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REVIEW

The Mycobiome in Health and Disease: Emerging Concepts, Methodologies and Challenges

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Abstract Fungal disease is an increasingly recognised global clinical challenge associated with high mortality. Early diagnosis of fungal infection remains problematic due to the poor sensitivity and specificity of current diagnostic modalities. Advances in sequencing technologies hold promise in addressing these shortcomings and for improved fungal detection and identification. To translate such emerging approaches into mainstream clinical care will require refinement of current sequencing and analytical

platforms, ensuring standardisation and consistency through robust clinical benchmarking and its validation across a range of patient populations. In this state-of-the-art review, we discuss current diagnostic and therapeutic challenges associated with fungal disease and provide key examples where the application of sequencing technologies has potential diagnostic application in assessing the human 'mycobiome'. We assess how ready access to fungal sequencing may be exploited in broadening our insight into host–fungal interaction, providing scope for clinical diagnostics and the translation of emerging mycobiome research into clinical practice.

Keywords Mycobiome · ITS sequencing · Metagenomics · Bioinformatics · Clinical

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Introduction

Fungal disease affects over 300 million people worldwide causing over 1.6 million deaths annually [1]. Despite their natural environmental abundance, few fungi are human pathogens, and while fulminant fungal infection is uncommon in the healthy individuals, invasive fungal disease is a concern in the immuno-compromised host with significant associated morbidity and mortality [2]. Increasing numbers of patients are at risk of invasive fungal disease

including those with human immunodeficiency virus (HIV), malignancy and transplant recipients on immunosuppressive or immunomodulatory therapies, each contributing to the rising global trend of fungal infections among susceptible populations. Global warming and climate change also have significant impacts on the pathogenicity and survival of fungi [3]. Acclimatisation to higher temperatures increases their ability to replicate in the human body with high basal temperature, resulting in increased pathogenic potential even for species previously reported as non-pathogenic [3]. This impacts fungal distribution where increases in heat-resistant species facilitate human interaction, infection and transmission through skin contact, inhalation and/or ingestion [3]. Fungal infection therefore can manifest as superficial infections of the skin, nail, hair and mucous membranes, by dermatophytes and *Candida* spp. or as invasive disease caused by opportunistic and endemic mycoses [4]. Superficial disease is generally mild and easily diagnosed with readily available treatment. However, in the immuno-compromised individual, such infections may progress to invasive disease. Opportunistic fungal disease is primarily seen in the immuno-compromised with aspergillosis, candidiasis and cryptococcosis most commonly reported. The annual incidence ranges from 200,000 to 1 million, with high and variable mortality rates ranging from 20 to 90% [5]. Table 1 and Fig. 1 summarise the most common invasive fungal diseases and the anatomical sites involved. Endemic fungal diseases occur in distinct geographic regions, apparently driven by environment and climate. Irrespective of underlying host immunity, individuals residing in endemic regions are at higher risk of life-threatening disease. Blastomycosis and histoplasmosis are endemic in the Midwestern United States (US), coccidioidomycosis in the Southwestern US, paracoccidioidomycosis in Brazil and talaromycosis in Southeast Asia [5]. Worryingly, endemic fungal disease continues to propagate even expanding beyond traditional accepted boundaries, again driven at least in part by climate change, urbanisation, land development and ease of travel [6]. A key example is the outbreak of *Cryptococcus gattii* at Vancouver Island and the Pacific Northwest of the USA [7]. This poses significant and emerging challenges for diagnosis, which in turn delays treatment initiation with adverse clinical consequence.

Diagnostic and Treatment Challenges in Fungal Infection

The early diagnosis and initiation of therapy for fungal infection is critical, influencing outcome and mortality, particularly in the immuno-compromised [8–10]. Current available diagnostic modalities lack sensitivity and specificity, and therefore the diagnosis of invasive fungal disease relies on a combined clinical, microbiological and radiological approach with empirical treatment often initiated based on clinical judgment alone pending diagnostic confirmation [11]. Patient characteristics including the presence or absence of neutropenia or use of antifungal prophylaxis impact the diagnostic accuracy of tests, adding additional uncertainty [12–14]. The ability to distinguish infection from colonisation poses an additional and important clinical challenge. Conventional culture methods provide species-level identification and information on antifungal susceptibility. However, culture-based methods are poorly sensitive and delay time to diagnosis [15–17]. Their accuracy is contingent on sample source, type of fungus, as well as disease and host immune status. For instance, *Pneumocystis* species are unable to grow on culture media [18, 19] and isolation of *Aspergillus* in blood is uncommon and ineffective for the diagnosis of invasive aspergillosis [16, 20]. In addition, positive cultures obtained from non-sterile body sites may not be representative and should be interpreted with caution. Direct microscopic examination is useful in providing rapid and more accurate diagnosis of fungal infection allowing differentiation by their morphology and staining [21]. However, as with culture, the diagnostic yield is contingent on several factors and exhibits variable accuracy [22]. Non-culture-based techniques are increasingly employed to aid the diagnosis of invasive fungal disease. For example, detection of β-glucan, a component of the fungal cell wall, identifies patients with invasive disease with sensitivities ranging from 50–100% and specificities of 44–98% [15, 23, 24]. The high false positive rate, however, limits the usefulness of this approach particularly in high-risk patient groups including patients with haematological malignancy, stem cell and lung transplant recipients, where co-existing bacterial infection is present and in those receiving haemodialysis [25–27]. While β-glucan is a useful diagnostic target, it is not specific to any single fungal

Table 1 Review of major invasive fungal disease with organ involvement, disease manifestation, susceptibility, diagnosis and management

Fungal infection	Mortality	Organisms	Organ involvement	Disease manifestations
Aspergillosis	30–95% [228]	*A. fumigatus*	Pulmonary	Pneumonia, cavity, aspergilloma, tracheobronchitis [229]
		A. niger	CNS	Meningitis, meningoencephalitis, cerebral infarction, haemorrhage, mycotic aneurysm [232]
		A. flavus		
		A. terreus	GIT	Intestinal oedema, obstruction, haemorrhage, perforation [233]
			Cutaneous	Pustules, haemorrhagic bulla, ulcerative lesion, cellulitis, abscess [234]
Candidiasis	19–30% [235, 236]	*C. albican*	Blood	Candidaemia [237]
		C. tropicalis	Pulmonary	Pneumonia [237]
		C.glabrata	CNS	Meningitis, abscess [232]
		C. krusei	Eye	Chorioretinitis, endophthalmitis [238]
		C. parapsilosis	GIT	Peritonitis, oesophagitis, liver abscess [237]
			Mucosal	Oral, vulvovaginal candidiasis [237]
Cryptococcosis	20–70% [228]	*C. neoformans*	Pulmonary	Cryptococcoma, pneumonia [239]
		C. gattii	CNS	Meningitis, cerebral cryptococcoma [232]
			Cutaneous	Nodules, ulcerated lesion [239]
Mucormycosis	46–96% [241]	*Rhizopus* spp.	Rhino-cerebral	Sinusitis, meningitis, orbital oedema, cellulitis, nasal necrotic/ulcerative lesion [242]
		Mucor spp.		
		Rhizomucor spp.	Pulmonary	Pneumonia, cavity, nodules, mass [243]
		Lichtheimia spp.	Cutaneous	Nodules, ulcer, scaly plaques, cellulitis, necrotic eschar [244]
			GIT	Necrotising enterocolitis, intestinal perforation, ulceration [245]
Pneumocystis	20–80% [228]	*P. jirovecii*	Pulmonary	Pneumonia [9]
			Extra-pulmonary	Eye, ear, thyroid, GIT, bone marrow, muscle, lymph node involvement [247, 248]
Blastomycosis	0.06–68% [228, 249]	*B. dermatitidis*	CNS	Meningitis, brain and epidural abscess [250]
		B. gilchristii	Cutaneous	Papulopustular, ulcerative, verrucous, crusted lesions
			GUT	Prostatitis, epididymitis, tubo-ovarian abscess, endometritis, salpingitis
			Pulmonary	Pneumonia
			Bone	Osteomyelitis
Histoplasmosis	5–60% [9, 228, 252]	*H. capsulatum*	Pulmonary	Pneumonia, nodule, cavity, fibrosing mediastinitis, broncholithiasis [225, 253, 254]
			Cutaneous	Erythema nodosum, erythema multiforme [227]
			CNS	Meningitis, parenchymal lesion [227, 232]
			GIT	Ulceration, stricture [227]
Coccidioidomycosis	1–70% [228]	Coccidioides immitis	Pulmonary	Pneumonia, nodules, cavity [256]
		Coccidioides posadasii	Cutaneous	Erythema nodosum, erythema multiforme, nodule, plaque, papules [258]
			Bone and Joint	Osteomyelitis, arthritis, abscess [259, 260]
			CNS	Meningitis [256]

