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Bacterial-Fungal Interactions and Their Impact on Microbial Pathogenesis

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Abstract

Microbial communities of the human microbiota exhibit diverse effects on human health and disease. Microbial homeostasis is important for normal physiological functions and changes to the microbiota are associated with many human diseases including diabetes, cancer, and colitis. In addition, there are also many microorganisms that are either commensal or acquired from environmental reservoirs that can cause diverse pathologies. Importantly, the balance between health and disease is intricately connected to how members of the microbiota interact and affect one another's growth and pathogenicity. However, the mechanisms that govern these interactions are only beginning to be understood. In this review, we outline bacterial-fungal interactions in the human body, including examining the mechanisms by which bacteria govern fungal growth and virulence, as well as how fungi regulate bacterial pathogenesis. We summarize advances in the understanding of chemical, physical, and protein-based interactions, and their role in exacerbating or impeding human disease. We focus on the three fungal species responsible for the majority of systemic fungal infections in humans: *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*. We conclude by summarizing recent work in mining microbes for novel antimicrobials and antivirulence factors, highlighting the potential of the human microbiota as a rich resource for small molecule discovery.

Introduction

Microbial communities are ubiquitous throughout nature, occupying diverse ecosystems and exhibiting a range of interactions within and between species, from symbiosis to competition and predation[1]. One ecological niche of particular interest is the human body, as diverse collections of bacteria, fungi, archaea, and viruses living on and within this mammalian host interact to govern diverse aspects of human health[2–5]. Specifically, these communities of microbes play pivotal roles in maintaining normal physiological functions, as dysbiosis of the microbiota is associated with many diseases, including inflammatory bowel disease[6],

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irritable bowel syndrome[7], diabetes[8], obesity[9], and cancer[10,11], as well as allergies[12,13]. Such commensal organisms can be acquired through birth, food consumption, and exposure to diverse environments, and thus can have a profound impact on human development and health throughout an individual's lifetime[14,15]. Importantly, members of the microbiota interact with each other as well as with the host to influence diverse phenotypic traits. Yet, despite the importance of these communications, our understanding of how these players interact remains in its infancy.

In addition to those members of the microbiota that play important roles in maintaining human health, there are also many microorganisms capable of causing diverse pathologies. While traditionally human microbiome studies have focused on bacterial species, advancements in sequencing technology have facilitated the identification and characterization of other members of these microbial communities in the human gut[3,16,17], and other anatomical sites[18–20]. Among these commensal organisms are opportunistic fungal pathogens. These eukaryotic pathogens are reported to infect over 1 billion people annually, leading to approximately 1.5 million deaths worldwide[21,22]. Most fungal pathogens of humans take advantage of local or systemic suppression of the immune system to cause diverse maladies, including but not limited to: superficial skin, hair, and nail infections; chronic fungal lung infections; and systemic infections with mortality rates as high as 90%[23]. The three main opportunistic invaders capable of causing systemic disease in humans include species of *Candida, Cryptococcus,* and *Aspergillus.* Humans are exposed to some of these organisms through an environmental reservoir, including *Aspergillus fumigatus* and *Cryptococcus neoformans*[24]. Other fungal infections are caused by constituents of the human microbiota, including *Candida albicans*, which colonizes the gastrointestinal tract, urogenital tract, and skin[24]. Not surprisingly, bacterial pathogens capable of residing within a human host are also responsible for significant morbidity and mortality in humans, including but not limited to *Pseudomonas aeruginosa* in the lungs[25], *Staphylococcus aureus* in the nasopharyngeal cavity[26], and *Clostridioides difficile* in the gut[27]. While recent work has begun to elucidate the role of bacterial-fungal interactions in opportunistic infections[25,28,29], the mechanisms by which these interactions contribute to pathogenesis remain largely unknown. Several recent publications have also reviewed bacterial-fungal interactions in the context of the human host[3,30–38].

The scope of this review will focus on bacterial-fungal interactions and their role in microbial pathogenesis in the context of human health and disease (Figure 1). In recent years, bacterial-fungal interactions have been reviewed. We provide an overview of current work evaluating bacterial-fungal interactions in the human body, including examining the mechanisms by which bacteria govern fungal growth and virulence, as well as how fungi regulate bacterial pathogenesis. We summarize advances in understanding chemical, physical, and protein-based interactions, and their role in exacerbating or impeding human disease. We conclude by exploring the potential of bacterial-fungal interactions as a rich source for antimicrobial discovery and the identification of anti-virulence strategies to thwart infectious disease.

Modulation of fungal proliferation and virulence by bacteria

Fungal pathogens of humans employ a range of adaptive mechanisms and virulence factors to facilitate growth and survival during infection of the host (Figure 2A). These include secretion of proteases and toxins, as well as switching between diverse morphological states that assist with adhesion, penetration of host tissue, and evasion of the host immune system[39–42]. By affecting the virulence of fungal pathogens, commensal and pathogenic bacteria can modulate the ability of fungi to thrive and cause disease in the host, as well as influence the host-response to the fungus.

Candida albicans

C. albicans is a natural member of the human mucosal microbiota and is a commensal in approximately 50% of healthy adults[43]. As an opportunistic pathogen, *C. albicans* is responsible for mucosal infections such as oral and vaginal candidiasis, as well as life-threatening systemic disease in immunocompromised individuals[23,44,45]. In its host niche, *C. albicans* interacts with bacterial commensals, which influence diverse fungal phenotypic traits[25]. Many bacterial species inhibit *C. albicans* growth, while others secrete factors that inhibit fungal virulence traits, including filamentation and biofilm formation (Figure 2A)[34,46]. In contrast, some bacteria promote fungal growth or enhance *C. albicans* virulence attributes. Naturally, a thorough understanding of the factors and conditions that govern *C. albicans* commensalism and pathogenesis is critical to understand how this organism can cause disease.

Proliferation—The ability of *C. albicans* to proliferate in the host relies on the fungus' ability to adapt to various environmental perturbations including exposure to elevated temperatures, alternative carbon sources, reactive oxygen species, cell wall stressors, and diverse pH ranges[47]. These responses can all be influenced through interactions with commensal and pathogenic bacteria. In the oral and vaginal microenvironments, *C. albicans* interacts with *Lactobacillus* spp., which secrete lactic acid and other weak organic acids that inhibit *C. albicans* proliferation[48,49]. Additionally, *Lactobacillus* spp. secrete cyclic dipeptides that inhibit the growth of the fungus[50]. This particular inter-kingdom interaction appears to be important in maintaining a healthy physiological state as individuals with reduced colonization by key *Lactobacillus* spp. are at increased risk for vulvovaginal candidiasis[51,52]. Beyond *Lactobacillus* spp., other commensal bacteria found in the oral mucosa inhibit *C. albicans* growth in co-culture assays, including *Actinomyces israelii* and *P. aeruginosa,* as well as high concentrations of *Prevotella nigrescens* and *Porphyromonas gingivalis*[53]. In the gut, *C. albicans* interacts with bacteria and other members of the microbiota that also influence its ability to proliferate. Recent work explored interactions between *C. albicans* and the gut commensal *Escherichia coli* (strain MG1655) *in vitro*. *E. coli* was found to secrete a soluble factor that directly kills the fungus in a magnesium-dependent manner[54], as depletion of magnesium in a *C. albicans-E. coli* co-culture rescued growth of the fungus[54]. Notably, the authors indicate that the magnesium levels present in serum of healthy humans are well above the depleted levels used in the study. Therefore, for the inter-kingdom interaction to be physiologically relevant *in vivo* it would need to occur in microenvironments with depleted magnesium, which may occur at the sites of infection.

