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Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease

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Abstract

Background and Aims: The scope of this working group was to review (1) ecological interactions at the dental biofilm in health and disease, (2) the role of microbial communities in the pathogenesis of periodontitis and caries, and (3) the innate host response in caries and periodontal diseases.

Results and Conclusions: A health-associated biofilm includes genera such as *Neisseria*, *Streptococcus*, *Actinomyces*, *Veillonella* and *Granulicatella*. Microorganisms associated with both caries and periodontal diseases are metabolically highly specialized and organized as multispecies microbial biofilms. Progression of these diseases involves multiple microbial interactions driven by different stressors. In caries, the exposure of dental biofilms to dietary sugars and their fermentation to organic acids results in increasing proportions of acidogenic and aciduric species.

Key words: caries; dental biofilm; dysbiosis; innate host responses; microbial interactions; periodontal diseases; symbiosis

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Conflict of interest and source of funding statement

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In gingivitis, plaque accumulation at the gingival margin leads to inflammation and increasing proportions of proteolytic and often obligately anaerobic species. The natural mucosal barriers and saliva are the main innate defence mechanisms against soft tissue bacterial invasion. Similarly, enamel and dentin are important hard tissue barriers to the caries process. Given that the present state of knowledge suggests that the aetiologies of caries and periodontal diseases are mutually independent, the elements of innate immunity that appear to contribute to resistance to both are somewhat coincidental.

Oral biofilms are present on all intra-oral surfaces. Biofilm composition varies between healthy sites and between different between healthy and diseased sites. Dental caries and periodontal diseases are among the most prevalent diseases of mankind. These oral diseases have a negative impact on quality of life, and dental caries and periodontitis are the main causes of tooth loss. The biofilm is required for the development of these oral diseases. The microbial composition of the dental biofilm has been extensively studied using cultural methods, which limit the identification and cultivation of all microorganisms in the environment. Current approaches, however, use genome sequencing to identify and quantify the microorganisms in dental biofilms, which is now known to be more complex and variable from site-to-site than previously appreciated. The potential interactions between individual species, between the biofilm, the environment, and the host have been mainly studied using in vitro model systems. Whereas these models cannot completely mimic the host environment, they do provide a basis to raise and test mechanistic hypotheses of microbe–microbe and microbe–host interactions contributing to oral health and disease.

To better understand the role of microbial biofilms in the maintenance of oral health and the development of dental caries and periodontal diseases, the following working terms have been applied:

- *Biofilm*: microorganisms attached to a surface embedded in an extracellular matrix in contact with a fluid phase. The properties of the microorganisms in the biofilm are generally different from those in planktonic state. Plaque on a tooth surface is a classical example of a biofilm and is termed: dental biofilm. Microorganisms function in dental biofilms as interactive microbial communities. Biofilms also exist on other surfaces in the mouth and can shed into the fluid phase (saliva, gingival crevicular fluid).
- *Microbial Interactions*: microorganisms in biofilms are in physical proximity, which facilitates a range of interactions, which may be synergistic or antagonistic.
- *Symbiosis* is a mutually beneficial relationship among members of the microbial community and between the microbial communities and the host, with varying degrees of benefit.

- *Dysbiosis* is a change in the microbial communities associated with health, resulting in a breakdown of the beneficial relationship with the host, which is deleterious to health.
- *Host defence* includes host responses and physical barriers.
- *Physical barriers*, such as enamel and epithelium, together with oral fluids, protect the underlying tissues from microbial insults.
- *Host response* is an active process that includes innate and adaptive immunity, which commonly arises from a microbial challenge.
- *Innate host response* (*versus* adaptative immunity) is a protective mechanism against microbial challenge that is immediate and of limited specificity.
- *Adaptive host response* is an acquired response to a specific microbial challenge.
- *Functional fluids* are fluids produced by the body that carry innate and adaptive immune mediators.

This consensus report will provide a foundation for designing studies to elucidate the actual interactions between the biofilm and the host with the potential to design novel strategies to improve oral health.