Table 1 continued

Fungal infection	Mortality	Organisms	Organ involvement	Disease manifestations
Paracoccidioidomycosis	65–27% [228, 261]	*P. brasiliensis*	Pulmonary	Pneumonia, nodule, cavity, emphysema, fibrosis, pleural effusion, miliary-like shadow [262, 263]
		P. lutzii	Cutaneous	Ulcerated lesion, abscess [227]
			Mucosa	Oral ulcer, gingivostomatitis [227]
			Lymph node	Lymphadenopathy [227]
			CNS	Meningitis, CNS granuloma, abscess [227, 232]
Talaromycosis	2–75% (6)	*T. marneffe*	Cutaneous	Subcutaneous abscesses, papule-like ulcers, molluscum-contagiosum-like lesion [264]
			Lymph node	Lymphadenopathy [9]
			Pulmonary	Diffuse reticular infiltrates, cavity, localised alveolar infiltrates [9, 264]
			GIT	Hepatosplenomegaly, Intestinal [267, 268]

Fungal infection	Susceptible host	Diagnosis (specific assays) [9, 15, 24, 28, 32, 225–227]	Diagnosis (general assays) [28]	Treatment
Aspergillosis	Immuno-compromised	Galactomannan (serum, BAL, CSF)	Serum beta glucan	First line: voriconazole [230], isavuconazole [24, 231]
	ICU/critically ill	Lateral-flow device antigen detection (serum, BAL)	Histopathology	Severe: liposomal amphotericin
	COPD	PCR	Culture	Alternative: itraconazole, posaconazole, micafungin [230]
Candidiasis	Immuno-compromise	*Candida albicans* germ tube antibody (CAGTA) assay	Histopathology	First line: echinocandin [30]
	Invasive procedure and surgery	Mannan-Ag and mannan-Ab	Culture	Alternative: fluconazole, voriconazole, amphotericin [30]
	Trauma or burn	PCR	Serum beta glucan	
	Prolong ICU stay			
Cryptococcosis	Both immune-compromised and immunocompetent host	Cryptococcal antigen test (CSF, serum)	Histopathology Culture	First line: amphotericin +flucytosine, fluconazole [9, 240]
				Alternative: itraconazole
Mucormycosis	Diabetes	PCR	Histopathology	First line: liposomal amphotericin
	Immuno-compromised		Culture	Alternative: posaconazole [226], isavuconazole [237]
	Trauma or burn			
Pneumocystis	Immuno-compromised	PCR	Histopathology	First line: trimethoprim/sulfamethoxazole [9]
			Serum Beta glucan	Alternative: primaquine + clindamycin, pentamidine [9, 246]
Blastomycosis	Both immune-compromised and immunocompetent host at the endemic region	Antibody test (serum) Antigen test (urine)	Histopathology Culture	Severe: amphotericin Mild to moderate: itraconazole [251]

Table 1 continued

Fungal infection	Susceptible host	Diagnosis (specific assays) [9, 15, 24, 28, 32, 225–227]	Diagnosis (general assays) [28]	Treatment
Histoplasmosis	Both immune-compromised and immunocompetent host at the endemic region	Antibody test (serum) Antigen test (urine, serum)	Histopathology Culture	Severe: liposomal amphotericin [225] Mild to moderate: itraconazole [255] Mild disease treatment is usually not require [255]
Coccidioidomycosis	Both immune-compromised and immunocompetent host at the endemic region	Antibody test (serum)	Histopathology Culture	First line: fluconazole/itraconazole [257] Severe: amphotericin [257]
Paracoccidioidomycosis	Both immune-compromised and immunocompetent host at the endemic region	Antibody test (serum)	Histopathology Culture	First line: itraconazole [225, 227] Severe: amphotericin [225, 227] Alternative: trimethoprim/sulfamethoxazole, voriconazole, posaconazole, isavuconazole [227]
Talaromycosis	Immuno-compromised	PCR Serology (not widely available)	Histopathology Culture Galactomannan	First line: liposomal amphotericin and itraconazole [9, 265] Alternative: voriconazole [9, 266]

CNS central nervous system, *GIT* gastrointestinal tract, *ICU* intensive care unit, *COPD* chronic obstructive pulmonary disease, *BAL* bronchoalveolar lavage, *CSF* cerebrospinal fluid, *PCR* polymerase chain reaction

