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## Interactions of *Candida albicans* Cells with Aerobic and Anaerobic Bacteria during Formation of Mixed Biofilms in the Oral Cavity

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### Abstract

Biofilm is a compact coating formed on various artificial and physiologic surfaces by a population of microorganisms which in this habitat establish a close cooperation, exploiting both the physical interactions that stabilize the community and chemical cooperation, engaging numerous agents to modify the environment, i.e., to influence the acidity, nutrient acquisition, or oxygen availability. Microorganisms can also communicate using quorum-sensing molecules carrying specific messages. Some microbes temporarily dominate, while others are constantly replaced by different community members. But these co-operations or competitions have a deep sense—they serve to protect the whole community against the defense system of the host to assure survival. The oral cavity is inhabited by diverse microorganisms, including bacteria, but also yeast-like fungi from the genus *Candida* that stay under a tight control of the host as long as its immune system is not weakened; then these relatively mild commensals convert to dangerous pathogens that start the invasion often in collaboration with other microbes. Elongated hyphal forms of fungal cells favor the biofilm type of growth and communication with other microbes supporting immune resistance of the biofilm. In this chapter, we discuss the mechanisms of interactions between bacteria and *C. albicans* in the oral cavity, their communication, host responses, and possible strategies of anti-biofilm treatment.

**Keywords:** *Candida albicans*, biofilm, aerobic and anaerobic bacteria, quorum-sensing, host responses, anti-biofilm therapies

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## 1. The oral cavity: a common place for polymicrobial biofilm formation

The oral cavity comprises the most complex niches of the human body colonized by a wide variety of bacteria and fungi species. These commensal or often opportunistic and pathogenic colonizers tend to form biofilms—structured microbial communities, attached to natural or artificial surfaces, which are directly attributable to the virulence of these microorganisms and their ability to cause infections [1].

The variety of microbial species inhabiting the oral cavity results from the presence of two different functional surfaces, the mucosal surface and the teeth, representing various conditions in terms of nutrient and oxygen availability [2]. The microorganisms that early colonize the salivary pellicle on the tooth surface are streptococci such as the oral commensal—*Streptococcus gordonii*. Further biofilm formation involves some bridging microorganisms such as *Fusobacterium nucleatum* [3]. As the biofilm extends below the gum line and becomes subgingival plaque, more pathogenic, Gram-negative anaerobes such as *Porphyromonas gingivalis* or *Tannerella forsythia* are embedded [4].

For polymicrobial growth and survival in the human oral cavity, establishing a well-functioning community, i.e., biofilm, is essential. The formation of biofilm increases a resistance to antimicrobial agents and nutritional changes. However, the transition from planktonic type of growth to biofilm community requires many transcriptional and proteomic changes. Most of them concern co-aggregation/-adhesion processes, sensing diffusible signals, and metabolic interactions. Such a developed biofilm is still exposed to changes of nutrition and oxygen availability, pH fluctuation, antimicrobial properties of saliva, and is also modified by the contact with host tissues [5].

The latest studies of oral microbiome pointed at an opportunistic inhabitant of oral mucosa—the *Candida albicans* yeast-like fungus—as an important biofilm player among microbiota that contacts with mucosal tissues of the host. Under colonization or infection conditions, *C. albicans* adheres to tissues, interacting with a variety of host extracellular matrix molecules that promote adhesion to the host surfaces [6]. The adherence is strictly dependent on *C. albicans* ability to switch morphology between yeast and hyphal forms [7, 8].

Numerous observations supported a hypothesis that fungi have a beneficial or favorable role in maintaining the healthy balance between microbes and the host. On the other hand, *C. albicans* well adapted to constantly changing demands in the human host environment [9] seems to be able to use the different colonizing strategy under situations that emerge in the pathological disparities.

Tracking the yeast oral infections showed that the same initiating and bridging microorganisms composing biofilms were involved in the interaction with hyphal filaments of *C. albicans*, promoting co-colonization of these surfaces by yeast [10]. Moreover, the interactions between yeast and streptococci appear to be synergistic. In addition to providing adhesion sites, streptococci excrete lactate that can act as a carbon source for yeast growth [11]. On the other hand, *C. albicans* may provide bacteria with growth stimulatory factors, resulting from the nutrition metabolism [12] and can reduce the oxygen pressure to the level, preferred by streptococci.

