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Dental Biofilm: Ecological Interactions in Health and Disease

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Abstract

The oral microbiome is diverse and exists as multi-species microbial communities on oral surfaces in structurally- and functionally-organised biofilms. **Aim.** To describe the network of microbial interactions (both synergistic and antagonistic) occurring within these biofilms, and assess their role in oral health and dental disease. **Methods.** PubMed database was searched for studies on microbial ecological interactions in dental biofilms. The search results did not lend themselves to systematic review and have been summarized in a narrative review instead. **Results.** 547 original research articles and 212 reviews were identified. The majority (86%) of research articles addressed bacterial-bacterial interactions, while inter-Kingdom microbial interactions were the least studied. The interactions included physical and nutritional synergistic associations, antagonism, cell-to-cell communication and gene transfer. **Conclusions.** Oral microbial communities display emergent properties that cannot be inferred from studies of single species. Individual organisms grow in environments they would not tolerate in pure culture. The networks of multiple synergistic and antagonistic interactions generate microbial inter-dependencies, and give biofilms a resilience to minor environmental perturbations, and this contributes to oral health. If key environmental pressures exceed thresholds associated with health, then the competitiveness among oral micro-organisms is altered and dysbiosis can occur, increasing the risk of dental disease.

Clinical relevance:

Scientific rationale: Micro-organisms persist in the mouth as multi-species biofilms that deliver important benefits to the host. Microbes will interact because of their physical proximity, and the outcome will influence oral biofilm composition and activity. **Principal findings:** A literature review confirmed that numerous synergistic and antagonistic interactions occur among the resident microbes, resulting in tightly integrated communities that are resilient against minor environmental perturbations, which contributes to oral health. **Practical implications:** Treatment strategies should also include reducing environmental pressures that drive dysbiosis so that a favourable ecological balance is maintained.

Introduction

The mouth supports the growth of diverse communities of micro-organisms - viruses, mycoplasmas, bacteria, Archaea, fungi and protozoa (Wade 2013). These communities persist on all surfaces as multi-species biofilms and form the resident oral microbiome, which generally exists in harmony with the host, and delivers important benefits that contribute to overall health and well-being. The micro-organisms found within these oral biofilms live in close proximity with one another, which results in a wide range of potential interactions, which can be synergistic or antagonistic. The composition of the microbiome is influenced by the oral environment, and changes in local conditions can affect the microbial interactions within these oral communities and determine, in part, whether the relationship between the oral microbiome and the host is symbiotic or potentially damaging (dysbiotic), thereby increasing the risk of diseases such as caries or periodontal diseases (Marsh 2003; Roberts & Darveau 2015). Our aim was to review systematically the literature on microbial interactions in dental biofilms in health and disease. However, the search strategy and outcomes, presented below, led to a conclusion that the topic is too broad for a systematic report and so the results are presented as a narrative review, highlighting the main microbial interactions in dental biofilms in health and introducing the environmental drivers for ecological dysbiosis towards disease.

Literature search

A PubMed search procedure was performed on 19-07-2016. The query combined four separate search items: 1) 'microbiota', including either bacteria, viruses, Archaea, fungi, protozoa or mycoplasma; 2) 'oral', including distinct oral niches; 3) interactions, including either 'ecology', 'interaction', 'synergy', 'inhibition', 'co-occurrence', 'communication', 'metabolism', 'nutrients', 'gene transfer' or 'quorum sensing' and 4) 'plaque', 'biofilm', 'community' or 'consortium' (Supplementary Table S1). This resulted in 3758 hits. Of these, 3593 passed the English language filter. After the screening of the titles and abstracts, the entries that did not relate to the topic were excluded, leaving 759 articles. Among these were 212 reviews.

The vast majority (86%) of the original research articles (N=547) addressed bacterial interactions (Table 1). These included physical (e.g., co-aggregation, co-adhesion) and nutritional synergistic interactions, antagonistic interactions such as production of bacteriocins and other inhibitory substances, cell-to-cell communication and gene transfer. The bacterial species involved ranged from primary colonizers to taxa associated with caries and periodontal disease. Only 45 (8.2%) of the studies involved fungi, while interactions involving viruses (18 studies), Archaea (4 studies) and protozoa (3 studies) were the least studied. Inter-kingdom interactions were addressed in 71 studies, with the majority of these focusing on Candida albicans and oral streptococci (Table 1).

Due to the high number of articles included and the broad range in the methods and the outcomes among the studies found, it was not possible to report on the results in the form of a systematic review or meta-analysis. Instead, the articles that were identified by the described search procedure were used as the basis of the narrative review below.

Microbial interactions in health

The close physical proximity of micro-organisms within oral biofilms inevitably increases the probability of interactions occurring. The most common types of interaction are listed in Table 2, and can be synergistic or antagonistic to the participating species (Diaz 2012; Guo et al. 2014; Hojo et al. 2009; Huang et al. 2011; Jakubovics 2015a; Kolenbrander 2011; Ng et al. 2016; Nobbs and Jenkinson 2015).