Dental Biofilm: Ecological Interactions in Health and Disease

Multispecies biofilms form naturally on all oral surfaces. The proximity of microorganisms within these biofilms creates opportunities for species to interact. Many types of intermicrobial interactions have been described, but it should be noted that most of these interactions have been investigated in laboratory systems, and occasionally animal models, and therefore, some caution should be exercised when extrapolating these findings to events in humans (Marsh & Zaura 2017).

Saliva is the primary source of nutrients for health-associated biofilms. The metabolism of host glycoproteins and proteins by the oral microbiota requires microbial cooperation and the sharing of a broad repertoire of glycosidases and proteases. The catabolism of these salivary molecules facilitates the growth of oral microorganisms but with minimal changes in environmental pH. In the presence of a stable pH, microbial species that grow optimally in the pH range 6.5–7.5 (neutrophilic microorganisms) results in biofilm homoeostasis and generation of a range of nutritional inter-dependencies among the bacteria.

Dental biofilms develop as functionally and structurally organized communities of interacting microorganisms. These interactions can be synergistic or antagonistic and produce a biofilm that prevents colonization by non-oral microorganisms and provides protection to the dental surfaces. A health-associated biofilm is associated with an active balance between slow rates of acid production and compensatory alkali generation, resulting in an environment with a broadly neutral pH. Such conditions help to stabilize the composition of health-associated species while restricting the growth of microorganisms associated with caries and periodontal diseases. Microbes in a health-associated biofilm can produce substances including H_2O_2 and bacteriocins that may suppress the growth of microorganisms associated with disease.

In addition to saliva, dietary sugars are a major source of nutrients. Among them, sucrose has unique properties. It is considered the most cariogenic dietary carbohydrate, because fermented to acids by the bacteria biofilm, but also because it can be metabolized to intracellular and extracellular polysaccharides (IPS and EPS). In the presence of dietary sugars, the biofilm is characterized by saccharolytic, acidogenic and aciduric species, that also synthesize extracellular polysaccharides that contribute to the biofilm matrix. The saccharolytic and acidogenic microorganisms generate a low pH from the fermentation of dietary sugars, which inhibits "neutrophilic microorganisms" associated with enamel health. In the absence of dietary sugars, a caries-associated biofilm can generate acids from the metabolism of extracellular glucans and fructans, and intracellular storage compounds.

Gingival crevicular fluid (GCF) is a main source of nutrients for microorganisms in biofilms associated with periodontal diseases, and the biofilm is characterized by proteolytic and often obligately anaerobic species. Inter-microbial interactions are necessary for the concerted and sequential catabolism of host proteins and glycoproteins for nutritional purposes. These interactions are also essential for growth factors required by fastidious microorganisms present in these biofilms; examples include the release of haemin from haemoglobin, and the sharing of siderophores. Complex food webs develop, whereby the products of one organism are further degraded by neighbouring species, and organisms scavenge oxygen creating highly reduced environments suitable for obligately anaerobic organisms.

To facilitate survival or persistence, some microorganisms subvert the host defences through the release of certain molecules, such as proteinases, leukotoxins, modified lipopolysaccharides (LPS). Some of these microorganisms are not only capable of enduring the host response, but have adapted to exploit the altered environmental conditions, and are termed inflammophiles "inflammophiles" (Hajishengallis, 2014).

Microorganisms associated with both caries and periodontal diseases are metabolically highly specialized and organized as multispecies microbial biofilms. Progression of dental caries and periodontal diseases involves positive feedback loops, but are driven by different stressors. In caries, the frequent exposure to dietary sugars (carbohydrates) and fermentation to organic acids can result in a positive feedback loop resulting in ever increasing proportions of acidogenic and aciduric species which enhances the acidity of the environment. In gingivitis, plaque accumulation at the gingival margin leads to inflammation and a positive feedback loop resulting in ever increasing proportions of inflammophiles. Some highly specialized members of the community can subvert and dysregulate the host immune response, which may result in destruction of periodontal tissues in susceptible individuals.

