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Review Article:

Recent Advances in Studies of Polymicrobial Interactions in Oral Biofilms

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Abstract

The oral cavity supports a complex and finely balanced consortia of microbial species, many of which co-operate within structured biofilms. These communities develop through multitudinous synergistic and antagonistic inter-species relationships. Changes in the dynamics of oral microbial populations are associated with the transition from healthy teeth and gums to dental caries, gingivitis and periodontitis. Understanding the ecology of oral biofilm communities, and how different species communicate within a given host, will inform new strategies for treatment and prevention of oral diseases. Advances in sequencing technologies have fuelled an increasing trend towards global genomic and proteomic approaches to determine the key factors that initiate oral diseases. Whilst metabolic profiling seeks to identify phenotypic changes of whole microbial communities; transcriptomic studies are exploring their complex interactions with each other and the host. This review discusses the most recent *in vitro* and *in vivo* studies of interspecies interactions within polymicrobial oral biofilms.

Key Words (25)

Polymicrobial communities; biofilm models; inter-species interactions; microbiome; proteomics;

Introduction:

Oral diseases such as dental caries and gingivitis affect 3.9 billion people globally [1]. The involvement of microorganisms in the development of these diseases has long been recognised. Over 700 microbial taxa have been detected in dental plaque, attached to tooth surfaces and in the gingival crevice [2]. In any one individual, 1 gram of mature dental plaque may contain around 10^9 bacterial cells, belonging to 200 species [3]. These complex microbial communities form highly structured biofilms that develop through a succession of physical and chemical inter-species interactions. Whilst some bacterial species, such as *Streptococcus mutans*, are major etiological agents of tooth decay [4]; others, such as *Porphyromonas gingivalis*, have traditionally been more commonly linked to periodontitis [5]. This chronic condition is characterized by inflamed and bleeding gums that lead to bone re-adsorption and tooth loss. Detailed analyses of oral microbial ecology have shown that changes in these species ratios are associated with a shift from health to disease [2, 6].

Recent metagenomic studies, including the human microbiome project, have highlighted further complexity in the relationship between polymicrobial oral communities and human health [3, 7, 8] and the distinction between “commensal” and “pathogenic” species is no longer clear [9, 10]. Advances in experimental model systems and *in situ* analyses of dental biofilms have identified important inter-species interactions that determine the composition of the oral flora and the interplay between polymicrobial communities and their individual hosts.

This review focuses on recent advances in the study of oral microbial ecology and the interactions between species that govern community development.

Microbial Community Development in the Oral Cavity:

Biofilms can be described as communities of microbial cells that are usually attached to a surface, and protected by an extracellular matrix of secreted polysaccharides, proteins, nucleic acids and lipids [11]. This extracellular polymeric substance (EPS) offers protection, concentration of nutrients and structure to microbial populations [12]. Gradients in pH, O₂ and nutrient concentrations have been observed at different biofilm depths [13], creating micro-environments that support the co-existence of diverse microorganisms. Bacterial cells within biofilms have been shown to exhibit slower metabolic rates and increased resistance to antimicrobial agents and immune attack [14, 15]. Significant increases in antibiotic resistance have been demonstrated (4-250 fold) by several oral bacteria when grown as biofilms [16]. The close proximity of microbial cells within biofilms allows for efficient cell-cell signaling; metabolic co-operation and horizontal transfer of genetic material [17].

The striated species composition of mixed biofilm communities in the oral cavity have been reviewed extensively [2, 18-22] and a number of key interacting players have been well described. The specific communities found within each individual host will depend on the outcomes of numerous synergistic and antagonistic relationships as well as host factors. Oral biofilms develop through a series of successive physical, metabolic and signaling inter-species interactions that have traditionally been grouped into a set of dynamic stages:

