Environmental effects on oral biofilm communities

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Background: Periodontitis is associated with shifts in the balance of the microbial composition of subgingival biofilms. Many species that predominate in disease have not been isolated from healthy sites or are found in low abundance, raising questions as to the reservoir or origin of these putative pathogens.

Aims: This project aims to generate an *in vitro* model of dysbiosis to demonstrate whether it is possible to observe the outgrowth of low abundance disease-associated species from biofilms taken from healthy sites and subjects by mimicking a disease-promoting environment.

Materials and Methods: The Calgary Biofilm Device and several types of protein-rich media were used to culture five-species microbial communities. Then, the optimised model was used to culture complex biofilms using an inoculum of plaque and saliva from healthy young adult volunteers in media mimicking the nutritional status of the inflamed periodontal pocket. Later, three-week complex biofilms were cultured just in sterile human saliva to see whether changes in the enriched biofilms could be reversed. Metagenomics was used to characterise the taxonomy and functional potential of biofilms, and longitudinal comparisons were performed on biofilms and the inoculum.

Results: The inoculum consisted mainly of health-associated genera, such as *Streptococcus, Actinomyces* and *Haemophilus*. After culture in various media for one or three weeks, the biofilm composition shifted and numerous fastidious and periodontal disease-associated species belonging to genera *Bacteroidetes, Fretibacterium, Prevotella* and *Alloprevotella* were enriched. These enriched biofilms, subsequently cultured solely in human saliva, showed a minor decrease in disease associated-species. There was a shift in functional activities, with cultured biofilms having a greater abundance of genes associated with virulence.

Conclusion: The results suggest that the source of the periodontal pathogens is the healthy human mouth, and that these species can be enriched at the expense of health-associated species in a nutritional environment resembling inflammation.

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List of Abbreviations

AAA	Amsterdam Active Attachment
ANOVA	Analysis of variance
BHI	Brain Heart Infusion
bp	Base pair
CBD	Calgary Biofilm Device
CDFF	Constant Depth Film Fermenters
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
FISH	Fluorescence in situ hybridisation
GCF	Gingival crevicular fluid
HOMD	Human Oral Microbiome Database
H_2O_2	Hydrogen peroxide
KH_2PO_4	Monopotassium phosphate
KCl	Potassium chloride
LCA	Lowest common ancestor
MEGAN	MEtaGenome Analyzer
MBEC	Minimum Biofilm Eradication Concentration
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
OUT	Operational Taxonomic Units
PBS	Phosphate buffer saline
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction

QIIME	Quantitative insights into microbial ecology
qPCR	Quantitative Polymerase Chain Reaction
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
Rpm	Rotation per minute
rRNA	Ribosomal RNA
SEM	Scanning electron microscopy
TNF-α	Tumor necrosis factor alpha
VPS	Vibrio Polysaccharide

Chapter 1 Introduction

Large, multicellular organisms have co-existed with small (invisible to the naked eye) cells for millions of years, but it was only in 1677 that a Dutch merchant, Antonie van Leeuwenhoek, improved the existing microscope lenses to discover a new form of life – bacteria. He investigated human supragingival dental plaque and faecal samples and noted a great variety of bacterial shapes that were distinct between these two distinct habitats (Ursell et al., 2012). Today – more than 300 years later – rapid advances in DNA sequencing techniques have made possible the in-depth investigation of the human microbiome in health and in disease. Multiple microbiome projects are underway worldwide to characterise the complex microbiota associated with humans and to understand the role of microbiota in human health and disease (Turnbaugh et al., 2007).

1.1 Approaches for studying the oral microbiome

Since the discovery of bacteria, researchers have been trying to increase our knowledge about this ubiquitous and diverse group of microorganisms. A diverse array of microorganisms in the oral cavity can be found, including bacteria, fungi, Archaea, viruses and even protozoa, although the largest group are bacteria. All together the microflora of each person is termed microbiota, while all the genes these cells harbour are termed microbiome.

For decades, bacterial culture or microscopy were the only approaches to study the oral microbiota that later were appended with diversity of methods. So far, varying complexities of laboratory culturing techniques are used to isolate and identify members of oral microbiota. These culturing techniques range from simple culture on selective and non-selective media to the use of other methods to culture strictly anaerobic bacteria (e.g. the use of anaerobic chambers, and in the past, Hungate roll tubes) (Attebery and Finegold, 1969). However, still about 30% of the oral bacteria cannot be cultivated (Chen et al., 2010). While these methods have been important in cataloguing the oral microbiome, more sophisticated approaches are needed to investigate how these complex communities develop, and individual organisms cooperate and respond to environmental stimuli. Other culture methods include complicated

(elaborate) multispecies models, such as mixed culture chemostats, Constant Depth Film Fermenters (CDFF) and artificial mouths (see sections 1.12 and 1.13 for more details), but even these have limitations when attempting to replicate the complexity of oral environment. Nevertheless, these approaches have been of great value in isolating a wide array of species from the oral cavity, advancing our understanding of how they interact with each other and communicate with the host (Syed and Loesche, 1973; Bradshaw et al., 1994). The utility of various models for different purposes are reviewed in greater detail in section 1.12.

Imaging in microbial research has been of great value in investigating the morphology of individual bacteria and the structure of complex biofilms such as dental plaque. The simplest approach is bright field microscopy; for example, Gram staining allows major distinctions in the composition of the bacterial cell wall, while phase contrast microscopy enables live bacteria from the mouth to be visualised with minimal processing. SEM (scanning electron microscopy) is usually used to investigate major bacterial structures and can resolve details smaller than those seen through light microscopes. However, SEM requires specific sample preparation, that might distort the architecture of biofilms. More complex methods, requiring species sample preparation without the distortion of biofilm architecture include FISH (fluorescence *in situ* hybridisation) or confocal microscopy. Techniques such as FISH use specific probes (antibody or nucleotide type) or specific dyes, reacting with bacterial DNA or biofilm matrix components (Guggenheim et al., 2001) to resolve details in biofilm structure and organisation.

More recently, our knowledge of the diversity of the oral microbiome has been increased markedly by the development of culture-independent molecular techniques based on genomic data (Figure 1.1). These approaches have enabled microbial detection and identification without labour-intensive culturing experiments. These techniques are mainly based on a spontaneous pairing of DNA sequences and a complementary probe based on their homology. The most common closed-end genetic identification techniques are PCR (Polymerase Chain Reaction) and qPCR (Quantitative Polymerase Chain Reaction). They allow the identification and quantification of genomic material (DNA and cDNA(Complement Deoxyribonucleic acid)). When using optimised

protocols, even low quantities of oral bacteria or their expressed RNA converted to cDNA can be detected, quantified and monitored in the oral samples (Suzuki et al., 2004; Ammann et al., 2013b). Another technique, DNA-DNA hybridisation, was initially used by Socransky et al. (1998) to cluster oral bacteria into complexes. Utilization of DNA probes against 40 common oral species enabled complexes of bacteria to be defined that were associated with health or higher risk of periodontal disease. Sometimes the previously mentioned FISH is used not only to locate the bacteria in the structures, but also to detect certain species in the samples (Vartoukian et al., 2009).

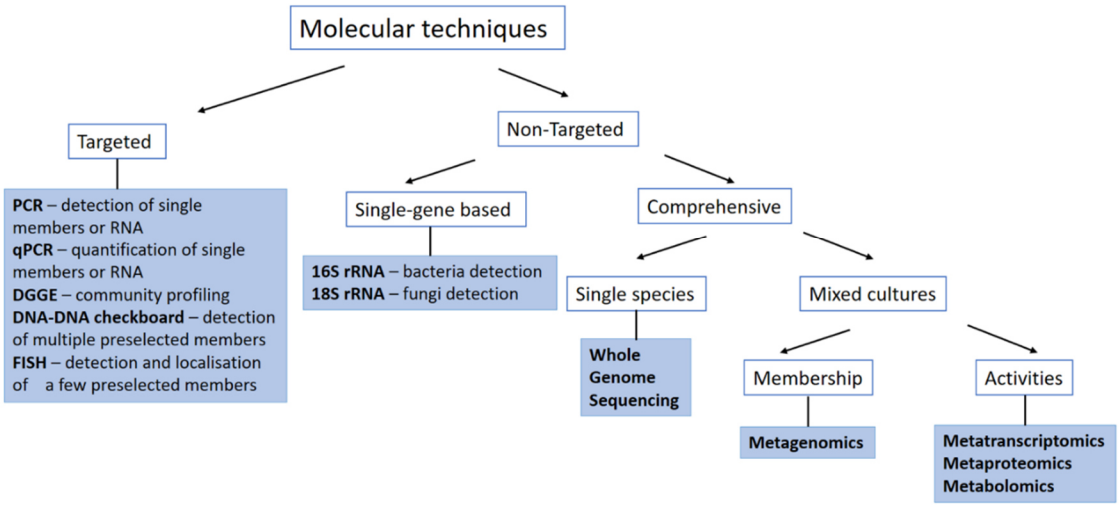

Figure 1.1. Molecular techniques most commonly used in microbiology for identification and community profiling.

Modified and adapted from Marsh et al. (2016b).

However, the targeted identification of species concentrated only on a subset of already known pathogens and possibly still leads to bias in our understanding of oral diseases. Current understanding proposes that caries and periodontal disease are dysbiotic conditions of the normal and symbiotic oral microbiome. Therefore, tools that enable monitoring of the complete microbial profile and its shifts during different stages of disease are necessary. Subsequently, open-end approaches emerged that offered the non-selective evaluation of all genetic material within the sample. In 1980 it became possible to obtain sequences of nucleotides of bacterial genomes by Sanger sequencing based on dye-terminator chemistry (Sanger et al., 1977). First generation sequencing was expensive and low-throughput, but further developments over the next four decades provided second and third generation high-throughput sequencing tools that allow the rapid acquisition of massive amounts of genetic data at affordable price. This has accelerated research into the human microbiome, including the oral microbiome.

Sequencing of hypervariable regions of the bacterial housekeeping 16S rRNA gene facilitated the discrimination between different taxonomic groups. In this approach, offered by, for example, Roche 454 pyrosequencing, Illumina MiSeq, and Ion Torrent sequencers, 16S rRNA genes from biological samples (from humans, animals or environment) are amplified using conserved primers and then retrieved during the sequencing run. In the case of mycobiome sequencing, the 18S rRNA gene is targeted. Studies exploiting 16S and 18S rRNA sequencing revolutionized our understanding about oral microbiome by revealing a broad diversity of species, many of which were not isolated using conventional culturing methods.

However, these techniques also have limitations. For example, DNA library preparation, which is a multi-step process, possibly introduces potential errors and alters the results at any library preparation step. Because hypervariable region amplification relies on primers of conserved regions, variability within primer-binding conserved regions might exclude some bacteria from the analysis (Hong et al., 2009). Another issue is actual variability within the hypervariable 16S rRNA region. Some species, for example, from the *Streptococcus* genus, do not diverge enough to discriminate between species based on the 16S rRNA gene alone (Lal et al., 2011). Moreover, adequate DNA

read-length is not always achieved with currently available methods for reliable identification beyond genus level.

Metagenomics, sometimes called shotgun metagenomics, was developed to study the genomes of the whole microbial community, rather than concentrating on a single marker gene. The technique uses all of the DNA of the sample which is randomly sheared and sequenced. Comprehensive reads are then analysed by: 1) binning - grouping of conserved gene sequences that represent individual genomes of different microorganisms, or 2) assembled in contigs to obtain longer, thus more specific, genomic sequences; the contigs are later identified by computational algorithms. Because many genes are included in metagenomic analyses, insights into both taxonomy and functional potential of the bacterial community could be obtained, providing a more comprehensive understanding of the oral microbiome. Several studies have used this technique to capture microbial diversity of a core and healthy oral microbiome, and helped define the microbial profiles and potential metabolic pathways of caries and periodontal disease-affected patients (Zaura et al., 2009; Liu et al., 2012; Abusleme et al., 2013; Dabdoub et al., 2016). These second-generation techniques provided good resolution for accurate taxonomic assignment. However, similar disadvantages are of concern, such as multi-stage library preparation, short read length, difficult analysis of partial rather than full genomes. As PCR amplification for the library preparation is involved, this also can bias the final analysis. Moreover, some of the reference genomes in the databases are incomplete.

Both, 16S rRNA and metagenomic sequencing expanded the knowledge about many bacterial species that have not been identified using conventional culturing techniques or targeted detection methods and concentrated on the profiling of the entire oral microbiota rather than concentrating on single representatives revealing the full scale of the complexity of the oral microbiome. Moreover, second generation sequencing enables an exploration of bacterial functions by RNA sequencing. Metatranscriptomics is sequencing of a complete transcriptome of a bacterial community. After the isolation of microbiome RNA, its enrichment, fragmentation and reverse transcription, the sequencing of cDNA enables the profiling of the active microbial community. For example, recently it was proposed that bacterial proteases in dentin caries

might contribute to the degradation of the host tissues (Do et al., 2017). Other advanced molecular techniques provide insights into the bacterial metabolome or metaproteome. These studies use a combination of ‘omics’ methods to expand the understanding about the composition of a microbial community and its functions. For instance, a study of the oral metaproteome revealed potential biomarkers for caries disease (Belda-Ferre et al., 2015), while Zaura et al. (2017) stratified a group of people into five ecotypes, based on salivary microbiome and metabolome differences. Different ecotypes might show either a saccharolytic or proteolytic potential and indicate the risk for developing either caries or periodontal disease, respectively.

Recently developed third-generation sequencing technologies provide ways to study genomes, transcriptomes and metagenomes at an unprecedented resolution (van Dijk et al., 2018). Depending on the technique, it allows single-molecule sequencing and generation of ultra-long reads. Quick and simple library preparation, that does not require sophisticated equipment, enables samples to be sequenced in the field in the remote locations (Arias et al., 2016). The first developed third-generation method used single-molecule real-time sequencing technology by Pacific Biosciences that could sequence 10k bp long reads at extremely high accuracy. Later, nanopore sequencers were developed by Oxford Nanopore Technologies for example, minION. The pocket-sized device generated extremely long reads, if good-quality DNA was provided, however it suffered from a high error rate. The advancement of third-generation techniques undoubtedly benefited medical research, for instance, the complete human genome was sequenced and assembled from ultra-long reads (Jain et al., 2018). So far, metagenome analysis using long read sequencing allows the high resolution of microbiome up to species level (Kuleshov et al., 2016).

1.2 Primary microbial colonisation of the human body

Contact with microorganisms starts at the very earliest stages of human life, and even during intrauterine human foetus development. Although, after birth, the initial neonatal microbiome is derived mainly from the mother, it is as yet unknown if the principal transfer that further structures the microbiome of babies happens before, during or after birth. For example, between 36% and

70% of amniotic fluid samples obtained from pregnant women were found to contain some bacteria, possibly of oral origin, which support the hypothesis that early colonization could begin during intrauterine life (Jalava et al., 1996; Hitti et al., 1997; Bearfield et al., 2002). However, the primary colonisers of infants are mostly determined by the mode of delivery. Babies born by vaginal delivery have bacteria similar to mother's vaginal bacterial communities: predominantly *Lactobacillus, Prevotella,* and *Sneathia spp*., while babies delivered by Caesarean section have bacterial communities similar to those that predominate on the mother's skin, such as *Staphylococcus, Corynebacterium,* and *Propionibacterium spp.* (Dominguez-Bello et al., 2010). After birth, bacterial communities are essentially undifferentiated across different body sites. During the transition from infancy to childhood and adulthood these communities mature to become more complex and specific to a particular body niche and these are shaped by local environmental differences (Sampaio-Maia and Monteiro-Silva, 2014).

1.2.1 Primary microbial colonisation and maturation of the oral cavity

The oral cavity is defined by the lips, the cheeks, the palate, the tongue, teeth, and the floor of the mouth. Its principal functions are to receive, process food, and participate in breathing and communication, by speech or facial expressions. It is also a gateway to the gastrointestinal tract, and plays a dominant role in structuring the microbiota within it. The gastrointestinal microbial community has a significant role in digestion, energy generation, metabolism of harmful compounds, synthesis of beneficial substances, prevention of pathogen colonisation etc. Therefore, the development of normal oral microbiota is crucial for the well-being of the entire human body. In contrast, disrupted transition of the microbiota from the mother to the baby is associated with obesity, type I diabetes and asthma in later life (Gomez-Arango et al., 2017).

The neonatal oral microbiota profile comprises principally of maternal oral (65%) and placental (3%) microbiota, while 32% is of unknown origin. None of the maternal gut microbiota has been detected in neonatal oral cavities so far (Gomez-Arango et al., 2017). The significance of delivery mode on primary oral bacteria was observed by Gomez-Arando et al. (2017). Families of

Streptococcaceae, Gemellaceae and order *Lactobacillales* were all significantly enriched in neonates who underwent natural vaginal delivery. In contrast, in those born by Caesarean section, a lower bacterial diversity and higher abundance of species from Proteobacteria phylum was detected. These observations are often regarded as a signature of dysbiosis and inflammation. However, the authors suggest that these changes might be determined by the antibiotic use during the C-section rather than the actual delivery mode (Gomez-Arango et al., 2017).

Within the first hours after birth oral cavity becomes exposed to microorganisms through breathing, breastfeeding and contact from parents and medical staff. At this stage the primary colonisers of the new-born child comprise of Gram-positive facultatively anaerobic species. The *Streptococcus* genus, particularly *Streptococcus salivarius*, predominates in new-borns, because of its ability to adhere to epithelial cells (Hegde and Munshi, 1998). However, more recent sequencing studies suggest that the microbial composition of the mouth of babies is more complex than previously thought (Cephas et al., 2011; Gomez-Arango et al., 2017). Communities of high diversity are reported in edentulous (lacking teeth) infants, although these are not as complex as those found in adults (Cephas et al., 2011). As the baby develops, the microbiota evolves and later in life the environment of the mouth has the greatest impact on the oral composition. For instance, the numbers of health-associated *Lactobacillus* species are determined by feeding mechanism: greater proportions of these bacteria are found in breast-fed rather than formula-fed babies (Holgerson et al., 2013). At five months, infants usually have a distinct microbiota from their mothers, primarily due to environmental exposure (Cephas et al., 2011). At this age the microbiota consists mostly of bacteria from six phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Spirochaetes*. At the genus level, *Streptococcus, Veillonella, Neisseria, Rothia, Haemophilus, Gemella, Granulicatella, Leptotrichia* and *Fusobacterium* are predominant in infants, while adults have a high abundance of *Haemophilus, Neisseria, Oribacterium, Rothia,* and *Actinomyces* species and higher proportions of Gram-negative species from genera *Veillonella, Fusobacterium* and *Treponema*. Moreover,

even before the eruption of teeth rich diversity is reported in infants (Cephas et al., 2011).

The composition of the oral bacterial community significantly changes after eruption of the first teeth; these hard, non-shedding surfaces provide a distinct ecological habitat facilitating attachment and biofilm formation. The abundance of mucosa-associated *S. salivarius* decrease, while *Streptococcus mitis* and *Streptococcus oralis* and *Actinomyces* become more dominant (Nyvad and Kilian, 1987; Sarkonen et al., 2000). It was previously thought, that some cariogenic species, such as *Streptococcus mutans*, become established in the oral cavity only following the eruption of teeth. However, late colonisers related to caries or periodontal disease have been detected in infants even before the eruption of teeth, such as periodontal disease-associated *Fusobacterium nucleatum, Porphyromonas gingivalis, Treponema denticola* and *Prevotella intermedia* and caries-associated species including *Veillonella parvula, Streptococcus cristatus and S. mutans* (Kononen et al., 1999; Cephas et al., 2011). Even after the complete set of teeth erupt at around 3 years old, the maturation of the oral microbiome gradually continues until adulthood. Microbiomes of children with deciduous and mixed dentition have a high abundance of health-associated species from *Streptococcus* and *Actinomyces* genera, while increase of *Bacteroidetes*, *Fusobacterium* and *TM7* genera is driven by age (Crielaard et al., 2011). Once established, the oral microbiome of the adult is relatively stable and specific to an individual, unless the breakdown of homeostasis takes place (Utter et al., 2016). This gradual development of microbial communities in human life leads to the formation of a normal microbiota that lives in harmony with the host and constitute the normal oral microbiome.

1.3 Biofilm formation

Oral bacteria persist in the mouth by forming biofilms. A biofilm is a structurally- and functionally-organised microbial community immersed in an extracellular polymeric matrix, adhered to a surface. Bacteria that primarily form a biofilm rarely come in contact with a clean tooth surface but rather interact with a conditioning film (Jakubovics, 2015). Within minutes after the complete removal of the dental plaque, the surface of a tooth becomes covered in a pellicle

derived mainly from saliva. This pellicle is a complex multi-layered matrix consisting of salivary glycoproteins, phosphoproteins, lipids and enzymes of host and bacterial origin that have a high affinity to hydroxyapatites (HA) (Hannig, 1999). Depending on the site, pellicle can also contain GCF (gingival crevicular fluid)-derived components. This pellicle partially protects the tooth surface from acids and mechanical wear, but it also actively participates in the formation of dental plaque.

Bacteria are passively transported by the flow of saliva to the pellicle, and can be held reversibly near the surface by electro-chemical interactions between the charge of molecules in the pellicle and those on the microbial cell surface. This interaction can become more permanent if specific adhesins on the micro-organism can bind to complementary receptors in the pellicle. Various adhesins, depending on a bacterial species, participate in this initial adherence. For instance, *Streptococcus* species bind through Antigen I and II, glucan binding protein and lipoteichoic acid to specific receptors in the pellicle (Marsh et al.). First, only sparse bacteria can be identified on a pellicle-coated surface and these are health-associated species (Lourenco et al., 2014) and mainly belong to the genera *Streptococcus* and *Actinomyces* (Jakubovics et al., 2014). Once attached, the early colonisers start to divide and synthesise extracellular matrix, while salivary glycoproteins continue to be absorbed. These early colonisers are important constituents of the healthy oral microbiome - they are known to produce hydrogen peroxide, in the presence of oxygen, or antimicrobial substances (e.g. bacteriocins) that inhibit the growth of late-colonisers and pathogenic bacteria in the initial stages of biofilm formation (Hillman et al., 1985; Duran-Pinedo et al., 2014b; Loozen et al., 2014).

If plaque remains on the enamel surface for longer periods, its quantity continuously increases following the orderly and predictable pattern of change in microbial community over time; this process is called microbial succession. FISH technique, described in previous section (1.1), showed that 4h - 8h plaque is dominated by *Streptococcus, Gemella, Granulicatella, Prevotella, Neisseria* and *Rothia* (Diaz et al., 2006). More sensitive sequencing techniques reveal the presence of pathogenic bacteria in fresh biofilm, but these species are in low-abundance (Abusleme et al., 2013). Microbial succession and compositional changes occur through co-aggregation – a process involving the

recognition and attachment of genetically distinct cells that result in mutually beneficial pairings of species. These co-aggregation partnerships are highly specific, and for example, happen rarely between early and late colonisers. A key bridging species between early and late colonisers which co-aggregates with both is *F. nucleatum* (Scott and Baxter, 1987; Rickard et al., 2003). It provides the 'bridge' for the further development and pathogenicity of the microbial community. On the other hand, in the absence of *F. nucleatum*, many secondary colonisers are unable to integrate in dental biofilms (Rickard et al., 2003).

As the biofilm matures, the microbial community becomes more diverse. The metabolism of the early colonisers alters the environment within the biofilm making conditions suitable for the growth of more fastidious organisms. A significant increase in obligate anaerobic and therefore potentially pathogenic bacteria is observed as soon as seven days after the biofilm formation (Teles et al., 2012). Masticatory movements of tongue, cheeks and lips, and desquamation, result in the constant shedding of the biofilms. However, as teeth are non-shedding surfaces, they permit the accumulation of large masses of micro-organisms, especially at stagnant sites, unless removed regularly by effective oral hygiene. Bacteria can also actively detach themselves from biofilms by producing enzymes that cleave their adhesins from the cell surface.

1.4 Symbiotic relationship with the host

Recent calculations suggest that the number of bacteria inhabiting the human body at least equals the number of human body cells (Sender et al., 2016). This immense component of the human body – the human microbiome - displays a wide array of symbiotic relationships with native human cells and is proposed to participate in the evolution of its host (Sapp, 1994). Co-existence between the host and the microbiome led to the adaptation and evolution of bi-directional beneficial relationships. While some members of the microbiome can be transitional, and have little effect on the host, numerous studies reveal that the host is totally dependent on some of its long-term symbionts. O'Hara and Shanahan (2006) were the first to label the gut microbiota as an 'additional organ', stressing that gastrointestinal tract microbiota is necessary for a wide

array of human body functions, such as digestion, immune development, production of vitamin K and other activities.

Symbiotic relationships between the host and the microbiota developed during evolution. In 2008, Zilber-Rosenberg and Rosenberg proposed a holobiont theory of the host and its residing microorganisms (Zilber-Rosenberg and Rosenberg, 2008). The term ”holobiont” describes the host with its symbiotic microbiome, while the term ”hologenome” defines the sum of the genetic information of the host and its microorganisms (Zilber-Rosenberg and Rosenberg, 2008). These two components (holobiont with hologenome) are considered as one unit of selection in evolution. The hypothesis posits that a dynamic relationship exists between symbiotic microorganisms and host under different environmental conditions. Evolution selects for the most advantageous holobiont in the context of the prevailing conditions and a great part of genetic variation within the hologenome depends on microbial genomes (Rosenberg et al., 2007). For example, an increased consumption of carbohydrates through human history resulted in microbial population shifts and selection of specific bacterial genes. Two major dietary changes occurred during human evolution. The first was when hunter-gatherers transferred to a carbohydrate-rich Neolithic diet (agricultural revolution ~10,000 years ago). The second transition occurred after the industrial revolution with the advent of processed flour and sugar. Sequencing of calcified dental plaque samples from these eras demonstrate shifts and decreased bacterial community diversity in more recent plaque samples (Adler et al., 2013). Interestingly, the rise of *S. mutans*, the major caries-associated pathogen, started about 10,000 years ago, co-occurring with the agricultural revolution (Cornejo et al., 2013). Authors identified a set of genes in *S. mutans* that were under positive selection, most of which were involved in either sugar metabolism or acid tolerance. Acid produced though carbohydrate metabolism dissolves HA crystals of enamel causing caries. Consequently, gradually increasing consumption of carbohydrates led to the current high incidence of caries found in most developed countries (Whittaker et al., 1981; Kassebaum et al., 2017).