Biofilms associated with caries and periodontal diseases display emergent properties; that is, the properties of the community are more than the sum of the individual species. For example, most oral

bacteria can grow only poorly or not at all in pure culture on glycoproteins in saliva or GCF, but can thrive when metabolizing these complex host molecules as part of a microbial community. Microorganisms that persist in dentin caries and periodontal diseases require the cooperative degradation of complex host molecules (proteins and glycoproteins) as nutritional and energy sources.

Many of these interactions have been defined in vitro, using simple combination(s) of species. Additionally, the majority of the interactions studied involve bacteria only, ignoring other segments of the oral microbiota (fungi, *Archaea*, viruses, protozoa). Current technological advances enable the study of more complex community level interactions (Edlund et al. 2015), including those among members of the microbiota from different kingdoms (Diaz et al. 2016). Rather than just cataloguing which microorganisms are present, attention needs to be focussed on what they are doing within these microbial communities (Takahashi 2015).

In the future, a more complete understanding of the microbial "interactome" (Jenkinson 2011) could facilitate discovery of novel ecological preventive and therapeutic approaches. Such approaches could be applied to disease control by promoting health-associated communities through nutritional or therapeutic strategies, and interfering with the interactions associated with disease.

Role of Microbial Communities in the Pathogenesis of Periodontitis and Caries

Until recently, the microbial community structure of healthy dental biofilms was based on cultural microbiology, DNA:DNA hybridization and polymerase chain reaction (PCR) investigations. From these studies, the microbial community was suggested to be of relatively low diversity dominated by Gram-positive organisms including species belonging to the genera *Streptococcus* and *Actinomyces*. More recent studies using alternative high throughput non-cultural approaches suggest the diversity in health is higher than previously thought (Mira et al. 2017). However, at present, it is not possible to define a "core" microbiome or specific community structures associated with health because of the following:

- dental biofilms in healthy individuals vary substantially within and between individuals and may reflect differing susceptibility to disease;
- during the course of life from birth through old age, community variations change, reflecting ageing oral anatomy, ecology and immune function; and
- most studies to date using the new technologies have been performed on relatively few individuals and few sites.

Nevertheless, there is an emerging consensus that several genera are normally associated with health, including *Neisseria*, *Streptococcus*, *Actinomyces*, *Veillonella* and *Granulicatella*. Understanding the role of these health-associated organisms may have utility in the application of pre- and probiotic approaches for the prevention and treatment of disease.

Given high inter-individual variability in bacterial composition in health, functional properties of bacterial communities should be a conceptual focus rather than taxonomic composition. Indeed, whole-DNA sequencing studies show that different healthy individuals may harbour extremely variable bacterial communities but their gene content and functional capability are remarkably similar (Human Microbiome Project Consortium 2012).

As the microbial composition shifts from health to caries, the community structure varies substantially from site-to-site and between individuals and with developing disease. Nevertheless, there are some unifying characteristics of caries-associated biofilms. Overall microbial diversity appears to be lower in disease than health, which may reflect the ecological pressure of lowered environmental pH. Characteristic features of these organisms include acidogenicity, acid tolerance and the formation of extracellular polysaccharides from dietary sucrose. In addition to the classically recognized organisms such as the mutans streptococci and lactobacilli, more recent studies implicate a wider range of organisms in a dynamic and potentially interactive community structure. These include *Bifidobacterium dentium* (Mantzourani et al. 2009) and *Scardovia wiggsiae*, the latter associated with Early Childhood Caries (Tanner et al. 2011). DNA from other bacteria such as *Schlegelella* or *Pseudoramibacter* appears to be present in dentin caries lesions (Simón-Soro & Mira 2015), but the lack of experimental studies on these organisms has precluded evaluation of their cariogenic potential.

