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REVIEW

Medically Important Fungi in Multi-Species Biofilms: Microbial Interactions, Clinical Implications and Therapeutic Strategies

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Abstract

Purpose of Review This review aims to elucidate clinically important sites where multi-species biofilms are formed. We highlight key *in vitro* and *in vivo* studies, discuss the clinical implications of these biofilms, and explore strategies for their prevention and eradication.

Recent Findings Multi-species biofilms significantly enhance antimicrobial resistance and pathogenicity. Synergistic interactions, such as those between *Candida albicans* and *Staphylococcus aureus* or *Pseudomonas aeruginosa*, illustrate how fungal biofilms can elevate bacterial drug resistance. Innovative treatments, including combination therapies and targeting specific biofilm components, show promise in disrupting these resilient communities.

Summary Understanding the molecular and environmental factors driving multi-species biofilm formation is crucial for developing effective therapies. Future research should emphasize in vivo interactions, host responses, and the potential of natural substances and polymeric devices to improve treatment outcomes and reduce the clinical burden of multi-species biofilm-associated infections.

Keywords Polymicrobial interactions · Clinically important fungi · Multi-species biofilms · Therapeutic

Introduction

The concept of community extends beyond humans; microorganisms, although historically investigated as isolates, are social beings that exist in communities and significantly impact the health and disease states of their hosts. Some of these microbes form biofilms within the human microbiota, found on the skin, mouth, vagina, intestine, and medical devices [1]. Biofilms are microbial communities embedded in a self-produced extracellular polymeric substance (EPS), facilitating surface adherence and protection against environmental and biological stressors [1, 2]. Although the formation of monospecies biofilms is well understood, the phases of multi-species biofilm development render a cliffhanger that challenges understanding these complex communities. Herein, we show a hypothetical representation of multi-species biofilm formation (Fig. 1A).

Once microorganisms coexist with different species, complex ecological interactions are established within the community. Cooperative and antagonistic interactions govern the survival of a type in a multi-species community and the success or collapse of microbial systems. Biofilms coordinate through cohesive gene expression, quorum sensing (QS) molecules, and metabolic cooperation, which provides resistance to antimicrobials [2]. Among these ubiquitous-complex communities, fungi remain neglected. However, their ability to colonize the human microbiota without signs of inflammation and transition into true pathogens determines an underestimated opportunism [3]. Interactions between bacteria and other pathogenic fungi have been investigated recently [4], revealing

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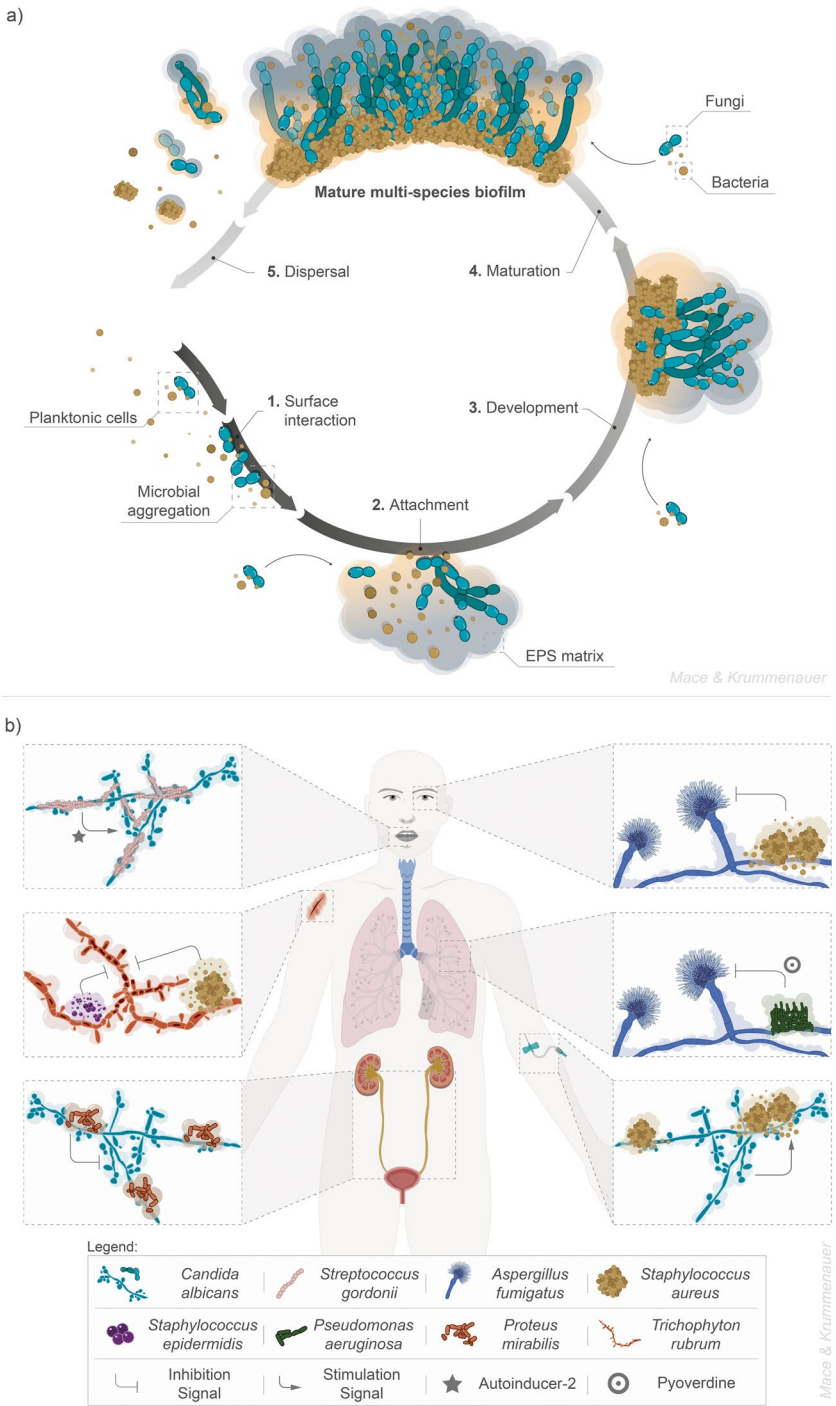