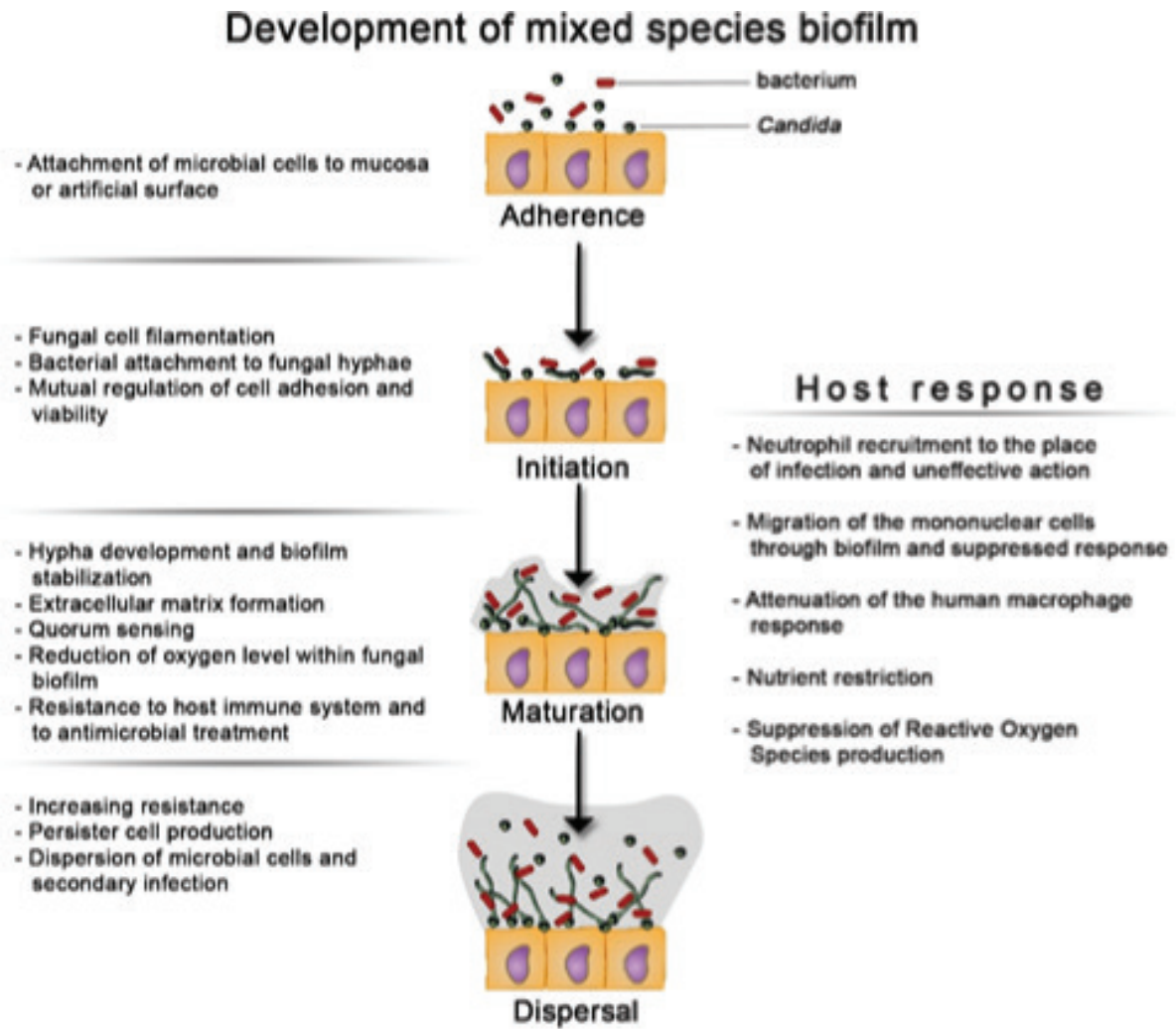
*C. albicans* also co-aggregates with an obligatory anaerobe, *F. nucleatum* [13], or a facultative anaerobe—*Actinomyces oris* [14].

Current studies [15–17] report that *C. albicans* biofilms protect the obligatory anaerobes, like *P. gingivalis* and *T. forsythia*, under aerobic culture conditions. It is possible because oxygen depletion within the structured fungal biofilm or its fast consumption by fungal cells results in creation of anaerobic micro-niches that help strict anaerobic bacteria to survive and proliferate. The observed depletion of oxygen could depend on the number of *C. albicans* cells or their respiratory rate.