species and may be detectable across a range of fungal infections including invasive aspergillosis, candidiasis, and cryptococcosis. Galactomannan (GM) is another commonly used marker for the diagnosis of invasive aspergillosis in patients with haematological malignancy and/or neutropenia with sensitivities and specificities of 79–96% and 74–99%, respectively [23]. However, sensitivity decreases with use of antifungal agents and among non-neutropenic patients [12, 24]. Serological testing is another option available for a range of fungal infections including candidiasis, cryptococcosis and endemic mycoses with diagnostic accuracies varying based on the type of fungal organism and sample source. While providing a good diagnostic yield for histoplasmosis and coccidioidomycosis, it is not particularly useful for the diagnosis of invasive aspergillosis [22, 28]. Polymerase chain reaction (PCR) provides an alternative, more rapid and accurate diagnostic tool for the detection of fungal infection [29]. Numerous PCR assays are described with variations in primer sequence, DNA extraction method, positivity thresholds and assay performance, which coupled with limited multi-centre clinical validation, has curtailed widespread adoption [28–31]. Importantly, false positive results are common as fungi are ubiquitous and contamination possible at various sample processing stages necessitating rigorous and costly quality control processes [32]. Next-generation sequencing (NGS) is increasingly used in epidemiological studies and during infectious disease outbreaks for resolution of species, virulence and antibiotic resistance, to assist infection control and its management [33–35]. In addition, NGS may be employed in the resolution of cryptic epidemiological cases where it has demonstrated superiority to traditional diagnostic methods in the detection of rare infections [36–38]. However, demonstration of its utility within the medical diagnostic setting is currently limited. Low abundance of fungi in relation to the overall microbial community ($\leq 0.1\%$) poses challenges to their detection via metagenomic sequencing in human samples, necessitating targeted amplicon sequencing approaches, which are still in their infancy
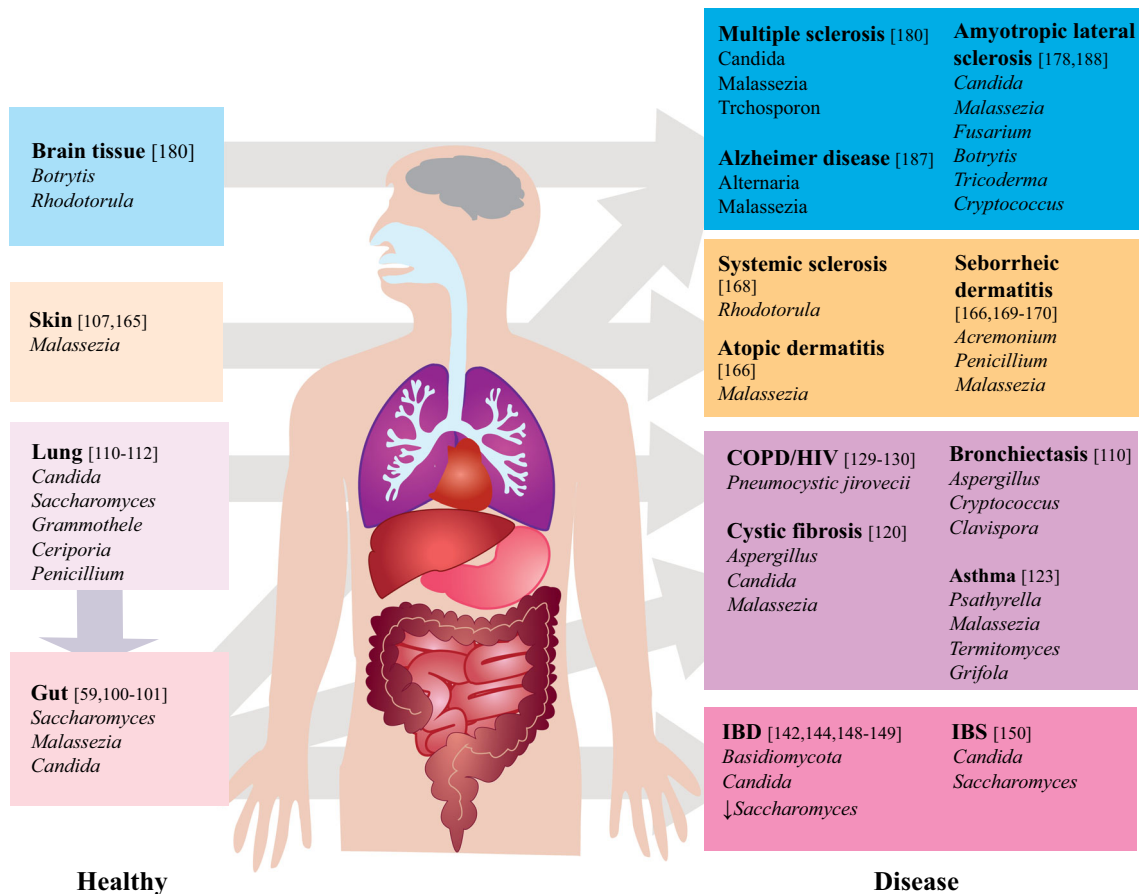
Brain tissue [180]
Botrytis
Rhodotorula

Skin [107,165]
Malassezia

Lung [110-112]
Candida
Saccharomyces
Grammothele
Ceriporia
Penicillium

Gut [59,100-101]
Saccharomyces
Malassezia
Candida

Multiple sclerosis [180]
Candida
Malassezia
Trchosporon

Alzheimer disease [187]
Alternaria
Malassezia

Amyotropic lateral sclerosis [178,188]
Candida
Malassezia
Fusarium
Botrytis
Tricoderma
Cryptococcus

Systemic sclerosis [168]
Rhodotorula

Atopic dermatitis [166]
Malassezia

Seborrheic dermatitis [166,169-170]
Acremonium
Penicillium
Malassezia

COPD/HIV [129-130]
Pneumocystic jirovecii

Cystic fibrosis [120]
Aspergillus
Candida
Malassezia

Bronchiectasis [110]
Aspergillus
Cryptococcus
Clavispora

Asthma [123]
Psathyrella
Malassezia
Termitomyces
Grifola

IBD [142,144,148-149]
Basidiomycota
Candida
↓*Saccharomyces*

IBS [150]
Candida
Saccharomyces

Healthy

Disease

Fig. 1 Overview of the mycobiome in healthy and diseased states. Purple arrow: cross-talk between lung and gut; grey arrow: dysbiosis. COPD: chronic obstructive pulmonary disease; HIV: human immunodeficiency virus; IBD: inflammatory bowel disease; IBS: irritable bowel syndrome; down arrow: decreased abundance

[29, 39, 40]. Further refinement and standardisation of reproducible wet-lab and bioinformatic workflows coupled to automated and scalable user-friendly reporting systems remain key challenges for the field, which unless addressed, will continue to impede widespread adoption and translation into clinical practice [41–43].

Four distinct classes of antifungal drugs are currently prescribed: the polyenes, flucytosines, azoles and echinocandins. Despite treatment, mortality rates for invasive fungal disease remain high with factors contributing to poor prognosis including delayed diagnosis and initiation of antifungal treatment, host factors, site of infection, emerging antifungal resistance and drug toxicity. Clinical presentation of fungal infection is largely non-specific, and therefore a high index of suspicion is necessary for early diagnosis and initiation of treatment, particularly in the immuno-compromised population. Host factors of relevance include underlying comorbidities and long-term medications that interact with antifungal treatment. This is most marked in concomitant antifungal and antiviral treatments in HIV-related cryptococcus infection, which results in life-threatening inflammation secondary to immune reconstitution. The available tissue concentrations of antifungals also differ according to their chemical and pharmacokinetic properties leading to sub-optimal concentrations, therapeutic failure and the development of resistance in many settings, highlighting the importance of infection site to the appropriate selection of antifungal agent [44]. Drug toxicity is a further consideration as treatment is often prolonged and, in those with other comorbidities, increases the risk of drug–drug interactions [45].

Antifungal resistance is emerging rapidly, both intrinsic resistance and that driven by the widespread use of antifungal and environmental fungicides [46]. Resistance, however, varies based on geographic location, patient demographics and prior drug exposure [47, 48]. Azole resistance in *Candida* and *Aspergillus* spp. ranges from 2.3–40 to 0.85–28%, respectively [47, 49–52]. Resistance to echinocandins—first-line antifungal agents for invasive candidiasis—is also described in invasive *Candida glabrata* infections [48, 51, 53–55]. The development of new antifungals with decreased toxicity and broad-spectrum coverage is urgently required to reduce mortality rates, and a better understanding of host–fungal interaction is necessary to facilitate future therapeutic development pipelines. Furthermore, advances in NGS enable identification of antifungal resistance genes, informing our understanding of the resistome and guiding antifungal drug development [56–58]. In the following sections, we aim to describe our current understanding of the mycobiome in the context of recent NGS advances and the methodological challenges and barriers that exist in terms of its clinical implementation.