Synergistic interactions

Physical interactions and biofilm architecture

Oral micro-organisms must attach to surfaces if they are to persist in the mouth and avoid being lost by swallowing. Evidence primarily derived from laboratory studies suggests that early colonisers adhere via specific adhesin-receptor mechanisms to molecules in the conditioning films that coat oral surfaces (Hojo et al. 2009), though, ultimately, microbial growth is the major contributor to the increase in biofilm biomass (Dige et al 2007). Oral micro-organisms have a

natural tendency to adhere to other microbes and this process (co-adhesion – the adherence of planktonic cells to already attached organisms on a surface) facilitates the formation of multi-species biofilms (Kolenbrander 2011). In addition to anchoring a cell to a surface, co-adhesion also promotes microbial interactions by co-locating organisms next to physiologically-relevant partner species, thereby facilitating nutritional co-operation and food chains, gene transfer and cell-cell signalling. Substantial changes in gene expression occur when cells are in close proximity or physical contact with one another (Wright et al. 2013), while functional consequences can result, such as the protection of obligately anaerobic bacteria in aerobic environments by neighbouring species that either consume oxygen (Bradshaw et al. 1994) or are oxygen-tolerating (Diaz et al. 2002). Candida albicans can also co-aggregate with oral streptococci, and can form synergistic partnerships in which the yeast promotes streptococcal biofilm formation while streptococci enhance the invasive property of Candida (Diaz et al. 2012; Xu et al. 2014). These physical and functional associations can manifest themselves in some of the complex multi-species arrangements observed in oral biofilms formed in vivo, such as 'corn cob', 'test-tube brush' and 'hedgehog' structures (Dige et al. 2014; Mark Welch et al. 2016; Zijnge et al. 2010).

Nutritional interactions

The primary nutrients for oral micro-organisms are host proteins and glycoproteins, and these are obtained mainly from saliva for organisms in supragingival plaque (for a review, see: Jakubovics 2015b) and from gingival crevicular fluid (GCF) for those located in subgingival biofilms (Wei et al. 1999). Pure cultures of oral micro-organisms grow poorly or not at all on these structurally complex substrates, and consortia of interacting species are needed for their catabolism. Proteins are broken down by the action of mixtures of proteases and peptidases, but the catabolism of glycoproteins (consisting of a protein backbone decorated with linear or branched oligosaccharide side chains) involves the sequential removal of terminal sugars from side-chains before the protein backbone becomes accessible to proteolytic attack (Takahashi et al 2015). Oral bacteria express glycosidases with different specificities so that the

concerted action of several species is necessary for the complete degradation of host glycoproteins (Bradshaw et al. 1994). Similarly, combinations of mutans streptococci, Streptococcus oralis and Fusobacterium nucleatum degraded albumin more effectively than any of the three species alone (Homer and Beighton 1992). The biofilm matrix is another potential source for carbon and energy for interacting consortia of oral bacteria. Fructans and soluble glucans in dental plaque can be metabolised by combinations of bacteria that produce exo- and/or endo-hydrolytic enzymes (Bergeron and Burne 2001; Koo et al. 2013). Individual bacteria are dependent, therefore, on the metabolic capability of other species for access to essential nutrients.

Further complex nutritional inter-relationships develop in microbial communities when the products of metabolism of one organism (primary feeder) become the main source of nutrients for another (secondary feeder), resulting in the development of food-chains or food webs (Hojo et al. 2009) (some examples are illustrated in Figure 1). These food webs can result in the complete and energetically-efficient catabolism of complex host molecules to the simplest end products of metabolism (e.g. CO_2, CH_4, H_2S). Numerous synergistic metabolic interactions occur among bacteria in subgingival biofilms in order to enable them to degrade host proteins and glycoproteins as nutrient sources (ter Steeg & van der Hoeven 1989; ter Steeg et al 1987). These interactions are discussed in more detail later in the section on 'Ecological drivers towards dysbiosis and disease'.

Nutritional inter-dependencies such as those described above contribute to the temporal stability and resilience of oral microbial communities, while a consequence of the reliance of resident oral bacteria on the metabolism of these complex substrates is that species avoid direct competition for individual nutrients, and hence are able to co-exist and maintain a stable equilibrium, also termed microbial homeostasis (Alexander, 1971; Marsh, 1989). This has been elegantly demonstrated in a computational study on KEGG pathway-based metabolic distances between 11 oral bacteria that are known to interact (Mazumdar et al. 2013). Metabolism was a major factor driving the order of colonization, with specific metabolic pathways associated with different layers in the biofilm, resulting in a functionally structured community. However, in such a

structured community, there was an optimal trade-off between their resource sharing and functional synergy (Mazumdar et al. 2013).

