need to be considered. Espinosa-Ortiz *et al.* (2016) report inferior mixing and oxygen supply, foaming and fungal growth on the bioreactor walls, agitators and baffles as identified operational problems. A loss of the extracellular enzymes and mediators with discharged water, and excessive growth of fungi are mentioned by Hai *et al.* (2006). These papers also report the problem of a bacterial attack when non-sterile conditions are employed, which will be the case in a real-life WWTP. Summarizing, to better understand the potential use and operational challenges in full scale, more advanced research is still needed, in which the same operational non-sterile conditions for the same effluent will be applied and compared for different reactor configurations.

Like other biological systems, fungal WWT technologies are not flawless and thus may pose health and environmental risks. Therefore, any kind of possible negative impact of fungal bioreactors should be considered in risk assessment procedures. Unfortunately, this issue seems to be overlooked and ignored in scientific discussions, as there are only a limited number of relevant publications, in which only little attention is devoted to such analysis, and mainly such aspects as the prevention of the production of mycotoxins and wastewater disinfection have been considered. The production of secondary metabolites by fungi, such as mycotoxins, should be continuously monitored as this could introduce new toxic compounds into wastewater (Venkatesh and Keller, 2019). The release of such harmful and dangerous compounds under relevant cultivation and stress conditions should be validated and controlled both in lab-scale tests followed by large-scale experiments. This has to be an obligatory step for each single strain introduced into the system, and for every change noticed in wastewater composition. Moreover, such monitoring should be extended to the whole existing system and microbial community into which new strains are introduced. Analyzing the interactions between mycotoxins and other chemical compounds is undoubtedly challenging and time-consuming. However, such data are necessary for ensuring safe WWTP operation. Another issue, strongly related to mycotoxins, is to develop a control system that will ensure fast response for any threat caused by the presence of toxins in the effluent from the bioreactor. Such action may involve, e.g., internal recirculation of wastewater or additional purification step where harmful compounds will be efficiently oxidized. Above mentioned technical solutions should be considered during technology design. For the unexpected and uncontrolled release of the fungal cells with the effluent, several methods have been recommended for water disinfection, e.g., application of chlorine dioxide, ozonation and UV radiation, and many other different substances, like alcohols, iodophors, peracetic acid, aldehydes, peroxygen-based compounds or a mixture of those chemicals (Korukluoglu *et al.*, 2006; Hojnik *et al.*, 2019). Furthermore, to prevent fungal biomass wash-out into the environment, bioreactors can be equipped with micro-screens, what simultaneously supports bacterial decontamination (van Leeuwen *et al.*, 2003; Asif *et al.*, 2017b; Silva *et al.*, 2019). The last, but equally important issue is whether pathogenic or genetically modified microorganisms should be tested for application in WWT bioreactors at all, even when achieving high efficiency in biodegradation of the most persistent contaminants. To date this question remains unanswered.

Conclusions

Based on the present knowledge, employment of fungi for WWT is a feasible alternative or addition to currently operated bacterial systems, since apart from removal of both organic and inorganic pollutants, they allow the recycling of the cell biomass and present a high potential for the recovery of a wide spectrum of added-value products. Despite all those advantages, together with the increasing

amount of feasibility studies concerning fungal WWT, such technologies are not commonly applied at industrial scale yet. Future success in their implementation depends on overcoming some of the most important barriers, such as system nutrition, stability of enzymes and their activity, high required HRT and bacterial contamination. Nevertheless, especially in the case of micropollutants and xenobiotics, WWT by fungi shows a potential that should be explored as a viable alternative for bacterial or chemical processes. Taking into account the rate of discovering new fungal species and fundamental differences between employed microorganisms, it is a real possibility that in the near future the development of a symbiotic fungal–bacterial consortium for the removal of variety of industrial pollutants will dominate the research in the area of WWT. This underlines the great need for further research and new strategies for an effective introduction of fungal wastewater systems into the industrial and commercial market.

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Antitumor and Immunomodulatory Compounds from Fungi

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Introduction

In the last decades, elucidation of the human genome and the manifold achievements in molecular biology have enabled the development of novel therapeutic strategies based on the employment of finely targeted pharmaceuticals. In this evolving context, the need for finding more effective drugs has increased, with a stronger pulse for the exploitation of natural sources. Fungi are particularly interesting in this search because of their pervasive occurrence in every environment, and their suitability for laboratory manipulation and use of selected strains in industrial production. In fact, fermentative processes generally afford higher outputs of bioactive substances, in comparison with the more costly extraction from plant biomasses. Even when it is not possible to optimize their direct pharmaceutical application, fungal products may represent valuable models inspiring the creation of new drugs through combinatorial synthesis (Gordaliza, 2007; Cragg *et al.*, 2009).

Fungal secondary metabolites display very diverse chemical structures, ranging from small molecules to polymers (Brakhage, 2013; Bills and Gloer, 2017; Keller, 2019). Unlike the latter, which in most instances are structural components of fungal cell walls, low molecular mass metabolites, or extrolites, usually have an outward-oriented function finalized to interactions with other organisms, serving as antibiotics, regulators, or chemical messengers in developmental processes. At some extent, these bioactivities reflect antitumor and immunomodulatory properties, which are examined in this chapter with reference to possible pharmaceutical applications.

Interrelations Between Immunomodulatory and Antitumor Properties

Immunomodulation is the process of modifying immune response, in terms of its induction, expression, amplification or inhibition, through the administration of drugs. Immunomodulators are used for the suppression of normal or excessive immune function (immunosuppressants, as for the treatment of graft rejection or autoimmune diseases), or the reconstitution of immune deficiency (immunostimulators, as for the treatment of AIDS). However, both kind of products may also find application in cancer chemotherapy.

An effective treatment of tumor diseases should aim to combat the primary focus and prevent the formation of metastases. In this respect, there is a growing trend to integrate products directly targeting cell proliferation and migration with immunomodulators in order to stimulate the innate capacity of the organism to contrast cancer progression (Corradetti *et al.*, 2019). The immune system may react through an acute inflammatory response where the early-generated tumor cells are eliminated by major agents, such as killer cells, dendritic cells, macrophages, polymorph nuclear cells, mast cells, and cytotoxic T cells. When neoplastic cells resist this basic surveillance, an equilibrium between their proliferation and apoptosis is established, which can limit tumor aggressiveness. However, this equilibrium can be disrupted by any factor reducing immunogenicity of tumor cells, with the release of anti-inflammatory mediators inhibiting the effector T lymphocytes and other steps of the immune response. Moreover, the release of reactive oxygen species (ROS) and cytokines may further induce mutations in the normal cells, and the tumor transition (Castaldo *et al.*, 2016). Reactive oxygen and nitrogen species are generated in response to proinflammatory cytokines and growth factors, and downregulated by tumor suppressor genes and anti-inflammatory cytokines. The release of high level of these products is observed in almost all cancer types due to activity of oxidases, increased metabolic rate, mitochondrial dysfunction, and the infiltration of inflammatory cells in the tumor microenvironment. Their proapoptotic effect is exerted through activation of either the extrinsic or the intrinsic mitochondrial pathway after disruption of the mitochondrial membrane potential and downregulation of antiapoptotic proteins, leading to release of cytochrome *c*. Cytokines and other growth factors may also promote angiogenesis, which is a fundamental step for progression of many solid tumors (Galdiero *et al.*, 2018).

Inflammation of tissues represents an immune response against any invading pathogen and injury. However, chronic inflammation leading to oxidative stress and DNA damage may be conducive to initiation and progression of cancer. Activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein complex represents a crucial step in the initiation of the inflammatory response through genetic regulation. Stimulation of this signaling pathway promotes tumor growth as well as angiogenesis and metastasis, so that NF-κB represents one of the main targets for anticancer drugs (Ravi and Bedi, 2004; Umezawa, 2006). Other proteins involved in the complex signaling system influencing cancer progression are the mitogen-activated protein kinases, which regulate cell proliferation, differentiation, invasion, stress response and apoptosis after external stimulation by cytokines and growth factors, and AP-1, a transcription factor which is activated by proinflammatory cytokines and oxidative stress (Braicu *et al.*, 2019). Other targets considered in the treatment of many types of cancer due to their role in tumor initiation and progression are enzymes such as cyclooxygenase (COX-2) and nitric oxide synthase (iNOS). Besides its role in inflammation, overexpression of COX-2 leads to upregulation of Bcl-2, an anti-apoptotic protein family, and angiogenic mediators, such as the vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs), while iNOS induction leads to release of nitric oxide, with ensuing carcinogenic effect due to DNA damage and mutation (Ghosh *et al.*, 2010).

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Finding Fungi Producing Antitumor and Immunomodulatory Drugs

The primary function of fungal extrolites is to improve the ecological fitness of their producers. In this respect, a logical and fruitful ground of investigation is represented by contexts conducive for antibiosis, undoubtedly the most powerful weapon used by fungi in competitive biocenotic interactions. At a certain extent, the biochemical factors responsible for inhibitory effects on growth and viability of the target organisms can display their activity against any kind of cell type. However, unlike the proper antibiotic drugs which are effective on specific classes of pathogens with minor side effects on mammalian cells, antitumor and immunomodulatory agents are required to target neoplastic cells or the immune system in a selective manner. More constraints concern safety of the notorious fungal products known as mycotoxins, which are mostly considered for their occurrence in fodder and foodstuffs. Although functionally related to the antibiotics and responsible for cytotoxic effects which are directly or indirectly implicated in cell division, these products are usually not regarded as candidate drugs by reason that their bioactivity is incompatible with a pharmaceutical application. In fact, toxicological studies on human and mammalian cell lines have shown that compounds such as citrinin, sterigmatocystin, aflatoxins, ochratoxins, fumonisins, penitrems and related tremorgenic toxins can be responsible for substantial genotoxic, carcinogenic and/or teratogenic effects (Marin *et al.*, 2013; Dellafiora and Dall'Asta, 2017). However, there can be exceptions; in fact, products such as gliotoxin, sporidesmin, patulin, secalonic acid D, alternariol, chaetoglobosin, some trichothecenes and several anthraquinones have displayed interesting bioactivities in specific assays, introducing opportunities for their possible exploitation as antitumor or immunomodulatory drugs (Ali *et al.*, 2013; Scharf *et al.*, 2016; Abastabar *et al.*, 2017; Schmutz *et al.*, 2019).

Nutritional implications also characterize many mushroom forming fungi, whose edible carpophores are commonly used in traditional medicine worldwide. Thereby a number of species in the Basidiomycota have a potential as functional food (prebiotics), representing a vehicle for the assumption of beneficial molecules which may interfere with cancer onset and progression, and the immune system. Following the pioneering observations on the sarcoma 180 line in mice treated with extracts of *Boletus edulis* and other homobasidiomycetes (Byerrum *et al.*, 1957), antitumor effects have been reported at some extent for hundreds of mushrooms (Wasser, 2010; Popovic *et al.*, 2013). Besides the relevant properties of polysaccharides (Meng *et al.*, 2016b) and trace elements such as selenium, which are known to accumulate in these fungi (Sinha and El-Bayoumy, 2004), low molecular weight compounds are considered to integrate the beneficial dietary contribution.

Other bioactive extrolites have been reported as specific and non-specific toxins of fungi behaving as plant pathogens. In fact, after having been characterized for their role in plant disease onset/progression, compounds such as fusicoccin, ophiobolins, cochlioquinones, sphaeropsidins and pyrenocines have aroused pharmaceutical interest based on their consistent cytostatic effects (Lallemand *et al.*, 2012; Bury *et al.*, 2013; Tian *et al.*, 2017; Wang *et al.*, 2016a; Myobatake *et al.*, 2019).

Interactions between fungi and plants are more often asymptomatic, leading to the establishment of a symbiotic relationship known as endophytism. This condition sometimes implies a mutual benefit, which on the plant side may consist in improved protection against pests and pathogens. This effect possibly derives from the ability by the endophytes to produce bioactive compounds which stimulate plant defense reaction, or even directly affect the development of pathogens through the mechanisms of antibiosis. Such hypotheses are corroborated by a huge amount of new natural products characterized by endophytic fungi, many of which have been found to possess antiproliferative and/or immunomodulatory properties in assays on human cell systems. Considering the astonishing number of fungal species which have been estimated to occur endophytically (Ganley *et al.*, 2004), this category is regarded as a gold mine of undescribed chemodiversity attracting the attention of researchers involved in drug discovery. In this investigational context most noteworthy are endophytes which have been found to produce bioactive compounds formerly characterized from plants (**Table 1**). The best-known example is represented by taxol (paclitaxel), the first one-billion-dollar natural antitumor drug originally characterized by *Taxus* species (Wani *et al.*, 1971; Vidensek *et al.*, 1990), but later reported as a secondary metabolite of an increasing number of endophytic fungi (Uzma *et al.*, 2018).

The biological reasons of this extraordinary biosynthetic convergence are still hypothetical (Aly *et al.*, 2013). The high number and diversity of endophytic strains which developed this aptitude and the occurrence in clusters of genes involved in the biosynthesis of many classes of fungal secondary metabolites may call into question horizontal gene transfer as a possible explanation (Gao *et al.*, 2014; Soucy *et al.*, 2015; Marcet-Houben and Gabaldón, 2016). However, analyzing this topic more in depth goes beyond the theme of this chapter.

Besides the above more or less direct biological associations where these compounds find a functional *raison d'être*, reports in the literature show that successful discovery of fungi producing new antitumor and immunomodulatory drugs operates in other diverse contexts. In fact, promising strains have been recovered from extreme environments, such as Antarctic soil (Godinho *et al.*, 2015), deep sea vents and sediments (Pettit, 2011), and contaminated sites (Stierle *et al.*, 2004; Stierle *et al.*, 2012). In the end, every fungal isolate is a potential source of new bioactive products. Therefore, even the isolates that do not outstand in screening programs for bioactivities are sometimes re-evaluated after altering their metabolism through stress factors or co-culturing with other microbial strains, to activate silent or cryptic gene clusters (Brakhage, 2013; Fang *et al.*, 2014; Keller, 2019). Moreover, less active fungal products may represent models for the development of synthetic drugs. This is the case of plinabulin, a synthetic derivative of phenylahistin, which is currently in phase 3 of clinical trials (Cimino *et al.*, 2019).