Morphogenesis—*C. albicans* undergoes a transition from yeast to filamentous morphologies in response to a variety of host-relevant cues including exposure to serum, nutrient limitation, neutral pH, and elevated temperature[55]. This transition is important for virulence as *C. albicans* mutants locked in either morphological state are largely avirulent in mouse models of infection[56,57]. The current paradigm is that the filamentous, hyphal morphology is important for tissue invasion and adherence, whereas the yeast morphotype is imperative for dissemination. The filamentous form is also associated with additional virulence factors, including secretion of the cytolytic peptide candidalysin, as well as proteases[41,58]. Importantly, previous work demonstrated an inverse relationship between filamentous growth and commensalism, as mutations in key regulators of the yeast-to-filament transition lead to enhanced fitness in the mouse gut[58]. Finally, in addition to filamentous states and the standard 'white' round-to-oval yeast morphology, *C. albicans* can also transition into several elongated yeast-like cell types (opaque, grey, and GUT) that exhibit distinct *in vitro* properties and interactions with the host[56]. Thus, modulating the diverse morphological states adopted by *C. albicans* can have a profound consequence on its ability to cause disease.

Commensal and pathogenic bacteria play key roles in regulating the *C. albicans* yeast-to-hyphal transition, with both inhibitors and enhancers of filamentation identified. One key bacterial-fungal interaction occurs between *P. aeruginosa* and *C. albicans* in the human lung (Figure 3A). Previous work found that *P. aeruginosa* can physically adhere to *C. albicans* filaments in response to the quorum sensing molecule N-(3-Oxododecanoyl)-L-homoserine lactone, leading to fungal cell death[59,60]. Notably, production of this quorum sensing molecule increases resistance of *C. albicans* to the most widely deployed antifungal, fluconazole, by upregulating efflux pump expression and activating stress response pathways[61]. The secretion of additional compounds with antifungal activity by *P. aeruginosa* is also well characterized, including the production of phenazines, which inhibit *C. albicans* growth at high concentrations[62], and inhibit hyphal morphogenesis at sub-inhibitory concentrations[63]. Phenazine production is at the core of the chemical interaction between *C. albicans* and *P. aeruginosa* and elicits a myriad of effects on the host. During growth, *C. albicans* secretes ethanol as a by-product of fermentation, which enhances the production of phenazine compounds, such as pyocyanin, by *P. aeruginosa*[63]. Increased phenazine production further increases fungal ethanol secretion in a positive feedback loop by compromising mitochondrial function[64]. To add further complexity to this interaction, the production of phenazines and ethanol affect the host response to both pathogens, as ethanol reduces the ability of macrophages to clear *P. aeruginosa*[65], while phenazines cause direct damage to epithelial tissues[66]. Finally, the host detects phenazines through the aryl hydrocarbon receptor (AhR), upregulating antimicrobial defences and proinflammatory cytokines, which lead to the degradation of virulence factors and eventual clearing of the microorganisms[67]. Interactions between *P. aeruginosa* and *C. albicans* are also important upon phagocytosis by host immune cells. Co-infection of macrophages with *P. aeruginosa* and *C. albicans* decreases fungal survival, reduces fungal escape from macrophages, and reduces *C. albicans* filamentation in response to host cells relative to macrophage infection with *C. albicans* alone[68]. This is dependent on the presence of

phenazines, as *P. aeruginosa* strains defective in compound production are unable to enhance fungal killing by macrophages.

Over time, *C. albicans* and *P. aeruginosa* have evolved to adapt to the negative pressures each pathogen exerts on one another. For example, clinical isolates of *C. albicans* from the lungs of cystic fibrosis patients have been identified that are resistant to the filament-repressive effects of *P. aeruginosa*[69]. Genome sequencing of the *C. albicans* clinical isolates revealed that most of the constitutively filamentous strains harbored mutations in the transcriptional repressor gene *NRG1*; such mutations were necessary and sufficient for the filamentous phenotype. Six independent *nrg1* mutations arose in *Candida* isolates from different cystic fibrosis patients[69], providing a poignant example of parallel evolution in the context of the human host.

In the oral cavity, *C. albicans* interacts with the oral commensal and opportunistic pathogen, *Streptococcus mutans*. This microbe along with other *Streptococcus* spp. secrete *trans*-2-decenoic acid, a small molecule that inhibits *C. albicans* hyphal morphogenesis without affecting fungal growth[70]. *S. mutans* also secretes mutanobactin A, another small molecule that blocks *C. albicans* hyphal morphogenesis[71].

Another mucosal site frequently colonized by *C. albicans* is the human vagina[18,72]. Within this environment, *C. albicans* is the leading cause of vaginal candidiasis[73] and interacts with commensal bacteria on the vaginal mucosa, including *Lactobacillus* spp. (Figure 3B).[74,75]. The ability of *Lactobacillus* spp. to affect *C. albicans* morphogenesis is dependent on white-opaque cell type switching. The white cell morphology has been best studied for its interaction with *Lactobacillus* spp. Cellular proteins secreted by this genus include major secreted protein 1 (Msp1), which is readily produced by *Lactobacillus rhamnosus* GG and acts as a chitinase to break down the fungal cell wall, blocking filamentous growth in white cells[76]. In addition to chitinase, other factors resistant to protease and heat treatment within *Lactobacillus*-conditioned medium inhibit *C. albicans* hyphal morphogenesis in white cells[77–79], although the molecular entity responsible for this effect remained elusive for some time. Fortunately, recent work identified a component of the *Lactobacillus* secretome, 1-acetyl-beta-carboline, that inhibits *C. albicans* hyphal morphogenesis and biofilm formation via inhibition of the DYRK1-family kinase, Yak1[80]. However, the same conditions that block filamentation in white cells do not block morphogenesis in opaque cells, highlighting the broad phenotypic plasticity *C. albicans* displays in response to both its environment and inter-kingdom interactions[81].

Streptococcus agalactiae is another bacterium found in the vaginal microenvironment that is commonly isolated from individuals with recurrent vulvovaginal candidiasis[82]. Similar to *Lactobacillus, S. agalactiae*-conditioned medium inhibits *C. albicans* hyphal morphogenesis. However, co-inoculation of mice with *C. albicans* and *S. agalactiae* increased fungal burden in a mouse model of recurrent vulvovaginal candidiasis due to a decreased Th17 immune response [82], highlighting the complex connections between the fungus, bacterium, and host in governing pathogenesis.