Cell-cell signalling

Laboratory studies have shown that microbial cells are able to communicate with, and respond to, neighbouring cells in biofilms by means of small, diffusible, effector molecules. Gram-positive cells produce peptides that generally have a narrow spectrum of activity. In S. mutans, two peptides (competence-stimulating peptide, CSP, and sigmaX-inducing peptide, XIP) promote genetic competence in other cells of S. mutans; production of these peptides is influenced by the local pH (Guo et al. 2014) and carbohydrate source (Moye et al. 2014). CSP-mediated quorum sensing has also been identified in S. gordonii and S. intermedius. The function of CSPs is to alter gene transcription and protein synthesis involved in biofilm formation, competence development, bacteriocin synthesis, stress resistance, and autolysis (Guo et al. 2014; Senadheera and Cvitkovitch 2008). Some streptococci can inactivate CSPs, and thereby inhibit biofilm formation by S. mutans (Wang et al. 2011). CSP produced by S. gordonii can also inhibit biofilm formation by C. albicans (Jack et al. 2015), so it is possible that a complex network of signalling interactions will exist in a multi-species biofilm such as dental plaque.

Autoinducer-2 (AI-2) is produced by several genera of oral Gram-positive and Gram-negative bacteria, and may be a 'universal language' for inter-species and inter-kingdom communication in dental biofilms, and the efficiency of signalling might be enhanced by co-adhesion. Biofilm formation with two co-adhering species - S. oralis and Actinomyces naeslundii - was inhibited when an AI-2 knockout of S. oralis was used instead of the wild type (Rickard et al. 2006), while AI-2 produced by Aggregatibacter actinomycetemcomitans inhibited hyphae formation and biofilm formation by C. albicans (Bachtiar et al. 2014). AI-2 produced by F. nucleatum had a differential effect on biofilm formation when cultured with two different species of oral streptococci; biofilm formation was enhanced with S. gordonii but reduced with S. oralis (Jang et al. 2013). Some of these responses are dependent on the concentration of the signalling molecules. These cell–cell signalling strategies could enable cells to sense and adapt to

various environmental stresses and, thereby, regulate (and coordinate) the expression of genes that influence the ability of pathogens to cause disease.

Gene transfer

The close proximity of cells in biofilms provides ideal conditions for horizontal gene transfer (HGT). HGT involves either acquisition of DNA from co-resident species or from exogenous sources (Petersen et al. 2005; Roberts & Kreth 2014). DNA can be transferred through: transduction by bacterial viruses (bacteriophages), conjugation by bacterial pili, and transformation by DNA uptake involving naturally competent bacteria; in addition to the mechanisms above, DNA can also be transferred via membrane vesicles in Gram-negative bacteria (Olsen et al. 2013). HGT allows oral bacteria to sample from an immense metagenome, and in this way increase their adaptive potential to changes in the oral environment (Roberts & Kreth 2014). For instance, metabolic adaptability to carbohydrate-rich environments such as the oral cavity and gut has been found in a Lactobacillus salivarius strain carrying a plasmid with genes involved in glycolysis (Roberts & Kreth 2014). HGT is thought to be the main mechanism in acquiring antibiotic resistance genes (ARGs), which are richly present in the oral cavity (Sukumar et al. 2016).

As described earlier, signalling molecules such as competence-stimulating peptide (CSP) markedly increase the ability of recipient cells to take up DNA (Senadheera and Cvitkovitch 2008). Extracellular DNA (eDNA) is a component of the biofilm matrix and plays a critical role in adhesion and in possible nutrient storage and as a potential source of phosphate and other ions (Jakubovics & Burgess 2015). eDNA release has been demonstrated in dual species experiments with S. mutans and S. gordonii through S. mutans competence-induced bacteriocin production (Kreth et al. 2005); Gram-negative bacteria also release eDNA, including Veillonella spp (Hannan et al. 2010), Porphyromonas gingivalis and F. nucleatum (Ali Mohammed et al. 2013).

Evidence for horizontal gene transfer in dental biofilms has come from the discovery that both resident (S. mitis, S. oralis) and pathogenic (S. pneumoniae) bacteria isolated from the naso-pharyngeal area possess genes conferring penicillin resistance that display a common mosaic structure (Chi et

al. 2007). Similar evidence suggests sharing of genes encoding for penicillin-binding proteins among resident oral and pathogenic Neisseria species (Bowler et al. 1994), and IgA protease encoding genes among a range of oral streptococcal species (Poulsen et al. 1998).

Antagonistic interactions

A considerable number of studies addressed antagonistic interactions involving inter-species and inter-kingdom competition or "warfare". The production of antagonistic compounds such as bacteriocins, hydrogen peroxide, organic acids, different enzymes and release of lytic phages are just a few examples of "weapons" that can give an organism a competitive advantage during colonisation and when competing with other microbes (Table 3).