Table 1 Plant secondary metabolites with antitumor and immunomodulatory properties which have also been reported as products of endophytic fungi

Compound[a]	*Main producers*		*Reference*
	Plants	*Fungi*[b]	
Aconitine	*Aconitum* spp.	*Cladosporium cladosporioides*	Yang *et al.* (2013c)
Astins	*Aster tataricus*	*Cyanodermella asteris*	Schafhauser *et al.* (2019)
Berberine	*Berberis* spp.	*Neocosmospora solani*	Vinodhini and Agastian (2013)
Bilobalide	*Gingko biloba*	*Pestalotiopsis uvicola*	Qian *et al.* (2017)
Camptothecins	*Camptotheca acuminata*	*Entrophospora infrequens*	Kai *et al.* (2015)
Costundide	*Saussurea costus*	*Pestalotiopsis uvicola*	Qian *et al.* (2017)
Diosgenin	*Discorea* spp.	*Acremonium* sp.	Cao *et al.* (2007)
Emodin	*Rheum emodi*	*Penicillium simplicissimum*	Marinho *et al.* (2005)
Ephedrine	*Ephedra sinica*	*Schizophyllum commune*	Su *et al.* (2014)
Eremophilanolides	*Senecio* spp.	*Xylaria* sp.	Isaka *et al.* (2010)
Gingkolide B	*Gingko biloba*	*Fusarium oxysporum*	Cui *et al.* (2012)
Hypericin	*Hypericum perforatum*	*Canariomyces subthermophilus*	Kusari *et al.* (2008)
Myrtucommulones	*Myrtus communis*	*Neofusicoccum australe*	Nicoletti *et al.* (2018)
Peimisine	*Fritillaria* spp.	*Fusarium redolens*	Pan *et al.* (2015)
Physcion	*Rheum officinale*	*Aspergillus* spp.	Gessler *et al.* (2013)
Piperine	*Piper nigrum*	*Periconia* sp.	Verma *et al.* (2011)
Podophyllotoxins	*Podophyllum* spp.	*Trametes hirsuta*	Puri *et al.* (2006)
Questin	*Cassia* spp.	*Aspergillus* spp.	Gessler *et al.* (2013)
Rhein	*Rheum palmatum*	*Neocosmospora solani*	You *et al.* (2013)
Rohitukine	*Amoora rohituka*	*Fusarium* spp.	Kumara *et al.* (2014)
Sanguinarine	*Sanguinaria canadensis*	*Fusarium proliferatum*	Wang *et al.* (2014b)
Silybins	*Silybum marianum*	*Aspergillus iizukae*	El-Elimat *et al.* (2014)
Tanshinones	*Salvia* spp.	*Trichoderma atroviride*	Ming *et al.* (2012)
Taxanes	*Taxus* spp.	*Taxomyces andreanae*	Stierle *et al.* (1993)
Ursolic acid	*Malus pumila*	*Pestalotiopsis uvicola*	Qian *et al.* (2017)
Vinca alkaloids	*Cataranthus roseus*	*Talaromyces radicus*	Palem *et al.* (2015)

[a]Polyphenols, flavonoids and other antioxidant compounds, such as resveratrol, which can be common products of endophytic fungi (Nicoletti and Fiorentino, 2015), are not considered in this table.

[b]Fungal species listed in **Tables 1** and **2** have been updated considering the last accepted names reported in Mycobank (www.mycobank.org); therefore they do not necessarily correspond to the ones used in the corresponding references.

Chemodiversity of Antitumor and Immunomodulatory Products from Fungi

Besides origin, taxonomy and ecological interactions of the producing strains, the term "diversity" is even more appropriate for defining chemical structures of these compounds. Indeed, their skeleton and biosynthetic schemes are very variable (**Fig. 1**), offering a wide array of molecular models which make their classification problematic. Some products, such as mycoepoxydiene (**34**) (Cai *et al.*, 1999; Prachya *et al.*, 2007), have been even proposed as the founders of novel classes of secondary metabolites. Moreover, several products, particularly alkaloids and terpenes, present a hybrid structure, which further complicates their assimilation to a definite class. A tentative arrangement is proposed in **Table 2**, which however is not exhaustive as a number of products are placed in a provisional miscellaneous category.

A total of 289 entries are included in **Table 2**, each of them corresponding to either single definite compounds, or groups of analogs, or more or less assorted compound families. Actually, the analysis of properties of every single member within the latter groupings is a matter for dedicated reviews, considering that their structural model affords a high number of possible intramolecular substitutions, originating variants with uneven bioactive effects. In this respect, an example is represented by the sorbicillinoids (or vertinoids), whose structure is based on the hexaketide scaffold of sorbicillin. This group includes about 90 monomeric, dimeric or trimeric compounds, and a number of hybrid products such as sorbicillactone A, whose cytotoxic effects are quite variable with reference to whether or not a double bond is present in the sorbyl side chain (Meng *et al.*, 2016a). Less numerous and almost exclusive of *Talaromyces* species, the funicones are structured on a γ-pyrone moiety linked to an aromatic ring through a ketone function. Their most studied representative, 3-*O*-methylfunicone (**24**), has been characterized as a prospect antitumor drug based on valuable antiproliferative, proapoptotic and antimetastatic properties (Nicoletti *et al.*, 2014).

Mostly known as mycotoxins, the trichothecenes consist of a family of over 200 compounds grouped in four types sharing a common tricyclic 12,13-epoxytrichothec-9-ene core nucleus (McCormick *et al.*, 2011). Although some members have disclosed consistent antitumor and immunomodulatory properties (Peres de Carvalho *et al.*, 2016), it is difficult to provide univocal indications concerning their bioactivity profile and possible pharmaceutical interest. In fact, trichothecenes can induce both cell-survival and cell-apoptosis pathways. Moreover, they have complex effects on the immune system of mammals, both immunosuppressive

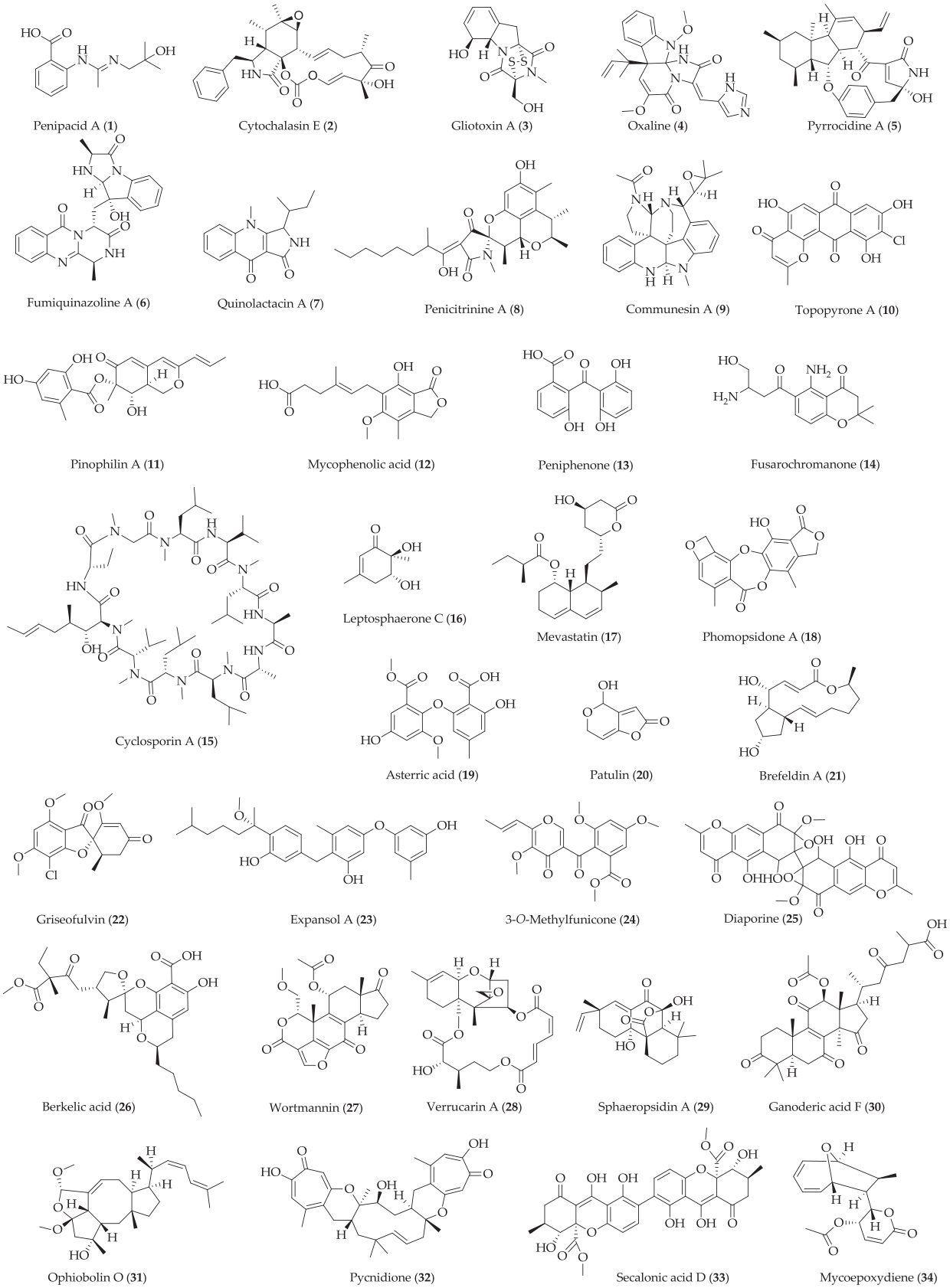

Fig. 1 Chemodiversity of antitumor and immunomodulatory products of fungi. Structures are numbered with reference to the compound list in Table 2.

Table 2 Basic activities of antitumor and immunomodulatory compounds from fungi

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Alkaloids							
Amides							
Gymnastatins, dankastatins	*Gymnascella dankaliensis*	■					Amagata *et al.* (2008)
Penipacid (**1**)	*Penicillium paneum*	■					Li *et al.* (2013)
Cytochalasans							
Chaetochalasin A	*Ovatospora brasiliensis*	■					Oh *et al.* (1998)
Chaetoglobosins	*Chaetomium globosum*	■	■	■	■		Li *et al.* (2014b)
Cytochalasins (**2**)	*Aspergillus* spp.	■	■	■	■		Trendowski (2015)
Periconiasins	*Periconia* sp.	■					Zhang *et al.* (2013b)
Phomacins	*Phoma* sp.	■					Alvi *et al.* (1997)
Epipolythiodioxopiperazines							
Adametizine A	*Penicillium adametzioides*	■					Liu *et al.* (2015b)
Brocazines	*Penicillium brocae*	■					Meng *et al.* (2014)
Chaetocins	*Chaetomium* spp.	■	■	■		■	Han *et al.* (2017)
Chaetocochins	*Chaetomium* spp.	■	■				Wang *et al.* (2015a,b)
Chetomins	*Humicola seminuda*	■	■			■	Fujimoto *et al.* (2004)
Chetracins	*Oidiodendron truncatum*	■					Li *et al.* (2012)
Emestrins	*Aspergillus nidulans*	■	■				Nursid *et al.* (2015)
Gliocladicillins	*Gliocladium* sp.	■	■				Chen *et al.* (2009b)
Gliotoxins (**3**)	*Aspergillus fumigatus*	■	■	■		■	Scharf *et al.* (2016)
Leptosins	*Leptosphaeria* sp.	■					Yanagihara *et al.* (2005)
Penicisulfuranols	*Penicillium simplicissimum*	■					Zhu *et al.* (2017)
Sporidesmins	*Pithomyces chartarum*	■				■	Mullbacher *et al.* (1986)
T988	*Tilachlidium* sp.	■					Feng *et al.* (2004)
Thiosilvatins	*Penicillium* spp.	■					Salvatore *et al.* (2019)
Verticillins	*Bionectria* sp.	■	■	■		■	Zewdu *et al.* (2016)
Prenylated diketopiperazines							
Aurantiamine	*Penicillium aurantiogriseum*	■					Hayashi *et al.* (2000)
Deoxymycelianamide	*Gliocladium* sp.	■					Yao *et al.* (2009)
Echinulins	*Aspergillus oryzae*	■					Gao *et al.* (2012)
Fumitremorgins	*Aspergillus fumigatus*	■					Abraham (2018)
Fungerin	*Metarhizium anisopliae*	■					Koizumi *et al.* (2004a)
Meleagrins, oxalines (**4**)	*Penicillium oxalicum*	■	■		■	■	Koizumi *et al.* (2004b)
Notoamides	*Aspergillus ochraceus*	■	■			■	Hu *et al.* (2019)
Penicillivinacine	*Penicillium vinaceum*	■			■		Asiri *et al.* (2015)
Penicimutanins	*Talaromyces purpurogenus*	■					Fang *et al.* (2014)
Penicitroamide	*Penicillium* sp.	■					Feng *et al.* (2016b)
Penipalines	*Penicillium paneum*	■					Li *et al.* (2014a)
Phenylahistin (halimide)	*Aspergillus ustus*	■	■	■			Kanoh *et al.* (1999)
Tryprostatins	*Aspergillus fumigatus*	■	■				Usui *et al.* (1998)
Pyrrocidines							
Embellicines	*Embellisia eureka*	■	■			■	Ebrahim *et al.* (2013)
Pyrrocidine A (**5**)	*Neonectria candida*	■	■				Uesugi *et al.* (2016)
Pyrrospirones	*Neonectria candida*	■	■				Shiono *et al.* (2008)
Quinazolines							
Auranomides	*Penicillium aurantiogriseum*	■					Song *et al.* (2012)
Aurantiomides	*Penicillium aurantiogriseum*	■					Xin *et al.* (2007)
Chetominine	*Chaetomium* sp.	■					Jiao *et al.* (2006)
Fumiquinazolines (**6**)	*Aspergillus fumigatus*	■					Takahashi *et al.* (1995)
Penipanoids	*Penicillium paneum*	■					Li *et al.* (2011a)
Quinolones							
Penicinoline	*Penicillium* sp.	■					Shao *et al.* (2010a)
Quinolactacins (**7**)	*Penicillium* spp.	■	■			■	Kakinuma *et al.* (2000)