## 2. Mechanisms and structures involved in formation of *Candida albicans* biofilms

The ability of *C. albicans* to form a biofilm is closely related to the virulence potential of pathogenic form of *C. albicans* and is characterized by a high heterogeneity among different clinical isolates [18, 19]. This form of fungal community depends on the cell surrounding and proceeds via sequential steps. The process starts with the initial adhesion of single yeast-like cells to the artificial or mucosal surface (**Figure 1**) and formation of a basal yeast cells layer [20–22]. During this phase both the surface properties on which microorganism form aggregate, its structure, charge, hydrophobicity, or roughness, as well as the structure of molecules present at the surface of pathogen cells play important roles [23]. In the further cell proliferation step, the fungi develop the filamentous hyphal form of the cells accompanied by production of an extracellular polysaccharide matrix in mature biofilm, which protects them, and strengthens the biofilm structure [24]. These processes lead to a significant increase in the thickness of the biofilm, and its maturation is controlled by at least nine master transcription regulators (Ndt80, Bcr1, Efg1, Rfx2, Flo8, Rob1, Brg1, Gal4, and Tec1) that supervise the network of about 1000 of targeted genes involved in biofilm formation [21, 25]. Then, the dispersion of biofilm-associated yeast-like cells can occur with further fungal cell dissemination, often associated with invasive diseases [24, 26].

Numerous different mechanisms and molecules are involved in the overall complex process of *C. albicans* biofilm formation [27]. Initially, the general physicochemical properties of *C. albicans* cell surface and subsequent activity of cell wall adhesive proteins play extremely important roles, allowing the cells to adhere to the targeted substrates or materials [28]. This essential group of molecules responsible for in vitro and in vivo biofilm development includes several proteins covalently bound to the fungal cell wall and equipped with a signal peptide for classical secretion and glycosylphosphatidylinositol (GPI)-anchor site, i.e., hyphal cell wall protein Hwp1 [29], proteins from agglutinin-like sequence (Als) protein family, such as Als1 and Als3 [28], and hyphally regulated cell wall protein Hyr1 [28]. Their transcription is regulated by the transcription factor Bcr1 [30] and is primarily associated with the morphological transition from yeast cells to filamentous forms, thus implicating their association mainly with the cell wall of hyphae [31–33]. Additionally, other adhesins are required for *C. albicans* adhesion and proper biofilm formation, including Eap1 (enhanced adhesion to polystyrene 1) protein present at the cell surface of both yeast cells and hyphal forms [34, 35]. Adhesion-related proteins are important not only for the binding of fungal cells to the receptors on host



**Figure 1.** Polymicrobial biofilm: stages of development and host responses.

tissues or to the artificial surfaces, but also for maintaining the cell-cell interactions within the biofilm that allow further stabilization of the structure and avoiding the removal of fungal cells by the action of host defense mechanisms such as the salivary flow. In the process of aggregation and intracellular interactions between fungal cells, fragments of adhesins that consist of amino acid sequences predicted to form amyloid-like  $\beta$ -aggregates and mediating amyloid formation may participate, as described for the protein Rbt1 (repressed by TUP1), a GPI-anchored cell wall protein with a similarity to Hwp1 [36, 37].

After the adhesion step, further proliferation of cells and production of filamentous forms lead to the enhanced development of biofilm [38], processes related not only to the change in the surface properties of fungal cells and the increase of their adhesiveness, but also to the production of further virulence factors and biofilm matrix components [24]. Among the large repertoire of extracellular hydrolytic enzymes produced by *C. albicans* that play a pivotal role during the invasion on host tissues during the infection and are involved in biofilm-related pathogenesis, representatives of families of lipases, phospholipases, and secreted aspartyl proteases (Saps) can be included [39]. The major biofilm-associated Saps are hypha-specific Sap5

and Sap6, responsible for the acquisition of nutrients, aggregation of fungal cells, intracellular communication, and production of extracellular matrix during biofilm development [40–42].

The highly complicated and heterogeneous extracellular biofilm matrix (ECM) is composed of numerous proteins (55%), carbohydrates (25%), mainly branched  $\alpha$ -1,6-mannans, unbranched  $\beta$ -1,6-glucans, and  $\beta$ -1,3-glucans, as well as lipids (15%), including neutral glycerolipids, polar glycerolipids and sphingolipids, and nucleic acids (5%) [43]. The matrix that strengthens the biofilm significantly contributes to the development of biofilm resistance to adverse environmental conditions and penetration of antifungal drugs [43–45]. Several matrix-associated proteins have been identified, both involved in the basic cellular metabolism, as well as the proteins responsible for the rearrangement of the matrix structure and maintenance of its functionality (glucan-modifying enzymes and protein mannosyltransferases, i.e., Xog1, Exg1, Bgl2, Pmt1, Pmt2, Pmt4, Pmt6) [46, 47]. Extracellular DNA (eDNA) detected in *C. albicans* biofilm matrix is probably mainly responsible for the structural integrity [48].