C. albicans also resides in the human gut[83] where it interacts with commensal and pathogenic bacteria, including *E. coli, Salmonella enterica,* and *Enterococcus faecalis.* Previous work found that secretory products found during culture of *E. coli* biofilms were able to inhibit *C. albicans* hyphal morphogenesis and the expression of key hyphal-associated genes[84]. Additionally, *S. enterica* serovar Typhimurium was able to inhibit *C. albicans* filamentation in a *Caenorhabditis elegans* model of infection and directly kill *C. albicans* hyphae through a mechanism that relied on the inositol phosphatase, *sopB*[85]. Interestingly, *C. albicans*-conditioned medium increased the expression of *sopB* and other genes important for *S. enterica*-mediated hyphal-killing[85], highlighting a compensatory mechanism adopted by the bacterium to regulate fungal virulence. Finally, the interaction between *E. faecalis* and *C. albicans* has been well studied, where early work found that co-infection with both organisms resulted in attenuated virulence in a *C. elegans* model[86]. *E. faecalis* secretes EntV, a bacteriocin with antifungal and anti-filamentation activity, which protects *C. elegans* from infection with *C. albicans*[87]. Recent work found that post-translational modifications are important for the regulation of EntV, including identifying the requirement for gelatinase activity to cleave EntV into its active form[88]. Many other bacteria have also been reported to produce soluble factors that govern *C. albicans* morphogenesis, including the opportunistic pathogens *C. difficile* and *Burkholderia cenocepacia,* which secrete para-Cresol[89], and *cis*-2-dodecnoic acid (BDSF)[90], respectively.

More broadly in the gut, short-chain fatty acids (SCFAs) are metabolites produced in the colon by bacterial fermentation of dietary fibers, and these lipids play a key role in maintaining a healthy microbiota. SCFAs inhibit growth, filamentation, and biofilm formation of *C. albicans in vitro*[91]. Consequently, antibacterial-treated mice susceptible to *C. albicans* gut infection exhibit significantly reduced levels of SCFAs in the cecum and higher fungal loads in the feces[91], providing *in vivo* evidence that SCFAs are important in controlling *C. albicans* overgrowth. Additionally, recent work assessed the role of *C. albicans* in governing pathogenicity in a chemical-induced colitis mouse model, and observed that vaccination with the *Candida* NDV-3A vaccine protected mice from fungal-induced damage during colitis[92]. In the future, it will be interesting to see if these protective effects are also observed in a bacterial-induced colitis model.

In contrast to the examples highlighted above, commensal bacteria are also implicated in enabling *C. albicans* morphogenesis in the mammalian gut[93]. Serial passage of *C. albicans* through the gastrointestinal tracts of antibiotic-treated mice led to the rapid generation of low-virulence strains unable to form hyphae due to mutations in a gene encoding a transcription factor that positively regulates filamentation, *FLO8*[93]. These evolved lineages stimulated proinflammatory cytokines and conferred transient cross-protection against several other gut inhabitants. However, if an intact microbiota was present, only the virulent hyphal form persisted, suggesting that bacterial commensals play a critical role in promoting the filamentous or virulent form. The reason for this contradiction remains unclear but may be due to the commensal status of organisms as opposed to inter-kingdom interactions in disease contexts.

Biofilm Formation—Another important *C. albicans* virulence trait is its ability to form intrinsically drug-resistant biofilms, or surface-associated communities, which colonize

medical devices such as catheters in healthcare settings[94]. While the yeast-to-filament transition is intricately linked to the ability of *C. albicans* to form these structures, there are numerous other facets that contribute to their formation. *C. albicans* is frequently isolated in polymicrobial biofilms that are also comprised of *Streptococcus* spp., *P. aeruginosa*, and *S. aureus*[95–97]. These primarily occur on healthcare devices such as catheters and pacemakers, as well as in the oral cavity, where co-colonization with bacteria is associated with dental cavities, periodontitis, and denture stomatitis[98]. In the mouth, *C. albicans* adheres to oral *Streptococcus* spp., providing additional surface for fungal colonization[99]. *Streptococcus gordonii*, a common commensal of the oral mucosa, exerts physical force and produces chemical signals that lead to enhanced fungal morphogenesis and biofilm formation in *C. albicans*[100]. Additional work found that the competence regulation system ComDE in *S. gordonii* is important in the early stages of dual-species biofilms, but inhibits *C. albicans* biofilm formation in later stages[101]. *Streptococcus oralis* colonization of the oral mucosa also leads to increased biofilm formation, enhanced dissemination of *C. albicans* in mice, and increased expression of proinflammatory cytokines that result in enhanced tissue inflammation and immunopathogenesis[102]. Other bacterial members of the oral microbiota also enhance *C. albicans* virulence in early stages of biofilm formation, including *Streptococcus sanguinis, Actinomyces odontolyticus,* and *Actinomyces viscosus*[103].

In contrast, the opportunistic pathogen and oral commensal *Aggregatibacter actinomycetemcomitans* secretes the quorum sensing molecule autoinducer-2 that inhibits *C. albicans* biofilm formation by blocking filamentation[104]. As well, *P. aeruginosa* secretes factors that inhibit *C. albicans* biofilm formation, although through a mechanism that is independent of morphogenesis[105]. Using a strain of *P. aeruginosa* that does not secrete homoserine lactone, it was discovered that the bacterial supernatant could inhibit biofilm formation in the constitutively filamentous *tup1* deletion strain of *C. albicans*, implying an effect beyond inhibition of hyphal morphogenesis[105].

Cryptococcus neoformans

While studies examining fungal–bacterial interaction have mainly been performed with *C. albicans*, the impact of bacteria on *C. neoformans* growth and virulence has also been investigated. *C. neoformans* is an opportunistic human fungal pathogen and causative agent of cryptococcosis[106]. While immunocompromised individuals are most vulnerable to cryptococcal infections, there are also reports of *C. neoformans* causing systemic infections in immunocompetent hosts[107,108]. An estimated 223,100 cases of cryptococcal meningitis occur globally each year, leading to 181,100 deaths[109]. These staggeringly high mortality rates are due to numerous factors including a limited antifungal arsenal, the frequent development of antifungal resistance, and the fact that these infections predominantly occur in resource-poor settings where proper medical care is inadequate. As a human fungal pathogen, *C. neoformans* relies on several unique virulence traits to survive in a human host including the ability to proliferate at mammalian body temperature, as well as the capacity to form a polysaccharide capsule, produce melanin, and form the atypical titan cell morphology (Figure 2)[110]. Titan cells are cryptococcal cells with enormous dimensions and clinical relevance due in part to being refractory to phagocytosis by human immune cells[111].

Proliferation—Given the diverse environmental niches that *C. neoformans* is capable of inhabiting, there are many reports of diverse bacterial species exerting anti-cryptococcal activity[31]. *C. neoformans* is commonly isolated from pigeon guano suggesting that its gastrointestinal tract is at least temporarily colonized with this fungus, despite this species being recalcitrant to cryptococcal infection[112]. Early work that co-incubated *C. neoformans* with seven species of bacteria found within the pigeon microbiota observed a complete inhibition of *C. neoformans* growth, speculating that a specialized avian microbiota may at least partially protect birds from infections by *C. neoformans*[113]. Follow-up investigations found that the growth-inhibitory activity was mainly exerted by only two bacterial species, *P. aeruginosa* and *Bacillus subtilis*[114]. *P. aeruginosa* can inhibit *C. neoformans* growth through both a contact-dependent mechanism as well as contact-independent mechanisms that include the secretion of pyocyanin and other phenazine derivatives[115]. *S. aureus* is also reported to kill *C. neoformans* through a mechanism that involves attachment to the capsule[116] as the anti-proliferative effects are specific to *C. neoformans* and not to other fungal pathogens that do not produce the polysaccharide layer. While these examples all highlight inter-kingdom interactions that impair *C. neoformans* growth, the soil bacterium *Acinetobacter baumanii* was observed to increase *C. neoformans* survival in biofilms and stimulate the formation of capsule[117]. The exact molecular mechanism of this interaction remains to be determined; however, physical contact was not required, at least for the biofilm-inducing activity, suggesting that *A. baumanii* likely secretes specific factors that affect the fungus either at the cell surface or inside the cell. Overall, the impact of these inter-kingdom interactions on *C. neoformans* proliferation is highly complex and dependent on the organism involved.