(*Continued*)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Tetramic acids							
Aspergillines	*Aspergillus versicolor*	■					Zhou *et al.* (2014)
Epicoccamide D	*Epicoccum* sp.	■					Wangun *et al.* (2007)
Penicillenols	*Penicillium* sp.	■					Lin *et al.* (2008a)
Penicitrinine A (**8**)	*Penicillium citrinum*	■	■		■		Liu *et al.* (2015a)
Other alkaloids							
Citrinadins	*Penicillium citrinum*	■					Tsuda *et al.* (2004)
Communesins (**9**)	*Penicillium marinum*	■					Pompeo *et al.* (2019)
Isohericerin, isoherocerone	*Hericium erinaceus*	■					Kim *et al.* (2012)
Peniproline	*Penicillium decumbens*	■					Wang *et al.* (2017)
Perinadine	*Penicillium citrinum*	■					Sasaki *et al.* (2005)
Pyridoxatin	*Tolypocladium cylindrosporum*	■	■		■		Li *et al.* (2015)
Terretriones	*Penicillium vinaceum*	■			■		Asiri *et al.* (2015)
Trichodermamides	*Trichoderma virens*	■					Garo *et al.* (2003)
UCS1025	*Acremonium* sp.	■					Agatsuma *et al.* (2002)
Xanthocillin X	*Penicillium commune*	■	■				Zhao *et al.* (2012)
Anthraquinones							
Alterporriols	*Alternaria* spp.	■	■				Huang *et al.* (2012)
Altersolanols	*Stemphylium globuliferum*	■	■		■		Teiten *et al.* (2013)
Aspergiolides	*Aspergillus glaucus*	■	■		■		Wang *et al.* (2014c)
Averantins	*Penicillium glabrum*	■					Ren *et al.* (2007)
Averufins, nidurufin	*Penicillium glabrum*	■					Ren *et al.* (2007)
Dactylariol	*Pleospora* sp.	■					Ge *et al.* (2005)
Deoxybostrycin	*Pleospora* sp.	■					Ge *et al.* (2005)
Endocrocin	*Aspergillus fumigatus*					■	Berthier *et al.* (2013)
Haloroquinone (SZ-685C)	*Halorosellinia* sp.	■	■				Wang *et al.* (2015b)
Norsolorinic acid	*Aspergillus nidulans*	■	■				Wang *et al.* (2008)
Quinofuracins	*Staphylotrichum boninense*	■	■				Tatsuda *et al.* (2015)
Rubrocristin	*Microascus tardifaciens*	■				■	Fujimoto *et al.* (1999a)
Saintopin	*Paecilomyces* sp.	■					Yamashita *et al.* (1990)
Skyrins	*Cyanodermella asteris*	■	■				Jahn *et al.* (2017)
Topopyrones (**10**)	*Phoma* sp.	■					Kanai *et al.* (2000)
UCT1072s	*Aspergillus* sp.	■					Asai *et al.* (1999)
Variecolorquinones	*Aspergillus variecolor*	■					Wang *et al.* (2007b)
Versicolorins	*Penicillium glabrum*	■					Ren *et al.* (2007)
Azaphilones							
Berkchaetoazaphilones	*Pleurostomophora* sp.	■					Stierle *et al.* (2015)
Chaetomugilins	*Chaetomium globosum*	■					Yasuhide *et al.* (2008)
Chaetoviridins	*Chaetomium globosum*	■					Phonkerd *et al.* (2008)
Comazaphilones	*Penicillium commune*	■					Gao *et al.* (2011a,b)
Coniellins	*Coniella fragariae*	■			■		Yu *et al.* (2018)
Epiaustdiols	*Talaromyces* sp.	■					Liu *et al.* (2010)
Luteusin, RP-1551	*Penicillium* sp.			■			Toki *et al.* (1999)
Pinophilins (**11**)	*Talaromyces pinophilus*	■					Myobatake *et al.* (2012)
Sclerotiorins	*Cephalotheca faveolata*	■					Giridharan *et al.* (2012)
Sequoiamonascins	*Aspergillus parasiticus*	■					Stierle *et al.* (2003)
Benzofurans							
Awajanoran	*Acremonium* sp.	■					Jang *et al.* (2006)
Diaporthelactone	*Diaporthe* sp.	■					Lin *et al.* (2005)
Mycophenolic acid (**12**)	*Penicillium brevicompactum*	■	■	■		■	Dun *et al.* (2013)
Photinides	*Pestalotiopsis photiniae*	■					Ding *et al.* (2009)
Benzophenone derivatives							
Arugosins A-B	*Aspergillus nidulans*	■					Kralj *et al.* (2006)
Penicillenone	*Penicillium* sp.	■					Lin *et al.* (2008b)
Peniphenone (**13**)	*Talaromyces* sp.					■	Liu *et al.* (2016)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Chromenones, isocoumarins							
Acetophthalidin	*Penicillium* sp.	■					Cui *et al.* (1996)
Alternariols	*Alternaria alternata*	■	■			■	Palanichamy *et al.* (2019)
Bipenicilisorin	*Penicillium chrysogenum*	■					Chen *et al.* (2017)
Chaetocyclinone B	*Diaporthe* sp.	■					Ding *et al.* (2017)
Chromosulfine	*Talaromyces purpurogenus*	■					Yi *et al.* (2016)
Cochliones	*Cochliobolus* sp.	■					Wang *et al.* (2010a)
Coniochaetone D	*Fimetariella* sp.	■					Deng *et al.* (2013)
Conioxepinols	*Coniochaeta* sp.	■					Wang *et al.* (2010b)
Desmethyldiaportinol	*Ampelomyces* sp.	■					Aly *et al.* (2008)
Epiremisporine B	*Penicillium* sp.	■					Liu *et al.* (2017b)
Fusarochromanone (**14**)	*Fusarium equiseti*	■	■	■	■		Mahdavian *et al.* (2014)
Fusidienol	*Fusidium griseum*		■				Singh *et al.* (1994a)
Oxalicumones	*Penicillium oxalicum*	■	■				Bao *et al.* (2014)
Penimethavone A	*Penicillium chrysogenum*	■					Hou *et al.* (2016)
Pestaloficiols	*Pestalotiopsis fici*	■					Liu *et al.* (2009b)
Pestalotiopsones	*Pestalotiopsis* sp.	■					Xu *et al.* (2009)
Preussochromones	*Preussia africana*	■					Zhang *et al.* (2012)
Rhytidchromones	*Rhytidhysteron rufulum*	■					Chokpaiboon *et al.* (2016)
Cyclic peptides and depsipeptides							
Beauvericin, enniatins	*Beauveria bassiana*	■	■			■	Sood *et al.* (2017)
Chlamydocins	*Tolypocladium* sp.	■	■			■	Du *et al.* (2014)
Chlorofusin	*Microdochium caespitosum*		■				Clark *et al.* (2009)
Clavatustide B	*Aspergillus clavatus*	■	■				Ye *et al.* (2014)
Cyclosporins (**15**)	*Tolypocladium inflatum*	■	■	■		■	Muñoz *et al.* (2010)
Destruxins	*Metarhizium anisopliae*	■	■			■	Liu and Tzeng (2012)
Fusaristatins	*Fusarium* sp.	■	■				Shiono *et al.* (2007)
IB-01212	*Clonostachys* sp.	■	■				Cruz *et al.* (2006)
Paecilodepsipeptide A	*Paecilomyces cinnamomeus*	■					Isaka *et al.* (2007)
Phalloidin	*Amanita phalloides*	■					An *et al.* (2010)
Pullularins	*Clonostachys rosea*	■					Ebrahim *et al.* (2012)
Psychrophilin D	*Penicillium* sp.	■					Dalsgaard *et al.* (2005)
Roseotoxin B	*Trichothecium roseum*	■				■	Wang *et al.* (2016b)
Sansalvamides	*Fusarium* sp.	■					Belofsky *et al.* (1999)
Scopularides	*Scopulariopsis brevicaulis*	■					Yu *et al.* (2008)
Stevastelins	*Penicillium* sp.					■	Morino *et al.* (1994)
Trapoxins	*Helicoma ambiens*		■			■	Kijima *et al.* (1993)
Trichomides	*Trichothecium roseum*					■	Zhang *et al.* (2013a)
WF-3161	*Petriella guttulata*	■					Umehara *et al.* (1983)
Zygosporamide	*Zygosporium masonii*	■					Oh *et al.* (2006)
Cyclohexenoids							
Leptosphaerone C (**16**)	*Penicillium* sp.	■					Lin *et al.* (2008b)
Pericosine A	*Periconia byssoides*	■					Yamada *et al.* (2007)
Trichocladinols	*Pleotrichocladium opacum*	■					Guo *et al.* (2009)
Decaline derivatives							
Epoxyphomalins	*Paraconiothyrium* sp.	■	■				Mohamed *et al.* (2010)
Fusarielins	*Fusarium graminearum*	■		■			Fujimoto *et al.* (2008)
Phomopsidin	*Phomopsis* sp.	■					Namikoshi *et al.* (1997)
Statins (**17**)	*Penicillium* spp.	■	■	■	■	■	Jakobisiak and Golab (2010)
Tanzawaic acids	*Penicillium citrinum*	■	■				Cardoso-Martínez *et al.* (2015)
Depsidones							
Aspergillusidone A, unguinol	*Aspergillus unguis*	■					Chottanapund *et al.* (2017)
Paeciloxocin A	*Paecilomyces* spp.	■					Wen *et al.* (2010)
Phomopsidone A (**18**)	*Diaporthe* sp.	■					Zhang *et al.* (2014b)

(*Continued*)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Diphenylethers							
Asterric acids, sulochrins (**19**)	*Penicillium glabrum*	■	■	■			Nicoletti *et al.* (2008)
Barceloneic acids	*Penicillium albocoremium*		■				Overy *et al.* (2005)
Penicillides	*Talaromyces pinophilus*	■					Zhao *et al.* (2015)
Sinopestalotiollides	*Pestalotiopsis palmarum*	■					Xiao *et al.* (2018)
Furanones							
Annulosquamulin	*Annulohypoxylon squamulosum*	■					Cheng *et al.* (2012)
Kobifuranones	*Gelasinospora kobi*					■	Fujimoto *et al.* (1998b)
Patulin (**20**)	*Penicillium expansum*	■	■			■	Abastabar *et al.* (2017)
Macrolides							
Benquoine	*Diaporthe* sp.	■					Adelin *et al.* (2011)
Brefeldin A (**21**)	*Penicillium brefeldianum*	■	■				Shao *et al.* (1996)
Clavilactones	*Ampulloclitocybe clavipes*	■	■				Miyazaki *et al.* (2016)
Colletodiol	*Diplogelasinospora grovesii*					■	Fujimoto *et al.* (1998a)
Curvularins	*Curvularia* sp.	■	■	■			Greve *et al.* (2008)
Cytospolides	*Cytospora* sp.	■					Lu *et al.* (2011)
Galiellalactone	*Galiella rufa*	■	■	■			Rudolph *et al.* (2013)
Lasiodiplodins	*Lasiodiplodia theobromae*	■	■				Hazalin *et al.* (2013)
Oxacyclododecindione	*Exserohilum rostratum*			■			Rudolph *et al.* (2013)
Radicicol, pochonins	*Pochonia chlamydosporia*	■	■	■			Gadelle *et al.* (2006)
Sch 642305	*Penicillium canescens*	■					Nicoletti *et al.* (2008)
Phenol-methoxyphenyl derivatives							
Asperflavin	*Microascus tardifaciens*	■	■			■	Fujimoto *et al.* (1999a)
Bacillisporins, duclauxin	*Talaromyces bacillisporus*	■					Dethoup *et al.* (2006)
Cladosporols	*Cladosporium cladosporioides*	■	■				Koul *et al.* (2017)
Cylindrol A	*Thelonectria lucida*		■				Singh *et al.* (1995)
Gerronemins	*Gerronema* sp.	■					Silberborth *et al.* (2002)
Ginsenocin	*Penicillium melinii*	■					Zheng *et al.* (2013)
Griseofulvin (**22**)	*Penicillium* spp.	■	■	■		■	Rebacz *et al.* (2007)
Hericenones	*Hericium erinaceus*	■					Kim *et al.* (2012)
Hispolon	*Tropicoporus linteus*	■	■		■		Lu *et al.* (2009)
Hypoxylonols	*Annuloxypoxylon truncatum*			■			Fukai *et al.* (2012)
Pestalols	*Pestalotiopsis* sp.	■					Sun *et al.* (2014)
Sorbicillinoids	*Penicillium* spp.	■	■				Meng *et al.* (2016a,b)
Sordarial	*Gelasinospora* spp.					■	Fujimoto *et al.* (1999b)
Stemphyperilenol	*Talaromyces* sp.	■					Liu *et al.* (2010)
Truncatones	*Annulohypoxylon* spp.	■					Sudarman *et al.* (2016)
Ulocladol	*Alternaria botrytis*		■			■	Höller *et al.* (1999)
Polyphenols							
Expansols (**23**)	*Penicillium expansum*	■					Lu *et al.* (2010)
Peniphenylanes	*Penicillium dierckxii*	■					Zhang *et al.* (2016)
Terrestrols	*Penicillium crustosum*	■					Chen *et al.* (2008a,b)
Vialinins, thelephantins	*Thelephora aurantiotincta*	■	■			■	Norikura *et al.* (2011)
Pyrone derivatives							
Allantopyrone A	*Allantophomopsis lycopodina*	■	■			■	Yokoigawa *et al.* (2015)
Funicones (**24**)	*Talaromyces* spp.	■	■	■	■		Nicoletti *et al.* (2009)
Hispidins	*Tropicoporus linteus*	■	■	■		■	Gonindard *et al.* (1997)
Macrophin	*Diplogelasinospora grovesii*	■				■	Fujimoto *et al.* (1998a)
Multiforisins	*Gelasinospora* spp.					■	Fujimoto *et al.* (1999b)
Pestalrone	*Monochaetia karstenii*	■					Luo *et al.* (2012)
Pyrenocines	*Setophoma terrestris*	■					Myobatake *et al.* (2019)
Quinones, hydroquinones							
Anhydrofusarubin	*Fusarium* sp.	■					Shao *et al.* (2010b)
Antroquinonols	*Taiwanofungus camphoratus*	■	■		■	■	Angamuthu *et al.* (2019)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Asterriquinones	*Aspergillus terreus*	■	■				Wijeratne *et al.* (2003)
Chaetopyranin	*Chaetomium globosum*	■					Wang *et al.* (2006)
Cochlioquinones	*Bipolaris* spp.	■	■	■			Wang *et al.* (2016a)
Cycloepoxydon	*Xylaria* sp.		■			■	Gehrt *et al.* (1998)
Diaporine (**25**)	*Diaporthe* sp.	■	■		■	■	Feng *et al.* (2016a)
Epiepoxydon	*Apiospora montagnei*	■					Klemke *et al.* (2004)
Epoxyquinols	*Pestalotiopsis sp.*	■	■	■	■		Li *et al.* (2002)
Herbarins	*Chaetosphaeronema sp.*	■					Osman *et al.* (2018)
Hydroxy-methyl-butenyl-hydroquinone	*Piptoporus betulinus*				■		Kawagishi *et al.* (2002)
Pestaloquinols	*Pestalotiopsis* sp.	■					Ding *et al.* (2011)
Phleichrome	*Heterosporium phlei*	■					So *et al.* (2018)
Tauranin	*Phyllosticta spinarum*	■	■				Wijeratne *et al.* (2008)
Torreyanic acid	*Pestalotiopsis microspora*	■					Lee *et al.* (1996b)
Spiroketals							
Berkeleydione, berkeleytrione	*Penicillium* sp.	■			■		Stierle *et al.* (2004)
Berkelic acid (**26**)	*Penicillium* sp.	■			■		Stierle *et al.* (2006)
Chloropestolide A	*Pestalotiopsis fici*	■					Liu *et al.* (2009a)
Chloropupukeanolides	*Pestalotiopsis fici*	■					Liu *et al.* (2011)
Massarigenins	*Massarina* sp.	■					Sun *et al.* (2011)
Preussomerins	*Preussia* sp.		■				Singh *et al.* (1994b)
Spiropreussione A	*Preussia* sp.	■					Chen *et al.* (2009a)
Steroids							
Dankasterones	*Penicillium* sp.	■					Qi *et al.* (2014)
Demethylincisterol A3	*Pestalotiopsis* sp.	■	■				Zhou *et al.* (2018)
Ergosterol and derivatives	*Most fungi*	■	■	■		■	Merdivan and Lindequist (2017)
Penicisteroids	*Penicillium chrysogenum*	■	■				Gao *et al.* (2011a)
Wortmannin (**27**)	*Talaromyces wortmanni*	■	■	■		■	Akter *et al.* (2012)
Terpenoids							
Sesquiterpenes							
Adametacorenol B	*Penicillium adametzioides*	■					Liu *et al.* (2015b)
Antrocin	*Taiwanofungus camphoratus*	■	■		■		Chiu *et al.* (2016)
Emeremophiline	*Aspergillus aurantiobrunneus*					■	Fujimoto *et al.* (2000)
Eurochevalierine	*Eurotium chevalieri*	■				■	Schnekenburger *et al.* (2018)
Fischerindoline	*Neosartorya pseudofischeri*	■					Masi *et al.* (2013)
Fudecadione A	*Penicillium* sp.	■					Pittayakhajonwut *et al.* (2011)
Fumagillin	*Aspergillus fumigatus*	■	■	■			Sheen *et al.* (2005)
Hirsutanol A	*Chondrostereum* sp.	■	■				Yang *et al.* (2013a)
Illudins	*Omphalotus illudens*	■	■				Schobert *et al.* (2011)
Insulicolide A	*Aspergillus insulicola*	■					Rahbæk *et al.* (1997)
Ligerin	*Penicillium* sp.	■					Vansteelandt *et al.* (2013)
Panepoxydone	*Lentinus crinitus*	■	■			■	Arora *et al.* (2014)
Talaperoxides	*Talaromyces flavus*	■					Li *et al.* (2011b)
Terrecyclic acids, quadrones	*Aspergillus terreus*	■	■			■	Turbyville *et al.* (2005)
Trichothecenes (**28**)	*Fusarium* spp.	■	■		■	■	Wu *et al.* (2017)
Xylarenic acid	*Xylaria* sp.	■					Hu *et al.* (2008)
Diterpenes							
Cercosporene F	*Cercospora* sp.	■					Feng *et al.* (2014)
Conidiogenones	*Penicillium chrysogenum*	■					Du *et al.* (2009)
Diaporthein B	*Eutypella scoparia*	■					Sun *et al.* (2012a)
Emindole DA, paspaline	*Aspergillus nidulans*	■			■		Sallam *et al.* (2013)
Fusicoccin A	*Diaporthe amygdali*	■	■		■		Bury *et al.* (2013)
Geopyxin B	*Geopyxis majalis*	■					Wijeratne *et al.* (2012)

