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PEARLS

The gut mycobiome: The overlooked constituent of clinical outcomes and treatment complications in patients with cancer and other immunosuppressive conditions

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Extensive efforts have focused on investigating the contributions of the intestinal microbiome to health and disease, including immunomodulation [1, 2]. While the term "microbiome" technically refers to microorganisms including bacteria, fungi, viruses, protozoa, and parasites, the majority of studies focus on the bacteriome [3]. Although the bacteriome constitutes >99% of the microbiome [4] (which is potentially the reason most studies focus on the bacteriome), it is irrefutable that the commensal fungi, or the "mycobiome," alongside the other microorganisms, coexist and interact ways that can be beneficial or detrimental to the host [5–7]. Emerging research has focused on how the bacteriome relates to gastrointestinal (GI) disorders, cancer therapy–related toxicities, and stem-cell transplantation outcomes; including correlations with infection, graft-versus-host disease (GvHD), tumorigenesis, cancer relapse, and mortality [8–11]. Thus far, there has been a lack of dedicated research focusing on the influence of mycobiome-associated immunomodulation in patients with cancer and other states of immunosuppression. Herein, by focusing on the gut ecosystem, we discuss the role of fungi in various patient populations, the importance of bacterial-fungal dysbiosis, and offer ideas for future investigations regarding the role of mycobiome.

Current implications of gut fungi in patients with cancer or critical illness

Fungal diversity and density are low in healthy subjects [7], although the factors for colonization resistance against fungi in the gut are inadequately understood. It has been long known that commensal bacteria limit fungal colonization via activation of mucosal innate immunity by bacterial derived metabolites [12], while antibacterial agents can predispose individuals to *Candida albicans* colonization and infections [13]. For example, antibiotic-induced dysbiosis of intestinal microbes, such as *Bacteriodes spp*., was linked to a reduction in the cathelicidin antimicrobial peptide (CRAMP), which resulted in the outgrowth of intestinal *Candida* spp. [12]. Recently, it was found that an antibiotic-induced reduction in the levels of bacterial derived short-chain fatty acids (SCFAs) in the cecum enhanced GI colonization of *C. albicans* [14].

Antibiotics, however, are not the only factor that can potentially result in increased fungal burden in the gut. In addition to the known effects of proton pump inhibitors (PPIs) as

promoters of *Candida* gut colonization [15], it has been shown that high-intensity chemotherapy results in reduced diversity of the GI microbiota, reduction of anaerobes [16], and a shift in the Firmicutes to Bacteriodetes ratio [17]. Given that decreases of anaerobic bacteria have been shown to promote *Candida* overgrowth [18], and Bacteroidetes have been shown to be negatively associated with fungi [19, 20], it stands to reason that the gut mycobiome is altered during cytotoxic chemotherapy.

In addition to the known fact that *Candida* colonization precedes *Candida* invasion in the bloodstream [21], enrichment of the fungal consortium in the gut could also affect cancer treatment-related complications and oncological outcomes. It was previously shown that prolonged administration of fluconazole for 75 days after hematopoietic stem cell transplantation (HSCT) was not only associated with protection against invasive candidiasis and *Candida*-related death, but also with decreased gut GvHD [22]. Indeed, *Candida*-colonized patients have significantly higher incidence of severe GvHD [23]. C-type lectin receptors (such as Dectin-1 and -2) of dendritic cells recognize fungal cell wall polysaccharides, which trigger protective antifungal T helper 17 cell (Th17) responses in the GI mucosa [24]. In fact, the induction of Th17/interleukin-23 (IL-23) responses via activation of pattern recognition receptors by *Candida* has been suggested as a potential mechanism of GvHD pathophysiology [25].

The alterations in gut bacterial metabolites in the setting of antibiotics, chemotherapy, and HSCT might have indirect effects on fungal fitness and morphogenesis. It has been shown that antibiotics with activity against anaerobic organisms can reduce gut SCFA levels [26] and that low fecal butyrate and propionate levels correlate with decreased microbial diversity and higher incidence of GvHD post HSCT [27]. Interestingly, SCFAs have been demonstrated to induce transcriptional changes in *C. albicans* [28] and butyric acid has been found to inhibit yeast–hyphal transition [29]. Furthermore, it is possible antibiotics may have an indirect role on adverse GvHD outcomes by affecting *Candida* physiology, such as promoting yeast-to-hyphae transition [30].

