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Polymicrobial Biofilm Studies: From Basic Science to Biofilm Control

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Abstract

Microbes rarely exist as single species planktonic forms as they have been commonly studied in the laboratory. Instead, the vast majority exists as part of complex polymicrobial biofilm communities attached to host and environmental surfaces. The oral cavity represents one of the most diverse and well-studied polymicrobial consortia. Despite a burgeoning field of mechanistic biofilm research within the past decades, our understanding of interactions that occur between microbial members within oral biofilms is still limited. Thus, the primary objective of this review is to focus on polymicrobial biofilm formation, microbial interactions and signaling events that mediate oral biofilm development, consequences of oral hygiene on both local and systemic disease, and potential therapeutic strategies to limit oral dysbiosis.

Keywords

Polymicrobial; multi-species; biofilm; microbiome; oral health; quorum sensing

Introduction

The oral cavity is the beginning of the gastrointestinal tract and is colonized by a multitude of diverse microorganisms, including viruses, bacteria and fungi [1]. This moist, warm, and nutrient rich environment is ideally suited for thriving microbial growth. Composition of the oral microbiome varies significantly between individuals and based on intra-oral geography [2, 3]. Enormous differences in salivary flow and pH between teeth and even on surfaces

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Compliance with Ethics Guidelines

Conflict of Interest

Hubertine ME Willems, Zhenbo Xu, and Brian M Peters declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

from the same teeth in close proximity [4], creates multiple selective micro-niches where pH, oxygen, temperature, and redox potential can influence the settling and attachment of micro-organisms and thus community development [5]. Adaptations within polymicrobial consortia often confers resistance to these environmental stressors, thereby enhancing growth, replication, and fitness [6]. Maintained adherence to the tooth surface results in the formation of dental plaque, otherwise known as an oral biofilm [7, 8].

More specifically, an oral biofilm is a polymicrobial 3-dimensional community of numerous microbial species, embedded in a matrix that consists of microbial metabolic products and/or host components, such as salivary glycoproteins [1]. A typical oral biofilm starts with formation of the enamel pellicle covering the tooth surface, resulting from a layer of proteins and carbohydrates derived from selective adsorption of salivary components [9]. The first microorganisms, often streptococci [10], to actively attach to the tooth surface do so via selective adhesin-receptor binding [11, 12]. Although the initial adhesion to the tooth surface can happen as quick as 5 min [13, 14], it takes up to hours for a typical oral biofilm to fully develop, starting with colonization of the surface by *Streptococcus mitis* and *Streptococcus oralis* [15], and quickly followed by *Actinomyces*, *Gemella*, *Neisseria* and *Veillonella*. In time, the composition of the biofilm changes. Early colonizers remain but the diversity of species increases when anaerobic bacteria such as *Porphyromonas*, *Fusobacterium*, *Prevotella*, *Veillonella* and *Capnocytophaga* are introduced into the plaque and increase in number [16].

In absence of proper oral hygiene, this plaque remains undisturbed and accumulates on tooth surfaces and in the gingival sulci, eventually leading to caries formation and chronic inflammation, respectively. With the advent of rapid and comprehensive sequencing technologies, we are now only beginning to understand the complex relationship between oral polymicrobial communities and human health and disease.

The oral microbiome

The oral microbiome is a complex ecosystem represented by bacterial and fungal Kingdoms [17]. The many microorganisms found in the oral cavity have been altogether referred to as the oral microbiota (also known commonly as the oral microbiome [18]) and defined as all microorganisms found on or in the human oral cavity and its contiguous extensions [18]. The first characterizations of the dental plaque microbiome were performed by selective culturing, and identified mostly known dominant community members, including *Streptoccocus* spp, *Neisseria* spp. and *Veillonella* spp. All three species were found at the initial stages of tooth colonization, followed by the introduction of Gram-negatives into the biofilm, like *Fusobacterium* spp [19]. However large-scale studies of the oral microbiome were extremely difficult, and many bacteria were unable to be detected or analyzed until the arrival of culture-independent techniques. Among the first and most widely used of these techniques was 16S rRNA gene-based cloning, which identified approximately 700 species or phylotypes in the oral cavity [20] and represents one of the best characterized communities within the total human microbiome [21].