(*Continued*)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Kampanols	*Stachybotrys kampalensis*		■				Singh *et al.* (1998)
Libertellenones	*Libertella* sp.	■					Oh *et al.* (2005)
Myrocins	*Arthrinum* sp.	■		■			Ebada *et al.* (2011)
Periconicin B	*Periconia atropurpurea*	■					Teles *et al.* (2006)
Radianspene C	*Coprinellus radians*	■					Ou *et al.* (2012)
Scopararanes	*Eutypella scoparia*	■					Sun *et al.* (2012a)
Sphaeropsidins (**29**)	*Diplodia cupressi*	■					Lallemand *et al.* (2012)
Triterpenes							
Clavaric acid	*Hypholoma sublateritium*		■				Jayasuriya *et al.* (1998)
Fasciculols	*Hypholoma fasciculare*	■					Kim *et al.* (2013)
Irpexans	*Irpex* sp.		■			■	Silberborth *et al.* (2000)
Kobiin	*Gelasinospora kobi*					■	Fujimoto *et al.* (1998b)
Lanostanoids (**30**)	*Ganoderma* spp.	■	■	■	■	■	Rios *et al.* (2012)
Shearinines	*Penicillium simplicissimum*		■				Smetanina *et al.* (2007)
Sesterterpenes							
Ophiobolins (**31**)	*Aspergillus ustus*	■	■				Yang *et al.* (2012)
Variecolins	*Aspergillus aurantiobrunneus*					■	Fujimoto *et al.* (2000)
Meroterpenoids							
Andrastins	*Penicillium* sp.		■				Uchida *et al.* (1996)
Aszonapyrone, sartorypyrone	*Neosartorya* spp.	■					Eamvijarn *et al.* (2013)
Chartarolides	*Stachybotrys chartarum*	■	■				Liu *et al.* (2017a)
Chevalones	*Eurotium chevalieri*	■					Kanokmedhakul *et al.* (2011)
Eupenifeldin	*Penicillium brefeldianum*	■					Mayerl *et al.* (1993)
Macrophorins	*Eupenicillium crustaceum*					■	Fujimoto *et al.* (2001)
Montadial	*Bondarzewia mesenterica*	■					Sontag *et al.* (1999)
Phenylpyropenes	*Penicillium concentricum*	■					Ding *et al.* (2015)
Phomoarcherins	*Phomopsis pittospori*	■					Hemtasin *et al.* (2011)
Pycnidione (**32**)	*Durotheca rogersii*	■					Hsiao *et al.* (2012)
Pyripyropenes	*Aspergillus fumigatus*	■		■			Hayashi *et al.* (2009)
Radarins	*Aspergillus sulphureus*	■					Laakso *et al.* (1992)
Ramiferin	*Kionochaeta ramifera*	■					Bunyapaiboonsri *et al.* (2008)
Xanthones							
Acremoxanthones	*Acremonium* spp.	■					Meléndez-González *et al.* (2015)
Anomalins	*Arthrinum* sp.	■	■	■		■	Ebada *et al.* (2011)
Brocaenols	*Penicillium brocae*	■					Bugni *et al.* (2003)
Conioxanthone, pinselin	*Talaromyces* sp.					■	Liu *et al.* (2016)
Ergoflavin	*Claviceps purpurea*	■	■				Deshmukh *et al.* (2009)
Globosuxanthone A	*Chaetomium globosum*	■	■				Wijeratne *et al.* (2006)
Norlichexanthone	*Talaromyces* sp.	■					Liu *et al.* (2010)
Paeciloxanthone	*Paecilomyces* sp.	■	■				Wen *et al.* (2008)
Phomoxanthones	*Diaporthe longicolla*	■	■			■	Frank *et al.* (2015)
Secalonic acids (**33**)	*Penicillium oxalicum*	■	■	■			Zhang *et al.* (2009)
New xanthone derivatives	*Phomopsis* sp.	■					Yang *et al.* (2013b)
Miscellaneous							
Atpenins	*Penicillium atramentosum*	■		■			Kawada *et al.* (2009)
Aranorosins	*Gymnascella dankaliensis*	■					Hammerschmidt *et al.* (2015)
Chaetomellic acids	*Chaetomella acutiseta*		■				Lingham *et al.* (1993)
Cordycepin	*Cordyceps militaris*	■	■		■	■	Tuli *et al.* (2013)
Epolactaene	*Penicillium* sp.	■	■				Mizushina *et al.* (2000a,b)
Mycoepoxydienes (**34**)	*Diaporthe* sp.	■	■		■	■	Wang *et al.* (2012)
Mycosporulone	*Coniothyrium sporulosum*	■					Guiraud *et al.* (1999)
Myriocin	*Isaria sinclairii*	■	■			■	Lee *et al.* (2011)

Table 2 Continued

Compound[a]	*Main producing species*	C	P	A	M	I	*Reference*
Myrotheciumone A	*Paramyrothecium roridum*	■	■				Lin *et al.* (2014)
Penicimutanolones	*Talaromyces purpurogenus*	■	■				Fang *et al.* (2014)
Penostatins	*Penicillium marinum*	■	■				Iwamoto *et al.* (1998)
Rubratoxin B	*Talaromyces* spp.	■			■	■	Wang *et al.* (2007a)
Sequoiatones	*Aspergillus parasiticus*	■					Stierle *et al.* (1999)
Zaragozic acids	*Sporormiella intermedia*		■				Huang *et al.* (1995)

[a]Numbers in parentheses refer to the structures reported in **Fig. 1**.
Note: C: antiproliferative/cytotoxic; P: pro-apoptotic; A: antiangiogenic; M: antimetastatic; I: general immunomodulatory properties.

and immunostimulatory, which vary according to the challenging pathogen and/or the administered dose. These effects likely depend on their capacity to bind to ribosomes, thereby inhibiting protein synthesis, impairing membrane functions, and altering intercellular communication (Wu *et al.*, 2017).

Lanostanoids are widespread secondary metabolites of many basidiomycetes, representing the main bioactive compounds of mushroom extracts used in traditional medicine in many countries. So far about 200 such compounds have been identified, including lanosterols, ganoderiols, ganoderic and lucidenic acids, lucialdehydes, polyporenic and poricoic acids, whose bioactivities highlight valuable properties against various targets in tumor chemotherapy (Rios *et al.*, 2012; Bhat *et al.*, 2019).

Within the alkaloids, the diketopiperazines (DKPs) include dozens of bioactive products whose scaffold results from the condensation of two amino acids. Therefore, they can be classified in various ways, according to their basic constituents and some relevant structural features, such as prenylation and the presence of di- or polysulphide bridges (Jiang and Guo, 2011; Li and Hua, 2015; Ma *et al.*, 2016). Along with the quite controversial gliotoxin (**3**) (Scharf *et al.*, 2016), the latter products include the homogeneous family of thiosilvatins, deserving further bioactivity assessments (Salvatore *et al.*, 2019). Within the prenylated DKPs, another homogeneous family includes several indole products deriving from L-tryptophan and L-histidine or anthranilic acid, such as roquefortines, meleagrins, glandicolins and oxalines (Martín *et al.*, 2014). Cytochalasans represent another numerous group of alkaloids with more than 60 different compounds, whose structure is based on a substituted isoindole scaffold fused with a macrocyclic ring deriving from a highly reduced polyketide backbone and an amino acid. They are classified into various subgroups, basically with reference to the amino acid constituent (Scherlach *et al.*, 2010; Trendowski, 2015).

Mechanisms of Action of Antitumor and Immunomodulatory Compounds

Properties of antitumor and immunomodulatory compounds are very varied with reference to the complex biomolecular events involved in disease initiation, promotion and progression, which can be targeted for immunomodulation and the treatment of cancer. In most instances, these products possess multiple mechanisms of action, ranging from general cytostatic/cytotoxic properties to more complex effects on gene expression and enzyme operation.

Information on bioactivity of novel compounds is usually limited to remarks concerning assays carried out for the preliminary characterization of the producing strains. Hence, following the development of methods for in vitro culturing of mammalian and human cell lines, which has enabled researchers from many chemical laboratories to directly perform bioassays, the majority of the recently discovered compounds have been reported for antiproliferative or cytotoxic effects on different cell types (**Table 2**). Besides what previously introduced with reference to the deleterious effects of mycotoxins, it is questionable if cytotoxicity actually represents an evidence for considering a product as a candidate drug, regardless to its potency. In this respect, attempts to classify cytotoxic products with reference to the effective dose resulting in preliminary assessments (Liu *et al.*, 2017c) have proved not to be completely reliable, considering that quite a wide variation emerges depending on the cell type and other experimental conditions. However, in the last decade advances concerning genes and related factors controlling apoptosis and other processes related to tumorigenesis and functioning of the immune system have refined the possibility to attain to a more accurate evaluation of biological properties of natural products at an early stage after discovery (Otto and Sicinski, 2017; Terracciano *et al.*, 2018).

Antimitotic Agents and Cell Cycle Regulators

Antimitotic agents are compounds that affect the course of cell division. Their effects generally consist in perturbations in cell cycle progression, and/or DNA synthesis, and/or microtubule organization. In fact, many antitumor drugs interfere with microtubule dynamics through either inhibiting tubulin polymerization, or stabilizing the microtubule bundles (Desbène and Giorgi-Renault, 2002; Jordan and Wilson, 2004). Taxol, the best known representative of the latter type, basically acts by impairing microtubule dynamics (Zhao *et al.*, 2005).

Natural products known for their capacity to inhibit the formation of the mitotic spindle are classified according to whether or not their tubulin binding site is the same as colchicine (Desbène and Giorgi-Renault, 2002). Many compounds in the first subclass

present a trimethoxyphenyl moiety able to react with the sulphydryl groups of aminoacids. This kind of active site is present in 3-*O*-methylfunicone and related compounds (De Stefano *et al.*, 1999; Nicoletti *et al.*, 2009). Inhibition of tubulin polymerization also shapes bioactivity of phomopsidin (Namikoshi *et al.*, 1997), and some DKPs binding at the colchicine site, such as phenylahistin (Kanoh *et al.*, 1999), aurantiamine (Hayashi *et al.*, 2000), oxaline (**4**) and neoxaline (Koizumi *et al.*, 2004b), fungerin (Koizumi *et al.*, 2004a), and tryprostatin A (Usui *et al.*, 1998). Griseofulvin (**22**) is known to affect organization and function of the mitotic spindle (Panda *et al.*, 2005), and to inhibit centrosome coalescence. The latter is a selective mechanism for tumor cells containing multiple centrosomes, where the formation of multipolar mitotic spindles is responsible for defects in chromosome segregation (Rebacz *et al.*, 2007). Recently, investigations on the cytotoxic properties of pyrenocine A have disclosed a new mechanism qualifying candidate anticancer drugs, consisting in the induction of monopolar spindle formation (Myobatake *et al.*, 2019).

