6 Fungi—A Component of the Oral Microbiome Involved in Periodontal Diseases

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Abstract

The human oral cavity is a diverse ecological niche favorable for colonization by hundreds of different species of microorganisms. They include not only bacteria but also numerous species of fungi, many of which are able to cause opportunistic infections when the host's immunity is impaired, predominantly by systemic and chronic diseases like diabetes, pulmonary diseases, renal disorders, or acquired immunodeficiency syndrome. Within the dental biofilm and subgingival sites, fungi of the genus *Candida* are often found, also in individuals affected with periodontitis. Moreover, fungal species of other genera, including *Malassezia*, *Aspergillus, Penicillium*, and *Rhodotorula* were identified in the oral cavity as well. The wide range of various virulence factors and mechanisms displayed by fungal pathogens allows them effectively invading host tissues during periodontal infections. These pathogenicity-related mechanisms include firstly the fungal ability to adhere successfully to the host tissues closely related to the formation of hyphae, the increase in the surface hydrophobicity, and the surface display of a wide variety of adhesins. Further mechanisms include biofilm formation and secretion of an armory of hydrolytic enzymes and toxins enabling the attack on host cells, modulation of the local inflammatory state, and evading the host immune system. In the pathogenesis of periodontitis, the significant role of fungal co-existence with key bacterial periodontopathogens has been demonstrated, and such interactions were primarily confirmed for *Candida albicans* and *Porphyromonas gingivalis*, where the presence of fungi ensured the survival of strictly anaerobic bacteria under unfavorable aerobic conditions. However, several other mechanisms, including those related to the production of quorum sensing molecules, might also be indicated as particularly important for synergistic or antagonistic interactions with a variety of bacterial species within mixed biofilms. These interactions constitute an extraordinary challenge for applying effective methods of combating biofilm-related infections in the periodontium without the risk of the development of drug resistance, the recur-

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rence of disease symptoms, and the progress of life-threating systemic complications.

Keywords

Fungi · *Candida* · *Aspergillus* · Virulence factors · Biofilm · Fungal-bacterial interaction · Periodontium

Abbreviations

Als	Agglutinin-like sequence protein family
ECM	Extracellular matrix
EPM	Extracellular polysaccharide matrix
GAG	Galactosaminogalactan
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hwp1	Hyphal wall protein 1
NET	Neutrophil extracellular trap
NO	Nitric oxide
ROS	Reactive oxygen species
Sap	Secreted aspartic proteinase
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
QSM	Quorum-sensing molecules

Highlights

- Several fungal species from genera *Candida*, *Rhodotorula*, *Penicillium*, *Aspergillus*, and *Malassezia* belong to the human oral microbiome and some of them may be important in the pathogenesis of periodontitis
- Conditions associated with decreased immunity may lead to the development of periodontal disease associated with the presence of fungi in subgingival sites
- Several fungal virulence factors, including adhesins, toxins, and hydrolytic enzymes, may contribute to the development of periodontitis
- The coexistence of fungi with key bacterial periodontopathogens within the periodontium may affect the pathogenesis of periodontitis

Considerations for Practice

- The documented relationship between periodontal diseases and a healthy lifestyle confirms that proper oral hygiene and smoking cessation may increase the chances of long-term oral health
- Controlling and systematically removing the biofilm from supragingival sites is an effective method of maintaining healthy gums
- In patients with chronic diseases like diabetes mellitus, chronic pulmonary diseases, or chronic kidney diseases, the prevention and treatment of periodontitis should be planned individually

Patient Summary

The most common periodontal diseases are caused by various species of bacteria that colonize the oral cavity along with a broad spectrum of fungal pathogens. Bearing in mind the noticeable role of fungi in the pathogenesis of periodontal diseases, the effective treatment should consider the increased resistance of mixed subgingival dental biofilm to antibiotics and antimycotics. The use of appropriately selected therapy increases the chances of a complete cure for periodontal diseases and reduces future recurrence risk.

6.1 Fungal Species as a Part of the Microbiome of the Human Oral Cavity

The human oral cavity has one of the most diverse microbiomes, comprising about six hundred species of bacteria and about one hundred fungal species (Ghannoum et al. 2010; Dewhirst et al. 2010; Baumgardner 2019). Its humid, warm, and nutrient-rich environment is divided into several niches, including the tongue, cheeks, palate, tonsils, gingival pockets, teeth, and saliva, which enable the coexistence of numerous microorganisms within the complex and protecting structure which is a biofilm. A well-known example is a dental biofilm in which various bacterial species may collectively coexist and form communities both above the gingival line (supragingival sites) and below the gingival line (subgingival sites) (Könönen et al. 2019). Collaborating, they can modulate the host's defense response, thus ensuring successful colonization associated often with the development of two of the most common oral cavity disorders—caries and periodontitis (Marsh 2006; Murakami et al. 2018; Valm 2019).

Contrary to bacteria, fungal microbiome profile has been so far poorly characterized in particular with regard to species belonging to genera other than *Candida*. Furthermore, their presence in the oral cavity is often limited to diseases associated with mucosal infections (Hellstein and Marek 2019). The first study characterizing the fungal microbiome in healthy people's oral cavity was presented a decade ago and identified 101 species of fungi from oral wash samples using molecular biology methods (Ghannoum et al. 2010). The most frequently isolated genera were *Candida*, *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, and *Cryptococcus*. The later studies additionally indicated *Penicillium*, *Schizophyllum*, *Rhodotorula*, and *Gibberella* as fungi commonly found in the oral cavity of healthy people (Monteiro-da-Silva et al. 2014; Peters et al. 2017).

Despite the widespread use of oral hygiene products, the periodontal disease remains one of the most common diseases affecting adults and children (Kinane et al. 2017). Supragingival microbes are responsible for the mild course of gingivitis, and root caries disease. Subgingival species accelerate the destruction of the tissues that support the teeth and might cause severe periodontitis (Shi et al. 2018a; Könönen et al. 2019). The main species in the supragingival sites are *Candida*, *Malassezia*, *Cryptococcus*, *Saccharomyces*, *Trichoderma*, and *Cladosporium*, but their abundance fluctuates when caries occurs (Fechney et al. 2019; Baraniya et al. 2020). Despite the diverse microbiome of individual niches in the oral cavity, the only species isolated from the subgingival sites of healthy individuals were *Candida albicans* and *C. dubliniensis* (Reynaud et al. 2001; Urzúa et al. 2008; Canabarro et al. 2013; Babitha et al. 2018), which suggests that periodontal pockets are not a favorable site for fungal microorganisms (Urzúa et al. 2008; Jewtuchowicz et al. 2008). However, in patients with chronic periodontitis, *C. dubliniensis*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* were abundantly isolated (Babitha et al. 2018; De-la-Torre et al. 2018), but there was no correlation between the degree of colonization and the depth of periodontal pockets (Babitha et al. 2018). Moreover, species such as *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis*, and *Rhodotorula* spp. have been identified in the subgingival biofilm of patients with severe chronic periodontitis but always as associated with *C. albicans* (Canabarro et al. 2013). The diversity of fungal communities in the oral cavity is summarized in Table 6.1.