However, the oral cavity harbors many different structures and tissues, e.g. teeth, gingiva, tongue, and palate, and those structures provide different niches suitable for the growth of

various microbes [21]. Accordingly, it has been demonstrated that microbial composition varies significantly between these oral structures [20] and the oral microbiome can therefore be viewed as a group of diverse, site-specific microbial biofilms [21]. This is an extremely important point to consider when analyzing sequencing analyses of oral microbial communities in order to avoid sweeping, generalized conclusions of community composition.

Regarding abundance, fungi are a relatively minor component of the oral microbiome as compared to prokaryotes, and thus assumed to be functionally inconsequential [17]. In part, this assumption was biased [17] by available detection methodologies, such as 16S rRNA gene sequencing pipelines and extensively curated prokaryotic sequence databases enabling accurate oral bacterial taxonomic assignment. Therefore the fungal component of the oral microbiome, in both health and diseases (e.g. periodontitis and caries) remains an important and understudied frontier in oral biology [22, 23]. It is known that fungi are a medically important component of the oral microbiome, given the fact that opportunistic fungal infections commonly afflict the oral mucosa of immunocompromised hosts. The majority of these infections are caused by *Candida* species and are assumed to result from an overgrowth of indigenous species in a permissive host environment [24]. Also, in immunocompetent hosts, it has been demonstrated that the presence of *Candida albicans* is associated with higher caries rates, especially in children [25]. However, to date there have been few studies investigating fungal species present in the oral cavity or how these fungal populations shift during fungal or bacterial oral infection. Studies by Ghannoum, et al. and Dupuy, et al. have attempted to define the composition of fungal communities in healthy individuals using approaches to sequence the variable internal transcribed spacer region for species-identification [26, 27]. Although limited in breadth (20 and 6 participants, respectively) over 100 unique species of fungi were found in the oral cavity—a much higher number than initially anticipated. Up to 20% of individuals harbored the five of the most common genera of pathogenic fungi, including *Candida*, *Aspergillus*, *Fusarium*, *Cryptococcus*, and *Malassezia*. Because these examined samples were saliva, it is still unclear whether there is more fungal diversity associated with oral tissues or precisely where fungal communities are located within the oral cavity. With the rise of novel genomics technologies, studies of the human mycobiome and its interaction with the bacterial microbiota will be fully realized, shedding insight on the incompletely defined role of fungi in oral health and disease.

Oral polymicrobial biofilm formation and structure

It is well known that oral bacteria can cause several diseases, most commonly caries, periodontitis and endodontic infections [21]. In the absence of a proper oral health regime, oral bacteria form a robust biofilm over the tooth surface by attaching to deposited sugars and salivary proteins. The colonization of oral surfaces occurs temporally by several different mechanisms. One species may serve as an early colonizer recruiting others species to bind via direct microbe-microbe interactions—a process referred to as coaggregation. This process is under strict control of specific cell surface-associated receptor-ligand interactions, often resulting in synergistic increases in multi-species biofilm formation. Alternatively, bacterial species may bind one another and induce phenotypic changes that result in enhanced binding to oral surfaces. As shared surface area and nutrients are at a

premium in the oral cavity, polymicrobial biofilm formation enables competitive advantages for these valuable resources.

The mature biofilm plaque matrix consists mainly of glucan and fructan [28]. The glucan is insoluble and composed of α1-3-, α1-4-, and α1-6-linked glucose, forming an ideal physical and chemical barrier for saliva [29]. The majority of the bacteria in dental plaque are acidogenic and aciduric [30] and the acid produced by these bacteria is normally neutralized by the buffering capacity of saliva. However, when plaque accumulates on the tooth surface, saliva cannot penetrate the thick outer glucan matrix to reach deeper layers of the plaque where the acid producing bacteria reside [31]. Streptococci located within deep plaque layers are considered to be prototypical carious agents. They produce glucosyltransferases B, C and D (GtfB, GtfC and GtfD), which add to the virulence of the biofilm in several ways. Secreted GtfB can exert its activity *in trans* with neighboring oral bacteria, conferring the ability to produce glucan when exposed to sucrose [28]. Also, GtfB has a high affinity for the fungus *Candida albicans* and facilitates the colonization of dental plaque by this fungus, especially in the presence of sucrose [32]. GtfC has the highest affinity for saliva coated surfaces and adheres to other species via indirect binding-mechanisms [33]. Although GtfD is present in high concentrations in saliva it has a low affinity for the saliva-coated tooth surface. Therefore its role in plaque formation is considered to be a primer substratum for the other isoforms [34]. The chemical structure of the plaque is dynamic, with mature plaque exhibiting differences from young plaque in both matrix composition and microorganisms present [31, 28]. These properties make therapeutic enzymatic digestion of matrix components a challenge for efficient biofilm removal.