Other fungal products can impair cytoskeletal organization by interference on actin polymerization. This category includes phalloidin, which binds actin filaments more tightly than actin monomers and essentially blocking actin depolymerization, and cytochalasins, which form a complex with actin monomers inhibiting association/dissociation of the subunits (Cooper, 1987; Trendowski, 2015).

Mitosis can be also affected through direct interferences with DNA structure and synthesis. As an example, bioactivity of illudins is basically related to alkylating effects on DNA and proteins, impairing their development as anticancer therapeutics (Kelner *et al.*, 1987). However, some illudin derivatives, such as irofulven, acylfulvene and dehydroilludin M, present increased histiospecific toxicity towards malignant cells (McMorris *et al.*, 1996a,b). Particularly, irofulven displayed proapoptotic effects ensuing the activation of the extracellular signal-regulated kinases (ERK) and the c-Jun N-terminal kinases (JNK) (Wang *et al.*, 2002), making it an interesting drug prospect (Poindessous *et al.*, 2003a,b). Similar properties were later reported for ophiobolin O (**31**) (Yang *et al.*, 2012).

Protein kinases (PKs), which modify the active/inactive status of other functional proteins through phosphorylation, are able to regulate cancer progression not only in terms of cell proliferation, but also as a result of effects on angiogenesis, invasion and metastasis. Moreover, PKs have a potential role in cancer immunotherapy (Xue *et al.*, 2015), introducing additional relevance of their inhibitory products as immunomodulators. Inhibitory properties against protein tyrosine kinases (PTKs) have been reported for ulocladol (Höller *et al.*, 1999), leptosin M (Yamada *et al.*, 2002), dideoxyverticillin (Zhang *et al.*, 2005), and clavilactones (Cassinelli *et al.*, 2000).

Similar to hispidin (Gonindard *et al.*, 1997), emodin is a specific inhibitor of protein kinase C (PKC) and PTK $p56^{lck}$ which are deregulated in tumor malignancy (Jayasuriya *et al.*, 1992; Fredenhagen *et al.*, 1995). Moreover, in hepatoma cells this compound was found to induce overexpression of specific oncogenes modulating the cell cycle, and to increase levels of p21, p53, Fas and caspase-3 inducing cell cycle arrest and apoptosis (Shieh *et al.*, 2004). Inhibitory effects on DNA synthesis have been reported in HeLa cells, where emodin promotes apoptosis through the activation of caspase-3 and -9, and cleavage of poly(ADP-ribose) polymerase (Srinivas *et al.*, 2003). Cytotoxicity of emodin and other anthraquinones is related to interference with electron transportation altering the cell redox status, and their use has been proposed to enhance chemotherapeutic efficacy of standard anticancer drugs (Srinivas *et al.*, 2007). Emodin and/or chrysophanol may also occur in fungal extracts after decomposition of dimeric bisanthraquinones, such as hypericin (Kubin *et al.*, 2005), rugulosin, skyrin, luteoskyrin and rubroskyrin which also possess direct inhibitory effects (Fouillaud *et al.*, 2016).

Due to its low toxicity and the observed synergism with nocodazole, therapeutic use in combination with other antitumor agents has also been proposed for griseofulvin (Ho *et al.*, 2001). This product increased cyclin B1/cdc2 kinase activity, and induced apoptosis following caspase-3 activation, Bcl-2 hyper-phosphorylation, and inhibition of the normal function of Bcl-2 associated with Bax. Similar properties have been reported for the azaphilone compound sclerotiorin (Giridharan *et al.*, 2012).

The signaling pathway mediated by phosphatidylinositol-3-kinase (PI3K) represents another valuable chemotherapeutic target. In association with protein kinase B (PKB or Akt), PI3K is involved in cell proliferation, apoptosis, motility and adhesion. In myeloid leukemia, levels of PI3K/Akt products are increased, and their dephosphorylation promotes suppressive effects (Martelli *et al.*, 2006). In *ras*-transformed fibroblasts chaetoglobosin K induces overexpression of a gene encoding for a large plus-end of tensin, an F-actin capping protein, and apoptosis occurs through PKB inhibition (Tikoo *et al.*, 1999). This cytochalasan is also responsible for inhibition of phosphorylation of c-Jun NH_2-terminal kinase (Ali *et al.*, 2013). Other fungal products reported to suppress the PI3K/Akt pathway are haloroquinone (Xie *et al.*, 2010; Wang *et al.*, 2015b), leptosins (Yanagihara *et al.*, 2005), and wortmannin (**27**) (Powis *et al.*, 1994). The chemotherapeutic use of the latter compound has been impaired by remarkable systemic toxicity (Boehle *et al.*, 2002); however, the nanoparticle technology has recently revived the clinical potential of this steroid, particularly in view of exploiting its radiosensitizing properties (Karve *et al.*, 2012).

In murine mesenchymal cells, secalonic acid D (**33**) was found to downregulate cyclin E, to inhibit cdk 2, and to promote the cdk inhibitor p21. The same effects were observed in the corresponding human cell type, along with higher level of the cdk inhibitory protein p57 and decreased levels of cyclins A, D1, D2, D3, E and cdk 4/6 (Dhulipala *et al.*, 2005). This mycotoxin also induced apoptosis after cell cycle arrest at the G1 phase in HL60 and K562 cells (Zhang *et al.*, 2009).

The above-mentioned tumor suppressor p53 is a transcription factor promoting cell cycle arrest and apoptosis in response to DNA damage. Its activity is modulated by MDM2, a ligase which prevents p53 functioning and is overexpressed in many cancers. The cyclopeptide chlorofusin is able to bind a hydrophobic cleft of MDM2 disrupting its interaction with p53, which is restored in its regulatory functions and inhibitory effects on tumor growth (Clark *et al.*, 2009). MDM2 downregulation has also been reported as a property of hispolon (Lu *et al.*, 2009). Besides p53, gliocladicillins are able to upregulate p21 and cyclin B, and to activate caspases, with ensuing proapoptotic effects (Chen *et al.*, 2009b). General properties as cell cycle inhibitors are displayed by other DKPs, such as the meleagrins (Du *et al.*, 2010), the fumitremorgins and some related products (Cui *et al.*, 1997).

Brefeldin A (**21**) induces apoptosis through a p53-independent pathway, unrelated to upregulation of cyclin B1/cdc2 kinase (Shao *et al.*, 1996), making it a chemotherapeutic candidate for prostate cancer (Wallen *et al.*, 2000). Moreover, in PC3 cells this macrolide acts as a direct cell cycle modulator, reducing cdk 2/4 and cyclin D1 expression, and promoting dephosphorylation of the retinoblastoma protein (Mordente *et al.*, 1998).

The p53-independent pathway is also relevant in apoptosis induction by 3-*O*-methylfunicone in HeLa cells, along with downregulation of cyclin D1 and cdk4 mRNA, and promotion of p21 mRNA expression (Buommino *et al.*, 2004). The latter effect characterizes cell cycle arrest caused by this funicone compound in melanoma cells, along with a marked decrease in the cyclin B1/$p34^{cdc2}$ complex (Baroni *et al.*, 2009). Moreover, in breast cancer cells it inhibits the expression of survivin, a typical marker of tumor progression, and the formation of mammospheres (Buommino *et al.*, 2007, 2011). Likewise, cladosporol A is responsible for cell cycle arrest and apoptosis induction after overexpression of p21, reduction of Bcl-2, cyclin D1, cyclin E, cdk2, and cdk4, and increase of ERK and JNK (Zurlo *et al.*, 2013; Koul *et al.*, 2017). Decreased expression of cyclins D1-E also determines the proapoptotic effects by the tropolone compound pycnidione (**32**), along with inhibition of survivin and activation of caspase-3 and -8 (Hsiao *et al.*, 2012).

The statins, including widely used anticholesterolemic agents such as mevastatin (or compactin, **17**) and lovastatin, possess complex antitumor properties (Graaf *et al.*, 2004; Gauthaman *et al.*, 2009). They have been reported to cause p53-independent apoptosis ensuing cdk 2 downregulation, along with induction of cdk inhibitors (p21 and/or p27) (Dulak and Jozkowicz, 2005). Synergism with butyrate has been documented in colon carcinoma cells, arrested in proliferation after downregulation of cdk 4, cdk 6, and cyclin D1 (Wächtershäuser *et al.*, 2001). Moreover, statins contrast the antiapoptotic effect of VEGF (Zhang *et al.*, 2013c). Further investigations on bioactivity of statins have also disclosed extensive immunomodulatory properties, stimulating consideration for their potential in the treatment of autoimmune diseases. In this regard, modulation of post-translational protein prenylation seems to represent a key mechanism by which statins alter immune function (Greenwood *et al.*, 2006). Following their increasing pharmaceutical importance, attempts have been done to obtain novel statin analogs through the improvement of fermentation processes, even by employing genetically engineered fungal strains, leading to the release of a series of patents (Nicoletti *et al.*, 2012).

Cyclosporin is another blockbuster drug of fungal origin, mostly employed because of its immunosuppressive properties. The name actually identifies a family of lipophilic cyclic undecapeptides comprising many analogs obtained by regulating fermentation by the producing agent through the addition of selected amino acids. The founder product cyclosporin A (**15**), available in the market with a number of brand names, acts by binding to an immunophilin called cyclophilin which catalyzes the folding of ribonuclease. However, its properties also depend on the induction of the potent immunosuppressive cytokine TGF-β whose direct effects are estimated to be at least 10,000 times higher than cyclosporin A itself. Moreover, cyclosporin selectively inhibits production of interleukin 2 and other cytokines, arresting T lymphocytes in G0/G1 phase (Survase *et al.*, 2011).

Many fungal products have been reported to possess modulatory properties on NF-κB, such as irpexans (Silberborth *et al.*, 2000), the trichothecene verrucarin A (**28**) (Oda *et al.*, 2005), terrecyclic acid A (Turbyville *et al.*, 2005), embellicines (Ebrahim *et al.*, 2013), altersolanol A (Teiten *et al.*, 2013) and allantopyrone A (Yokoigawa *et al.*, 2015), and the epoxidic compounds panepoxydone (Erkel *et al.*, 1996), cycloepoxydon (Gehrt *et al.*, 1998) and epoxyquinol B (Kamiyama *et al.*, 2008b). Mycoepoxydiene, also possessing an epoxide function, disrupts proliferation of MCF-7 cells through the suppression of NF-κB, besides inducing apoptosis through p53 activation. It also induces ROS production, resulting in cell cycle arrest due to DNA damage and activation of phosphorylation of PKs, and inhibits NF-κB transactivation by the tumor necrosis factor (TNF-α) (Wang *et al.*, 2012). Similar effects on NF-κB and TNF-α have been reported for cordycepin which is also known for antiproliferative, proapoptotic and antimetastatic effects (Tuli *et al.*, 2013). Inhibitory properties on cytokines and TNF-α responsible for antiproliferative effects have been also observed for ergoflavin (Deshmukh *et al.*, 2009), and enniatins A1 and B1, which in turn display proapoptotic properties deriving from activation of caspase-3/7 and inhibition of an extracellular regulated kinase (p44/p42) (Wätjen *et al.*, 2009). Beauvericin, also belonging to the cyclodepsipeptide family of enniatins, possesses cytotoxic properties towards human leukemic cells, with a yet to be completely elucidated mechanism of action (Wang and Xu, 2012).

Roseotoxin B is another cyclopeptide able to downregulate NF-kB signaling in activated T cells, along with increased autophagy. It causes G0/G1phase arrest in the cell cycle, suppresses production of proinflammatory cytokines, and inhibits the activation of Akt. Observations carried out on mice indicated that roseotoxin B acts in autophagy-dependent manner in T lymphocyte-mediated skin diseases, with a unique anti-inflammatory mechanism introducing possible use of this compound in the treatment of immune-related dermatitis (Wang *et al.*, 2016b).

Diverse Enzyme Inhibitory Properties

Modulation of NF-κB is again a mechanism of action of gliotoxin, a mycotoxin which is attentively considered for its multiple anticancer and immunosuppressive properties (Zhou *et al.*, 2000; Scharf *et al.*, 2016). Antiproliferative and proapoptotic effects by this compound are also related to the inhibition of geranylgeranyl transferase and farnesyl transferase, representing a viable therapeutic target in oncogene-mediated tumors (Vigushin *et al.*, 2004). Similar properties have been reported for antroquinonol (Angamuthu *et al.*, 2019), and the triterpenoid compound clavaric acid with reference to the latter enzyme (Jayasuriya *et al.*, 1998). More fungal products reported to decrease farnesylation of Ras protein are chaetomellic acid (Lingham *et al.*, 1993), fusidienol (Singh *et al.*, 1994a), preussomerins (Singh *et al.*, 1994b), cylindrol A (Singh *et al.*, 1995), andrastins (Uchida *et al.*, 1996),

barceloneic acid (Overy *et al.*, 2005), zaragozic acids (Huang *et al.*, 1995), kampanols (Singh *et al.*, 1998), and statins (Graaf *et al.*, 2004; Zhang *et al.*, 2013c).

In addition, gliotoxin and other epipolythiodioxopiperazines containing a disulfide bond have displayed inhibitory effects on histone methyltransferases, which are aberrantly expressed in some tumors indicating an association between histone methylation and malignancy (Sun *et al.*, 2012b; Cherblanc *et al.*, 2013). Likewise, acetylation/deacetylation of histones induce remarkable effects on cell cycle progression and epigenetic regulation. Particularly, proapoptotic effects and upregulation of several tumor repressor genes may derive from inhibition of histone deacetylases (HDs). Moreover, HD inhibitors possess immunomodulatory properties, extending their possible pharmaceutical application (Licciardi *et al.*, 2013). Fungal products reported for such effects are the terpenoid eurochevalierine (Schnekenburger *et al.*, 2018), and cyclic tetrapeptides bearing an expoxyketone side chain (Cheng *et al.*, 2017), such as trapoxins (Kijima *et al.*, 1993) and 1-alaninechlamydocin (Du *et al.*, 2010).