Virulence—*C. neoformans* melanization is an important virulence trait to help protect the fungus from oxidative damage, antifungal assault, and high temperature, while also functioning to modulate host immune responses[118]. To better understand how bacteria regulate *C. neoformans* melanin production, a screen was performed using 40 microorganisms found in environmental niches occupied by the fungus[119]. This work identified several species of the *Bacillus* genus that were able to inhibit melanization without affecting growth. *Bacillus safensis* was further investigated and found to inhibit other virulence traits including capsule production and biofilm formation, in part via the action of chitinase activity[119]. There are also examples of bacteria enhancing *C. neoformans* virulence. The opportunistic bacterial pathogen *Klebsiella aerogenes* promotes melanization of *C. neoformans* cells during co-cultivation through the bacterial production of dopamine, a precursor for cryptococcal melanin biosynthesis[120]. Finally, it was recently shown that the mouse microbiota has the capacity to induce titan cell formation by *C. neoformans*[121]. The *in vivo* significance of the microbiota in promoting titan cell formation was established by observing that mice pre-treated with antibiotics prior to infection with *C. neoformans* had significantly less fungal cells with the titan morphology compared to antibiotic-free mice[121]. Further analysis of the titan cell-inducing mechanisms revealed that bacteria such as *E. coli,* and *Streptococcus pneumoniae* trigger cryptococcal titanization via shedding of peptidoglycan, a component of the bacterial cell wall[121].

Aspergillus fumigatus

Aspergillus fumigatus is a saprotrophic fungus ubiquitous in the environment and a leading cause of invasive aspergillosis[122]. *A. fumigatus* is a significant cause of invasive infections in individuals with impaired immune function, including those with neutropenia, solid organ transplant recipients, and patients on immunosuppressive therapies, such as high-dose corticosteroids. It is estimated that more than 200,000 cases of invasive aspergillosis occur each year, with staggering mortality rates of up to 50% with treatment and 100% if left undiagnosed[24]. *A. fumigatus* relies on several virulence traits during infection of a human host, including the production of gliotoxin and germination of conidia into hyphae[123].

One of the most consequential bacterial-fungal interactions occurs between *A. fumigatus* and *P. aeruginosa*, which can be deadly in individuals with chronic lung conditions, such as cystic fibrosis[97,124]. It is well established that *P. aeruginosa* secretes antifungal compounds with activity against *A. fumigatus*, including pyocyanin and other phenazines[125,126]. Expanding beyond these well-characterized secreted compounds, Sass *et al.* evaluated 24 *P. aeruginosa* mutants with deletions in genes important for virulence[127]. The authors found that the *P. aeruginosa* siderophore pyoverdine was effective at inhibiting *A. fumigatus* biofilm formation. By capturing extracellular iron, the authors predicted that pyoverdine limits *A. fumigatus* growth and biofilm formation by creating a nutrient-limited environment[127]. Pyoverdine was later found to work synergistically with the *Pseudomonas* quinolone signal (PQS) quorum sensing molecule that is also responsible for iron chelation and inhibition of biofilm formation under low iron conditions[128]. Paradoxically, under high iron conditions, PQS enhances *A. fumigatus* biofilm formation and this process is dependent on the *A. fumigatus* iron siderophore, ferricrocin[128]. Finally, *P. aeruginosa* produces volatiles that stimulate *A. fumigatus* to invade the lung parenchyma when the two organisms are physically separated[129]. However, as soon as the organisms come into direct contact, their relationship becomes antagonistic as they compete for nutrients, including iron[129]. This highlights the complex inter-kingdom interactions displayed between these two opportunistic pathogens that have a profound impact on the human host, as well as the key role of iron in modulating this dynamic relationship.

In other mucosal sites of the human body, an important fungal-bacterial interaction occurs between *A. fumigatus* and *S. aureus*. During polymicrobial biofilm formation, *S. aureus* inhibits *A. fumigatus* conidiation, filamentation, and biofilm maturation[130]. Another study found that *E. coli* DH5α secretes a 60 kDa protein with activity against *A fumigatus*. This activity was then linked to a siderophore-based inhibition of fungal growth via limitation of iron acquisition[131].

Overall, these studies demonstrate that different bacteria have disparate effects on *C. albicans, C. neoformans*, and *A. fumigatus* either promoting or preventing growth, and either enhancing or blocking the production of virulence factors. As further studies continue to explore the inter-kingdom interactions that occur in the human host, more mechanistic insights will be gleaned as to how fungal pathogenesis is impacted by other inhabitants of the human microbiota.

Modulation of bacterial proliferation and virulence by fungi

Similar to fungi, bacteria employ a range of virulence traits to facilitate infection in the host, including the use of secretion systems for the release of toxins and other effectors[132,133]. Some fungi exhibit broad effects on the virulence of multiple bacterial pathogens. For example, *C. albicans* biofilms create a hypoxic microenvironment that facilitates the growth of anaerobic bacteria including *Clostridium perfringens* and *Bacteroides fragilis*[134]. Here, we summarize current research efforts to evaluate the effect of fungi on bacterial growth and virulence in the context of the human host.

Pseudomonas aeruginosa

P. aeruginosa is a leading cause of hospital-acquired infections, including pneumonia and urinary and wound infections[135], and is frequently detected in the lungs of cystic fibrosis patients[97]. In fact, over 75% of CF patients over 18 years of age are chronically colonized with *P. aeruginosa*, which often persists throughout the life of the patient. Previous work found that *C. albicans* impacts *P. aeruginosa* virulence, biofilm formation, and secretion of antifungal compounds. Specifically, *C. albicans* produces the quorum sensing molecule farnesol, which inhibits transcription from the pqsA–E operon, blocks production of the quinolone signal PQS, and ultimately inhibits the expression of phenazine biosynthetic genes[136]. However, in *P. aeruginosa–C. albicans* biofilms, where *P. aeruginosa* and PQS concentrations are high, the presence of the fungus leads to increased production of phenazines through an uncharacterized pathway, suggesting that the effects of *C. albicans* on *P. aeruginosa* are complex[62,137]. Interestingly, *C. albicans* can also impact *P. aeruginosa* virulence by inhibiting iron acquisition. *C. albicans*-secreted proteins inhibit the expression of *P. aeruginosa* genes important for iron acquisition and virulence, including pyochelin and pyoverdine[138]. Oral administration of *C. albicans* secreted proteins was sufficient to protect mice from *P. aeruginosa* infection and oral iron supplementation rescued bacterial virulence in the presence of *C. albicans*. *P. aeruginosa* and *A. fumigatus* are also frequent co-colonizers in the lungs of cystic fibrosis patients[97] and gliotoxin produced by *A. fumigatus* inhibits *P. aeruginosa* growth and biofilm formation[139]. Other work found that *A. fumigatus* secretes isocyanides that bind copper and exhibit broad-spectrum antimicrobial activity, including activity against *P. aeruginosa*[140].