Mycobiome Analysis: Methodologies and Challenges

Sample Processing

Sample processing including storage conditions and various DNA extraction protocols may inadvertently introduce confounders that affect experimental results (Fig. 2). To date, studies comparing various methodologies of sample processing demonstrate conflicting results and varying efficacies, with implications for cross-comparisons of published mycobiome research. Freezing samples within 12 h to prevent overgrowth by fast-growing fungi and avoiding multiple freeze–thaw cycles has been advocated [59, 60]. The addition of RNAlater decreases the relative abundance of certain fungal taxa particularly in faecal samples suggesting possible introduction of bias in relative abundance assessments [61]. Nevertheless, taxonomic diversity generally remains assessable and largely unaffected by the freezing process [61–63]. When extracting fungal DNA for mycobiome sequencing, the highly rigid fungal cell wall, rich in glucans, chitin,

mannans and glycoproteins, has necessitated additional steps that involve rigorous bead-beating or enzymatic lysis prior to DNA extraction [61, 62, 64–67]. Notably, enzymatic lysis provides an alternative option for increasing DNA yields [68–70]. The harsher bead-beating method, however, is observed to have conflicting efficacies likely due to DNA degradation [71]. Various DNA extraction methods also significantly affect fungal DNA yield and quality with phenol/chloroform extraction often outperforming the various available commercial kits (Fig. 2) [61, 62, 65, 70, 71]. This loss of DNA using commercial kits is likely related to use of silica column purification [71]. DNA extraction kits illustrate inconsistent results when performed with different sample types and produce variable fungal DNA yields [70, 72]. While extraction methods affect DNA yield and quality, their impact on mycobiome composition and diversity appears minor [62, 65, 71]. However, recent work from our group, using spontaneous induced sputum, has demonstrated more consistent amplification of the ITS region from samples subjected to mechanical disruption as compared to enzymatic lysis [40]. This was observed despite higher overall DNA yields achieved by enzymatic lysis suggesting preferential liberation of fungal DNA by mechanical lysis [40]. Extraction methods are critical and must be applied consistently and carefully both within and between mycobiome studies. Ultimately, sample-specific mycobiome protocols are required and a need exists to incorporate specific methods adjusted to sample type or disease-specific application, importantly, with validation for routine use in future mycobiome research.

Amplicon Sequencing

In contrast to high-throughput sequencing (HTS) of the bacterial microbiome, taxonomic classification and primer choice are not well established for the fungal mycobiome (Fig. 2) [60, 73]. As amplicon sequencing is well recognised to be subject to selective amplification bias, careful consideration of primer selection is crucial [74]. Key factors include taxonomic resolution, coverage, accuracy and amplicon length [74]. The ITS region, located between genes encoding ribosomal subunits 18S and 23S, has been proposed as the universal genetic fungal barcode, primarily because these regions have high
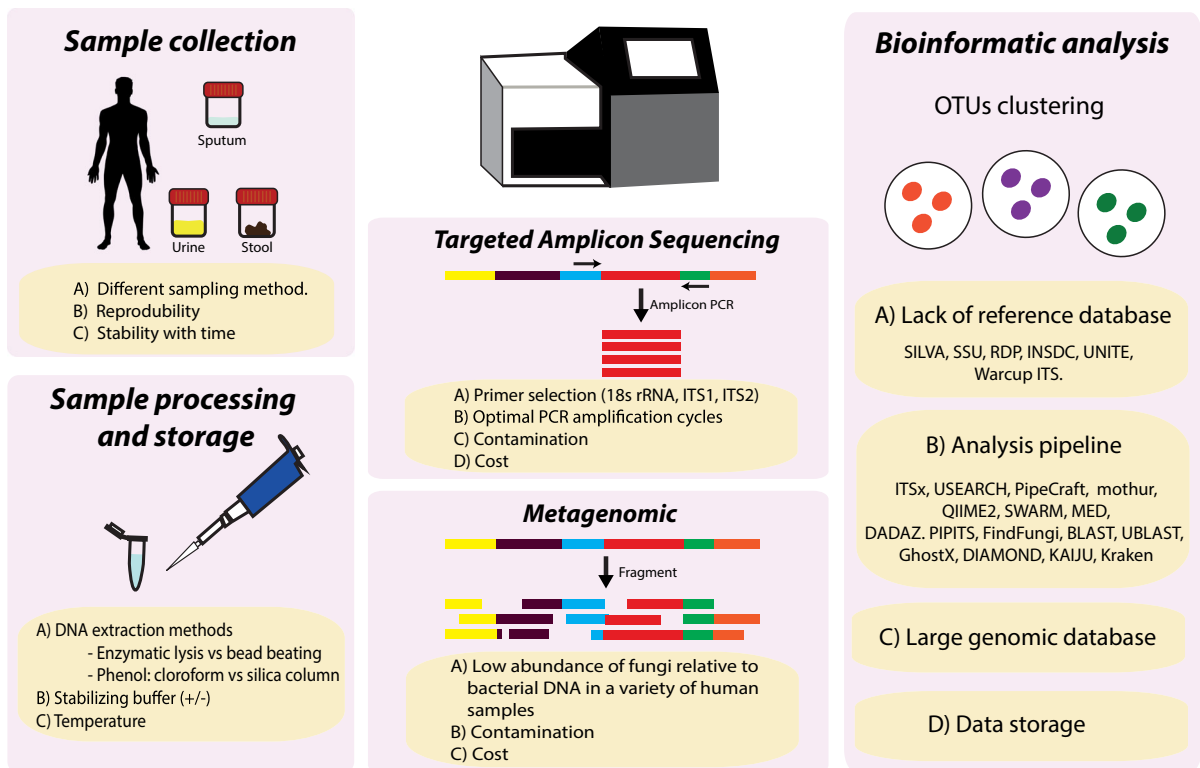
Fig. 2 Diagram summarising the key challenges in mycobiome sequencing and analysis. PCR: polymerase chain reaction, rRNA: ribosomal ribonucleic acid, ITS: internal transcribed spacer, DNA: deoxyribonucleic acid, OTU: operational taxonomic unit

evolutionary rates and are flanked by highly conserved regions serving as suitable target sites for universal primers [60, 73, 75, 76]. The ITS region typically spans 500–700 bps, and consists of two sub-regions, ITS1 and ITS2, which are separated by the conserved 5.8S region [77]. Although primers targeting ITS1 (i.e. ITS1F and ITS2) have been used for decades in several large-scale microbiome projects, to date, it is still unclear which ITS fragment is optimal. Recent findings demonstrate that these primers suffer from primer bias, amplification bias and fail to accurately profile mock communities analysed, leading to inconsistent representation of synthetic fungal communities of known taxonomic abundance [40, 74, 78–82]. Based on these observations, Nilsson and colleagues provide a list of fungus specific, HTS-oriented primers for amplification of ITS region, and recommend targeting the ITS2 sub-region by using degenerate forward primers gITS7ngs and the reverse primer ITS4ng owing to their superior coverage of the fungal kingdom [60]. Advantages of the ITS2 sub-region include a more universal primer site and lower length

variation, leading to less taxonomic bias compared to ITS1 [60, 83, 84]. While this primer set (gITS7ngs and ITS4ng) has a superior coverage of fungal kingdoms, its accurate taxonomic resolution has yet to be evaluated experimentally [82]. Further confirmatory studies are required before the field can advance and eventually reach consensus on methodology for targeted amplicon sequencing of the mycobiome, as has been the case for 16 s rRNA sequencing of bacterial microbiomes. In addition to primer choice, care must be taken to yield fewer compromised amplicon sequences. These include careful consideration of number of PCR cycles and dilution of DNA samples where possible (Fig. 2) [85, 86]. High fidelity proofreading PCR polymerase with low GC content bias is also necessary to reduce chimeras and random errors that accumulate in later cycles [85, 86]. It is also essential to include appropriate negative controls and a mock community into the analysis. The former indicates sources of potential contamination, while the latter allows assessment of chimera formation, quantification of index switching and operational

taxonomic unit (OTU) inference stringency (Fig. 2) [85, 87, 88].