Bacteriocins and bacteriocin-like substances are produced by both Gram-positive and Gram-negative bacteria, with the most studied oral species being streptococci, and examples include mutacin produced by S. mutans (Merritt and Qi 2012), sanguicin by S. sanguinis and salivaricin by S. salivarius (Jakubovics et al. 2014). Two types of mutacin have been detected; lantibiotics, which have a broad spectrum of activity, and the more common non-lantibiotics, which have a narrower antimicrobial range (Merritt and Qi 2012). Lactobacilli also produce bacteriocins, and are being evaluated as potential oral probiotics largely due to their antimicrobial properties; for example, reuterin from Lactobacillus reuteri was active against selected periodontal and cariogenic bacteria (Kang et al. 2011).

Bacterial "warfare" implies that one of the interacting partners benefits at the expense of the other. This has been shown with two taxa occupying the same niche - S. gordonii and S. mutans, where S. gordonii had a competitive advantage over S. mutans when using amino sugars from salivary glycoproteins as an energy source: S. gordonii released hydrogen peroxide that inhibited transcription of S. mutans genes responsible for the metabolism of these compounds (Zeng et al. 2016). Indeed, hydrogen peroxide is one of the most studied agents produced in dental biofilms but its impact on the oral microbiota is complex and difficult to predict. Under aerobic conditions (as could occur during early stages of biofilm formation), Streptococcus sanguinis produces high

concentrations of hydrogen peroxide that are capable of inhibiting a range of Gram-positive species (Holmberg & Hallander 1972; Holmberg & Hallander 1973; Kreth et al. 2016); much lower concentrations are generated during anaerobic growth. Streptococcus mutans is susceptible to hydrogen peroxide, but strains that produce mutacin are able to inhibit other streptococci (Ashby et al. 2009; Ryan & Kleinberg 1995). Hydrogen peroxide production has been proposed as a major mechanism for controlling the levels of putative periodontopathic bacteria in dental plaque (Hillman & Shivers 1988; Hillman et al. 1985). However, other bacteria in the supragingival biofilms (e.g. Neisseria, Haemophilus and Actinomyces species) are also able to degrade hydrogen peroxide, and little free peroxide can be detected in plaque (Ryan & Kleinberg 1995). Thus, there may be varying concentrations of hydrogen peroxide in different regions of the biofilm, and the balance between symbiosis and dysbiosis may depend on the complex interplay between multiple antagonistic microbial interactions.

Counter-intuitively, antagonistic interactions might also be beneficial to both partners involved and might even stimulate the fitness of the microbial community (Stacy et al. 2014). In the presence of oxygen, A. actinomycetemcomitans that cross-feeds with lactate produced by S. gordonii, has to survive high concentrations of hydrogen peroxide released by S. gordonii (Figure 2). To ameliorate oxidative stress, A. actinomycetemcomitans not only expresses catalase (H_2O_2-detoxifying enzyme), but also responds to elevated H_2O_2 by induction of Dispersin B – an enzyme that promotes dispersal of A. actinomycetemcomitans biofilms, resulting in increased physical distance between the A. actinomycetemcomitans and the H_2O_2-producing S. gordonii. On the other hand, S. gordonii, which does not make its own catalase, is cross-protected by A. actinomycetemcomitans from self-inflicted oxidative stress.

A highly diverse oral bacteriophage gene pool has been discovered through a metagenomics approach (Dalmasso et al. 2015; Edlund et al. 2015a; Naidu et al. 2014; Pride et al. 2012). Phages are bacterial viruses that may lyse competing cells. The production of antagonistic factors will not necessarily lead to the complete exclusion of sensitive species as the presence of distinct microhabitats within a biofilm such as plaque enable bacteria to survive under

conditions that would be incompatible to them in a homogeneous environment. Noteworthy, although parasitic by their nature, phages might have beneficial role in the oral ecosystem: a recent comparison of the bacteria-phage network revealed that phages supported a complex microbial community structure in health that was absent during periodontal disease (Wang et al. 2016).

Antagonism will also be a mechanism whereby exogenous species are prevented from colonizing the oral cavity (bacterial interference or colonization resistance). Oral streptococci have been shown to interfere with colonization by Pseudomonas aeruginosa through nitrite-mediated interference (Scoffield & Wu 2015; Scoffield & Wu 2016), while a sophisticated colonization resistance structure has been described in an in vitro murine oral microbial community with the 'Sensor' (Streptococcus saprophyticus) sensing the intruding non-oral Escherichia coli strain and producing diffusible signals to the 'Mediator' (Streptococcus infantis) that de-represses the capacity of the 'Killer' (Streptococcus sanguinis) to produce hydrogen peroxide, resulting in inhibition of the invading E. coli (He et al. 2014).