Early birds

Initial colonization of tooth surfaces is mediated by mostly Gram positive pioneer species, mainly belonging to the genera, *Streptococci*, *Veillonella*, and *Actinomyces* [21-26]. Bacterial surface adhesins bind to receptors within the salivary pellicle [18, 20, 22, 27]. Streptococci exhibit wide ranging adhesive properties and metabolic capabilities, enabling them to fill several niches on tooth surfaces, and the surrounding soft tissues [23]. Multiplication and

growth of these organisms marks the early stages of oral biofilm development. Both synergistic and antagonistic interactions occur between these early arrivals [24-26], to establish successful mixed biofilm communities that alter the environmental conditions in the mouth. In particular, the oral streptococci produce a plethora of general and specific antimicrobial agents in the form of hydrogen peroxide (H₂O₂), bacteriocins, mucins and lantibiotics in fierce competition for adhesion sites [28, 29]. Conversely, *A. naeslundii* produces arginine, which is required for growth by oral streptococci [25] and the type II fimbriae of *Actinomyces* have been shown to bind phosphopolysaccharides on streptococcal surfaces [30]. Host and dietary factors also play a major role in early oral biofilm development. Initial colonization of tooth surfaces can be dramatically altered in the presence of dietary carbohydrates such as sucrose [31]

Regardless of the specific composition of early dental biofilms in the oral cavity of individual hosts, they usually result in reduced pH and oxygen concentrations and provide a new surface, with unique receptor sites, for the attachment of other microbial species [18].

Veillonella species are central to the development of multispecies plaque communities. Whilst they utilise the lactic acid produced by streptococcal species, they produce vitamin K that enhances growth of *P. gingivalis*, *A. actinomycetemcomitans* and *Prevotella* spp [21]. *Actinomyces naeslundii* and *Fusobacterium nucleatum* have been termed “bridging species” as they play a pivotal role in the development of mature dental biofilms [32]. *F. nucleatum* can grow in a wide pH range (5.0 -7.0) and neutralizes acidic environments [33]. *A. naeslundii* has been shown to co-aggregate with, and protect many oral species by consuming H₂O₂ via protein oxidation [34]. The resultant reduction in oxygen, thus creates an permissive environment for the growth of several anaerobes.

Fashionably late

The late colonisers of oral biofilms are dominated by obligate anaerobes such as *P. gingivalis*; *T. forsythia*; *T. denticola* [19, 35] and *P. intermedia* [33] [36] that produce degradative enzymes and induce inflammatory responses and the destruction of periodontal tissue *in vivo*. These species have been shown to dramatically increase in numbers in the deep periodontal pockets that characterize advanced periodontitis. Other late colonisers, such as *Aggregatibacter actinomycetemcomitans* play a role in the development of localised aggressive periodontitis (LAP) [37].

Cell-cell signalling plays a decisive role in the incorporation of late colonisers into oral biofilm communities. Quorum sensing is a density-dependent signaling system, in which diffusible auto-inducers (AI) regulate the expression of multiple genes. The *luxS* gene expressing AI-2 is conserved among both Gram-negative and Gram-positive species of oral bacteria, including *S. mutans*, *S. gordonii*, *S. oralis*, *P. gingivalis*, and *A. actinomycetemcomitans* [38, 39]. The *luxS* gene is required for the establishment of mixed biofilms (e.g. between *S. oralis* and *A. naeslundii* [18, 40] and between *S. gordonii* and *P. gingivalis*) [41]. Streptococcal *luxS* signalling has also been implicated in the formation of fungal *C. albicans* biofilms [42].

The synergistic interaction between *S. gordonii* and *P. gingivalis* is multifaceted and has been studied extensively. Physical, metabolic and chemical interchanges between the 2 species have been well characterised. The major fimbriae of *P. gingivalis* bind to surface expressed streptococcal glyceraldehyde-3-phosphate dehydrogenase [43], whilst the minor fimbriae bind to SspA/B [44]. *P. gingivalis* is asaccharolytic, and does not compete with *S. gordonii* for sugars. It has been suggested that *S. gordonii* may deplete oxidants, allowing the survival of the anaerobic *P. gingivalis*, which secretes several proteases that may breakdown peptides for *S. gordonii* metabolism [45]. *S. gordonii* AI-2 is essential for dual species

biofilm formation with *P. gingivalis in vitro* [41] under the control of the transcription factor CdhR [46]. This synergism has also been demonstrated *in vivo*, leading to increased bone loss in a mouse model of periodontitis [44].

The new kids on the block

Studies of healthy individuals have confirmed that, although each individual mouth harbours unique microbial communities, they share many conserved genera [47]. In addition to *Streptococci*, and *Veillonella* spp, *Gemella*, *Granulicatella*, *Abiotrophia*, *Selenomonas* and *Capnocytophaga* spp were consistently identified on healthy tooth surfaces [3].