Oral-biofilm associated disease

The recognition of acid as the major etiological agent in dental caries led to the identification of the Gram-positive bacterium *Streptococcus mutans* in the early 1960s, as the microbe predominantly responsible for dental caries [35, 36]. As described earlier, *S. mutans* adheres to the tooth surface primarily via Gtfs [33] and co-aggregates with other bacteria [28, 34]. After this initial phase, microbes will proliferate and spread to other sites in the oral cavity, eventually penetrating into deeper tissues and gingival crevices, where generated acid leads to dissolution of hydroxyapatite crystals in enamel and dentin. This process results in tooth cavitation [37, 38], inflammation of the tissue surrounding the affected tooth (gingivitis), and eventually tooth loss. *S. mutans* has been thought to be the primary etiologic agent of dental caries. However, recent evidence indicates that nearly all cases of caries are polymicrobial, and the role of other co-colonizing microbes in caries development is only beginning to be appreciated. For example, there is a high prevalence of *C. albicans* in dental biofilms where *S. mutans* resides, suggesting that the interaction between these diverse species may mediate cariogenic development [39, 40]. An in vitro study has demonstrated additive increased biomass during dual-species biofilm growth of these microbes, but unexpected suppression of extracellular matrix (ECM) production by *S. mutans* [41]. Using a transcriptomic approach, it was found that during dual-species biofilm formation with *C. albicans*, *S. mutans* diverts its glucan synthesis pathway from ECM production to intracellular glycogen storage, while also up-regulating potential cariogenic virulence factors. These results are contrary to findings described by Falsetta and colleagues,

in which co-cultures of *C. albicans* and *S. mutans* exhibited robust extracellular matrix production, with streptococci enmeshed in a thick layer of fungal-derived β-1,3-glucan [32]. Furthermore, these in vitro findings were recapitulated in a murine model of caries development, in which co-infection resulted in enhanced infection and elevated plaque biomass. It is possible that bacterial and fungal strain-dependent differences or variability in experimental systems may account for discrepancy between in vitro results.

Gingivitis is the inflammation of the soft tissues surrounding the teeth and one of the most common oral diseases in humans [42, 40]. It results from plaque overgrowth into the gingival margins [42], facilitating a robust inflammatory response [43, 44] resulting in red, swollen gums that bleed upon probing. Symptoms of gingivitis can be reversible and disappear relatively quickly, since the disease is triggered by numerous internally or externally imposed disturbances including oral hygiene, transient immunosuppression, injury, or dietary factors [45, 46]. When untreated, gingivitis can become chronic and eventually lead to periodontitis, an irreversible and much more severe oral manifestation. Polymicrobial consortia are thought to disrupt tissue homeostasis by manipulating host signaling pathways, compromising innate mucosal immunity. This imbalance causes a shift in the relative abundance of pathogenic microbes, resulting in localized inflammation [47, 48], characterized by deep gingival pockets and alveolar bone loss, progressing to eventual tooth loss [48-50].