Ecological drivers towards dysbiosis and disease

When the oral environment changes, the ecology of the ecosystem is affected. This has an impact on the outcome of the interactions among the micro-organisms in the biofilms, which will affect the proportions of the members of the community, and can increase the risk of disease (dysbiosis). Two scenarios will be dissected below: one leading towards a cariogenic and the other towards a periodontopathogenic ecosystem.

Dental caries is associated with an increased frequency of dietary sugar intake. These sugars are metabolised rapidly to acid (mainly lactic acid) and a low pH is generated within the biofilm. Lactate can be utilised by Veillonella spp., and other species, e.g. Neisseria (Hoshino & Araya 1980), Haemophilus (Traudt & Kleinberg 1996), Aggregatibacter (Brown & Whiteley 2007), Porphyromonas (Lewis et al. 2009), and Actinomyces (Takahashi & Yamada, 1996), and converted to weaker acids. Fewer carious lesions and less lactate in plaque was measured in rats inoculated with S. mutans and Veillonella alcalescens than in animals

infected with S. mutans alone (van der Hoeven et al. 1978). Higher proportions of Veillonella spp. have been detected in samples from caries lesions when compared to plaque from healthy enamel (Gross et al. 2012), perhaps because of the increased glycolytic activity and higher levels of lactate at these sites. Symbiosis between Veillonella and S. mutans has been demonstrated in mixed cultures: when Veillonella parvula was added to the pair of antagonists (S. mutans and S. gordonii), it mitigated the inhibitory effects of S. gordonii on sugar metabolism and growth of S. mutans (Liu et al. 2011).

The frequent conditions of low pH in biofilms associated with caries are inhibitory to the growth of many of the bacteria associated with enamel health, resulting in decreased microbial diversity (Gross et al. 2012; Jiang et al. 2011; Li et al. 2007; Peterson et al. 2013). Repeated conditions of low pH alter the competitiveness of members of the biofilm community and select for increased proportions of acidogenic and acid-tolerating bacteria including mutans streptococci, lactobacilli (Bradshaw et al. 1989), low-pH non-S. mutans streptococci and bifidobacteria (Marsh 1994; Takahashi & Nyvad 2008). Sucrose-induced dysbiosis results not only in reduced taxonomic diversity, but also in a changed metaproteome, as recently shown in microcosms where proteins involved in acid tolerance and acid production dominated the dysbiotic biofilms (Rudney et al. 2015).

A counter mechanism against acidification of the ecosystem is alkali production by the members of the community, mainly through ammonia production from arginine and urea (Burne & Marquis 2000; Huang et al. 2015; Liu et al. 2012; Shu et al. 2003; Takahashi 2015). Recently, by applying a metatranscriptomics and metabolomics approach, a much higher diversity in alkali-generating pathways within complex oral biofilms has been discovered, including glutamate dehydrogenase, threonine and serine deaminase, and upregulation in membrane proteins involved in ammonia gas conduction besides the urease activity and arginine deiminase system (Edlund et al. 2015b). Additionally, this study revealed that Veillonella species are well adapted towards acid stress by upregulating various pathways that contributed to pH recovery.

Thus, unlike health, dental caries is associated with a shift in the composition of the biofilm to a community that is dominated by a strongly saccharolytic and acid-tolerant microbiota leading to a loss of diversity, and a reduction in levels and activity of beneficial bacteria (Gross et al. 2012; Jiang et al. 2011; Li et al. 2007; Peterson et al. 2013), although the diversity may increase when the lesion penetrates dentine, perhaps reflecting important environmental changes (Simón-Soro et al. 2014).

In contrast, the accumulation of microbial biomass around the gingival margin induces an inflammatory response. This results in an increased flow of GCF, which delivers not only components of the host defences (e.g. immunoglobulins, complement, neutrophils, cytokines, etc) (Ebersole 2003), but, inadvertently, host molecules that can act as substrates for proteolytic bacteria. Some of these host molecules also contain haemin (e.g. haptoglobin, haemopexin, haemoglobin), which is an essential cofactor for the growth of potential periodontopathogens such as P. gingivalis (Olczak et al. 2005). The change in local environmental conditions associated with inflammation will alter the competitiveness and outcome of multiple interactions among the microbes that make up the subgingival microbiota, leading to substantial changes in the microbial composition of the biofilm. Although there is agreement that there are major changes in the proportions of individual species in biofilms from inflamed sites (for examples, see reviews by Diaz et al., 2016; Pérez-Chaparro et al. 2014), there are conflicting reports on whether the diversity of the resultant microbial communities is altered. The diversity may increase in gingivitis (Kistler et al., 2013; Schincaglia et al., 2016), but the evidence for chronic periodontitis is more contentious (Abusleme et al., 2013; Hong et al., 2015; Kirst et al., 2015; Park et al., 2015).