In the process of biofilm dispersion, the molecular chaperone, heat shock protein 90 (Hsp90) is strongly involved [49], affecting the morphogenetic transition from yeast cells to hyphal forms and repressing Ras1/PKA (cAMP-dependent protein kinase) signaling cascade [50]. Furthermore, the conserved histone deacetylase complex, including Set3, Hos2, Snt1, and Sif2 proteins also participates in the dispersal of biofilm, modulating the transcription kinetics of the genes that regulate biofilm maturation [51].

### **3. *C. albicans* interactions with bacteria during mutual biofilm formation**

*C. albicans* colonizes the oral cavity, presenting the commensal or pathogenic properties that can be modified by direct or indirect interactions with different types of bacteria, depending on the localization of the microbial communities, such as the supragingival plaque, subgingival plaque, and tongue coating. The metabolic activity of microorganisms that colonize the supragingival sites, i.e., nonmutans streptococci and *Actinomyces* enriches the environment in lactic acid, creating a temporarily acidic environment that favors the entrance of the more cariogenic microorganisms, mutans streptococci into the ecosystem. Conversely, at subgingival sites, colonized by *Fusobacteria* and *Prevotella*, a neutral pH and anaerobic environment dominate and facilitate the establishment of a less acid-tolerant but periodontopathogenic bacterium, *P. gingivalis* [52].

The mitis group of streptococci (MGS), including *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus gordonii*, and *Streptococcus sanguinis* [53, 54] belongs to the early colonizers of oral cavity and appears to interact synergistically with *C. albicans* hyphal filaments providing the physical and metabolic interactions by the exposition of specific adhesion sites and excreting lactate that serves as a carbon source for yeast growth [55]. On the other hand, *C. albicans* can provide bacteria with growth stimulatory factors, resulting from the fungal nutrition metabolism [12] and reduces the oxygen pressure to the level, preferred by streptococci. Moreover, a mutual collaboration of *C. albicans* and *S. oralis* increases the inflammatory host responses

compared to monospecies infections [56, 57]. The consequence for the host is provided by the precise interactions between the cell surface proteins of *S. oralis*, *S. mitis* and *S. gordonii*, especially SspA and SspB [58, 59] and the candidal cell wall adhesins of agglutinin-like family, mainly Als3, presented on the surface of fungal hyphae [60]. Some parts of these proteins seem to be particularly important for the interactions but a precise mechanism of the interaction requires further research, especially to clarify a significance of cell wall mannosylation [57, 61] in this process.

Moreover, the polysaccharides of both types of interacting microorganisms that compose ECM not only protect the cells, but also create a new platform for mutual interactions between fungal glucans and mannans and bacterial glucosyltransferases within the ECM matrix structure [46, 62, 63].

The best example of microbial cooperation for increased pathogenic properties of mixed biofilm is represented by the interactions of *C. albicans* with *S. aureus*, identified in oral cavity and being a source of systemic infection [64–67]. The bacteria prefer hyphal filaments of *C. albicans* [68, 69] for adhesion, but its localization within the biofilm seems to depend on the surface colonization sequence. When bacteria are the first colonizer, the development of fungal biofilm is slower and bacteria cells are spread in whole three-dimensional biofilm structure [68]. On the other hand, a simultaneous contact of microorganisms with the surface favors the rapid formation of the mixed biofilm with *S. aureus* localization within upper layers of fungal biofilm and with involvement of multiple microbial proteins. However, the role of fungal adhesins from Als family in these interactions has been questioned [70]. The formed biofilm and its ECM with extracellular fungal DNA protect the bacteria against the antibiotic treatment [71–73].

*C. albicans* co-aggregates with an obligatory anaerobe *F. nucleatum* [13], with engagement of a mannan receptor on the *C. albicans* surface [74]. A facultative anaerobe—*A. oris*—makes its own carbohydrate-containing surface molecules available to the interaction with *C. albicans* [14]. Last studies have also shown that *C. albicans* is able to interact with keystone pathogen of subgingival plaque—*P. gingivalis*—the obligatory anaerobe. However, it is difficult to judge whether the pathogens apply a synergistic or concurrence style of interactions. It was demonstrated that *P. gingivalis* suppressed *Candida* biofilm formation by a reduction of fungal cell viability [75]. Recently, the gingipain activity has been suggested to be the main destructive force influencing fungal cells wall within the mixed bacterial-fungal biofilm (unpublished data). On the other hand, *P. gingivalis* was also shown to induce germ-tube formation by *C. albicans* cells, generating a more invasive phenotype of fungal cells [76]. But such an effect could also result from fungal protection toward contacting bacteria and their virulence potential (unpublished data). The mutual interactions are supported by the involvement of adhesins, especially fungal Als3, Mp65 and surface-located enolase, and a bacterial internalin, InlJ or gingipains ([77], unpublished data). The important role in the interaction between *C. albicans* and *P. gingivalis* has been also assigned to peptidylarginine transferase of *P. gingivalis* (PPAD), the enzyme capable of modifying Arg residues to citrullines. Its action can directly contribute to the change in the spatial structure of the molecule [78]. A bacterial mutant deprived of PPAD forms a reduced mixed biofilm compared to the wild-type strain. Potential molecules whose citrullination may affect the effectiveness of biofilm formation include arginine-specific cysteine proteinase (RgpA) and adhesive Mfa1 fimbriin [17, 79].