Metagenomic Shotgun Sequencing

An alternative approach to targeted amplicon sequencing is the use of whole-genome shotgun (WGS) metagenomics to study the mycobiome. This entails the sequencing of all extracted DNA in a given sample without the use of targeted PCR amplification of the ITS or other specific target sequences. Here, all DNA from resident microbes and host cells present in a sample is assessed. Sequence reads are processed and classified relative to a reference database through either assembly-based or assembly-free methods [89]. Analysis will usually also include steps for removal of host DNA sequences, which may contribute to a significant proportion of sequence data if removal of human cells is not attempted prior to DNA extraction [90]. While, in principle, metagenomic sequencing should provide a superior, unbiased assessment of the mycobiome, studies to date employing WGS metagenomics highlight the low abundance of fungi relative to bacterial DNA across a variety of human samples (Fig. 2) [39]. This represents a significant cost barrier given the sequencing depth therefore required to detect fungi in samples where bacteria predominate while lower sequencing coverage also hinders assembly-based analysis of fungi [89]. Currently, it appears that low fungal abundance in human samples hinders the widespread application of metagenomic WGS in human samples, a finding that appears unrelated to DNA extraction methods and reflects a genuinely low overall in vivo fungal abundance [63].

Bioinformatic Challenges

The development of tools for the analysis of ITS-derived amplicon sequences is still a nascent area of research, and many options are available for the analysis of metabarcoding amplicon data sets [29]. While the field still has no consensus on standards and best practices, further studies are clearly required, and no optimal, broadly applicable protocol has been yet established. Further, it is probable that amplicon sequencing and its accompanying analysis largely depend on the samples under investigation due to variable primer coverage, as demonstrated for comparable bacterial microbiome analysis targeting the

16 s rRNA gene [91]. The increased variability of the fungal ITS region exacerbates this problem in the case of mycobiome research, and this requires careful consideration when interpreting ITS-derived mycobiome profiles. A further analytic hurdle of mycobiome metagenomic analyses remains the sparsely populated fungal databases on which read classifications are based (Fig. 2). This leads to large numbers of unclassified reads or unidentified operational taxonomic units (OTUs), which may be addressed by generation of more high-quality metagenomic and whole-fungal genome assemblies [63, 92]. Major fungal ITS reference databases include the INSDC, UNITE and Warcup ITS [60]. Although accurate species-level classification is not possible using the ITS regions, there are currently no known alternate markers offering comparable lineage separation, while reported taxonomic thresholds for filamentous fungal identification at the genus, family, order and class levels are estimated at 94.3%, 88.5%, 81.2% and 80.9%, respectively [60, 93]. The increased accessibility of third-generation long-read sequencing offers promise in this regard as the large stretches of repetitive and non-coding DNA as well as intron-containing genes found in fungal genomes can be resolved by these technologies which may also be applied to targeted sequencing of the ITS [60, 63]. Although the literature on fungal metagenomics is lacking, two key studies (Nash et al. and Donovan et al.) represent the most in-depth assessment to date in terms of metagenomic identification of fungi in human samples [63, 94]. Nash et al. performed a large assessment of gut samples from the human microbiome project, performing both metagenomic analysis and comparative ITS sequencing. Importantly, they showed that DNA extraction methods did not have appreciable effects on the levels of fungi detected, re-affirming the apparent low abundance of fungi in these samples. Secondly, they performed a comparative analysis of ITS and 18 s targeted sequencing protocols demonstrating that ITS2-targeting primers showed greater resolution of low-abundance taxa [63]. The work of Donovan and colleagues is notable for their bioinformatic dissection of the challenges inherent to analysis of the fungal metagenome. They present a solution in the form of the 'FindFungi' pipeline, which highlights the challenges of fungal metagenomic analysis including false positive detection, for which they developed an important step to identify uneven

read distribution across reference genomes indicating spurious fungal detection [94]. Finally, an additional challenge that remains is the implementation of these bioinformatic pipelines, which, necessitates execution on high-performance computing clusters due to the requirement to hold large genomic databases in memory (Fig. 2). Such computation requires domain expertise that may not be widely available to fungal research laboratories and remains an operational and logistical challenge.

Comparison with Culture and Other Fungal Detection Methodologies

Culture-based methods of detection require a priori selection of fungal growth conditions and are susceptible to bacterial overgrowth hindering the proliferation of selected fungi leading to bias. In addition, culture-based methods are prone to cross-contamination of spore-producing fungi, which can be minimised by the use of horizontal laminar flow clean bench [95, 96]. While the addition of antibiotics to culture media is commonly employed to prevent bacterial contamination, its drawbacks include limited antibacterial spectrum, and its potential inhibitory effect on fungi [97–99]. Results from culture methods may be difficult to cross compare with the existing literature given heterogeneity of methodology and study designs as well as sample pre-processing protocols, thereby requiring a standardised protocol for detecting key fungal pathogens [100, 101]. However, culture-based methods do allow for an assessment of antifungal susceptibilities and the isolates obtained by culture provides scope for further molecular characterisation. Numerous culture-independent approaches have been developed to overcome the limitations of culture-based methodology but are not without their disadvantages. Internal transcribed spacer-polymerase chain reaction (ITS-PCR) approaches could be used to detect mixed infections; however, careful target selection is necessary [76, 102–104]. In some cases, ITS-PCR may appear even less reliable than culture for fungal detection [95]. Multiplex PCR assays allow for greater efficiency but require strict primer design [101]. However, PCR-based approaches are susceptible to false-negative results where sub-optimal sample storage or DNA extraction methods are employed or where PCR inhibitors are present. The use of oligonucleotide array

platforms represents a possible alternative but is expensive and subject to batch effects [101]. Arguably, sequencing of individual amplified ITS regions remains the gold standard for fungal identification, allowing for sensitive detection of unculturable or low-abundance fungi but may not be practical for all laboratories [105]. The use of mycobiome profiling as an alternative to culture-based and molecular diagnostic detection methods represents potentially valuable applications for this technology, but one that requires further validation and research [95].

The Mycobiome in Health and Disease

Host–Fungal Interaction

The term mycobiome is used to describe the fungal component of the microbiome. It is yet unclear whether a core mycobiome, analogous to the bacteriome, exists in humans [106]. Relative to the bacteriome (> 99% of the total microbiota), the human mycobiome is less diverse (generally < 20 OTUs per sample) and present at a lower abundance ($\leq 0.1\%$ of the total microbiota) [39, 63]. Targeted sequencing approaches reveal that infants are colonised by fungi shortly after birth, predominantly by members of the genera *Candida*, *Saccharomyces*, *Cladosporium*, *Cryptococcus* and *Malassezia* [107, 108]. Where correlated, stool mycobiomes from healthy adults reveal profiles predominated by *Saccharomyces*, *Malassezia* and *Candida* suggesting that these genera are the key constituents of a healthy mycobiome throughout life. Critically, however, and in contrast to human bacteriome studies, little correlation with host phenotype metadata is firmly established in healthy individuals and, as such, our knowledge of fungal mycobiome–host interaction remains limited to that in fungal colonisation and/or disease. It is clear from existing studies and their subsequent molecular analysis that intricate immunological systems have evolved to regulate fungal homoeostasis and, when perturbed, lead to infection or other pathologies [109]. Immunity to fungi is underpinned by innate signalling pathways recognising both spores and fungal cell wall components and include adaptive T cell responses that orchestrate tolerance and appropriate immune surveillance of ubiquitous fungi encountered daily [109, 110]. Immune responses to fungi are protective

when functioning, however, where over-zealous, can manifest as allergy. Consequently, the evolution of immuno-regulatory mechanisms in vivo is perturbed across a range of fungal-driven allergy. Given the parallels with the human bacteriome, where host immunity acts as gatekeeper, it is arguable that similar ecological interactions occur with fungi [111]. Perhaps the strongest evidence for a conserved human mycobiome is evidenced by the restricted numbers of fungi that actually inhabit the human body, suggestive of niche specialisation [112]. Despite over 50 described bacterial phyla, the human bacteriome is characterised by four: *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. In corollary, fungal taxa exhibit lower diversity in vivo compared to that seen environmentally and associations between fungi and specific body sites indicate the likely existence of selection and adaptation [113, 114]. Given the limited available data, formal meta-analysis of clinical mycobiome studies, while important, is currently challenging to perform. However, emerging associations based on culture-independent mycobiome studies in humans are summarised in Fig. 1.