Staphylococcus aureus

S. aureus and *C. albicans* are frequently co-isolated in biofilm-associated diseases such as keratitis and urinary tract and wound infections[141]. *S. aureus* adheres to *C. albicans* hyphae through the adhesins FnpB, SasF, and Atl to facilitate tissue penetration and seed dissemination of the bacteria[142]. In a mouse model of oral candidiasis, co-infection of *C. albicans* and *S. aureus* results in the establishment of systemic infection as opposed to symptoms of oral candidiasis with the fungus alone or no symptoms with the bacteria alone[142]. A mouse model of intra-abdominal infection also found that coinfection with *C. albicans* and *S. aureus* results in synergistic lethality[143,144]. Interestingly, this synergism is not dependent on the ability of *C. albicans* to undergo hyphal morphogenesis and non-*albicans* species of *Candida* also enhance infection[145,146]. Additionally, recent work established that during polymicrobial growth of *C. albicans* and *S. aureus*, the fungus

elevates extracellular pH to enhance the production of alpha toxin, the major cytotoxic agent released by the bacterium[147]. As well, when exposed to the *C. albicans* quorum sensing molecule farnesol, *S. aureus* exhibits enhanced tolerance to antimicrobial agents due to increased expression of drug efflux pumps[148].

Other Examples—*S. mutans* and *C. albicans* are frequently co-isolated in plaques and biofilms in the oral mucosa, contributing to caries and other tooth decay and damage, particularly in children. While high levels of farnesol can inhibit *S. mutans* growth, lower concentrations that are found in *S. mutans-C. albicans* conditioned medium actually increase growth and enhance biofilm formation in *S. mutans*[149]. Farnesol also increases the expression of glucosyltransferases in *S. mutans* that contribute to the robust exopolysaccharides found in the extracellular matrix of biofilms[149]. Thus, there is a dynamic relationship between farnesol production and *S. mutans* growth and virulence.

To investigate the mechanisms underlying the high rate of mucosal and systemic candidiasis in cancer patients receiving chemotherapy[150], Bertolini et al. developed a chemotherapy-immunosuppressed mouse model of oral and gut mucosal breach by *C. albicans*[151]. The authors showed that infection with *C. albicans* led to changes in the oral mucosa that contributed to disease[151]. These changes in the microbial community led to an increase in prevalence of *Enterococcus* spp., which reduced the integrity of the epithelial barrier and promoted invasion of *C. albicans* and immunopathology associated with candidiasis.

In contrast to most of the mechanistic studies published over the past decade, recent work established that positive interactions between microbes were much more common than previously predicted. This was determined by using a high-throughput co-culture platform that examined over 180,000 different interactions between 20 soil-dwelling bacteria in 40 different environment conditions[152]. It would be interesting to use a similar platform in the future to examine bacterial-fungal interactions in a high-throughput manner to systemically evaluate both positive and negative effects on proliferation and virulence.

Targeting Virulence as an Antimicrobial Strategy

Microbe-derived biomolecules, including natural products, are a rich source of antimicrobial compounds[153,154]. About 70% of antibacterial agents used in the clinic are of natural product origin, and 97% of these compounds originate from either fungi or bacteria[155,156]. However, a vast majority of these antimicrobials target essential gene products or processes required for pathogen viability. A relatively underexplored area of study is the therapeutic potential of anti-virulence compounds to thwart infectious disease[157,158]. A challenge of canonical antimicrobial agents is their relatively non-selective inhibition of microbial growth. This leads to negative effects on commensal microbes in the human body and contributes to antimicrobial resistance[159–161]. One of the exciting possibilities of anti-virulence strategies to combat disease is the ability to specifically prevent organisms from causing infections, as opposed to directly killing microbes. This reduces selective pressure on the organism to evolve resistance as cells are still able to grow and survive, without employing virulence factors that damage the host[162]. Inhibiting virulence factors also extends potential drug targets beyond those involved in essential processes in pathogens.

This is especially important in fungi, as these eukaryotic pathogens share many essential processes with their human hosts. However, there are limitations to anti-virulence strategies, as elimination of the pathogen is necessary in the case of many infections. Anti-virulence strategies alone could be employed for common opportunistic infections such as oral and vaginal candidiasis. However, for bloodstream infections or complicated disease, combination therapy could be employed that uses both an antimicrobial and anti-virulence-based strategy[163].

While this review has described several secreted compounds produced by members of the human microbiota that modulate key virulence attributes, thus far, the only FDA-approved anti-virulence therapeutic to date has used antibodies to bind and neutralize toxins in bacterial pathogens. The first study to demonstrate efficacy of an anti-virulence strategy against a pathogen involved treatment of infant botulism with antibodies purified from adult donors that neutralize the botulism toxin[164]. Building on the success of this approach, a separate study successfully employed an anti-virulence treatment in stage three clinical trials to combat recurrent *C. difficile* infection[165]. The authors administered two monoclonal antibodies, actoxumab and bezlotoxumab, that bind and neutralize the *C. difficile* toxins A and B, respectively. Treatment with both antibodies was associated with a significant reduction in recurrent *C. difficile* infection in at-risk patients[165]. Additionally, another monoclonal antibody, raxibacumab, binds and neutralizes a component of the anthrax toxin and confers improved survival and clinical outcomes following anthrax exposure in rabbits and monkeys[166]. Probiotic bacteria have been extensively investigated for their ability to treat infections that lead to microbial dysbiosis, including diarrheal disease and vaginal candidiasis[167,168]. However, only one fungal species is established as a treatment for bacterial infection, including diarrhea caused by *C. difficile* in adults and children. Previous work found that *Saccharomyces boulardii* CNCM I-745 secretes a 54-kDa protease which digests the *C. difficile* toxin A[169]. Oral administration of the probiotic fungus has no effect on the microbiota of healthy humans, but it can rescue eubiosis of the intestinal microbiota following diarrheal disease[170] and infection with *Helicobacter pylori*[171]. These examples highlight the potential of targeting virulence factors to combat *C. difficile* infections, including the potential application of microbiota-derived factors.

