Gut mycobiota under scrutiny: fungal symbionts or environmental transients?

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The human gastrointestinal tract is home to a thriving community of microbes including the fungal 'mycobiota'. Although sequencing methodology has enumerated diverse fungal genera within this niche, discerning persistent symbiotic residents from contaminants and purely environmental transients remains a challenge. Recent advances in culturomics and sequencing employing metagenomics, metatranscriptomics and longitudinal studies have begun to reveal a human symbiont 'core mycobiome' that may contribute to human health and disease. Trans-kingdom interactions between the bacterial microbiota and evolution within the niche have defined *C. albicans* as a true symbiont, setting a bar for defining other fungi. Additionally, elegant investigations of mammalian antifungal immunity have examined mononuclear phagocytes, neutrophils, antigen-specific recognition by T cells and other mechanisms important for local and systemic effects on the host, providing further evidence supporting gut persistence. In this review we discuss current research aimed at investigating the symbiotic mycobiota and propose four criteria aiding in the differentiation of fungal symbionts from environmental transients.

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Introduction

The barrier surfaces of the human body serve as habitats to a variety of fungal organisms collectively known as the human 'mycobiota'. The intestinal mycobiota has been linked to maintenance of healthy homeostasis and prevention of systemic infection [1•,2,3•,4••]. Dysbiosis of the human mycobiota and expansion of *Candida* spp., fungal species highly represented in the healthy gastrointestinal tract, have been identified as signatures of inflammatory bowel disease (IBD) and several other inflammatory and non-inflammatory diseases [5,6•,7–9]. Patients with genetic polymorphisms in receptors involved in fungal recognition [9] display increased intestinal disease severity, while a deficiency in CX_3CR1, a fractalkine receptor flagging a population of mononuclear phagocytes, is associated with decreased IgG responses to fungi in patients with Crohn's Disease [10••]. Moreover, overgrowth of opportunistic pathogens, such as *C. albicans* or *C. parapsilosis*, has been linked to an increased risk of translocation and systemic infection in patients with compromised immune function [11]. The growing consensus on the importance of the intestinal mycobiota has prompted the investigation of which fungi are capable of surviving, residing and replicating in the gastrointestinal tract and thereby maximally able to influence the host over a prolonged period.

The mycobiota poses unique challenges in terms of identifying and validating constituents of this intestinal community. In contrast to bacteria, fungi are found in relatively low abundance in the gut, but are common in various food sources and the environment (cheese, beer, bread, wine, airborne spores, etc.). While the development of deep sequencing approaches have provided researchers with utmost opportunities to investigate the fungal variety making up the gastrointestinal (GI) 'mycobiome', the combination of low fungal DNA abundance in the gut and high dietary/environmental abundance provides data sets susceptible to serendipitous contamination from sources outside of the body and from other bodily surfaces (i.e. face, hands, sweat etc.). In addition to outnumbering fungal organisms, many members of the bacterial community are dependent on the anaerobic environment provided by the gut, greatly increasing their probability of persisting within the intestines. Fungi which are typically either facultative anaerobes or able to withstand hypoxic environments lack this certainty. These discrepancies between bacterial and fungal communities highlight the urgent need for more specific mycobiota-tailored criteria to differentiate resident fungi, referred here as fungal symbionts (Box 1) from transient passengers and contaminants.

**Box 1 Fungal symbionts of the gastrointestinal tract.**

Symbiont refers to any organism persistently living in contact with another. In the context of this review, this term specifies fungi living and replicating within the gastrointestinal tract. Symbionts include all manner of non-transient relationships including: mutualism, parasitism and commensalism, among others. In delineating symbionts from transients, or passing environmental/dietary organisms, specific relationships may act as qualifiers justifying potential symbiont classification. The precise type of symbiosis may be altered between fungi and the host depending on body site, extent of colonization and immune integrity, as is the case for pathobionts and opportunistic pathogens which transition from a neutral or beneficial relationship to one of a pathogenic nature.

We propose the use of four primary lenses for examining suspect fungal symbionts : 1) Detection by both sequencing and culturomics, 2) Activation of host immune responses, 3) Ability to populate and evolve in the gastrointestinal niche through fungal adaptation, and 4) Evidence of trans-kingdom interactions between bacteria and fungi. (Figure 1) Herein we review these strategies for studying distinct members of the mycobiota, focusing on the last two years of efforts to define the fungal symbiotic relationships in the mammalian gut.

