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**In sickness & in health – what does the oral microbiome mean to us?
An ecological perspective.**

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Abstract

The oral microbiome is natural, and has a symbiotic relationship with the host by delivering important benefits. In oral health, a dynamic balance is reached between the host, the environment and the microbiome. However, the frequent intake of sugar and/or reductions in saliva flow results in extended periods of low pH in the biofilm, which disrupts this symbiotic relationship. Such conditions inhibit the growth of beneficial species and drive the selection of bacteria with an acid-producing/acid-tolerating phenotype, thereby increasing the risk of caries (dysbiosis). A more detailed understanding of the interdependencies and interactions that exist among the resident microbiota in dental biofilms, and an increased awareness of the relationship between the host and the oral microbiome, is providing new insights and fresh opportunities to promote symbiosis and prevent dysbiosis. These include: modifying the oral microbiome (e.g. with prebiotics and probiotics), manipulating the oral environment to selectively favour the growth of beneficial species, and moderating the growth and metabolism of the biofilm to reduce the likelihood of dysbiosis. Evidence is provided to suggest that the regular provision of interventions that deliver small but relevant benefits, consistently over a prolonged period, can support the maintenance of a symbiotic oral microbiome.

Humans have co-evolved with micro-organisms, and it is now estimated that we are comprised of equal numbers of prokaryotic and eukaryotic cells (Sender et al. 2016). These micro-organisms [termed the human microbiome] are not mere passengers on our bodies but play an intimate and essential role in our health and well-being. While it has been known for some time that this natural microbiome acts as a barrier to colonisation by exogenous micro-organisms, contemporary research is demonstrating additional functions that range from the normal development of the host defences and gut mucosa, to vitamin and energy production, and regulation of the cardio-vascular system (Chow et al. 2010; Relman 2012). The relationship between the microbiome and the host is dynamic, and therefore susceptible to change if there is any major perturbation to the environment. As an example, the microbiota of the forearm is relatively sparse and is dominated by staphylococci; however, if the forearm is deliberately occluded to increase moisture levels then the change in conditions drives a massive rise in the biomass (by several orders of magnitude) and a shift to a corynebacterial-dominated community (Marples 1965). It has also become apparent that disruption of this intimate relationship can lead to deleterious consequences for the host, and even pathology [a process termed dysbiosis]. Detrimental effects can range from auto-immune or inflammatory-mediated diseases (such as inflammatory bowel disease or Crohns Disease) to malnutrition or obesity and even to neurological disorders and cancer (for examples, see (Cho 2012; Chow et al. 2010; Dinan and Cryan 2017; Relman 2012).

The oral microbiome in health

The mouth is colonised naturally by a diverse range of micro-organisms, and the composition of this oral microbiome is characteristic of the site and distinct from that of neighbouring sites, such as the skin and the digestive tract, in spite of the regular

and repeated transfer of micro-organisms between these habitats (Aas et al. 2005; Krishnan 2017; Papaioannou et al. 2009). This confirms the key role played by the local environment in determining which species can colonise, grow and become either major or minor components of the microbiota at a site. Like elsewhere in the body, the oral microbiome has a symbiotic relationship with the host. The host provides a nutritious and warm habitat, with a prevailing pH and a range of gaseous atmospheric conditions that are suitable for the growth of a wide range of microbial genera. In return, the oral microbiota delivers some key functions that provide important benefits to the host. The resident oral microbiota also acts as a barrier to exogenous organisms but, in addition, some members of the microbiota [e.g. certain streptococci] play an immunomodulatory role, and down-regulate unwanted potentially pro-inflammatory responses to beneficial indigenous organisms (Devine et al. 2015). Other resident oral bacteria have also been shown to participate in an entero-salivary nitrate reduction cycle in which dietary nitrate that reappears in the mouth via saliva is reduced to nitrite [which has beneficial effects for blood pressure control and vascular health]; furthermore, when the nitrite is swallowed it is converted in the stomach to acidified nitric oxide, which is antimicrobial and stimulates gastric mucus production (Kapil et al. 2013; Kapil et al. 2014). The different surfaces within the mouth support distinct combinations of consortia of oral micro-organisms (Aas et al. 2005; Papaioannou et al. 2009); the composition of these consortia are a response to and reflect the prevailing ecological determinants at each site, especially in terms of nutrient supply, degree of anaerobiosis and pH. Once established at a site, the overall composition of the microbiota can remain relatively stable over time (Richards et al. 2017).