The interaction of both microbes seems to exert marked consequences to the host. However, it was presented that both microbes appear to have antagonistic effects on one another, as *P. gingivalis* inhibited the adhesion of *C. albicans* to buccal epithelial cells [80]. But the presence of *C. albicans* did not enhance adhesion of *P. gingivalis* to gingival epithelial cells or gingival fibroblasts. On the other hand, a pre-exposure of gingival epithelial cells and fibroblasts to *C. albicans* enhanced the cell invasion by *P. gingivalis* [81].

#### 4. Host responses to the candidal biofilms

Clinical candidal oral biofilm inhabiting mucosal surfaces or artificial devices may trigger more or less similar host responses (**Figure 1**). Regardless of the biofilm origin, it remains under the influence of immune factors produced by contacting epithelial cells [82].

Dongari-Bagtzoglou et al. [83] analyzed the candidal biofilm in a murine model and found that this fungal cell community induced a hyperkeratotic response and epithelial cell desquamation. Moreover, the matrix that surrounded the fungal biofilm was enriched in keratin and desquamated cells.

Also, in a rat model of chronic denture gingival dermatitis, the host proteins were prominent in the extracellular matrix, including amylase, hemoglobin, and antimicrobial peptides [84, 85].

The oral biofilm elicits responses of human immune cells (**Figure 1**). The neutrophil migration and their deeper localization within the biofilm were identified, but these defense cells were not effective in clearing these infections [82]. An analysis of neutrophil responses in the ex vivo models of their contact with *C. albicans* biofilm also showed a diminished activity of neutrophils against this structured fungal community, compared to the responses against the planktonic form of fungal cells [86].

Upon a contact with pathogens, neutrophils can activate many mechanisms of response to suppress the infection. These include degranulation, phagocytosis, and neutrophil extracellular traps (NETs) formation [87]. The latter process aims at entrapment of large objects such as fungal hyphae [88]. Nevertheless, a group of Johnson showed that neutrophils failed to release NETs in contact with fungal biofilm [89]. These results were described as *an immunological silence*, where host immune system ignored contacting biofilm because of its shielding by the matrix components [90]. The biofilm matrix prevents the exposition of so called pathogen-associated molecular patterns (PAMPs) that can be recognized by highly specialized pattern recognition receptors (PRRs) of human immune cells [91, 92].

Another explanation proposed to interpret the evasion of host response by the biofilm was *an immune deviation* that could result from action of yet unidentified fungal compounds. They could act directly or indirectly by triggering host immunomodulatory factors that transform the immune response into ineffective form [93]. Such hypothesis was supported by the observation that *C. albicans* cell wall components were able to induce the expression of Il-10 influencing Th2 response [94].

A further explanation of biofilm survival was represented by a model of *immune resistance* proposed in [95], where GPI-anchored cell wall protein Hyr1 could play an important role



[96]. Moreover, all of proposed mechanisms or their combinations could be involved in local paucity of PMN responses. Katragkou et al. [86, 97] and Xie et al. [98] documented that developed biofilm covered by ECM exposed fungal  $\beta$ -glucans that were involved in hindered neutrophil responses to cytokine priming of PMNs or fungal cell opsonization. Such an argument was also strengthened by the observation that pre-treatment of PMNs with interferon- $\gamma$  or granulocyte colony-stimulating factor (G-CSF) did not significantly enhance their activity against opsonized or nonopsonized *C. albicans* biofilms. Moreover, neutrophils contacting the mature biofilm did not produce reactive oxygen species, necessary for triggering of phagocytosis, or one of the pathways of NET production [99]. Nevertheless, the precise mechanism of this phenomenon remained to be clarified.

Similar, diminished responses were also observed for a contact of fungal biofilm with mononuclear cells, compared to their co-culture with fungal planktonic form [100]. Although the migration of the mononuclear cells through biofilm was detected with their main compaction in the basal part of biofilm, their phagocytosis properties were suppressed, and the production of pro-inflammatory cytokines in response to biofilm decreased. Surprisingly, the mononuclear cells augmented biofilm proliferation, increasing the biofilm thickness over two-fold [97, 101].