At diseased sites, species diversity has been shown to be more complex and increased numbers of several novel species have been detected. The dominant causative agents of dental caries have traditionally been thought to be mutans streptococci and *Lactobacilli*. In contrast, Gram-negative anaerobes such as *P. gingivalis* and *T. denticola* have been thought of as major etiologic agents of periodontitis. However, newly recognised potential pathogens have triggered a paradigm shift in concepts of pathogenic oral communities [45].

Metagenomic analysis of caries samples suggest a significant role for *F. nucleatum*, *Bifidobacterium* species [48] and *Scardovia wiggsiae* [49]. Studies of subgingival plaque taken during periodontitis have identified potentially pathogenic roles for many more species (e.g. *Selenomas* spp, *Eubacterium* spp, *Synergistes*, *Desulfobulbus*, TM7, *Filifactor alocis* [3, 10, 50, 51]). In light of these discoveries, new theoretical models are moving away from the idea of individual causative agents of oral disease, towards the suggestion that intricate interactions between oral inhabitants and their specific host may involve a shift in whole community behaviour to become more pathogenic [9, 10, 52].

Advances in Model Systems for Studying Oral Biofilms

Despite the recent advances in metagenomic profiling of plaque samples, relevant, simple and well controlled *in vitro* models are still required to decipher the physical, spatial, metabolic and chemical basis of these interactions [35, 37]. Dental biofilms have been characterised using *in vitro* and *in vivo* models with increasing complexity (reviewed by Palmer *et al.*, 2010 [53]). Table 1 summarises the most recent findings on inter-species interactions in model systems.

The simplest models involve the growth of static cultures in microtitre plates. Improved biofilm formation on microtitre well surfaces has been achieved using low nutrient concentrations, and hydroxyapatite (the main component of tooth enamel) has been used as a removable substratum. Pre-treatment of surfaces with processed saliva can mimic the salivary pellicle, providing relevant receptors for the adhesion of pioneer species [54]. The multi-well format of these models allows the simultaneous screening of many species combinations for co-operative interactions; the effect of changes in media composition and the comparison of different strains [55].

Polymicrobial biofilms containing *A. naeslundii*, *V. dispar*, *F. nucleatum*, *S. sobrinus*, and *S. oralis* have been developed on hydroxyapatite discs [56]. Mixed communities of *Streptococcus* and *Veillonella* species, micromanipulated from dental plaque, have been developed using saliva as a sole source of nutrients [57]. More recently, these model systems have been used to identify new mechanisms of co-operation involving protease interactions between *T. denticola* and *P. gingivalis* [58, 59] and AI-2 signalling between *S. gordonii* and *C. albicans* [42].

Flow cell systems involve the continuous flow of media across the model biofilm. They provide adherent cells with replenished nutrients, removing waste products and unattached

cells. These systems more closely mimic the environment in the oral cavity and can be used for longer experiments. Palmer *et al.*, 2001 used a flowcell model with 25% saliva as the sole nutrient source to demonstrate a mutualistic relationship between *S. oralis* and *A. naeslundii*, that can only grow as biofilms when co-cultured [60]. *V. parvula*, *A. actinomycetemcomitans* and *F. nucleatum* have, more recently, been shown to require co-culture for biofilm growth under flow [37]. By contrast, *P. gingivalis*, *A. oris* and *V. parvula* have been shown to grow in dual-species partnerships, but not as a tri-species community in flow systems.

Adding complexity

Simple biofilm models comprising two or three species have proved to be an excellent method to determine individual inter-species relationships [61] [58, 59] [62] [35]. However, many interactions are complex and involve multiple community members. These relationships may not be captured in simple dual or tri-species models. Polymicrobial models could provide a fuller picture of such communities. Bradshaw *et al.* established a 10-species biofilm model almost 20 years ago using a stirred vessel reactor [63]. More recently the 10-species Zurich biofilm model, in microtitre plates, has demonstrated that human serum in the growth medium yields more stable biofilms that are representative of species' ratios found in periodontitis patients [64].

Ex-vivo models: Other biofilm models have been developed by seeding the growth media directly from *in vivo* oral samples. Biofilms derived from mouse oral microbiota have been shown to inhibit the integration of *F. nucleatum*, upon addition to the system, due to cell contact-dependent induction of hydrogen peroxide [32]. Others have developed polymicrobial biofilms, using human pooled saliva as the inoculum. The authors reported that

60-80% of the diversity of the original inoculum was maintained for 48 hours, including many uncultivable species [65].