1.5 Benefits of the oral microbiome

The cooperation between the host and the microbiota often contributes to the fitness of the holobiont. While the host provides the habitat, the microbiota offers many health benefits (Leser and Molbak, 2009; Kilian et al., 2016). These include the:

- protection against infections through pathogen exclusion and induction of IgA,
- production of anti-inflammatory substances,
- immune system development,
- assistance with metabolic functions of gastrointestinal tract (vitamin synthesis, ion absorption),
- ensuring optimal epithelial functioning, and
- participation in cardiovascular health.

Although much of the research on the beneficial relationships is related with gut microbiota and might not be applicable to other body sites, some benefits of oral microbiota are well known. A major benefit of symbionts in the oral cavity is to confer resistance to colonisation by pathogens. Commensal oral bacteria occupy the habitat and have evolved to be highly competitive for nutrient acquisition and also to produce inhibitory molecules, and so prevent colonisation by pathogenic microorganisms. In a healthy population, the levels of *Candida* within the oral cavity are controlled by commensal bacteria through competition for dietary substrates and adhesion to epithelial cells. The prolonged use of wide spectrum antibiotics can diminish these commensal populations and thereby predispose a site to fungal overgrowth and infection (Farah et al., 2010).

Oral bacteria participate in nitrogen metabolism and contribute to cardiovascular health. 25% of dietary nitrate (NO_3^-) is extracted from blood by salivary glands and reappears in saliva (Koch et al., 2017). Commensal oral facultative anaerobic bacteria use salivary nitrate as an electron acceptor and reduce it to nitrite (NO_2^-). Also, afterwards nitrite is metabolised to nitric oxide (NO) which acts as a vasodilator and contributes to vascular homeostasis. The disturbance in this cycle can have a minor, yet potentially important implications in cardiovascular health (Kapil et al., 2013). For example, enrichment of salivary *Prevotella* and *Veillonella* is linked to poor maintenance of nitrogen metabolism, and thus a higher association with cardiovascular disease

(Vanhatalo et al., 2018). Moreover, NO has more beneficial links with general health, including inhibition of platelet aggregation, maintenance of pulmonary vascular health and improved mitochondrial efficiency (Kapil et al., 2013; Koch et al., 2017). NO_2^- also participates in nitric acid production in the stomach which elicits antibacterial and mucus-generating properties (Lundberg et al., 2008).

Another form of symbiotic relationship is elicited between bacteria and epithelial cells. For example, the primary coloniser, *S. salivarius*, can modulate the immune response by inhibiting the activation of NF-kB – the key intracellular inflammatory response regulator, thus reducing any undesirable pro-inflammatory responses by oral epithelial cells to the oral microbiota (Cosseau et al., 2008; Kaci et al., 2014).

An altered balance of the commensal bacteria is also associated with some systemic diseases. Dysbiotic oral bacterial communities are detected in oral cancer patients as well as some distant gastrointestinal tract cancers, such as oesophageal and pancreatic cancer, while *F. nucleatum* is extensively associated with colorectal cancer (He et al., 2015; Shang and Liu, 2018). It is proposed that bacterial metabolism might contribute to the cellular damage and initiation of tumorigenesis (Chocolatewala and Chaturvedi, 2009). Epidemiological studies also reveal associations between diabetes mellitus and a dysbiotic oral microbiome in that diabetes patients harbour more *P. gingivalis* and *P. intermedia*. Consequently, these patients experience a three-fold greater risk for developing periodontal disease than a healthy population (Preshaw et al., 2012). Interestingly, the treatment outcomes of diabetes are more favourable if patients do not experience periodontal disease (Simpson et al., 2010). In fact, performing scaling and root planning on periodontitis and diabetes mellitus patients improves their glycaemic control (Simpson et al., 2010). The associations between a dysbiotic oral community and obesity, preterm birth, bacterial pneumonia and rheumatoid arthritis (RA) have also been demonstrated (Offenbacher et al., 2006; Leech and Bartold, 2015; Vinturache et al., 2016). While some mechanisms have been proposed to explain these links, the actual biological basis for associations between the human microbiome and disease is still to be fully elucidated.

1.6 The diversity of oral microbiota in health

The oral cavity is a habitat suitable for the growth of many microbial species; these organisms can occupy different niches of the mouth to form distinct communities. It has been shown that there is a characteristic prevalence of microbiota at distinct sites within the mouth - a phenomenon, termed the microbiota signature (Lourenco et al., 2014). For instance, a high prevalence of *Streptococcus* spp. and *Actinomyces* spp. is observed either in newly formed biofilms or in biofilms adjacent to periodontal tissues with no inflammatory signs (Diaz et al., 2016). At the same time, different mouth sites accumulate specific communities: the fissured and grooved tongue dorsum facilitates the growth of obligately anaerobic species, enamel surfaces facilitate the growth of species able to attach and form biofilms, while the bacterial profile of saliva is similar to the microbiota of the tongue, but somewhat distinct from other mucosa-associated communities (Zaura et al., 2009; Belda-Ferre et al., 2015).

1.6.1 Salivary microbiota

Saliva supports a relatively stable ecosystem regarding temperature and nutrient supply. It is rich in glycoproteins, amino acids and urea - all these compounds can be degraded by oral microbiota as nutrients (Marsh et al., 2016a). It is also a major determinant of oral health: through buffering and its antimicrobial properties the microbiota is maintained in a homeostatic phase. First, bicarbonates/ hydrogen carbonate ions are principal salivary buffers and, together with phosphate ions and some proteins, they are responsible for buffering the environment and keeping stable levels of pH around neutrality (Vuletic et al., 2014). This pH is favourable to the growth of beneficial oral bacteria and prevents the outgrowth of acidophilic species. Second, the abundance of salivary antimicrobials ensures an effective regulation of salivary microbial composition (Carpenter, 2013). These antimicrobials can be grouped into two categories (Marsh et al., 2016a). Directly acting antimicrobial agents, for example, histatins, lysozyme, lactoperoxidase, act as cationic proteins, lyse bacterial cells walls or oxidise enzymes and other key proteins, respectively. Indirectly acting agents are mucins, proline-rich proteins, that bind and agglutinate the microorganism, lactoferrins, that bind iron or bactericidal permeability inducing proteins, that act through lipopolysaccharide binding

proteins. Their antifungal, antiviral, and antimicrobial properties manifests through reduced growth or killing of microorganisms in a planktonic and biofilm form.

More than 70% of species detected in saliva belong to the genera *Streptococcus*, *Prevotella*, *Veillonella*, *Neisseria*, *Haemophilus*, *Rothia*, *Porphyromonas*, and *Fusobacterium* (Nasidze et al., 2009). The microbial composition varies significantly between healthy individuals and is specific for each person, but displays a high inter-individual stability during a period of at least one-year (Stahringer et al., 2012; Hall et al., 2017). However, saliva does not support the growth of a natural microbiota, because microorganisms are swallowed quicker than they can divide. Saliva represents the communities found on other oral surfaces, especially the tongue. On the other hand, the compositional changes in salivary microbiota might indicate underlying systemic conditions (Rathnayake et al., 2013). For example, an increase in salivary proportions of *P. gingivalis* is associated with a greater risk of periodontal disease, while increases in salivary mutans streptococci indicate a risk of caries (Guo and Shi, 2013; Belstrom et al., 2017b). Based on a potential connection with several diseases, saliva is an attractive fluid to monitor the pathological condition of human body due to easy and non-invasive sampling.

1.6.2 Surface-associated microbiota

Most microorganisms in the oral cavity are surface-associated and variation in the local environmental conditions at distinct oral surfaces ensures an abundance of different habitats for colonisation. Histologically, the mouth is divided into three types of mucosa: 1) lining mucosa covered by stratified squamous non-keratinized epithelium, 2) masticatory mucosa, lined by stratified squamous keratinised epithelium, and 3) specialised mucosa, covered by a mucous membrane that contains nerve endings for general sensory reception and taste perception. Although oral bacteria can be associated with host tissues through low specificity electrostatic and hydrophobic forces, they have highly developed recognition systems (adhesins) that interact with specific macromolecules on host epithelium cells or components of underlying extracellular matrix (termed ‘receptors’). For example, fibril adhesins enable Gram-positive and Gram-negative bacteria to attach to epithelial cells, type I

collagen, fibronectin and salivary pellicle (Nobbs et al., 2011). Different species demonstrate specific tropism towards mucosal surfaces. For example, *S. mitis* and *Streptococcus sanguinis* possess sialic acid binding receptors, and sialic acid is three times more likely to be found on buccal epithelium than on other locations (Gibbons, 1989). *S. salivarius* colonises the tongue better than the buccal mucosa (Gibbons et al., 1976). Due to the complex surface architecture of the tongue, it is considered as a reservoir for perio-pathogenic bacteria; more Gram-negative obligate anaerobic species are detected on the dorsum of the tongue than other epithelial surfaces (Roldan et al., 2003). The adherence of bacteria to soft tissues is restricted by constant shedding and desquamation of epithelial cells and masticatory movements.

Adults have 28 – 32 teeth; their hard and non-shedding surfaces provide a distinct ecological habitat for oral bacteria. For example, supragingival biofilms have access to oxygen through oxygenated saliva and air, which boosts the growth of aerobic and facultatively anaerobic species and restricts the growth of obligate anaerobes, although, deeper layers of the supragingival biofilms can harbour obligate anaerobes due to the development of oxygen gradients (Wessel et al., 2014). Biofilm bacteria residing above the gingival margin principally rely on saliva, food debris and cellular components of epithelium as nutrient sources (Marsh et al., 2016a). Salivary glycoproteins, or mucins, are complex, highly glycosylated polypeptides that require cooperation among species with different enzyme profiles for its degradation (Bradshaw et al., 1994; Wickstrom et al., 2009). For example, species from *Actinomyces* genus grow on saliva only in mixed cultures (Zhou et al., 2016). For this reason, a healthy supragingival biofilm exhibits high bacterial diversity. Different studies indicate that supragingival plaque of healthy individuals contain around 200 OTUs and the core supragingival microbiome consists of *Streptococcus, Neisseria, Actinomyces* and *Capnocytophaga* species*, F. nucleatum, H. parainfluenzae, Rothia dentocariosa Corynebacterium durum, Eikenella* spp*.* and others (Hall et al., 2017; Chen et al., 2018). The supragingival microbiota preferentially ferments sugars, producing acids that can dissolve hydroxyapatite and cause caries (Marsh, 2003a). Continuous access to saliva allows a buffering effect, through which the enrichment of acidophilic species is

reduced. However, caries develops if the homeostatic balance is disturbed for prolonged periods.

1.7 Caries and its related microbiota

In healthy individuals, supragingival microbial communities are in balance with the host; they do no harm and even provide benefits for the local and general health of the host, (these are described in section 1.4). Under certain circumstances, however, the composition of plaque can be disrupted, driven by a change in oral conditions (Marsh, 2003a). The increased consumption of dietary sugars, poor oral hygiene and salivary dysfunction are responsible for the enrichment of acidogenic and acidophilic species. Oral bacteria produce acids following the metabolism of dietary fermentable sugars, and reduce the environmental pH in the biofilm. When the pH drops to around 5.5, the inorganic components of teeth start to dissolve, leading to appearance of a demineralised spot under the accumulated plaque which later progresses to a cavity (Moye et al., 2014). This pH is termed the ‘critical pH’.

Caries is the most prevalent disease in the wold (Marcenes et al., 2013). The key factor for the appearance of disease is the pathogenic potential of the supragingival community. Lactobacilli, bifidobacteria, *Atopobium* and *Slackia exigua* together with two major etiological agents of dental decay, *S. mutans* and *Streptococcus sobrinus,* are usually found in plaque covering caries lesion (Tanner et al., 2016; Nascimento et al., 2017). The two latter members of the oral community are able to produce acid in unprecedented quantities even at low pH (de Soet et al., 2000). These streptococci are found in low numbers in healthy communities, as they are not competitive under neutral pH conditions of the normal oral cavity (Kohler et al., 1995; Colby and Russell, 1997). However, when there is a surplus of fermentable carbohydrates, especially when ingested frequently, the environmental pH drops, and the biofilm spends longer periods under acidic conditions, increasing the competitiveness of these acidophilic species while simultaneously inhibiting the growth of beneficial bacteria (Takahashi and Nyvad, 2011). In their favoured acidic environment their abundance increases, concomitantly bringing the pH further down and leading to an ecological catastrophe in the mouth (Bradshaw et al., 1996b; Marsh, 2003a).

High consumption of carbohydrates has a strong relationship with caries development. Rapidly fermentable ‘free sugars’ (mono- or di-saccharides detected in sugary foods) pose the greatest risk for caries induction (Moye et al., 2014). Quantity- and frequency-dependent relationships have been discovered between sugar intake and new caries lesions (Sheiham and James, 2014). Any tooth surface exposed to the critical pH for prolonged periods (usually as a result of bacterial sugar metabolism) is prone to caries (Dorozhkin, 2012; Kianoush et al., 2014). Another risk factor for caries development is poor oral hygiene and accumulation of dental plaque (Fejerskov, 2004). Location-wise, caries can affect tooth crown or root. Anatomy of the tooth crown plays a role in plaque accumulation; approximal areas, fissures and grooves that are protected from mechanical forces (toothbrushing, chewing, tongue movements) are more prone to caries (Kutsch, 2014). If low levels of pH remain in retentive areas for prolonged periods, enamel crystals gradually dissolve (Dorozhkin, 2012). Salivary dysfunction also introduces teeth to the risk for caries development (Kutsch, 2014). Bicarbonates, phosphates and proteins detected in saliva can cope with some fluctuation in pH; however, an excess of acids from the fermentation of dietary carbohydrates and simultaneous reduced salivary flow exhausts its buffering properties, leading to a comprehensive drop in pH.

Root caries is another type of oral decay, which establishes after the gingiva recedes to expose the root surface and facilitate the accumulation dental plaque. The pathogenesis of root caries is the same as for other type of caries (Do et al., 2017). It involves the dissolution of hydroxyapatite crystals by acids produced by the fermentation of dietary carbohydrates; however, cementum is more susceptible to acid than enamel. The slightly different ecological niche found on the root surface results in a distinctive microbiota. The most commonly isolated species from active root caries are saccharolytic facultative anaerobes, for example, species from genera *Streptococcus, Lactobacillus* and *Actinomyces* (Nyvad and Kilian, 1987; Preza et al., 2008). However, the proximity of exudate from gingival sulcus enables the growth of proteolytic species. Studies report a higher abundance of Gram-negative capnophilic proteolytic species in root surface plaque than in fissure caries; these include, for example, species from the genera *Prevotella, Fusobacterium,*

Capnocytophaga and *Campylobacter* (Preza et al., 2008). Moreover, it has been postulated that Gram-positive and Gram-negative species directly contribute to the degradation of tooth organic matrix through microbial-derived collagenases, gelatinases and peptidases (Do et al., 2017).

1.8 Periodontal disease

Periodontal diseases are a complex group of diseases of the periodontium. Although being only the 6th most prevalent global disease, it affects 20% – 50% of population in developing and developed countries, having a significant impact on both human health and quality of life (Marcenes et al., 2013; Nazir, 2017). Epidemiological studies demonstrate that the prevalence of periodontal disease differs between countries, ethnic and age groups with a tendency to be more prevalent in older populations (Kim et al., 2012). The high prevalence of periodontal disease inevitably places a heavy burden on health care systems as oral diseases are the fourth most expensive diseases to treat among industrialized countries (Petersen et al., 2005). There are several types of periodontal disease; gingivitis and chronic periodontitis are the most common, but there are other forms of periodontitis, such as aggressive or ulcerative necrotizing periodontitis, that induce early tooth loss among young populations.

Particular lifestyles and underlying conditions – smoking, obesity, systemic diseases, stress, genetic predisposition, deficiencies in neutrophil function, and increased plaque accumulation due to poor oral hygiene, are all associated with the induction of periodontitis. Disease manifests itself in the inflammation of periodontium (gingiva, cementum, periodontal ligament and bone) and eventually resorption of alveolar bone that finally results in tooth loss (Van Dyke and Sheilesh, 2005; Taba et al., 2012). Moreover, studies investigating the impact of periodontitis on general health have demonstrated an association with cardiovascular system disorders and diabetes mellitus (Tsai et al., 2002). Current treatment options for periodontitis include mechanical plaque removal and antibacterial therapy, and treatment of complications of the disease are limited and very expensive. The successful control of periodontal disease can therefore be a challenge for a dental practitioner.

1.9 Gingivitis

Gingivitis is characterised by plaque build-up in the gingival crevice, and it is a transitory state between health and periodontitis. Clinically, gingival inflammation exhibits colour changes to the gingivae, contour alteration, increased sulcular exudate and bleeding upon probing, but without the resorption of bone and adjacent tissues. At this stage the disease is non-finite; thorough cleaning and plaque removal reverse the inflammation, otherwise, gingivitis can subsequently progress to periodontitis. Unique taxonomic groups are found in plaque from healthy patients and those with gingivitis, especially increased abundance of Gram-negative species. Early culture studies identified that plaque from gingivitis areas was inhabited by Gram-negative rods, that constituted about 25% of total isolates and Gram-positive species, that belonged to genera *Actinomyces* and *Streptococcus* (26.8%) (Slots et al., 1978). An experimental gingivitis study showed that 13 genera were enriched in sites with gingivitis and two genera concomitantly reduced (Huang et al., 2014). After sequencing, authors identified species *Tannerella forsythia, Parvimonas micra, F. nucleatum, Haemophilus paraphrophilus* and *Capnocytophaga* sp*. oral clone CZ006* as gingivitis-drivers; however, these are also associated with periodontitis. The taxonomic shift from health to gingivitis was also accompanied by a functional shift. The same study found that the microbiota of gingivitis patients expressed more proteins involved in flagellar biosynthesis that samples from healthy sites (Huang et al., 2014). Flagella are related with increase in virulence of the microbiota as they are related to bacterial movement and invasion of host tissues.

1.10 Periodontitis

If good oral hygiene is re-instated during gingivitis, then plaque levels return to normal, and the bacterial profile becomes similar to that found in oral health, and clinical symptoms of gingival inflammation disappear (Huang et al., 2014). However, if plaque control remains poor, gingivitis can be followed by periodontitis. In periodontitis, the supporting tissues of the teeth (gingiva, periodontal ligament, cementum, bone) are exposed to inflammation, followed by the migration of junctional epithelium down the root of the tooth resulting in the formation of a periodontal pocket. This creates a different ecological habitat distinct to that of other sites in the mouth. The flow of GCF is increased, and this introduces components of the host defences (Huynh et al., 2015). Host cell

debris, low oxygen levels and a slightly alkaline pH are features of the periodontal sulcus (Eggert et al., 1991; Bosshardt, 2018). As deepening of the pocket is caused through inflammation, this results in a slight rise in local pocket temperature, cell breakdown, and enrichment of iron and haem-containing molecules, such as transferrin and haemoglobin (Barros et al., 2016). If this inflammatory response fails to reduce the microbial 'insult', then the change in environment can promote the growth of pathobiont bacteria by providing additional nutritional sources.

1.10.1 Risk factors for periodontal disease

Periodontitis is a disease with a complex aetiology acting on many levels. On one level it is caused by a commensal dysbiotic microbial community and its effects on host tissues; on another level, the host displays either a genetic predisposition or resistance to the disease. Poor oral hygiene is linked with periodontal disease, but actually some individuals do not experience periodontitis despite having significant tooth-associated biofilm accumulation. A genetic basis for periodontal disease is supported by twin studies (Stabholz et al., 2010); moreover, a genome-wide association study discovered three host loci (14q21, 7p15 and 6p21.1) being suggestive for severe periodontitis (Divaris et al., 2013).

Some systemic conditions predispose the host to periodontitis. For example, a systematic review which looked into 29 studies on periodontitis and diabetes mellitus links, found that 27 published papers supported evidence of adverse effects on periodontal tissues in diabetes mellitus patients, especially in terms of poor metabolic control (Negrato et al., 2013). It was suggested that a rise in advanced glycosylation end products and a subsequent increase in pro-inflammatory cytokines in saliva and gingival crevicular fluid favours the development of an inflammatory environment, which might lead to the development of a dysbiotic microbiota (Llambes et al., 2015).

Another example of oral dysbiosis triggered by a systemic condition is inflammatory bowel disease. Patients with inflammatory bowel disease are more likely to experience periodontitis, and of a higher severity, than a comparable healthy population (Lira-Junior and Figueredo, 2016). Inflammatory bowel disease, especially in its active form, is linked with increased peripheral

neutrophil activity and high levels of pro-inflammatory mediators in saliva and gingival fluid. Also, increased levels of species from *Prevotella, Treponema, Capnocytophaga, Rothia,* and *TM7* genera were reported in the mouth of these patients, all of which are indicators of poor periodontal health (Lira-Junior and Figueredo, 2016).

A link between rheumatoid arthritis and periodontitis has been recognised for many decades. Common features of both diseases include the chronic nature of both diseases and shared environmental and genetic risk factors (Leech and Bartold, 2015). RA patients are noted to have oral and gut microbiome that is distinct from that of a healthy population. In the oral cavity, RA patients had more species from the genera *Veillonella, Atopobium*, and fewer health-associated *Haemophilus, Neisseria, Kingella and Eikenella* species (Scher et al., 2012). Higher proportions of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* found in severe periodontitis and might play a role in initiation and propagation of localised and systemic RA autoimmunity (Cheng et al., 2017). Moreover, treatment of RA with methotrexate (immune suppressant) partially restores a healthy oral microbiome (Zhang et al., 2015). It has also been established that smoking has a negative impact on chronic diseases such as RA, inflammatory bowel disease, multiple sclerosis and periodontitis (Johannsen et al., 2014). Higher levels on *P. gingivalis, T. forsythia, T. denticola* and *A. actinomycetemcomitans* and species from the *Fusobacterium* genus were detected in subgingival plaque of smokers in comparison to non-smokers (Haffajee and Socransky, 2001). Increased systemic levels of interleukins, C-reactive protein and haptoglobin have been reported in smoking patients, and many of these substances and mediators are associated with periodontitis (Johannsen et al., 2014).

1.10.2 Periodontitis is an inflammation-mediated disease

Periodontal disease is mediated by the inflammatory response to bacteria in the dental biofilm. Under normal conditions, neutrophils are abundant in the gingival sulcus lining the junctional epithelium, which acts as a physical barrier for bacteria (Shimono et al., 2003). At a histological level, bacteria residing in

the gingival sulcus secrete metabolites and toxins, and bacterial antigens can penetrate through junctional epithelium and stimulate the immune system. That causes junctional epithelium to produce cytokines, leading to vasodilation of local blood vessels and recruitment of more neutrophils. Neutrophils are important for gingival health but also constitute the majority of recruited cells in periodontal disease (Dutzan et al., 2016). They cause periodontal tissue destruction through the release of degradative enzymes, for example matrix metalloproteinases or cytotoxic substances, such as reactive oxygen species (Hajishengallis, 2014a). Lymphocytes, granulocytes and macrophages are all present in gingival tissue and an increase in multiple inflammatory cell types is associated with periodontal disease. The proportions of infiltrating cells alters in diseased gingival tissue, with increased numbers of lymphocytes, specifically T cells and antibody secreting plasma cells seen, in addition to the dramatic surge in neutrophil numbers (Dutzan et al., 2016). Activated macrophages within gingivae can release TNF-alpha that can induce collagenase release from variety of connective tissue cells. If the bacterial triggers are not removed from the site, the chronic lesion may develop (Cekici et al., 2014; Huynh et al., 2015). However, this environment offers a plentiful nutrient source for proteolytic bacteria. For example, the flow of gingival crevicular fluid in periodontitis increases more than 30 times compared to health and provides inflammatory mediators, proteins, tissue breakdown products (Goodson, 2003). Moreover, Hajishengallis and colleagues conducted a variety of studies leading to the proposal that commensal bacteria work synergistically to evade immune-mediated killing. At the same time, the subgingival microbiota can tolerate inflammation and obtain essential nutrients from both GCF and from tissue breakdown products such as degraded collagen peptides and haem-containing compounds (Hajishengallis, 2015). He also suggests that once inflammatory environment is established by “keystone” pathogen *P. gingivalis*, other periodontitis-associated inflammophilic bacteria provoke that inflammation to secure nutrients which causes further tissue damage (Hajishengallis, 2014b).

The synergistic effects between genetic predisposition, environmental factors (such as oral hygiene and smoking) and systemic conditions all play a role in the development of the disease. However, the main aetiological trigger are bacteria.

1.11 Microbiota in periodontitis

Periodontitis is driven by dysbiosis of the subgingival plaque. In total, around 700 bacterial species have been detected in the mouth and 100 – 300 species can be detected in a single individual (Chen et al., 2018). The species demonstrate specific tropism towards different oral environments and exhibit different abundances in oral health and disease. For example, bacteria detected in oral health are less frequently detected in caries and periodontal diseases, and early studies identified certain bacteria as potential aetiological agents.