6.2 Factors Predisposing to Fungal Infections in the Periodontium

Among several factors predisposing to the development of periodontitis associated with an oral fungal infection should be considered specific genetic conditions, primarily increasing the susceptibility of particular individuals to the progression of this inflammatory-related infectious disease. However, other risk factors are important, including acquired systemic diseases that weaken the human organism's ability to defend

Table 6.1 The most common species of fungi isolated from selected niches within the oral cavity

	Dental biofilm	Subgingival sites	Root canals
Candida			
C. albicans	+ [a]	+, # [b-k]	+ [l, m]
C. glabrata		# [b, f-i, k, n, o]	+ [p, r]
C. tropicalis		# [b, e, h, i, k, n, o]	
C. parapsilosis		# [b, e, k, n]	
C. dubliniensis	+ [a, s]	+, # [b, c, e, t]	
C. krusei	# [u]	# [k, o]	
other *Candida* spp.	# [u]	# [k]	+ [p, r]
Malassezia	+ [a]		
Saccharomyces	+ [a]		
Aspergillus	+ [a]	# [j]	# [w, x]
Penicillium	+ [a, s]		# [w]
Rhodotorula	+ [a, s]	# [e]	+ [m]

+ fungi identified in healthy people's oral cavity, # fungi identified in patients with periodontal disease, [a](O'Connell et al. 2020); [b](Babitha et al. 2018); [c](Urzúa et al. 2008); [d](Reynaud et al. 2001); [e](Canabarro et al. 2013); [f](Melton et al. 2010); [g](Hammad et al. 2013); [h](Sardi et al. 2011b); [i](Matic Petrovic et al. 2019); [j](Kamma et al. 1999); [k](Brusca et al., 2010); [l](Baumgartner et al. 2000); [m](Egan et al. 2002); [n](De-la-Torre et al. 2018); [o](Santhana Krishnan et al. 2020); [p](Waltimo et al. 1997); [r](Waltimo et al. 2003); [s](Fechney et al. 2019); [t](Jewtuchowicz et al. 2008); [u](Brusca et al. 2010); [w](Gomes et al. 2010); [x](Gomes et al. 2015)

against oral opportunistic pathogens. The specific environmental risk factors should not be underestimated either (Fig. 6.1) (Page and Kornman 1997; Johansson and Dahlén 2018).

One of the main medical conditions that might contribute to the onset or exacerbation of pre-existing symptoms of fungal-related periodontal disorders is the infection with the human immunodeficiency virus (HIV) and the subsequent development of acquired immunodeficiency syndrome (AIDS) (Aas et al. 2007). The analysis of samples taken from HIV-positive patients with periodontal lesions showed intense colonization of the oral cavity by yeasts *C. albicans* and *S. cerevisiae* (Aas et al. 2007).

The development of periodontal disease may also be influenced by cancer diseases leading to general immunosuppression. Qualitative and quantitative changes in the oral microbiome have been reported in patients with hematological neoplasms, where, in addition to *C. albicans*, have been found other *Candida* species—*C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* or *C. kefyr* (Schelenz et al. 2011; Aslani et al. 2018).

The incidence of prolonged inflammation related to periodontitis has been frequently reported as associated with chronic systemic diseases, and the observed relationship was often suggested to be bidirectional, where the occurrence and severity of one condition correlated with an increased risk of the other disorder (Wahid et al. 2012; Casanova et al. 2014; Hou et al. 2017). Although more extensive research on this issue is still required due to the high heterogeneity of the previously reported studies, the interdependence of periodontitis and diabetes mellitus, chronic pulmonary diseases, or chronic kidney diseases has been suggested so far (Taylor et al. 2004; Shi et al. 2018b; Zhao et al. 2018; Kapellas et al. 2019; Norhammar et al. 2019). Under such concomitant conditions weakening the immune system, *Candida* yeasts were often identified within the group of microorganisms isolated from the subgingival dental biofilm in individuals suffering from periodontitis (Javed et al. 2009; Bastos et al. 2011; Sardi et al. 2012a; Matić Petrović et al. 2015). In the case of patients with well-controlled and poorly controlled diabetes mellitus, both the most common *Candida* species—*C. albicans*—and other species of the genus, including *C. glabrata*, were isolated from periodontal tissues affected by inflammation (Melton et al. 2010; Hammad et al. 2013). It was also previously reported by Sardi et al. (2011b) that patients with co-morbid chronic periodontitis and diabetes showed greater colonization of the periodontal pockets and furcation sites by various species of the genus *Candida*, with the predominance of *C. dubliniensis* and *C. albicans*, in comparison to non-diabetics. Also, in these studies, two other *Candida* species—*C. tropicalis* and *C. glabrata*—were identified in subgingival biofilm exclusively in diabetic patients (Sardi et al. 2011b). Other studies also indicated that the prevalence of *Candida* species in subgingival sites was higher in diabetics with poor glycoregulation, and the most frequent species isolated

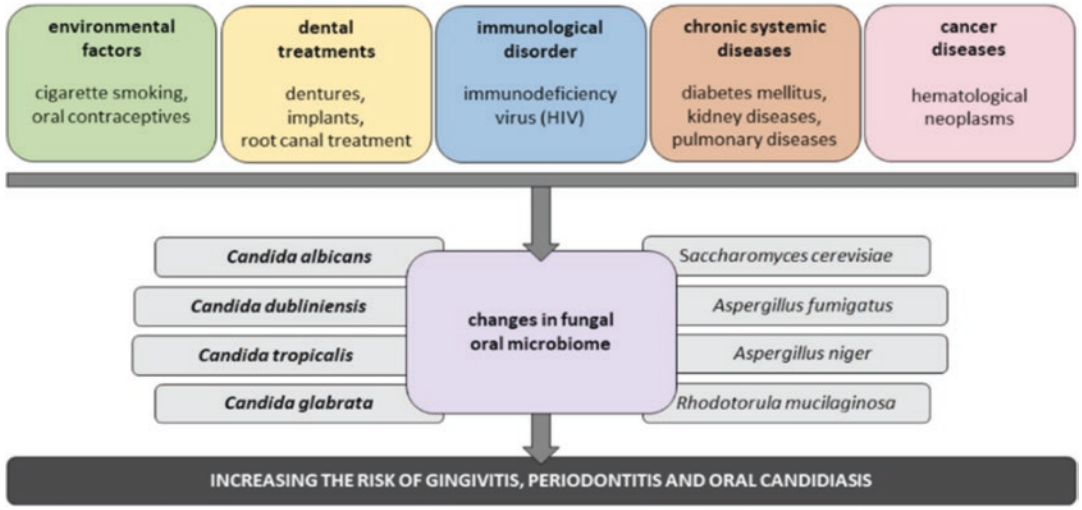

Fig. 6.1 Factors predisposing to oral fungal infections Environmental factors, dental treatments, chronic and immunological diseases contribute to the differentiation of the oral microbiome profile. Qualitative and quantitative changes in the fungi present in the oral cavity are associated with an increased risk of periodontal diseases. The species of fungi whose relationship with the development of periodontal diseases is discussed in the text below are in bold

from subgingival areas in such patients was *C. albicans* followed by *C. glabrata* and *C. tropicalis* (Matic Petrovic et al. 2019). Furthermore, it was also shown that in the case of patients with chronic kidney disease, periodontitis was characterized by greater severity and increased frequency of *C. albicans* and red-complex bacteria, compared with a control group of patients (Bastos et al. 2011).