The Lung Mycobiome

While fungal lung disease is now widespread and a growing global concern, the lack of dedicated mycobiome studies assessing the effects of fungi in chronic lung disease is hampering the field. In this particular organ system, the prior presupposition of sterility in a healthy lung puts back microbiome studies in this area for a number of years, and consequently, our understanding of the respiratory microbiome lags significantly behind that at other anatomical sites [115]. The advent of culture-independent sequencing technologies has uncovered the diversity of organisms inhabiting the lung, including that in the healthy state [116, 117]. Through the use of internal transcribed spacer (ITS) sequencing, it was reported that *Ceriporia lacerata*, *Saccaromyces cerevisiae* and *Penicillium brevicompactum* were possible pulmonary fungi, with the main pathogen being *Aspergillus fumigatus* [118, 119]. Lung-dwelling microbiota include bacteria, fungi and viruses, which together maintain a cross-kingdom ecological network [120]. Interactions between lung and gut microbiomes, through the lung–gut axis, have further immunological consequence, and changes in the gut mycobiome can influence allergic airway disease [121–125]. The healthy lung mycobiome differs from that observed in patients suffering with chronic inflammatory respiratory disease such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and bronchiectasis [126, 127]. The lungs in asthma, COPD and CF all exhibit lower fungal diversity, which in turn is linked to poorer lung function postulated to be influenced by fungal overgrowth and/or loss of some fungal species [128]. In asthma, higher loads with increase percentages of *Psathyrella candolleana, Malassezia pachydermatis, Termitomyces clypeatus* and *Grifola sordulenta* and a greater fungal diversity were observed compared to that of a healthy airway [129, 130]. In children with severe asthma, a higher abundance of *Rhodosporidium, Pneumocystis, Leucosporidium* and *Rhodotorula* was detected when compared to those without asthma [131]. Bronchiectasis, a permanent and irreversible dilatation of airways, places patients at the higher risks of fungal acquisition and colonisation [132]. This is reflected by airway mycobiomes demonstrating a higher abundance of potentially pathogenic taxa including *Aspergillus, Penicillium* and *Cryptococcus* and the detection, in many patients, of an unfavourable allergic sensitisation and immune response profile associated with *Aspergillus* [117, 133]. In view of the dysfunctional mucociliary clearance inherent in bronchiectasis, the airway is susceptible to inhaled airborne fungi and therefore colonisation [134]. In COPD, *Aspergillus* spp. has been isolated in 17% of patients and particular fungal taxa such as *Pneumocystis jirovecii* associated with a COPD airway have also been found in HIV and COPD-associated HIV suggestive of immunological predisposition [135, 136]. The overrepresentation of *Pneumocystis* and other fungal species in HIV-related COPD further illustrates the relationship between fungal communities in the lung and corresponding lung function and homoeostasis, warranting more detailed investigation by culture-independent sequencing [137]. Significant fungal variability is seen in cystic fibrosis (CF), a genetic disease caused by abnormal Cystic Fibrosis Transmembrane conductance Regulator (CFTR) function, where fungal colonisation and infection for *A. fumigatus* range between 6 and 60% [138]. While dedicated CF mycobiome research is nascent, a number of groups have nevertheless started to characterise its main components. Stable fungal

communities are detected and exist in parallel to the more ephemeral fungal species that remain transient when assessed longitudinally [95, 127, 128, 139–143]. Targeted sequencing studies of the CF mycobiome reveal that in addition to the fungi detected by mycological culture alone, that (unculturable) fungal taxa are also present [95, 139, 142]. Overall, an increased abundance of *Aspergillus* species (particularly *A. fumigatus*), *C. albicans*, *C. parapsilosis* and *Malassezia* is identified [127]. In both asthma and CF, an allergic response may be induced when *Aspergillus* is encountered, with the key form being allergic bronchopulmonary aspergillosis (ABPA) [144]. The application of mycobiome sequencing approaches to the lung has started to gain significant traction and research interest with the transition from smaller pilot-scale optimisation studies to a more detailed and comprehensive assessment in well-phenotyped cohorts of patients with respiratory disease [117, 139, 145, 146].

The Gut Mycobiome

Efforts to examine gut mycobiomes have largely focused on gastrointestinal (GI)-associated disease, and key findings echo trends of mycobiome alteration in other non-GI diseased states. In inflammatory bowel disease (IBD), for instance, initial work using 18S rRNA and rDNA sequencing showed an increased fungal diversity in Crohn's disease (CD) but not ulcerative colitis (UC) [147, 148]. Further differences were also identified between inflamed and non-inflamed tissue [147]. More recently, however, application of ITS2 sequencing has found distinct fungal microbial dysbiosis in IBD with increased Basidiomycota/Ascomycota ratios accompanied by higher proportions of *C. albicans* but reduced levels of *S. cerevisiae* [149]. In CD, ITS2 pyrosequencing also confirms increases to overall fungal load during disease flares and a higher prevalence of the Cystofilobasidiaceae family and *C. glabrata* species. In correlation, the application of ITS1 sequencing in another study revealed an increased *Candida tropicalis* in CD as compared to non-CD relatives [150]. Interestingly, *S. cerevisiae* and *Filobasidium uniguttulatum* are associated with non-inflamed tissue in CD [151]. *Malassezia restricta* was found to increase CD severity in a subset of patients with *CARD9* polymorphisms, suggesting that the targeting of specific fungi

in certain patient phenotypes may have value [152]. It is further demonstrated that antifungal drugs lead to increases in colitis severity in animal models where ITS1 amplicon sequencing revealed gut fungal dysbiosis characterised by a reduction in *Candida* spp. and concurrent increase in *Aspergillus, Wallemia* and *Epicoccum* spp. [153]. Similarly, antibiotics influence gut inflammation through modification of the gut bacteriome with a consequential impact on fungal colonisation [154]. Here, alteration in *Enterobacteriaceae* modulates fungal colonisation and colitis severity [154]. Although the mouse gut mycobiome does not fully represent that in humans, these findings lend support to the implication of fungal dysbiosis in IBD. Paediatric IBD has also been assessed providing insight into potential age-associated effects, and 18S rDNA pyrosequencing illustrates Basidiomycota dominance [155]. In other paediatric IBD works, ITS1 pyrosequencing reveals high *Candida* burdens and a reduced fungal diversity [156]. While dedicated study of the healthy gut mycobiome is limited and much required, Nash et al. observed the healthy human gut mycobiome to be lacking in diversity and dominated by yeast genera such as *Saccharomyces*, *Malassezia* and *Candida*, albeit with significant inter- and intra-individual variability [63]. This suggests active and dynamic change to the gut mycobiome over time, further influenced by host immunity, even in the absence of disease. In Irritable Bowel Syndrome (IBS), a functional gastrointestinal disease, ITS1-based metabarcoding of faecal samples reveals a gut mycobiome dysbiosis with a significant loss of diversity. *Saccharomyces* and *Candida* were dominant in IBS and healthy controls, but higher proportions were observed in the former [157]. Visceral hypersensitivity is linked to IBS with fungi implicated in animal models, suggesting that targeted manipulation of the mycobiome may provide scope for therapeutic intervention [158, 159]. Mycobiome signatures clearly differ between hypersensitive and normally sensitive IBS [158]. While these findings are clearly of clinical interest, more compelling evidence and biological validation is required to further clarify mechanistic underpinnings of these early observations [157]. Interestingly, in the oncological setting, antifungal treatment is associated with decreased tumour progression in pancreatic ductal carcinoma, while post-therapeutic repopulation by *Malassezia* accelerates tumour growth [160]. In addition, an increased