The infectious nature of periodontitis necessitated the identification of pathogenic bacteria involved and therefore lead to the initiation of plaque culture studies. Early culture studies identified a marked increase in Gram-negative anaerobic species in periodontal pockets compared to healthy sites (Slots, 1979). Gram-negative anaerobic rods comprised about 75% of all culturable species found in the periodontal pocket, especially *Bacteroides melaninogenicus ss. Asaccharolyticus (*now *P. gingivalis, Porphyromonas asaccharolyticus, Prevotella nigrescens* and *P. intermedia*) (Slots, 1979). Later, Socransky used DNA-DNA hybridisation technique on subgingival plaque and found that many bacterial species tend to cluster together, depending on the status of the site (Socransky et al., 1998). For example, orange complex, consisting of mainly Gram-negative anaerobes, was closely associated with red complex comprising of *P. gingivalis, T. denticola* and *T. forsythia.* These three species were usually found together in deep periodontal pockets. Moreover, they were rarely found in the absence of the orange complex. Increased colonisation by orange complex was also associated with the increased numbers of red complex (Socransky et al., 1998). These milestone findings gave rise to the hypothesis that multiple species cooperate in periodontal disease.

More recently, the development and application of advanced sequencing technologies have enabled the breadth of the populations of bacteria at different stages of periodontal disease to be characterised. While bacterial communities in gingivitis are quite similar in composition to plaque from healthy sites, microbiota of deep periodontal pockets tend to cluster separately based

on taxonomical similarity (Perez-Chaparro et al., 2018). This suggests than the breakdown of microbial homeostasis starts during gingivitis and further progresses to the more pronounced dysbiosis that is detected in periodontitis. An increase in bacterial load, species richness and diversity are features of bacterial communities in periodontal disease (Abusleme et al., 2013).

Periodontitis-compromised areas are usually inhabited by proteolytic, fastidious and unculturable species. At the phylum level, Spirochetes, Synergistetes and Bacteroidetes are enriched in periodontitis (Liu et al., 2012; Abusleme et al., 2013; Meuric et al., 2017). Species belonging to the genera *Fusobacterium, Treponema, Campylobacter, Eubacterium*, and *Tannerella,* are often identified in subgingival plaque. Periodontal tissue damage is strongly dependent on the presence of the red complex bacteria: *P. gingivalis*, *T. forsythia*, *T. denticola*, which are found in the later stages of periodontitis (Holt and Ebersole, 2005; Colombo et al., 2009; Loozen et al., 2014; Perez-Chaparro et al., 2018). Moreover, deeper periodontal pockets have more *Treponema, Porphyromonas, Tannerella* and *Fusobacterium* than shallow ones (Perez-Chaparro et al., 2018). Numerous studies have tried to define the microbiota of periodontal pocket. The most commonly detected species, apart from red complex, are *Filifactor alocis, Porphyromonas endodontalis, Treponema socranskii, Peptostreptococcus stomatis, Pseudomonas alactolyticus, Mogibacterium timidum, P. micra* and others (Abusleme et al., 2013). A diversity of sequencing studies suggested a variety of species associated with periodontitis. (Table 1.1). Conversely, some commensal genera, such as *Streptococcus, Corynebacterium, Cardiobacterium, Rothia, Capnocytophaga* and *Veillonella* are found in decreased abundance in chronic periodontitis subjects (Perez-Chaparro et al., 2018).

Table 1.1. Species associated with periodontal disease.
Species associated with periodontal disease were determined using open-end sequencing studies in a systematic review. (–) Gram-negative species, (+) Gram-positive species, a - not yet cultured. Adapted from Perez-Chaparro et al. (2014).

Phylum	Species
Bacteroidetes	*Bacteroidales* [*G-2*] *sp. oral taxon 274* HOT 274 (–)
	Porphyromonas endodontalis HOT 273 (–)

	Prevotella denticola HOT 291 (–)
	Alloprevotella tannerae HOT 466 (–)
Firmicutes	*Eubacterium* [*G-5*] *saphenum* HOT 759 (+)
	Mogibacterium timidum HOT 042 (+)
	Peptostreptococcus stomatis HOT 112 (+)
	Filifactor alocis HOT 539 (+)
	Anaeroglobus geminatus HOT 121 (–)
	Selenomonas sputigena HOT 151 (–)
	Enterococcus faecalis HOT 604 (+)
Proteobacteria	*Desulfobulbus sp. oral taxon 041* HOT 041[a]
	Johnsonella sp. oral taxon 166 HOT 166[a]
	Eubacterium [*X1*] [*G-3*] *brachy* HOT 557 (+)
	Peptostreptococcaceae [*XIII*] [*G-1*] *sp. oral taxon 113*[a]
	Lachnospiraceae [*G-8*] *sp. oral taxon 500*[a]
	Dialister pneumosintes HOT 736 (–)
Spirochaetes	*Treponema lecithinolyticum* HOT 653 (–)
	Treponema medium HOT 667 (–)
	Treponema vincentii HOT 029 (–)
	Treponema phylogroup II (–)
Synergistetes	*Fretibacterium sp. oral taxon 360* HOT 360[a]
	Fretibacterium sp. oral taxon 362 HOT 362[a]
	Fretibacterium fastidiosum HOT 363 (–)
	Fretibacterium sp. oral taxon 359 HOT 359[a]

Candidate division TM7	*TM7* [*G-5*] *sp. oral taxon 356* HOT 356[a]
	TM7 [*G-1*] *sp. oral taxon 346* HOT 346[a]
	TM7 [*G-1*] *sp. oral taxon 349* HOT 349[a]
Proteobacteria	*Acinetobacter baumannii* HOT 554 (–)
	Escherichia coli HOT 574 (–)
Candidate division SR1	*SR1* [*G-1*] *sp. oral taxon 345*[a]

Different functional profiles are concomitantly detected in periodontal plaque compared to heath. The metabolic shifts that occur in bacteria during bone destruction are poorly known, however, metagenomic studies reveal that the metabolic functions of the disease-associated microbiome are consistent with an increase in lysine fermentation to butyrate, histidine catabolism, nucleotide biosynthesis, pyruvate fermentation, iron acquisition, flagellar motility, peptides transport and beta lactam degradation (Duran-Pinedo et al., 2014b; Jorth et al., 2014). A metatranscriptomic study by Yost et al. (2015) revealed increased levels of chaperones in pathogenic biofilms, inferring that the level of stress is higher in disease conditions under which biofilm is formed and exists. They also indicated that there are particular genes over-expressed by bacteria growing in biofilm, compared with planktonic bacteria, thus identifying genes that are important in biofilm formation, growth and survival within biofilm. It seems that an increased rate of metabolism in biofilms is at least partially explained by the high expression of genes specific to biofilm formation and elevated levels of stress in microbiota, when pathogens are present (Frias-Lopez and Duran-Pinedo, 2012). Periodontal disease-associated communities are more ribosome enriched, which may signify an increased metabolic rate and, possibly, more complicated bacterial interactions with the host or each other (Jorth et al., 2014). Interestingly, transcription signatures are well conserved in disease-associated biofilms, but organisms carrying out these processes do vary between individuals (Jorth et al., 2014).

In the middle of the last century, the phenomenon of dysbiosis was not regarded as a key factor in periodontitis. The main theory for disease rather emphasised that plaque amount acted as a driver for the disease. A Non-specific Plaque Hypothesis suggested that accumulation and overgrowth of a wide range of bacteria can cause inflammation leading to the development of periodontitis (Miller, 1890). It was determined that a load of plaque accounted for pathogenicity without discriminating about bacterial virulence (Rosier et al., 2014). However, this could not explain why some patients with significant plaque retention were not experiencing periodontitis. Later, a Specific Plaque Hypothesis was proposed (Loesche, 1976). The early investigations on periodontal disease observed a dramatic compositional change in bacterial composition (Slots, 1979). Higher abundance of few species, most commonly detected in disease but rarely observed in health, implied that some specific pathogens directly cause disease through their virulence traits. This finding pointed to a bacterial specificity suggesting that a few species are aetiological agents for periodontitis (the specific plaque hypothesis). However, some patients were undergoing periodontitis, even if no *P. gingivalis* or other pathogens were detected with current methods, and so alternative explanations were sought.

1.11.1 Reasons for the dysbiosis

Neither of these two early hypothesises explained the drivers for the dysbiosis during the transition from health to gingivitis and periodontitis. Marsh (1994b) proposed an Ecological Plaque Hypothesis, to include the role of the local environment in driving dysbiosis. In this theory, it is accepted that putative pathogens may be present at low levels in plaque from healthy sites, but these species are not competitive under the prevailing conditions, and therefore are not clinically significant. However, if plaque accumulates and generates a non-specific inflammatory response, then the environmental change in the periodontal environment (the increased flow of GCF, rise in pH and temperature, the surplus of proteins, cell breakdown products, reduction in redox potential, etc) favours the growth of the putative periodontal pathogens at the expense of the commensal bacteria. Proteolytic species become more competitive and are enriched, making the community dysbiotic. The proteases of bacterial origin fuel this harmful cycle by deregulating the host immune

response, which further leads to periodontal tissue damage. Periodontitis-associated bacteria feed on nutrients derived through this destruction, aggravating the immune response still further by thriving in inflammatory environment and perpetuating inflammatory tissue destruction (Hajishengallis, 2014b). The Ecological Plaque Hypothesis differs from the previous concepts by arguing that any species that induces and thrives in an inflammatory environment can contribute to the development of periodontal disease. The low abundance of periodontitis-associated species in healthy plaque and their enrichment in disease suggests that periodontitis results mostly from ecological shifts in community structure, rather than a shift in membership (Abusleme et al., 2013). Specific environmental factors drive the selection of the proteolytic and inflammatory bacteria, that indirectly participate in destruction of tissues.

More recently, a polymicrobial synergy and dysbiosis model was suggested by Hajishenallis and Lamont. (Hajishengallis and Lamont, 2012). Polymicrobial synergy is termed as a process when one microorganism enhances the colonisation/virulence of another. Hajishengallis and Lamont proposed that the polymicrobial biofilm community that accumulates on the healthy gingiva has a disease-inducing potential through keystone pathogens. Under healthy circumstances, the growth and pathogenicity of the bacterial community is controlled by the host inflammatory response; the same inflammatory homeostatic state has been described in the gastrointestinal tract (Lozupone et al., 2012). However, the pathogenic potential of a biofilm can be increased by these keystone species. In the mouth, colonisation of *P. gingivalis* elevates the pathogenicity of the community by manipulating host immune response, but some other pathogens, such as *F. alocis,* can also evade immune response (Aruni et al., 2011). For example, gingipains of *P. gingivalis* can inactivate complement, enhance the inflammation but reduce the immune clearing targeted at bacteria (Olsen et al., 2017). In general, *P. gingivalis* virulence factors (such as capsule, gingipains, fimbria, lipopolysaccharides, surface anionic polysaccharides) are able to modulate the immune response, avoid the elimination and allow the pathogen to survive on debris of other microorganisms (Slaney et al., 2006; Wilensky et al., 2015). Through interactive communication with accessory pathogens, such as mitis group streptococci that are otherwise commensal bacteria, *P. gingivalis* can elevate the virulence

of the entire community. The orchestrated disruption of the commensal community causes dysbiosis and destruction of periodontal tissues (Hajishengallis and Lamont, 2012), but again the change in environmental conditions associated with inflammation plays a role in dysbiosis.

1.12 Model systems for studying oral dysbiosis

The approaches to study the oral microbiota in biofilm form have been presented in section 1.1. The cultivation of complex biofilm communities in the laboratory is challenging and multiple systems have been developed to culture standardized biofilms. These systems vary considerably based on their complexity, controllability of environment, sophistication of employed equipment, and expertise needed. The models reviewed in more detail below are categorised as *closed* and *open* systems based on nutrient and metabolite turnover.

Microbial culture models in *closed* systems have a finite supply of nutrients. Under these conditions, bacterial growth is faster than in the oral cavity, resulting in different patterns of bacterial behaviour than *in vivo*. Therefore, experiments based on closed or batch culture are of shorter duration than those in open culture models. However, they are widely used by researchers due to their greater simplicity and productivity, speed and repeatability, controllability of experimental conditions and less contamination (Yu et al., 2017). The common microbial models in closed system are microtitre plates and their modifications.

The simplest systems used in oral biofilm research are based on microtitre plates. They are usually made of a polystyrene and have a flat surface. Biofilms are usually formed directly in the wells although, alternatively, surfaces can be introduced, such as hydroxyapatite discs or coverslips. Each well contains some millilitres of liquid and allows the independent growth of biofilm in each well. A well-known modification of the microtitre plate is the ‘Zurich biofilm model’ introduced by Guggenheim in 2001 and later developed by the same laboratory group in multiple studies (Guggenheim et al., 2001; Hofer et al., 2015). Ten species are sequentially introduced into the microtire plate containing HA discs; during biofilm formation a few media (supplemented with either glucose of serum) are used to represent different stages of biofilm

formation. The biofilms are later examined with FISH or inoculated onto selective agars for quantification. The model was used to investigate the formation, species interaction, and maturation of subgingival communities (Thurnheer et al., 2016; Bao et al., 2018). Although very useful, these systems suffer from disadvantages. For example, during replacement of media, biofilms can be easily distorted; the systems are also labour intensive, especially if sequential inoculation of many species is needed.

The Calgary biofilm device (CBD) was originally developed in 1999 to test the susceptibility of biofilms to antimicrobial or toxic compounds, and has been widely used since (Ceri et al., 1999). CBD is also known and marketed as MBEC (Minimum Biofilm Eradication Concentration) assay. The model consists of two parts: a lid with 96 pegs, which sits in the wells of a standard 96-well microtitre plate. Hydroxyapatite-coated pegs are available for experiments with oral biofilms. The model allows a selective attachment of bacteria to the peg surface, as pegs are immersed in the growth medium with the inoculum. Later, sterile medium is provided which supplies nutrients for the attached species. Fresh medium can be introduced without damage to the biofilms by simply replacing a standard 96-well plate. Each peg can be broken from the lid and investigated further at any stage of an experiment. The model has been used in oral microbial research to investigate the susceptibility to antibiotics of complex microbial communities, isolation of unculturable species, and culturing and microbial profiling of saliva (Kistler et al., 2015; Soares et al., 2015; Wade et al., 2016). A derivation of the CBD is the Amsterdam Active Attachment (AAA) model. It consists of a stainless steel lid with 24 clamps that each holds a glass coverslip or hydroxyapatite disc. A standard 24-well plate is used as a receptacle to house the growth media (Krom and Willems, 2016). In a similar manner as the CBD, multiple biofilms can be cultured simultaneously.

Open or *continuous* culture systems have several advantages over closed systems because they enable the constant supply of fresh nutrients and provide removal of metabolites; hence concentrations of nutrients and metabolic compounds remain constant. Nevertheless, the possibility of contamination is high due to the complexity of model construction. Chemostats, constant depth film fermenters and a variety of ‘artificial mouths’ have been used in oral research.

Chemostats provide the careful control of environmental conditions and allow variation of single parameter at a time. This continuous culture approach does not attempt to reproduce all of the physical properties of the habitat; it rather enables to demonstrate a cause-and-effect relationships established unequivocally and permits the modelling under highly controlled and reproducible conditions, of specific events that occur *in vivo* (Bradshaw and Marsh, 1999). The simplest chemostat systems consist of a single stage reactor. However, for biofilm studies, modified equipment is necessary to create extra ports through which surfaces for biofilm formation can be introduced and removed aseptically (Bradshaw et al., 1996b). The bacterial communities have to grow as a steady-state in order to examine the stresses applied. A steady-state is when bacterial growth, the levels of medium components, waste compounds and bacterial enzymes are constant. Depending on the experimental design and inoculum, it can be induced in 3 – 10 days. Single, mixed and complex cultures can be investigated, and large volume of culture can be taken repeatedly during the time-course of an experiment. However, complex communities, such as saliva or plaque, are extremely diverse, and so are difficult to analyse, quantify and characterise. Experiments of such complex cultures cannot be adequately reproduced, so the use of a defined inoculum has been used for some chemostat experiments (Bradshaw et al., 1996b). The application of chemostats in cariology enhanced the understanding of caries aetiopathogenesis. Bradshaw and Marsh investigated the effects of glucose pulses on bacterial communities to observe that low pH from carbohydrate metabolism, rather than just carbohydrate availability, was responsible for the shifts in microbiota. The fall in pH to between 4.5 - 5.5 favoured the growth of acidogenic and aciduric cariogenic species. A further drop in pH enhanced the competitiveness of cariogenic species and inhibited the growth of commensal bacteria, leading to the complete breakdown of homeostasis in the community (Bradshaw and Marsh, 1998b).

Another complex system, the Constant Depth Film Fermenter, is particularly suited for studies of biofilms of oral bacteria in that the environment factors such as the substratum, nutrient source, and gas flow can be altered (Jonathan, 2007). For example, biofilm growth occurs on a solid substratum

with nutrients being provided in a thin film of liquid, that is continuously replenished. The CDFF system is advantageous because it provides multiple biofilms in a single run and allows sampling at various stages or time points during the experiment. It also allows pulsing of nutrients or other substances, such as antimicrobials. During the run either supragingival or subgingival biofilms can be mimicked, depending on the composition of the atmosphere used for experiment. As with chemostats, the experiments using complex cultures cannot be reproduced to satisfactory extent, so a defined inoculum is usually used.

The Artificial Mouth Model is a multiple state model, more complex than other models discussed above. It consists of a few independent micro-stations, and different environments can be applied simultaneously. Well-controlled temperature and gas-phase allowing the replication of *in vivo* conditions (Rasiah et al., 2005). The system provides long-term, independent, controlled evaluation of plaque culture (Sissons et al., 1991). However, the system disadvantages from difficulty of repeated sampling.

Other systems have also been used, such as the Flow Cell Biofilm model, the Drip Flow Biofilm reactor or the multiple Sorbarod Model, all of which have been reviewed by Yu et al. (2017), however they are used less frequently in oral biofilm research. Although providing invaluable information about oral diseases, open or continuous culture systems use complex equipment which requires specific handling knowledge, thus they are used less frequently. These experiments are long and laborious. The complex nature of the oral microbiota can be a challenge to establish and analyse; moreover, continuous culture can be demotivating when contamination occurs.

1.13 Growth environment of oral biofilms used in in vitro models

Culture-independent methods have detected a wide variety of species in the mouth while current culturing techniques only allow the isolation and cultivation of two thirds of those species. Hence, around one third of oral bacteria are yet-to-be cultured (Vartoukian et al., 2016). Numerous strategies have been used for the cultivation of these extremely fastidious members of the oral microbiota. For example, medium supplementation with exogenous compounds, co-culture

with helper cells and mimicking of the natural habitat have helped to isolate many fastidious species. Recently, siderophores introduced in the growth medium resulted in the isolation of five previously uncultured species (Vartoukian et al., 2016). However, culturing of the diverse and complex microbiome in biofilm form requires specific conditions as well, especially because some oral bacteria are slow-growing, nutritionally fastidious and at a disadvantage to fast-growing species. The environment used in oral biofilm models should support the growth of complex communities and maintain its natural diversity. Some environmental variables such as medium type and its supplementation, atmosphere and pH are important for biofilm culture.

A variety of general purpose growth media are used to culture mixed communities. For example, Brain Heart Infusion broth (BHI), Trypticase Soy Broth, or Cooked Meat Broth are suitable for the growth of diverse communities. These nutrient-rich media consist of an assortment of hydrolysed proteins and glucose, supporting the growth of both saccharolytic and proteolytic species; however due to the high nutrient content they are not physiologically relevant and favour fast-growing species. However, Kistler et al. (2015) used BHI in a CBD to culture biofilms from pooled saliva samples and obtained complex biofilms of high diversity in two weeks.

However, oral biofilm communities might not be adapted to grow in high concentrations of nutrients; in their natural habitat supragingival biofilms rely on saliva, which is 98 - 99% water with the remainder being electrolytes, proteins and glycoproteins, antibacterial compounds and enzymes. Nutrient-rich media favour fast-growing species that out-compete slow growing ones. After a low nutrient medium was adopted, some slow growing species of soil and water have been isolated for the first time (Connon and Giovannoni, 2002). In oral research, some continuous culture models use media with a low nutrient concentration to establish stable multispecies biofilms (Bradshaw et al., 1989; Bradshaw et al., 1994). First chemostat experiments were performed in full strength Basal Medium with high protein content, and the rational for the medium switch was in order to replicate the natural growth environment (McKee et al., 1985). The Zurich 10 species model use modified fluid universal medium, which consists of 30% of proteinaceous medium and 60% of natural saliva to culture consistent subgingival biofilms (Ammann et al., 2013b).

Oral bacteria grow in complexes in order to satisfy the nutritional and growth requirements for the plethora of species. In order to fulfil the nutritional needs *in vitro*, medium supplementation is usually practiced in the models. Saliva, and mainly mucins, are the main source of food for oral bacteria (Jakubovics, 2015). Mucins represent around 26% of salivary proteins; they are heavily glycosylated glycoproteins of high molecular weight. Two types of mucins are found in the oral cavity: cell-bound mucins MUC1 and MUC4 and two types of secreted mucins: high molecular weight MUC5B and low molecular weight MUC7 (Derrien et al., 2010). Polypeptide (or apomucin) forms the backbone of the molecule, with O-linked glycans around it. The glycans make up 50% - 90% of the molecular weight (Derrien et al., 2010). Mucins support the growth of both saccharolytic and proteolytic bacteria as the glycans and protein core can be used as an energy source by complex communities of oral bacteria (Jakubovics, 2015). Bradshaw et al. (1994) demonstrated that nutrient-poor medium supplemented with mucin provided the growth to a complex community of nine or 10 oral bacteria. The higher community diversity enhanced the viable counts of the system, probably by introduction of new degradative enzymes. These new enzymes enabled the establishment of new species in a model community, but also enhanced the growth of existing species (Bradshaw et al., 1994). Apart from their nutritional role, mucins can also serve to support the attachment of bacteria to oral surfaces and promote biofilm formation (Jakubovics, 2015).

Other supplements widely introduced in the models are haem and vitamin K_1. Haem is a prosthetic group of several proteins and functions as a cofactor mediating oxygen transport, energy generation, and mixed-function oxidation (Lee, 1995). In addition, the iron chelated in the porphyrin ring may serve as an iron substrate for bacterial growth. Vitamin K_1 participates in electron transport. Both, haemin and vitamin K_1 have been reported as necessary for the growth of *Prevotella* and *Porphyromonas* species (Mayrand and Holt, 1988).

Serum is used in models as a proteinaceous nutrient source to favour the growth of proteolytic species (Ammann et al., 2013b). Moreover, in the culture of subgingival biofilms, it has been used to mimic GCF due to similarities in their chemical composition. Continuous culture studies showed that serum, like mucin, is degraded by the synergistic and concerted actions of subgingival

bacterial populations (Gramer et al., 2013). For example, *T. denticola* was unable to grow in pure culture in serum, and its growth depended on the presence of accompanying species (ter Steeg and van der Hoeven, 1990). It was suggested that single periodontal organisms generally lack the enzymatic diversity of glycosidases and proteases required to degrade complex serum glycoproteins; therefore, serum is a valid substrate to culture a diverse subgingival community (ter Steeg and van der Hoeven, 1990). Finally, some media also incorporate arginine and urea, to provide a natural buffering or pH-modifying effect because some bacteria metabolise these substrates to ammonia, which increases the pH (Wijeyeweera and Kleinberg, 1989).

Also, the gas mixture used for modelling biofilm community can affect its final bacterial composition. The cultivability of plaque bacteria greatly improved with the application of anaerobic methods. Anaerobic chambers, providing 80% N_2, 10% H_2 and 10% CO_2, were developed to culture obligate anaerobes (Cox and Mangels, 1976). Some natural habitats, such as the periodontal pocket, are anaerobic, and the majority of species residing there are also oxygen-sensitive. The presence of oxygen in the growth environment can be lethal for many species; however, species residing in biofilms are less susceptible to oxygen (Wessel et al., 2014). Environmental pH levels are also important for the growth of some species. For example, the pH in the periodontal pocket is known to be around neutrality with the potential to become alkaline during inflammation, different to supragingival areas, where high pH fluctuations are observed (4.5 – 7) (Eggert et al., 1991; Vroom et al., 1999). Other factors, such as light or temperature fluctuation, may also be important in modelling the *in vivo* environment for oral biofilm growth.

The employment of simple *in vitro* systems ensures reproducible microbial behaviour in a simulated environment, where the host with its immune response is excluded. Although *in vitro* models never reflect the precise environment for bacterial growth, they are used to investigate patterns in biofilm development, species interactions in complex communities, and the response of established communities to external stimuli (Bradshaw et al., 1994; Ammann et al., 2013b; Kistler et al., 2013).

In vitro models were very useful to show that an altered environment, low pH precisely, was a key factor for the enrichment of acidophilic species and the

development of cariogenic community (Marsh, 2003a). Periodontal disease is defined as dysbiosis of the oral microbiota, resulting in the enrichment of proteolytic Gram-negative bacteria compared to the consortium of streptococci, lactobacilli, *Veillonella* and *Haemophilus* species detected in periodontal health (Diaz et al., 2016).

Overall aim and hypothesis

It is well established that the microbiota from an inflamed periodontal pocket is markedly different from that found naturally in a healthy gingival crevice. A key question in periodontal microbiology, therefore, is what is the source of the fastidious and unusual bacteria seen in disease, and what are the drivers that enable them to become predominant at the expense of our health-associated species? Recent high resolution microbial DNA sequencing studies have shown that some of these periodontitis-associated species can be detected in low numbers in healthy plaque (Abusleme et al., 2013), but not all of the putative periodontal pathogens have been reported, so what is their origin? The Ecological Plaque Hypothesis proposes that environmental changes taking place in the periodontal pocket would favour the growth of previously uncompetitive minor constituents of the microbiota, including putative periodontal pathogens. The periodontal pocket creates a specific habitat, with an absence of oxygen, surplus of proteins and cell breakdown products, a shortage of rapidly fermentable carbohydrates and a slightly alkaline pH that could selectively favour the growth of these pathogens.