Fig. 1 Hypothetical model of multi-species biofilm formation. **a** Multi-species biofilm formation key stages: (1) microbial aggregation on a biotic or abiotic surface, and recognition of hydrophobicity profile, charge distribution, roughness, and the presence of other microbes; (2) adhesin-mediated attachment coupled with extracellular polymeric substances (EPS) production and microbial growth; (3) biofilm development through nutrient exchange, EPS production, expulsion of toxic substances via diffuse channels, and modulation of metabolite secretion by quorum-sensing (QS) molecules, with the potential incorporation of other microorganisms into the established community; (4) formation of a complex and exclusive microenvironment that hinders removal and facilitates extensive genetic exchange; the final plot occurs when (5) cells disseminate to other sites; **b** Multi-species biofilms containing medically relevant fungi found in human anatomical sites

mechanisms that could clarify potential tools for controlling these populations and their effects on the human host. However, there is a growing need for studies that summarize and discuss key findings on multi-species biofilms containing medically important fungi.

This review aims to investigate the main microbes in the human body in the form of multi-species biofilms containing fungi of clinical relevance. Herein, we highlight the occurrence of these biofilms in human anatomical sites, discuss their clinical implications, and explore potential therapeutic strategies to combat these resilient microbial populations.

Clinically Important Sites for Inter-kingdom Biofilms

Ocular Site

Multi-species biofilms in the eye pose significant challenges for clinical diagnostics and therapeutic interventions, requiring targeted treatment approaches for each microbial species involved [5]. *Candida albicans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* contribute to ocular infections such as keratitis, blepharitis, and endophthalmitis. They form mixed biofilms on *ex vivo* human corneas. Simultaneous incubation or pre-formed biofilms of *C. albicans* with *S. aureus* and *S. epidermidis* result in increased cell viability and metabolic activity compared to their monomicrobial counterparts, as measured by XTT assay. Adding *C. albicans* in pre-forming *S. aureus* biofilms increases ampicillin's minimal biofilm eradication concentration, likely due to reduced antimicrobial permeation, nutrient-limited slow growth, and persister cells [6]. Increased cellular metabolism and biofilm formation leading to drug resistance present a threat to patients with mixed infection in the ocular tract, given its rapid progress and severe symptoms like intense pain, light sensitivity, and vision loss, often more severe than those from isolated fungal or bacterial infections [7].

Aspergillus fumigatus was tested for biofilm formation with *S. aureus* in primary cultures of human limbo-corneal fibroblasts [8]. Scanning Electron Microscopy (SEM) of clinical samples from patients with keratitis caused by both pathogens revealed antagonistic interactions, showing structural damage during mixed biofilm formation [9]. Similar antagonistic interactions, such as mycophagy by *Staphylococcus* spp., have been reported [10–12] and both fungal and bacterial structures were observed within the EPS [9]. Additionally, damage to fibroblasts was observed, including perforation by hyphae growth and bacterial intracellular invasion, although fibroblasts also exhibited antagonistic responses, such as pro-inflammatory reactions mediated by exosome-like structures [8]. This study highlights the complex interactions in mixed ocular infections and emphasizes the need to consider factors like immune response, microbiota, and underlying health conditions to fully understand disease onset, progression, and treatment. Ocular infections by *A. fumigatus* are particularly concerning in immunocompromised patients due to their proximity to brain tissue and the associated high mortality rate [13]. Thus, prompt diagnosis and individualized therapeutic approaches are critical for a favorable prognosis, especially in cases of mixed infections.

Fusarium solani was studied for biofilm formation with *S. aureus* and *S. epidermidis* on ex vivo human corneas. Multi-species biofilms exhibited greater metabolic activity through the XTT assay and biomass than monospecies, demonstrating greater cell viability and proliferation. These results demonstrate the potential for establishing severe infections that require timely intervention to avoid complications. Confocal microscopy showed mixed biofilms were twice as thick as bacterial biofilms alone, with SEM confirming thickness. Although an initial equal fungus-bacteria ratio was observed, bacterial cells decreased after 48 h, likely due to increased EPS secretion. Notably, mixed biofilms of *S. aureus* and *F. solani*, as well as *S. epidermidis* and *F. solani*, showed increased sensitivity to antifungals (itraconazole and fluconazole) [14]. This suggests that interspecies interactions can modulate antimicrobial susceptibility, potentially enhancing sensitivity rather than just increasing tolerance.