Considering the host: The development of oral microbial biofilms and the advancement of periodontal disease is not solely dependent on microbial community dynamics. The *in vitro* models discussed so far are unable to account for the major contribution of host inflammatory and immune responses [66]. Recent advances in biofilm model systems have included the co-culture of multi-species biofilms with primary gingival epithelial cell lines [67]. Co-culture of a nine-species biofilm with gingival epithelial cells invoked apoptosis, release of pro-inflammatory cytokines and rapid degradation of cytokines. The addition of other relevant host cells, such as neutrophils to these models, will provide further insights into the interaction between polymicrobial biofilms and host factors.

In recent years, a number of polymicrobial infection models have been used to assess the synergistic behaviour of oral microorganisms *in vivo*. Co-culture of oral streptococci with *A. actinomycetemcomitans* has been shown to elicit enhanced resistance to killing by host innate immunity in a mouse model [68]. This relationship was also demonstrated to increase pathogenesis in the polymicrobial infection model [69]. Similarly, simultaneous infection of mice with *P. gingivalis* and *S. gordonii* confirmed that inter-species binding was important for *P. gingivalis* colonization and increased bone loss [44]. By contrast, *S. cristatus* has been shown to interfere with the pathogenicity *P. gingivalis* in mice [70]. In another study, low levels of *P. gingivalis* have been shown to impair innate immunity and promote the dysbiosis of the symbiotic microbiota, leading to bone loss *in vivo*. Interestingly, *P. gingivalis* did not cause periodontitis in germ-free mice. These findings prompted the labelling of *P. gingivalis*

as a “*keystone pathogen*” [9]. Synergistic virulence has also been demonstrated between *P. gingivalis* and *T. denticola* in a murine periodontitis model [71].

These studies provide evidence that interaction between community members is capable of mediating resistance to host innate immunity and increased virulence, and reinforces the need to understand how polymicrobial growth affects interaction with the host immune system.

Imaging polymicrobial oral communities

Fluorescent *in situ* hybridisation (FISH) has enabled investigators to visualise snapshots of the spatial arrangements of mixed biofilms either grown within specialised microtitre plates, on removable discs, within flow-cell chambers or *in vivo*. Similar studies, using a Culture Well chambered coverglass system have recently been used to determine the physical interaction of the new potential pathogen *F. alocis* with *P. gingivalis*, *S. gordonii*, *F. nucleatum* and *A. actinomycetemcomitans* [72]. The 3D topology of the biofilm structure and detailed spatial arrangements of many interacting species have been visualised [56, 57, 64, 73-75].

Xiao *et al.*, 2012 use novel imaging techniques to provide insight into the functionality of oral biofilm architecture. They observed how small populations of *S. mutans* assemble a diffusion-limiting 3D matrix scaffold, which creates localized and highly acidic microenvironments throughout the biofilm. This model could explain the rapid accumulation of cariogenic plaque in the presence of sucrose, even if the initial *S. mutans* population is numerically and proportionally lower than other co-habitants [76]

Others have begun to use 3D- printing to create bacterial communities that are physically distinct, yet able to communicate through chemical interactions. This approach shows

promise for deciphering the importance of spatial arrangements in polymicrobial communities [77].

The next generation of model oral biofilm research:

Rapid advances in -omics technologies promise to identify new inter-species interactions through metagenomic, gene expression; proteomic and metabolic profiling of real patient samples and more relevant biofilm models [19, 45, 61, 78-81].

Developing new paradigms through metagenomics:

Over half of the bacterial species identified so far in the human oral cavity are not cultivable *in vitro* [2]. It is clear, therefore, that classic models based on culture dependent methods can only provide a “drop-in-the-ocean” view of oral microbial communities *in vivo* (the oral microbiome). Metagenomic approaches are better able to describe the microbial ecology of oral biofilms *in situ*. Sequencing across highly variable areas of the *16S rRNA* gene, enables the identification of all the species present in a sample. This approach can provide a comprehensive snap shot of an oral community at a given point in time. DNA microarrays have been used successfully as a high throughput means of screening large numbers of samples for the presence of known species [82]. Further advances in next generation sequencing (NGS) technologies have revolutionised our view of oral microbial communities, and have revealed that overall species diversity may be double than previously thought [45]. As NGS becomes cheaper and faster, it is now possible to perform longitudinal studies of oral biofilm development in large cohorts providing a more robust picture of the core human oral microbiome.