Other factors that increase periodontal disease risk are dental procedures, such as endodontic treatment, which expose recessed dental structures. The most commonly isolated species from infected root canals were *C. albicans*, *R. mucilaginosa, C. glabrata*, *C. guilliermondii* and *C. inconspicua*; however significant correlation with the presence of these yeasts in saliva was found (Waltimo et al. 1997, 2003; Baumgartner et al. 2000; Egan et al. 2002). *C. albicans*, as a dentinophilic species with an invasive affinity for the dentin smear layer and dental tubules, can form a complex biofilm in root canals. The possibility to dentin colonization provides yeasts access to nutrients and protects them from the action of antifungal drugs, thus these species becoming one of the causes of persistent cases of apical periodontitis (Siqueira and Sen 2004; Ghogre 2014). In addition to the *C. albicans* species, in root canals with pulp necrosis and periapical lesions, filamentous fungi of the genus *Aspergillus*, *Penicillium*, and *Fusarium* were also identified (Gomes et al. 2010, 2015); however, they have not yet been identified as dentinophilic species (Siqueira et al. 2002). Besides, the implant sites have been shown to provide an attractive microenvironment that fosters uncontrolled microbial biofilms (Øilo and Bakken 2015); however, the fungal profile depends on the implant's clinical condition. *C. dubliniensis* and *Cladosporium cladosporioides* predominate in healthy implantation sites. In contrast, in samples taken from peri-implantation sites, dominate *C. albicans*, *C. boidinii*, *Penicillium* spp., *R. laryngis*, *Paecilomyces* spp., *Saccharomyces* and *Cl. cladosporioides*. The coexistence of fungi with *Parvimonas micra* and *Tannerella forsythia* was found in healthy and diseased peri-implant sites (Schwarz et al. 2015).

One of the most important risk factors for periodontitis associated with environmental conditions and the chosen lifestyle is cigarette smoking (Tomar and Asma 2000). The samples of

subgingival sites obtained from smokers with early-onset periodontitis were characterized by the noticeably higher incidence of bacterial periodontopathogens and fungi *A. fumigatus* and *C. albicans* (Kamma et al. 1999). Current research confirms the presence of other species of the *Candida* genus in the subgingival sites and saliva of smokers with chronic periodontitis, including *C. krusei*, *C. tropicalis* and *C. glabrata* (Santhana Krishnan et al. 2020). Moreover, it has been demonstrated that both cigarette smoke and e-cigarette vapor might significantly increase the overall virulence potential of *C. albicans* and enhance the adhesion of fungal cells to the gingival epithelial cells and fibroblasts, thereby increasing the pathogenicity of fungi during infections localized in the oral cavity and periodontium (Alanazi et al. 2014, 2019).

Other environmental factors may also contribute to the increased colonization of subgingival sites by *Candida* fungi, including changes in the hormonal balance caused by oral contraceptives. As reported by Brusca et al. (Brusca et al. 2010), the oral administration of ethinyl estradiol, gestodene, and drospirenone, combined with cigarette smoking, caused a statistically significant increase in the incidence of severe periodontitis, and under such conditions, different species of the genus *Candida*, including *C. parapsilosis*, *C. glabrata, C. tropicalis, C. krusei*, *C. albicans*, and *C. guilliermondii* were isolated from the gingival pockets. In most cases, yeast isolates were identified together with bacterial periodontal pathogens, such as *Actinobacillus actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis* (Brusca et al. 2010).

6.3 Virulence Factors Involved in the Fungal Invasion of Periodontal Tissue

The participation of fungi in the development of periodontal disease might be related to the exploitation of a number of virulence factors by fungal pathogens that allow them to efficacious adhesion to host tissues followed by their invasion and destruction as well as effective avoiding the host's immune response (Fig. 6.2). The mechanisms related to the pathogenicity of fungi are based on the morphological polymorphism and the formation of adhesive filamentous forms, production of protective cellular envelopes, exposition of surface adhesins, biofilm formation and the ability of intercellular communication within it, enhancement of environmental stress resistance, and secretion of hydrolytic enzymes and toxins (Karkowska-Kuleta et al. 2009). Also, the coexistence of fungi with bacterial key periodontopathogens in the subgingival sites is considered as a factor contributing to the pathogenicity of fungi identified in the periodontal lesions (O'Donnell et al. 2015; Delaney et al. 2018).

6.3.1 Adhesion and Biofilm Formation

The adhesion of pathogen cells to the host tissues or the surface of artificial materials located in the oral cavity is one of the first and crucial steps in initiating colonization and further invasion by a microorganism (Nikawa et al. 2006). In fungi, there are several mechanisms associated with the enhancement in cell adhesiveness and surface hydrophobicity, including the presentation of a wide range of different adhesive proteins and N-linked glycans on their cell surface (Glee et al. 1995; Masuoka and Hazen 2004; El-Kirat-Chatel et al. 2015; Hoyer and Cota 2016). These properties are also associated with the microbial ability to form biofilms as structures more resistant to adverse environmental conditions and the host's immune system activity (Kucharíková et al. 2015; Silva-Dias et al. 2015).

The most frequently described yeast adhesins, that are typically multidomain proteins equipped with N- and O-linked polysaccharide chains and a glycosylphosphatidylinositol (GPI) anchor, are agglutinins and flocculins of *S. cerevisiae*, lectin-like Epa (epithelial adhesin) protein family of 17 members, six adhesins Awp (adhesin-like wall proteins) and seven Pwp proteins (PA14-containing wall protein) of *C. glabrata* and *C.*

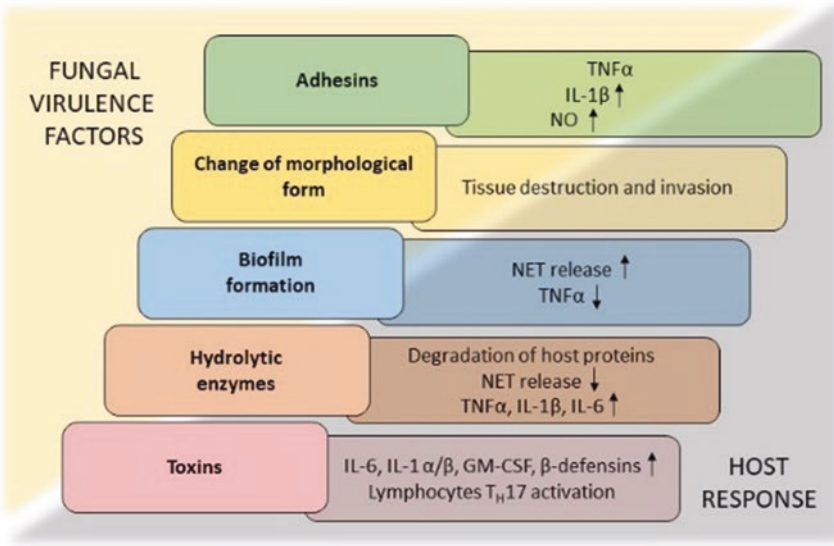

Fig. 6.2 Host responses induced by the fungal virulence factors
The mechanisms related to the pathogenicity of fungi include morphological polymorphism, production of surface adhesins, biofilm formation, and secretion of hydrolytic enzymes and toxins. These mechanisms influence the invasion of the host's tissues, their destruction, and modulation of the host's immune system

albicans adhesins from Als (agglutinin-like sequence) protein family consisting of eight members (Als1–7 and Als9), (Hoyer 2001; Verstrepen and Klis 2006; Dranginis et al. 2007; de Groot et al. 2008; Kraneveld et al. 2011; Timmermans et al. 2018). Other important *C. albicans* adhesins are Hwp1 (hyphal wall protein), Eap1 (enhanced adhesion to polystyrene), and Csh1 (cell surface hydrophobicity), while the latter is also indicated as essential for *C. dubliniensis* cell surface hydrophobicity and virulence (Singleton et al. 2001, 2005; Li and Palecek 2003; Hazen 2004; Naglik et al. 2006; Ene and Bennett 2009). In the case of *C. albicans*, *C. parapsilosis* and *C. glabrata* it was shown that cell surface hydrophobicity correlates with adhesion to human buccal epithelial cells and denture acrylic surfaces (Panagoda et al. 2001; Luo and Samaranayake 2002). Also, it was reported that cells of *C. albicans* strain, isolated from patients with chronic periodontitis and diabetes that showed high surface hydrophobicity had a greater ability to adhere and invade human gingival fibroblasts (Sardi et al. 2012b).