Multi-species biofilms pose a significant challenge in treating eye infections, as the delicate ocular surface is easily compromised by microbial invasions and physiological imbalances. Innovative microbiological control strategies are crucial for preserving ocular health and improving treatment outcomes. These approaches should target biofilm disruption and enhance the efficacy of existing antimicrobial therapies to address the complexities of multi-species biofilms.

Oral Cavity

The mouth cavity harbors more than 700 bacterial species and over 100 fungal species [15]. Although saliva promotes the clearance of microorganisms and fermentable carbohydrates, it also provides nutrient for the growth of microbes. In both healthy and periodontal patients, the oral microbiome commonly includes *Candida* spp. and *Aspergillus* spp., alongside other less prevalent fungi [16–18].

Tri-species biofilms of *Actinomyces naeslundii*, *S. mutans*, and *C. albicans* in the human oral cavity showed reduced metabolic activity compared to bi-species biofilms, likely due to products of cell metabolism decreasing the cell viability of one of the microorganisms by XTT assay, as proposed by the study. Tri-species biofilms of these microbes showed increased biomass, indicating that more diverse biofilms exhibit reduced metabolism [19]. In addition, *Actinomyces oris* and *Streptococcus oralis* are also common in the oral cavity and can associate with *C. albicans* in pathogenic biofilm formation on dental plaque. Co-cultivation of *C. albicans*, *A. oris*, and *S. oralis* on saliva-coated resin discs led to microbial coverage and increased bacterial adhesion in irregular areas, forming a prominent biofilm layer with *C. albicans* hyphae. Although microbial abundance increased for all strains, the specific contributions of each species remain unclear due to complex interactions within the biofilm. Both bacteria showed increased colony-forming units (CFU) when associated with *C. albicans* [20]. Still, the

biofilms of *S. oralis* and *A. oris* alone were not investigated, making it difficult to determine if bacterial traits contributed to the biofilm's success. In this sense, it is not feasible to determine whether the results of these interactions derive from the interplay of more complex communities or whether they can also be observed in simpler microbial populations.

The mechanisms behind the prevalence of *C. albicans*, *A. oris*, and *S. oralis* in biofilms are not well understood. However, excessive *C. albicans* growth can lead to oral conditions such as dental stomatitis and candidiasis [21]. The interactions between *C. albicans* and *Aggregatibacter actinomycetemcomitans* explored the role of the QS molecule autoinducer-2 (AI-2). *A. actinomycetemcomitans* lacking the AI-2-producing gene (*luxS*) induced *C. albicans* hyphae and biofilm formation. Wild-type *A. actinomycetemcomitans* co-cultivation significantly reduced *C. albicans* biofilm formation compared to monoculture. Treating established *C. albicans* biofilms with *A. actinomycetemcomitans* metabolites from early growth stages led to biofilm disruption, while treatment after 8 h did not affect them [22]. This suggests that AI-2 production during early growth stages may inhibit *C. albicans* biofilms, whereas inhibition is effective only in the initial stages of infection. If the fungal burden remains high, the biofilm may eventually re-establish itself [23].

Streptococcus gordonii expresses AI-2 and can interact with other microorganisms, potentially posing health risks [24, 25]. Co-cultures of *C. albicans* and AI-2-deficient *S. gordonii* showed reduced *C. albicans* hyphae formation, highlighting AI-2's role in biofilm development [26]. *C. albicans* hyphae not only serve as a scaffold but also shape the biofilm structure [27]. Unlike *A. actinomycetemcomitans*, AI-2 is a key driver of *S. gordonii-C. albicans* biofilm formation (Fig. 1B). The *C. albicans* gene regulatory proteins also play roles in these biofilms, influencing filamentation, adherence, biofilm formation, and cellular structure. Knocking out genes involved in filamentation, decreased biofilm formation and reduced *S. gordonii*'s tolerance to ampicillin and erythromycin [28]. In *C. albicans-S. gordonii* biofilms showed increased glucosyltransferases (GHs) synthesis, resulting in higher bacterial biomass and reduced fungal biomass. Knocking out the *tec1* gene in *C. albicans* reduced GH synthesis by *S. gordonii*, suggesting that *C. albicans* hyphae facilitate *S. gordonii* survival in low-carbohydrate environments [29]. Understanding the molecular mechanisms behind *C. albicans-S. gordonii* biofilm formation offers insights into infection dynamics and potential strategies for its controlling.

S. mutans, a major cause of caries in children, interacts extensively with *C. albicans*. *C. albicans CHK1*-deleted mutants reduce *S. mutans* colonization in a co-infected caries rat model. The *CHK1* gene is crucial for cell wall synthesis, QS, and virulence [30], making it a potential target for controlling caries in mixed biofilms. Additionally, *C. albicans* mannans, cell wall components, and EPS interact with the *S. mutans* exoenzyme GtfB, enhancing EPS production and biofilm formation [31, 32]. The suppression of genes involved in fungal mannan synthesis and the deficiency of GtfB in *S. mutans* impaired the formation of mixed biofilm [31].