The classic conceptual model for periodontal disease is that the bacteria *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* found in the mature plaque are responsible for disease pathogenesis and are collectively referred to as the ‘red complex bacteria’ [51, 52]. However, knowledge about periodontal disease has changed significantly since the use of 16S sequencing techniques, outdating the ‘red complex’ paradigm [53]. For example, ‘red complex’ bacteria are also found in the oral cavities of people without periodontal disease [52] and moreover *P. gingivalis* is only found in 50% of the patients with periodontitis [54]. Interestingly, independent studies have found that individuals with periodontitis harbor Archaeans as part of the complex biofilm consortium in the subgingival pocket; a finding not observed in healthy populations. Most recently, Yost, et al. utilized metatranscriptomic analyses of subgingival biofilms of patients with oral dysbiosis in an effort to define prognostic molecular signatures of periodontitis [55]. It was found that oral sites linked to progressing disease were associated with increases in cell motility, lipid A and peptidoglycan synthesis, iron transport, and non-classical periodontal pathogens (including *Streptococcus* spp, *Veillonella* spp., and *Pseudomonas fluorescens*) [55]. Therefore to fully understand the role of complex oral biofilms in periodontal disease, more mechanistic research and comprehensive sequencing surveys are desperately needed.

Do oral biofilms play a role in systemic diseases?

Biofilms are associated with nearly two third of all bacterial infections [56], including direct potential effects of periodontitis-associated biofilms on general health outcomes [57]. As described earlier, the deep pockets observed in periodontitis allow the biofilm to be in direct contact with oral connective tissues, leading to the disruption of these tissues and creating a

direct portal for bacteria and bacterial products, such as lipopolysaccharide, (LPS) to enter the bloodstream. This triggers a local pro-inflammatory immune response and, in extreme cases, can result in bacteremia or sepsis [47-49]. Inflammatory mediators produced in the periodontal tissues, (including proinflammatory cytokines, chemokines, and gingiva-derived C-reactive protein (CRP) [58, 57]) and specific bacterial extracellular proteases, such as *P. gingivalis* peptidylarginine deiminase (PPAD) and arginine gingipains (RgpA and RgpB) [59, 60] that can cleave substrates at lysine or arginine residues [61], can also be disseminated systemically through the bloodstream.

Rheumatoid arthritis (RA) is the world's most common autoimmune disease, affecting approximately 1% of the adult population [62]. The disease is characterized by chronic synovial inflammation that leads to a breakdown of articular cartilage and progressive joint destruction, resulting in warm, swollen and painful joints and as the disease progresses, articular deformities [63]. RA is associated with periodontal disease, and more specifically to the capacity of *P. gingivalis* to deaminate host proteins via extracellular PPAD activity. This directly leads to the production of autoantibodies targeting these citrullinated proteins (ACPA). Thus, it is proposed that one mechanism for the development of autoinflammation in RA occurs via ACPA generation. The ACPA's formed locally in the gingiva can spread though the blood stream and cross-react with citrulline epitopes located in the joints, resulting in RA [64-67].

Recently, the American Heart Association endorsed findings suggesting an association between periodontal disease and atherosclerotic vascular disease (ASVD) [68]. As mentioned earlier, periodontal disease is a chronic inflammation of the periodontal tissue and the resultant inflammatory mediators can affect distant biological sites after entering the bloodstream. Thus, not surprisingly, there is a strong correlation between systemic inflammation and endothelial dysfunction [69, 70]. Recently, Gangula et al., established periodontal infection in mice with *P. gingivalis*, *T. denticola* and *T. forsythia*, and demonstrated altered systemic vascular and gastrointestinal smooth muscle relaxation, focusing on differences in tetrahydrobiopterin (BH4) /neuronal nitric oxide synthase (NOS) pathways between infected mice and healthy controls. For the first time it was clearly demonstrated that experimental polymicrobial periodontal infection results in changes in the BH4/NO/NRF2 pathway in mice, which could directly impact endothelial function leading to hypertension [71]. Reichert and co-workers (2015) investigated whether oral hygiene habits, severe periodontitis, and presence of periodontal pathogens in the subgingival biofilm represent independent risk factors for the incidence of new cardiovascular events in patients suffering from coronary heart disease. They found that use of dental floss and/or interdental brushes (removal of dental plaque) was significantly associated with an adjusted decreased hazard ratio for new cardiovascular events among patients with congestive heart disease within a 1-year follow-up period. However severe periodontitis, number of missing teeth, amount of detected bacterial species were not [72], suggesting that microbial composition or low grade chronic oral inflammation may be more intimately linked with ASVD. Interestingly, similarities in microbial diversity were found in periodontal pockets and atheromatous plaques of cardiovascular disease patients [73], indicating that periodontal pathogens may “migrate” from the oral cavity to distant biological sites, including in the development of atherosclerosis. These findings were recapitulated using a murine model of

P. *gingivalis* periodontal infection in which *P. gingivalis* could be recovered from the oral epithelium as well as aortal plaque demonstrating the *in vivo* tissue translocation potential of oral pathogens [74]. Despite these studies demonstrating a role for oral pathogens in the development of cardiac diseases more research will be required to determine whether periodontal disease is an independent risk factor for cardiac disease or is a result of underlying meta-inflammation, co-morbidities, or innate immunomodulation (for a review see Janket *et al.*, 2015) [75].