In periodontal diseases, the community structure is historically viewed as associated largely with Gram-negative proteolytic anaerobic species. A limited number of species were considered to be major pathogens, including: *Porphyromonas gingivalis, Treponema denticola, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans*. Using more recent technologies, the microbial community structure in periodontal diseases appears to undergo major alterations. Dysbiosis is recognized as follows: the loss or reduction in potentially beneficial organisms, increased proportions of species in health, and emergence of many organisms that were undetected using previous technologies. Unlike caries, dysbiosis in periodontal diseases is associated with an increase in microbial diversity, which could be the result of impaired local immune function, increased availability of nutrients or a reflection of the diverse environmental niches at the sampling site at the periodontal pocket (Dewhirst et al. 2010, Camelo-Castillo et al. 2015). This view does not preclude the possibility of occasional instances of periodontitis caused by a more restricted spectrum of organisms as evidenced by the specific role of the JP2 clone of *A. actinomycetemcomitans* in cases of aggressive periodontitis in individuals of West African descent (Haubek et al. 2008). In general, the functional characteristics of microbial communities in periodontal diseases include the ability to withstand/deregulate the immune and inflammatory response, to prevail in an anaerobic environment and to take advantage to the altered nutritional availability of potential substrates in gingival crevicular fluid flow and blood.

The microbial communities associated with periodontal health and caries-free dentition are in symbiosis with the host. Maintenance of this symbiotic state will be dependent upon on factors/processes derived from both bacteria (for example nutritional interdependencies between different bacteria) and from the host (for example salivary glycoproteins which provide a continuous supply of nutrients to the dental biofilms). That the microbial community requires these factors and processes would appear to stabilize the symbiotic state with the host and the maintenance of health.

Stressors applied to the symbiotic state can perturb homoeostasis and lead to dysbiosis wherein the health-associated microbial populations are significantly altered and consistent with the development of disease (Mira et al. 2017). In dental caries will include dietary sugars and reduced salivary flow or altered salivary composition. Stressors in the case of periodontal diseases may include alterations to the effectiveness of the immune response (for example impaired function or reduced numbers of neutrophils) and the activities of keystone bacterial species (for example *P. gingivalis*) able to manipulate the overall bacterial population structure. The dysbiotic state may also resist reversion to symbiosis dependent upon factors derived from both bacteria and the host. Understanding the factors and process that are required to maintain symbiotic and dysbiotic states and the stressors that drive one state into another could inform new development of preventive and therapeutic strategies in caries and periodontal diseases.

To reliably characterize the extensive within and between individual variations in the microbial communities in dental biofilms in both health and disease, studies with larger population sizes and more refined sampling methods are necessary. To elucidate the dimensional and changeable presentation of these diseases, longitudinal investigations will be required on a range of different populations. Functional analysis of microbial populations may prove to be a more valuable approach than taxonomic analysis. The application of -omics technologies coupled with methods for integration of large data sets will allow a holistic overview of these diseases which combines the contribution of not only microbial populations but also the host and the environment. Such a holistic overview may allow the translation of the multifactorial aetiology of these oral diseases into integrated diagnostic and therapeutic approaches.

With regard to the functional roles of microbial populations in dental biofilms, the development and application of the following methods will be important:

- advanced imaging at the microscopic and submicroscopic level to elucidate, for example, community structures and cell–cell communication in both model and natural biofilms;
- analysis of gene expression at the microbial community level in both symbiotic and dysbiotic conditions; and
- application of microbiological endpoints into randomized clinical trials.

The historical paradigm that both caries and periodontal diseases were a consequence of only a limited repertoire of microorganisms led to diagnostic, preventive and therapeutic strategies with targeted specificity. Based on our more recent understanding that both groups of diseases are associated with complex and variable communities in a dysbiotic state, future approaches should recognize this multifactorial and variable microbial aetiology and the potential functional importance of microbial populations in dysbiosis with their host. The ultimate challenge will be to translate this new knowledge into novel, practical approaches for prevention, diagnosis and therapy of caries and periodontal diseases to the benefit of human populations.