In this project, the CBD will be employed to simulate this subgingival environment in order to investigate bacterial growth and enrichment of minor species. Its advantages over other systems include its convenience, simplicity of use and ability to generate highly reproducible multi-species and complex biofilms (Kistler et al., 2015). A growth medium consisting of 20% high protein medium and 80% artificial saliva, which had been used previously in numerous *in vitro* systems to culture subgingival communities, will be modified by adding physiological levels of urea and arginine (Bradshaw and Marsh, 1999). The first part of the project will concentrate on the optimisation of the *in vitro* model while later stages will focus on the culture of a natural inoculum taken from healthy

subjects in environments relevant to the periodontal pocket and determine their effect on the bacterial composition of the developing biofilms. The rationale behind the project was to determine whether the minor fastidious slow growing constituents found in healthy plaque can become competitive under different (and physiologically relevant) environments linked to the inflamed periodontal pocket and out-compete fast-growing species that dominate in healthy plaque. The study would also establish whether the fastidious species that have recently been implicated in periodontal disease were present in biofilms taken from sites in periodontally healthy adults, but at low or undetectable levels (Perez-Chaparro et al., 2014).

Hypothesis

Biofilms taken from non-diseased sites in healthy individuals harbour bacteria that are implicated in periodontal disease, and these putative pathogens can be detected and become prevalent if samples are cultured under environmental conditions that reflect nutritional conditions found in the inflamed subgingival environment.

Specific Aims

The driving idea behind the thesis was to deepen the knowledge on the links between oral dysbiosis observed in periodontal disease and environment as a trigger. The aim was to understand if minor components of healthy plaque can be enriched in a biofilm community in response to alterations in the growth environment. The imposed growth conditions will replicate some aspects of the periodontal pocket during inflammation. The characterisation of inoculum and biofilms will be carried out using modern open-end sequencing techniques and established analysis pathways.

This main objective was subdivided into the following specific aims:

- Develop a simple multi-species model to allow the screening of different environmental conditions on defined oral biofilms.
- Investigate the effects of biofilm composition, culturing time and supplementations of serum on the growth of defined multi-species biofilms.
- Apply the simple multi-species model to culture complex biofilms from pooled plaque, tongue and saliva samples of healthy young adults.
- Perform a longitudinal characterisation and comparison of the natural inoculum and the biofilms that develop after one and three weeks of culture in two growth environments using open-end sequencing techniques.
- Assess the impact of different nutritional conditions on the growth of complex oral biofilms and the selection of putative periodontal pathogens derived from an inoculum from healthy adult volunteers.

- Determine whether deleterious changes in the microbiota could be reversed by returning the dysbiotic biofilms to a health-associated nutritional environment based on human saliva.

Chapter 2 Materials and Methods

2.1 Multi-species biofilm model

2.1.1 Bacterial strains

Bacterial species used in the culture experiments were selected based on their relevance to oral health and disease (Ammann et al., 2012). The following species were used to form multi-species biofilms: early colonisers used in the experiments were *S. oralis* ULCP86 (clinical isolate), *S. salivarius* ULCP97 (clinical isolate) and *A. naeslundii* M42 (clinical isolate) (Henssge et al., 2011). Species that are associated with mature plaque included *F. nucleatum* ssp*. polymorphum* ATCC® 10953™*, P. intermedia* OMZ 248 and *P. gingivalis* W83 (Hong et al., 2015). The selection was based on: 1) ease of species identification by culture; 2) species were reported to be detectable in periodontal pockets; 3) species were reported to grow together in multi-species models (Ammann et al., 2012). Stocks of selected species were taken from the -80°C freezer and inoculated onto anaerobically pre-reduced Columbia Blood Agar (Oxoid) plates supplemented with 5% (v/v) of oxalated horse blood (Thermo Scientific) (CBA) in an anaerobic work station (Don Whitley Scientific; Shipley) in 10% H_2, 10% CO_2, 80% N_2 at 37°C for 24h - 72h, depending on the species being grown. Morphology of colonies was evaluated together with Gram staining to confirm the absence of stock contamination.

2.2 Media composition

Three types of media were prepared for biofilm cultures (Table 2.1). Different concentrations of serum were tested for the growth of biofilms. The detailed composition of Basal Medium and Artificial Saliva used in experiments are provided in Tables 2.1 and 2.2. Basal medium, which is nutrient-rich medium, was successfully used to culture oral multi-species bacterial communities previously (McKee et al., 1985). However, later it was modified by diluting it with artificial saliva by (Bradshaw et al., 1989). In the experiment described in Chapter 3, the modification of Basal Medium was used.

Table 2.1. Different types of media used to culture multi-species biofilms.

	Protein-rich medium	Protein-rich medium 10% serum	Protein-rich medium 20% serum
Basal medium	200 (mL/L)	200 (mL/L)	200 (mL/L)
Artificial saliva	800 (mL/L)	700 (mL/L)	600 (mL/L)
Foetal bovine serum* (Sigma)	0 (mL/L)	100 (mL/L)	200 (mL/L)

Table 2.2. Composition of media used in experiments.
The pH of the media were adjusted to the level of 7.4 by adding 2 M NaOH, autoclaved at 121°C for 30 min.

Basal Medium	Proteose peptone (Oxoid)	10 (g/L)
	Tryptose peptone (BDH)	5 (g/L)
	Yeast extract (Oxoid)	5 (g/L)
	L-Cysteine hydrochloride (Sigma)	0.5 (g/L)
	Haemin (Sigma)*	0.0002 (g/L)
	Menadione (Sigma)*	0.00004 (g/L)
Artificial saliva	Hog gastric mucin (Sigma)	2.5 (g/L)
	NaCl (Sigma)	0.381 (g/L)
	KCl (Sigma)	1.114 (g/L)
	KH_2PO_4 (BDH)	0.738 (g/L)
	Ascorbic acid (Fisher Scientific)	0.002 (g/L)
	Urea (Fisher Scientific)**	9 mM
	Arginine (Sigma)**	5 mM

*added after autoclaving. The solution of haemin was prepared by dissolving haemin chloride in 0.1 M KOH, ethanol and distilled water. Menadione was dissolved in ethanol.

** filter-sterilised, added after autoclaving.

2.2.1 Sterilisation of human saliva

Sterile human saliva preparation followed the protocol proposed by Palmer et al., (2001).One volunteer was asked to drool saliva into a sterile polystyrene tube at least 1.5h after eating, drinking and tooth brushing. Saliva was stimulated by mastication of Parafilm balls and stored on ice. DL-dithiothreitol (Sigma) was added to give a 2.5 mM final concentration, and the mixture was gently stirred for 10 min to reduce protein aggregation. Later it was centrifuged at 4°C at 27,000 × *g* for 20 min (Palmer et al., 2001). The clarified saliva supernatant was decanted and diluted to 50% in sterile phosphate buffer saline (PBS) (Lonza). Diluted saliva was filtered through a 0.22-µm-pore-size low-protein-binding filter (Corning Incorporated, Corning, NY) and frozen at -20^{o} C in 10 mL aliquots. The efficiency of the protocol was checked by inoculating 50 µL

of saliva onto CBA plates and incubating them anaerobically and aerobically at 37^{o} C for 72h (Sanchez et al., 2011).

2.2.2 Heat-inactivation of serum

All sera, used as culture media ingredients, were heat-inactivated following a standard procedure to inactivate the complement cascade. Serum was heated to 56°C for 30 min in a water bath, aliquoted to 50 mL tubes and kept at -20°C until further use.

2.2.3 Culturing conditions

The Calgary Biofilm Device was used to generate biofilms. CBD is a commercially available MBEC™ Assay System, available through MBEC Biofilms Technology Ltd., Calgary Alberta, Canada (Figure 2.1). This device was described earlier by Ceri et al., (1999) and is essentially 96 pegs mounted on the inside surface of the lid of a 96-well microtitre plate. When the CBD is placed over a microtitre plate, each peg is lowered into its corresponding well without contacting the well surface. The pegs used to culture biofilms in the following experiments were coated with hydroxyapatite by the manufacturer to mimic the enamel and dentine surface.

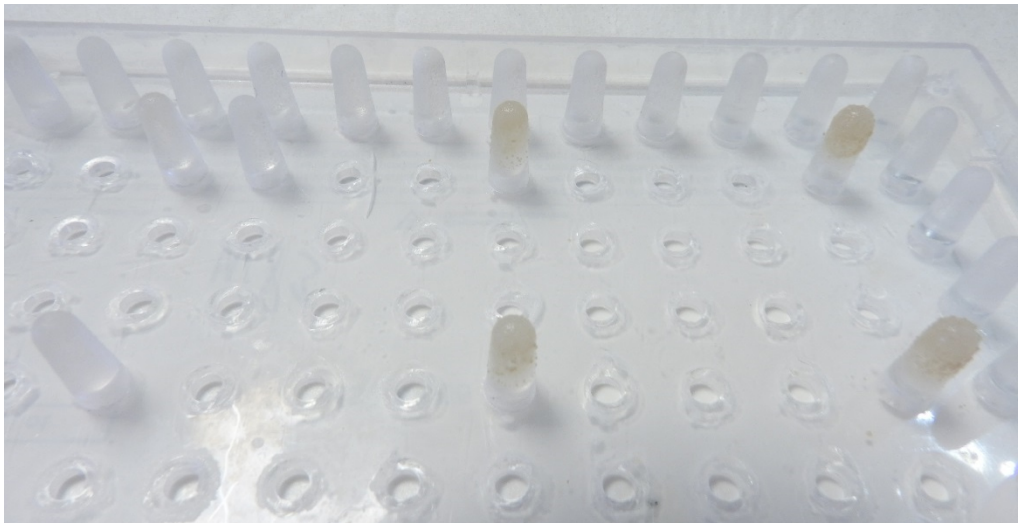

Figure 2.1. Calgary Biofilm Device at the end of the experiment with a few biofilms and unused pegs.

2.2.4 Inoculum preparation for five-species biofilms.

In order to know how colony forming units/mL (CFU) counts correlate with OD_{600} values, standard curves were prepared as follows: for each species, optical densities at 600 nm (OD_{600}) of overnight cultures in BHI broth (Oxoid) were measured with a Jenway 6305 spectrophotometer (MRS Scientific). Serial doubling dilutions were prepared in serum free medium to a maximum dilution of 1 in 16, and OD_{600} values for each were recorded using sterile medium as a

blank. Serial tenfold dilutions of individual bacterial suspensions were inoculated onto CBA and incubated anaerobically for 4 days. After recording the corresponding CFU counts, the CFU – OD_{600} standard curves were prepared for three separate biological replicate experiments.

For inoculation, the cultures were grown separately in BHI for 16 hours at 37°C anaerobically, diluted to OD_{600} = 0.2. Depending on the species-specific OD-CFU standard curves prepared previously, different volumes of diluted cultures (3 - 20µL) were pooled together to contain CFU counts that are provided in Tables 2.3 and 2.4. Later this mixture was inoculated into the growth media in 96-well plate of CBD.

2.2.5 Five-species biofilm model development and evaluation of growth media.

Several protocols were compared during the development of the five-species *in vitro* model. The inoculation frequency, length of biofilm culture and inoculum compositions were tested during the model development. The final protocol which was used to investigate the effects of serum in the growth medium is depicted in Figure 2.2. During the model development, several inoculation times and different bacterial strains were compared, and these are described below in sections 2.2.5 - 2.2.7.

The optimised biofilm culture experiment was performed anaerobically for 14 days. Three types of media were used to culture five-species into the model. The layout of the CBD plates for this experiment is shown in Figure 2.3. The biofilm culture was performed in this sequence: 5h prior to bacterial inoculation, each well was inoculated with 175 µL of sterile human saliva and the CBD plate was incubated at 37°C with gentle shaking at 65 rpm (MaxQ Mini 4450, Thermo Fisher Scientific) to form a salivary pellicle on the hydroxyapatite surfaces (Kistler et al., 2015). Afterwards, the CBD was transferred to an anaerobic environment (section 2.2.3) where sterile saliva was replaced with 175 µL of medium containing early colonisers *S. salivarius* and *A. naeslundii*. The compositions of media are detailed in Table 2.1 and viable counts of inocula for *S. salivarius* and *A. naeslundii* are provided in Table 2.3. After 12h, the biofilms were supplied with 175 µL of fresh medium and cultured for an additional 12h. Then each well was inoculated with the medium containing obligate anaerobes: *F. nucleatum, P. intermedia* and *P. gingivalis* (bacterial counts provided in Table 2.3). The inoculation of obligate anaerobes was repeated after 24h. After this, the media were changed every 24h for additional 12 days. At the end of the experiment, biofilms were harvested and plated on different agars as described in section 2.2.8.

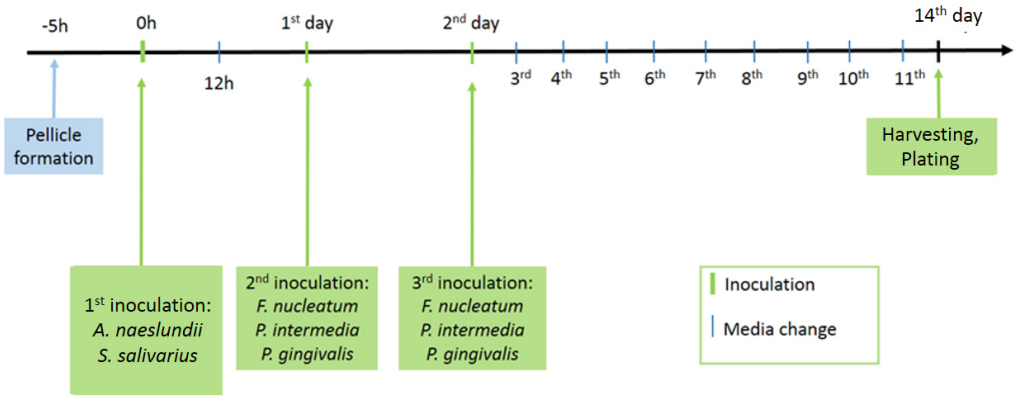

Figure 2.2. Experimental plan for two-week biofilm incubation.
Schematic representation of the experiment used to investigate the effects of different media on five-species biofilms. The pellicle formation, and timings of inoculations and media changes are depicted.

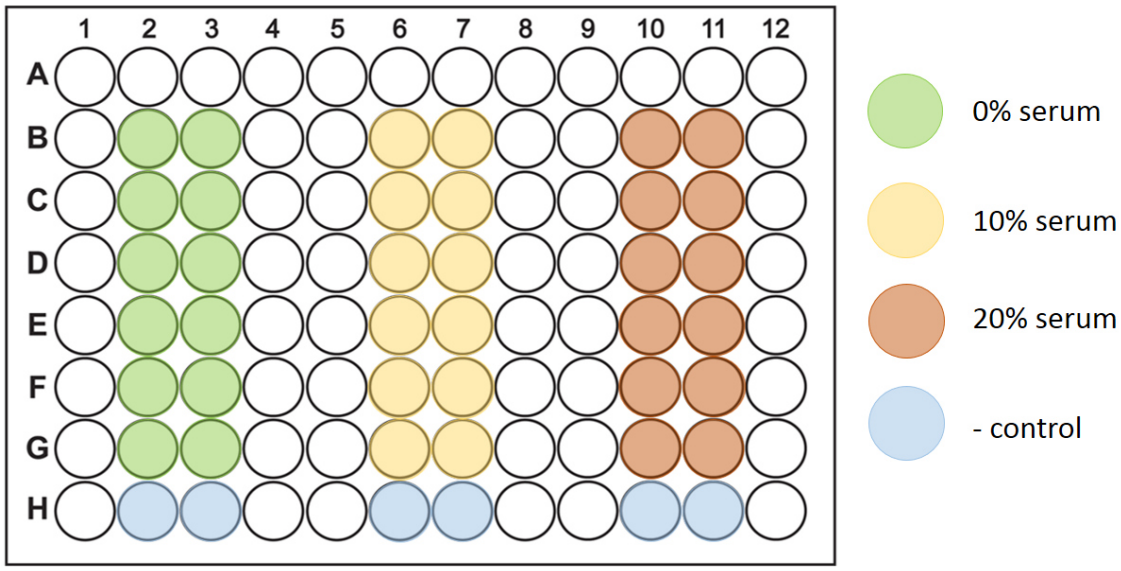

Figure 2.3. The layout of Calgary biofilm plate used to culture five-species biofilms.
Green, yellow and brown wells show the media used to culture biofilms, blue wells show where sterile water was used for inoculation as a negative control. All other wells were inoculated with 200 µL of sterile PBS to avoid cross-contamination between media types.

Table 2.3. Inoculum size for the two-week biofilm formation experiment. During inoculation, each well was inoculated with the mixture of bacteria, diluted in protein-rich medium with or without serum. CFU counts of each species/biofilm/inoculation are provided for day 0, day 1 and day 2.

	S. salivarius* OR *S. oralis	***A. naeslundii***	***F. nucleatum***	***P. intermedia***	***P. gingivalis***
Day 0	1×10^5	2×10^5			
Day 1			6×10^6	6×10^6	6×10^7
Day 2			6×10^6	6×10^6	6×10^7

2.2.6 Five-species biofilms using *Streptococcus oralis* ULCP 86

During the development of the five-species model, we also used *S. oralis* ULCP 86 instead of *S. salivarius* ULCP 97 and recorded pH values and CFU counts at the end of experiment.

2.2.7 Experimental plan for four-week biofilm study to evaluate growth media

The development of the five-species model included four-week biofilm culture experiments that are detailed below. Figure 2.4 demonstrates the experimental plan for a four-week biofilm culture. Three types of media, shown in Table 2.1, were simultaneously used to grow biofilms.

Biofilms were cultured following these steps: 5h prior to bacterial inoculation, each well was inoculated with 175 µL of sterile human saliva and the CBD plate was incubated at 37°C with stirring at 65 rpm to form a salivary pellicle on the hydroxyapatite surfaces. After each well was inoculated with 175 µL of media containing *S. oralis* and *A. naeslundii* (counts shown in Table 2.3), the CBD was incubated in 10% CO_2 at 37°C. Then, media were changed 24 hours after inoculation and subsequently every 2 days. On day six, the CBD plate was transferred to an anaerobic environment (section 2.2.3). At day seven, the model was inoculated with *A. naeslundii* and *F. nucleatum*. The media were changed the next day and then, every two days. At day 14, the model was inoculated with *F. nucleatum*, *P. intermedia* and *P. gingivalis*. At day 21, *P. intermedia* and *P. gingivalis* were again inoculated. At day 28, the pegs were harvested as described in section 2.2.8 and plated on agars for species identification.

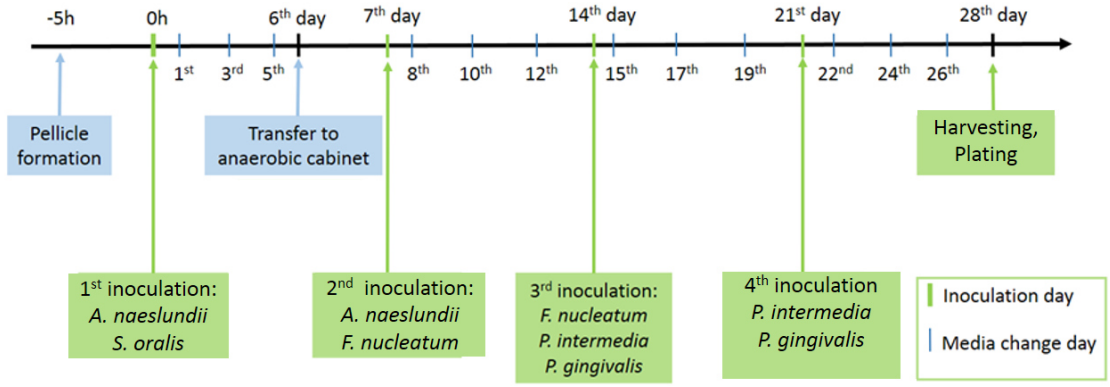

Figure 2.4. Experimental plan for four-week biofilm incubation.
During the four-week experiment, bacteria were inoculated at one-week intervals on four occasions. The diagram summarises inoculation, media changes and culturing environment.

Table 2.4 Inoculum size of individual species used during the four-week experiment.
CFU counts of each species/biofilm/inoculation are provided at day 0, day 7, day 14 and day 21.

	S. oralis	***A. naeslundii***	***F. nucleatum***	***P. intermedia***	***P. gingivalis***
Day 0	1×10^5	2×10^5			
Day 7		2×10^5	6×10^6		
Day 14			6×10^6	6×10^6	6×10^7
Day 21			6×10^6	6×10^6	6×10^7

2.2.8 Bacteriological analysis of the biofilms

After culturing, the selected pegs were snipped off the CBD plate with sterile pliers and washed by dipping each peg into a well with 200 µL of sterile PBS three times. Then, each peg was transferred to 500 µL of pre-reduced sterile PBS and harvested by carefully scraping each peg with a sterile dental scaler. Attached biofilms were scraped by the same person for 5 min, by going around each three times.

2.2.8.1 Viable counts of each species using different media.

Collected biofilms were vortex mixed for 30s and serial dilutions of suspended biofilm bacteria were prepared in sterile PBS and 100 µL aliquots of 10^3-10^6 dilutions were inoculated onto agar plates. To estimate total CFU counts of *Streptococcus* species and *A. naeslundii,* the serial dilutions were inoculated onto CBA and incubated in a 10% CO_2 atmosphere at 37°C for 48h. To

estimate the total CFU counts of *F. nucleatum*, *P. gingivalis and P. intermedia,* CBA + 0.0075 g/L vancomycin (CBA + V) plates were used, as they prevent the growth on Gram-positive species, and incubated anaerobically for 5 days.

Species identification was achieved by observation of colony morphology and Gram staining. After 48h of incubation, *Streptococcus* species were dark green and displayed alpha haemolysis, while *A. naeslundii* appeared as dark brown small colonies (Figure 2.5). After 5 days of anaerobic incubation, *F. nucleatum* appeared as yellowish white colonies with irregular margins, *P. intermedia* formed circular dark brown umbonate colonies, *P. gingivalis* grew as circular brown convex colonies (Figure 2.6). Data were scored for each species as CFU per biofilm.

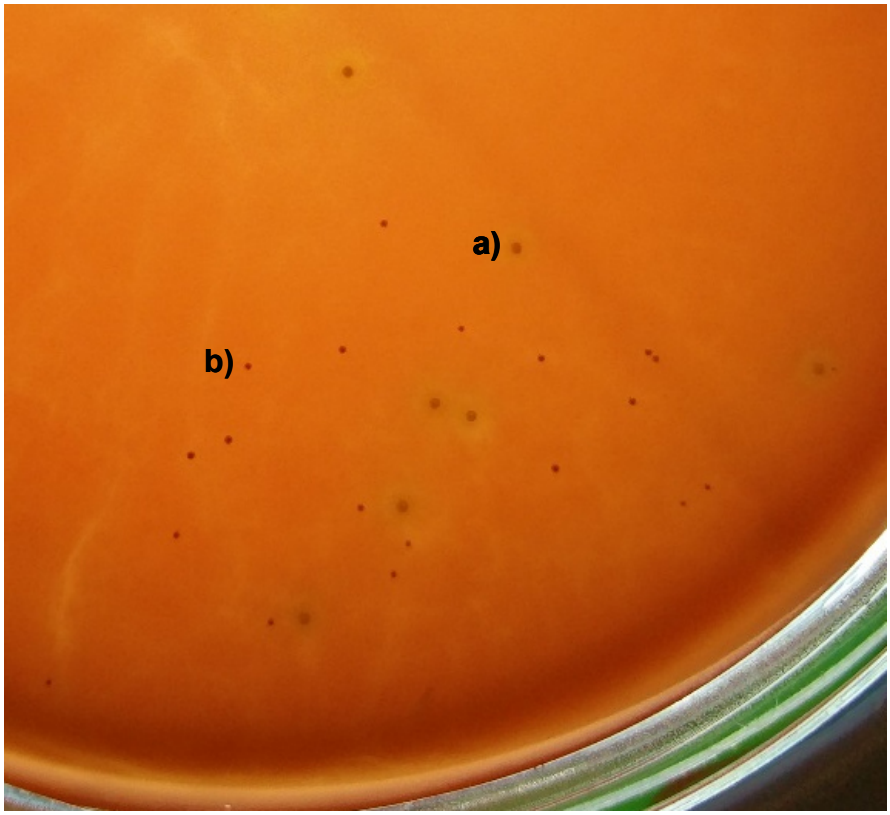

Figure 2.5. Columbia Blood Agar plate with *Streptococcus oralis* and *A. naeslundii* after 48h of inoculation.
a) Dark green *S. oralis colonies* displaying alpha haemolysis, b) Small dark brown *A. naeslundii* colonies.