The mouth supports the growth of a diverse array of micro-organisms including viruses, fungi, *Archaea*, and even protozoa, but the predominant group are bacteria, of which approximately 700 species have been identified (Aas et al. 2005; Wade 2013). Of these 700, only about half have been given an official name, while 30% have yet to be cultivated in the laboratory (Dewhirst et al. 2010). On average, a person may harbour approximately 100-200 individual species.

The microbiota exists in the mouth as multi-species biofilms, the composition and metabolic activity of which is determined by host and environmental factors (Filoche et al. 2010). Biofilms do not form randomly, but develop via a number of waves of microbial succession in which the diversity and richness of the microbiota increases over time (Jakubovics and Kolenbrander 2010). Early colonisers modify the environment enabling more fastidious species to attach and become established at a later time point. As the biofilm matures, some of the bacteria synthesise extracellular polymers [especially from sucrose] and these contribute to the biofilm matrix (Koo et al. 2013). This matrix functions as more than as a physical scaffold, and has important functions such as preventing desiccation and retaining extracellular products including enzymes (Flemming and Wingender 2011). The matrix also contains extracellular DNA, derived from lysed bacteria, and this also contributes to the physical structure of dental biofilms (Jakubovics et al. 2013). Thus, microbial biofilms are both structurally- and functionally-organised, and exist as highly interactive microbial communities (Mark Welch et al. 2016). These microbial interactions can be both synergistic and antagonistic, and create a series of inter-dependencies that provide stability and a resilience to change (Jakubovics 2015; Marsh and Zaura 2017). These interactions enable consortia of organisms to catabolise structurally-complex host substrates, such as salivary mucins, in a

concerted and sequential manner; these molecules would generally be recalcitrant to the action of single species (Bradshaw et al. 1994; Byers et al. 1999). Similarly, obligately anaerobic bacteria thrive in an overtly aerobic habitat by co-existing with oxygen-consuming species (Bradshaw et al. 1998). In this way, oral microbial communities exhibit emergent properties, in that the attributes of the community are more than the sum of the individual species (Konopka 2009). A feature of microbial biofilms that is of clinical significance is their reduced sensitivity to antimicrobial agents. This tolerance is due to a number of factors that include: a lack of penetration of charged molecules into the depths of the biofilm, the slow growth rate of bacteria when on a surface, sub-optimal conditions for drug activity within the biofilm, and inactivation of the agent by neighbouring organisms (Olsen 2015). In addition, gene transfer is an efficient process in biofilms because bacteria are in close proximity to one another, and there is evidence for the transfer of drug resistance genes in dental plaque (Roberts and Mullany 2010).

In summary, dental biofilms are natural, and play a positive role in maintaining oral health, with many of the resident bacteria delivering important benefits. A complex network of interdependencies exists among the members of the biofilm, and these contribute to maintaining community stability and resistance to change.

The oral microbiome and disease

On occasions, this symbiotic relationship between the oral microbiome and the host can breakdown, and disease can be a consequence (dysbiosis). For the purpose of this article, the subsequent discussion will be focussed on the role of the microbiome in dental caries; the role of the oral microbiome in periodontal diseases has been reviewed extensively elsewhere (Diaz et al. 2016; Mira et al. 2017; Perez-Chaparro

et al. 2014).

Numerous studies of people of different ages, from a variety of countries and with different diets, have shown that there are substantial difference in the composition of the microbiota in biofilms overlying caries lesions, with an enrichment of species with an acidogenic and acid-tolerating phenotype. Early culture-based studies had shown that enamel caries was associated with increases in the numbers and proportions of mutans streptococci (Loesche 1986), with lactobacilli being recovered from more advanced lesions (Caufield et al. 2015). However, such studies always reported caries sites in which these organisms were not detected, and the presence of these bacteria on surfaces that were caries-free at the time of sampling (for examples see (Loesche et al. 1975; Loesche and Straffon 1979).