The inflammatory response can influence the subgingival microbiota in two ways: (1) via the impact of the host defences, and (2) by the resultant changes to the environment. The innate defences will inhibit susceptible species, but a number of periodontal pathogens, such as P. gingivalis, can subvert the host response, for example, by degrading complement, interfering with neutrophil function, and blocking phagocytosis (for reviews, see

413 Hajishengallis & Lamont, 2014; Mysak et al, 2014; Slaney & Curtis, 2008). Thus,
414 sensitive species will be eliminated (though some may survive due to cross-
415 protection from neighbouring organisms), but those that can tolerate the
416 inflammatory response will flourish. It has been argued that the microbial
417 consortia that are associated with periodontitis are 'inflammo-philic' in that they
418 have adapted to not only endure inflammation but also to exploit the altered
419 environmental conditions (Hajishengallis, 2014), such as small rises in pH and
420 temperature (Eggert et al. 1991; Fedi & Killoy 1992; Haffajee et al. 1992; Nyako
421 et al. 2005). Such small changes to the local environment can alter gene
422 expression and increase the competitiveness of species such as P. gingivalis
423 within microbial communities (Marsh et al., 1993). However, a more substantial
424 change to the inflamed pocket is the altered nutrient status as a result of the
425 increased flow of GCF. In order to study the impact of this, laboratory studies
426 have been performed using serum as a surrogate for GCF, and complex
427 nutritional inter-relationships among subgingivally-derived microbial consortia
428 have been observed (ter Steeg & van der Hoeven 1989; ter Steeg et al. 1987).
429 When biofilms from patients with chronic periodontitis were inoculated into
430 pre-reduced (i.e. anaerobic) heat-inactivated human serum, the microbial
431 composition of the consortia changed over time and these changes correlated
432 with distinct stages in glycoprotein breakdown involving bacteria with different
433 metabolic capabilities. Initially, carbohydrate side-chains were removed by
434 organisms with complementary glycosidase activities; this was followed by the
435 hydrolysis of the protein core by obligately anaerobic bacteria leading to
436 extensive amino acid fermentation. Significantly, individual species grew only
437 poorly in pure culture on serum (ter Steeg & van der Hoeven 1989).

438 Numerous nutritional inter-dependencies and physical
439 interactions will develop among the species coping with the array of novel host
440 factors produced during the inflammatory response. For example, a complex but
441 symbiotic metabolic relationship has been demonstrated in laboratory studies of
442 P. gingivalis and T. denticola (Grenier, 1992; Tan et al., 2014). Early studies
443 demonstrated that isobutyric acid produced by P. gingivalis stimulated the
444 growth of T. denticola, while succinic acid generated by T. denticola enhanced the

445 growth of P. gingivalis (Grenier, 1992). More recent studies have shown that the
446 biomass is higher when both species are grown in co-culture, and glycine
447 produced by P. gingivalis is utilised by the spirochaete (Tan et al., 2014). Both
448 species respond to the presence of the other as seen by changes in global gene
449 expression in both species. Similarly, the growth of certain species that have
450 been previously described as being 'unculturable' (e.g. Fretibacterium
451 fastidiosum, Prevotella HOT-376, Tannerella HOT-286) has been shown recently
452 to be due to their dependence on siderophores and to the close physical
453 proximity of 'helper' strains (Vartoukian et al. 2016a; Vartoukian et al. 2016b).
454 Other studies have demonstrated the importance of close physical associations
455 to biofilm formation by interacting species of Gram-negative anaerobic bacteria
456 (Sharma et al., 2005; Okuda et al., 2012).

457 Periodontal diseases may be an example of 'pathogenic synergism' (van
458 Steenbergen et al. 1984), in which disease is a consequence of the combined
459 activity of an interacting consortium in which each member is only weakly
460 virulent. Different species would undertake a distinct role or function in order
461 for the consortium to persist, and cause disease. This is consistent with the
462 recent concept of low abundance species ('keystone pathogens') having a
463 disproportionate effect of the virulence of the whole community (Hajishengallis
464 & Lamont 2012; Hajishengallis et al. 2011). Gene transfer can occur within these
465 communities; this can include not only mobile elements that code for drug
466 resistance but also larger stretches of DNA that effect the virulence of recipient
467 cells, for example, P. gingivalis possesses a 'pathogenicity island' (Curtis et al.
468 1999).

469 Evidence for the role of the entire community and not just a few
470 pathogens in dysbiosis has recently been delivered by metatranscriptome
471 analysis of dental biofilms from sites with active periodontal disease (Yost et al.
472 2015): various streptococci, Veillonella parvula and Pseudomonas fluorescens
473 were highly active in transcribing putative virulence factors besides periodontal
474 pathogens such as Tannerella forsythia and P. gingivalis. The genes that were
475 over-represented at these sites were related to cell motility, lipid A and
476 peptidoglycan biosynthesis, and transport of iron, potassium and amino acids.