Several subtypes of DNA polymerases are known to play different interactive roles in DNA synthesis. Particularly, telomerases represent an important subgroup of these enzymes stimulating tumor progression by enabling neoplastic cells to escape replicative senescence, and to proliferate indefinitely. Therefore, they are considered as useful diagnostic and prognostic markers, and interesting therapeutic targets (Sampedro Camarena *et al.*, 2007). Inhibitory properties on telomerase have been reported for the pyrrolizidine alkaloids UCS1025A and B (Agatsuma *et al.*, 2002), and 3-*O*-methylfunicone, which is able to downregulate expression of human telomerase reverse transcriptase and the telomerase protein component-1 in several tumor cell lines (Buommino *et al.*, 2007, 2011; Baroni *et al.*, 2009). An alternariol analog, dehydroaltenusin (Kuriyama *et al.*, 2008), and two azaphilones, pinophilins A (**11**) and B (Myobatake *et al.*, 2012), have also been characterized as DNA polymerase inhibitors.

Inhibitory properties against DNA polymerases are often associated to similar effects on DNA topoisomerases. These enzymes have a modulatory role on DNA structure through the creation of temporary strand breaks, and represent a target in the treatment of several cancer types (Topcu, 2001). Activity of topoisomerase I is quite uniform throughout the cycle of proliferating cells, while levels of topoisomerase II present a peak at the G2/M phase. Two lanostanoid compounds, dehydrotrametenonic acid and dehydroebriconic acid, have been characterized as DNA topoisomerase II and DNA polymerase inhibitors (Mizushina *et al.*, 2004; Akihisa *et al.*, 2004). Besides general cytotoxic properties (Wu *et al.*, 2001; Rios *et al.*, 2012), more products of this class, such as ganoderic acid X and fomitellic acids, are reported as inhibitors of DNA topoisomerase I and II (Li *et al.*, 2005; Mizushina *et al.*, 2000a). The latter also have inhibitory effects on DNA polymerases α and β (Mizushina *et al.*, 2000a), along with the related lucidenic acids, while the ergostanoid compound cerevisterol is only active on the alpha isoform (Mizushina *et al.*, 1999).

DNA topoisomerase I inhibitory effects of herbarin (Osman *et al.*, 2018) are similar to camptothecin (Li *et al.*, 2006), while, unlike the latter drug, secalonic acid D does not form covalent complexes with the enzyme (Hong, 2011). Ambivalent activity against both topoisomerases has been reported for topopyrones (**10**) (Khan *et al.*, 2008), saintopin (Yamashita *et al.*, 1990, 1991), fusaristatins A-B (Shiono *et al.*, 2007) and leptosin F, while the related leptosins C and M, respectively, inhibited DNA topoisomerases I and II (Yamada *et al.*, 2002; Yanagihara *et al.*, 2005). Radicicol (Gadelle *et al.*, 2006), pericosine A (Yamada *et al.*, 2007), and the anthraquinones UCT1072s (Asai *et al.*, 1999) and aspergiolide A (Wang *et al.*, 2014c) are selective towards topoisomerase II, while alternariol has a broader spectrum of activity against DNA topoisomerase I, IIα and IIβ, particularly targeting the isoform IIα (Fehr *et al.*, 2009). Finally, epolactaene has been characterized as an inhibitor of human DNA topoisomerase II and mammalian α-β DNA polymerases (Mizushina *et al.*, 2000b). However, due to structural unstableness it is mainly considered as a model for the synthesis of derivatives with improved properties (Nakai *et al.*, 2002).

Additional enzyme complexes are considered as possible targets for the treatment of several tumor types. This is the case of DNA methyltransferase 1, catalyzing DNA methylation, which is overexpressed in cancer. Besides effects on proliferation and migration, antroquinonol D was found to be able to inhibit DNA methylation, to reactivate tumor suppressor genes and to restore tumor suppressor pathways (Wang *et al.*, 2014a). Known to promote prostaglandin synthesis, COX-2 is overexpressed in prostate, breast and esophageal cancer (Lieberman, 2002). Its inducible expression is reported to be inhibited by a few anti-inflammatory products, such as cordycepin (Tuli *et al.*, 2013), radicicol (Chanmugam *et al.*, 1995) and gerronemins (Silberborth *et al.*, 2002).

Besides causing depletion of guanine nucleotides, mycophenolic acid (**12**) interferes with DNA synthesis through inhibition of inosine monophosphate-dehydrogenase, an enzyme which is particularly active in tumor cells and represents a valuable chemotherapeutic target (Franchetti and Grifantini, 1999; Dun *et al.*, 2013). In nanomolar concentrations this compound was found to block proliferation of T and B lymphocytes (Eugui *et al.*, 1991), stimulating its consideration as an immunosuppressive agent in such a way that a synthetic derivative (mofetil ester) has found widespread application as an anti-rejection drug in organ transplantation. Likewise, use of mycophenolic acid is indicated for the treatment of lymphomas and monocytic or lymphocytic leukemia (Lipsky, 1996).

Myriocin is known as an inhibitor of serine palmitoyltransferase, which is involved in sphingolipid biosynthesis. In melanoma cells it was found to arrest cell cycle in the G2/M transition after activation of p53 and p21, and inhibition of cyclin B1 and cdc2 (Lee *et al.*, 2011). Sphingolipids are known for proinflammatory effects and a role in immune response. In fact, several inflammatory diseases are associated with increased ceramide content, and reduction of ceramide helps recovering from inflammation damage. These effects can be exploited in the treatment of cystic fibrosis which is characterized by hyper-inflammation and excessive innate immune response (Caretti *et al.*, 2014). Moreover this compound is reported for unique immunoregulatory properties deriving from the capacity to prevent immune cell exit from the lymphoid tissue, on which is based the development of a synthetic derivative (fingolimod) for the treatment of relapsing-remitting multiple sclerosis and other autoimmune diseases (Ingwersen *et al.*, 2012).

Angiogenesis Inhibitors

Tumors may directly regulate angiogenesis by means of stimulatory and inhibitory factors which operate on the extracellular matrix, endothelial cell proliferation, and differentiation of capillaries (Beecken *et al.*, 2000). Antiangiogenic products are strategic in cancer therapy also because they are unlikely to develop drug resistance. By directly targeting endothelial cells, they usually promote the expression of endogenous angiogenesis inhibitors, or counteract proangiogenic peptides (Hayes *et al.*, 1999; Madhusudan and Harris, 2002). This is the case of the previously mentioned TGF-β, a cytokine also known to play a role in cell proliferation, differentiation and apoptosis, which is inhibited by several fungal lactones, such as galiellalactone, curvularins and oxacyclododecindione (Rudolph *et al.*, 2013).

A main position in this category pertains to products inhibiting VEGF, a protein also known for oncogene promotion and inactivation of some suppressive genes (Xie *et al.*, 2004). Such activity has been reported for chaetocin (Isham *et al.*, 2012) and the related verticillins (Chen *et al.*, 2005), fusarochromanone (**14**) (Mahdavian *et al.*, 2014), ganoderic acid F (**30**) (Kimura *et al.*, 2002), asterric acid (**19**) and its derivatives (Lee *et al.*, 2002), pyripyropenes (Hayashi *et al.*, 2009), chaetoglobosin K (Luo *et al.*, 2013), and 3-*O*-methylfunicone (Buommino *et al.*, 2012). More particularly, antiangiogenic effects of hispidin derive from inhibitory properties on PKCβ, which is involved in the signal transduction pathway induced by VEGF (Gonindard *et al.*, 1997; Yoshiji *et al.*, 1999).

The above-mentioned epoxyquinol B has been regarded as a model for the development of novel antiangiogenic drugs. As a result of VEGF inhibition, this epoxide reduces both vessel and tumor growth, and blocks the activation of the fibroblast, the epidermal and the platelet-derived growth factor receptors (Kamiyama *et al.*, 2008a). The latter effect has also been reported for luteusin and other analogs of the RP-1551 family of azaphilones (Toki *et al.*, 1999). An epoxidic group is again determinant for bioactivity of cytochalasin E (**2**), responsible for selective antiproliferative effects on bovine capillary endothelial cells, and able to reduce angiogenesis through inhibition of the basic fibroblast growth factor (Udagawa *et al.*, 2000).

An accurate investigational activity has been carried out on the antiangiogenic properties of fumagillin (Furness *et al.*, 2005; Sheen *et al.*, 2005; Chen *et al.*, 2007). However, because of several negative side effects discouraging its direct use in chemotherapy, this spiroepoxide is mainly regarded as a structural model for the synthesis of novel drugs (Lefkove *et al.*, 2007).

Inhibition of tumor vascularization is the most relevant property of ergosterol (Takaku *et al.*, 2001), and integrates the profile of products such as cochlioquinones (Jung *et al.*, 2003; Phuwapraisirisan *et al.*, 2007), emodin (Kwak *et al.*, 2006), mycophenolic acid (Domhan *et al.*, 2008), and DKPs. Particularly, compounds in the latter class displayed potent effects against the plasminogen activator inhibitor-1, known to promote angiogenesis and metastasis (Martins and Carvalho, 2007). Finally, a peculiar type of antioangiogenic activity was found to contribute to the broad-spectrum antitumor properties of cyclosporin A. In fact, this cyclopeptide behaves as a selective tachykinin receptor antagonist inhibiting proliferation of endothelial cells. It has also been found to act synergically with other cytostatic drugs and to reverse the multidrug resistance of several cancer and leukemia cell lines, so that it can be considered for chemotherapeutic integration in the treatment of recalcitrant tumors (Muñoz *et al.*, 2010). Similar application could be considered in breast cancer for a few products, such as tryprostatin (Jain *et al.*, 2008), the related fumitremorgin C (Rabindran *et al.*, 2000), ophiobolin O (Yang *et al.*, 2012), and the lanostanoid trametenolic acid B (Zhang *et al.*, 2014a), reported to be able to reverse resistance to taxol and other antitumor drugs.

Antimetastatic Products

Metastasis is the process by which cells from a primary tumor spread to other organs, and establish multiple foci responsible for uncontrolled disease progression. In order to become invasive and produce metastases, tumor cells must degrade proteins forming the extracellular matrix (fibronectin, collagens, laminin and glycoproteins). This biochemical process is governed by MMPs, a class of membrane-bound zinc-dependent endopeptidases playing a basic role in cell proliferation, differentiation, migration and angiogenesis. Therefore, the use in chemotherapy of MMP-inhibitors may provide multiple beneficial effects. There are many fungal products characterized as MMP-inhibitors, such as statins (Gauthaman *et al.*, 2009; Zhang *et al.*, 2013c), pyridoxatin (Lee *et al.*, 1996a), ganoderic acid T (Chen *et al.*, 2010), polyporenic acid C and 2-(4-hydroxy-3-methyl-2-butenyl)-hydroquinone (Kawagishi *et al.*, 2002), rubratoxin B (Wang *et al.*, 2007a), berkeleydione and berkeleytrione (Stierle *et al.*, 2004), berkelic acid (**26**) (Stierle *et al.*, 2006), and penicitrinine A (**8**) (Liu *et al.*, 2015a). The anti-invasive properties of altersolanol A have also been ascribed to effects on MMPs deriving by primary interference in the NF-κB signaling pathway (Teiten *et al.*, 2013), likewise later reported for coniellins (Yu *et al.*, 2018).

3-*O*-Methylfunicone represents a significant example of how MMP-inhibitory properties integrate the chemotherapeutic profile of anticancer drugs. In breast cancer cells, this product was reported to affect proliferation selectively, and to modify the distribution of tubulin fibers. Moreover, it impaired their motility through downregulation of $\alpha v\beta 5$ integrin and inhibition of MMP-9 (Buommino *et al.*, 2007). Likewise, 3-*O*-methylfunicone was found to damage and inhibit motility of mesothelioma cells, by altering the signaling activity of ERKs, and downregulating $\alpha V\beta 5$ integrin and MMP-2. Again, these effects were not observed in normal mesothelial cells (Buommino *et al.*, 2010). Moreover, a synergistic effect resulted in combination with cisplatin, indicative a possible application to overcome resistance against the latter drug (Buommino *et al.*, 2012).

Antimetastatic effects of other products are independent on MMP inhibition. In this regard hispolon, known to induce apoptosis in several cancer types (Chen *et al.*, 2008b, 2013; Lu *et al.*, 2009), has been reported to inhibit cell motility through downregulation of the lysosomal protease cathepsin S and ERK-triggered autophagy (Hsin *et al.*, 2017). Moreover, meleagrin has displayed inhibitory activity against the hepatocyte growth factor, known to promote migration and invasion of breast cancer cell

lines (Mady *et al.*, 2016). Finally, the antimigratory properties reported for some products, such as aspergiolides C-D (Du *et al.*, 2011), fusicoccin A (Bury *et al.*, 2013), fusarochromanone (Mahdavian *et al.*, 2014), penicillivinacine and terretriones (Asiri *et al.*, 2015), require further assessments concerning the biomolecular bases in view of eventual pharmaceutical exploitation.

Future Perspectives

This overview highlighted diversity in origin, structures and bioactivities of antitumor and immunomodulatory compounds from fungi, which represent a treasure awaiting to be accessed by the pharmaceutical industry. Still, a low number of these compounds have effectively been entered into clinical use, which at least for a proportion of them is due to inaccurate characterization of their real therapeutic relevance. In this respect, the definition of guidelines for a more thorough evaluation of novel products at an early stage after discovery is determinant in the aim to select compounds which can be employed in human medicine (Kawada *et al.*, 2018; Richter *et al.*, 2019). Besides improvements at the screening stage, the extent at which this unexploited potential is going to deliver new drugs having a positive impact in the treatment of cancer and immune diseases will depend on the capacity to balance therapeutic properties and intrinsic toxicity. To this purpose, a fundamental contribution can be provided by nanotechnologies and their capacity to increase bioavailability, to access tumor microenvironment, and to hit crucial molecular targets (Choi *et al.*, 2019; Ansari *et al.*, 2020). Moreover, a notable pulse is expected to derive from the combined use or the concomitant operation of the several mechanisms of action of these products (Yin *et al.*, 2018).

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Mycelium Materials

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A Non-Sustainable Economy

Resource management in current socio-economic systems is often based on a "take-produce-consume-discard" model. This model assumes abundance of resources and unlimited waste disposal with no negative side effects (Jurgilevich *et al.*, 2016). However, natural resource consumption is higher than its regeneration rate and discharged waste streams outpace the remediation capacity of ecosystems. Environmental consequences of this extractive model are resource depletion and waste accumulation in the biosphere leading to environmental problems such as climate change and desertification (Clark and York, 2005; Watson *et al.*, 2016).