The cariogenicity of the bacteria implicated with dental caries has been linked to their ability to rapidly convert dietary sugars to acid [and lower the pH and demineralise the tooth structure], and importantly, to be able to continue to grow and metabolise sugars under these acidic conditions (Harper and Loesche 1984; Loesche 1986). Organisms such as mutans streptococci can also synthesise intracellular and extracellular polysaccharides from sucrose (Bowen and Koo 2011; Loesche 1986); the former provides a carbohydrate reserve which could be used to generate acid in the absence of dietary sugars while the latter makes a major contribution to the plaque matrix. In contrast, many of the beneficial resident bacteria preferentially grow at neutral pH and are unable to grow under acidic conditions. If such conditions of low pH are repeated on a regular basis, then the acidogenic/aciduric species are eventually able to increase their proportions and drive the plaque pH even lower, and out-compete the beneficial species (Bradshaw

et al. 2002; Bradshaw et al. 1989).

The bacterial traits linked to cariogenicity are not unique to mutans streptococci, however, and over time studies have shown that a number of other species have properties that are relevant to the caries process. Also, laboratory studies have shown that there is heterogeneity in terms of expression of these attributes among clinical strains belonging to a species, so that some strains of mutans streptococci can be less acidogenic than isolates of other streptococcal species (de Soet et al. 2000) (Burne et al, this issue). Recent culture-based studies have correlated more diverse communities of bacteria with caries, including reporting on the association of *Actinomyces* and *Bifidobacterium* species with lesions, often with mutans streptococci comprising a relatively small percentage of the microbiota at diseased sites (Mantzourani et al. 2009a; Mantzourani et al. 2009b; Tanner et al. 2016)(see Tanner et al, this issue). The more recent application of molecular-based [culture-independent] techniques have confirmed a much wider diversity of species associated with caries including newly described organisms such as *Scardovia wiggsiae* and *Slackia exigua* (Henne et al. 2015; Richards et al. 2017; Tanner et al. 2011). Thus, although there may be a lack of apparent specificity in the aetiology of caries in terms of bacterial name, there is a definite specificity in terms of biochemical function. Characterising oral biofilms by metabolic activity rather than by listing the predominant species will become an increasingly common approach in the future when defining plaque biofilms in health and disease.

Approaches to manipulate the microbiome to favour oral health

The accumulative body of evidence, therefore, suggests that, in contrast to classical infectious diseases in which a specific pathogen is acquired and disease is a consequence, caries is associated with a dysbiotic shift in the composition of a natural microbiome. This involves increases in the number and/or proportions of acidogenic and acid-tolerating species within the biofilm, all of which can also be detected in health (albeit in low numbers). These concepts have been captured in the various iterations of an Ecological Plaque Hypothesis (Filoche et al. 2010; Kleinberg 2002; Marsh 1994; 2003; Takahashi and Nyvad 2008; 2011), in which caries is a result of a shift in the composition of the biofilm microbiota driven by environmental change. An enrichment of acidophilic bacteria will occur if the biofilm spends increasing amounts of time under acidic conditions as a result of the frequent intake of fermentable carbohydrates in the diet and/or an impairment in saliva flow. Implicit in the original Ecological Plaque Hypothesis is that disease could be prevented not only by targeting the implicated organisms directly but also by reducing or interfering with the drivers of dysbiosis (Marsh 1994; 2003). This is consistent with the established view that the control of caries, as a multi-factorial disease, requires an holistic approach of effective mechanical plaque control, diet modification and modulation of the microbiota. Given that the oral microbiota is natural and provides benefits, then it is logical to consider complementary approaches in order to modify the microbiota and promote the growth of beneficial bacteria (Table 1). It also follows that, if caries is a consequence of an altered environment, then a healthy microbiome might be maintained or even restored if the drivers of dysbiosis are inhibited.

Modify the composition of the microbiome. A number of approaches to favourably manipulate the composition of the oral microbiome are being investigated.

Probiotics are live micro-organisms that deliver health benefits, and a number of dairy strains of lactobacilli and bifidobacteria have been developed for human use, and incorporated into a range of delivery vehicles. There is evidence that their regular consumption provides benefits to the biology of the gut, and so, by extrapolation, similar bacterial species are now being evaluated for comparable health benefits in the mouth (Devine and Marsh 2009). A major difference in their proposed application, however, is that these dairy strains are not adapted for growth in the mouth, and unlike the gut, the amount of time for them to deliver any benefit in the oral cavity is short, unless effects are systemically derived following swallowing. Evidence for health benefits from the use of these strains is equivocal (Laleman et al. 2014), and a systematic review has concluded that there is insufficient evidence to support the use of probiotics in reducing caries, but there is a small benefit for the management of gingivitis and periodontitis (Gruner et al. 2016).