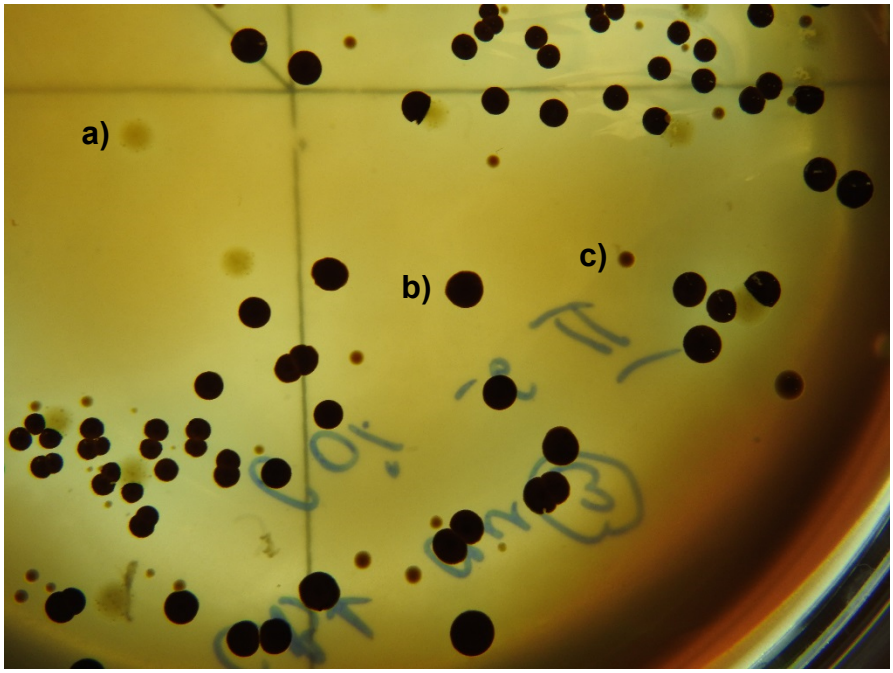

Figure 2.6. Columbia Blood Agar + V plate with *F. nucleatum, P. intermedia* and *P. gingivalis* after five days of anaerobic incubation.
a) *F. nucleatum*, b) *P. intermedia*, c) *P. gingivalis*.

2.2.8.2 pH evaluation of biofilm supernatants

pH values of supernatants were recorded using an Orion 9110DJWP Double Junction Micro pH electrode (Thermo Scientific Orion). The values of pH were compared between supernatants of different biofilms.

2.2.8.3 Cross-streak cultures to evaluate bacterial antagonism

During the biofilm modelling experiments, it was noticed that some *Streptococcus* strains were not growing in co-culture with *P. gingivalis*. In order to investigate the ability of *Streptococcus* and *P. gingivalis* strains to grow in close proximity, some cross-streak cultures were performed. CBD plates were inoculated with 50 µL of 8h broth cultures of different *Streptococcus* strains and dried for 10 min anaerobically. Then, the overnight broth *P. gingivalis* W83 culture was streaked across the middle of the agar. The plates were incubated anaerobically for 5 days and evaluated for bacterial growth.

2.2.8.4 Prussian Blue agar to detect hydrogen peroxide production

Some *Streptococcus* strains produce hydrogen peroxide, and this can inhibit the growth of some anaerobic bacteria (Ashby et al., 2009). Prussian Blue agar is known to change colour from yellow to blue when exposed to hydrogen peroxide (Saito et al., 2007). *Streptococcus* strains were plated on pre-reduced Prussian Blue agar plates and incubated in a CO_2 incubator for 2 days. The medium was prepared using (g/L): BHI 37, agar-agar technical 15 (Oxoid),

$FeCl_3$*$6H_2O$ 1.0 (Alfa Aesar), potassium hexacyanoferrate(III) 1.0 (Alfa Aesar) following the published protocol (Saito et al., 2007).

2.2.8.5 Biofilm imaging - Scanning Electron Microscopy

For SEM, pegs coated with biofilms were washed by dipping them in sterile PBS to remove loosely adherent cells and fixed in 2.5% (v/v) glutaraldehyde solution at room temperature for 3h and later washed three times in PBS. Afterwards, samples were dried sequentially in 30%, 60%, 80%, 97%, and 100% (v/v) ethanol for 15 min in each step. Drying in 100% ethanol was repeated three times. After air-drying, the biofilms were sputter-coated with gold and imaged using a SEM (Hitachi S-3400N, Hitachi Science and Technology) in secondary electron mode, using 30 mA for 90s. Imaging was performed under high vacuum at 10.00 kV, with current of 3 pA and working distance of 5.2 mm – 7.5 mm.

2.2.9 Statistical analysis

The experiments for biofilm culture were repeated three times with inoculum freshly prepared for every experiment. After collecting data, the normality of each data set was checked using the Shapiro-Wilk test and homogeneity of variance was checked using Levene's test from *car* package in R (Fox and Weisberg, 2011). Depending on the assumptions met, tests for normal ANOVA (Analysis of Variance) with Bonferroni correction or Welch-F test) or non-normal distribution (Kruskal-Wallis) were applied to make the comparisons. A significance threshold of $p < 0.05$ was applied.

2.3 *In vitro* models to culture complex oral biofilms

After finishing the five-species modeling to compare different culture media, the culture of complex biofilms was attempted, using natural plaque and saliva as the inoculum. For biofilm culture, CBD plates with hydroxyapatite-coated pegs were used. Separate experiments were performed to culture plaque and saliva.

2.3.1.1 Volunteer recruitment and sample collection

Ethics approval for sample collection was granted by University of Leeds Dental Research Ethics Committee (020915/MN/175). Eight dentally-healthy volunteers (mean age 31 ± 8 y, 50% male: 50% female) were recruited to provide the samples of supragingival buccal molar plaque, biofilm from the tongue dorsum and 5 mL of unstimulated saliva. Inclusion criteria were routinely brushing teeth twice daily, and attending regular dental check-ups. Exclusion criteria were currently undergoing any treatment for dental caries or periodontal disease, having had antibiotics in the previous three months or

undergoing any treatment for systemic disease at the time of experiment. Informed consent was obtained from the participants. Two plaque collections were performed at 9 month intervals. At both times the same volunteers were recruited, except that one volunteer opted out from the second sampling, but was replaced with another age- and gender-matched volunteer. The opted out person was pregnant during the second plaque collection and did not want to refrain from tooth brushing for 16h.

Volunteers were asked to refrain from tooth brushing for 16h and not to eat or drink 1.5h before sample collection. Plaque was collected into 3 mL sterile pre-reduced protein-rich medium (Table 2.1, medium without serum), then each volunteer drooled at least 5mL of saliva into a sterile 20 mL tube. The samples were transferred to an anaerobic work station (section 2.2.3) within 60 min after collection. Each plaque and saliva sample was homogenised by vortex mixing for 60s, and pooled to obtain separate saliva, tongue and supragingival plaque samples, then vortex mixed for an additional 60s; the pooling was performed under anaerobic conditions to prevent the death of obligate anaerobic species.

2.3.1.2 Culturing conditions and media

Plaque and saliva of healthy volunteers were cultured in separate experiments using different media and culturing conditions. The experimental plans for these two experiments are detailed in sections 2.3.2 and 2.3.3. For both experiments, the same sample collection, harvesting, DNA processing and metagenome analyses were performed on biofilms and inocula, and these are detailed in section 2.3.4.

2.3.2 Investigation of the effects of time and protein-rich media, with and without serum, on the development of complex oral biofilms

Pooled inoculum was cultured for three weeks, before harvesting and analysing the biofilms (Figure 2.3).

2.3.2.1 Media composition and culturing conditions

The inoculum of pooled plaque, tongue biofilm and saliva was cultured in protein-rich medium and protein-rich medium supplemented with 20% (v/v) foetal bovine serum. The composition of the media is detailed in Table 2.1, experimental plan is depicted in Figure 2.7 and layout of the CBD plate is presented in Figure 2.8.

The hydroxyapatite-coated pegs were preconditioned with 200 µL sterile human saliva 5h prior to inoculation. Later, each well was filled with 91 µL pooled saliva, 54.5 µL of pooled supragingival plaque and 54.5 µL of tongue

biofilms, to produce 200 µL of inoculum per well. Four wells per medium type were inoculated with sterile water as a negative control. After inoculation, the plates were incubated anaerobically at 37°C (section 2.2.3). The medium in each well was changed after 24h and subsequently twice a week. After one, two and three weeks of incubation, biofilms were harvested by snipping pegs from the lid. Half of the samples were treated with PMA (propidium monoazide) to obtain DNA from intact cells prior to DNA isolation as described in section 2.3.4.1. The experiment was carried out simultaneously in triplicate using three separate CBD plates.

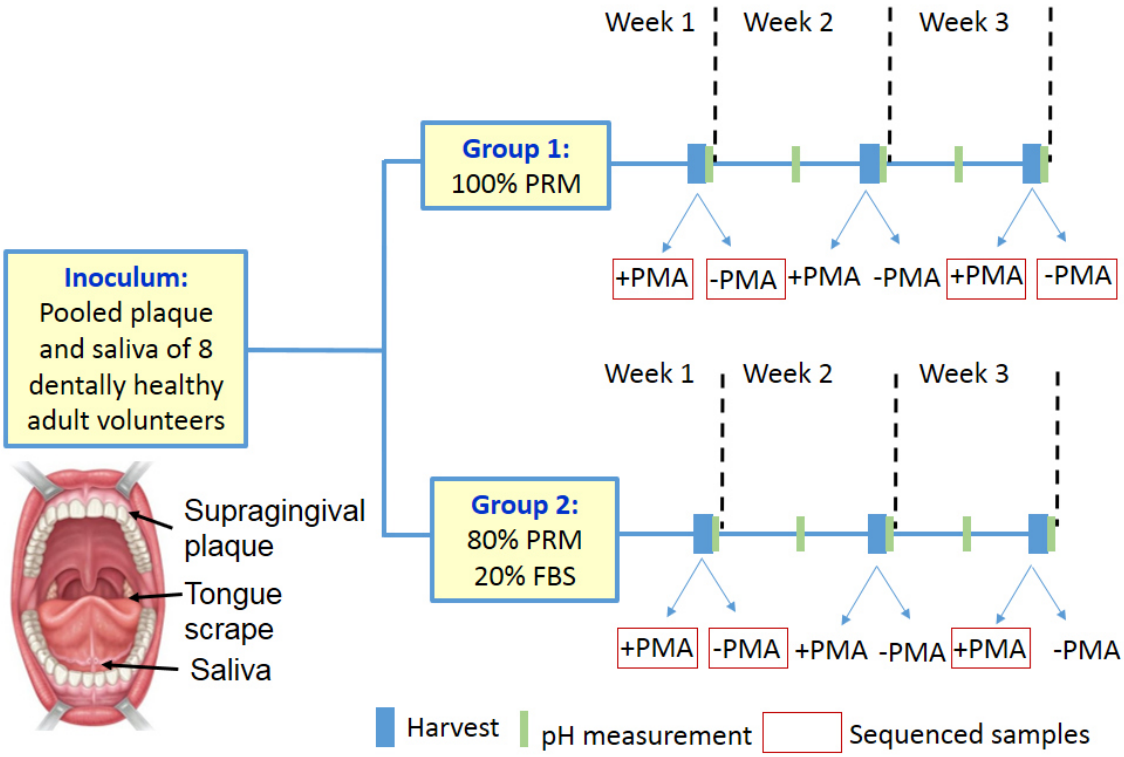

Figure 2.7. Experimental design for the *in vitro* culture and analysis of pooled natural inoculum in protein-rich media with and without serum. The diagram summarises the culture media used, harvesting time, pH measurements, propidium monoazide treatments and shows which samples were sequenced and analysed for microbial content and functional potential by metagenomics. Supragingival plaque, tongue biofilm and saliva samples from eight volunteers were pooled and cultured either in protein-rich medium (PRM) or PRM with 20% (v/v) foetal bovine serum for three weeks, and harvested at week one, week two, and week three. Half of the samples were treated with PMA and the metagenomes of inoculum, week one and week three samples were selected for the evaluation of taxonomy and functional potential. As the majority of species that are associated with periodontal disease are fastidious and slow growing species, biofilms cultured for three instead of two weeks were sequenced for longitudinal comparison.

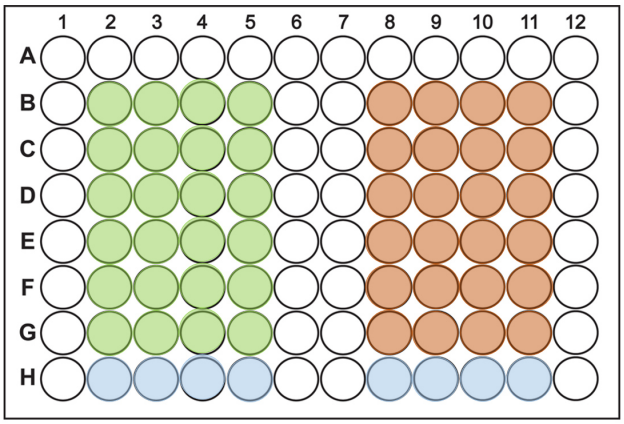

Figure 2.8. The layout for the plates used to culture complex oral biofilms in two types of media.
Green shows the biofilms that were cultured in protein-rich medium and brown represents the biofilms cultured in protein-rich medium with 20% serum. Blue wells were inoculated with sterile water as a negative control and white wells show where sterile PBS was used throughout the experiment.

2.3.2.2 qPCR to quantify the absolute counts of *P. gingivalis* in the samples.

Absolute counts of *P. gingivalis* were determined in each biofilm sample and inoculum using qPCR to confirm that the numbers of *P. gingivalis* changed throughout the experiment before we embarked on sequencing. We targeted *P. gingivalis*, as pilot five-species experiments revealed problems when this organism tried to grow in multi-species system. Primer pairs used in a study targeted *P. gingivalis* specific *waaA* gene encoding for lipid A synthesis (Hyvarinen et al., 2009) (Table 2.5). Serial dilutions of DNA from *P. gingivalis* W83 in nuclease free water was used at defined concentrations of 10 ng - 0.001 ng to generate a standard curve for qPCR reaction. (Appendix Figure 1). For generation of a standard curve, qPCR was run in a total reaction volume of 10 µL, containing 5 µL of LightCycler® SYBR Green I Master mix (Roche Diagnostics Penzberg, Germany), 4 µL of DNA, 0.5 µL of 10mM primers. To quantify the numbers of *P. gingivalis* in the inoculum and biofilms, 4 µL containing 10 ng of DNA were used with 0.5 µL of 10mM primers and 5 µL of Master mix. Amplification was performed on a LightCycler 480 by initial denaturation for 5 min at 95°C, 40 cycles of 15s at 95°C and 60s at 60°C. Melting curve analysis was performed at 65°C for 1 min. *P. gingivalis* DNA concentration in each sample was calculated from the obtained crossing point (Cp) values and the abundance was calculated using the theoretical genome weight (Ammann et al., 2013b).

Table 2.5. Primer sequences and properties.

Organism	gene	Sequence	T_m (°C)	Product length (bp)
P. gingivalis	*waaA*	F: TGGTTTCATGCAGCTTCTTT R: TCGGCACCTTCGTAATTCTT	60	145

Formula used to quantify No of copies of *P. gingivalis*

$$PG = (x\ ng * 6.0221 * 10^{23})/ (N * 660g/mol * 10^{9}ng/g)$$

PG – number of copies of *P. gingivalis*

x – the amount of DNA that was extrapolated from standard curve

N – genome size based on published genome sequence for *P. gingivalis* (2355000 MB) (Ammann et al., 2013b)

After qPCR data evaluation, only inoculum, week one and week three samples were submitted for sequencing, considering that fastidious species need sufficient time to enrich in the biofilm community due to their slow growth.

2.3.3 The influence of human saliva and human or bovine serum on biofilm composition, and the effect of human saliva on reversing enrichment.

For the second plaque and saliva culture experiment, sterile human saliva and human serum were used as media components to replicate the oral environment in health and during inflammation (periodontal pocket). In this set of experiments, the influence of saliva and the source of serum (bovine versus human) was investigated. After three weeks of enrichment, the biofilms were incubated with sterile human saliva to see whether any changes to the microbiome could be reversed. The significance of this was to investigate, if bacterial population induced by growth in protein-rich media with or without serum can be reversed by changing the medium to saliva.

Table 2.6 summarises the growth media that were used to culture biofilms. The composition of protein-rich media is detailed in Table 2.1 and the saliva sterilisation protocol was described in section 2.2.1. Human serum was obtained from Sigma-Aldrich and heat-inactivated following the procedures described in section 2.2.2. The selection of these media types allowed

investigation of: 1) effects of human serum in comparison with bovine serum 2) the enrichment of biofilms when cultured in sterile human saliva and 3) comparison of the effects on enrichment of sole saliva vs medium with human serum.

Table 2.6. The composition of the four growth media used to culture pooled inoculum in *in vitro* model.
Four different media were used to culture biofilms for three weeks. After three weeks, only sterile human saliva was used as a nutrient supply, except for Group 3, for which culturing beyond three weeks was discontinued. PRM – protein-rich medium, FBS – foetal bovine serum, all percentages mean v/v.

Group 1 PRM + human saliva + FBS	**Group 2** PRM + human saliva + human serum	**Group 3** Human saliva	**Group 4** Human saliva + human serum
20% protein-rich medium	20% protein-rich medium	100% sterile human saliva	80% sterile human saliva
60% sterile human saliva	60% sterile human saliva		20% human serum
20% foetal bovine serum	20% human serum		
Media after week 3			
100% sterile human saliva	100% sterile human saliva		100% sterile human saliva

The hydroxyapatite-coated pegs were preconditioned with 200 µL sterile human saliva 5h before the inoculation. Each well was filled with 91 µL of pooled saliva and 54.5 µL of pooled supragingival plaque and 54.5 µL of tongue biofilms, to produce 200 µL of total inoculum per well. The layout of the CBA plates is shown in Figure 2.9. The collection and processing of inocula is detailed in section 2.3.1.1 and Figure 2.10 shows the experimental plan for the biofilm cultures.

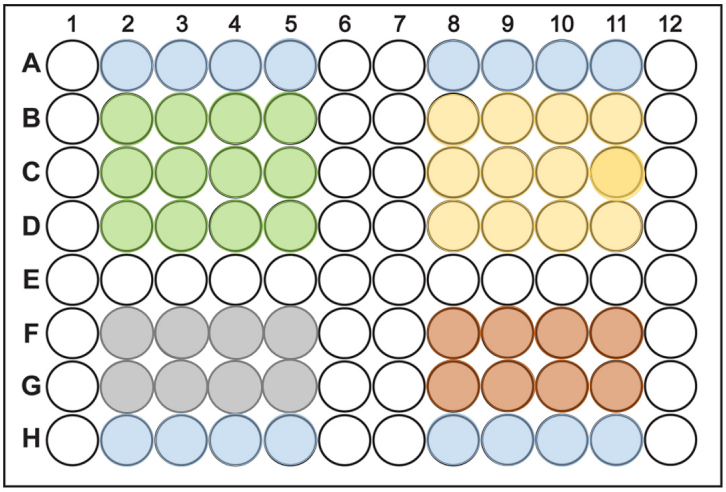

Figure 2.9. The layout of Calgary Biofilm Device plate used to culture complex biofilms in sterile human saliva and human serum.
Four different growth media were used in this experiment, each represented by a different colour. Blue wells show where sterile water was used as a control in place of the inoculum. While wells were inoculated with sterile PBS. The experiment was performed in triplicate.

After inoculation, the media were changed after 24 hours and later twice a week. Four wells per medium type were inoculated with sterile water as a negative control. After inoculation, the plates were incubated anaerobically (section 2.2.3). Half of the biofilms were harvested after three weeks of culture. For the remaining biofilms in groups 1, 2 and 4, the growth media were switched to sterile human saliva and cultured for two more weeks by supplying fresh sterile saliva twice a week. The biofilms were then harvested, treated with PMA and processed as described in section 2.3.4.

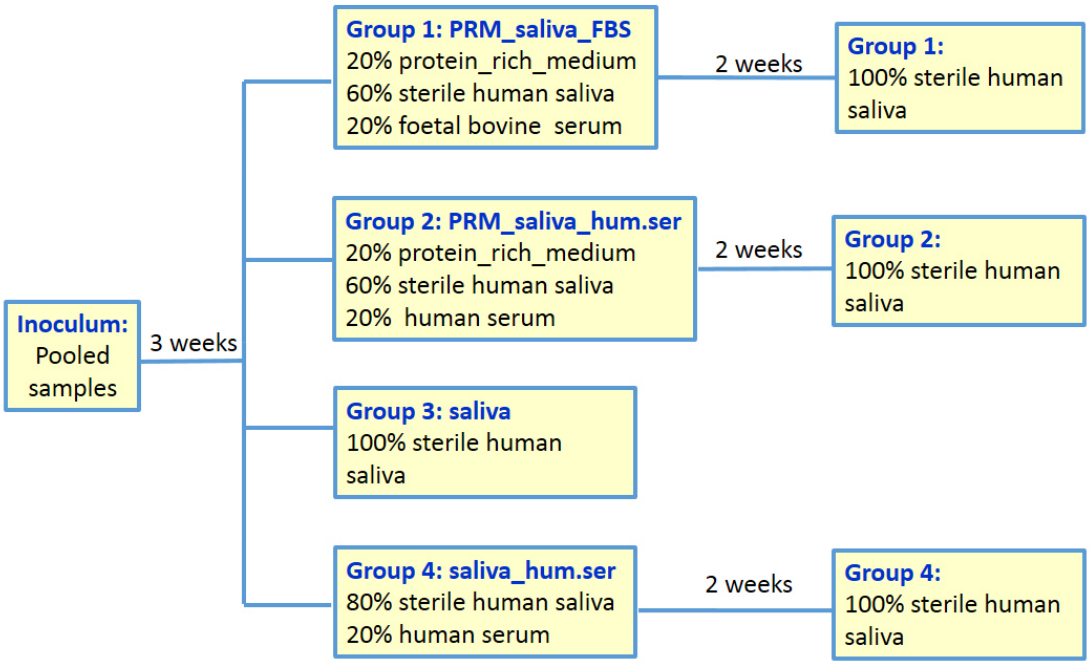

Figure 2.10. Experimental plan for the *in vitro* model experiment that compared sterile human saliva and human & bovine serum as nutrients. After enrichment, the effect of a recovery phase was investigated using solely human saliva as a nutrient source. PRM – protein-rich medium, FBS – foetal bovine serum.

2.3.4 Biofilm sample preparation

After the pegs were removed from the CBD, each biofilm was washed three times by dipping each peg in 200 µL sterile PBS to remove loosely adherent bacteria. Then pegs were transferred to 500 µL of sterile PBS and carefully scraped with a sterile dental scaler as detailed in section 2.2.8. Biofilms from three pegs were pooled together to obtain one sample as described by Kistler et al. (2015).

2.3.4.1 PMA treatment of biofilms and DNA isolation

Prior to DNA isolation, biofilm samples were treated with 1.5 µL PMA (final concentration 50 µM) according to the manufacturer's instructions (Biotium, Fremont, CA) to obtain DNA from intact cells (Exterkate et al., 2015). The quality and quantity of DNA was assessed using the Pico Green Kit (Molecular probes, Eugene).

DNA was isolated from the biofilm samples using the UltraClean® DNA Isolation Kit according to the manufacturer's instructions (Mo Bio, Carlsbad) except that shearing time was extended from 10 min to 30 min.

2.3.4.2 DNA library preparation

DNA was fragmented with the Covaris system (Covaris, Woburn) to obtain 200 bp DNA fragments. Analysis of samples on the Agilent D1000 ScreenTape (Agilent Genomics, Santa Clara) showed, that the majority of fragmented DNA was 240-280 bp long (Appendix Figures 2 and 3). During bead size selection, the reads of 250 bp were used for further processing.

The libraries were prepared using NEBNext Ultra DNA Library Preparation Kit for Illumina® or NEBNext Ultra DNA Library Preparation Kit for Illumina® (New England BioLabs, Ipswich, MA), depending on DNA yield, according to the manufacturer's instructions. PCR enrichment of adaptor-ligated DNA was performed using 6 cycles for denaturation, annealing and extension.

2.3.4.3 DNA sequencing

After assessing the quality of the libraries, size selection was performed if required. 100 ng of each library was pooled and submitted for 150 bp paired-end sequencing on an Illumina HiSeq 3000 (Illumina, San Diego).

2.3.5 Evaluation of sequencing depth

The latest literature suggests a redundancy based approach to evaluate the depth of sequencing (Rodriguez and Konstantinidis, 2014), (Dabdoub et al., 2016) for metagenomics data. The Nonpareil program examines the degree of overlap among individual sequence reads to compute the fraction of reads with no match, which is used to estimate the abundance-weighted average coverage (Rodriguez and Konstantinidis, 2014). Minimum overlap percentage was set at 70%. To our knowledge, it is the only program which assesses the sequencing coverage for metagenomics studies.

2.3.6 Quality control

Adapters were removed with cutadapt (Martin, 2011) and sickle v.1.33 was used to quality-trim the paired-end reads. The Phred quality threshold was set to 28, where a score of 30 provides a 1 in 1000 possibility of incorrect base call.

2.3.7 The analysis of metagenomes

Paired-end reads were mapped against the non-redundant bacterial protein database (downloaded November 2017 from National Centre for Biotechnology Information (NCBI)) using default parameters in diamond (Buchfink et al., 2015). The outputs from diamond were uploaded to The MEtaGenome ANalizer (MEGAN) (v.6.8.9) (Huson et al., 2011) for the analysis of taxonomy using the recommended parameters (*Min-score* threshold was set to 80 and

top-percent filter was set to 10%, min support 0.005%, meaning that the detection threshold for species was 0.005% of total reads).

2.3.7.1 Alpha diversity

Absolute counts of the reads from MEGAN were used for alpha diversity comparisons. Alpha diversity is characterized by using a total number of organism within a sample, such as richness, which is measured as the number of Operational Taxonomic Units (OTUs), the relative abundance of the organisms (evenness), or indices that combine these two dimensions. Observed number of OTUs, Chao and Inverse Simpson indexes were compared between sample groups.