Detection-sequencing and culturomics

Just as detectives wear gloves to protect the crime scene, mycobiome researchers also pay close attention to their choice of methods, aiming to accurately elucidate a microscopic reality in the fungal world hidden inside the mammalian GI tract. High throughput sequencing (HTS) for the gastrointestinal mycobiome has become a mainstay and the first step toward studying fungal symbiosis in the gut. The most common HTS method is amplicon sequencing of the Internal Transcribed Spacer region 1 or 2 (ITS1/2) (Table 1). The convenience of amplicon sequencing results from the amplification of fungal DNA, aiding in the identification of fungi in microbial communities residing the GI tract that are naturally dominated by bacteria. However, several caveats of this approach have also been demonstrated, such as primer bias, amplification preference and choice of optimally curated database for fungal identification [12–14]. Reverse transcriptomic sequencing of rRNA has been proposed to decrease the primer bias but has thus far been shown more suitable for bacterial identification [15]. In contrast, shotgun metagenomic sequencing omits targeted PCR amplification, thus improving result fidelity [16]. An intrinsic disadvantage to this approach is that fungal sequences can be buried by the overwhelming abundance of fecal bacterial DNA [17•]. The present hope is that this issue is partially resolved as the cost-to-sequencing depth ratio decreases over time, and databases record more complete fungal genomes. Alternatively, metatranscriptomic analysis has been performed on environmental mycobiomes and has the advantage of

Figure 1

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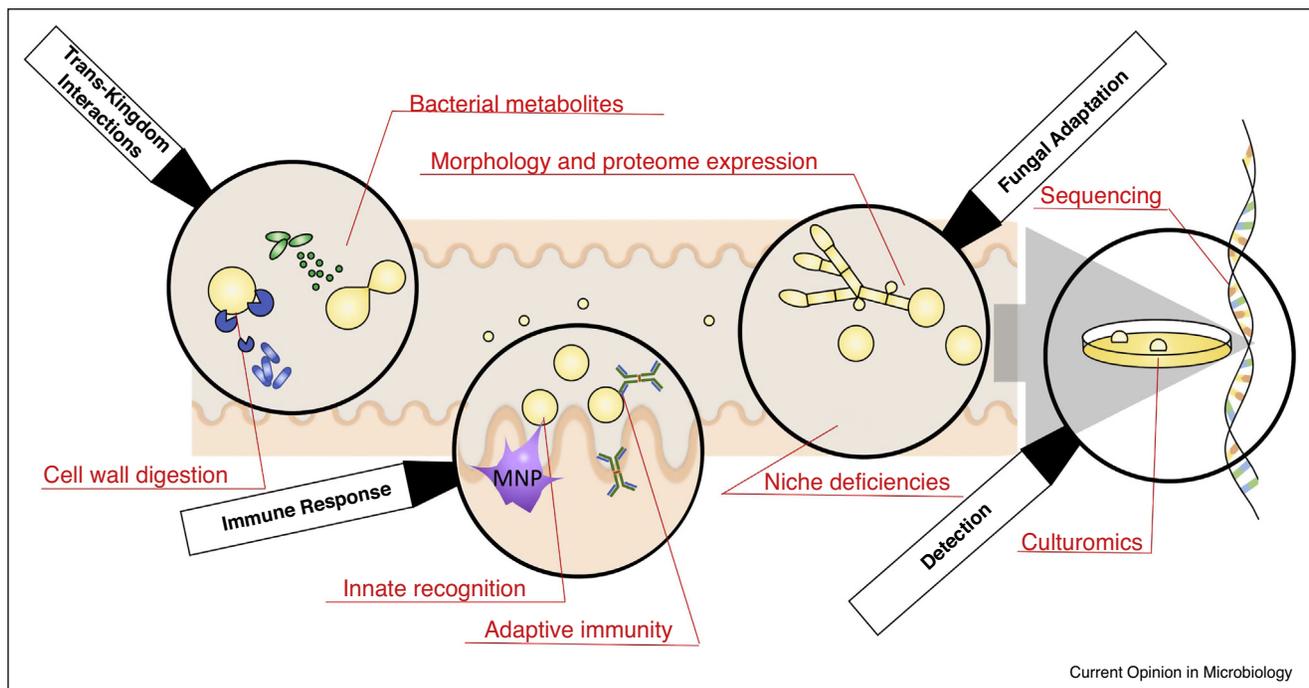
Maturing perspectives for the investigations of symbiotic members of the mycobiota. Both bacterial-fungal and host immunity interactions supplement the direct observations of species evolution, sequencing and culturomics observed in gut fungi. Together these tools have been used to validate *C. albicans* as a model intestinal symbiont.