Recently, oral streptococci with potentially useful properties have been identified in caries-free individuals, and these strains may form the basis of more effective oral probiotics. *Streptococcus* A12 was isolated from a caries-free individual and shown to express the arginine deiminase system while also being able to inhibit the growth and block key functions of *S. mutans* (Huang et al. 2016). Similarly, *S. dentisani* has been recovered from a high proportion of caries-free individuals, and is also arginolytic, and produces a bacteriocin that can kill mutans streptococci (Lopez-Lopez et al. 2017). Both of these bacteria are found naturally in the mouth, possess the ability to raise the pH in biofilms, and have evolved to colonise and compete in oral biofilms. In the future, these strains may form the basis of bespoke oral probiotic strains, as they should be more successful than dairy or gut probiotic organisms at colonisation if implanted in the oral cavity.

A complementary approach is to selectively boost the growth of resident beneficial bacteria using prebiotics. Some species of commensal streptococci generate energy from arginine; the metabolism of arginine also leads to ammonia production and a rise in environmental pH (Huang et al. 2016; Lopez-Lopez et al. 2017). Many of these arginolytic bacteria also produce hydrogen peroxide that is antagonistic to other plaque bacteria, including species associated with periodontal diseases (Hillman et al. 1985). Arginine has been formulated into a toothpaste, and a short-term, small pilot study reported no change in the microbial composition of the biofilm but a favourable shift in the salivary microbiome and a reduced capacity to convert sucrose to lactate (Koopman et al. 2017).

Recently, studies have been initiated to systematically screen a wide range of compounds to identify additional molecules that can exclusively stimulate the growth of beneficial bacteria and either inhibit or have a neutral impact on potentially pathogenic organisms. Beta-methyl-D-galactoside and N-acetyl-D-mannosamine were shown in a number of model systems to be able to boost the growth and metabolism of streptococci linked to oral health (Slomka et al. 2017). Challenges lie ahead in identifying affordable compounds that could be formulated appropriately, and then delivered to, and retained in the mouth for, sufficiently long periods to drive favourable changes to the composition of the biofilm. The prospect of the development of novel and effective pre- and probiotics could eventually lead to new therapeutic options to maintain a beneficial oral microbiome.

Manipulate the local oral environment. If dental caries is driven, in part, by a deleterious environmental change in the biofilm which selects for acidogenic and acid-tolerating bacteria, then it follows that these microbial shifts could be prevented

or reduced if the oral environment is maintained under conditions that favour dental health.

Saliva plays a central role in oral health, especially in protecting against dental caries. Saliva has a number of important functions, and these include: acting as a buffer and maintaining a favourable pH for the resident oral microbiome; removal of substrates, fermentation products and loosely attached bacteria; delivery of components of the innate and adaptive host defences; and providing substrates that support the growth of beneficial oral micro-organisms (Marsh et al. 2016). A reduced flow of saliva dramatically increases the risk of dental caries, and this can be as a consequence of ageing, a side-effect of medications, or following head and neck radiation treatment. Any approach that stimulates the flow of saliva will help to maintain an environment that supports growth of the natural and beneficial oral microbiome. Strategies can include the use of sugar-free gums, and encouraging patients to avoid regular snacking on sugar-containing drinks and food, and, where appropriate, using products containing non-fermentable sweeteners. Sugar alcohols such as erythritol and xylitol have been incorporated into a variety of products, including those specifically designed for oral care, and shown to reduce the incidence of caries; these polyols stimulate the flow of saliva but cannot be fermented by oral bacteria to acid, while there is some evidence that they possess some antibacterial properties (Falony et al. 2016; Makinen 2010) (Decock this issue). As discussed earlier, the supplementation of oral care products with base-generating compounds such as arginine can help to foster a favourable oral environment (and by extrapolation, a beneficial microbiome) by promoting alkali generation within the biofilm (Koopman et al. 2017).

Recently, a metagenomic study has been performed on the effect of an oral care product formulated with components of the innate host defences [lactoferrin, lysozyme, lactoperoxidase system] as well as a number of other proteins. The short term regular use of this product by dentally-healthy subjects led to small but arguably favourable shifts in the balance of the oral microbiome. There were small but significant increases in 12 taxa associated with dental health including *Neisseria* spp. and a decrease in 10 taxa associated with periodontal disease including *Treponema* spp. (Adams et al. 2017).