Microbial interactions in such complex consortia could influence treatment outcomes. Although not advocated for routine use in periodontal disease, antibiotics are frequently used as adjunctive treatment to mechanical debridement in cases with severe or recurrent disease (Jepsen & Jepsen 2016). However, care needs to be taken as, apart from the existence and inter-species transfer of resistance genes within microbial communities, β-lactamase producing bacteria are commonly present in subgingival biofilms and they could protect neighbouring organisms that should be susceptible to the action of the drug (Rams et al. 2013; van Winkelhoff et al. 1997; Walker et al. 1987).

Attempts have also been made to exploit antagonistic interactions to resolve both periodontal disease and caries. For periodontal therapy, either bacterial interference has been applied by deliberately implanting beneficial oral bacteria into a treated pocket (Teughels et al. 2013; van Essche et al. 2013) or by using predatory protozoa, such as Bdellovibrio species (Dashiff and Kadouri 2011; Loozen et al. 2015; Van Essche et al. 2011), or bacteriophage [reviewed by Allaker & Douglas (2009)], while for caries prevention, different approaches (e.g., lozenges, milk, yoghurt) with probiotic bacteria that are antagonistic against S. mutans have been tried (Cagetti et al. 2013). A recent systematic review on the use of probiotics in managing oral diseases concluded that there is sufficient evidence for supporting the use of probiotics in the case of gingivitis and periodontitis but not for caries (Gruner et al. 2016), though this is an area in which more research is required.

Conclusions

Microbial communities, such as those found in dental biofilms, display 'emergent properties', i.e. their properties are more than the sum of the component species, and their characteristics cannot be inferred from studies of individual organisms (Diaz et al. 2014). The microbiota is structurally and functionally organised, and it has been argued that such microbial communities could be considered as primitive multi-cellular organisms (Caldwell et al. 1997; Ereshefsky & Pedroso 2015). In health, numerous interactions contribute to stability and resilience of

507 the ecosystem against environmental perturbations (Alexander, 1971; Marsh,
508 1989).

509 If certain key environmental pressures exceed thresholds that vary from
510 patient to patient, then the competitiveness of certain bacteria is altered and
511 dysbiosis can occur, leading to caries or periodontal diseases. In caries and
512 periodontal diseases, changes in the nutrient status at the site due to increases in
513 fermentable carbohydrates (and the resultant acidic conditions) and host
514 proteins (including haemin-containing molecules), respectively, disrupt the
515 microbial interactions that control the balance of the microbial communities in
516 health. Effective prevention of dental disease will require interference with
517 these factors that drive dysbiosis (Marsh 2003), and a greater understanding of
518 microbial interactions could lead to strategies to actively promote oral health.

519 The current literature search led us to the following conclusions: 1) oral
520 microbial interactions belong to a highly studied and diverse topic, which was
521 too broad for a systematic review; 2) most oral microbial interactions have been
522 investigated in laboratory systems, and occasionally animal models, and
523 therefore some caution should be exercised when extrapolating these findings to
524 events in humans; 3) the majority of the interactions studied involve bacteria
525 only, while other segments of the oral microbiota (fungi, Archaea, viruses,
526 protozoa) are understudied; 4) current technological advances (e.g.
527 metagenomics, metatranscriptomics, metaproteomics, metabolomics, spectral
528 imaging fluorescence in situ hybridization, etc) enable the study of more complex
529 community level interactions, including those among members of the microbiota
530 from different kingdoms (Diaz et al. 2014) rather than just the conventional dual
531 species studies; 5) both synergistic and antagonistic interactions contribute to
532 the ecological stability of the microbial community that characterises oral health;
533 and 6) more attention needs to be focussed on what micro-organisms are doing
534 within these microbial communities (Takahashi 2015), rather than just
535 cataloguing which ones are present. The oral microbiome in health and disease
536 might be better described by a series of functions and interactions, rather than as
537 a list of individual organisms, as these functions might not be provided by the
538 same microbes in different people (Lloyd-Price et al. 2016).

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896

897 **Table 1.** Details on original research articles (N=547) obtained in PubMed

898 search described in Supplementary table S1.