Table 1

Comparisons between common mycobiome identification methods.

Method	Technology	Target	Advantages	Disadvantages
High throughput sequencing (HTS)	Amplicon sequencing	Internal Transcribed Spacer 1 and 2 (ITS1/2) 18S/26S rRNA genes Reverse transcribed rRNA Metagenome	Database availability; amplification of target sequences Decreased primer bias No amplification preference	Primary bias; amplification preference Amplification preference Low cost efficiency due to fungal DNA exiguity
	Shotgun sequencing	Metatranscriptome	Identification of live organisms	Low cost efficiency; classification mostly reliable at phylum level
Culturomics	Culturing in selective media	Live organisms	Identification of live organisms	Potential to miss anaerobic or fastidious fungi (e.g. anaerobic fungi from ruminant gut)
Mass spectrometry (MS)	Matrix Assisted Laser Desorption/Ionization Time-Of-Flight (MALDI-TOF)	Protein	Fast process; strain-level identification	Limited and often proprietary databases

identifying living, actively transcribing organisms. However, the current classification of fungal metatranscriptomic contigs is chiefly reliable only at the phylum level [18].

To eliminate the possibility of identifying fungal nucleic acids from dead or non-proliferating cells, culturomics is still used as the gold standard for identifying live fungi when they are culturable [19]. Fungal isolation is traditionally coupled with morphological analysis and whole genome sequencing but recently these methodologies have been supplemented with mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been applied for yeast identification in both academic settings as well as for clinical isolates [20]. Better curated databases in the future will improve the power and accuracy of this rapid identification method, allowing for greater expansion of fidelity to strain-level identification [21]. Further development of culturomic approaches that capture a vast majority of fungal species found in GI tract will further advance the field of gut mycology, mirroring success recently achieved for intestinal bacteria [22].

Recent efforts are aiming to broadly define the human intestinal fungal communities with established sequencing platforms. Nash and colleagues studied the fecal mycobiome of 317 healthy donors from the Human Microbiome Project and characterized a high prevalence of the genera *Saccharomyces*, *Malassezia* and *Candida*, with the highest abundance represented by the species *S. cerevisiae*, *M. restricta* and *C. albicans*, respectively [17•]. The authors applied amplicon sequencing of the ITS2 region and verified their results via sequencing 18S rRNA gene and shotgun metagenomics. Despite the generally large variability in mycobiomes, these taxa were commonly identified both across individuals and

longitudinally within individuals. Previously published work by Hoffman and colleagues sequenced ITS1 of 98 individuals. They identified many fungal taxa in common, with the top three being *Saccharomyces*, *Candida*, and *Cladosporium* [23]. In addition to these identified most prevalent taxa, both studies respectively reported another 177 and 63 fungal genera while only 29 genera among them were shared. These findings suggest a 'core mycobiome' across populations of healthy humans, with a highly diverse remainder of less represented fungi. The latter diversity arises either from environmental/dietary transient variability or from rare fungal symbionts (a less plausible possibility).

Candida species, including *C. albicans*, are generally accepted as true gut symbiotic fungi. *C. albicans* is frequently sequenced and isolated from the feces of healthy humans. However, the symbiotic status of *S. cerevisiae* and *M. restricta* are still debated, mainly due to their abundance in food sources and skin mycobiota, respectively [24,25]. Recent studies have gone as far as to challenge the origin of fecal *C. albicans*, suggesting that it arises from the oral mycobiota or food sources [26,27]. As these questions remain unanswered, another direction of evaluating symbiosis is to study the sources of fungi found within the GI tract. Boix-Amorós and colleagues have conducted two studies on breast milk mycobiomes [28,29]. By combining 28S rRNA sequencing with culturomics, they first identified *Candida*, *Saccharomyces* and *Malassezia* as the most prevalent genera. They further expanded the study to healthy volunteers spanning across four countries. Despite different environmental and dietary factors, *Malassezia* maintained an average of 40% abundance across four cohorts. While these studies do not answer the question whether *Malassezia* is a true gut symbiont, they do reveal a feasible route for transient early-life inoculation of the infant gut through mother breast skin-derived *Malassezia*

during breastfeeding [30]. Indeed, transient *Malassezia* presence in infant fecal samples have been detected by ITS sequencing until four months of age whereupon it is overtaken by mycobiota members belonging to the *Saccharomycetales* order with the development of stable intestinal microbiota later in life [30].