2.3.7.2 Differences in relative abundance

Absolute read counts were rarefied to an even depth by randomly subsampling each sample to the even depth in *phyloseq* package using the *rarefy_even_depth* function (McMurdie and Holmes, 2013). Rarefied values were compared between the sample groups to find the differently abundant taxa using the DESeq2 package (Love et al., 2014).

2.3.7.3 16S rRNA gene analysis

Together with analysis of metagenomes, 16S rRNA analysis was performed. It is based only on one housekeeping gene and can offer some preliminary data in a relatively short time, while metagenomics assesses full sets of genes and can be computationally very challenging. For 16S rRNA analysis, the quality-filtered reads (detailed in section 2.3.6) were aligned against the Human Oral Microbiome Database (HOMD) (Dewhirst et al., 2010) (version 14.5) using the bowtie2 programme (Langmead and Salzberg, 2012). Mapped reads were clustered at 98.5 % identity using usearch61 (Edgar, 2010) (version v6.1.544) option in QIIME (Quantitative insights into microbial ecology) (Caporaso et al., 2010). *Phyloseq* (McMurdie and Holmes, 2013), *DESeq2* (Love et al., 2014), and *vegan* (Crist et al., 2003) packages in R were used for analysis.

2.3.7.4 Rarefaction curves

To evaluate if adequate depth was achieved by sequencing, rarefaction curves for 24 samples were prepared using the *vegan* package in R on 16S rRNA data.

2.3.7.5 The composition of inoculum samples.

To visualize the taxonomy of the inoculum, *metacoder* was used in R (Foster et al., 2017).

2.3.7.6 UniFrac distances

Unifrac distances in the GUniFrac package (Chen et al., 2012) in R were used to visualize the phylogenetic relatedness of the samples. UniFrac distance is a robust method for comparing the differences between microbial communities between samples; it measures the proportion of shared branch lengths on a phylogenetic tree between samples.

2.3.8 Functional analysis - read annotation

For differential analysis of potential functions between samples, the reads were aligned against the non-redundant NCBI bacterial protein database (downloaded November 2017) with diamond aligner. MEGAN was used to provide SEED (not an acronym) annotations for reads using the lowest common ancestor algorithm and recommended parameters (Overbeek et al., 2005). The SEED method organises genes into subsystems based on their functional roles. The recommended parameters were: m*in-score* threshold was set to 80 and *top-percent* filter was set to 10%, 0.005% min support.

2.3.8.1 Analysis of functional potential between different biofilms

After annotating the metagenomes with SEED annotations in MEGAN, the root squared counts of functional data were uploaded into the DeSeq2 package for the comparison.

2.3.9 Statistical analysis

After evaluating the normality of the data sets, ANOVA with Bonferroni correction, *post hoc* after Kruskal-Wallis test or Welch F tests, were used to detect the significant differences between groups. The significance threshold was set to $p < 0.05$.

Chapter 3 Development of a mixed species *in vitro* biofilm model – pilot studies on the effects of inoculation time, inoculum and media composition

3.1 Introduction

The oral biofilm community is highly dependent on a complex web of symbiotic and antagonistic bacterial interactions. Maintaining homeostasis among species is a key factor for periodontal health. However, due to reasons that are not fully understood, this balance can breakdown resulting in an altered bacterial profile, triggering a cascade of gingival and periodontal inflammation, finally leading to bone resorption and tooth loss (Huang et al., 2004). During periodontal disease, some changes in local conditions in the periodontal pocket are detected, such as a rise in pH, an increased flow of gingival crevicular fluid, and a decreased availability of oxygen (Kenney and Ash, 1969; Eggert et al., 1991). These environmental changes can have a significant effect on shaping the microbial community that resides within the periodontal pocket. However, our knowledge about the bacterial community dynamics in response to these environmental aspects remains limited.

Longitudinal *in vivo* studies can provide detailed information about plaque formation and its changes during the development of periodontal disease (Kistler et al., 2013). However, there are only small numbers of such studies due to the methodical challenges and ethical aspects of this type of research. Inter-individual variation in oral bacterial composition, varying clinical and microbial expression of disease, low quantities of subgingival plaque in healthy sites and the high cost of data analysis impede the characterisation and comparison of bacterial communities.

For these reasons, simplified *in vitro* models are widely used, especially for preliminary studies aiming to understand questions about biofilm formation, maturation, and the response of microbial communities to antimicrobials (Kistler et al., 2015; Soares et al., 2015). Although, these multi-species models do not reflect the precise bacterial behaviour or local environment in the mouth, they have been of value in understanding sequential biofilm formation patterns, biofilm susceptibility to antibiotics, species cooperation and antagonism while

growing in the community (Periasamy and Kolenbrander, 2009; Duran-Pinedo et al., 2014a). The CBD is a convenient and simple to set up system, which has been used to culture single and multispecies bacterial or fungal biofilms (Kistler et al., 2015; Wade et al., 2016). The CBD is versatile, allowing many parameters to be investigated, and has been used to answer questions about bacterial unculturability, sensitivity to antimicrobials, etc. (Wade et al., 2016).

The experiments described in this Chapter aim to develop a simple multi-species model using the CBD in conjunction with an inoculum of five representative oral bacteria. This simplified system was used to evaluate the effects of the environment on biofilm community. Protein-rich media were used, with and without serum supplementation, and biofilm composition and structure was characterised. Moreover, one of the aims was also to check the suitability of selected growth conditions for the subsequent development of more complex subgingival biofilms.

3.2 Aims

To establish a simple *in vitro* biofilm model using the CBD, with five species commonly associated with periodontal disease for preliminary investigations of the effect of the growth environment on the composition of an oral bacterial community.

Specifically:

To observe the effects of a different media on the growth of multi-species biofilms.

To characterise biofilms cultured under different conditions in terms of composition and 3D structure.

To ascertain the suitability of the CBD to culture bacterial species for prolonged periods.

3.3 Results

During the development of the five-species *in vitro* model, a number of protocols were tested that varied in the inoculum composition and frequency of fresh medium supply. The biofilms developed under the optimised protocol are described in section 3.3.4 while the outcomes of other trialled protocols are detailed in sections: 3.3.1 and 3.3.2.

3.3.1 Four-week multi-species biofilm development

One of the aims of the experiment was to develop an *in vitro* biofilm model that would sustain viable multi-species bacterial community for long periods. The literature suggests that biofilms become stable after 3 – 7 days of culture (Jiang et al., 2015). During the first attempt to establish a multi-species model, bacterial species were sequentially inoculated once a week and cultured for four weeks in total. Section 2.2.7, Figure 2.3, and Table 2.3 provide details of the culturing protocol for this *in vitro* model. The composition of the *in vitro* model described by Ammann et al., (2012) was modified and consisted of sequential inoculations of *S. oralis* ULCP86*, A. naeslundii* M42*, F. nucleatum* ssp. ATCC® 10953™*, P. intermedia* OMZ 248 and *P. gingivalis* W83.

3.3.1.1 Viable counts of multi-species biofilms after 28 days of culture

Visual evaluation before harvesting showed that biofilms cultured in the media supplemented with serum were thicker, compared with biofilms cultured in a medium without serum. Growth on agar plates showed that at day 28 all biofilms, irrespective of medium, were dominated by *S. oralis* and *A. naeslundii* (Figure 3.1).

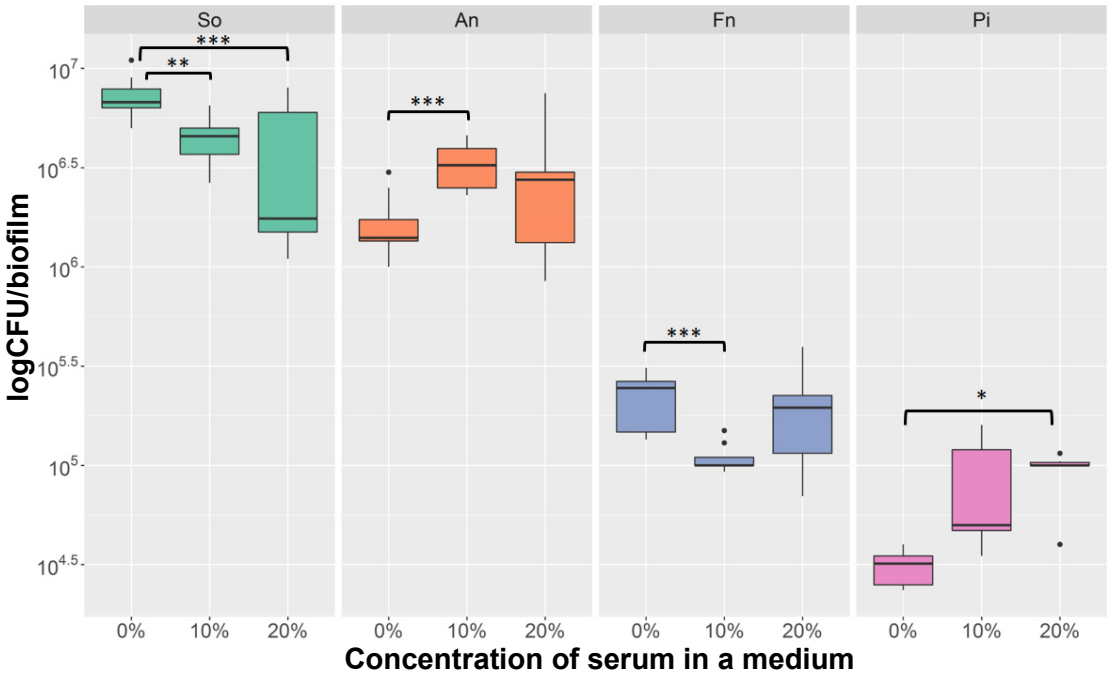

Figure 3.1. Viable counts of four-week biofilms in protein-rich medium supplemented with different concentrations of serum.
So – *S. oralis*, An – *A. naeslundii*, Fn – *F. nucleatum*, Pi – *P. intermedia*. * - p = 0.05,** - p = 0.01, *** - p = 0.001, *post hoc* after Kruskal-Wallis test, n = 3 individual experiments, with 5 technical replicates each.

S. oralis and *A. naeslundii* comprised 95.5% - 97.6% of all bacteria within the biofilms. Biofilms cultured in protein-rich medium without serum had low numbers of *F. nucleatum (*2.4% ± 0.7) and *P. intermedia* (0.3% ± 0.06). Moreover, numbers of *F. nucleatum* and *P. intermedia* were relatively stable throughout the experiment, irrespective of medium type. *Post hoc* analysis showed that a protein-rich medium with 10% serum was unfavourable for the growth of *F. nucleatum*, p = 0.001, while 20% serum favoured the growth of *P. intermedia, p* = 0.05. Moreover, not all biofilms had viable *P. intermedia* at the end of the experiment. In general, the supplementation of 10% or 20% serum in a growth medium was unfavourable for the growth of *S. oralis* but it boosted the growth of *A. naeslundii.* However, viable counts of these two species varied greatly between the biofilms cultured in 20% serum medium. *P. gingivalis* was not detected in 28 day biofilms, irrespective of the growth medium

3.3.1.2 Changes in pH of supernatants throughout the four-week experiment.

During the four-week experiments, pH values of culture supernatants were recorded at day seven (after two-species were inoculated), day 14 (three-species inoculation) and day 28 (five-species inoculation). Starting pH was adjusted to the level of 7.4 prior the experiments (Figure 3.2).

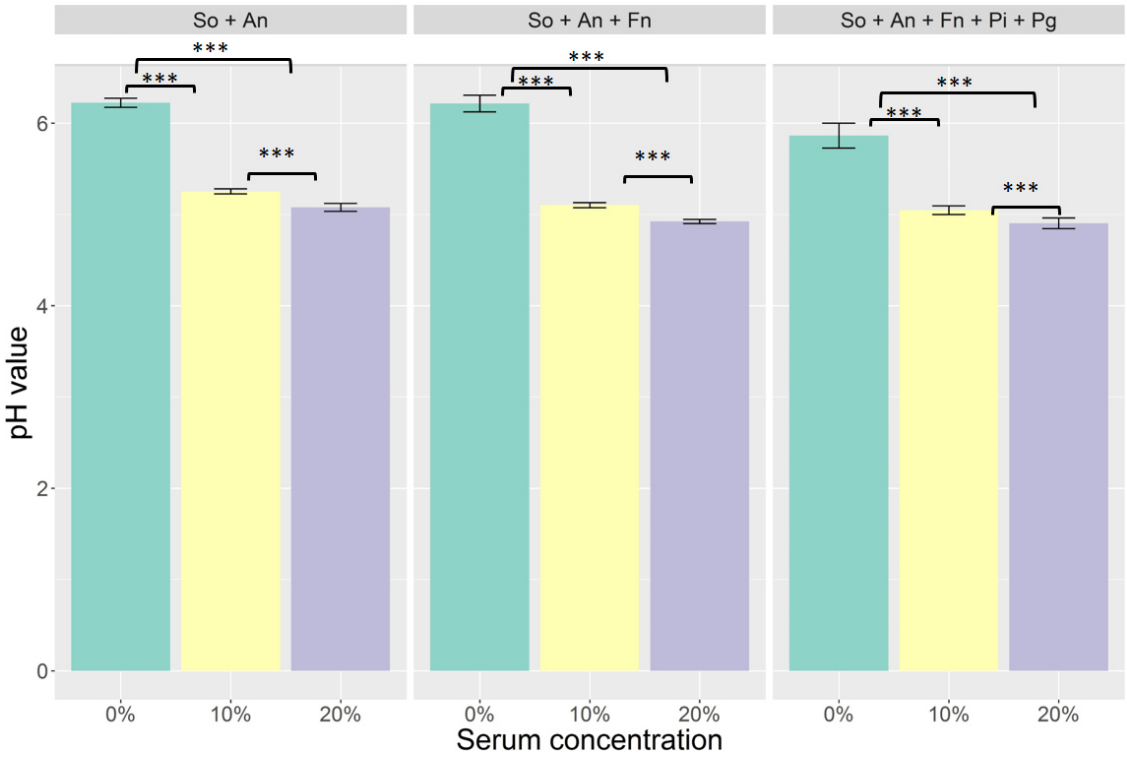

Figure 3.2. Measurements of the pH of growth medium at various points in the development of 28 day biofilms.
Measurements were made at day seven (So + An), day 14 (So + An + Fn) and day 28 (So + An + Fn + Pi + Pg). So – *S. oralis*, An – *A. naeslundii*, Fn – *F. nucleatum*, Pi – *P. intermedia*. The significance of differences in pH values between medium types was evaluated using *post hoc* analysis after Kruskal-Wallis test, *** - $p < 0.001$, n= 3 individual experiments, each with 5 technical replicates.

The pH values of two species biofilms at day seven varied greatly between media types, $p < 0.001$, Kruskal-Wallis. Protein-rich media without serum had the highest pH values in supernatants (6.2 ± 0.02), $p = 0.001$, *post hoc* after Kruskal-Wallis, while pH values of media with 10% and 20% serum were significantly lower (pH 5.3 ± 0.1 and 5.1 ± 0.2, respectively), $p = 0.001$. Similarly, the pH values of the supernatants of three and five species biofilms (at day 14 and day 28) were the highest if no serum was used in the growth medium (6.2 ± 0.04 and 5.8 ± 0.2, respectively), $p = 0.001$. The lowest pH values were observed in five-species biofilms cultured in protein-rich medium supplemented with 20% serum (i.e. - at day 28 it was pH 4.9 ± 0.04).

3.3.2 Two-week multi-species biofilm development – effect of inoculum composition

Viable counts of the four-week experiment showed an enrichment of *S. oralis* and *A. naeslundii*, poor growth of other species and a drastic decline in pH of supernatants. Newly designed experiments performed a more frequent inoculation and shortened incubation times, as described for other models

(Guggenheim et al., 2011; Ammann et al., 2012). The more frequent inoculation might prevent the enrichment of early colonisers before the slower growing species could become established in the model. Moreover, during the *in vitro* model development, the inclusion of different streptococci was compared. The methods are detailed in section 2.2.5, Figure 2.2 and Table 2.2. Section 3.3.4 describes the results of a protocol where *S. oralis* was replaced with *S. salivarius.*

3.3.2.1 Viable counts of two-week biofilms containing *S. oralis*

Five-species biofilms were cultured in protein-rich medium with different concentrations of serum, and viable counts were recorded after two weeks of incubation (Figure 3.3). When 10% or 20% of serum was used to supplement the protein-rich medium, higher counts of *A. naeslundii,* p = 0.01, and lower counts of *F. nucleatum* and *P. intermedia* were detected, p = 0.001, *post hoc* after Kruskal-Wallis. However, if serum was not used, *F. nucleatum* and *P. intermedia* comprised on average 0.09% and 0.02% of the community, respectively. *P. gingivalis* could not be detected in any of the biofilms with *S. oralis* after two weeks of incubation.

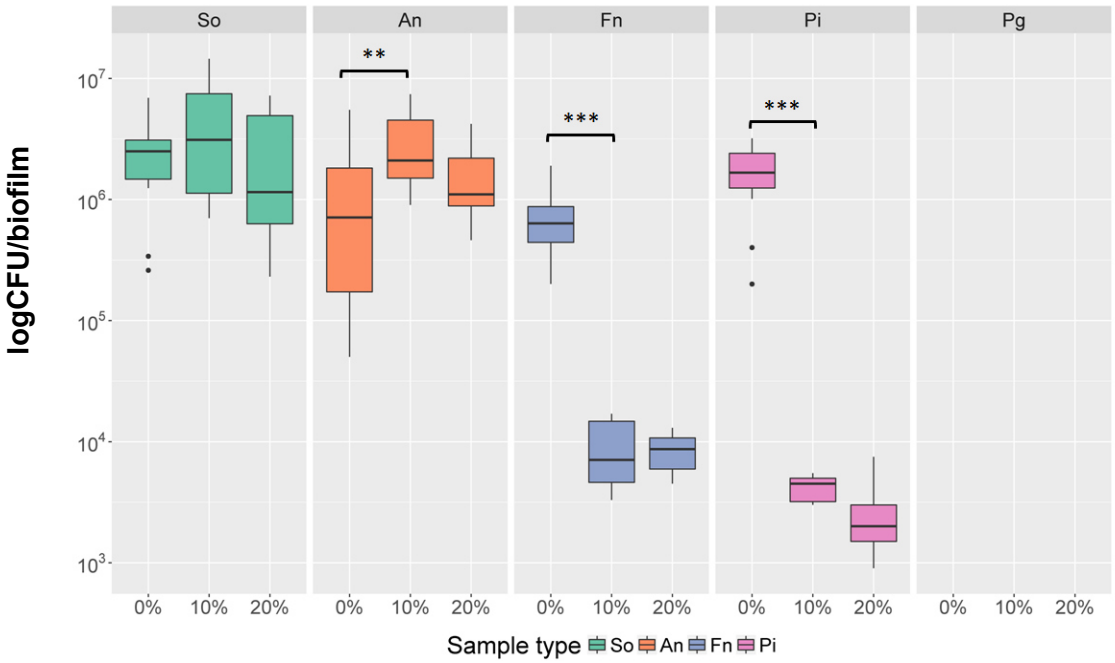

Figure 3.3. Viable counts of two-week old five-species biofilms with *S. oralis* cultured in protein-rich medium, supplemented with different concentrations of serum.

Post hoc after Kruskal-Wallis test,** - p = 0.01, *** - p = 0.001, n= 3 individual experiments, each with 5 technical replicates.

3.3.2.2 Changes in pH values of *S. oralis* multi-species biofilms over two weeks.

The supernatants during the two-week experiment were measured twice: at day two, when biofilms comprised of two species (*S. oralis* and *A. naeslundii*) and at day 14, after all five species were inoculated (Figure 3.4).

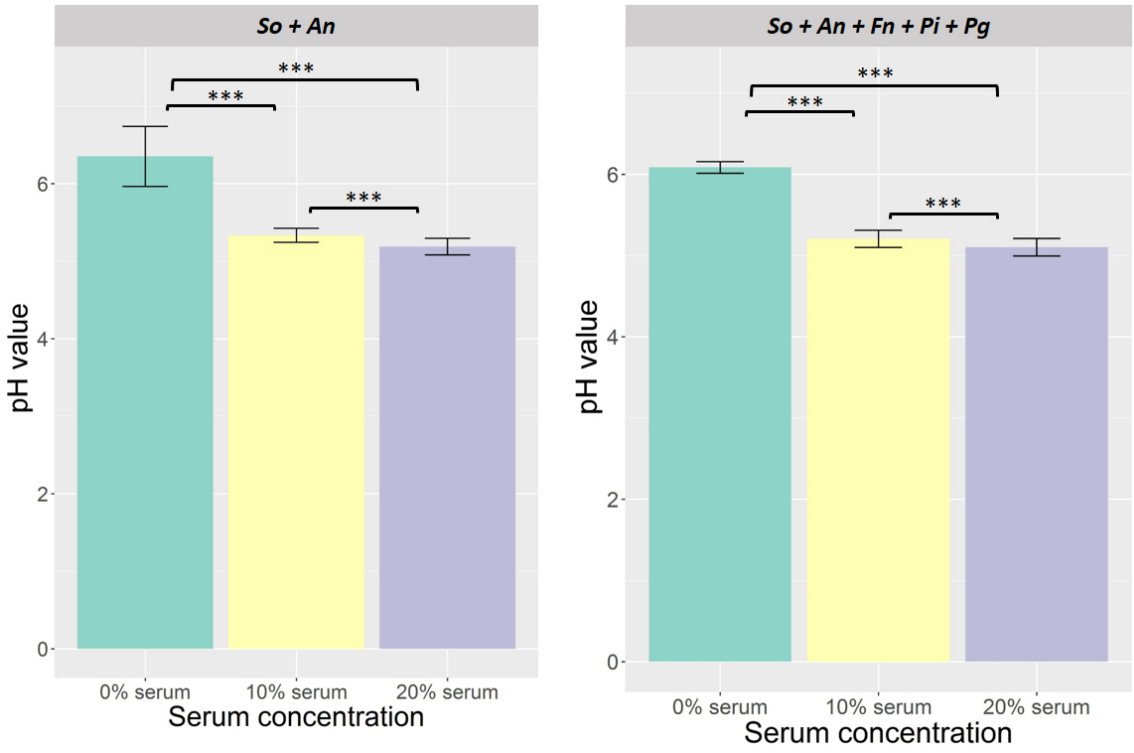

Figure 3.4. Measurements of the pH of growth medium at various points in the development of two-week biofilms with *S. oralis*.
Measurements were made at day two (So + An) and day 14 (So + An + Fn + Pi + Pg). So – *S. oralis*, An – *A. naeslundii*, Fn – *F. nucleatum*, Pi – *P. intermedia, Pg – P. gingivalis*. The significance of differences in pH values between medium types was evaluated using *post hoc* analysis after Kruskal-Wallis test, *** - p = 0.001, n= 3 individual experiments, each with 5 technical replicates.

At day two, pH values of supernatants without serum were higher than supernatants with 10% or 20% serum, p = 0.001, *post hoc* after Kruskal-Wallis test. Similarly, at day 14, the pH of supernatants without serum were higher than supernatants with 10% or 20% serum.

3.3.3 Investigation into antagonistic interactions among *Streptococcus* species and *P. gingivalis W83*

During biofilm development and experiments to determine viable counts, it was observed that some *Streptococcus* species and *P. gingivalis* did not grow in

close proximity to each other, implying that some species may produce an inhibitor that is antagonistic to *P. gingivalis*.

3.3.3.1 Co-culture of *Streptococcus* species with *P. gingivalis W83*

In order to overcome this an allow for the inclusion of this important periodontal pathogen in the biofilm model a simple experiment was designed to investigate which *Streptococcus* species can be co-cultured with *P. gingivalis* (Figure 3.5).

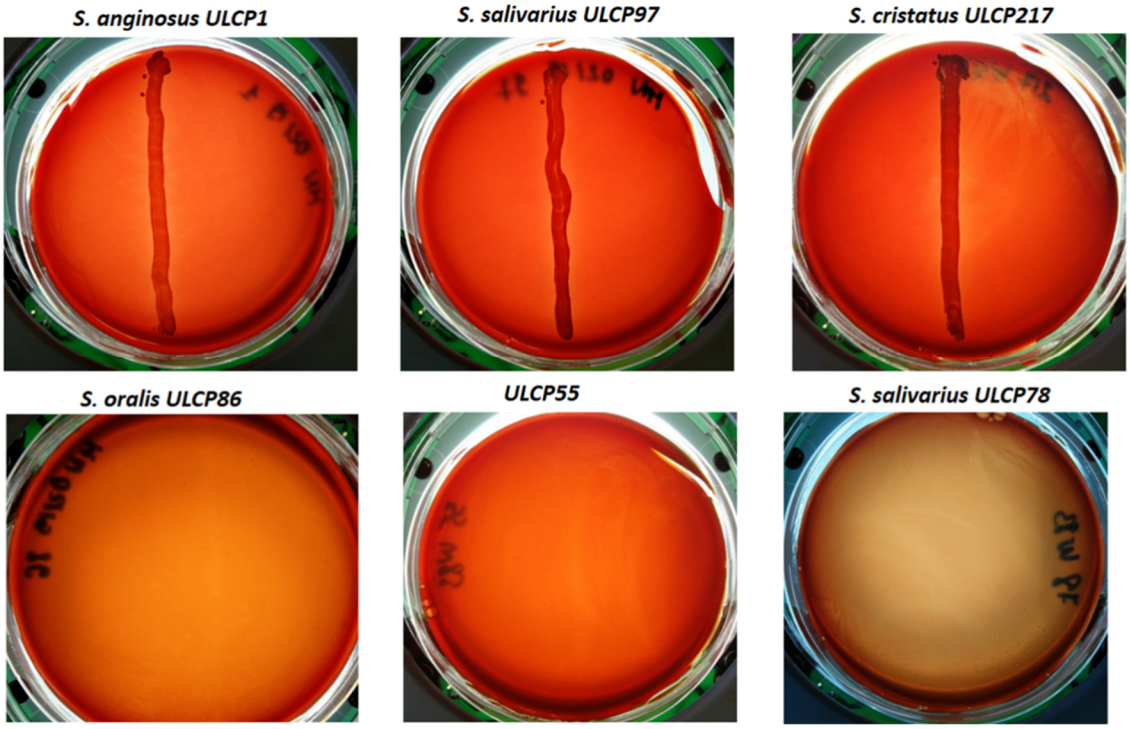

Figure 3.5. Different *Streptococcus* species cross-streaked with *P. gingivalis*.
CBA plates were inoculated with different *Streptococcus* species, cross-streaked with *P. gingivalis* W83 and incubated anaerobically. The visible growth of *P. gingivalis* was evaluated after 72h.