In terms of tumorogenesis, the mycobiome has been implicated in the pathogenesis of colon adenomas [31] and, most recently, pancreatic ductal adenocarcinoma [32]. It was shown that fungi migrate from the gut to the pancreas and that pancreatic tumors are infiltrated by *Malassezia* spp. Removal of the mycobiome was protective against tumor growth. Mechanistically, fungi promoted the progression of pancreatic cancer by inducing the complement cascade via activation by mannose binding lectin. Similarly, researchers have also recently shown increases in Malasseziomycetes and decreases in Saccharomycetes in patients with colorectal cancer, but no mechanism has been proposed [33].

The mycobiome and chronic inflammatory bowel disorders

In addition to the cancer and critically ill setting, the gut mycobiome has also been implicated in inflammatory GI disorders, to include Crohn disease and ulcerative colitis. It was first observed that patients with inflammatory bowel disorders (IBDs) had a higher GI colonization rate by *C. albicans* compared to healthy individuals [34]. Furthermore, Sokol and colleagues observed an imbalance in the Basidiomycota to Ascomycota ratio in IBD compared to healthy subjects [35]. Further mechanistic studies implicated the mycobiome as a key contributor to initiation of inflammation and pathogenesis of IBD, where dectin-1–deficient mice had more severe IBD symptoms and colonization by pathogenic fungi [36]. It was also shown that *C. tropicalis* could exacerbate colitis severity in dectin-1–deficient mice. However, the association of antifungal selection pressure to the constitution of the gut mycobiome and its direct or indirect consequences to the underlying GI pathology and microbiome are rather complex. For example, prolonged treatment with fluconazole led to decreased levels of *Candida* gut colonization

at the expense of increased levels of gut colonization by opportunistic molds, such as *Aspergillus amstelodami*, resulting in elevated colitis severity [37]. Interestingly, susceptibility to colitis occurring in the presence of commensal bacteria eradication was revered by colonization with either *C. albicans* or *Saccharomyces cerevisiae* [6]. This shows that in the appropriate context, fungi can also confer protection against mucosal injury by tuning immune response.

Fungal–bacterial interactions to consider in the patient with cancer or GI disorder, or critically ill patient

Many bacterial–fungal interactions that have been reported to influence the colonization and pathogenesis of both kingdoms (Table 1). However, most studies were derived from in vitro experiments or murine models that have a mono-microbial view of alterations in fungal biology as a result of interactions with bacteria. These interactions have been shown to provide synergy in commensalism, as in the case of *C. albicans* with enterococci [38], or are mutually antagonistic, such as the case between *C. albicans* and *Pseudomonas aeruginosa* [39] (Table 1). Interestingly, fecal microbiota transplantation (FMT) efficacy was reduced in patients with *Clostridioides difficile* colitis who had dominance of *Candida* in the gut [40]. One possible reason why patients with *C. difficile* and co-colonized with *Candida* species may not respond to FMT is that *C. albicans* has also been shown to affect gut bacterial reconstitution or recolonization after antibiotic administration [19]. Given that FMT has become an attractive treatment strategy, not only for *C. difficile* infection or GI disorders but also to mitigate other treatment-related toxicities such as GvHD, immune checkpoint inhibitor–associated colitis, and antibiotic resistant infection, one must consider the fungal contribution to the effectiveness of this strategy [41–44].

Fungal implications for immunomodulation

There are data analogous to the bacteriome which suggest the immunomodulatory role of fungi colonizing the GI tract in both innate and adaptive immunity [24]. It is well characterized that gut colonization by *Candida* or other fungi elicits Th17 and Th1 responses [45, 46]. In fact, among 30 taxa of the human mycobiome, *C. albicans* is the major inducer of systemic Th17 cells [47]. Additionally, *Candida*-specific Foxp3$^+$ regulatory T (Treg) cells, which are implicated in the maintenance of mucosal immune homeostasis, have been detected in the peripheral blood of healthy individuals [48]. Depending on the morphogenetic state of *S. cerevisiae*, both subsets from human CD4$^+$ T cells can be induced; thus, *S. cerevisiae* yeasts induce Th1 CD4 differentiation, while *S. cerevisiae* spores promote Th17 CD4 expansion. These differential effects of fungi on T-cell responses appear to be dependent on the influence of fungal mannans on dendritic cells [49]. Moreover, as discussed above, inoculation with *S. cerevisiae* and *C. albicans* were sufficient to alleviate the severe colitis as well as reduced levels of protective CD8$^+$ T cells in antibiotic-treated mice infected with influenza virus [6].