The change in the morphological form and the formation of hyphae, on which surface typical adhesive proteins are abundantly exposed, may also increase the adhesiveness of *Candida* yeasts (Kumamoto and Vinces 2005; Tronchin et al. 2008; Mayer et al. 2013). Hyphal growth of *C. albicans* cells was previously considered as essential for penetration into gingival tissues in chronic periodontitis (Jarvensivu et al. 2004). The cigarette smoke condensate promoted the hyphae formation by *C. albicans*, increased expression of *HWP1* and *EAP1* genes, biofilm-forming ability, and fungal adhesion to human gingival fibroblasts, whereas e-cigarette vapor stimulated the binding of fungi to gingival epithelial cells and elongation of hyphae (Alanazi et al. 2014, 2019; Semlali et al. 2014).

In the case of *A. fumigatus*, the mechanisms of fungal adhesion are not sufficiently well understood. Initially, it was pointed out that some proteins of the conidia surface, including conidial hydrophobin RodA, 37-kDa allergen Asp f 2, and extracellular thaumatin domain protein (AfCalA) may bind to host ligands (Thau et al. 1994;

Banerjee et al. 1998; Upadhyay et al. 2009). However, the major role in the adhesion of these fungi to plastic, fibronectin, intact basal lamina, and the epithelium is generally assigned to surface-exposed negatively charged carbohydrates and galactosaminogalactan (GAG) (Wasylnka and Moore 2000; Gravelat et al. 2013; Rambach et al. 2015).

6.3.2 Fungal Hydrolytic Enzymes and Toxins

The hydrolytic activity is essential for fungal pathogens to obtain nutrients, degrade host tissues and proteins during the invasion, and evade the immune system response. Therefore fungi secrete extracellularly or present on their cell surface a variety of hydrolytic enzymes, including lipases, phospholipases, phosphatases, proteases, and hemolysins (Barrett-Bee et al. 1985; Robinson et al. 1990; Monod et al. 1994; Hube et al. 2000; Luo et al. 2001; Wartenberg et al. 2011).

One of the groups of yeast hydrolases that have been most thoroughly described regarding their involvement in the pathogenesis is secreted aspartic proteinases (Sap). Two catalytic Asp residues being a part of an Asp-Ser-Gly/Asp-Thr-Gly motif are responsible for the Sap hydrolytic activity at acidic pH as optimal (Koelsch et al. 2000; Rapala-Kozik et al. 2018). In the case of *C. albicans*, there are identified ten members of the *SAP* gene family (*SAP1–10*), three for *C. parapsilosis* (*SAPP1–3*), and four for *C. tropicalis* proteinases (*SAPT1–4*) (Zaugg et al. 2001; Hube and Naglik 2001; Singh et al. 2019). The broad substrate specificity of Saps ensures the participation of these proteins not only in the acquisition of nutrients, adhesion, and tissue damage but also additionally in many processes related to the interaction with host proteins, including the degradation of antimicrobial peptides, the modulation of inflammatory state or the deactivation of the complement system (Villar et al. 2007; Gropp et al. 2009; Bras et al. 2012, 2013; Kozik et al. 2015; Svoboda et al. 2015; Bochenska et al. 2015, 2016; Yu et al. 2016; Singh et al. 2019). The genome of *C. glabrata* contains at least 11 genes encoding yapsins (*YPS*)—extracellular glycosylphosphatidylinositol-linked aspartyl proteases also involved in fungal adhesion, virulence and cell wall maintenance (Kaur et al. 2007; Rasheed et al. 2018).

Other pathogenic fungi—*A. fumigatus* also produce aspartyl proteinases, including 38 kDa Pep1 protein (Reichard et al. 1994), 32 kDa serine protease Alp1 (Reichard et al. 1990) and metalloproteinase Mep1 (Shende et al. 2018). The latter two were identified as responsible for the degradation of the complement system components (Behnsen et al. 2010; Shende et al. 2018).

Apart from proteolytic activity, also other hydrolytic enzymes are essential for fungal virulence. In *C. albicans*, such enzymes also include the lipase family consisting of 10 members (*LIP1–10*) (Hube et al. 2000) and different phospholipases. Of the *Candida* family of phospholipases, the best described is the extracellular phospholipase Plb1—a lipolytic enzyme considered as an important virulence factor of *C. albicans* (Leidich et al. 1998; Hruskova-Heidingsfeldova 2008). Phospholipase activity was also detected for *A. fumigatus*, and assigned for proteins afPLB1, afPLB3, and phospholipase D (Shen et al. 2004; Li et al. 2012). Furthermore, for pathogenic representatives of different fungal genera, extracellular hemolytic activity is very important in virulence since it allows them to obtain iron from host erythrocytes in the infectious site (Manns et al. 1994; Luo et al. 2001; Kaveemongkonrat et al. 2019).

In addition to enzymatic proteins, also toxins play a significant role in the pathogenesis of fungal infections. *C. albicans* secreted cytolytic toxin—candidalysin, which is released from the Ece1 (extent of cell elongation) protein by the Golgi-located Kex2 protease, has been identified recently as one of the key virulence factors (Moyes et al. 2016). This secreted candidal toxin is involved in the direct damage of the epithelial cells by their permeabilization but can also activate pro-inflammatory host protection by mono-

nuclear phagocytes (Naglik et al. 2019; König et al. 2020).

A wide variety of toxins are produced also by fungi other than *Candida* species, including *A. fumigatus*, for which several toxic secondary metabolites have been shown to contribute significantly to fungal pathogenicity (Rementeria et al. 2005). Among the best described mycotoxins of *A. fumigatus* are gliotoxin and fumagillin. Gliotoxin is an immunosuppressive and cytotoxic molecule highly affecting fungal virulence and responsible for the inhibition of the respiratory burst and phagocytosis, and the stimulation of apoptosis in leukocytes (Reeves et al. 2004; Sugui et al. 2007; Gayathri et al. 2020). Whereas fumagillin exhibits antiangiogenic properties through the irreversible inhibition of methionine aminopeptidase (MetAP) type 2 (Sin et al. 1997), it has also the ability to reduce the activity of neutrophils (Fallon et al. 2010) and is involved in the damage of the epithelial cells during infection (Guruceaga et al. 2018).