The Innate Host Response in Caries and Periodontal Diseases

The oral mucosa, salivary environment, transmigrating polymorphonuclear leucocytes (PMNs) and gingival crevicular fluid form the first lines of defence against the microbial challenge arising from the dental biofilm. Antimicrobial peptides and proteins derived from epithelial cells, salivary epithelial duct cells, neutrophils and fibroblasts provide protection for periodontal and enamel health (Meyle et al. 2017). In addition, antimicrobial peptide-rich neutrophils are found in both saliva and gingival crevicular fluid (Gorr & Abdolhosseini 2011, Dommisch & Jepsen 2015). Bioactive lipids, such as resolvins, may be protective mediators in periodontal health (Hasturk & Kantarci 2015). Mucins and agglutinins in saliva and pre-existing specific antibodies (IgG) in GCF contribute to the clearance of microbes. Other antimicrobial factors such as activated complement also function in GCF.

The dental biofilm may be affected by the relative abundance and composition of proteins in the salivary pellicle or film coating the teeth, which could protect against caries. The salivary pellicle also contains calcium-binding proteins such as statherin, which can make the pellicle supersaturated with calcium and phosphate salts in hydroxyapatite. The pellicle can, therefore, contribute to maintaining mineralization of the enamel surface and aid in resistance to demineralization. In response to chewing and the sensory quality of foods (gustatory stimulation), the salivary flow rate increases. The stimulated saliva has increased buffering capacity and altered composition. The shear forces from increased flow tend to reduce adhesion of microbes and microbial complexes to the tooth surface. The protein composition of stimulated saliva can inhibit microbial growth, whereas the ionic content reduces enamel solubility. Collectively these mechanisms provide protection against caries.

Enamel and dentin are important hard tissue barriers to the caries process. Fluoride interferes with the carious process by reducing demineralization and enhancing remineralization. Certain developmental defects of enamel result in breakdown of the barrier and may increase the risk of carious lesions. The prevalence of root caries increases in the presence of gingival recession, suggesting that intact junctional epithelium and proximal gingiva are protective. During the caries process, odontoblasts proximal to dentinal tubules may stimulate intra-

tubular mineralization resulting in closure (Garces-Ortiz et al. 2013) and reduced diffusion of intra-tubular fluid. In response, the dental pulp releases soluble proinflammatory mediators. For example, pulpal cells release interleukin-1β and defensins. The defensin hBD-2 and IL-1β stimulate the expression of other mediators including dentin sialophosphoprotein, contributing to dentin repair.

The incidence of gingivitis increases in proximity to cervical carious lesions. At these sites, the soft tissue inflammation may be promoted because dental plaque accumulates. During inflammation, the integrity of the epithelial barrier prevents bacterial penetration. Inflammation stimulates turnover of gingival and junctional epithelial cells. As the superficial epithelial cells are shed into the salivary environment and swallowed, adherent and intracellular bacteria are effectively disposed. The self-renewing quality of squamous epithelia is a valuable protective mechanism against infection.

Gingival epithelial cells also produce soluble proinflammatory mediators – cytokines – in response to oral microbes. For example, Gram-positive and Gram-negative bacterial pathogen-associated molecular patterns engage Toll-like receptors (TLR) to signal to regulate IL-1α, IL-6 and IL-8. TLR signalling and other signalling pathways work to increase innate antimicrobial resistance of the epithelium. For example, in response to the pathogenic bacteria, epithelial cells release IL-1α to stimulate epithelial cells via an autocrine loop, upregulating the expression of antimicrobial peptides, including hBDs, calprotectin and cathelicidin.

Bacterial chemoattractants and activated complement components stimulate activation and emigration of PMNs through the junctional epithelium into the GCF. As phagocytes are storehouses of antimicrobial proteins, PMNs represent a major innate cellular response. PMNs are prominent during gingivitis and early periodontitis. A preponderance of PMNs also appears during acute exacerbations of periodontitis. Diseases affecting normal function of PMNs typically increase the risk of periodontitis. For example, periodontitis is a prominent comorbidity of leucocyte adhesion deficiency syndrome and chronic granulomatous disease.