The balance and interactions of the local microbiota heavily influence the health of the oral cavity. Factors such as salivary flow, pH, diet, underlying diseases, and mechanical trauma can disrupt this balance, leading to harmful interactions and disease. Among clinically important oral fungi, *Candida* spp. predominate, often forming resilient biofilms that are challenging to treat. These biofilms, particularly when associated with bacteria, can develop resistance to treatments.

Wounds

Wound recovery involves four phases: coagulation, inflammation, proliferation, and remodeling [33]. Acute wounds typically heal within days to weeks, while chronic wounds often stagnate in the inflammation stage, taking weeks or months to heal [34]. This impaired healing is linked to bacterial and fungal colonization and biofilm formation, which decreases antimicrobial susceptibility [34, 35]. Fungi significantly contribute to chronic wound biofilms as opportunistic organisms, exacerbated by extensive antibiotic use that increases selective pressure and resistance [33, 35]. Their prevalence is often underestimated as they can evade infection sites, leading to fungemia and invasive fungal disease [33].

C. albicans and *S. aureus* are common components of human skin microbiota that can colonize tissue without harm [36]. However, they can also cause nosocomial blood, catheter-associated, and burn wound infections [37, 38]. In mixed communities, *S. aureus* and *C. albicans* exhibit increased antimicrobial resistance and virulence factor expression [39, 40]. *C. albicans* acts as a scaffold for *S. aureus* adhesion and biofilm formation, creating robust structures with increased miconazole resistance. Co-infection also leads to lower survival rates in murine models [41]. When co-cultured with *C. albicans*, a green fluorescent protein-expressing *S. aureus* exhibited increased fluorescence and biofilm formation. Transcriptome analysis showed upregulation of virulence pathways in *S. aureus*, including gamma-hemolysin, staphylocoagulase, enterotoxin, and hemolysin production. *C. albicans* enriched pathways related to ergosterol biosynthesis and drug transmembrane transport. Infected mice treated with fluconazole showed defective skin healing compared to single-species infections, and they responded better to methicillin and vancomycin [42]. Additionally, *S. aureus-C. albicans* co-culture increased staphylococcal protein pathways like L-lactate dehydrogenase 1, which protects against

host oxidative stress [43]. These findings partially explain the healing process of wounds infected with *C. albicans-S. aureus* biofilms treated with common antimicrobials. However, the complex human skin microbiome complicates the translation of laboratory findings to real-world scenarios. For example, *Malassezia globosa* decreases *S. aureus* biofilm formation by secreting aspartyl protease [44]. Moreover, *C. albicans* adhesins facilitate *S. aureus* dissemination through host immune phagocytosis. Interestingly, immunosuppression-induced leukocyte depletion protected mice from bacterial spread [45].

S. epidermidis is a skin commensal known for its biofilm-forming capacity, which can complicate wound care [46]. Communities of *S. epidermidis*, *S. aureus*, and *Trichophyton rubrum* were evaluated for cell viability and biofilm formation [47, 48]. While these infections typically resolve within weeks or months, they can combine with other microorganisms to penetrate deep tissue layers, complicating treatment and recovery [49]. A study found that adding *S. aureus* after *T. rubrum* adhesion induced well-structured hyphae, while simultaneous inoculation inhibited *T. rubrum* growth (Fig. 1B). Similarly, *S. epidermidis* inhibited *T. rubrum* hyphae projection [50]. Although *S. epidermidis* and *S. aureus* are common in chronic wounds [51], their role is debated compared to other pathogens like dermatophytes [46, 52]. The commensal relationship between *S. aureus* and *S. epidermidis* is a double-edged sword, inhibiting fungal pathogens but increasing bacterial abundance [50]. These microorganisms can shift from symbionts to pathogens, disrupting the microbial balance. Additionally, *T. rubrum* and *Rhinocladiella similis* - a yeast-like fungus causing chromoblastomycosis - were studied for biofilm formation on an *ex vivo* human nail model. The pathogens positively interacted, forming mature biofilms [53].

T. rubrum exhibits varying biofilm formation and metabolic activity when co-cultured with other skin commensals like *Candida parapsilosis*. In co-cultured biofilms, metabolic activity at 24 h was like *C. parapsilosis* alone, but by 72 h, it matched *T. rubrum* levels, likely due to the yeast's higher metabolism. SEM showed increased *C. parapsilosis* blastoconidia abundance and reduced *T. rubrum* hyphae growth when cultured. Conversely, pre-adhesion of *T. rubrum* resulted in greater hyphae formation [54], suggesting that colonization order affects microbial predominance. These data suggest that introducing a new species or the opportunistic behavior of local microbiota can significantly impact wound healing, offering strategies to recover inflamed skin tissue.