In summary, mycobiome studies shortlist core fungal taxa that are strongly suspected gut residents. However, mycobiome detection does not readily prove or disprove their symbiotic nature. Further studies on fungal interactions with the host and bacteria are needed to add confidence in making this distinction (Figure 1).

Immune response-antifungal immunity

For symbiotic fungi to persist and proliferate in the gut, they face the challenge of interacting with the host immune system. Hence, discovery of bidirectional interactions between fungi and the host immune system can serve as supportive evidence for symbiosis. On one hand, the host immune system monitors fungal expansion and penetration. On the other hand, symbiotic fungi may modulate the tone of immune responses locally or even distally [1•,31••]. In instances when direct exposure would endanger fungal persistence, fungal cell wall rearrangement strategies have been reported, although '*in situ*' evidence is still scarce [32••]. Most studies on intestinal immunity to mycobiota have been carried out on *C. albicans*, the most prevalent fungal pathobiont in the human gut. The specific immune system responses to *C. albicans* and some other fungi involve innate pattern recognition receptors (PRRs), CARD9/Syk signaling pathways and Th17 adaptive immunity, which have been previously reviewed [33,34]. In recent years, new discoveries on *C. albicans* and other potential fungal gut symbionts have added to our understanding of this dynamic, two-way relationship.

It was recently described that CX_3CR1^+ mononuclear phagocytes (MNPs) express several fungal-recognizing C-type lectin receptors, including dectin-1, dectin-2 and mincle. These phagocytes mediate fungal recognition and antigen-specific Th17 response to *C. albicans* in the mouse colon during the steady state and are crucial for control of *C. albicans* during intestinal disease [10••]. A further study showed the importance of CX_3CR1^+ MNPs in mediating the effect of gut fungal dysbiosis on house dust mite (HDM)-induced allergic airway disease (AAD), providing an explanation as to how intestinal fungal dysbiosis aggravates lung allergy [1•]. Intestinal fungal colonization of mycobiota-free altered Schaedler flora-colonized mice aggravated AAD without altering bacterial composition, suggesting the enhanced allergic responses are dependent on direct interaction with fungal constituents of the gut [1•].

Focusing on adaptive antifungal immunity, Bacher *et al.* [31••] found that among 30 mycobiota members, *C. albicans* was the strongest inducer of CD4$^+$ memory Th17

cells in humans, suggesting an 'immunological imprint' of this fungal symbiont. *C. albicans* induces Th17 responses in the mouse gut upon intestinal colonization independently of the preexisting gut mycobiome composition [10••,35,36]. Th17 cells induced by intestinal *C. albicans* colonization also exert a systemic effect by protecting mice against systemic *C. albicans* and *Staphylococcus aureus* infections [37•].

Researchers have expanded similar studies to minor components of gastrointestinal mycobiota identified by deep sequencing. Intestinal delivery of three fungal species, *A. amstelorami, E. nigrum* and *W. mellicola*, exacerbates HDM-induced allergic airway disease [38]. Oral gavage of *M. restricta* resulted in exacerbated DSS-induced colitis in mice in a CARD9-dependent manner [39•]. *M. restricta* also augmented Th1 and Th17 responses induced by acute DSS exposure in the mouse colon. However, whether these minor components of the mycobiota can naturally colonize the gut and whether they can prime intestinal immunity in the absence of inflammation remains to be determined.

The extensive new studies on mucosal immunity concerning the gut-residing *C. albicans*, together with other characteristics discussed later in this review, have begun to provide strong evidence supporting this organism's true symbiotic nature. Increasing findings pertaining to immune responses against *S. cerevisiae, M. restricta, W. mellicola, A. amstelodami, E. nigrum* and other *Candida* and non-*Candida* species do not by themselves assure their symbiotic status (Figure 1), but at least demonstrate scenarios where they promote persistent influences upon the host.