Plastics are an example of non-sustainable materials. Commercial development of plastics started in the 1940s and has led to great societal impact. Life standards improved greatly due to the wide applicability of these versatile polymers and gave rise to the term "plastic age" (Thompson *et al.*, 2009). The use of plastics was a driver of innovation in various industries like electronics, healthcare, construction and packaging (Thompson *et al.*, 2009). Despite the numerous benefits provided by plastics, their environmental impact is large. In 2015 alone, 380 million metric tons of plastics were produced worldwide (Geyer *et al.*, 2017). Production of plastics is energy-consuming and large volumes of greenhouse gasses are emitted. Moreover, these petrol-based materials cause economic dependence on fossil fuels. In total, 8% of global crude oil is used to produce plastics, thereby competing for feedstock for energy production (Harding *et al.*, 2007). Plastics are mainly used to produce disposables with a life span of less than a year. It is estimated that 42% of the plastics that were produced in 2015 were used as packaging, while 54% of total plastic waste that year originated from packaging (Geyer *et al.*, 2017). From these disposed plastics, less than one-third is recycled. This is partly caused by the mixing of different polymers that hampers separation and purification of plastic waste (Hopewell *et al.*, 2009). Most plastics end in landfills where they accumulate or from which they disperse into nature. This plastic waste fragments by sunlight with a rate depending on environmental conditions such as humidity, temperature, light and oxygen availability (Albertsson and Hakkarainen, 2017). The resulting microplastics affect wildlife and human health (Geyer *et al.*, 2017; Hermabessiere *et al.*, 2017; Revel *et al.*, 2018). Oil-based plastics are only one example of materials that have a negative footprint. Other industries that have proven to be unsustainable are the textile and building industry. Textile processing, dyeing and printing uses up to 200 l of water per kilogram of fabric and causes major pollution (Parisi *et al.*, 2015). On the other hand, the building industry consumes vast amounts of energy. Consumption of energy by the building industry is estimated to be up to 40% of total energy consumption in the EU and is responsible for up to 50% of total greenhouse gas emissions (Asif *et al.*, 2007; Zabalza Bribián *et al.*, 2009). Clearly, alternatives must be developed for these unsustainable processes and materials.

As Earth can be considered a closed system, resource management in nature largely depends on cyclic processes. Achieving a sustainable industrial system asks for the implementation of such cyclic processes (Greyson, 2007). A circular economy is a restorative system where resources are processed in cycles, either by society or by biogeochemical processes (Greyson, 2007). It mirrors natural principles such as production from waste, resilience through diversity and use of renewable energy sources. Circular economy focuses on closing the loop of materials, reducing resource consumption and waste entering the environment. This can be achieved by reusing, repairing, refurbishing and recycling existing materials and products (Jurgilevich *et al.*, 2016). This changes the concept of waste; used products become resources for the bio- or the techno-cycle (Gregson and Crang, 2015).

Two important factors in circular production of materials are renewable feedstocks and biodegradability. Renewable feedstocks refer to raw materials that are being extracted from nature and are replenishable, such as biomass used for producing biofuels (Melero *et al.* 2012), while biodegradability refers to the capability of being degraded by biological activity (Vert *et al.*, 2012). The relation between bio-based, biodegradable and sustainability is complex. Bio-based materials are materials that are synthesized by living organisms like plants, fungi and bacteria. Therefore, renewability is guaranteed. However, bio-based is not per definition sustainable. For instance, current production of palm oil or usage of tropical hardwood as feedstock is bio-based but not sustainable. There is also no relation between bio-based and biodegradability. Biodegradability of bio-plastics refers to, according to the American Society for Testing and Materials (ASTM) standards, the possibility "*to degrade by the action of naturally occurring microorganisms such as bacteria, fungi, and algae*", whereas compostability of bio-plastics refers to "*a bio-plastic that undergoes degradation by biological processes during composting to yield carbon dioxide, water, inorganic compounds, and biomass at a rate consistent with other known compostable materials leaving no visually distinguishable or toxic residues.*" (Kale *et al.*, 2007). There is an intuitive believe that materials produced from bio-based feedstocks are biodegradable (Garrison *et al.*, 2016). This is however not an origin-dependent property, but relies on the chemical composition of the material and the metabolic capacity of the environment (Song *et al.*, 2009). Examples of bio-based and bio-degradable materials are polylactic acid (PLA) and polyhydroxyalkanoates (PHA), whereas non-biodegradable bio-based polymers are bio-polyethylene (bio-PE) and bio-poly(ethylene terephthalate) (bio-PET) (Iwata, 2015). For these reasons, biodegradability of bio-based materials needs to be regarded as an important factor when using renewable resources as feedstock.

Bio-based materials are applied in an increasing range of applications (Álvarez-Chávez *et al.*, 2012). They may result from the use of the whole organism (e.g., wood) or from extraction and/or purification of its components (e.g., starch). The latter strategy is also used in

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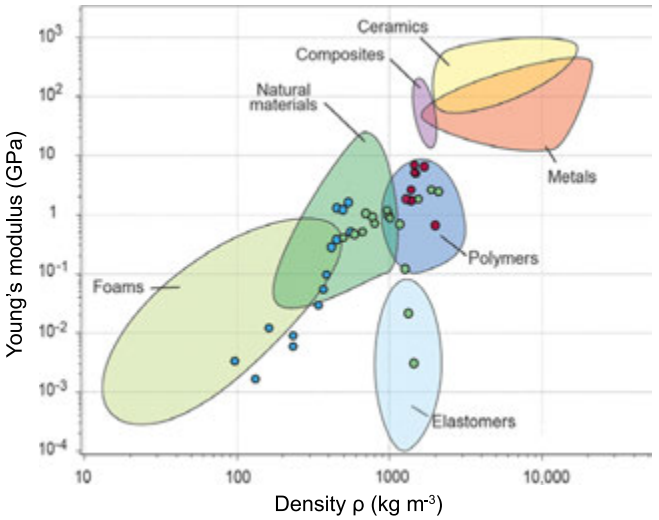

Fig. 1 Relation between the Young's modulus (GPa) and density (kg m^{-3}) of material families (Ashby, 2005) and of composite (Appels *et al.*, 2019; Appels, 2020) and pure fungal materials (Appels *et al.*, 2018; Appels, 2020) and chitin/glucan nanomaterials (Nawawi *et al.*, 2019b; Jones *et al.*, 2019b). Figure reproduced with permission from Granta Design, 2020, CES EduPack 2019, ANSYS Granta ©, all rights reserved.

the production of bioplastics. Examples are PLA and PHA that are for instance used as coatings, packaging, and in medical products (Chen, 2010; Arrieta *et al.*, 2015). These bioplastics result from bacterial fermentation of agricultural products (Tokiwa *et al.*, 2009). Apart from bacteria-derived bioplastics there are plant-based cellulosic- or starch-based bioplastics (Mohanty *et al.*, 2002). Another resource for bio-based polymeric materials may be fungal mycelium. So far, the whole organism is used to produce mycelium materials (see below). Yet, polymer extraction and purification from fungal mycelium and fruiting bodies appear also promising.

Composite and Pure Mycelium Materials

Fungi need organic compounds for their energy and carbon source. To this end, they degrade dead organic matter or establish a pathogenic, neutral or mutual beneficial symbiosis with plants, animals or microbes. So far, saprotrophic fungi have been used for the formation of mycelium materials. They colonize substrates by means of mm- to cm-long hyphae that have a width between 1–10 μm. By growing at their tips and branching sub-apically, a 3D network of hyphae is formed. The diameter of such a mycelium can vary in size ranging from sub-millimeter to kilometer dimensions. The basidiomycete mushroom forming fungi are known to colonize large surfaces and are therefore the fungi of choice to make mycelium materials. For instance, single individuals of *Armillaria* have been identified that had colonized $\geq$1000 ha of soil, making them the largest organisms on earth (Smith *et al.*, 1992; Ferguson *et al.*, 2003). Moreover, amongst the basidiomycetes are fungi capable of degrading lignin, which is a main component of agricultural waste and waste of the wood industry. By degrading this recalcitrant biopolymer, access is obtained to the cellulose and hemicellulose that is shielded by lignin. Many of the waste streams from agriculture and the wood industry cannot be used to feed animals. This explains why 0.25 billion tons of straw was burned in China alone in 2009 (Grimm and Wösten, 2018). Fungi can turn this low nutritional waste stream into high nutritional biomass by producing proteins and making cellulose available at the same time. This and the capability of fungi to transform low quality waste streams into materials will contribute to the circular economy.

Metals, polymers, elastomers, ceramics, glasses and hybrids are the main classes of materials (**Fig. 1**). The latter materials include composites such as reinforced plastics and aluminum, foams and natural materials such as wood, bone and skin (Ashby, 2005). Based on their properties (**Table 1**) fungal materials have foam-, polymer- or elastomer-like properties or properties resembling natural materials (Jones *et al.*, 2017; Appels *et al.*, 2018, 2019; Appels, 2020). Mycelium composite materials have been developed for use as packaging, insulation, and interior and construction materials (**Fig. 2**). On the other hand, pure mycelium can be used as leather or textile replacement, while materials produced from cell wall components of mycelium can be used for instance as coatings, membranes, packaging material, and paper.

Pure Mycelium Materials

Pure mycelium is obtained by growing a fungus on a solid or liquid substrate under static growth conditions (Lugones *et al.*, 2004; Haneef *et al.*, 2017; Appels *et al.*, 2018) or as a liquid shaken culture (Appels, 2020). When grown in liquid cultures, the total mycelium can be harvested and used to make mycelium material. In contrast, only the fungal skin can be used when grown on a solid substrate. This skin covers the substrate and contacts the air and is composed of densely packed hyphae. While only part of

Table 1 Examples of material properties

Property	*Unit*	*Definition*
Density (ρ)	kg m^{-3}	The amount of kilogram material per cubic meter
Young's modulus (E)	Pa	Measure of rigidity defined by the amount of stress (in Pa) causing a specific strain (in %)
Ultimate tensile strength (σ)	Pa	The amount of stress in longitudinal direction a material can withstand before breaking.
Flexural strength	Pa	Stress in a material just before it fractures in a flexure test
Compression strength	Pa	Capacity of a material to withstand loads tending to reduce size
Elongation at breaking (ε)	(%)	The proportional deformation determined by the change in length divided by its original length.
Water absorption capacity	(%)	Water retained by a material after adding water or an aqueous solution followed by centrifugation.
Fire resistance	h	Time a material is able to withstand a fire.
Thermal Insulation (R value)	°C m^2/W	Temperature difference per unit of heat flux needed to sustain one unit of heat flux between the warmer surface and colder surface of a barrier under steady-state conditions
Acoustic insulation	(%)	Reduction of a resonating frequency in a cavity

Fig. 2 Insulation panels (A), floor tiles (B) and multi-purpose pots (C) as examples of composite mycelium materials and mycelium leather (D) as an example of a pure mycelium material. Pictures obtained with permission from Mogu (Varese, Italy).

the mycelium can be harvested in the case of solid substrates, growth of static liquid cultures is labor intensive and challenging to upscale, while growth as liquid shaken cultures such as in bioreactors needs high financial investments. The way industries will grow the fungi in the future to produce pure mycelium materials will depend on both cost of production and material qualities.

The properties of pure mycelium materials not only depend on culture conditions, but are also the result of the type of substrate, the fungus, other environmental growth conditions and post-processing (Haneef *et al.*, 2017; Appels *et al.*, 2018; Appels, 2020). The latter can consist of physical, chemical, and/or biological treatments. Heat pressing and adding plasticizing agents are examples of physical and chemical treatments, respectively, while selective microbial degradation of components of a mycelium material is an example of a biological treatment.

A few reported examples show the impact of the substrate and the type of fungus on material properties of pure mycelium. The mycelium of the basidiomycetes *Pleurotus ostreatus* and *Ganoderma lucidum* is more elastic when these fungi are grown on a cellulose-based substrate supplemented with dextrose when compared to growth on cellulose alone. In both cases, the latter fungus forms a stiffer mycelium material when compared to the former fungus (Haneef *et al.*, 2017). Inactivation of a single gene can also impact material properties illustrated by the finding that the mycelium of a strain in which the hydrophobin gene *sc3* is inactivated (Δ*sc3* strain) is stronger when compared to the wild-type strain of *Schizophyllum commune* (Appels *et al.*, 2018) (see below). SC3 is an amphipathic protein and one of the most abundant proteins in cell walls of aerial hyphae of *S. commune*, while it also occurs at lower levels in cell walls of hyphae that grow within the substrate. SC3 attaches hyphae to hydrophobic surfaces (Wösten *et al.*, 1994a), mediates escape of hyphae from the aqueous environment into the air (Wösten *et al.*, 1999), and makes aerial structures hydrophobic (Wösten *et al.*, 1993, 1994b). The latter is illustrated by the 115° water contact angle of a wild-type

mycelium with aerial hyphae (which is similar to the hydrophobic surface of Teflon), while water immediately soaks into the mycelium of the Δ*sc3* strain (Wösten *et al.*, 1999).

Pure mycelium materials of *S. commune* have been studied best. Various examples illustrate the impact of growth conditions on the properties of pure mycelium of this basidiomycete. Dried mycelium films produced with static and liquid shaken cultures show mechanical material properties (i.e., Young's modulus, ultimate tensile strength and elongation at breaking; see **Table 1** for explanation) similar to materials that are classified in the group of natural materials (Appels *et al.*, 2018; Appels, 2020) (**Fig. 1**). The macrostructure of pure mycelium material produced with static and liquid cultures is different. The mycelium of static liquid cultures consists partly of submerged and partly of aerial mycelium. The submerged mycelium dries up very dense having almost no air voids with an appearance resembling that of paper. On the other hand, the aerial mycelium is less dense and has a cotton like appearance. Mycelium obtained from liquid shaking cultures has a more homogeneous appearance after drying, being most similar to the submerged mycelium from liquid static cultures. Still, some air voids are observed after drying mycelium from liquid shaking cultures. These air voids can be removed by vacuum drying, which is expected to increase strength of the mycelium material.

Stronger mycelium films of wild-type and Δ*sc3* strains of *S. commune* are obtained when dark-grown static liquid cultures are grown at ambient CO_2 (400 ppm) when compared to high CO_2 levels (i.e., 70,000 ppm). The opposite is observed when the strains are grown in the light. These results reflect events observed under natural growth conditions. Hyphae that grow within a solid substrate are exposed to darkness and high CO_2, while aerial hyphae will be exposed to light and ambient CO_2. Mycelium growing under the former condition have a higher maximal strength (6.5 MPa) when compared to mycelium grown under the latter condition (5.1 MPa). Intuitively, these results make sense since hyphae growing in wood will experience a higher resistance from the substrate. Yet, mycelium of *S. commune* is even more strong when grown in the light at high CO_2 (9.5 MPa) or grown in the dark under low CO_2 (9.6 MPa). Together, it is not yet clear how to relate the mechanical properties of mycelium with the environment of the fungus.