Recent years have seen an explosion of *16S rRNA* sequence data that describe the oral microbiota in samples collected from wide ranging individuals [83] [50, 84-86] [47]. Reviews

of these data have suggested that the progression from health to disease might be more complicated than previously thought [10, 45]. NGS technologies are also enabling researchers to consider the role of fungi and viruses in oral community stratification [45].

Candida albicans is a key player in multiple oral disease conditions and novel cross-kingdom interactions have been described with streptococcal spp, resulting in increased pathogenicity [42]. A bacterial exoenzyme has been shown to mediate *S. mutans* interactions with *C. albicans* by readily attaching to the yeast cell surface. The surface-bound enzyme was found to be functionally active, producing a glucan-rich matrix directly onto the fungal cell wall surface, dramatically enhancing adhesive interactions [87]. Ghannoum et al (2010) describe the human oral mycobiome from the oral cavity of 20 healthy individuals. 74 culturable and 11 non-culturable fungal genera were common across all samples, with *Candida* the most frequently identified, followed by *Cladosporium*, *Aureobasidium*, and *Saccharomycetales* [88].

Not to be left out, the *in vivo* viral component of individuals has been termed the human ‘virome’. A robust bacteriophage population has been detected in human saliva [89] and shared living environments have been suggested to play a significant role in determining the ecology of human oral viruses [90].

Metagenomic studies are providing a picture of oral biofilm communities that is far richer than previously thought. However, these analyses are mainly descriptive and new approaches are required to determine the interactions between these newly identified players. There is an intricate network of complex inter-species cross talk in oral microbial communities. Physical ligand-receptor binding; metabolic co-dependencies; chemical signals that trigger global gene regulators, and chemical warfare combine with host factors to build unique communities in each individual mouth. It has been suggested that many species might be uncultivable because they depend on the secondary byproducts of another microorganism

for growth. Deciphering these key signals may identify media supplements that could be used for *in vitro* culture of these new species [45] or to combat potential pathogens *in vivo*.

Deep sequencing approaches can now generate whole genome sequences from microbial communities (not just the 16S rRNA gene). This technique has the potential to provide a comprehensive view of global genetic and metabolic changes associated with a given condition. Liu et al (2012) performed whole metagenome sequencing on subgingival plaque samples from 15 patients. They found that periodontal patient samples were enriched with virulence factors and detected a common species structure in diseased samples that was not observed in healthy sites. Finally, the depth of sequencing performed in this study enabled the characterization of the unculturable TM7 genome [8].

Transcriptomics:

Extraction of RNA from biofilm communities and conversion to cDNA enables determination of the levels of expression of specific genes. Realtime, reverse transcription (RT)-PCR and micro-array technologies have enabled the exploration of specific and global transcriptomes (detecting differentially expressed genes in response to a given stimulus). Investigation of the transcriptional responses of *P. gingivalis* to growth within an oral bacterial community detected that 18 % of the genome was differentially expressed compared to growth in mono-cultures [91]. Cell envelope biogenesis, DNA replication, energy production and fatty acid metabolism were down-regulated, indicating a decrease in growth and replication. In contrast, transport systems, adhesins, signal transduction and transcriptional regulation were up-regulated, indicating multi-level inter-species interactions [91].

There have been several transcriptomic studies of the partnership between *S. gordonii* and *P. gingivalis*. Differentially expressed genes in the dual-species environment have been mainly

involved in metabolism and energy production, but also potential virulence factors such as a tyrosine phosphatase [19, 46]. Similar studies have demonstrated that co-culture with *T. denticola* promoted increased expression of *P. gingivalis* adhesin genes and proteases [59]. In contrast, a negative interaction was found between *P. gingivalis* and *S. cristatus* that down-regulated expression of *P. gingivalis* fimbrial (*fimA*) genes, and interfering with biofilm formation [92].