After plating different species of *Streptococcus* and cross-streaking *P. gingivalis*, the growth of bacteria was evaluated after anaerobic incubation (Table 3.1). Some *Streptococcus species* depicted here (for instance *S. oralis* ULCP86, *S. salivarius* ULCP78 and *Streptococcus* ULCP55) inhibited the growth of *P. gingivalis* on CBA plates. However*, P. gingivalis* W83 was able to grow on agar plates with some species, for example: *Streptococcus anginosus* ULCP1*, S. salivarius* ULCP97, *S. cristatus* ULCP217. Further photographic evidence of inhibitory relationship between *Streptococcus* species and *P. gingivalis* is provided in Appendix Figure 4, and summarised in Table 3.1.

Table 3.1. Summary of ability of *P. gingivalis* to grow when co-cultured with different *Streptococcus* strains.

Strain	Inhibition of *P. gingivalis*?
S. anginosus ULCP4	No
S. salivarius ULCP11	No
S. salivarius ULCP97	No
S. cristatus ULCP94	No
S. constellatus ULCP4	No
S. oralis ULCP11	No
S. cristatus ULCP94	No
Streptococcus ULCP100	No
S. oralis ULCP86	Yes
Streptococcus ULCP55	Yes
S. salivarius ULCP78	Yes
S. salivarius ULCP85	Yes
S. oralis ULCP10	Yes
S. oralis ULCP12	Yes
S. oralis ULCP92	Yes

3.3.3.2 The production of H_2O_2 by some *Streptococcus* stains

Some streptococci are known to produce hydrogen peroxide, which can be inhibitory to anaerobic bacteria (Kreth et al., 2005; Jakubovics et al., 2008). Therefore, Prussian blue agar plates were prepared as this medium will change colour from yellow to blue in the presence of hydrogen peroxide (Saito et al., 2007).

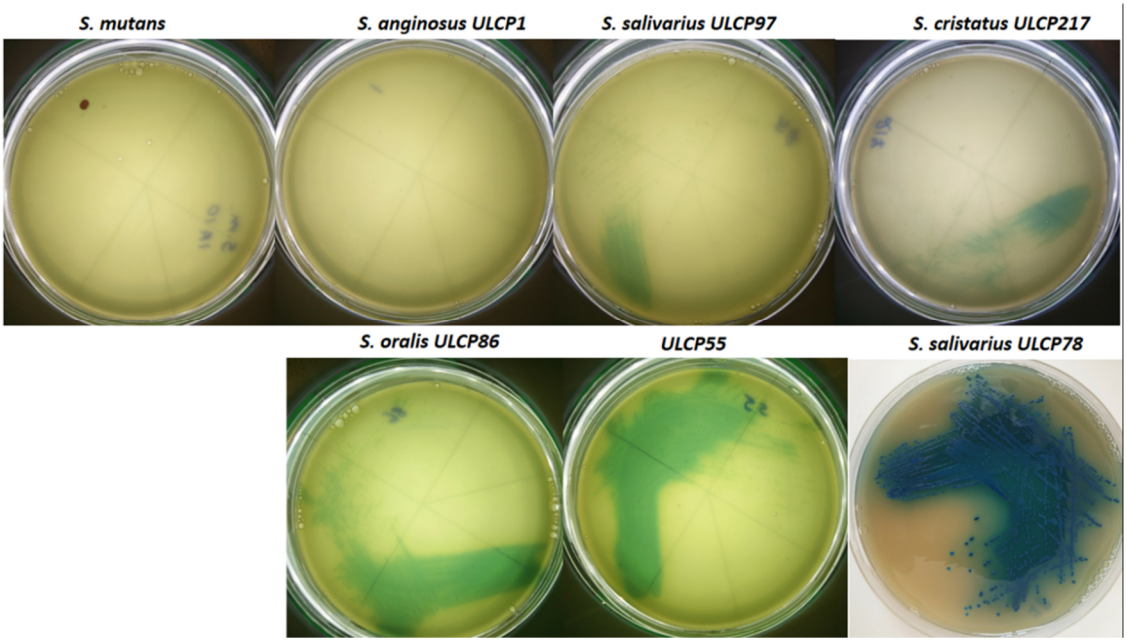

Figure 3.6. Qualitative evaluation of H_2O_2 production by selected *Streptococcus* strains.
Prussian blue agar plates change colour from yellow to blue when in contact with hydrogen peroxide. Clinical isolates being considered for inclusion in biofilm models were assessed for hydrogen peroxide production. Different *Streptococcus* isolates were inoculated onto Prussian Blue agar and incubated at 37°C for 24h. *S. mutans* OMZ70 was included as a negative control.

After 24 hours incubation, *S. oralis* ULCP86 formed blue colonies surrounded by blue areas on Prussian blue agar. *S. salivarius* ULCP 97 also changed the colour of the agar plates after 24 hours; however, the blue colour was less intense. Areas, less densely inhabited by *S. salivarius* ULCP97*,* remained yellow. Some *Streptococcus* strains, for instance *S. anginosus* ULCP1 and *S. mutans* OMZ70, which were used as negative controls, formed clear white colonies and did not change the colour of the agar (Figure 3.6).

3.3.4 Two-week multi-species biofilm development – *S. oralis* replacement with *S. salivarius*

After noting that *S. salivarius* ULCP97 was producing only low concentrations of hydrogen peroxide, grew in close proximity with *P. gingivalis* and, as suggested in the literature, had ureolytic activity (Sissons and Hancock, 1993), this species was selected to replace *S. oralis* ULCP98 in the multi-species model.

3.3.4.1 Viable counts of two-week biofilms with *S. salivarius*

The two-week experiment described in section 2.2.5 was carried out using a sequential inoculation of *S. salivarius* ULCP97*, A. naeslundii*, *F. nucleatum*, *P. intermedia* and *P. gingivalis*. The final protocol is depicted in Figure 2.2 and

Table 2.2. At the end of two weeks, cultured biofilms consisted of all five species (Figure 3.7).

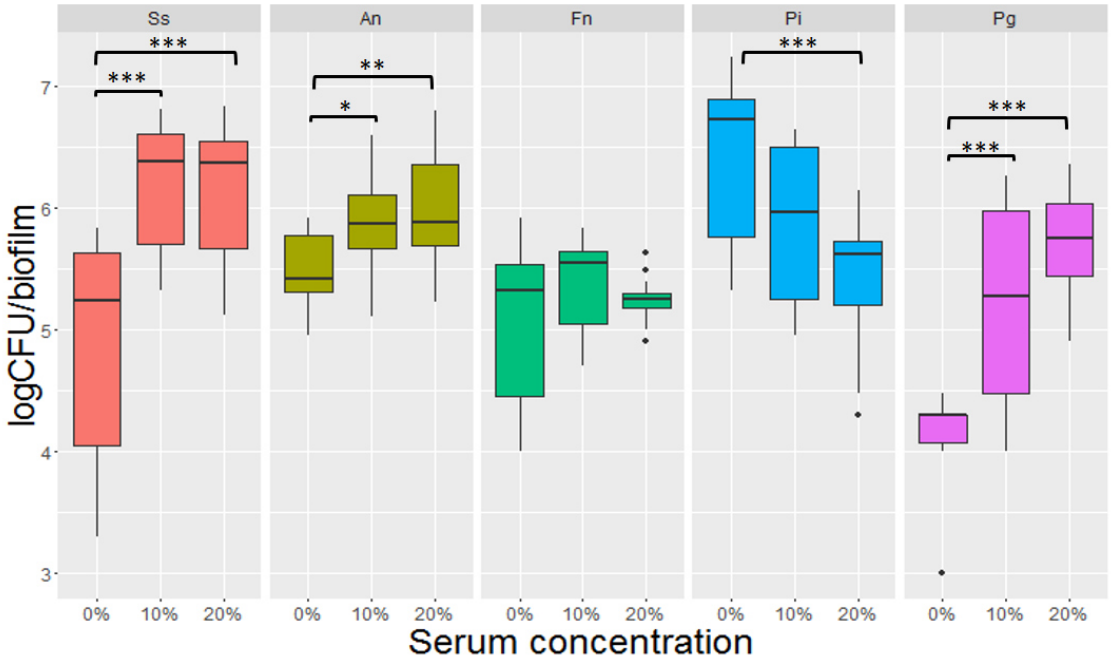

Figure 3.7. Viable counts of five-species biofilms containing *S. salivarius* at day 14 cultured in protein-rich media, supplemented with different concentrations of serum.
Ss – *S. salivarius*, An – *A. naeslundii*, Fn – *F. nucleatum*, Pi – *P. intermedia,* Pg – *P. gingivalis*. * - p = 0.05,** - p = 0.01, *** - p = 0.001, *post hoc* after Kruskal-Wallis test, n = 3 individual experiments, each with 5 technical replicates.

Culture on selective media showed that, when *S. salivarius* ULCP97 and four other species were used as the inoculum, all five species were detected at day 14. A higher serum concentration increased the growth of *S. salivarius* ULCP97 *(*$p < 0.001$*), A. naeslundii (*$p < 0.05$) and *P. gingivalis (*$p < 0.001$*)*, although a medium with 20% serum showed a reduced growth of *P. intermedia*, $p < 0.001$. Viable counts of *F. nucleatum* did not change significantly irrespective of the levels of serum in the culture medium, Kruskal-Wallis, $p = 0.2$.

3.3.4.2 Changes in pH levels in *S. salivarius* multi-species biofilms over two weeks.

The pH of supernatants was measured at two time points: at day two (before inoculating *F. nucleatum, P. intermedia* and *P. gingivalis)* and at day 14 (at the end of the experiment) (Figure 3.8).

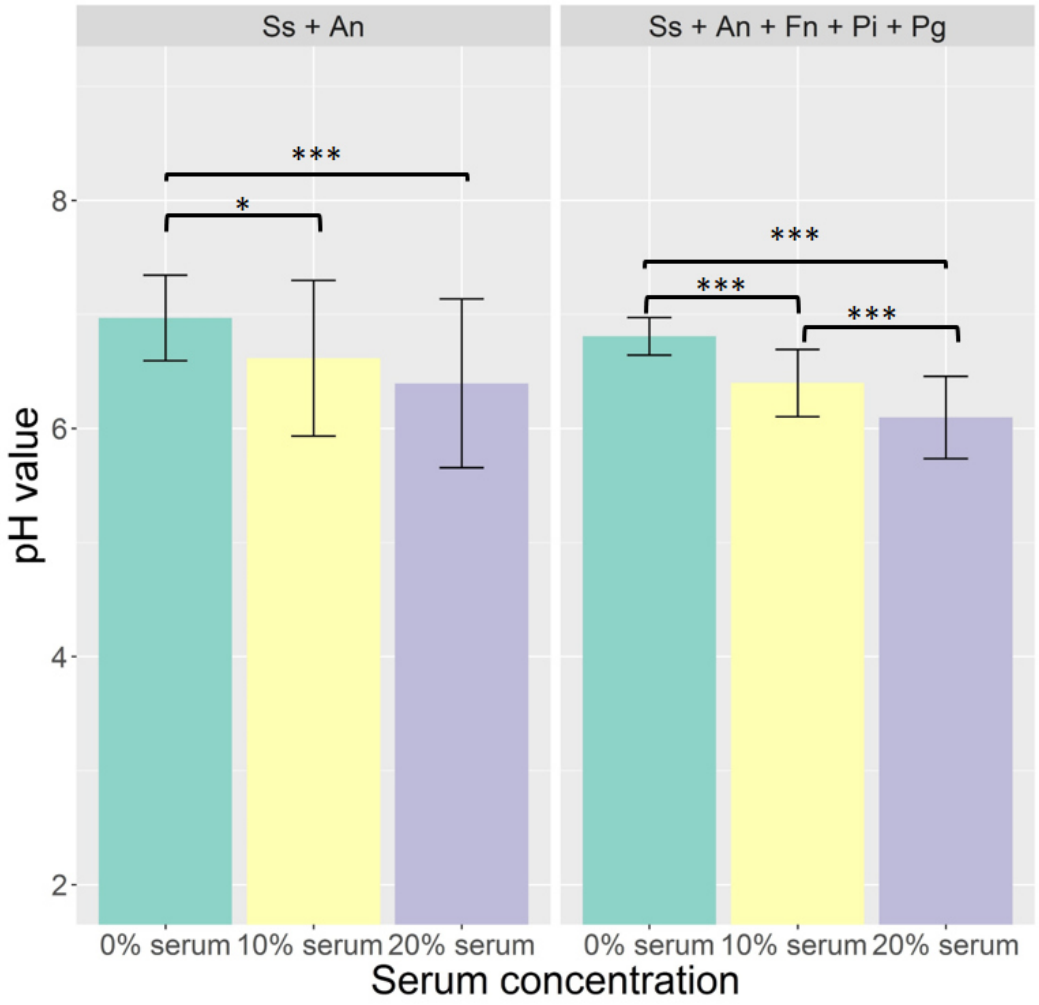

Figure 3.8. Measurements of the pH of growth medium at various points in the development of two-week biofilms with S. salivarius.
Measurements were made at day two (Ss + An) and day 14 (Ss + An + Fn + Pi + Pg). Ss – *S. salivarius,* An – *A. naeslundii*, Fn – *F. nucleatum*, Pi – *P. intermedia,* Pg – *P. gingivalis* * - $p < 0.05$, *** - $p < 0.001$, n= 3 individual experiments, each with 5 technical replicates.

The pH of supernatants without serum was higher than in supernatants with 10% or 20% serum. At day two, the mean pH of supernatants without serum was around neutral (7.0 ± 0.2). Lower pH values were recorded when 10%, $p < 0.05$, or 20% of serum was used in the medium, $p < 0.001$, *post ho*c for Kruskal Wallis test. A reduction in pH was observed in all media at day 14. The lowest pH values were detected when higher percentages of serum were used in the growth medium, Bonferroni correction for *post hoc* in one-way AVOVA, $p < 0.001$. Higher pH values of supernatants were recorded if *S. salivarius* rather than *S. oralis* was used in five-species biofilms (Figure 3.4).

3.3.5 Scanning electron microscopy of multi-species biofilms harvested at week two, containing either *S. oralis* or *S. salivarius*

grown on a protein-rich medium with or without supplementation with 20% serum.

After 14 days of incubation, the pegs with five-species biofilms cultured in two types of media were imaged by SEM to confirm the presence and uniformity of biofilms (Figures 3.9 and 3.10). All biofilms imaged showed a multi-layered and complex structure, and high-coverage of the pegs. After 14 days of incubation, SEM showed biofilms consisting of islands and aggregates of cocci and rods attached to a surface, irrespective of media type and biofilm composition. In the medium supplemented with 20% serum, biofilm aggregates were more three-dimensional and formed peaks consisting of rod-shaped, coccoid and fuso-shaped bacteria.

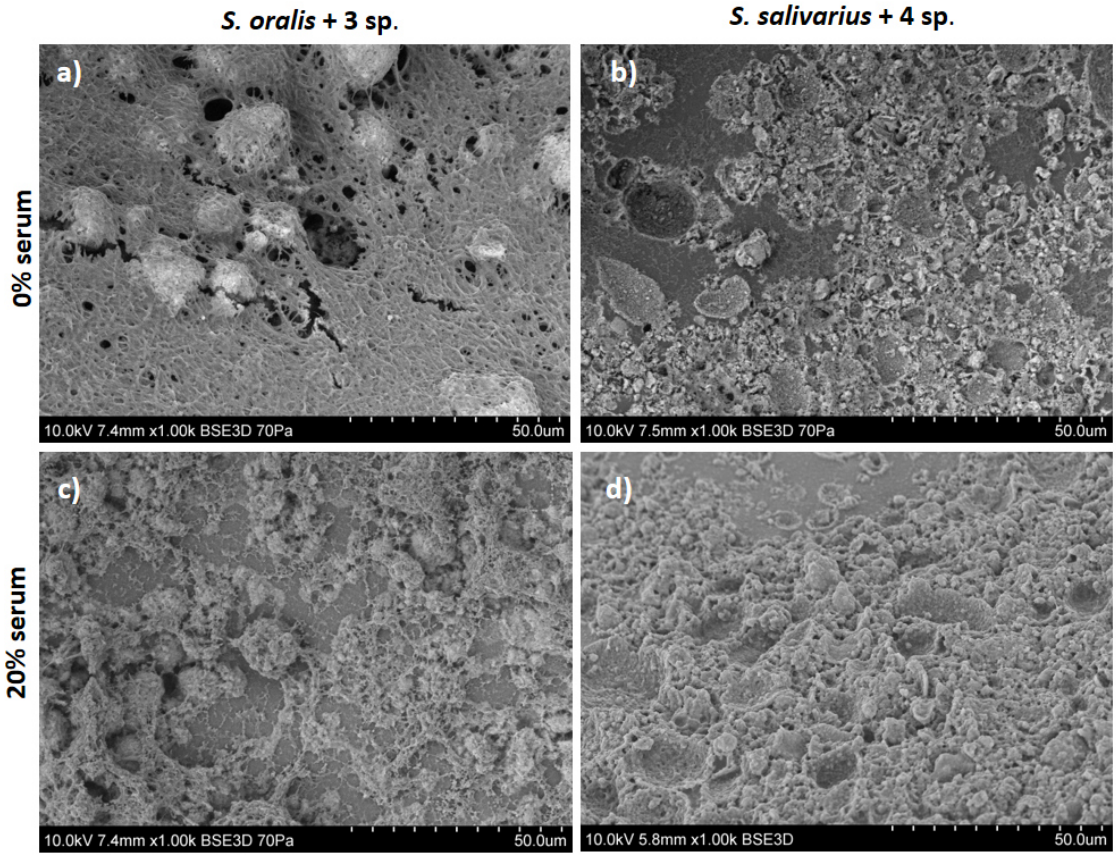

Figure 3.9. Scanning electron microscopy of five-species biofilms cultured in the Calgary Biofilm Device using two types of media for 14 days.
Images a) and c) show *S. oralis* + three species biofilms cultured in protein-rich medium without serum and with 20% serum, while b) and d) show *S. salivarius* + four species biofilm cultured in protein-rich medium without serum and with 20% serum.

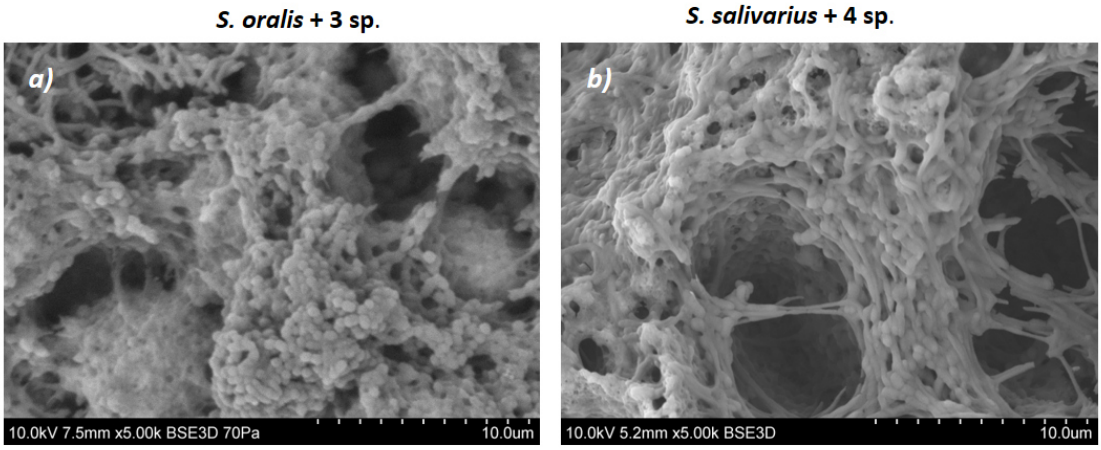

Figure 3.10. Scanning Electron Microscopy.
Images of a) *S. oralis* + three species in protein-rich medium with 20% serum and b) S. salivarius + four species in protein-rich medium with 20% serum.

Biofilms consisting of *S. oralis* and three other species (*A. naeslundii, F. nucleatum, P. intermedia*) mainly consisted of rod-and cocci-shaped bacteria, aggregated in round shapes (Figure 3.10a), while biofilms with *S. salivarius* and three other species consisted of nets of fuso-shaped bacteria with rod- and cocci-shaped bacteria attached to this net (Figure 3.10b). This method confirmed the dominance of coccoid and rod-shaped bacteria in the biofilms, especially if *S. oralis* instead of *S. salivarius* was used for the inoculum, whilst biofilms with *S. salivarius* had a more diverse composition.

3.4 Discussion

In this Chapter, a biofilm model consisting of five bacterial species commonly detected in the oral cavity in health and/or periodontal disease was established. This was then used to explore parameters that might affect the development of more complex oral microbial communities which will be investigated in subsequent Chapters. The CBD with hydroxyapatite-coated pegs was used as a relevant surface for biofilm formation. A protein-rich medium was chosen to investigate bacterial growth because it has been successfully used in other studies to culture complex oral bacterial communities and has been showed to satisfy the growth requirements of diverse bacterial species (Bradshaw et al., 1996b). The medium was supplemented with haemin and menadione, as some commonly detected oral bacteria such as species belonging to the genera *Porphyromonas* and *Prevotella* require these cofactors for growth (Gibbons and Macdonald, 1960). During the development of the *in vitro* model, variations in inoculation times and the bacterial composition were tested. Once the basic model had been optimised to sustain the growth of a five-species bacterial community for prolonged periods, the supplementation of the growth medium with serum was evaluated and the biofilms were characterised by viable counts and SEM.

The developing periodontal pocket offers a markedly distinct ecological niche compared to that found in supragingival habitats. In addition to an abundance of tissue breakdown products, inflammatory mediators, and host defence molecules, a periodontal pocket exhibits a slightly higher pH, a more anaerobic atmosphere, and has a distinct nutrient supply (Eggert et al., 1991). Supragingival sites are influenced more by saliva, and the presence of fermentable sugars in the diet, and, usually harbour many Gram-positive aerotolerant acidogenic and acidophilic species, whereas subgingival populations are generally predominated by obligate anaerobic, Gram-negative proteolytic species (Kenney and Ash, 1969; Diaz et al., 2016). In order to mimic aspects of the subgingival environment, the *in vitro* model therefore employed a protein-rich medium, supplemented with haemin and menadione, with a starting pH that was slightly alkaline, an anaerobic atmosphere and the absence of added fermentable carbohydrates in the growth medium.

The sequential inoculation of species that was used for the establishment of the biofilm model attempted to mimic the natural sequence of plaque formation as described in models by Guggenheim et al. (2011), and Ammann et al. (2012). In the mouth biofilm formation begins with species from *Streptococcus* and *Actinomyces* genera co-aggregating and adhering to the surface, as

demonstrated by FISH in an *in vivo* study (Palmer et al., 2003). Specific adhesins from the species of these genera can bind distinct salivary receptors in the acquired pellicle to obtain an initial attachment to the hydroxyapatite surface (Ruhl et al., 2004). The same FISH technique was used to investigate *in vivo* biofilms and confirmed the gradual increase of *F. nucleatum* in maturing biofilms (Al-Ahmad et al., 2007). *F. nucleatum* provides an adhesive and metabolic support for developing periodontal communities. Finally, *P. intermedia* and *P. gingivalis*, periodontal pathogens frequently detected in subgingival plaque, were inoculated. Experiments culturing naturally occurring human plaque samples showed that biofilm composition varies in immature and mature plaque, the latter being associated with the gradual emergence of periodontal pathogens (Al-Ahmad et al., 2007; Kistler et al., 2015). For these reasons, we cultured the biofilms for 14 - 28 days to allow time for the microbial communities to mature.

A wide variety of *in vitro* subgingival plaque models have been described which vary in duration, method of culture, or type and number of species included in the inoculum (Ammann et al., 2012; Soares et al., 2015; Bradshaw et al., 1996b). However, a high number of species, complex equipment and constant care make these models difficult to handle and to obtain reproducible results. Previously described *in vitro* systems modelling the subgingival pocket consisted of 10 - 40 species (Thurnheer et al., 2016; Bradshaw et al., 1996b; Soares et al., 2015). We aimed to develop a simple subgingival *in vitro* biofilm model to allow the investigation of the effects of environment on subgingival biofilm community in preliminary studies. The limited number of five representative species made the model easy to handle and more reproducible. The successful five-species biofilm culture lasted for two weeks and required daily media changes; however, after the last inoculation at day three, the maintenance of the model was relatively simple. At the end of the experiment the system yielded highly reproducible biofilms of large biovolume and high surface coverage of the pegs which was confirmed by SEM.