Modulate the growth of dental biofilms. It has been argued that dental caries is a consequence of a change in the oral environment that selects for micro-organisms that have an acid producing and acid-tolerating phenotype, eventually resulting in a shift in the composition and metabolism of dental biofilms. It follows, therefore, that approaches that restrict the enrichment of microbes with these traits in dental biofilms will support the maintenance of a beneficial microbiome. Approaches described in the preceding sections could be used in the future to manage the biofilm, but many existing strategies are based around the use of antimicrobial agents. Originally their use and mode of action was discussed in the same way as those used in medicine (Marsh 2010; 2012), but a consideration of the way these compounds are delivered to the mouth may shed fresh insights into the way they function when delivered from oral care products.

Firstly, the target for these antimicrobial agents is oral biofilms and, as discussed earlier, these structures are less susceptible than planktonic bacteria (Gilbert et al. 1997). Secondly, the pharmacokinetics of delivery of antimicrobial agents from oral care products (twice daily; short delivery time) means that active compounds will be present at high concentrations (at or above the MIC or MBC) for

relatively brief periods (perhaps minutes) but retained at sub-lethal levels for a much longer time (probably hours). At these latter concentrations, many antimicrobials are able to inhibit traits linked to cariogenicity such as sugar transport, acid production and glucosyltransferase activity (Marsh 2010). It has been argued that this mode of action can augment the benefits of mechanical plaque control by restricting the metabolic activity and growth of dental biofilms, and that this level of activity is appropriate for antimicrobials delivered indefinitely and unsupervised from oral care products. The potential favourable impact of small but regular inhibitory effects on biofilm composition has been modelled in computer simulations (Head et al. 2014; Marsh et al. 2014; 2015a; Marsh et al. 2015b). A small reduction in terminal pH or a selective inhibition of the metabolism of the aciduric populations in the biofilm following carbohydrate metabolism prevented the acidogenic/aciduric bacteria outcompeting beneficial (non-aciduric) populations over a simulation of several weeks (Marsh et al. 2015a; Marsh et al. 2015b) (Figure 1). These *in silico studies* demonstrate that small but accumulative effects can prevent dysbiotic changes in dental biofilms and help maintain a beneficial oral microbiome - the 'nudge' approach (Filoche et al. 2010).

In conclusion, the oral microbiome is natural and provides important benefits to the host. Dental caries is a consequence of a deleterious shift in the composition and activity of the dental biofilm, driven by acid production from the metabolism of fermentable carbohydrates, especially sucrose. This results in increased proportions of bacteria with an acid-loving phenotype and a suppression of beneficial species that prefer a neutral pH. As we increase our understanding of the interplay between the environment and the oral microbiome it will become possible to identify new

strategies to combat disease by actively promoting our natural microbiota and reducing the impact of the drivers of dysbiosis.

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Table 1. Approaches to manipulate the oral microbiome to promote a symbiotic relationship with the host and prevent caries (dysbiosis).

Approach	Examples
Modify the oral microbiome: <ul style="list-style-type: none"> - Probiotics - Prebiotics 	Dairy strains; Streptococcus A12; <i>S. dentisani</i> Arginine; N-acetyl-D-mannosamine
Manipulate local environment: <ul style="list-style-type: none"> - boost saliva - boost innate defences 	Sugar-free chewing gum Oral care products containing innate defences
Modulate biofilm growth & metabolism: <ul style="list-style-type: none"> - reduce acid production - inhibit enzymes [GTF;enolase] - reduce bacterial growth rates - promote alkali production - reduce biofilm accumulation 	Oral care products + antimicrobial agents* “ “ “ “ “ “ “ “ “ “ “ “ “ “ Arginine or urea supplements Antiplaque agents

*The antimicrobial agents can deliver these effects at sub-lethal concentrations.

Legend

Figure 1 [colour version]. The predicted effect of reducing the terminal pH from pH 5.0 to pH 5.5 following sugar metabolism in a computer model of a biofilm comprised of two bacterial populations [green = non-aciduric; red = aciduric] and exposed to a pulse of glucose four times each day for 100 days.

Data are based on studies described in detail elsewhere (Head et al. 2014; Marsh et al. 2015b).

Figure 1. The predicted effect of reducing the terminal pH from pH 5.0 to pH 5.5 following sugar metabolism in a computer model of a biofilm comprised of two bacterial populations [white = non-aciduric; grey = aciduric] and exposed to a pulse of glucose four times each day for 100 days.

Data are based on studies described in detail elsewhere (Head et al. 2014; Marsh et al. 2015b).