Transcriptomic characterisation of a polymicrobial biofilm model has recently been reported, using microarray chips that include the genomes of 5 oral species (*S. sanguinis*, *S. mutans*, *F. nucleatum*, *A. actinomycetemcomitans* and *P. gingivalis*) [78]. It is now possible to compare global gene expression in more than one species in response to one another's presence (the "interactomes" of mixed microbial communities) using RNASeq . Frias-Lopez *et al.*, (2011) used this technique for metatranscriptomic analysis of a multispecies biofilm model (*A. naeslundii*, *L. casei*, *S. mitis*, *V. parvula*, and *F. nucleatum*). Addition of *P. gingivalis* and *A. actinomycetemcomitans* to the system induced up-regulation of chaperones, ABC transport systems and putative transposases. Alterations in the expression of small RNAs provided evidence of inter-species gene expression control [93].

In addition, Murine GeneChip(R) microarrays [94] have been used to determine the host response to polymicrobial infections (*P. gingivalis*, *T. denticola*, and *T. forsythia*) using a murine calvarial model of acute inflammation and bone resorption [95]

Proteomics:

Proteomic approaches are now being applied to the investigation of inter-species interactions using simple, well controlled, *in vitro* model systems. The technique is able to identify and quantify all of the peptides within a given sample by mass spectrometry (2D capillary HPLC/MS/MS analysis). Peptide sequences are processed and concatenated using a range of

sequence databases to identify each protein. The relative abundance of each protein can also be calculated to gain quantitative information about each interaction. By comparing the protein content of a single species community, with that of a mixed culture, it is now possible to identify all of the proteins that are differentially expressed in response to a second or third microbial population [61, 96-98]. Ontology analysis tools, such as DAVID [99, 100] can infer meaning to the lists of differentially expressed proteins by clustering them according to function in specific pathways.

This method has been used to compare the envelope proteins of *P. gingivalis* cultures with those that were co-cultured with an oral bacterial community [101]. 24 surface proteins, including RgpA were up-regulated during biofilm growth and 18 proteins were down-regulated. When compared to transcriptional profiles of the same system [91] correlation between differentially expressed mRNA and proteins was marginal, reflecting the multi-faceted regulatory networks that control bacterial responses to complex environments [19].

Whole cell quantitative proteomics have recently been applied to models of community interaction between *S. gordonii*, *F. nucleatum* and *P. gingivalis* (Sg-Fn-Pg) [96, 98] and between *P. gingivalis*, *T. denticola* and *T. forsythia* (Pg-Td-Tf) [79]. These studies reported extensive changes in *P. gingivalis* protein expression in response to the other species. In the Sg-Fn-Pg model, the cell envelope proteome and vitamin synthesis pathways were affected [98]. The *S. gordonii* proteome exhibited increased energy metabolism, amino acid biosynthesis, exopolysaccharide and lactate production and oxidative stress protection mechanisms in response to the mixed environment. A decrease in sugar transport, adhesion and ethanol production was observed. Several interactions were found to be species specific or more prominent in response to one species compared to another. Acetate levels were decreased in response to *F. nucleatum*, but increased in response to *P. gingivalis*, whilst

formate was down regulated only in the presence of *P. gingivalis* [96]. In the absence of exogenous nutrients, these proteomic profiles are consistent with *S. gordonii* utilising nutrients produced by the other community members and down regulating adhesins after initial contact. Analysis of mixed Pg-Td-Tf biofilm communities in flow cell systems identified differential expression of proteins involved in iron acquisition, nutrient sharing, flagella structure and the secretion of virulence factors.

A high-throughput quantitative proteomics approach has also been used to examine how *S. mutans* interacts within mixed-species biofilms. Klein et al (2012) used state-of-the-art MudPIT technology to describe the proteomic profile of *S. mutans* when cultured in biofilms with *A. naeslundii* and *S. oralis*. This study also quantified the abundance of *S. mutans* proteins produced in mixed species environments in the presence of sucrose. Proteins involved in Exopolysaccharide matrix assembly, metabolic and stress adaptation processes were shown to increase as the biofilms matured. Specific adaptation mechanisms were also up-regulated as an adaptive response to acidic environments. Fundamental differences in matrix assembly were observed in mixed versus single species environment [97].

The proteomics studies discussed here demonstrate the scale of inter-species interactions between common inhabitants of the oral cavity and the intricate physiological processes that are linked to expression of virulence within complex biofilms. Mixed communities showed significant changes in 45 to 54 % of the detected proteome compared to single species controls [96]. This highlights the relatively little we still know about the intricacies of communication within oral microbial communities. The proteomic approach has potential to identify a wide range of previously un-recognised mechanisms of interaction. As technologies and pathway deciphering tools become more sophisticated, these analyses will be able to elucidate these mechanisms in more complex communities.