Fungal adaption-evolution towards persistence

The mammalian gastrointestinal tract presents a balance of challenges and benefits for potential fungal residents. This intestinal niche, from proximal duodenum to distal colon, represents an increasingly hypoxic, hypercarbic, anemic and hypoglycemic residence. Additional stressors include the innate and adaptive immune systems (vide supra) and a diverse bacterial clientele (vide infra). Occupants able to navigate these challenges are rewarded with a warm, moist and dark environment equipped with a natural defense from eukaryote-targeting pathogens (i.e. the host immune system). As such, potential long-term clientele of the gut would be anticipated to adapt to residence, providing another clue useful in identifying true symbiotic fungi.

Many fungi are opportunistic pathogens, intrinsically harboring the cellular machinery necessary for host invasion and infection which poses a liability to gut symbiosis. Elucidating the mechanisms by which these fungi achieve an evolutionary equilibrium conducive to

symbiosis is a recently burgeoning field of mycobiota research. In this regard, Bennett *et al.* [4**] have revealed a novel mechanism by which *C. albicans* evolves to compete in the GI tract through utilization of hyphal-regulating transcription factors EFG1 and WOR1 via genetic mutation and epigenetic regulation, respectively. Authors reported human isolates displayed a high percentage (11%) of disruptive *efg1* mutations resulting in *efg1*$^{+/-}$ and *efg1*$^{-/-}$ genotypes uniquely able to switch to a gray colony state *in vitro* and exhibiting a competitive advantage in the mouse GI tract. This work aligns well with several observations of loss of *efg1* functionality often linked with hypercompetitive phenotypes in systemic and gastrointestinal candidiasis [40–42], although conclusions as to regulation of WOR1 must be viewed in the context of gut-focused research showing that expression of this transcription factor can bypass the heterozygosity in the mating locus *in vivo* [42].

A more systematic study relying on a *C. albicans* gene disruption library published by the Noble lab [32**] revealed a series of transcription factors (EFG1, BRG1, TEC1) targeting morphogenesis and virulence whose gene disruption increased competitive fitness for gastrointestinal colonization *in vivo*. Intriguingly, these transcription factors all influence UME6, a master regulator of hyphal programing. Functional deletion of UME6 resulted in reduced filamentation *in vitro*, restoration of competitive fitness and, surprisingly, no change to *in vivo* morphology by FISH-assisted microscopy analysis of gastrointestinal contents. Further nanostring, RNAseq and mutational analyses identified two UME6-regulated proteins, SAP6 (secreted aspartyl protease 6) and, to a lesser extent, HYR1 (hyphally regulated gene 1) as culpable in reducing gastrointestinal competition of *C. albicans* strains independently of gross morphology. SAP6 was previously shown [43] to be adept at activating the inflammasome in dendritic cells and macrophages, possibly targeting the fungus for elimination by the immune system. Additionally, SAP6 was differentially expressed in an independent experimental approach [3•] which identified hyper-competition in FLO8 mutants in a murine model of gastrointestinal colonization. Combined these studies highlight the intriguing evolution of one fungi as it adapts to the challenges posed by the intestinal niche through diminishing its own proclivities towards a more destructive, pathogenic character. Finally, this emerging evolutionary adaption approach may prove invaluable when expanded to other fungal members of the mycobiota, providing a new lens by which to judge suspected symbionts (Figure 1).

Trans-kingdom interactions-bacterial modulation of fungi

The environmental and host immunity-mediated challenges present in the mammalian intestinal tract are compounded by the abundance of the facultative and obligate anaerobic bacteria that grossly outnumber other eukaryotic microorganisms. While transient microorganisms might transition through the gut without notice, true symbiotic fungi, persisting in the gut and competing for resources, would be expected to induce a retaliatory or mutalistic response from the bacterial microbiota (Figure 1). In light of this constant recognition, specific trans-kingdom interactions serve as an additional means of qualifying possible fungal symbionts in the gastrointestinal tract.

Bacteria depend on chemical interactions with other organisms both through a suite of small-molecule metabolites as well as protein gene products. Their diffusible metabolites arise from both primary (such as fatty acids or polysaccharides) and secondary (such as polyketides, non-ribosomal peptides, terpenes) metabolic processes. Bacterial pathobionts of the GI tract have recently been shown to produce both fungal modifying compounds as well as secreted enzymes. Coulthurst *et al.* [44•] have recently elucidated and interrogated two new type VI secretion system proteins, Tfe1 and Tfe2, with antifungal properties against *Candida albicans*, *C. glabrata* and *S. cerevisiae* from *Serratia marcescens*. Interestingly, this relationship appears to run contrary to recent sequencing data in Crohn's disease showing a strong positive correlation between expansion of *Serratia* and *Candida*, possibly implicating an underlying symbiotic relationship *in vivo*, an altered dynamic during disease-induced inflammation, or spatially distinct niches for these organisms within the gastrointestinal tract [45].