Most of the presented observations were made concerning host response to contact with fungal biofilm, but the host immune system usually has to face an ongoing polymicrobial infection [102] about which the information are rather scarce [103]. An example of the cross-kingdom infection of the human host was represented by *C. albicans* biofilm contacting gingival anaerobic bacteria, *P. gingivalis*. In this case, an attenuation of the human macrophage responses was observed [17]. Moreover, some studies presented that the host responses can vary depending on the pathogen that contacts the fungal biofilm [104]. The pathogen interactions can be synergistic as well as antagonistic. For example, in a rat model, the colonization of the airway by *C. albicans* impaired functions of alveolar macrophages and, in consequence, led to the reduced clearance of *Pseudomonas aeruginosa* [105]. On the other hand, Lopez-Medina et al. [106] showed in a mouse gut model that the co-infection of *P. aeruginosa* with *Candida* cells suppressed the expression of bacterial genes responsible for iron acquisition, and thus suppressed the infection. Nevertheless, our understanding of host responses to mixed biofilm formed between different type of pathogens remains still at its infancy and needs many further studies.

## **5. Quorum sensing within the mixed biofilm and its significance for the host**

An important phenomenon occurring in the process of biofilm formation is also the transmission of signals between microbial cells located within the biofilm, thus stimulating them to further growth and dispersion of the cells or, in contrary, suppressing them. In addition, signaling molecules can also affect microbial cells of other species that inhabit the same niche in the host organism, and thus promote synergistic or antagonistic interactions between different pathogens which can result in clinical outcomes. This phenomenon of the communication between microorganisms through the secretion of low molecular weight compounds, referred

to as the quorum sensing (QS) [107], involves specific chemical compounds whose increasing concentration is a signal to change the expression of selected genes in the cells of the entire biofilm population [108].

*C. albicans* produces autoregulatory substances involved in quorum sensing (quorum-sensing molecules, QSMs) that affect important virulence traits, such as transformation of the morphological forms [109]. One of them is farnesol—an alcohol from the terpene group, secreted by *C. albicans* in the later stages of biofilm formation, with a function of blocking the formation of filamentous forms of this yeast [110]. A function opposite to farnesol has a second fungal QS compound, tyrosol, which stimulates the phase of active growth of the *C. albicans* cell population and the formation of hyphae in the initial phases of biofilm formation, thus increasing the thickness of the biofilm [111–113].

When the concentration of farnesol is higher than that of tyrosol, the conversion of yeast form to hyphae is inhibited and a release of individual cells from the biofilm is stimulated. Such effect indicates possible interactions between these two QS systems in the process of biofilm building [112]. Additionally, *C. albicans* secretes two aromatic alcohols, phenylethyl alcohol and tryptophol, also identified as QSM [113].

The role of QSM seems to be particularly important in mixed biofilms, in which the coexistence of fungi and bacteria is associated with their mutual communications. QSM secreted by the bacteria can exert both stimulatory and inhibitory effects on the cell morphology and biofilm formation by *C. albicans* cells. It is likely that a combination of these contradictory signals orchestrates the balance between the cellular and filamentous form in biofilms, preventing the excessive growth of *C. albicans* within these communities [114].

One example of cross-species communication using QS signals are biofilms formed between *C. albicans* and Gram-negative bacteria *P. aeruginosa*. It has been shown that the presence of farnesol produced by *C. albicans* inhibits functioning of bacteria, and suppresses the production of a bacterial quinolone signaling molecule—PQS, and the piocyanin—an important bacterial virulence factor [115]. On the other hand, under formation of mixed biofilms, *P. aeruginosa* produces homoserine lactone that may fulfill a role similar to farnesol, reducing the production of fungal hyphae *in vitro* [116].

Another example is the biofilm with participation of *S. mutans*, in which the inhibition of biofilm formation was observed in response to a high concentration of farnesol (>100  $\mu\text{M}$ ), while a low level of farnesol (~25  $\mu\text{M}$ ) promoted bacterial growth [117–119].

In other studies, *S. mutans* could both, reduce the farnesol production by *C. albicans* [119] and inhibit the formation of filamentous form of *C. albicans* by the competence-peptide CSP, produced on the early stages of biofilm development [120, 121].

The same peptide produced by *S. gordonii* inhibited the formation of *C. albicans* biofilm, but not the hyphal growth [122]. In contrast, other bacterial QSM—the autoinducer-2 (AI-2), as well as  $\text{H}_2\text{O}_2$  secreted by *S. gordonii* affected the morphogenesis and production of farnesol. The strains with the deletion of the LuxS quorum-sensing system responsible for AI-2 production in *S. gordonii* presented a reduced ability to stimulate the growth of *C. albicans* hyphae

and thereby a general reduction of biofilm biomass. The identified responses correlated with an invasion into the host epithelial cells [10, 27].