Metabolomics is the comprehensive identification and quantification of all the metabolites present in a given biological sample at a given time. Most metabolites are small, polar, ionic molecules (e.g. amino acids, nucleotides, carboxylic acids, phosphorylated sugars) and can be separated using chromatography or, more recently, capillary electrophoresis (CE), then identified and quantified using high-resolution mass spectrometry (MS) [81]. This technique can determine the activity of many important metabolic pathways. In particular, sugar metabolism in plaque samples is of interest to researchers in the field of oral microbiology. Furthermore metabolic profiles of bacterial co-culture supernatants can identify mechanisms of bacteria-bacteria interaction by metabolite exchange. This novel approach has the capacity to reveal global and dynamic biological information, and can be used to comprehensively analyze intracellular and extracellular metabolites. Takahashi et al (2010) compared the metabolic profiles of supragingival plaque samples, from healthy volunteers, with pure cultures of oral bacterial species *S. mutans*, *S. sanguinis*, *A. oris*, and *A. naeslundii* before and after glucose treatment. They showed that the EMP pathway, pentose-phosphate pathway and TCA cycle were active both in supra gingival plaque taken directly from healthy patients and in pure cultures of representative plaque bacteria (*Streptococcus spp* and *Actinomyces spp*). Metabolic profiles were shown to change considerably following treatment with glucose. These data validate the uses of metabolomic profiling on *in vitro* model systems to characterise inter-species interactions in dental plaque. Data obtained from metabolomic studies have been suggested to reflect phenotypic changes more closely than proteomics or transcriptomics. It is interesting to note that, while large numbers of oral pathogen genomes have been sequenced; little quantitative information is known about their metabolic functions. Mazumdar et al 2009 report the computational construction of a genome-scale metabolic network for *P. gingivalis*, including 679 metabolic reactions. The network enables prediction of the phenotypic effects of gene knockouts in given conditions. Predictive computer

modelling will provide clues towards the successful culture of new potential pathogens and may be able to map novel inter-species interactions.

Conclusions:

The new oral microbiology research summarised in this review illustrates an evolution of inter-species interaction studies, from heavily culture-based, investigations, to use of new high-throughput sequencing technologies. Metagenomic studies have further elucidated the immense diversity of oral microbial ecology. They have inspired new dogma that suggests a role for community, rather than individual species, in the delicate balance between health and disease [10, 52]. These data have, so far, been mainly descriptive and have generated unfathomable sequence information, a large portion of which is assigned to un-named and uncultivable micro organisms. The clinical implications of these large sequence data sets are still to be fully resolved. The mere presence or numerical superiority may not necessarily be linked with the pathogenesis of the disease. Deeper sequencing, along with transcriptomic and proteomic analysis of clinical samples could highlight changes in the behaviour of these microbial communities and their interplay with the host. Future directions include more systems biology approaches that have developed new bioinformatic tools for assigning function to genes [80, 99, 102]. These enable bio-synthetic pathways of newly recognised species to be determined and allow researchers to predict the behaviour of specific communities. Such information will provide clues about the pathogenicity of given communities. Identification of crucial biosynthetic pathways will instruct us as to how previously uncultivable species might be cultured and any novel therapeutic potential that they might harbour. In the same way, systems level analysis and molecular imaging of complex *ex vivo* communities [103] will inform state-of-the-art modelling of the spatial and

temporal relationships between oral biofilm members at different physical niches within the mouth.

Computational analysis promises to add meaning to big data sets by combining metagenomic, transcriptomic, proteomic, and metabolomic studies, and building pathways to investigate global processes within whole communities [80, 104]. At the same time, it is important not to forget the power of small, simple *in vitro* model systems that can illustrate the mechanisms of individual inter-species interactions that are relevant *in vivo* with elegance and class.