Obligate anaerobic bacteria predominate the mammalian gastrointestinal tract and offer a snapshot into the neighboring organisms fungi must tolerate to maintain their symbiotic status in the unique environment of the gut. From the phylum *Firmicutes*, the opportunistic pathogen *C. difficile* maintains its hold over the intestinal niche through its distinctive production of *p*-cresol, an antibacterial agent derived from tyrosine metabolism [46]. This phenol metabolite was shown [47] to inhibit the yeast-hyphae transition and biofilm formation in *C. albicans*. Moreover, in co-culture with *C. albicans*, *C. difficile* was able to tolerate aerobic growth conditions independently of adhesion or bi-species biofilm formation [47] as previously shown for the bacterium *C. perfringens* [48]. Interestingly, these studies also align with an examination of both fecal material transplants (FMT) in patients and mouse models of *C. difficile* infection correlating reduced mycobiota diversity and increased *C. albicans* abundance to poor FMT recipient outcomes [49•], further implicating the physiological relevancy of this cross-kingdom interaction. Sellam *et al.* [50] confirmed the relevance of the *Firmicutes-Candida* interaction as well as implicated a member of the phylum *Bacteroidetes* with their discovery of morphology-modifying and fungistatic properties from both *Roseburia* and *Bacteroides* spp., although the exact molecules at play remain unknown.

Bacteria of the gastrointestinal tract serve a vital role in processing dietary fiber to extract carbon and nitrogen from sources otherwise inaccessible to the host. Fungal cell walls contain a complex polysaccharide coating consisting of mannan, β-glucan and chitin, providing an alternative source of fiber in the gastrointestinal environment. Both β-glucan and chitin have been found to be modified by bacteria of the microbiota through small molecule signaling [51] and enzymatic degradation [52], respectively. Specifically, *Bacillus* spp. were found to harbor chitinases capable of altering *C. albicans* morphology, inhibiting biofilm formation and antagonizing growth [52]. Lactic acid, a metabolite produced by lactobacilli in the GI tract, was also shown to promote restructuring of *C. albicans* cell wall architecture, masking β-glucan exposure in the cell wall [51]. A recent surge of interest in polysaccharide utilizing loci (PUL) chiefly from *Bacteroidetes* spp. within the mammalian gut has revealed discrete PUL responsible for fungal β-1,6-glucan [53] and *S. cerevisiae* mannan [54] catabolism. Although all members of the fungal kingdom contain β-(1,6 or 1,3)glucan and chitin (polymeric 1,4-*N*-acetylglucosamine) within their cell wall, mannan serves as a means of concealing potential pathogen associated molecular pattern (PAMP) ligands from the host [55] and varies significantly in linkages and gross structure across species [56]. Thus, the further identification and evaluation of bacterial enzymes dedicated to the cleavage of genus/species-specific mannosyl linkages could serve to validate stable predator-prey interactions conserved in the native microbiota.

Conclusions

As mycobiome sequencing technology continues to mature, we are ultimately faced with the question: How do we best categorize the diverse members of gastrointestinal mycobiota in order to separate true residents from transient passersby? Sequencing and culturomics have profiled the possible suspect fungi, but there is a need for additional longitudinal work across a variety of geographic regions and dietary conditions to delineate environmental and nutritional constituents from less variant members of the mycobiota. Specific interactions with both the host (immunity) and bacteria (microbiota) may serve as traits to gauge the role of fungi in the gut, but the scope of study has thus far been limited to chiefly *C. albicans* and *S. cerevisiae*. Expanding these investigations as well as interrogating the propensity of each fungal species and even of specific strains to evolve within the gastrointestinal niche offer the most promising strategies forward in defining a symbiotic mycobiota. In this regard, *Candida* species and *C. albicans* specifically may serve vital roles as not only a prototypical symbiont, but also as a future road map for studying important fungal constituents and distinguishing true fungal symbionts of the human gut.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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