An interesting interspecies communication is presented by the Gram-negative *Aggregatibacter actinomycetemcomitans*, acting in periodontal disease, which can inhibit the formation of *C. albicans* biofilm by producing AI-2. Although AI-2 has been described as QSM of different bacteria, other species give off other AI derivatives, so that the results obtained for different species do not have to be identical to one another. Interestingly, *A. actinomycetemcomitans* is one of the bacterium having a dual inhibitory system acting toward *C. albicans* biofilm. In addition to QSM, it also includes cytolethal distending toxin (CDT). One of the emerging hypotheses suggests that secreted QSM is a warning signal for *C. albicans* against a competitor that secretes the toxins [120, 123].

QSM also plays an important role in a communication between *C. albicans* and the Gram-positive bacterium—*S. aureus*. Farnesol, secreted by *C. albicans* inhibits the formation of *S. aureus* biofilm and increases its susceptibility to antibiotics [124, 125]. There were also studies, indicating that *S. aureus* stimulated the growth of *C. albicans* biofilm possibly by QSM [69]. It was also proposed that in the presence of farnesol, *S. aureus* acquires a resistant phenotype that induces oxidative stress, resulting in the upregulation of bacterial drug efflux pumps [126].

QS production within the biofilm has also an impact on the efficiency and the functioning of host defense systems. The gingival epithelial cells presented an upregulation of the toll-like receptor TLR2, and a decrease of the expression of TLR4 and TLR6 upon treatment with farnesol, suggesting the resulting activation of antifungal defense. Considering the role of epithelial cells in the secretion of pro-inflammatory cytokines, it was also shown that farnesol increased the secretion of IL-6 and IL-8. Moreover, farnesol modulated the secretion of antimicrobial peptides by epithelial cells, including hBD1 and hBD2. *C. albicans* cells, via production of farnesol, suppressed the epithelial secretion of hBD1, with a simultaneous increase in hBD2 secretion. Since both peptides have a high efficacy in *C. albicans* killing, the results suggest that farnesol may be a key factor in promoting host defense [127]. An additional function performed by farnesol includes its ability to activate neutrophils and monocytes and to reduce the phagocytic activity of mouse macrophages. Farnesol also impairs the differentiation of monocytes into dendritic cells and decreases their ability to activate and expand T cells, which consequently reduce the induction of IL-12 [128].

In summary, mutual QS interactions between fungi and bacteria may play an important role as a virulence mechanism that mediates the communication between the host and the formed biofilm, and could inspire future applications in diagnostics and biofilm treatment.

## 6. Resistance of oral biofilm

The biofilm formed on mucosal or artificial surfaces in oral cavity is difficult to eliminate since the biofilm structure protects the pathogenic cells against antimicrobial drugs, especially against antifungal agents, and suppresses immune responses [129]. Moreover, cooperating invaders often present increasing virulence resulting from synergistic and complex

interactions between microorganisms [130]. It has been demonstrated that *Staphylococcus* adheres to yeast and hyphal forms, and this interaction benefits the growth and antibiotic resistance of *S. epidermidis*. In addition, the components of the biofilm extracellular matrix produced by the wild-type of *S. epidermidis* prevent the effective penetration of antimycotic molecules such as fluconazole into the biofilm and promote the spread of yeast infection [131].

The low susceptibility of biofilms to medical treatment is attributed to multifactorial events, represented by upregulation of efflux pumps, the presence of extracellular matrix and appearance of recalcitrant persister cells [132].

The two classes of fungal efflux pumps (FEP: Cdr1, Cdr2, and Mdr1) are activated in planktonic cells in contact with antifungal drugs but in biofilms FEP are upregulated probably in response to contact with other partners that compose the biofilm. Such an explanation was supported by an observation that FEP efficient function appeared shortly after the cell surface adhesion and remains upregulated during whole process of mixed biofilm formation [21].

Another contributor to mixed biofilm resistance is the extracellular matrix and its components. This three-dimensional complex structure effectively inhibits antibiotic and antimycotic diffusion [133]. Moreover, the biofilm-composing polysaccharides not only mask the biofilm against its recognition by the host receptors, but also can directly bind and inactivate the drugs, as it was presented in a case of antifungal-acting amphotericin B, sequestered by  $\beta$ -1,3-glucan, composing ECM [134].

Also, eDNA is an especially important biofilm component, whose viscosity and negative electric charge influences the structural integrity and stability of biofilms but also contributes to drug resistance via acting as drug chelator. eDNA also binds magnesium ions, whose decreased level serves as a signal, inducing PhoPQ and PmrAB systems, responsible for *P. aeruginosa* resistance to antimicrobial peptides and to aminoglycosides [135].