Respiratory Tract

In human physiology, the respiratory tract performs the critical function of exchanging oxygen and carbon dioxide [55]. Cystic fibrosis (CF) is a hereditary disease characterized by thick, viscous mucus obstructing airways, trapping microbes and facilitating colonization, inflammation, and infection. The condition is linked to polymicrobial biofilm formation [56]. *C. albicans* and *Pseudomonas aeruginosa* are frequently co-isolated in CF patients' lungs [57]. *C. albicans* biofilms contribute to antibiotic tolerance, such as increased *P. aeruginosa* tolerance to meropenem at clinically relevant concentrations. This tolerance is associated with *C. albicans* EPS, as glycosylation-deficient mutants did not enhance *P. aeruginosa* tolerance [58]. A classic example of *C. albicans* commensalism is its interaction with *S. aureus* in nosocomial respiratory infections and on medical devices. A study on *C. albicans-S. aureus* biofilms showed that polymicrobial biofilms significantly increase *S. aureus* resistance to vancomycin. Time-lapse confocal fluorescent microscopy revealed decreased drug diffusion through the biofilm matrix, with the enhanced drug tolerance linked to the β-1,3-glucan *C. albicans* cell wall component [40]. Another study on CF investigated the interaction of *C. albicans* and *S. aureus* in 67 sputum samples from 28 individuals. Of the 67 samples, 64 exhibited a positive culture, with 34% revealing changes caused by *C. albicans* and gram-positive bacteria. Six *Candida* spp. and *S. aureus* interactions have been identified, whereas *C. albicans* was the most prevalent species. SEM of *S. aureus-C. tropicalis* biofilms revealed that *S. aureus* tends to adhere within the hyphal filaments of *C. tropicalis*, resulting in a complex biofilm throughout the structure [59].

P. aeruginosa and *A. fumigatus* can interact competitively in the airways of immunocompromised patients and those with CF. Research indicates that *P. aeruginosa*, primarily through live cells, inhibits *A. fumigatus* biofilm formation by releasing pyoverdine, which chelates iron and deprives *A. fumigatus* of it (Fig. 1B) [60–63]. This inhibition varies by strain, with non-mucoid *P. aeruginosa* isolates generally being more inhibitory than mucoid ones [64]. Despite the competitive interaction, it is only sometimes beneficial to the host. Co-culturing *P. aeruginosa* with *A. fumigatus* biofilm significantly increased elastase synthesis in 60% of bacterial isolates, contributing to lung tissue-associated disease pathogenesis [65].

In CF patients, *A. fumigatus* and *Stenotrophomonas maltophilia* form mixed biofilms with EPS comprising fungal hyphae and bacterial cells. Microscopic analysis and measurements of biofilm formation revealed that *S. maltophilia* exhibits antibiosis against *A. fumigatus*. While bacterial growth was similar in both mono- and polymicrobial biofilms, fungal development and EPS formation were reduced in the mixed biofilm [66]. The interaction between *S. maltophilia* and *C. albicans* is another inter-kingdom antagonistic interaction. The *S. maltophilia* strain K279a genome contains a QS system that relies on the diffusible signal factor (DSF). This DSF was found to be a homolog of farnesoic

acid - QS signal from *C. albicans* that inhibits yeast hyphae expression. So, *S. maltophilia* interferes with essential virulence factors expressed by *C. albicans*, including the shift from yeast to hyphae and biofilm development [67].

P. aeruginosa and the black yeast *Exophiala dermatitidis* are also found in CF patients. When *E. dermatitidis* is cultivated with *P. aeruginosa*, the number and length of hyphae decreased, an effect not observed with QS mutant strains. The study suggests that *P. aeruginosa* QS molecules mediate the reduction in the yeast's filament and biofilm production [68].

Urinary Tract

Like other sites in the human body, the urinary tract presents a varied microbiome. Thus, isolating microorganisms in urine samples hinders the determination of the clinical significance [69, 70]. Urinary tract infections can also extend to kidneys, ureters, bladder, and medical devices such as catheters. Further, 86% of catheter-associated urinary tract infections (CAUTIs) are polymicrobial, making treatment more challenging due to biofilm formation and antimicrobial resistance [70].

In CAUTIs, *Proteus mirabilis* and *C. albicans* both showed lower CFU counts in mixed biofilms than in planktonic coculture, suggesting the inhibitory effect is biofilm-related. *P. mirabilis* also inhibited the formation of *C. albicans* hyphae, as observed in Fig. 1B. However, incubation of *C. albicans* with secreted bacterial products revealed that *P. mirabilis* metabolites were not causing this event [71], which can be explained by direct bacteria-fungus interaction leading to fungal inhibition [72]. For instance, *Proteus vulgaris* and *P. mirabilis* and their cell products inhibit *C. albicans* biofilm formation by inhibiting hyphae-specific genes, which would affect three-dimensional biofilm structure [73]. The interaction between *P. mirabilis* and *C. albicans* reduced each other's cell counts, indicating potential population control through microbial interactions.