6.3.3 Fungal—Host Interactions

C. albicans Als3 protein enables effective invasion of the oral epithelium through binding to E-cadherin, cell adhesion molecule forming adherens junctions (Phan et al. 2007). Such interaction induces microfilament rearrangement, which activates hyphal endocytosis through the endothelial layer, allowing the invasion of tissues (Phan et al. 2007). It has been shown that the contact of fungal adhesins with gingival fibroblasts induces the production of proinflammatory factors such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), interleukin 13 (IL-13) by the activation of toll-like receptor 2 (TLR2) expressed on the cell surface (Pinheiro et al. 2018). Additionally, data presented by Sardi et al. indicated that gingival fibroblasts respond to fungal adhesion by producing nitric oxide (NO). A high concentration of NO in the microenvironment of periodontal disease may have a cytotoxic and cytostatic effect against infected cells and results in rapid tissue damage (Sardi et al. 2012b).

Adhesion to the surface of host proteins is a critical step in the initiation of biofilm formation (Phan et al. 2007). The appearance of such complex structures is a considerable challenge for the immune system, and the host cell response is closely dependent on the phase of biofilm development. At the initial stage of infection, access to the fungal cells is easier, and the immune system is effectively activated, mostly by recognizing the elements of the microbial cell wall or the secreted proteins. However, to date, several examples of the attenuation of the host cell's innate response to mature biofilm infections have been described. The main barrier that effectively hinders contact of the immune system with pathogen cells is the dense extracellular matrix (ECM) layer surrounding the mature biofilm (Sandai et al. 2016). Neutrophils, the first white blood cells recruited to the infection site, are equipped with several killing mechanisms, among which the most important in the context of fighting extensive fungal infections is the netosis process (Urban et al. 2006, 2009). During netosis, activated neutrophils release decondensed chromatin fibers, decorated with antimicrobial proteins derived from granules (NET) (Brinkmann et al. 2004). The research revealed that netosis is an effective mechanism to prevent invasion by large-size microorganisms that are relatively difficult to neutralize by phagocytosis (Branzk et al. 2014), including the filamentous forms of *Candida* and *Aspergillus* spp. Johnson et al. indicated that contact of neutrophils with *C. albicans* biofilm impairs NET release, making biofilm more resistant to neutrophil attack (Johnson et al. 2016). This inhibitory effect appears to be related to the presence of a high concentration of polysaccharides—the main components of ECM. It has been shown that *C. albicans* biofilms also resist attack by monocytes. Studies conducted on monocyte-like cell line THP-1 indicated the downregulation of TNF-α production, compared to planktonic cells (Katragkou et al. 2010). Inhibition of the production of this cytokine contributes to a significant reduction of phagocytosis. Taking into consideration that TNF-α has been shown to directly inhibit *C. albicans* biofilm formation by

interaction with N,N′-diacetylchitobiose, this mechanism may represent an evolutionary adaption of fungal biofilm to immune system evasion (Kernien et al. 2017; Rocha et al. 2017). Similar results, indicating a protective role of ECM, were obtained for *Aspergillus* biofilms. In this case, the ECM's most essential components are galactomannan and GAG (Loussert et al. 2010). The secreted enzyme Agd3 deacetylates these ECM components. Such modification is a critical step in forming *A. fumigatus* biofilms, ensuring the adhesion to anionic surfaces, including host tissues (Lee et al. 2016). Moreover, cell wall-bound GAG has been shown to influence the resistance of *Aspergillus* to neutrophil killing, probably by inhibition of NET binding to hyphae (Lee et al. 2015).

The main consequence of fungal proteolytic activity for a broad spectrum of host peptides and proteins, including lactoferrin, lactoperoxidase, cathepsin D, albumin, hemoglobin, LL37, histatin, contact system components, and the extracellular matrix proteins (Naglik et al. 2003; Rapala-Kozik et al. 2010, 2015) is their hydrolysis by Saps. Sap1–3 has been shown to degrade complement components preventing yeast opsonization and phagocytosis (Gropp et al. 2009). Saps are also the main fungal proteins involved in the penetration of the epithelium layer through the degradation of E-cadherin, mainly due to the activity of Sap5, which contributes to the destruction of host tissues (Villar et al. 2007). These enzymes might also activate the innate immune response. It has been indicated that Saps exhibit strong chemoattractant properties and drive the influx of neutrophils and macrophages to the place of infection and these proinflammatory properties are rather independent of proteolytic activity (Pietrella et al. 2010; Gabrielli et al. 2016). Through the activation of Akt/NF-κB pathway, Saps activate the expression of a broad spectrum of cytokines such as IL-1β, IL-6, and TNF-α (Pietrella et al. 2010).

Furthermore, Saps are involved in the activation of ROS generation and have the potential to induce NET formation (Hornbach et al. 2009; Zawrotniak et al. 2017). This mechanism may be of particular importance in the context of effective protection against oral infections, as it has been shown that neutrophils isolated from the peripheral blood and gingival crevice from individuals with periodontitis have significantly reduced phagocytic properties (Asif and Kothiwale 2010). In contrast to Saps, the role of *C. albicans* phospholipases in pathogenesis is not so well elucidated. Phospholipases facilitate the adhesion of yeast to the tissue surface and degrade the components of biological membranes, which are mainly composed of phospholipids (Sardi et al. 2010).

Also, cadidalysin, the main toxin produced by *C. albicans*, plays a critical role in developing oral infections (Moyes et al. 2016; Pellon et al. 2020). Moyes et al. demonstrated for the first time that at the early stages of infection, this peptide induces a strong inflammatory response in epithelial cells, including the production of IL-6, IL-1 α/β, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), CCL20, antimicrobial peptides (β-defensins), and damage-associated molecular patterns (IL-1α and S100A8/9) via p-MKP1/c-Fos pathway (Moyes et al. 2010, 2014; Verma et al. 2017). Accumulation of candidalysin during the progression of the infection causes direct tissue damage through the mechanism of intercalation, permeabilization, and calcium influx (Moyes et al. 2016). The rapid release of IL-1α and its synergistic role with EGFR ligands on the innate activation of human oral epithelial cells being the effect of candidalysin was also confirmed (Hanaoka and Domae 2020) and it was proved that candidalysin induces the release of antimicrobial peptides hBD2, hBD3 and LL37 and epithelial alarmins ATP, ROS/RNS and S100A8 during oral yeast infection (Ho et al. 2020). Moreover, candidalysin-induced production of IL-1α/β drives the activation of a specialized subpopulation of lymphocytes T_H17 expressing IL-17 (Verma et al. 2017). IL-17 production plays a crucial role in modulating the antifungal response (Mengesha and Conti 2017).

6.4 The Mixed-Species Biofilm Formation in the Periodontitis: Mechanism of Mutual Interactions Between Fungi and Bacteria

For many years, the common oral fungal colonizers were considered to be of minor importance in periodontal diseases. However, the observation that fungi can form multispecies biofilms with different types of bacteria has shed new light on the role of these microorganisms in periodontitis (Peters et al. 2017; Sultan et al. 2018; Delaney et al. 2018). The mixed-species microbial biofilm has a supragingival and subgingival location, where metabolic diversity encourages different species to aggregate and compete for space and nutrients (Aruni et al. 2015).

The formation of a polymicrobial community starts with the attachment of early colonizers to the tooth surface. The bacteria are represented by oral streptococci and *Actinomyces* species, which form the best environment for further appearance of bridging colonizers such as *Fusobacterium nucleatum.* Finally, the late colonizers—the red complex species: *P. gingivalis, T. forsythia*, and *Treponema denticola* find the best condition for existence, becoming the keystone species of periodontal disease (Könönen and Müller 2014; Mira et al. 2017; Manji et al. 2018; Valm 2019). Among them, *P. gingivalis* is also identified as a significant risk factor for developing cardiovascular disease (Oliveira et al. 2015), diabetes (Lamont et al. 2018), rheumatoid arthritis (Koziel et al. 2014), and Alzheimer's or Parkinson's diseases (Dominy et al. 2019; Olsen et al. 2020).