abundance of *Malassezia* is described in colorectal cancer by faecal shotgun metagenomic sequencing, along-with *Moniliophthtora*, *Rhodotorula*, *Acremonium*, *Thielaviopsis* and *Pisolithus*, while a higher abundance of *Basidiomycota* is associated with more advance disease [161, 162]. The gut mycobiome may additionally influence other organ systems such as the lung and central nervous system through their respective axes, but the precise mechanisms are poorly understood. In particular, the lung-gut axis, which includes the mycobiome, is highlighted as a key and emerging player in determining susceptibility to chronic lung disease, and the gut microbiome composition in itself has also been independently associated with several chronic inflammatory lung diseases [118]. For instance, the presence of *C. albicans* in the gut influences Th17-related immune pathways, which in turn has implications for *Aspergillus*-associated pathology in chronic airways disease supporting a role for lung–gut cross-talk in the pathogenesis of respiratory disease [124]. Gut mycobiome dysbiosis is reported in multiple neurological disorders, where a higher abundance of *Candida* occurs in schizophrenia, autism and Rett Syndrome [163–166]. This has prompted the investigation of gut fungi in the microbiome–gut–brain axis, an emerging biological concept already firmly established with respect to bacteria [167]. While recent studies describe associations between fungal dysbiosis and IBS, the association with neurological symptoms such as anxiety and depression in IBS sufferers is intriguing, results that further support the role of gut fungi in the microbiome–gut–brain axis [168]. While these findings remain speculative at present requiring more study to explain clearer biological mechanism, they nevertheless do illustrate the importance and emergence of the fungal mycobiome in human disease.

The Skin Mycobiome

The skin remains a key organ and provides barrier protection against pathogens but is also home to resident fungi [169, 170]. Different skin components demonstrate differing fungal patterns, and fungal species also vary by age and gender. For instance, children have greater numbers and variety of fungi on their skin compared to adults, who are dominated by lipophilic *Malassezia* [115, 171]. This is likely explained by the changes in sebum composition and

sebaceous gland activation [171]. Most work reveals that healthy skin (across many body sites) is characterised by *Malassezia*, which produces an aryl hydrocarbon receptor (Ahr) ligand beneficial for epithelial cell health and UV protection [114, 115, 172]. Diverse domains of the skin mycobiome interact with one another, promoting connectivity and providing network stability. Comparable to lung mycobiomes, the communities in skin mycobiomes help maintain ecological network structure [120]. As with other organ systems and their microbiomes, the skin mycobiome associates with skin disease: *Malassezia*, when in higher abundance or where an inappropriate immune response is observed is seen in association with seborrhoeic or atopic dermatitis [115, 173]. Conversely in psoriasis, which involves the multiplication of skin cells at much higher rates, patients had a lower abundance of *Malassezia* when compared to healthy skin despite *Malassezia* remaining the dominant phylum in both groups [174]. Relationships between the skin mycobiome and systemic diseases demonstrating dermatological manifestations are established: for example, the role of fungi in systemic sclerosis (SSc), an autoimmune disease characterised by skin thickening where *Rhodotorula glutinis* is highly abundant. Average *R. glutinis* abundance in normal subjects compared to SSc patients was 0.02 and 5.04 per million reads, respectively, supporting this association [175]. Tinea versicolor (*Pityriasis versicolor*), a skin fungal infection, attributed to *Malassezia* and a consequence of *Malassezia* protein-induced melanocyte apoptosis and UV exposure has also been the subject of multiple investigations [115]. Affected lesions illustrate high burdens of *M. globosa, M. sympodialis* and *M. furfur*, and mycelial fungal forms predominate in contrast to yeast forms commonly seen in healthy skin [176]. A role for the mycobiome has been established in seborrhoeic dermatitis, a condition where scaly patches, red skin and persistent dandruff ensue. While Ascomycota and Basidiomycota are prevalent in both healthy and dandruff-affected human scalps, the latter illustrates higher densities of *Acremonium*, *Penicillium and Malassezia* [176, 177]. *Malassezia restricta* and *Malassezia globosa* are identified in seborrhoeic dermatitis, and the presence of new colonising strains when compared to the healthy scalp is indicative of disease onset [173]. *Malassezia* may also more directly cause dandruff as antifungal agents are

particularly effective as treatment [176]. Atopic dermatitis (AD) (eczema) is another important skin condition with established mycobiome associations: significant intra- and inter-individual variation is observed in AD where taxonomic diversity has been examined. However, consistently, as is the case in other skin conditions, *M. sympodialis*, *M. sloofiae* and *M. dermatis* all characterise disease and sometimes exacerbate it. AD patients demonstrate circulating IgE to *Malassezia* and on closer examination, secreted *Malassezia* vesicles from AD patients differ in their RNA content when compared to healthy individuals [115, 176, 178–180]. In addition, *Malassezia* may induce cutaneous inflammation via a Th-17 response, with increased CCR6 + Th17 memory T cells (*M.* specific) observed in AD [181]. While *Malassezia* is a key AD correlate, it is important to recognise that non-*Malassezia* fungi are also described [179]. Different methodologies have been employed to characterise and describe skin mycobiomes: ion-torrent sequencing has been used in AD, while other approaches include targeted amplicon sequencing of various fungal regions, such as the fungal 28S rRNA large subunit D1/D2 and the 18S rRNA, internal transcribed spacer (ITS) regions ITS1 and ITS2 [108, 114, 171, 173, 175, 176, 179, 182–184].

The Mycobiome and Neurological Disorders

Increasing evidence suggests the importance of the microbiome in neurological disease. Changes to gut bacteria are linked to multiple sclerosis (MS), a neuro-inflammatory disease characterised by demyelination and neurodegeneration, suggesting cross-talk via gut–brain microbiota in its pathogenesis [185–190]. Higher abundance of *Candida, Malassezia* and *Trichosporon* by targeted ITS1 sequencing is reported in MS compared to control brain tissue [187]. Similarly, metagenomic analyses of cerebrospinal fluid (CSF) from MS patients reveal that the presence of *Malassezia, Ascomycota, Funneliformis, Glomus, Cladosporium, Candida* and *Alternaria,* however as concluded by these works, likely reflects environmental contaminants [191]. In contrast, Jovel et al. using CSF from MS patients did not report any detection of fungal reads [192]. In Alzheimer's disease, fungal cells are detected in neurons through immunohistochemistry and confocal microscopy within brain tissue and these fungal species are further identified by

nested PCR, where *Candida, Cladosporium, Malassezia, Neosartorya, Phoma* and *Sacharomyces* are detected [193]. Using targeted amplicon sequencing, *Alternaria* and *Malassezia* are higher in Alzheimer's compared to normal brain tissue [194]. In other works, brain tissue from individuals with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease with progressive motor neuron dysfunction, contains an increased abundance of *Candida*, *Malassezia*, *Fusarium*, *Botrytis*, *Trichoderma* and *Cryptococcus* [185, 195]. The strong association between seborrhoeic dermatitis and Parkinson disease (PD) also raises the suspicion of specific *Malassezia* contribution to both disease states. Importantly, genetic polymorphisms associated with PD and levodopa use are known to promote *Malassezia* growth and invasiveness, and hence this fungal association is of clinical relevance [196]. Collectively, these studies implicate fungi in the pathogenesis of several neurological diseases underlining the importance of fungal biology and host–microbe interaction to overall human health and the inter-organ axis communication networks posited to promote disease onset.