Emerging data suggest that gut microbes may impact antitumor immunity during immunotherapy by priming innate effectors and the adaptive immune responses, inducing cytokine production by antigen-presenting cells or lymphocytes [32]. In the aforementioned data, gut mycobiota (specifically *Malassezia* species) are implicated in the pathogenesis of pancreatic adenocarcinoma by promoting pancreatic inflammation through the complement cascade [32]. Interestingly, several in vitro studies using myeloid or keratinocyte cell lines have shown that stimulation with *Malassezia* leads to the induction of mainly proinflammatory cytokines and chemokines [50]. Given the evidence for the immunomodulatory role of the gut mycobiota, it is important to consider the effects perturbation of the gut fungi may cause on human

Table 1. Important fungal–bacterial interactions altering pathogenesis.

Fungi	Bacteria	Relationship	Model	Observation	Reference
Candida albicans	*Enterococcus* spp.	Synergy	Germ-free and antibiotic-perturbed mice	Enterococcal species are found to dominate the gastrointestinal microbiome following the introduction of *C. albicans*	[38]
		Antagonistic	*C. elegans* coinfection model	*E. faecalis* can inhibit *C. albicans* hyphal morphogenesis and virulence	[38]
C. albicans	*Pseudomonas aeruginosa*	Antagonistic	In vitro models	*P. aeruginosa* lipopolysaccharide inhibits *C. albicans* biofilm and hyphal development	[39]
			In vitro models	*P. aeruginosa* excretes quorum-sensing molecules and quinolone signals, which repress hyphal and biofilm formation	[39]
			In vitro models	*C. albicans* secretes farnesol, which down-regulates the expression of *P. aeruginosa* virulence factors through modulation of the *Pseudomonas* quinolone signal system	[39]
			In vitro models	*C. albicans* inhibits the production of cytotoxic exotoxin A and pyoverdine	[39]
			Neutropenic co-colonized mice	Mice colonized with both *P. aeruginosa* and *C. albicans* had significantly lower mortality compared to those colonized with *P. aeruginosa* alone	[39]
C. albicans	*Clostridium* spp.	Synergy	In vitro models	*C. albicans* coculture promotes *C. difficile* and *C. perfringens* growth in aerobic conditions	[63]
			C. difficile mouse model	Oral *Candida* administration worsens *C. difficile* severity	[64]
		Antagonistic	In vitro models	p-cresol, produced by *C. difficile*, inhibits hyphal formation and virulence of *C. albicans*	[63]
			C. difficile mouse model	*C. albicans* reduces *C. difficile* growth and *C. difficile*–related mortality, which appears dependent on the alterations that *Candida* induces on the gut bacteriome composition	[40]

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health and various disease states, including possibly sites distant from the gut, such as lungs [47] and central nervous system [51].

The immunomodulatory role of fungi may not only have implications for chemotherapeutic/immunotherapy response and leukemia persistence, but also for infectious complications. It has been shown that in scenarios where antibiotics promote *Candida* intestinal domination, genetic changes occur that lead to increased fitness of *C. albicans* in the gut [52]. Interestingly, this gut-adapted *C. albicans* confers increased protection against systemic fungal and bacterial pathogens [52], likely due to the induction of systemic adaptive Th17 responses [46]. *C. albicans* and *S. cerevisiae* were both shown to be capable of stimulating innate immunological memory in myeloid cells [53]. Interestingly, mice treated with β-1,3-glucan or chitin were protected from a *C. albicans* challenge, suggesting a mechanism by which fungi can train mucosal or circulating monocytes [54].

Technical considerations for mycobiome studies

The mycobiome field is in its infancy, and thus many technical challenges need to be considered when performing these studies. First, compared to bacteria and viruses, the mycobiome comprises a relatively minor component of the overall microbiome [7]. Many commonly used fecal genomic DNA extraction protocols are tailored for extracting bacterial genomic DNA and are often imperfect for extracting fungal genomic DNA in regard to bead size for mechanical lysis, enzymatic lysis buffers, and neutralizing or stabilizing agents [55]. Moreover, different extraction kits favor particular fungal species, are biased against others, and are prone to contamination [56]. Thus, one must carefully consider DNA extraction methods based on whether the study is mycobiome specific, or if one needs to combine bacterial and fungal microbiota analyses.