The biofilm cell mass is covered by an extracellular matrix that includes polysaccharides, lipids, and glycoproteins, not only of microbial but also of host origin. The biofilm matrix plays a unique role in protecting the formed microbial consortium against external factors, i.e. host defense factors, antioxidants, or antibiotics used in anti-biofilm therapies (Bowen et al. 2018).

During multispecies biofilm progression, identified fungal species can interact with bacteria, and the interactions may vary from synergism to competition or antagonism. Moreover, depending on the population diversity, opportunistic organisms, like yeasts belonging to *Candida* species, may switch from commensals to pathogens, equipped with an additional set of virulence factors. In periodontitis, the main synergistic microbial interactions were identified primarily for *C. albicans*, where fungal biofilm supports the milieu changes from aerobic to anaerobic (Fox et al. 2014), favoring the growth of Gram-negative obligate anaerobes. The microbial quorum-sensing molecules (QSM) or metabolic by-products may be useful in mutual pathogen communication. Moreover, the first colonizer's primary host tissue infection may predispose host cells to facilitate colonization by the subsequent pathogen (Basavaraju et al. 2016). On the other hand, the competition between the interacting microbes for nutrition or binding sites can restrict early facultative aerobes' growth, giving the late colonizer space for propagation.

The changes in the bacterial composition within mixed biofilm create a necessity for the lifestyle adaptation for fungi and modifying their interactions with emerging new bacterial partners. The different omic approaches supported the knowledge about the interactions and metabolic responses of microbial community members (Shokeen et al. 2021). These mutual microbial interactions can affect health or disease progression in the host organism. But the host activity can also impact the biofilm composition and the interplay between coexisting microbes by the immune system responses or divergences in the nutrient supply (Marsh and Zaura 2017; Lamont et al. 2018).

6.4.1 Synergistic Interactions

The mutual interaction of bacteria with fungi may be considered on two significant levels. The first one concerns the physical contact, where fungal filamentous cell morphotype (hyphae) serves as a structural platform for a mixed-species biofilm and plays a synergistic role. The second focuses on chemical and metabolic interactions and may also be antagonistic. In this case, the

produced compounds modified the local environment's properties by changing pH and oxygen availability and enabling bacterial development in unfavorable conditions. It also includes the action of QSM that might regulate the microbial morphology and population abundance (Marsh and Zaura 2017; Diaz and Valm 2020).

The mechanical stabilization of biofilms is carried out using non-specific physical interactions and specific protein-protein contact that facilitate microbes' cooperation, especially in the teeth area, where pathogens are exposed to mechanical removal from the surface. Co-adhesion and co-aggregation ensure the proximity of microorganisms and enable collaboration within the mixed biofilm (Kolenbrander 2000). The important role of fungi in creating the specific interactions between microorganisms is related to the possible morphological changes of fungal cells, best documented for *Candida* yeasts, where formed elongated hyphae supported pathogens' interactions within mixed biofilm structure (Cui et al. 2013; Diaz et al. 2014; Janus et al. 2016). The best-known relationships occurred between yeasts and the primary colonizers of oral cavity—streptococci (Diaz et al. 2012; Xu et al. 2014b), especially *S. mutans* and *S. gordonii* (Gross et al. 2012; Metwalli et al. 2013), where the fungi promote streptococcal biofilm formation, while streptococci enhance the invasive property of *C. albicans* (Diaz et al. 2012; Xu et al. 2014a). The synergistic physical coexistence between *Streptococcus* sp. and *C. albicans* leads to intimate corn-cobb-like structures in the supragingival sites (Zijnge et al. 2010) where *S. gordonii* promotes hyphal development of *C. albicans* cells. The adhesive contact between the microbes occurs due to specific protein-protein interactions in which the streptococcal cell surface adhesins CshA, SspA, and SspB are involved (Holmes et al. 1996; Silverman et al. 2010; Xu et al. 2014a). Fungal adhesins responsible for the recognition of streptococci are Hwp1 and the members of the Als protein family (Klotz et al. 2007; Bamford et al. 2009; Silverman et al. 2010). Additionally, the interactions between microorganisms are mediated by salivary proline-rich proteins, which adsorbed on the surface of *S. gordonii* may be recognized as receptors by *C. albicans* cells (Holmes et al. 1995; Bamford et al. 2009; Silverman et al. 2010).

Another example of an interplay between bacteria and fungi is the interaction of internalin-family surface protein—InIJ of *P. gingivalis* and *C. albicans* Als3 in the dental pockets (Sztukowska et al. 2018). The same fungal adhesin can also form complexes with the hemagglutinin domains of RgpA and Kgp—the major *P. gingivalis* cysteine proteinases—gingipains. Moreover, gingipains interact also with another *C. albicans* surface mannoprotein—Mp65, overproduced during contact with this bacterium, and important for hyphal morphogenesis, membrane cell wall organization, and fungal biofilm formation. An interesting relation was also observed for fungal enolase, a cytosolic protein that appears on the fungal cell surface as "moonlighting protein" (Satala et al. 2020a, b). The binding affinity between RgpA and enolase was even threefold higher than between RgpA and Als3 (Bartnicka et al. 2019).

The adhesion of *P. gingivalis* to fungal cells was also dependent on the activity of bacterial peptidylarginine deiminase (PPAD)—an enzyme that can perform the citrullination of both bacterial and fungal surface proteins. The decrease in interactions between these microorganisms, observed for the bacterial mutant strain with PPAD depletion, demonstrated the significant role of PPAD-mediated modification in these interactions (Karkowska-Kuleta et al. 2018).

Gingipains produced by *P. gingivalis* activate the host immune responses, but the host cell's coinfection by bacteria and *C. albicans* cells leads to the alleviation of the inflammatory process, pointing to the possible protection of bacterial cells by fungal biofilm. Such a hypothesis was supported by the experiments performed using the mouse model, where the fungus initiated a sequential infection, and a reduction of mouse mortality was observed. On the other hand, mixed species biofilm assured more prolonged survival of bacteria within host tissues,

suggesting the dual-species infection's chronic nature (Bartnicka et al. 2020).

The interacting microorganisms are secured by the extracellular polysaccharide matrix (EPM) formed by both types of microbes (Xu et al. 2008). For example, *S. mutans* accompanied by *C. albicans* induces glucosyltransferase B (GtfB) gene (Falsetta et al. 2014), encoding enzyme that catalyzes the production of bacterial α-glucans. Their deposition on *C. albicans* cell surface leads to the expansion of mutual EPM and enhancing microbes adhesion (O'Sullivan et al. 2000; Falsetta et al. 2014; Hwang et al. 2017). Moreover, the presence of EPM is also beneficial for fungi, as EPM structures might trap an antifungal drug—fluconazole, thus protecting *C. albicans* from its effects (Kim et al. 2018). The mixed biofilm of *C. albicans* and *S. gordonii* can also be stabilized through the extracellular release of bacterial DNA (Jack et al. 2015).

The relationship between yeasts and bacteria related to metabolism and its by-products is an essential aspect of microbial cooperation responsible for the maintenance of appropriate environmental conditions and the proper development of biofilm above and below the gum line (Lof et al. 2017). Some specific adaptations of *C. albicans* metabolism to co-existence with *S. gordonii* were correlated with an increased expression of genes involved in fungal arginine biosynthesis, probably required to overcome the effects of the oxidative stress generated by these bacteria (Dutton et al. 2016).