Recently, similar findings were observed in a murine model of polymicrobial oral candidiasis, in which *Staphylococcus aureus* was coinoculated along with *C. albicans* [76]. Mice inoculated with both pathogens exhibited severe morbidity and mortality consistent with systemic infection within three days post-inoculation, as mice inoculated orally with either pathogen alone did not succumb at this time point. Analysis of microbial burden in the kidney indicated systemic spread of *S. aureus* from the oral cavity during polymicrobial infection. This phenomenon was dependent on expression of the *C. albicans* hyphal cell wall adhesin Als3, to which *S. aureus* is known to avidly bind [77]. Indeed, microscopic observation of oral tissue demonstrated *S. aureus* attached to invading hyphal elements of *C. albicans*. Thus, it is likely that other oral microbes may "hitchhike" onto invasive fungal or bacterial species to cause systemic illness, even in the absence of mechanical mucosal barrier failure. In fact, it has been recently demonstrated that patients with aseptic loosening of prosthetic hip joints harbored clonal DNA of oral microbes in both oral plaque and in synovial fluid [78].

Other studies also highlight the complexity of oral microbes in systemic disease. For example, it was demonstrated that periodontal bone loss due to oral polymicrobial infection is exacerbated in ovariectomized mice due to elevated systemic levels of tumor necrosis factor alpha (TNF-α), suggesting a role for hormone signaling in disease pathogenesis [79]. Oral bacteria may also contribute to acute respiratory disease, including the previously discussed gingipains produced by *P. gingivalis* as essential for clinical symptoms of *P. gingivalis*-induced aspiration pneumonia [80]. Although there is a seemingly transparent connection between oral dysbiosis and several systemic diseases, exact mechanisms, predisposing factors, and cause-effect relationships remain to be clearly defined.

Microbial Interaction in Biofilms

In nature, microbes are nearly always found in mixed-species communities, often as polymicrobial biofilms [81]. Interactions within these biofilms can be mutualistic, commensalistic, or antagonistic, and bacteria have evolved highly defined responses to sense and adapt to cues from neighboring species [82]. For instance, bacterial coaggregation is a main type of cooperative interaction encountered among oral bacteria that facilitates co-adhesion of bacterial pairs to oral surfaces [83]. Benefits of co-aggregation include production of protective extracellular matrices, nutrient production, toxin removal, growth enhancement, or a combination of these factors, among others [84]. Aside from physical interactions, microbes employ the secretion of diffusible chemical signals to facilitate "communication" amongst biofilm community members; this process is termed quorum sensing (QS). Microbes use QS as a density-dependent intercellular communication system

to orchestrate phenotypic changes at the population level via regulated intracellular signaling cascades. QS controls diverse important functions such as nutrient acquisition, redox modulation, and virulence. Bacteria can regulate gene expression in response to signaling molecules produced and released into the local environment by its own species (intraspecies QS) or by bacteria of different species (interspecies QS) [85]. Moreover, bacterial species may inhibit intraspecies QS by producing interfering QS signals that block native QS activity. Importantly, QS is an integral component to biofilm development and likely facilitates many of the interactions occurring within oral species consortia [86].