During periodontitis, the PMN response tends to reduce proportionally to other cells. Periodontitis is also characterized by activation of Langerhans dendritic cells and intraepithelial lymphocytes such as γδ-T cells. These innate immune cells capture and present bacterial antigens to CD4 and CD8 T cells of the adaptive immune system. Activated γδ-T cells produce IL-17, which is mechanistically associated with periodontal bone loss in periodontitis. The expression of antimicrobial peptides also differs in gingival tissues from gingivitis and periodontitis patients. In patients with periodontitis, hBD-1 levels are higher than in gingivitis. Levels of hBD-2 and hBD-3 appear similar in both infections.

Innate immunity during the caries process includes salivary and to a lesser extent soluble mediators in GCF. The salivary factors work at the enamel surface to increase surface mineralization and minimize bacterial adhesion and growth. Within the pulp, stimulated odontoblasts also function in innate reparative mechanisms promoting mineralization from within the tooth.

The antimicrobial role of PMNs in gingivitis tends to be replaced in periodontitis by Langerhans dendritic cells and γδ-T cells. Upon stimulation, these cells increase production of proinflammatory cytokines including IL-1, IL-6, IL-8, IL-17, TNF-α and IL-23. The Langerhans cells and γδ-T cells bridge the innate and adaptive immune responses.

Invading bacteria and their products can activate proximal capillary endothelial cells to increase expression of ICAM-1 and selectin receptors (Hajishengallis et al. 2016). Concomitantly, capillaries allow exudation and leucocyte transmigration, facilitating the presence of leucocytes in the tissues. The activated endothelial cells also express Del-1, which blocks leucocyte adhesion to LFA-1 receptors and inhibits diapedesis. In periodontitis, Del-1 production is reduced removing an impediment to PMN infiltration of the gingiva.

Commensal bacteria and putative pathogens engage TLRs and other pathogen-related receptors (PRRs) to signal for upregulation of key cytokines and antimicrobial peptides. For example, bacterial lipopolysaccharide (LPS) is bound to LPS-binding protein (CD14) and selects TLRs on neutrophils, monocytes, macrophages and mast cells. Engagement of LPS activates these leucocytes, which reside in the subgingival connective tissue. The antimicrobial peptide LL-37 may antagonize the action of LPS.

Antimicrobial peptides can also serve as danger-associated molecular patterns (DAMPs), which activate inflammasomes of the epithelial cells and PMNs. A major product of the inflammasome, superoxides are toxic to both microbes and host tissues. While antagonizing the growth of bacteria, superoxides cause local tissue damage and micro-abscess formation.

Prostaglandin E2 and related lipid mediators can activate immune cells and stimulate proinflammatory and pro-coagulant responses. In contrast, tissue destruction during inflammation in periodontitis appears to be somewhat reversible. Tissue damage appears to be reversed by resolving lipids such as the resolvins and maresins.

Complement proteins in GCF percolate through the junctional epithelium into the gingival sulcus. Rapidly activated by oral microbes, complement peptides elicit an inflammatory response. For example, C3b acts as an opsonin, facilitating phagocytosis and intracellular killing of microbes by neutrophils.

The present state of knowledge suggests that the aetiologies of caries and periodontal diseases are mutually independent. Nonetheless, elements of innate immunity appear common to both types of infections. Current understanding indicates that similarities in innate immunity to caries and periodontal diseases are coincidental. Longitudinal prospective studies are required to obtain data to identify cellular and/or soluble mediators of innate immunity that might serve as biomarkers of disease or therapeutic targets.

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Clinical Relevance

Scientific rationale for study: Caries and periodontal diseases are among the most prevalent diseases of mankind. Both have a negative impact on quality of life and the main cause of tooth loss.

Principal findings: The biofilm is an essential component involved in the development of caries and periodontal diseases, and hence, knowledge of the composition and intermicrobial interactions is fundamental for developing effective preventive and therapeutic measures.

Practical implications The knowledge of the microbe-host interactions involved in the maintenance of oral health and the initiation and progression of dental caries and periodontal diseases is key to improve preventive strategies and to design novel strategies to improve oral health.