A relatively unexplored and exciting area of current focus is the potential of small molecules to target virulence traits of human fungal pathogens. Several small molecule inhibitors of *C. albicans* hyphal morphogenesis have been described (Figure 4)[60,77,172–174]. This includes small molecules secreted by bacteria, such as 1-acetyl-beta-carboline secreted by *Lactobacillus* spp.[80] and the *C. albicans* quorum sensing molecule, farnesol[175]. Other work has screened collections of small molecules for activity against *C. albicans* hyphal morphogenesis. Specifically, a collection of 30,000 small molecules was assessed for their ability to inhibit adhesion of *C. albicans* to polystyrene plates, identifying a single molecule, filastatin, that inhibits *C. albicans* adhesion, hyphal morphogenesis, biofilm formation, and fungal virulence in a nematode model of infection[172]. Analogous screens also identified biaryl amide compounds that inhibit *C. albicans* hyphal morphogenesis and prevent virulence in oral and invasive murine models of candidiasis[176], as well as diazaspiro-decane analogs as inhibitors of *C. albicans* biofilm formation, hyphal morphogenesis, and virulence in both an oral and invasive model of mouse candidiasis[173]. Additionally, a screen

of 678 compounds pre-selected based on bioactivity against *Saccharomyces cerevisiae* identified Tri-Chloro-Salicyanilide (TCSA) as a top compound that blocks *C. albicans* hyphal morphogenesis and biofilm formation. Through transcriptional profiling, this activity was linked to fungal mitochondrial protein import[177]. However, none of these compounds have advanced to a clinical trial. The only anti-virulence strategy to combat fungal infection that is under clinical development is the NDV-3A *C. albicans* vaccine[178]. It was developed using the Als3 adhesin protein that is critical for *C. albicans* adherence, invasion, and virulence in the host[179]. The vaccine has demonstrated efficacy against both systemic and oral candidiasis in murine models[180–182], as well as recurrent vulvovaginal candidiasis in women in a double-blind, placebo controlled clinical trial[178]. Thus, although targeting virulence traits represents a promising therapeutic strategy to mitigate infectious diseases, future study is necessary to develop microbiome-derived compounds with efficacy against fungal pathogens.

Bacteria and fungi alike have been mined for the secretion of antimicrobial compounds, including many front-line anti-infective agents used clinically[183,184]. However, the current mining of antimicrobials is neither systematic nor comprehensive, and the re-discovery of antimicrobial agents is a significant hurdle that is yet to be overcome[185]. Current work is trying to establish efficient pipelines coupled with modern ‘omics’ technology to facilitate natural product discovery in diverse microbial backgrounds. Such advancements could be applied to those organisms living in the environment as well as those found within a human host. Bioinformatics now facilitates the discovery of silenced or cryptic biosynthetic clusters responsible for natural product production in both fungi and bacteria[186–188]. This includes recent work that developed a pipeline for identifying natural products from anaerobic fungi using genomics, transcriptomics, proteomics, and metabolomics, highlighting the untapped potential of anaerobic gut fungi as producers of natural products[189]. Whole-genome sequencing to identify unexplored biosynthetic gene clusters has also led to the identification of a known antibacterial complestatin and a new antibacterial corbomycin, that bind to bacterial peptidoglycan and inhibit autolysin activity to prevent essential bacterial cell wall remodelling[190]. Both compounds were effective in a murine model of skin infection and decreased methicillin-resistant *S. aureus* burden[190]. Finally, leveraging the microbiomes of marine animals and cutting-edge metabolomics and genomic tools, a novel antifungal turbinmicin was discovered that displays potent *in vitro* and *in vivo* activity against multidrug-resistant fungal pathogens through a fungal-specific mode of action, targeting Sec14 of the vesicular trafficking pathway[191]. Continued advances in antimicrobial discovery will help scientists and clinicians realize the potential of anti-virulence strategies to thwart infectious disease.

Conclusion

Dissecting the mechanisms underlying inter-kingdom interactions is important for furthering our understanding of human health and disease. The literature outlined in this review demonstrates that microbial interactions, including both antagonistic and synergistic interactions, have important implications for opportunistic infections and beyond. We highlight the extensive literature that describes the effects of bacteria on fungal growth and virulence in the mammalian host, as well as the effect of fungi on bacterial proliferation

and virulence. By focusing on opportunistic fungal and bacterial pathogens that colonize the human body, we highlight key microbial interactions that contribute to the onset and severity of diverse infections. Studies highlighted in this review that investigate the synergistic interactions between bacteria and fungi identify their important implications for polymicrobial infections and disease susceptibility. Additionally, work describing antagonistic interactions between bacteria and fungi demonstrate their potential for the identification of novel antimicrobial compounds and strategies to thwart infectious disease. These novel antimicrobial compounds offer a promising reservoir of untapped chemical diversity that remains largely unexplored. Beyond the medical applications of inter-kingdom interactions, characterizing these communications is important for our understanding of the development and maintenance of the human microbiota. Similar to studying other ecological environments, examining the microbial composition of healthy and disease states can further our understanding of these complex communities.

With the rising threat of antimicrobial resistance, the need for novel antibacterial and antifungal agents is reaching a critical point. Improved strategies for mining bacteria and fungi for natural products now enable researchers to revisit ecological bacterial-fungal interactions as a source of novel antimicrobials. Given that many microbes are opportunistic pathogens that rely on virulence traits to cause disease in a human host, small molecules that inhibit microbial virulence represent an exciting area of antimicrobial discovery. With antibody and vaccine-based anti-virulence strategies beginning to gain approval for clinical use, further research is required to expand the available repertoire of microbial virulence inhibitors.

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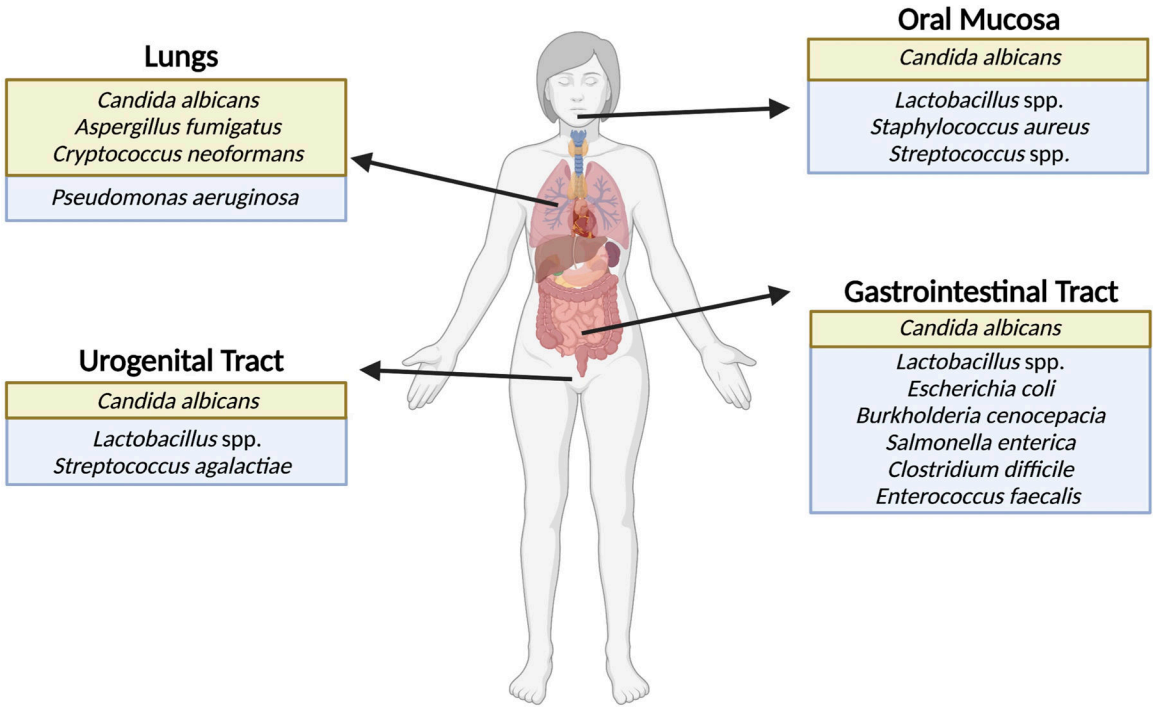

Figure 1: Bacterial-fungal interactions in the human body.