E. coli, involved in up to 80% of uncomplicated UTIs, and *C. albicans*, responsible for 17.8% of CAUTIs [74], were evaluated for mixed biofilm formation and antimicrobial susceptibility. SEM showed *C. albicans* forming hyphae with *E. coli* adhering, leading to increased *E. coli* tolerance to ofloxacin. After degrading biofilm EPS, adding laminarin, a β-1,3 glucan mimicking EPS, further increased *E. coli* survival against ofloxacin in polymicrobial biofilms [75]. These results suggest that *E. coli-C. albicans* communities could alter their susceptibility profile through EPS composition. EPS production is a key contributor to biofilm formation [76]. Modifying EPS properties such as composition, hydrophobicity, zeta potential, and protein-polysaccharide ratio could be a strategy to modulate pathogenic communities as these features determine cell surface charge, cell adhesion, and, therefore, the cohesion of the biofilm community.

A multi-species biofilm model with *Enterococcus faecalis*, *P. mirabilis*, *E. coli*, and *C. albicans* was developed to study interactions, revealing that *E. coli* growth was inhibited in all combinations involving *P. mirabilis* [77]. In a mouse infection model, mutants from clinical urinary isolates of *P. mirabilis* and *E. coli* with a deleted pentose phosphate pathway gene showed divergent outcomes. Co-inoculation of *P. mirabilis gnd* mutants and wild-type *E. coli* revealed no colonization disadvantage, but the *E. coli gnd* mutant was outcompeted by wild-type *P. mirabilis* [78]. These findings suggest altering metabolic processes can modulate competition between *P. mirabilis* and *E. coli* and their interactions. Furthermore, *C. albicans* had its growth affected in all combinations, possibly related to the presence of single microorganisms or their combinations. Although *E. faecalis* also showed notable growth, this event did not lead to the inhibition of the other species in the combination [77]. These results may be related to the cooperation between *E. faecalis* and *P. mirabilis*, in which the latter adheres to *E. faecalis*, facilitating biofilm formation [79]. Therefore, the combinations containing *E. faecalis* and *P. mirabilis* inhibited other species. In this specific case, *C. albicans* and *E. coli* raise the question of their actual pathogenic role in this community of four microorganisms since their growth was inhibited by *P. mirabilis*. *C. albicans* is a vaginal microbiota component, often misinterpreted in urine cultures [80]. However, in its opportunistic state, it exhibits significant virulence through pseudohyphae growth, adhesin production, and biofilm formation [81]. Microbial interactions, such as nutrient competition, contact-dependent inhibition, and metabolite production, greatly influence pathogenicity [82]. Thus, the infectious role of these inhibited species in competitive environments remains unclear.

Medical Devices

Medical device insertion is essential in clinical practice for therapeutic and diagnostic procedures, especially in managing critically ill patients [83]. The insertion of medical devices carries infection risks, prompting concern among healthcare professionals. Once infected, device removal is mandatory due to the impenetrable nature of microbial biofilms, which resist chemical, biological, and mechanical measures. Therefore, therapeutic regimens prioritize replacing implantable devices to prevent opportunistic or pathogenic microbes from entering [83].

The prevalence of microorganisms associated with medical devices differs according to the anatomical site where the material is implanted. For example, urinary catheters are mostly infected by *E. coli*, *P. mirabilis*, *E. faecalis*, and *K. pneumoniae*. On the other hand, central venous catheters

are more infected by skin microorganisms such as coagulase-negative *Staphylococcus* spp., *S. aureus*, and *C. albicans*. Voice prostheses are mainly infected by *C. albicans*, *Candida tropicalis*, and *Candida glabrata* [84]. Some studies evaluating the formation of multi-species biofilms containing fungi of clinical relevance on medical devices are discussed here in terms of the implications they may pose for the human host.

Titanium dental implants were assessed for their support of biofilm formation by *C. albicans*, *S. mutans*, *Streptococcus sanguinis*, and *P. gingivalis.* Biofilms were evaluated in trios, always including *C. albicans*. *C. albicans* showed increased aspartyl-proteinase expression in biofilms with *S. mutans* and *S. sanguinis* compared to when alone. Adhesin quantification also showed higher expression in biofilms with *C. albicans*, *S. mutans*, and *S. sanguinis*, as well as in the four-microbial biofilm. The study also found that the *HWP1* gene, expressed only in the hyphal form and mediating interactions with oral epithelial cells, was upregulated in *C. albicans*, *S. mutans*, and *S. sanguinis* biofilms but not in the quadruple biofilm [85]. These results suggest that *P. gingivalis* may inhibit the expression of this enzyme. However, the inhibition could also be due to interactions between *P. gingivalis* and other microorganisms in the study, potentially involving metabolites or molecular mechanisms that are not yet fully understood. Understanding interactions that trigger increased virulence in these microbes provides insights into therapeutic targets and the clinical implications, identifying which microorganisms enhance virulence in others. Greater expression of adhesins and proteinases by *C. albican*s facilitates biofilm formation and, consequently, its pathogenicity [86].