Table 1: Recent publication of inter-species interactions between oral microorganisms

Species	Interaction with	Mechanism of interaction	Ref
Physical dual-species interactions			
<i>F. nucleatum</i>	<i>S. sanguinis</i>	binding-dependent protection from H ₂ O ₂ produced by oral community	[32]
<i>P. gingivalis</i>	<i>S. gordonii</i>	Mfa-SSpB binding interaction increased bone loss in murine model of periodontitis	[44]
<i>T. denticola</i>	<i>P. gingivalis</i>	Improved adhesive capacities of <i>P. gingivalis</i> by increasing <i>rgpA</i> , <i>kgp</i> , and <i>hagA</i>	[59]
<i>P. gingivalis</i>	<i>S. gordonii</i>	Community signalling controlled by <i>CdhR</i> transcriptional regulator	[46]
Signalling dual-species interactions			
<i>S. mutans</i>	<i>L. casei</i> or <i>S. oralis</i>	Down-regulation of virulence genes <i>spaP</i> , <i>gbpB</i> , <i>luxS</i> and <i>gtfB</i>	[29]
<i>S. mutans</i>	<i>L. casei</i> or <i>S. oralis</i>	Down-regulation of virulence genes <i>spaP</i> , <i>gbpB</i> , <i>luxS</i> and <i>gtfB</i>	[29]
Dual-species interactions affecting pathogenicity <i>in vivo</i>			
<i>A. actinomycetemcomitans</i>	<i>S. gordonii</i>	Metabolite cross-feeding enhances virulence in an <i>in vivo</i> model of polymicrobial infection	[69]
<i>S. cristatus</i>	<i>P. gingivalis</i>	<i>S. cristatus</i> ArcA interferes with <i>P. gingivalis</i> pathogenicity <i>in vivo</i>	[70]
<i>T. denticola</i>	<i>P. gingivalis</i>	Synergistic virulence in a murine periodontitis model	[71]
-Omics analysis of dual and multi species interactions <i>in vitro</i>			
<i>S. gordonii</i>	<i>P. gingivalis</i> & <i>F. nucleatum</i> ,	Proteomics within a model developing oral microbial community	[96]
<i>S. mutans</i>	<i>A. naeslundii</i> & <i>S. oralis</i>	High-throughput quantitative proteomics of mixed-species biofilms	[97]
<i>P. gingivalis</i>	<i>T. denticola</i> ,	Proteomics: Differential expression of iron-acquisition proteins HusA, HusB and HmuY	[79]
<i>S. sanguinis</i> , <i>S. mutans</i> , <i>F. nucleatum</i> , <i>A. actinomycetemcomitans</i> & <i>P. gingivalis</i>		A five-species transcriptome array for oral mixed-biofilm	[78]
<i>S. mutans</i> , <i>S. sanguinis</i> , <i>A. oris</i> , & <i>A. naeslundii</i>		Metabolomics of supragingival plaque and oral bacteria	[81]
<i>A. naeslundii</i> , <i>L. casei</i> , <i>S. mitis</i> , <i>V. parvula</i> , & <i>F. nucleatum</i>)	<i>P. gingivalis</i> & <i>A. actinomycetemcomitans</i>	Metatranscriptome of a healthy multispecies biofilm model in the presence or absence of periodontal pathogens	[93]
Multi-species interactions <i>in vitro</i>			
<i>F. alocis</i> with – <i>F. nucleatum</i> , <i>P. gingivalis</i> & <i>A. actinomycetemcomitans</i>		Dual and tri-species co-aggregative interactions	[72]
<i>T. denticola</i> with - <i>P. gingivalis</i> , <i>F. nucleatum</i> , <i>P. intermedia</i> and <i>P. micra</i>		Chymotrypsin-like proteinase integrates spirochaetes within oral microbial communities	[58]
<i>S. oralis</i> , <i>S. anginosus</i> , <i>A. oris</i> (formerly <i>naeslundii</i>), <i>F. nucleatum</i> , <i>V. dispar</i> , <i>C. rectus</i> , <i>P. intermedia</i> , <i>P. gingivalis</i> , <i>T. forsythia</i> , <i>T. denticola</i>		Addition of 50% heat-inactivated human serum to 10-species subgingival Zurich biofilm model promoted more representative species ratios	[64]
Multi-species interactions <i>in vivo</i>			
<i>Lactobacillus</i> sp <i>Streptococcus</i> sp. <i>C. albicans</i> , <i>Actinomyces</i> sp., <i>T. forsythia</i> , <i>F. nucleatum</i> , <i>Spirochaetes</i> , & <i>Synergistetes</i>		Imaging of oral biofilm architecture on natural teeth	[75]
<i>P. gingivalis</i> , <i>T. denticola</i> , & <i>T. forsythia</i>		Polymicrobial periodontal pathogen affect transcriptomes of calvarial bone and soft tissue	[95]

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