In gingival pockets and dental canals, several bacteria are either facultative or obligate anaerobes. Maintaining a low oxygen concentration in such a milieu is essential for their proper growth and development. *C. albicans* survives in both aerobic and anaerobic conditions but possesses the ability to lower the local oxygen concentration (Fox et al. 2014; van Leeuwen et al. 2016; Janus et al. 2017), thus creates the conditions optimal for the development of a mixed-species biofilm with *C. perfringens*, *B. fragilis*, *P. gingivalis*, and *C. difficile* (Tamai et al. 2011; van Leeuwen et al. 2016; Karkowska-Kuleta et al. 2018).

6.4.2 Antagonistic Interactions

The interactions within the mixed biofilms depend on the fast adaptation to the coexisting microorganisms' immediate needs and infection progression. The antagonistic relationships for microbes involved in periodontic diseases were again best documented for *Candida* species, especially for dominating *C. albicans* (Krüger et al. 2019).

For one of the first bacterial colonizers involved in these infections—streptococci, a synergistic collaboration with *C. albicans* was documented as contributing to plaque formation (Metwalli et al. 2013; Koo et al. 2018). But some contrary effects were also observed. The streptococci acidify the environment excreting carboxylic compounds, like lactate, pyruvate, or α-ketoglutarate. Nonetheless, low pH is unfavorable for fungal hyphae development. That finding was supported by observing the diminished formation of fungal hyphae in the *Galleria mellonella* larval tissue treated with *S. mutans* cell supernatant, resulting in the reduction of fungal cell pathogenicity. On the other hand, the cariogenic potential of *S. mutans* was attenuated in the biofilm formed with *C. albicans* cells, where higher pH was detected in comparison to one species, bacterial biofilm (Willems et al. 2016). *C. albicans* can use lactate as a carbon source for cell growth, thus alkalizing the environment, and maintaining a local pH at 5.3–5.5 (Vylkova et al. 2011; Krom et al. 2014; Danhof et al. 2016). Moreover, *C. albicans* is also able to generate ammonia, as a product of amino acid catabolism, increasing environmental pH and the hyphae formation (Vylkova et al. 2011). The alcalification of mixed biofilm milieu by fungi could be the way to counteract a microbial switch towards a cariogenic community and prevent the demineralization of teeth (Jenkinson et al. 1990).

Competition for access to limited nutrients may also affect the population dynamics within the biofilm. A good example is the heme competition between *P. gingivalis* and *C. albicans*, where at the restricted access to heme, the promotion of bacterial virulence was observed, cor-

related with the increase in expression of bacterial genes involved in heme utilization (Guo et al. 2020).

For antagonistic interactions, microbes also used the QSM production system (Barbosa et al. 2016). Such compounds include *S. mutans* signaling molecules like S-*trans*-2-decenoic acid, which is relevant in shaping multispecies fungal—bacteria biofilms, suppressing germ tube formation (Vílchez et al. 2010).

Another is the competence-stimulating peptide (CSP) produced during the early stages of *S. mutans* cell growth and fungal interaction with *S. gordonii*, influencing the fungal filamentation (Jarosz et al. 2009; Jack et al. 2015). A similar effect was also observed for mutanobactin A, that induced formation of yeast forms by *C. albicans.* Such inhibition of fungal cell filamentation may prevent activation of the immune system and pro-inflammatory cytokine production by macrophages (Joyner et al. 2010).

Other oral cavity bacteria—*Streptococcus sanguinis* presented antibacterial activity towards periodontal pathogens by the production of hydrogen peroxide and bacteriocin, which is localized within the *S. sanguinis* cells (Zhu and Kreth 2010). The hydrogen peroxide also influences fungal QSM—farnesol production and thus hyphae formation. On the other hand, the cell extract containing bacteriocin has antifungal activity and suppresses *C. albicans* and *C. tropicalis* cell growth (Ma et al. 2014).

C. albicans isolate from the root canal, and periapical infections are also accompanied with *Enterococcus faecalis* (Dahlén et al. 2012)—the opportunistic pathogenic bacterium also using bacteriocin (EntV), to inhibit fungal hyphal growth, biofilm formation, and virulence, without effect on cell viability (Cruz et al. 2013; Graham et al. 2017).

Another bacteria of aggressive periodontal disease—*A. actinomycetemcomitans* produces a different QSM—an autoinducer-2 (AI-2)—to inhibit *C. albicans* cell filamentation and suppress biofilm formation (Baker et al. 2017; Bachtiar et al. 2014). The genetic analysis of multispecies biofilm formed by *A. actinomycetemcomitans* showed the suppression of fungal hyphal-associated genes (*ALS3* and *HWP1*) without any effect on gene representing yeast form (*YWP1*) (Bachtiar and Bachtiar 2020). However, the action of AI-2 released by *S. gordonii* presented an opposite effect on *C. albicans* cells, suggesting that different bacteria produce AI-2 derivates with a miscellaneous activity (Lof et al. 2017).

Antagonistic effects were also observed for *C. albicans* and *C. dubliniensis* collaborating with "bridging" bacteria—*F. nucleatum*, which is involved in colonization succession of the oral polymicrobial community (Grimaudo and Nesbitt 1997; Jabra-Rizk et al. 1999; Signat et al. 2011). The mutual influence of microbes is mediated by direct interaction between their surface proteins, fusobacterial membrane protein RadD and *Candida* cell wall protein Flo9 (Wu et al. 2015; Bor et al. 2016). Moreover, the supposed effects of their reduced virulence towards the host do not result from the secretion of regulatory particles or metabolic products. But they have the source in resulted inhibition of hyphal morphogenesis and fungal cell growth. It was proposed that the observed mutual attenuation of virulence may promote a rather commensal lifestyle of both biofilm-forming species (Bor et al. 2016).

6.4.3 The Periodontal Cells in the Face of Mixed Infections

With emerging evidence that the development of periodontitis is associated with the presence of both bacterial and fungal pathogens, understanding of the biological consequences of the cross-kingdom interactions of microbes for host response is crucial.

The most attention has been devoted to the model of mixed infections caused by *P. gingivalis* and *C. albicans*. In 2011 Tamai et al. demonstrated that heat-killed *C. albicans*, but also mannoprotein-β-glucan complex constituting a component of the yeast cell wall, enhanced invasion of human gingival fibroblasts and epithelial cells by *P. gingivalis* (Tamai et al. 2011). The mechanism underlying this phenomenon is unclear, but the authors put forward two hypoth-

eses. Firstly, pretreatment with *C. albicans* or cell wall components promotes the recruitment of clathrin and clathrin-mediated invasion of host cells by *P. gingivalis*, more efficiently than by *P. gingivalis* alone. On the other hand, *C. albicans* induces trafficking of cell receptors such as toll-like receptors (TLRs) to lipid rafts, membrane microdomains involved in the entry of *P. gingivalis* into the epithelial cells (Tsuda et al. 2008; Tamai et al. 2011). Additionally, Haverman et al. observed that the interaction between *P. gingivalis* and *Candida* spp. (*C. glabrata* and *C. kefyr*) also lead to the inhibition of oral epithelial cell migration more than either pathogen separately (Tsuda et al. 2008).