Most problems, however, lie in the lack of standardized methods for characterization of the mycobiota. When comparing amplicon sequencing, ITS1, ITS2, 18S, and 28S rRNA give slightly different results [56]. The 18S rRNA typically outperforms other markers in its ability to amplify and discriminate different species; however, because fungal rRNA copy numbers vary, there is a strong bias towards fungi with more copies. On the other hand, although the internal transcribed spacer (ITS) region represents the formal fungal barcode, it sometimes provides insufficient resolution to distinguish species. ITS primers show both amplification and sequencing biases related to the variable length of the product [56]. The length of the ITS1 and ITS2 markers vary from 50 bp to several kb. Incorrect mapping, and thus classification, leads to the inclusion of false positives or exclusion of valid operational taxonomic units (OTUs). Microbiome studies rely on well-curated reference databases in order to provide accurate taxonomic assignments of OTUs. Unfortunately, public repositories contain a high percentage of fungal sequences that are incomplete or even incorrectly annotated [57, 58]. Moreover, in regard to shotgun metagenomics, the number of annotated fungal genome sequences available in reference databases are sparse compared to the number of bacterial genome sequences available. Thus, for the mycobiome field to move forward, it would be critical to expand fungal sequencing efforts and improve fungal phylogenetics and taxonomic classification.

Another critical topic to consider in research moving forward is the potential for using mycobiome components as rapid diagnostic markers. Despite their promise, the diagnostic use of fungal biomarkers, such as galactomannan and beta glucan, are fraught with problems even in high-risk populations, such as acute myeloid leukemia (AML) and HSCT patients [59]. In contrast, mycobiome testing offers the promise of a holistic assessment of the fungal community in a particular site. As with all molecular-based clinical diagnostics in mycology, technical challenges include the sheer spectrum and number of fungi needed to be identified in immunocompromised patients, universal methods for preparing sample templates considering fungal morphology variability, lack of consistency in nomenclature, and the limitations of commercial platforms panels, reference libraries, and databases [60].

Conclusions

Although the impact of the microbiome in health and disease has been established, concurrent analysis of the bacterial/fungal consortium and its balance have been understudied [61]. This knowledge gap may be in part due to technical limitations within the metagenomic field; however, one can imagine that there is a vast number of cross-kingdom interactions that are important in the human host. To date, a small number of fungal-bacterial relationships have been studied in vitro or in model systems, but often this "one bacteria, one fungi" experimentation strips these insights of their complexity and the nuances of interactions in the setting of polymicrobial communities. This multifaceted *trans*-kingdom interplay is not only affected by the specific members that are present but also the complex immunological millieu of the human host. Fungal–bacterial combinations that may be neutral or advantageous in the human host may be detrimental in an immunocompromised patient. Thus, improved approaches in understanding the mycobiome are vital in order to provide a foundation for personalized medicine in the patient with cancer. A deeper comprehension of the fungal–bacterial–immunocompromised host triad may allow for identification of high-risk patients and improved treatment strategies [62]. We believe mycobiome is an underexploited field of investigation in the cancer field, and many fascinating research questions remain unanswered in both patients with hematologic cancer (Box 1) and other patients with chronic immunosuppressive conditions (e.g., recipients of solid transplant, chronic autoimmune disorders, and

Box 1. Mycobiome-related questions for future investigation in patients with malignancy.

1. What is the impact of mycobiome maintenance on a diverse, "healthy" microbiome following cancer therapy (or vice versa)?
2. What is the metabolic role of fungi within the gut microbiome? Are there any relationships between *Candida* dominance and microbial abundances of vancomycin-resistant enterococci (VRE), *Pseudomonas*, *Clostridia*, or streptococci in the patient with cancer? If so, can the mycobiome predict subsequent bacterial infections originating from domination in the gut?
3. Given that some chemotherapeutic agents have specific anti-*Candida* activities, are there any relationships between the mycobiome profiles and specific chemotherapies? Are mycobiome changes that occur as a result of chemotherapy GI specific, or do they occur elsewhere (e.g., nares, oral, lung)?
4. Are there any relationships between the baseline mycobiome with other clinical factors such as age, ethnicity, geographic, and leukemia cytogenetics? Are there any relationships of the mycobiome and key polymorphisms in pattern recognition receptors or other proteins involved in immune function (e.g., Toll-like receptors TLR2 and TLR4, dectin-1, NOD2 [nucleotide-binding oligomerization domain-containing protein 2], IL-17A, IL-22, or $Foxp3^{+}$)?
5. Are mycobiome changes related to probability and duration of remission?
6. Can microbiome manipulation with pro- or prebiotics result in a better mycobiome profile following cancer therapy–induced dysbiosis?

critically ill patients in the intensive care unit (ICU), where systematic mycobiome research is currently limited.

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