While this is not a comprehensive list of organisms found in the human microbiota, it highlights the key bacterial-fungal interactions summarized in this review, sorted by anatomical site. Figure made with biorender.com.

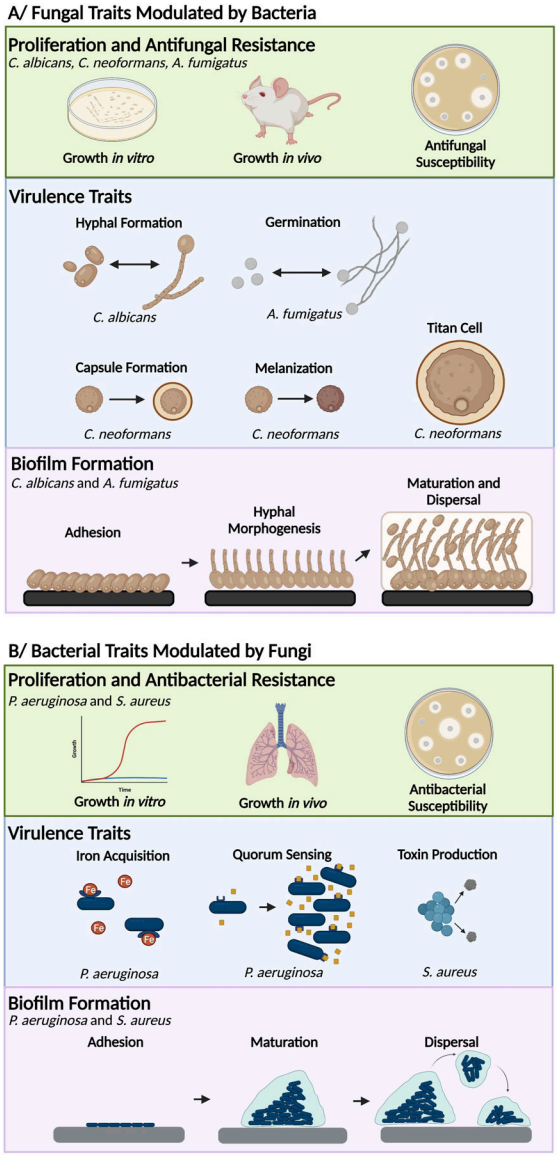

Figure 2: Microbial traits modulated by inter-kingdom interactions.

A/ Bacteria influence the ability of *C. albicans, C. neoformans,* and *A. fumigatus* to proliferate and modulate antifungal susceptibility, with certain inter-kingdom interactions enhancing these phenotypic traits and others impeding proliferation or compound susceptibility. This is examined *in vitro* by culturing fungi on solid (shown) or liquid medium in the presence or absence of bacteria or bacterial supernatants. It is also examined using mouse models, where the microbiota has been altered. Finally, the impact of bacteria on antifungal susceptibility can be assessed using a variety of approaches, including disc diffusion assays where the ability of bacterial supernatants to increase the efficacy (zone of inhibition) of antifungals can be assessed. Bacteria also modulate diverse virulence traits including *C. albicans* hyphal formation, *A. fumigatus* germination, and *C. neoformans* capsule formation, melanization, and titan cell induction. Finally, bacteria can modulate biofilm formation in *C. albicans* and *A. fumigatus*. Displayed are the developmental stages

of *C. albicans* biofilm formation, including adhesion to a solid surface, induction of hyphal morphogenesis, and maturation and dispersal where an extracellular matrix is produced and yeast cells are released. **B/** Fungi can influence the ability of *P. aeruginosa* and *S. aureus* to proliferate and can modulate antibacterial susceptibility through increasing the expression of efflux pumps in *S. aureus*. This has been determined through both *in vitro* co-culture assays on solid or liquid medium (shown) and through *in vivo* co-culture models of infection. Fungi can also modulate diverse bacterial virulence traits including inhibition of iron acquisition and quorum sensing in *P. aeruginosa*, as well as alpha toxin production in *S. aureus*. Finally, fungi can inhibit mono and polymicrobial biofilm formation in both *P. aeruginosa* and enhance biofilm formation in *S. aureus.* Figure made with biorender.com.