An important phenomenon that plays a key role in the development of drug resistance by oral microbiome is the horizontal gen transfer (HGT) [131]. The biofilm structure provides a suitable environment for gene exchange, because the microbial cells are in close proximity and the virulence genes are dynamically spread between different species of bacteria composing biofilm. The most popular mobile genetic element in oral microflora is the conjugative transposon *Tn916*, which contains genes encoding ribosomal protection proteins [131]. These proteins inhibit the action of tetracycline, the most popular antibiotic used in periodontal disease treatment, by preventing the binding of this antibiotic to the bacterial ribosome [136]. Another biofilm protective function is carried out by membrane vesicles (MVs), present in ECM [137], which protect bacteria against some antibiotics by the degradative properties of MV enzymes, such as  $\beta$ -lactamase [138].

The important factors that contribute to the biofilm resistance are the persister cells detected in bacterial and fungal biofilm [20, 139]. The persister cells are a minor subset of metabolically dormant cells presented within biofilms that possess extreme resistance to antimicrobial agents and are responsible for the severe chronic infectious disease. However, the mechanism of this resistance of persister cells remains to be discovered; they could possibly be a good target for further antimicrobial therapies.

## 7. The challenges for medical treatment of mixed oral biofilm

As no biofilm-specific drugs exist today, the treatment of infections caused by mixed species community remains a major challenge for contemporary medical biotechnology and the developing of new effective strategies for biofilm eradication becomes critical.

One of the strategies for combating biofilms formed by bacteria and yeasts can be a degradation of ECM. It has been demonstrated that enzymatic degradation of some biofilm-forming components facilitates the penetration of antibiotic and antimycotic molecules and affects the biofilm structural integrity [140, 141]. For example, a study demonstrated that a combined use of deoxyribonuclease and amphotericin B reduced the survival of *C. albicans* cells.

An effective alternative to antibiotic therapy may be a treatment with anti-biofilm peptides. These compounds easily penetrate the structure of multispecies biofilm and inhibit the growth of Gram-positive and Gram-negative bacteria. An example of such an anti-biofilm compound is a short synthetic peptide 1018 (amino acid sequence: VRLIVAVRIWRR), which blocks a stress response through an activation of the stress-signaling nucleotide degradation [142]. Another example of an anti-biofilm compound is D-enantiomeric peptide DJK-5 that has a similar mechanism of action to peptide 1018 [143]. The main advantage of the DJK-5 is its resistance to proteases produced by the host and bacteria. Moreover, DJK-5 possesses a higher biological activity than peptide 1018 and kills most of the oral biofilm-forming bacteria in a few minutes. It has been demonstrated that the use of anti-biofilm peptides in combination with conventional antibiotics both increases the effectiveness of treatment and reduces the required concentration of antibiotics [144].

Several natural products have been also proposed for fungal biofilm treatment. An example of plant metabolites with antifungal activity are terpenoids, such as xanthorrhizol extracted from *Curcuma xanthorrhiza* [145]. It has been demonstrated that this compound effectively inhibits the development of mature biofilms formed by various *Candida* species. Moreover, in contrast to commonly used antifungal drugs, xanthorrhizol is nontoxic to human cells even at very high concentrations.

Also, chemical signal molecules involved in quorum sensing possess a potential for the therapy of oral infections disease. There are two main mechanisms of action of the known QS inhibitors [146]. Some of these cause an enzymatic degradation of signaling molecules. The enzymes—AHL-lactonases and AHL-acylase can be classified to this group. Other inhibitors such as furanones that are produced by red marine algae are structural analogs that prevent bacterial biofilm development via binding to LuxR [147]. In the case of oral *C. albicans* infections, the use of farnesol has been proposed [148]. In vivo studies have shown that the addition of farnesol suppresses the hyphal growth on the mouse tongue at the first step of biofilm formation, and as a result prevents the invasion of mucosal membrane by the yeast and bacteria.

An interesting proposal for the treatment of mixed biofilm can be the photodynamic antimicrobial chemotherapy (PACT) that applies the nontoxic dye (photosensitizer) activated by visible light [149]. Singlet oxygen, which is effectively produced during this process, effectively kills pathogen cells. This novel method has been successfully used against *C. albicans* biofilm

and can be a promising antimicrobial therapy that has many advantages such as the high target specificity. What is more, the development of resistance to PACT is unlikely because microorganisms have no resistance mechanism against singlet oxygen [150].

A better understanding of the molecular mechanisms underlying the formation and maintenance of the mixed species biofilm is crucial for the development of their effective treatments in the future.

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