The Environmental Mycobiome

Fungi are ubiquitous and present in indoor (built) and outdoor environments [197]. While indoor and outdoor fungal communities are similar, indoor fungal particles are passively distributed and dominated by spores, hyphal fragments and other dormant fungi [198]. Fungi from indoor air may originate from outdoor air with air exchanges when windows are opened [199]. Outdoor airborne fungal composition is affected by location, temperature and humidity, with large numbers of fungi identified by metagenomic sequencing, with consequent and significant impact on indoor fungal communities [200]. Other sources of indoor fungi include humans, pets, plants, plumbing systems, heating ventilation and air conditioning systems (HVAC), and also dust re-suspension [201, 202]. Exposure to indoor fungi and fungal particles is linked to health-related outcomes including allergies, fatigue and asthma [197, 203]. In addition, damp dwellings pose an additional health risk due to fungal exposure [204]. The built mycobiome is therefore of interest from microbiological, environmental and clinical perspectives. Traditionally, morphology-based studies of fungal cultures and

their spores, derived from indoor sampling, have recognised many common indoor fungal species; however, with the application of high-throughput sequencing in the built environment, the much larger scale and depth of the indoor fungal diversity have been demonstrated [203, 205]. Using pyrosequencing of ITS2 and D1/D2 of the large ribosomal subunit (LSU), Amend et al. recovered approximately 4400 fungal OTUs at the species level in a global study of indoor dust samples, while Nonnenmann et al. applied the combination of quantitative PCR with the fungal tag-encoded flexible amplicon pyrosequencing technique and describe 450 fungal species from fifty indoor dust samples in the Yakima valley, WA (USA) [206, 207]. In other works, 986 fungal OTUs were detected in a homogeneous set of houses in a Californian family housing complex [199]. Considering culture-dependent and culture-independent approaches (including next-generation sequencing; NGS), substantial fungal diversity is now associated with the built environment. These indoor fungi, whether persistent residents or transient visitors, likely hold clues to the increasing numbers of human allergies and other health problems observed globally in previously healthy individuals [208]. One such example is 'sick building syndrome': associated with poor indoor air quality and fungal exposure and experienced by building occupants, with symptoms including headache, dizziness, nausea, eye, nose or throat irritation [209–211]. As part of the effort in reducing harmful exposure of indoor fungi, source-tracking of airborne fungi is necessary to locate and eliminate contaminated building surfaces that may serve as potential reservoirs, a major challenge given the highly dynamic movement of aerosols. Fungal growth on building surfaces may also be invisible to the naked eye, and it is impractical to swab all surfaces for examination. Therefore, future studies must instead attempt source-tracking by swabbing potential source surfaces in homes, classrooms and offices to compare them against air samples using probabilistic models [201, 212]. Some early works have reported mould and yeasts from the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhodotorula* and *Wallemia*, where *Rhodotorula mucilaginosa* generally dominate moist spaces such as toilets, *A. fumigatus* in drier spaces such as the living rooms and *Cladosporium* in almost every living space [212, 213]. Besides source-tracking, engineered building materials and surface coatings that prevent or minimise fungal colonisation are another area of active research [214, 215]. These solutions, however, are expensive, and it is almost impossible to maintain sterility in a built environment unless medically warranted, for example, in the intensive care units of healthcare facilities, which in themselves poses a challenge [216]. Alternatively, buildings should be constructed using sustainable fungal-resistant materials complemented with architectural designs that consider operating factors such as heating, ventilation and air conditioning (HVAC) systems for controlling indoor temperature and relative humidity, as well as plumbing designs with accessible sites for addressing water leaks, all of which can contribute to a safer indoor environment, minimal fungal contamination and potentially improved human health outcomes.

Translation of Mycobiome Research to Clinical Practice

The number of reported microbiome-related publications has increased exponentially in recent years and now includes numerous studies of the mycobiome [94, 185]. In parallel, a better understanding of the role of microbes in health and disease has emerged with studies increasingly investigating the importance of the mycobiome and its contribution to disease [117, 124, 133, 217–219]. It is likely that these organisms co-exist in the same environment and that interaction between bacterial, fungal and viral microbiomes achieves homoeostasis among healthy individuals, while dysbiosis leads to disease. Although increasingly investigated in association with pathological states, it is still too early to make conclusions about the true clinical importance and relevance of the mycobiome. Several challenges do remain including the validation and standardisation of robust sampling and analytic methodologies, which must be examined in larger-scale studies. Currently, there is no standardised method in mycobiome sequencing with variation in DNA extraction methods, primer selection, sequencing, reference databases and analytical pipelines (Fig. 2), precluding robust meta-analysis of clinical studies performed to date, which continue to increase, but remain limited in number. Nevertheless, the field of mycobiome research is evolving and the rapid adoption of sequencing and computational technologies may overcome current limitations and

allow a better definition of the mycobiome from a clinical context [29]. With an improved understanding of resident mycobiomes and their interaction with host immunity, as correlated with disease at difference stages, this potentially opens avenues for early diagnosis and more targeted therapeutic approaches. Moreover, the use of NGS to characterise resistome pathways may have relevance for surveillance, antifungal stewardship and the development of new antifungal drug approaches. Newer treatment approaches may emerge from better patient stratification based on mycobiome profiles, while manipulating or restoring a 'healthy' mycobiome also represents a potentially viable approach for precision medicine. These technological advancements combined with sequencing and analysis may be simplified and evolve into point of care diagnostics for fungal disease in the future.

Future Directions

The number of microbiome studies focused on bacteria far exceeds that of the fungal mycobiome (and virome). This is largely attributed to pioneering efforts towards the standardisation of methodologies for bacterial 16 s rRNA gene sequencing and analysis, allowing scalability. A standardised and reliable method of mycobiome sequencing combined with more comprehensive fungal database coverage is crucial to achieve similar scalability for fungal microbiomes [29, 92]. Despite known interaction between various microbes, studies integrating bacterial microbiomes, fungal mycobiomes, viromes and parasites are limited. Interaction between members of microbial kingdoms likely results in an alteration of function and behaviour of individual microbes, which in turn play important roles for disease pathogenesis. Microbes housed at key body sites exhibit cross-talk and interaction with host immunity resulting in systemic manifestations of disease outside the initial site of dysbiosis. Dysregulation of intestinal microbiome has been implicated in allergic airways disease, with increased intestinal *Clostridium* spp. in asthmatics and an emerging role for the gut mycobiome in regulating fungal airway responses through the lung–gut axis [118, 124, 220–222]. Such insight provides explanations for clinical phenomena including how disruption of the healthy intestinal mycobiome

composition with oral antifungals can lead to exacerbations of allergy airway disease [173]. Changes in the composition of intestinal microbiota are also implicated in diseases of the central nervous system including autism spectrum disorder, Parkinson disease and schizophrenia, further substantiating the key role of microbiome cross-talk, which is likely to include major fungal in addition to established bacterial players [165, 223, 224]. To better understand the precise role of the mycobiome and its contribution to disease pathogenesis, future studies must assess interactions between the various microbial kingdoms in tandem with host immunity across multiple body sites in a holistic systems-based approach. Across such complex and interlinked microbial systems, the mycobiome is clearly a recognised and emerging factor that contributes to a range of diseases and their pathogenesis, which if harnessed appropriately can represent an opportunity, one that improves disease prognosis and future approaches to precision medicine using the microbiome.

Compliance with Ethical Standards

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