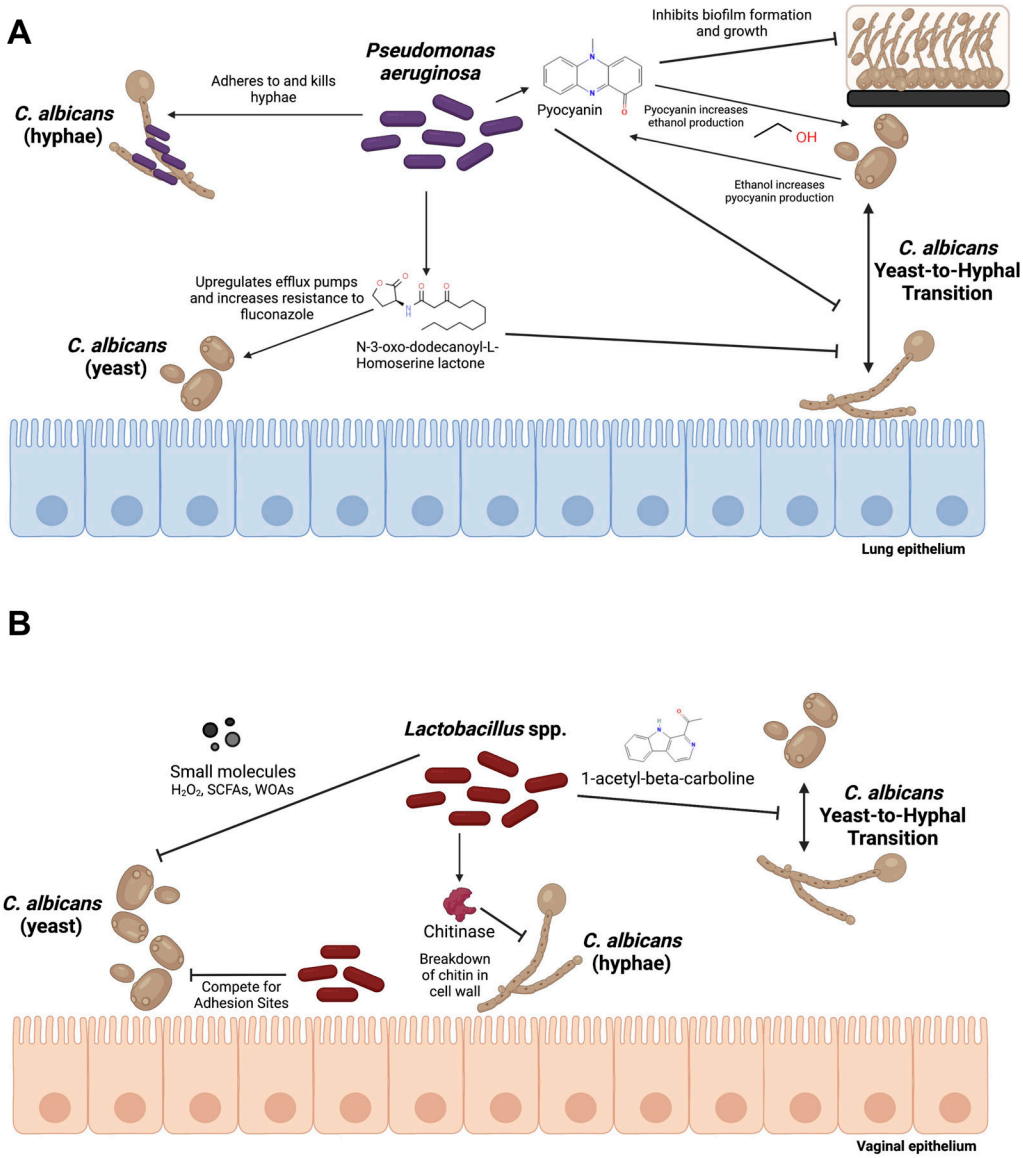

Figure 3: Interactions between bacteria and C. albicans modulate virulence of the fungus.

A/ Interactions between *C. albicans* and *P. aeruginosa* on the lung epithelium. *P. aeruginosa* secretes phenazines such as pyocyanin that inhibit *C. albicans* growth and biofilm formation, as well as the yeast-to-hyphal transition at concentrations below those inhibiting growth. Through a positive feedback loop, the production of ethanol by *C. albicans* stimulates phenazine production in *P. aeruginosa*, and phenazines then further increase ethanol production in the fungus through compromising mitochondrial function. *P. aeruginosa* quorum sensing (QS) molecules, including N-3-oxo-dodecanoyl-L-homoserine lactone, also inhibit the *C. albicans* yeast-to-hyphal transition. This QS molecule also upregulates efflux pumps in *C. albicans* leading to increased resistance to the antifungal, fluconazole. Finally, *P. aeruginosa* can adhere to *C. albicans* hyphae and directly kill them through the secretion of phenazines. **B/** Interactions between *C. albicans* and *Lactobacillus* spp. on the vaginal epithelium. *Lactobacillus* spp. secrete small molecules including hydrogen peroxide, short-

chain fatty acids (SCFAs), and weak organic acids (WOAs) such as lactic acid that affect fungal growth. *Lactobacillus* spp. also secrete 1-acetyl-beta-carboline that inhibits the yeast-to-filament transition, as well as chitinase, which breaks down the fungal cell wall. Finally, *Lactobacillus* spp. directly compete with *C. albicans* for adhesion sites on the vaginal epithelium. Figure made with biorender.com.

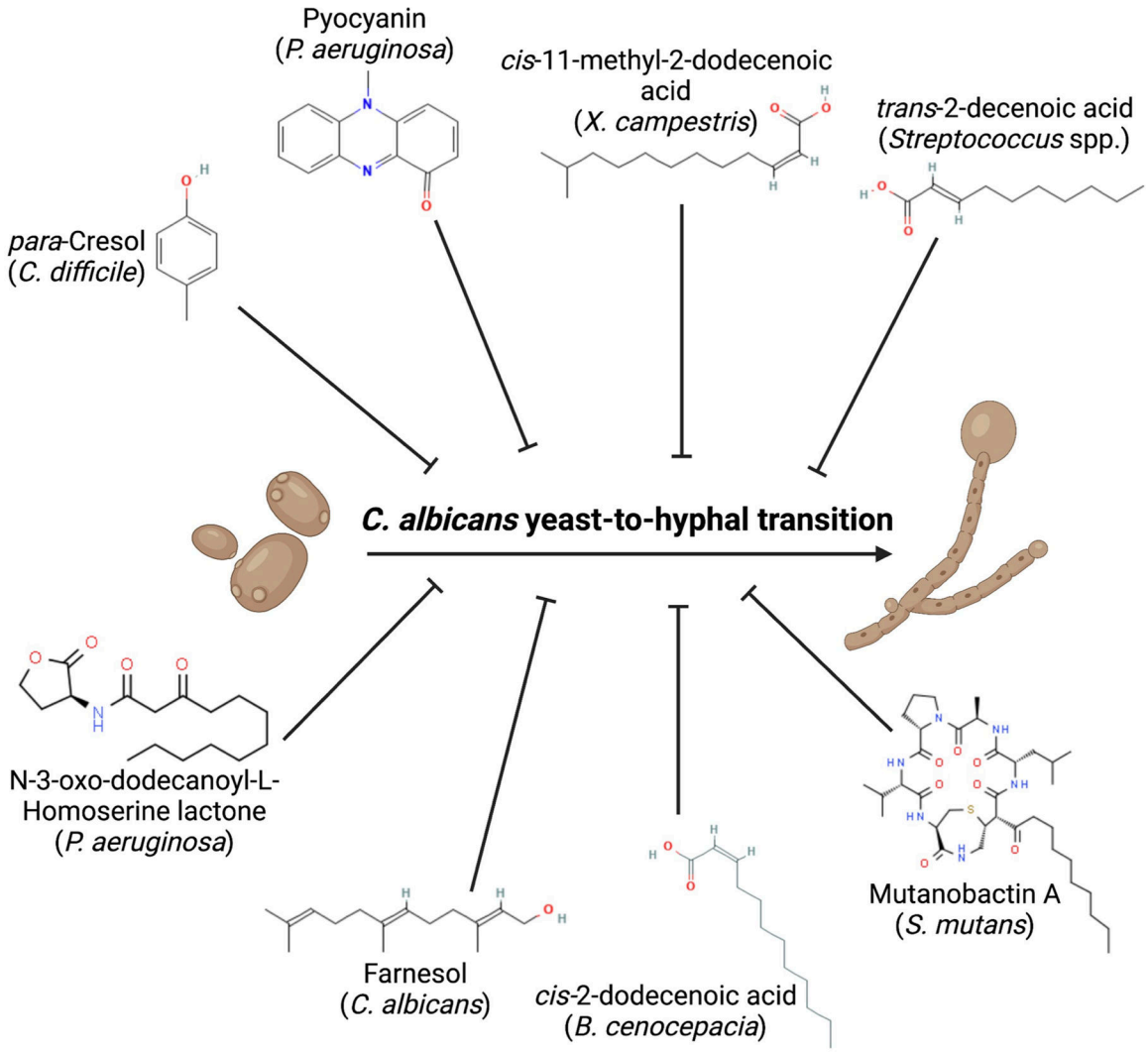

Figure 4: Bacteria secrete diverse compounds that inhibit the C. albicans yeast-to-hyphal transition.

The small molecules secreted by bacterial species highlight a diverse collection of chemical scaffolds capable of inhibiting hyphal morphogenesis in *C. albicans*. Figure made with biorender.com.