More frequently studied models, such as *C. albicans* and *S. aureus*, have shown that yeast supports mixed biofilm formation in catheters, facilitating bacterial adhesion [87, 88]. Furthermore, *C. albicans* with *C. glabrata* or *S. mutans* mixed biofilm formation was evaluated on different materials used in dentistry. The study showed that *C. albicans* biofilm alone and in pairs with *C. glabrata* and *S. mutans* grew the most on hydroxyapatite, followed by polymethyl methacrylate and soft denture liner. In addition, *S. mutans* was shown to inhibit the formation of hyphae by *C. albicans*. *C. glabrata* also showed a higher CFU count compared to *C. albicans*. However, covering the materials with saliva reduced the *C. glabrata* CFU count [89], indicating that host parameters significantly influence the modulation of components of these microbial communities.

Therefore, it is unfeasible to fully understand how these populations behave in the host, requiring more studies that encompass the variable nature of the human organism. Furthermore, medical device insertion into *in vivo* models is crucial to draw a closer picture of what happens in the human organism.

Therapeutic Approaches

Microbial biofilms are remarkable drivers of infection in the nosocomial setting, often associated with increased morbidity and mortality in patients linked to medical devices [3], respiratory infections - such as CF [90], and chronic wounds [91]. The challenge of treating infections produced by biofilms is well-established in clinical terms. Microbial interactions in mixed biofilms can impact therapy and clinical outcomes [18].

While multi-species biofilms are known to increase antimicrobial tolerance [92], recent studies show that approved drugs can effectively eradicate them. For instance, voriconazole disrupts *C. albicans* and *Actinomyces viscosus* biofilms in root caries by regulating the ergosterol pathway [93]. Another study revealed that caspofungin effectively reduces biomass and cell viability in mixed biofilms of *C. albicans* and *S. aureus* by an enlargement of *C. albicans* cells and disruption of the cell wall of both *C. albicans* and *S. aureus* in mixed biofilms [94]. Amphotericin was tested by targeting *C. albicans* before adding *S. aureus* to pre-formed biofilms. The study showed that liposomal amphotericin significantly reduced *S. aureus* and MRSA cells in mixed biofilms [95]. Some strains of *S. maltophilia* exhibit a stronger inhibitory effect on *A. fumigatus*, which increased multi-species biofilms susceptibility to amphotericin B compared to *A. fumigatus* biofilms alone [96].

Synergistic combinations have also been explored against multi-species biofilms. Polymyxin B and amphotericin B were investigated for ventilator-associated pneumonia caused by *P. aeruginosa* and *C. albicans* biofilms on endotracheal tube surfaces. The combination of amphotericin B (0.0156 µg/mL) and polymyxin B (256 µg/mL) successfully eradicated polymicrobial biofilms [97]. Although polymyxin B can cause nephrotoxicity [98], the combination therapy can be used to develop functionalized medical devices, such as the surface of endotracheal tubes.

Moxifloxacin, meropenem, and caspofungin were evaluated for synergism against *S. aureus*, *E. coli*, and *C. albicans.* Meropenem was more effective against *E. coli* in biofilms with *C. albicans*. Moxifloxacin reduced biofilm biomass of single-species *S. aureus* or *E. coli* biofilms, but not of multi-species ones. Caspofungin significantly reduced two-species biofilms, particularly *S. aureus-C. albicans*. In both single and dual-species biofilms, caspofungin-antibiotic combinations had a synergistic effect. Moxifloxacin was more effective against *S. aureus*, and meropenem was more efficient against *E. coli* [99].

Tobramycin-posaconazole combination was highly effective against *P. aerugiosa and A. fumigatus* mixed biofilms. In contrast, cefepime and posaconazole combination

showed remarkable activity against *P. aeruginosa* biofilm but was less effective against two-species biofilms [100]. In mixed biofilms of *A. fumigatus* and *S. maltophilia*, amphotericin B-levofloxacin or rifampicin combination was effective against polymicrobial biofilms [96].

Regarding biofilm-related treatments in oral health, nystatin - widely used for candidiasis, and chlorhexidine - prescribed primarily for periodontal disease involving *S. mutans* [101], were investigated. The nystatin-chlorhexidine combination is controversial due to the formation of an ineffective low-solubility salt [102]. However, patients are sometimes prescribed both drugs concurrently. Nystatin-chlorhexidine combination against *S. mutans* in planktonic cells and mixed biofilms with *C. albicans* showed no difference from chlorhexidine. However, nystatin administration followed by chlorhexidine yielded better results for biofilm treatment, suggesting that order of administration impacts biofilm outcomes [103]. These findings suggest that the concomitant use of nystatin and chlorhexidine should be avoided, but, if necessary, nystatin should be administered first. These results emphasize the importance of studying sequential drug administration to enhance pharmacotherapeutic efficacy.

A synergistic minocycline-fluconazol combination was unrevealed in *C. albicans-S. aureus* biofilms. The combination inhibited 80% of 12-hour-biofilms formed by fluconazole-resistant *C. albicans* and oxacillin-resistant *S. aureus*. Still, no remarkable effects were found in 24-hour biofilms. The addition of EGTA, a chelating agent, and benedipine, a calcium channel blocker, also increased the synergistic potential of the drugs [104]. This combination was previously shown to inhibit azole-resistant *C. albicans*. Minocycline enhanced fluconazole penetration by increasing intracellular calcium release [105].