Deletion of *sc3* results in a >2-fold increase in material rigidity compared to wild-type *S. commune*, irrespective of the environmental growth conditions (Appels *et al.*, 2018). Chemical analysis by ATR-FTIR did not show large differences in macromolecular composition of the material of the wild-type and the Δ*sc3* strain. On the other hand, scanning electron microscopy revealed that the Δ*sc3* material is more homogeneous and densely packed compared to the wild-type material. Indeed, density of the material is higher when produced with the deletion strain. This density increase correlates strongly with increased rigidity and ultimate tensile strength of the mycelium-based material. Together, Δ*sc3* material properties are similar to those of polymers, while properties of the wild-type are similar to those of natural materials (**Fig. 1**).

Post-processing of pure mycelium material can have a high impact on material properties. Plasticizers and crosslinking agents are commonly used chemicals to alter these properties. Plasticizers increase plasticity of a material by lowering the glass transition temperature (Cowie and Caleria, 2007). The plasticizer acts like a lubricant by improving the capacity of polymers to move. Plasticizers such as glycerol and water do so by filling the space between polymer chains, thereby increasing their distance (Cowie and Caleria, 2007). Indeed, elasticity of *S. commune* pure mycelium material is increased by treatment with glycerol and also reduces ultimate tensile strength and increases elongation at breaking (Appels, 2020). This shift in material properties from being brittle to ductile is reflected by a shift from being a natural-like material to a polymer-like material such as plastic (concentrations of glycerol up to 8%) or even an elastomer-like material like rubber (concentration of glycerol $\geq 16\%$) (**Fig. 1**). Crosslinkers are the opposite of plasticizers and reduce mobility of a polymer structure. Crosslinking enhances ultimate tensile strength and reduces elasticity. Ionic and hydrophobic interactions are examples of non-covalent crosslinks, while chemical crosslinking involves formation of covalent bonds. For instance, dialdehydes such as glutaraldehyde and glyoxal react with amino or hydroxyl groups in polysaccharides (Crini, 2005). Chemical crosslinks can also be the result of for instance gamma- or photo-irradiation or sulfur vulcanization (Bhattacharya *et al.*, 2009). Properties of biological materials, such as fungal mycelium, can be changed by introducing both non-covalent and covalent crosslinks with proteins and polysaccharides (Yang *et al.*, 2005). For instance, pure mycelium materials of *S. commune* become stronger and more brittle after cross-linking with glutaraldehyde (our unpublished results).

Composite Materials

Composite mycelium material is obtained by stopping growth of the fungus before the substrate (e.g., straw or sawdust) is fully degraded. In this case, hyphae bind organic fibers or particles together while colonizing the substrate (Jones *et al.*, 2018a). Fungal growth can be stopped by drying and/or heating the colonized substrate. Heating will kill the fungus, while drying will preserve the fungus in a "hibernated" state. In the latter case, growth can be reinitiated by wetting the substrate. Composite mycelium materials can also be obtained by mixing pure mycelium with organic fibers. This is a more controlled process compared to growth in a complex organic substrate. Apart from adding organic materials, pure mycelium can also be mixed with inert materials such as metals or ceramics.

Like for pure mycelium materials, properties of the mycelium composites depend on the fungus, substrate, growth conditions and processing of the material (Jones *et al.*, 2017; 2019a; Appels *et al.*, 2018, 2019; Appels, 2020). So far, composite mycelium materials are classified in the family of foams (also including expanded polystyrene), irrespective of the type of fungus, the organic substrate and the growth conditions (Pelletier *et al.*, 2013; Ziegler *et al.*, 2016; Yang *et al.*, 2017; Islam *et al.*, 2018; Appels *et al.*, 2019; Appels, 2020). The mycelium dominates the soft compression of the composite material at low strain levels, while the organic substrate particles cause rapid stiffening at higher strain levels. The impact of growth conditions on the properties of

mycelium material composites is illustrated by the finding that densely packed substrates result in higher Young's moduli and compressive strength when compared to loosely packed substrates (Yang *et al.*, 2017). The impact of the fungus and the substrate on material properties was described by Appels *et al.* (2019). In this study, the basidiomycetes *P. ostreatus* and *Trametes multicolor* were grown on beech sawdust, rapeseed straw and cotton waste. *T. multicolor* grown on beech sawdust showed the highest Young's modulus, while ultimate tensile strength was comparable to *T. multicolor* grown on rapeseed straw. Notably, even small modifications in the composition of a standard fungal growth medium induce significant differences in the morphology, the chemical, and the hydrodynamic properties of *G. lucidum* mycelium (Antinori *et al.*, 2020). Mycelium materials grown in glucose-enriched potato dextrose broth (PDB) are highly porous, are thicker, and can absorb more moisture when compared to mycelium materials grown in PDB with a small quantity of lignin. The latter medium, however, enables fast growth and results in a dense and less hydrophilic mycelium. The type of substrate also impacts acoustic properties of the mycelium composite material (Pelletier *et al.*, 2013). In general, mycelium composites of basidiomycetes grown on a variety of substrates such as cotton by-products, rice straw, sorghum stalks, flax shive, kenaf, and hemp show much better acoustic performance than standard foam insulation board, including improvements at the key automotive road noise frequency of 1000 Hz. Although cotton bur fiber is a relatively low-performing acoustic material when compared to other substrates it still absorbs 70%–75% of the sound at a peak frequency of 1000 Hz.

Post-processing has a higher impact on material properties than the fungal species, the environmental growth conditions, and the type of substrate (Appels *et al.*, 2019). Cold pressing of straw- or cotton-grown *P. ostreatus* increases density 2-fold, while heat pressing of straw-grown *T. multicolor* and cotton- and straw-grown *P. ostreatus* results in a >3-fold density increase. Heat-pressing also improves homogeneity, rigidity and ultimate tensile strength of the materials and shifts the performance of the materials from being foam-like (e.g., styrofoam) to natural-like (e.g., cork). The use of high temperature increases the incidence of the Maillard reaction. This complex non-enzymatic reaction known to be responsible for browning of food and flavor formation links amino acids and reducing sugars (Martins *et al.*, 2000). These cross-links are expected to enhance strength and rigidity of the fungal materials.

Water absorption is not related to the fungal species, substrate, or pressing condition used to produce the composite mycelium material (Appels *et al.*, 2019). Exposure to 60% relative humidity (RH) results in thickness increase for rapeseed straw-grown *P. ostreatus* that has not been heat- or cold-pressed. Similar results are found with cotton-grown *P. ostreatus* with no further pressing. Exposure to 80% RH results in weight increase of rapeseed-grown *P. ostreatus* material that has been cold- or hot-pressed. Exposure to 80% RH increases thickness only for hot-pressed *T. multicolor* grown on rapeseed straw. Placing straw- and cotton-based materials on water for 192 h results in a 4–5-fold weight increase for non-pressed materials and a 2–3-fold increase for cold- or heat-pressed materials (Appels *et al.*, 2019). Notably, weight increase of non-pressed sawdust amounts only 43%, possibly due to the reduced fibrous structure and/or higher lignin content. Also, the highest water uptake takes place in the first 3 h of the experiment (Appels *et al.*, 2019; data not shown). Together, mycelium composites need to be sandwiched between other materials or need to be coated when they are used in humid environments, preferably with biodegradable bio-based coatings like PLA or PHA (Iwata, 2015).

Mycelium material is less prone to ignition and combustion, and, consequently, safer to use when compared to poly (methylmethacrylate) (PMMA) and PLA (Jones *et al.*, 2018b). It was also shown that the presence of mycelium reduces fire reaction properties of wheat grains. This is due to a higher charring tendency of mycelium compared to wheat grain, which reduces the heat release rate by acting as a thermal insulator and by limiting the supply of combustible gases.

Cell Wall Composition and Materials of Cell Wall Components

Increased density improves the strength of mycelium materials. Increased density can be obtained by pressing the material (see above), but also by increased branching or by the formation of mycelial cords. The cell wall composition of the fungus used to produce the mycelium material is also expected to impact material properties. Cell walls make up 15%–25% of the mycelium biomass (Nevalainen *et al.*, 2005), play an essential role in interactions of fungi with their (a)biotic environment and are important for morphogenesis and mechanical strength of hyphae (Gow *et al.*, 2017). The composition of fungal cell walls is dynamic and varies between species, strains, environmental conditions and developmental stage (Bowman and Free, 2006; Erwig and Gow, 2016). The composition of the cell wall of the reference basidiomycete *S. commune* had been studied in the past by destructive enzymatic and chemical treatments (Sietsma and Wessels, 1977; Sietsma and Wessels, 1979; Sonnenberg *et al.*, 1982; van Wetter *et al.*, 2000). These studies revealed that the outer layer of the *S. commune* cell wall consists of a water-soluble mucilage of (1,3)(1,6)-β-glucan (Sietsma and Wessels, 1979). This so-called schizophyllan is also secreted into the culture medium (van Wetter *et al.*, 2000; Zhang *et al.*, 2007). An alkali-soluble (1,3)-α-linked glucan was found to be located beneath the mucilage, while the inner layer of the cell wall would consist of chitin cross-linked to a highly branched (1,3)(1,6)-β-glucan (Sietsma and Wessels, 1979, 1981). Recently, non-destructive solid-state NMR presented a novel view on the cell wall of *S. commune* (Appels, 2020). The flexible part of the cell consists of β-(1,3-1,6)-glucan, α-(1,3)-glucan, α-(1,4)-glucan and homo- or heteropolymeric mannose, while the rigid part of the cell wall consists of chitin, β-(1,3)(1,6)-glucan, α-(1,3)-glucan and homo- or heteropolymeric fucose. Studying the cell wall of the ascomycete human pathogen *Aspergillus fumigatus* with ssNMR also resulted in a dramatic change in our understanding of its composition (Kang *et al.*, 2018). Together, we are only at the beginning to understand the architecture and composition of the fungal cell wall and how this affects material properties.

Chitin is not only a major component of the fungal cell wall but also the main constituent of exoskeletons of crustaceans. As such, it is the second most produced biopolymer in nature after cellulose (Kumar, 2000). Chitin of exoskeletons has shown great potential to solve numerous problems in environmental and biomedical engineering such as wastewater treatment and wound dressing (Kumar, 2000). Lately, the use of fungal derived chitin nanofibrils has attracted attention. Fungal derived chitin is easier to purify than crustacean chitin due to the absence of resilient minerals such as calcium carbonate (Kurita, 2006). Fungal chitin can be partially purified by extracting homogenized mycelium or fruiting bodies with hot water and mild alkaline (Jones *et al.*, 2019b; Nawawi *et al.*, 2019a,b, 2020). Soft compression of the extracted material results in paper-like nanomaterials containing mostly alkali-resistant glucans and chitin/chitosan. Sugar analysis by Nawawi *et al.* (2019b) of alkaline extracted stalks and caps of *Agaricus bisporus* fruiting bodies showed a sugar content of about 30% glucosamine (representative of chitin/chitosan), 40%–45% glucose (representative of glucans) and smaller fractions of mannose, xylose and galactose. Young's moduli and ultimate tensile strength reached approximately 7 GPa and 200 MPa, respectively (Nawawi *et al.*, 2019b). These values were higher than those of crustacean (*Cancer pagurus*) chitin nanofilms showing an Young's modulus of 2.7 GPa and an ultimate tensile strength of 69.5 MPa (Nawawi *et al.*, 2019b). Jones *et al.* (2019b) found about 40% glucosamine and 50% glucose in alkali extracted materials obtained from *A. bisporus* fruiting bodies, whereas mycelium-derived alkali extracts of different fungal species resulted in glucosamine concentrations ranging between 12%–23% glucosamine and 42%–86% glucose. This decrease of chitin/chitosan in the obtained materials is most likely responsible for the change in mechanical performance of fruiting body and mycelium derived materials, decreasing from a Young's modulus of 6.5 GPa and an ultimate tensile strength of 97.6 MPa to Young's moduli between 0.7–1.9 GPa and ultimate tensile strengths between 0.9–24.7 MPa.

Tensile properties of chitin/glucan nanomaterials from fruiting bodies of *A. bisporus* are significantly higher than those of pure and composite mycelium materials (see **Fig. 1**). Most likely this is due to increased density, purity and homogeneity of the chitin/glucan nanomaterials (Appels *et al.*, 2018; Jones *et al.*, 2019b; Nawawi *et al.*, 2019b).

Conclusions

The number of peer-reviewed publications covering mycelium-derived materials is growing exponentially since 2015. This illustrates the novelty and societal demand of this field. A range of mycelium materials have already been produced with foam-, natural-, polymer-, and elastomer-like Young's moduli and densities. At this moment, pure mycelium materials have been produced that could replace materials with a negative footprint such as textiles and leather. Composite materials have been produced with properties ranging from foam-like to natural material-like. The foam like materials can be used for thermal insulation (Yang *et al.*, 2017) or acoustic absorbance (Pelletier *et al.*, 2013; Jones *et al.*, 2017). Natural material-like materials could replace particle boards such as oriented strand board (OSB) and medium density fiber board (MDF), often containing toxic compounds such as formaldehyde (Boran *et al.*, 2011). More research is needed to optimize properties and to scale up production. This research should focus on fungal species, substrates, growth conditions, and post-processing techniques. For instance, only few fungi have been used so far to produce mycelium materials. Some species such as *S. commune* are fast colonizers of the substrate but enter the stationary growth phase relatively fast (Appels, 2020). Possibly, these species have difficulties to reach and degrade less accessible polysaccharides. Other species such as *Trametes hirsuta* and *Lenzites betulina* have a longer lag phase but grow exponentially for a longer time. The latter species produces a denser mycelium within the substrate when compared to the fast colonizing strains, and resulting material properties should be studied in the future. Apart from the mycelium materials, cell wall polymers like chitin (either or not complexed to glucan) can be used to develop high-performing nanomaterials. First publications show that these materials are very strong compared to mycelium materials.

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Subject Index

Note
This index is in letter-by-letter order, whereby hyphens and spaces within index headings are ignored in the alphabetization, and it is arranged in set-out style, with a maximum of three levels of heading.

Cross-reference terms in italics are general cross-references, or refer to subentry terms within the main entry (the main entry is not repeated to save space).

Location references refer to the volume number, in bold, followed by the page number.

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