Members of the interaction(s)	Details
Bacteria-Bacteria (N=473)	– Oral health: N=205; Caries pathogen (CP): N=107; Periodontal pathogen (PP): N=149; CP & PP: N=6; Oral vs non-oral species: N=6 – Antagonism (A): N=116; Synergy (S): N=214; A & S: N=3; Metabolism: N=98; Communication: N=32; Gene transfer: N=10
Bacteria-Fungi (N=45)	– Candida albicans: N=40; C. albicans and other Candida species: N=3; undefined Candida spp.: N=2 – Bacteria involved: Streptococcus mutans: N=11; Streptococcus gordonii: N=10; other oral streptococci: N=9; Actinomyces: N=5; Staphylococcus aureus N=2; Aggregatibacter actinomycetemcomitans, Enterococcus faecalis, Fusobacterium nucleatum N=1 each; probiotic lactobacilli: N=1; microbial consortia or microcosm: N=8 – Antagonism: N=11; Synergy: N=33; Communication: N=5
Bacteria-Viruses (N=18)	– Bacteriophages: N=6; Herpes viruses: N=7; virome: N=3; CRISPR: N=3
Bacteria-Archaea (N=4)	– Vianna et al (2008; 2009), Horz et al (2012; 2015): Metanogenic archaea & periodontal pathogens
Fungi-Fungi (N=7)	– different Candida species: N=6; Pichia vs opportunistic fungi (Mukherjee et al., 2014)
Fungi-Viruses (N=1)	– Plotkin et al (2016): HSV enhances C. albicans adherence
Bacteria-Protozoa (N=3)	– Dashiff & Kadouri (2011); van Essche et al (2011); Loozen et al (2015): Bdellovibrio bacteriovorus – bacterial predator

899

900 **Table 2.** Types of synergistic and antagonistic microbial interactions that occur
901 among oral micro-organisms growing in dental plaque biofilms.

Interactions:	
Synergistic	**Antagonistic**
Enzyme complementation / enzyme sharing	Bacteriocin production
Food chains (food webs)	Hydrogen peroxide production
Co-adhesion	Organic acid production / generation of inhibitory pH conditions
Cell-cell signalling	Bacteriophage release
Gene transfer	Competition for essential nutrients
Environmental modification	Predation

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Figure legends:

Figure 1. Examples of nutritional interactions among oral micro-organisms (Figure modified from Figure 3 in Hojo et al, 2009).

Figure 2. Model for S. gordonii and A. actinomycetemcomitans interactions: hydrogen peroxide production by S. gordonii (Sg) supports lactate consumption by A. actinomycetemcomitans (Aa) (Figure S8 from Stacy et al 2014). A. actinomycetemcomitans expresses H_2O_2-detoxifying enzyme catalase (KatA), which also protects S. gordonii from self-inflicted oxidative stress. Dispersin B (DspB) is an enzyme that promotes dispersal of A. actinomycetemcomitans biofilms and results in increased distance between the A. actinomycetemcomitans and the H_2O_2-producing S. gordonii. The three zones (Peroxide killing zone, Synergy zone and Carbon starvation zone) correspond to different concentrations in oxygen, hydrogen peroxide and lactate in the biofilm, as indicated with the respective triangles.

Supplementary material:

Supplementary Table S1. PubMed query search terms and results.

PubMed Query 19-07-2016	**Items found/ included**
((((("Microbiota"[Mesh] OR "Metagenome"[Mesh] OR "Bacteria"[Mesh] OR "Archaea"[Mesh] OR Microbiot*[tiab] OR Metagenom*[tiab] OR Bacteria*[tiab] OR eubacteria*[tiab] OR microbiom*[tiab] OR microorganism*[tiab] OR micro organism*[tiab] OR commensal*[tiab] OR flora[tiab] OR floras[tiab] OR microflora*[tiab] OR colonisati*[tiab] OR colonizati*[tiab] OR microbial*[tiab] OR "Viruses"[Mesh] OR Virus*[tiab] OR viral[tiab] OR "Archaea"[Mesh] OR Archaea*[tiab] OR Archaeobacteria*[tiab] OR Archebacteria*[tiab] OR Archaebacteria*[tiab] OR Archaeon[tiab] OR "Fungi"[Mesh] OR Fung*[tiab] OR mold*[tiab] OR candida*[tiab]OR protozoa*[tiab]OR mycoplasma[tiab])) AND ("Mouth"[Mesh] OR Mouth*[tiab] OR oral[tiab] OR "cavitas oris"[tiab] OR saliva*[tiab] OR tongue*[tiab] OR dental[tiab] OR dentition[tiab] OR teeth[tiab] OR tooth[tiab] OR gum[tiab] OR palat*[tiab] OR lip[tiab] OR lips[tiab] OR gingiva*[tiab] OR periodont*[tiab] OR uvula*[tiab] OR "Cheek"[Mesh] OR cheek*[tiab] OR bucca*[tiab] OR "Palatine Tonsil"[Mesh] OR tonsil*[tiab] OR "Waldeyer ring"[tiab] OR crevic*[tiab] OR periodontal pocket*[tiab])) AND ("Ecology"[Mesh] OR ecolog*[tiab] OR interaction*[tiab] OR synerg*[tiab] OR co-occurren*[tiab] OR inhibition[tiab] OR communicat*[tiab]OR metabol*[tiab] OR metabolism[tiab] OR metabolic[tiab] OR "metabolism"[Mesh] OR nutrient[tiab] OR gene transfer[Mesh] OR quorum sensing[tiab])) AND (plaque OR biofilm OR community OR consortium)	3758
Language filter: English	3593
After initial screen of titles and abstracts	759