Polymyxin B-caspofungin combination was evaluated against *P. aeruginosa* (carbapenem-sensitive and resistant) and *Candida* spp. biofilms. An increase in minimum biofilm inhibitory concentration (MBIC) for single-drug treatments in multi-species biofilms compared to monobiofilms was observed. The caspofungin-polymyxin B combination significantly reduced total biomass in two-species biofilms, particularly those formed by *P. aeruginosa* and *C. glabrata* (70% reduction) [106]. Additionally, the synergistic effect of polymyxin B with azoles facilitates antifungal entry into cells, presenting a promising strategy for combined treatments.

The formation of *C. albicans-S. aureus* biofilms on catheters has been investigated *in vitro* and *in vivo* exploring the tigecycline-anidulafungin combination as alternative pharmacotherapy. The study demonstrated that anidulafungin effectively reduced *C. albicans* counts in single and mixed-species biofilms, while tigecycline reduced *S. aureus* counts in both biofilm types. However, tigecycline alone did not affect *C. albicans*, nor did anidulafungin affect *S. aureus*. Additionally, the study showed that anidulafungin enhances the antibacterial effect of tigecycline in *in vivo* biofilms. Furthermore, anidulafungin was shown to inhibit the synthesis of poly-β-(1,6)-N-acetylglucosamine (PNAG), a major component of the *S. aureus* biofilm matrix [107].

Here, most studies on multi-species biofilms involving clinically important fungi discuss the potential therapies employed in the nosocomial setting. However, other studies investigating the combination of FDA-approved drugs with natural substances [108–110] and polymeric devices [111–114] can be an innovative alternative approach to managing the multi-species biofilm infections and treating medical devices.

Conclusions

Biofilms pose a significant challenge in clinical settings due to their enhanced antimicrobial resistance and the protective microenvironment resulting less effective treatment. Here, the diverse interactions and implications of fungal-bacterial biofilms across various human anatomical sites were discussed. The interplay between fungi and bacteria within biofilms can alter susceptibility and virulence, hindering treatment. The synergistic effects of co-cultures of *C. albicans* with *S. aureus* or *P. aeruginosa* reveal how fungal biofilms can increase bacterial resistance to antibiotics [40, 58]. Controversially, not all inhibitory interactions in important pathogens lead to a positive prognosis for the host. The inhibition of *A. fumigatus* by *P. aeruginosa* led to enhanced elastase production, resulting in a significant increase in cytotoxicity [65], which may harm the host. These results suggest that the inhibitory nature of pathogens in clinical settings may not always benefit the patient outcome. Similarly, we can expect that synergistic interactions between microorganisms do not necessarily lead to negative consequences for the host.

Understanding the molecular mechanisms and environmental factors driving these interactions is crucial for developing effective therapeutic approaches. Advances in biofilm research have led to innovative treatments, including the use of combination therapies and targeting specific biofilm components to disrupt these resilient communities. The synergistic use of antibiotics and antifungals, as well as the development of new drug delivery systems, show promise in overcoming the challenges posed by multi-species biofilms. Despite these advances, more research is required to fully elucidate the mechanisms underlying biofilm persistence. Future studies should focus on the *in vivo* relevance of these interactions, considering the host's immune response, microbiota composition, and underlying health conditions. Additionally, exploring the potential of natural substances and polymeric devices as adjuncts to conventional therapies

could open new avenues for managing biofilm-associated infections.

Addressing the clinical implications of polymicrobial biofilms with medically important fungi requires a multifaceted approach, integrating molecular insights with innovative therapeutic strategies. Unravelling the complexities of these microbial communities, we can improve clinical outcomes and reduce the burden of biofilm-associated infections. Further, it will be possible to understand in a big picture the interactions that occur in biofilms involving fungi of medical relevance.

Key References

- Belizario JA, Bila NM, Vaso CO, Costa-Orlandi CB, Mendonça MB, Fusco-Almeida AM, et al. Exploring the Complexity of the Interaction between T. rubrum and S. aureus/S. epidermidis in the Formation of Polymicrobial Biofilms. Microorganisms. 2024;12.

– This study demonstrates how the timing of bacterial inoculation influences the biofilm structure, with early bacterial presence inhibiting fungal growth and later presence favoring fungal dominance.

- Vila T, Kong EF, Montelongo-Jauregui D, Van Dijck P, Shetty AC, McCracken C, et al. Therapeutic implications of C. albicans-S. aureus mixed biofilm in a murine subcutaneous catheter model of polymicrobial infection. Virulence. 2021;12:835–51.

– This study discusses how the presence of *C. albicans* in mixed-species biofilms stimulates the growth of *S. aureus*, leading to enhanced biofilm formation and increased bacterial biomass.

- Hernandez-Cuellar E, Guerrero-Barrera AL, Avelar-Gonzalez FJ, Díaz JM, Santiago AS de, Chávez-Reyes J, et al. Characterization of Candida albicans and Staphylococcus aureus polymicrobial biofilm on different surfaces. Rev Iberoam Micol. 2022;39:36–43.

– This study discusses how *C. albicans* contributes to the biofilm structure and stability, serving as a scaffold for *S. aureus* attachment, which preferentially adheres to the hyphal form of *C. albicans*.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of Interest The authors declare no competing interests.

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