Studies conducted on the human macrophage cell line (THP-1) have shown that contact with biofilm formed by *P. gingivalis* and *C. albicans* was characterized by a different level of production of the main proinflammatory cytokines—IL-1β, TNF-α and IL-8 (Bartnicka et al. 2020). The macrophage response to the supernatant obtained from a mono-species bacterial biofilm resulted in lower IL-1β production, compared to the reactions of THP-1 to *C. albicans* biofilm. Unexpectedly, the contact of THP cells with mixed biofilm resulted in a significant increase in IL-1β. On the other hand, a 24-h incubation of THP-1 with mixed biofilm caused a dramatic decrease in TNF-α and IL-8 levels compared to the treatment with mono-species biofilm. The reason for the observed changes in the level of cytokines may result from the activity of proteolytic enzymes produced by *P. gingivalis.* They can effectively degrade the cytokines produced by the host cells, impairing the immune response (Stathopoulou et al. 2009; Bartnicka et al. 2020), especially considering that their activity could be attenuated during contact with a fungal partner (Bartnicka et al. 2020). Another explanation might be that *C. albicans* cells prevent bacteria from being recognized by the immune cell receptors. Moreover, mixed biofilm also resulted in a reduced neutrophil response, as evidenced by a significantly lowered elastase activity compared to bacterial biofilm (Bartnicka et al. 2020).

An example of bacteria frequently co-isolated with *C. albicans* in periodontal pockets is *F. nucleatum* (Bor et al. 2016). *In vitro* studies have shown that their interaction retains *C. albicans* in morphology less sensitive to RAW macrophage killing. Moreover, the co-infection of *C. albicans* with *F. nucleatum* has been shown to inhibit the bacteria-induced production of proinflammatory molecules such as chemokine MCP-1 (monocyte chemoattractant protein 1) and cytokine TNF-α. The immune response's attenuation was more effective when *C. albicans* was grown in yeast-like morphology (Bor et al. 2016).

6.5 New Trends in Prevention and Treatment of Periodontitis

The high incidence of periodontal diseases, the unsatisfactory effect of commonly used antibiotics, and the reports about the acquisition of drug resistance by pathogenic microorganisms present in the oral cavity necessitate the search for novel, alternative methods of preventing and treating periodontitis (Shlezinger et al. 2017; Cheng et al. 2017; Kinane et al. 2017). An additional problem in periodontal diseases caused by both bacterial and fungal pathogens is the accurate diagnosis and the correct identification of fungal microorganisms in subgingival sites and, consequently, the necessity for the modification of the conventional treatment. The diagnosis of fungal involvement in periodontal disease depends on the proper collection of a sample from the periodontal pockets after careful removal of the supragingival biofilm (Jewtuchowicz et al. 2008; Urzúa et al. 2008). Further diagnosis includes conventional fungal identification methods involving the observation of morphological changes or growth on differential media, however, a method based on the amplification of the internal transcribed spacer (ITS) is currently recommended (Persoon et al. 2017). In the case of the presence of fungi in the periodontal pockets during periodontitis, together with traditional treatment based on proper oral hygiene, conventional periodontal therapy, the use of topical antiseptics, and systemic antibiotics, the use of antifungals should be considered, including nystatin or fluconazole

(Sardi et al. 2011a). Oral administration of antifungal drugs from other classes might also be required (De-la-Torre et al. 2017).

However, in view of the emerging resistance of pathogens in the biofilm (Sardi et al. 2011a; Jarvensivu et al. 2004), attempts are made to develop therapeutics based on microbial virulence mechanisms and targeted at specific pathogenic species. One of the attractive targets are *P. gingivalis* cysteine proteases—Rgp and Kgp, which play a significant role in developing periodontal diseases. It was reported that extract of *Aspergillus oryzae* S-03 grown on fat-free soybean inhibits *P. gingivalis* cell growth through gingipain inhibitors, probably derived from the digestion of soybeans by the *A. oryzae* protease (Danshiitsoodol et al. 2014). Another natural secondary bioactive metabolite is terrein from *Aspergillus terreus*. It has been shown that this compound inhibits osteoclastogenesis and related excessive bone resorption, correlated with periodontitis, by blocking the main osteoclast regulator's activity—the activated cytoplasmic T cell nuclear factor 1. It was postulated that synthetic terrein, due to its low molecular mass and short half-life, might be used as an oral agent to prevent periodontal diseases (Nakagawa et al. 2020). There were also reports on the secretion of substances inhibiting bacterial and fungal growth by *Malassezia* and *Pichia* species. *M. globosa* spent medium has been shown to have antibacterial properties against *S. mutans* and *S. mitis* (Baraniya et al. 2020), while *Pichia* spent medium inhibits the growth of *Candida*, *Aspergillus*, and *Fusarium* (Mukherjee et al. 2014).

Another promising treatment in the fight against periodontitis is antisense therapy based on the peptide, which may block the synthesis of microbial proteins due to their strong affinity for mRNA. It was reported that the use of antisense peptides effectively inhibits the growth of two pathogens associated with periodontitis, *P. gingivalis*, and *A. actinomycetemcomitans* (Sugimoto et al. 2019). Similarly, the use of cerium-doped nanoparticles also inhibits the *P. gingivalis* and *F. nucleatum*. The acidic environment of the gingival pockets of patients with chronic periodontitis promotes the hydrolysis of nanoparticles, which releasing of ions modulate the microenvironment's pH, consequently changing them to unfavorable for bacterial growth (Li et al. 2019). In turn, polylactic-co-glycolic acid (PLGA) nanoparticles, coated with a peptide derived from *S. gordonii* significantly reduce the virulence of *P. gingivalis* in a mouse model of periodontitis and strongly inhibit the adhesion of *P. gingivalis* to streptococci, preventing mixed infections (Mahmoud et al. 2019).

Potential candidates for alternative therapy are also sought among natural substances. For example, it has been shown that alcoholic red propolis extract has antifungal activity against *Candida* species isolated from patients with chronic periodontitis (Siqueira et al. 2015), while cardamom extract is bactericidal against periodontal pathogens—*P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *P. intermedia* (Souissi et al. 2020).

6.6 Conclusion and Perspectives

Dental biofilm, where diverse microbes form a compact structure of biofilm, could be, in states of dysbiosis, the primary source of dental caries and periodontal diseases, which can further implicate systemic diseases by translocation the included microbes by the bloodstream into a variety of host tissues.

Although biofilm-forming microbes sustain their repertoires of virulence factors, the interaction with the environment and surrounding microbes, often from different species, gives rise to the emergent new properties of the microbial community.

Here we highlighted the role of fungal species forming biofilm with periodontal bacteria, where physical, metabolic, and chemical interactions shape their properties. Although there is evidence of the presence of particular representatives of various fungal genera—including *Candida*, *Aspergillus*, and *Rhodotorula*—in the subgingival biofilm of patients with severe chronic periodontitis, the most data are available for *C. albicans*. Numerous studies show that fungi are

not an infection bystander but also an active member of the biofilm consortium. Fungal dimorphism and extracellular matrix, varied in the composition, may serve as bacterial shelter from the adverse environment or camouflage from host recognition, where synergistic or antagonistic interactions are presented. The understanding of cross-kingdom interplay that underlies the biofilm functioning, especially during contact with host cells, raises the need to search for new infection models that will enable the analysis of such diverse interactions. To meet this challenge, the development of organoids and organ-on-chip technology is the priority. It will create an opportunity to develop improved patient treatment strategies without the risk of infection recurrence.

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