Jack et al. demonstrated that *Streptococcus gordonii* produces competence stimulating peptide (CSP) a *ComC* gene product, that can potentially control *C. albicans* in a dual species oral biofilm by modulating extracellular DNA content and increasing fungal biomass [87]. Similarly, *P. gingivalis* uses the LuxS/Autoinducer-2 (AI-2) quorum sensing system to control both intra- and interspecies QS. Scheres *et al.* challenged periodontal ligament (PDL) fibroblasts from healthy donors with a *P. gingivalis luxS* mutant and compared its ability to induce an inflammatory response in these cells compared to the wild-type strain. They found that lack of *luxS* failed to induce a robust inflammatory response in PDL fibroblasts, suggesting that LuxS-signaling in *P. gingivalis* is required for periodontitis pathogenesis [88]. *F. nucleatum* also utilizes AI-2 to enhance attachment and biofilm formation to oral streptococci, while AI-2 of *A. actinomycetemcomitans* inhibits biofilm formation by *C. albicans* [89, 90]. Despite the structural similarity of AI-2 signaling compounds, these studies highlight the enormous functional diversity on polymicrobial biofilm development in the oral cavity. Moreover, classic QS signals (like LuxS) are only a fraction of secreted factors that may affect polymicrobial communication. Intense areas of future study will include the role of secondary metabolite, lipid, and carbohydrate signaling as facilitators of chemical crosstalk amongst biofilm members.

Novel therapeutic and control approaches

In a healthy host, microbial overgrowth is controlled by a functional and appropriate host immune response. The absence of adequate oral hygiene encourages overgrowth of oral pathogens (e.g. *P. gingivalis*), biofilm sustainment, and mucosal inflammation [91]. Oral biofilms may attach to the epithelial cells of the gingiva, where microbial structural and metabolic products activate innate and adaptive immune responses, including the production of secreted antimicrobial peptides (e.g. β-defensin family, cathelicidin, calprotectin, and adrenomedullin) exhibiting potent activity against oral pathogens [92]. Synthetic production of these peptides, or novel derivatives, could serve as a new therapeutic alternative in the treatment of oral disease.

As mentioned previously, oral microbes utilize quorum sensing (QS) to establish and maintain colonization and achieve full virulence. Therefore, recent efforts have been directed at designing QS inhibitors that target narrowly defined pathogenic oral species [93]. Such compounds could be administered in oral rinses, along with enzymatic treatments, to both inhibit and eradicate specific multispecies consortia. In fact, eliminating the pathogen entirely may be unnecessary if quorum-dependent virulence is sufficiently inhibited.

Phage therapy is another potential biotherapeutic approach to managing oral biofilm-mediated disease. Phages are viruses with tropism for bacterial cells and exert their antibacterial effects directly by lytic activity and by encoding depolymerases that degrade biofilm matrices. Moreover, phage can be genetically engineered to target species-specific bacterial receptors or cocktails of phage employed to reduce several community members simultaneously. Limited clinical trials in humans have demonstrated that phage therapy is seemingly safe, effective, and relatively inexpensive as a single treatment should theoretically self-replicate achieving total clearance [94].

Perhaps the most attractive option to limit oral disease, including caries and periodontitis, is the development of an inexpensive, efficacious, and long-lasting vaccine. However, this priority remains a significant challenge for several reasons. Due to the exceptional diversity among individuals and an incomplete picture of microbes responsible for oral disease, selection of appropriate antigenic vaccine targets becomes incredibly difficult. Even if some pathogens were to be eliminated, other microbial community members may assume a pathogenic role. Moreover, secreted factors from commensal microbes may act in trans with “pathogenic” microbes, further complicating antigen selection. Because canonical oral pathogens can be found in healthy individuals, important concerns arise regarding elimination of potentially normal microbiota and unintended consequences on oral health.

Concluding remarks

Despite fundamental advances from the first descriptions of oral polymicrobial plaque by van Leeuwenhoek, our true understanding of the complex biology governing multispecies consortia is in its infancy. The past decade utilized high-throughput 16S sequencing technologies to begin to define community composition, although much work is still needed to comprehensively profile resident fungi, Archaens, and viruses in the oral cavity. Now that a conceptual framework for defining microbial populations has been achieved, transcriptomic, metabolic, and proteomic studies will elucidate the functional relationships between community members. Sets of biologically and clinically relevant microbes can then be investigated in vitro and in disease models to elucidate specific molecular mechanisms that facilitate these microbial interactions. Ultimately, targeted therapeutic strategies and antimicrobial compound screening approaches can then be devised to limit polymicrobial disease and improve oral health.

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