However, the simplicity of the model also highlighted certain disadvantages: a five species *in vitro* biofilm, as with any *in vitro* biofilm, does not completely reflect the actual growth of a subgingival community. A five-species community can reflect only simple metabolic interactions, whereas hundreds of biofilm species in the oral cavity are linked through complex symbiotic and antagonistic relationships. Moreover, as for all *in vitro* models, it excludes the main component of periodontal pocket – a host and its response to a bacterial stimulus. During inflammation, the host immune system delivers a plethora of immune cells and molecules that concomitantly act on bacterial growth and

interactions. However, even simple *in vitro* models can be helpful in understanding bacterial mutualism, antagonism and biofilm formation patterns (Ammann et al., 2013a; Bradshaw and Marsh, 1998a; Thurnheer et al., 2016).

Five-species biofilm development experiments were started by inoculating new species every 7 days, which resulted in limited growth of late colonisers after four weeks. The main issue encountered during the development of these communities was a drastic drop in pH, which would be inhibitory for *F. nucleatum, P. intermedia* and *P. gingivalis*. *S. mutans* and *S. sobrinus* are well known acidogenic, acid-tolerant streptococcal species, but other streptococci, such as *S. oralis, S. mitis*, *S. sanguinis* and *S. anginosus,* also can exhibit acid-tolerance and, indeed, *S. oralis* can produce acid at rates exceeding that of some *S. mutans* strains (Senneby et al., 2017). Moreover, the reduced growth of *A. naeslundii* is reported only at pH values below 4 (Horiuchi et al., 2009). Thus, the pH values of around 5.1 – 6.3, recorded in our four-week experiment, facilitated the growth of early colonisers, but perio-pathogenic species would be disadvantaged by such an acidic environment (Takahashi and Yamada, 1999). For example, optimal growth of *F. nucleatum* occurs at pH 7.4 in continuous culture, while growth reduction by 50% is recorded at pH 6.8 (Rogers et al., 1991). The 100-fold decrease was recorded in CFU counts of *F. nucleatum* if pH declined below 6 in experiments of Chapter 3, while it was relatively stable if pH fluctuated around 6.8 – 7 while growing in biofilms.

More frequent inoculations were tested to see if this could prevent a drastic drop in pH leading to decline growth of late colonisers. The more frequent inoculation might prevent the enrichment of early colonisers before the slower growing species can become established in the model. However, inoculations performed every 24h did not result in a different pH of supernatants, which ranged around values of 5.0 – 6.6, thus still preventing the growth of *F. nucleatum, P. intermedia* and *P. gingivalis*.

Antagonism between species from *Streptococcus* genera and late colonisers, especially *P. gingivalis,* has been described in clinical and *in vitro* studies (Wang et al., 2009; van Essche et al., 2013). Streak-cultures in section 3.3.3.1 on CBA pates confirmed the inhibition of *P. gingivalis W83* by some *Streptococcus* species. Literature suggests that the main mechanisms for bacterial antagonism are: secretion of bacteriocins and other substances, organic acid production, bacteriophage release and competition for essential nutrients (Marsh and Zaura, 2017). Sections 3.3.1 and 3.3.2 of this Chapter refer to the extensive ability of *S. oralis* to produce acid and decline in the environmental pH. Another mechanism of antagonism, tested in Chapter 3, was

the production of hydrogen peroxide. The culture of *Streptococcus* species on Prussian Blue agar in section 3.3.3.2 showed a strain dependent ability to produce H_2O_2 (Figure 3.6). Moreover, under certain growth conditions, streptococci can exhibit an increased production of H_2O_2. For example, Duran-Pinedo et al. observed that *Streptococcus* species increased the production of reactive oxygen species in the presence of *P. gingivalis* up to 25-fold compared to growth of the anaerobe alone (Duran-Pinedo et al., 2014a). This concentration can have antagonistic effects on a wide variety of isolates (Kreth et al., 2005; Jakubovics et al., 2008). Although *P. gingivalis* can show inducible adaptation under oxidative stress, prolonged contacts with hydrogen peroxide are lethal (McKenzie et al., 2012). In Chapter 3 only the abilities to reduce the pH and produce hydrogen peroxide by different streptococci were investigated, however, when considering the development of the model, one should take into account potential mechanisms of antagonism as well.

Antagonism between early and late colonisers in biofilms was observed when *S. oralis* ULCP98 was used for inoculation, at least in part due to low pH and hydrogen peroxide production. For these reasons, ULCP98 strain was replaced with a different *Streptococcus* (*S. salivarius* ULCP97) that, based on cultures incubated on Prussian Blue agar, produced less H_2O_2. It also did not inhibit the growth of *P. gingivalis W83* on streaked agar plates. *S. salivarius* species has the ability to produce ammonia in the presence of urea, thus increasing the environmental pH in dental plaque (Sissons and Hancock, 1993). Even though, the levels of pH in *S. salivarius* biofilm supernatants again fell significantly, especially in serum supplemented media, but they were substantially higher than in *S. oralis* biofilms (Figure 3.3 vs 3.4). Moreover, the pH did not fall below pH 6.1, and as described by Sissons et al., (1993) stayed within optimum limits for urease activity, which is between pH 5 and 8 (Sissons and Hancock, 1993). At pH 6.1, *S. salivariu*s is able to utilise arginine and urea to produce ammonia, thus substantially raising the pH (Sissons and Hancock, 1993). Experiments reported in this Chapter showed that in the presence of *S. salivarius*, the drop in pH was not substantial enough to inhibit the growth of *F. nucleatum*, *P. intermedia* and *P. gingivalis.*

Once the pH control was achieved resulting in the growth of a five-species community, it was demonstrated that the presence of serum boosted the growth of *Streptococcus* species, *A. naeslundii* and *P. gingivalis*. The enrichment of early colonisers might have been associated with the increase of fermentable carbohydrates in the growth environment through higher serum concentrations. Foetal bovine serum can contain 0.85-1.81 mg/mL glucose, and this would promote the growth of *Streptococcus* and *Actinomyces*. On the

other hand, *P. gingivalis* is asaccharolytic and would benefit from the high protein and haemin content in the growth medium with serum.

It should be mentioned that once *in vitro* model was established that supported stable communities of five species growing in biofilms, their susceptibility to certain antimicrobials was tested. Different concentrations of host defense peptides, such as histatin 5 and LL-37, were used in CBD model with *S. salivarius* + four species. However, significant effects of histatin 5 or LL-37 have not been recorded on biofilm growth in terms of viable load and community's composition. This data is consistent to the results reported by unpublished data by J. Kistler, however he cultured natural saliva in CBD to investigate certain antimicrobials. One of the reasons for absence of effect might be the property of host derived peptides to stick to plastics; and microtitre plates are made from polystyrene.

In this Chapter, the CBD was confirmed to be a suitable model for growing mixed culture biofilms of oral bacteria for prolonged periods of between 2-4 weeks. The composition of both the inoculum and the growth medium were shown to be critical factors in determining whether biofilms comprising a diverse community of oral bacteria would become established. Sequential inoculation of bacteria created issues; for example, if the environment became acidic too quickly due to the metabolism of fast-growing bacteria, then the inoculation of later colonisers was unsuccessful; however, serum could be supportive of the growth of proteolytic organisms. Likewise, some early colonisers were antagonistic towards obligately anaerobic species. Based on these pilot studies, therefore, it was decided to attempt to grow more complex biofilms but using a natural inoculum from health human volunteers and in which all micro-organisms were introduced into the system at day one, and the protein-rich medium was supplemented with serum to support the growth of fastidious organisms.

Chapter 4 Effect of nutrients on the enrichment of putative periodontal pathogens from biofilms pooled from healthy sites

4.1 Introduction

Bacteria reside in the mouth embedded in an extracellular matrix in spatially structured multispecies biofilms that are attached to mucosal or hard surfaces. These complex biofilm communities are in balance with the host supplying a local and systemic benefit while exploiting the local environment. Although regular minor perturbations in the local environment (for example, in terms of pH, nutrients, temperature etc.) are frequent, biofilm communities stay relatively stable over time (David et al., 2014).

The term ‘core microbiome’ describes the characteristic microbiota present in plaque from healthy individuals. This microbiota maintain the functional stability and homeostasis necessary for healthy ecosystem. Despite variations in the bacterial composition of the mouth among the healthy population, it has been reported that many bacterial species are common among unrelated individuals (Zaura et al., 2009). For instance, species from the genera *Streptococcus, Actinomyces, Rothia, Veillonella, Haemophilus* are believed to constitute the core oral microbiome in health. Diaz et al. summarised recent sequencing studies, which suggest there might be around 60 bacterial species implicated in health (Diaz et al., 2016).

However, under certain conditions, the bacterial composition of these communities can change, leading to the development of two main oral diseases: caries and periodontal disease. In case of caries, the metabolism of dietary carbohydrates and the subsequent rapid drop in pH leads to the enrichment over time of acidogenic and aciduric species. This drop in pH also dissolves the hydroxyapatite crystals in enamel and leads to caries (Marsh, 2003b).

The most common form of periodontitis is adult choric periodontitis. The development of disease is associated with the enrichment of Gram-negative, obligate anaerobic and proteolytic bacteria (Liu et al., 2012), which induce host inflammatory pathways and subsequent neutrophil recruitment into the periodontium. The primary objective of neutrophils is to eliminate the bacterial challenge by numerous mechanisms, mainly by phagocytosis, but also by

release of antimicrobial substances and formation of neutrophil extracellular traps. Paradoxically, this persistent neutrophil activity can lead to immune overreaction and chronic inflammation resulting in damaged host tissues (Cortes-Vieyra et al., 2016).

Conditions such as stress, smoking, alterations in diet, or simply aging, contribute to an impaired host defence and progression of periodontal disease (Kononen et al., 2007; Akcali et al., 2013; Kilian et al., 2016; Jentsch et al., 2017). At the same time, chronic inflammation manifests in high expression of inflammatory molecules, and an increased flow of gingival crevicular fluid (a protein-rich serum-like exudate). GCF delivers not only components of the host response but it also contains proteins and glycoproteins that can act inadvertently as nutrients and cofactors for proteolytic subgingival plaque bacteria. Thus, an escalation in the host response can lead to an increased risk of self-induced tissue damage. A continuous sequence of positive feedback loops drives a vicious circle of an ever increasingly damaging immune response to an adapting and 'inflammophilic' microbiota (Hajishengallis, 2014).

Although the differences in bacterial composition are well documented between periodontal health and disease, the drivers for this change are unclear. Several theories have been postulated to explain the change of biofilm from symbiotic to dysbiotic relationship with the host (Rosier et al., 2014). The 'Ecological Plaque Hypothesis' suggests that minor components of the microbial community can be enriched over time in response to a change in local environment that increases their competitiveness.

In Chapter 3, it was shown that an environment that simulates the inflammatory environment found in the periodontal pocket can benefit the growth of some Gram-negative bacteria (e.g. *P. gingivalis*) and lead to their increase in a five-species community. However, a simplified five-species model can only very superficially represent the complex relationship of competitiveness and synergy of a natural biofilm community. Consequently, a more diverse and natural inoculum has been used to investigate if nutritional changes to the local environment can alter the composition of the entire oral bacterial community.

This Chapter aims to investigate if putative periodontal pathogens that are present in low numbers in biofilms at healthy sites of dentally-healthy young adult volunteers can increase in abundance under nutritional and environmental conditions that favour their growth and also reflect the environment that occurs in inflamed periodontal pockets (Perez-Chaparro et al., 2014).

4.2 Aims

The aim of the experiment described in this chapter was to determine whether culturing oral samples from healthy adult volunteers as biofilms in growth media that mimic aspects of the subgingival pocket could lead to the enrichment of species that have been associated with periodontal disease.

The experiment consisted of a longitudinal analysis of biofilm communities cultured in two types of media and a comparison of their microbial composition and functional potential profile at two time points using metagenomic techniques.

Absolute counts of *P. gingivalis* in the inoculum and in biofilms were monitored using qPCR, whilst the pH of supernatants were measured throughout the experiment.

4.3 Results

Supragingival plaque, tongue scrapings and saliva were collected and pooled from systemically and dentally healthy young adult volunteers (mean age 31 ± 8 y, 50% male: 50% female) and cultured in protein-rich medium with and without serum (for methods see sections 2.3.1 – 2.3.2 and Figure 2.6). Longitudinal analysis on the biofilms that developed after one, two or three weeks of incubation was performed to investigate *P. gingivalis* absolute counts and pH change in the supernatants (section 2.3.2.2). In addition, some samples were submitted for paired-end read sequencing to investigate the effects of time and growth medium on total biofilm composition and potential functions as measured by metagenomic approaches, and compared to the inoculum (section 2.3.4).

4.3.1 qPCR and *P. gingivalis* counts

In order to monitor *P. gingivalis* counts in the inoculum and in week one – week three biofilms, a standard curve was generated for a range of concentrations of DNA, from 10 ng to 0.001 ng, in which the primers showed high linearity (R^2 = 0.99) (Appendix Figure 1). Inoculum and biofilm DNA samples were diluted to contain 10 ng of total DNA per reaction and were always in the linear range of the standard curve. The melting curve of the PCR product showed that only one product was found, proving that the primers neither formed dimers nor showed amplification of regions outside the target gene (data not shown).

The abundance of *P. gingivalis* in each sample was determined from Cp value and theoretical genome weight (Ammann et al., 2013b). Significant differences in terms of absolute *P. gingivalis* counts were detected between inoculum and biofilm samples, $p < 0.001$, Kruskal-Wallis test (Figure 4.1). Pooled inoculum samples had fewer *P. gingivalis* ($7.9\times10^4 \pm 1.9\times10^4$) than any type of biofilm. For example, absolute *P. gingivalis* counts in the inoculum were approximately 15 times lower compared with week one biofilms ± serum ($2\times10^6 \pm 1.1\times10^6$). After one week, biofilms cultured in protein-rich medium without serum had significantly lower numbers of *P. gingivalis* ($1.3\times10^6 \pm 4.9\times10^5$) compared with biofilms cultured in protein-rich medium with serum ($3\times10^6 \pm 6.6\times10^5$), $p < 0.05$. Similarly, higher counts of *P. gingivalis* were observed in week two and week three biofilms in protein-rich medium supplemented with serum than in protein-rich medium alone, $p < 0.05$. A gradual increase in *P. gingivalis* counts was observed between inoculum, week one and week two samples, $p > 0.05$. Week three biofilms had slightly fewer *P. gingivalis* than week two biofilms; however, these changes were not statistically significant, $p > 0.05$. There was no

significant difference between PMA treated and untreated samples in terms of *P. gingivalis* $\log_{10}$ counts, $p > 0.05$.

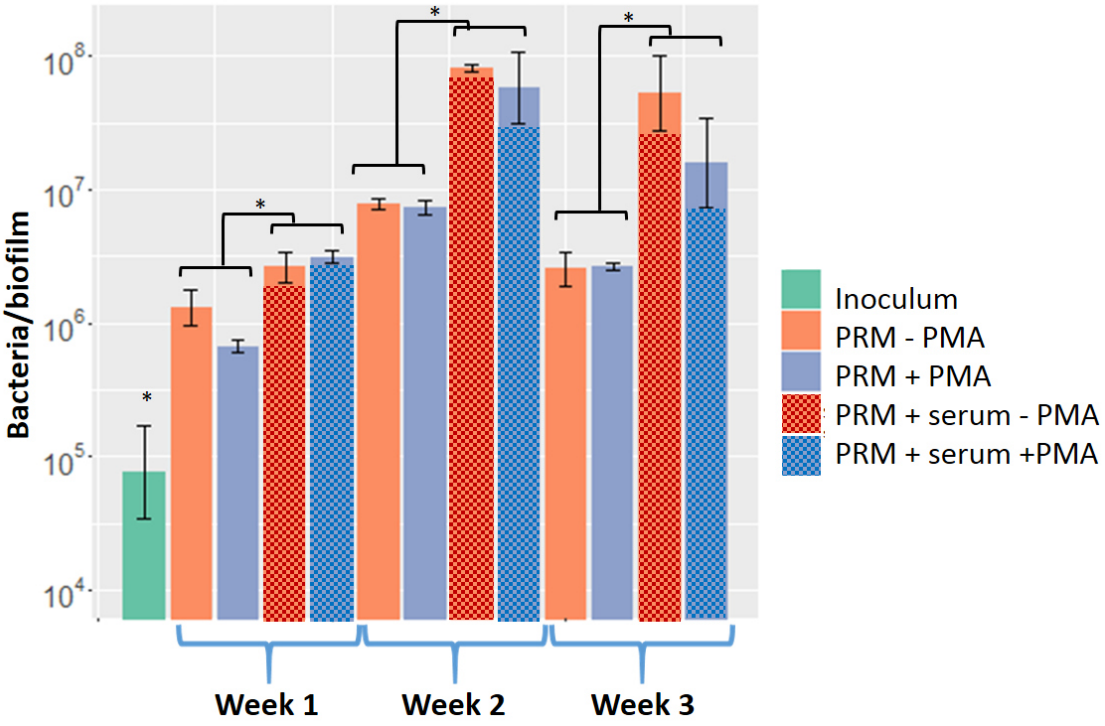

Figure 4.1. Abundance of *P. gingivalis* detected in inoculum and week one, week two and week three biofilms determined by qPCR.
Some samples were treated with propidium monoazide (PMA). The dotted bars represent biofilms cultured in protein-rich medium with 20% (v/v) serum, PRM – protein-rich medium. * - $p < 0.05$, *post hoc* after Kruskal-Wallis test, n = 3 individual experiments, with 3 technical replicates each.

4.3.2 pH changes in cultured biofilms

There were no significant changes in pH values between supernatants with and without serum and over time. Relatively stable pH values were observed throughout the experiment; however, there was a trend for a small rise in pH up to week 2.5 followed by a small decline at week three. These differences were not statistically significant ($p > 0.05$). Slightly lower values were observed in the supernatants where serum was used (Figure 4.2).

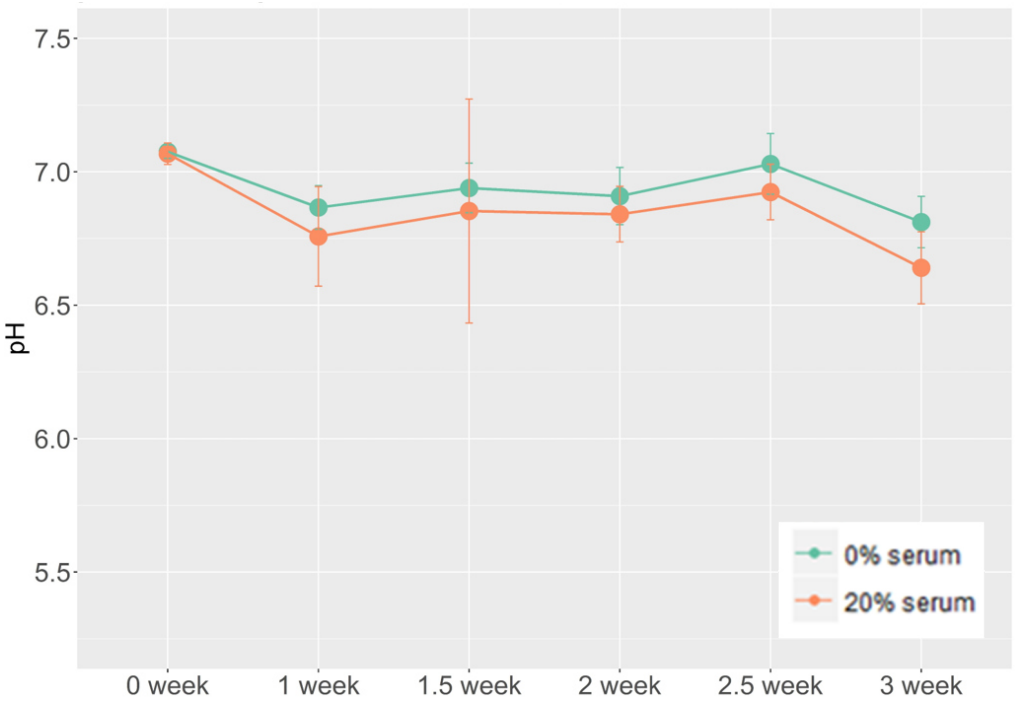

Figure 4.2. Changes in pH over time of the supernatants of the biofilms during the enrichment in protein-rich medium with and without serum. The pH was recorded before inoculation and after media changes, n = 3 individual experiments, with 3 technical replicates each.

4.3.3 The quality of sequenced samples

The DNA of inoculum and week one and week three biofilms were sequenced for a detailed analysis of the microbial composition and functional potential of these communities (see section 2.3.2 and Figure 2.6).

On average, 22.4 million sequences were obtained per sample (range of mean values 20.7 – 26 million). After quality processing and pair-end merging, the samples on average contained 22.5 million reads (range 16 – 25 million). About 0.5% of reads encoded for rRNA which corresponded to 690 OTUs at species level. Analysis of metagenomes with MEGAN showed that obtained sequences represented 659 OTUs through taxonomy levels and 4490 functionally annotated genes of bacterial origin. Only 28% - 35.5% of reads were assigned with a functional role.

4.3.4 Adequate coverage of metagenomes confirmed by Nonpareil curves

Nonpareil curves were used to determine the sequencing depth of the samples. They clustered in five groups, reflecting different levels of diversity between different samples (Figure 4.3). The curves indicated that the inoculum communities had the highest sequence complexity as the highest number of reads was required to reach the coverage for these samples. For the inoculum

samples, 75% ± 2.9 coverage was recorded. Biofilm communities cultured for three weeks in protein-rich medium displayed the lowest sequence complexity and displayed a coverage of 94% ± 0.9.

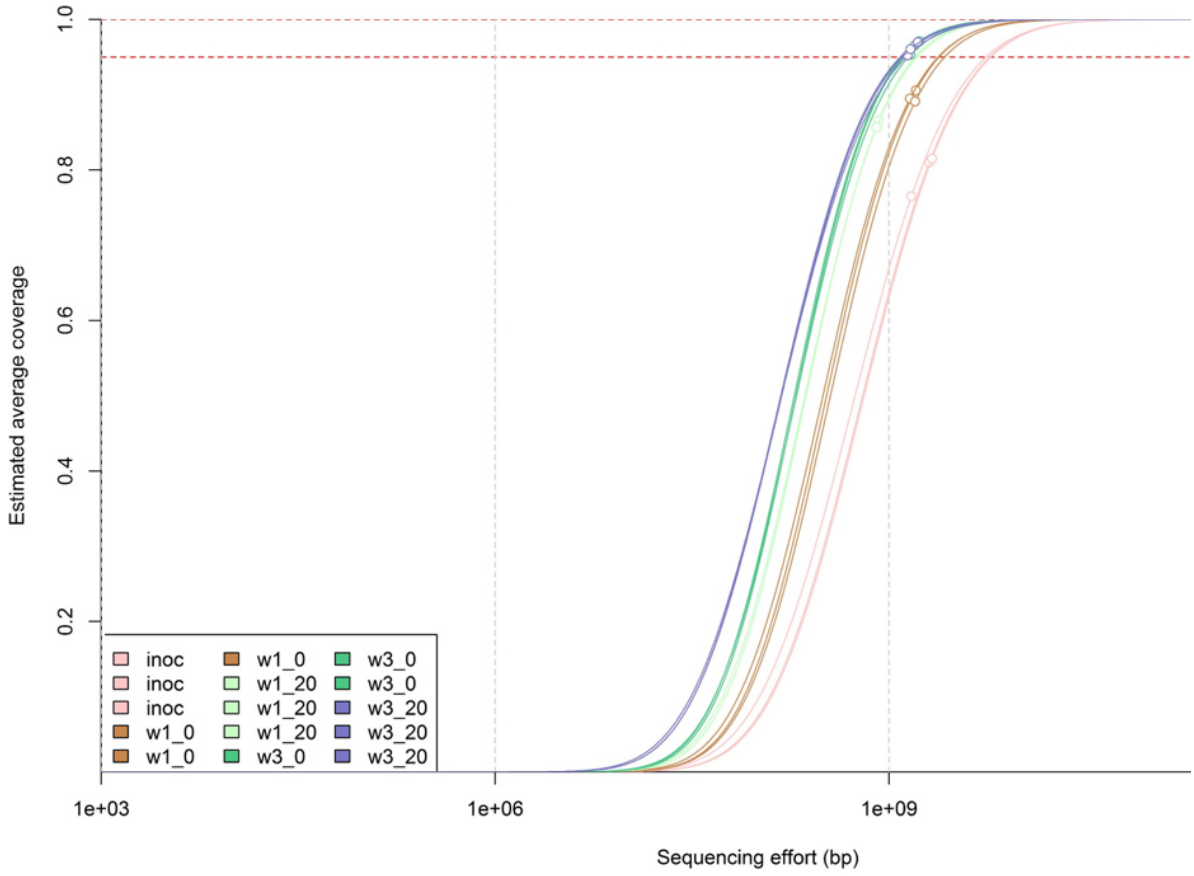

Figure 4.3. Comparison of nonpareil curves for metagenomes of inoculum and biofilm communities.
The horizontal dashed lines indicate 100% (pink) and 95% (red) coverage. The empty circles indicate the size of estimated average coverage of the datasets, and the lines after that point are projections of the fitted model. The rightmost group of inoculum represents samples largely dominated by a diversity of species. In the legend inoc – inoculum samples, w1 – week one, w3 – week three, 0 and 20 represent the supplementation with serum in the growth media.

4.3.5 Sequencing depth based on 16S rRNA

Rarefaction curves were generated for 16S rRNA data (at 1.5% distance) to evaluate if substantial sequencing depth was achieved. The samples were randomly rarefied to the depth of the smallest library size that accounted for 7672 reads (Figure 4.4).

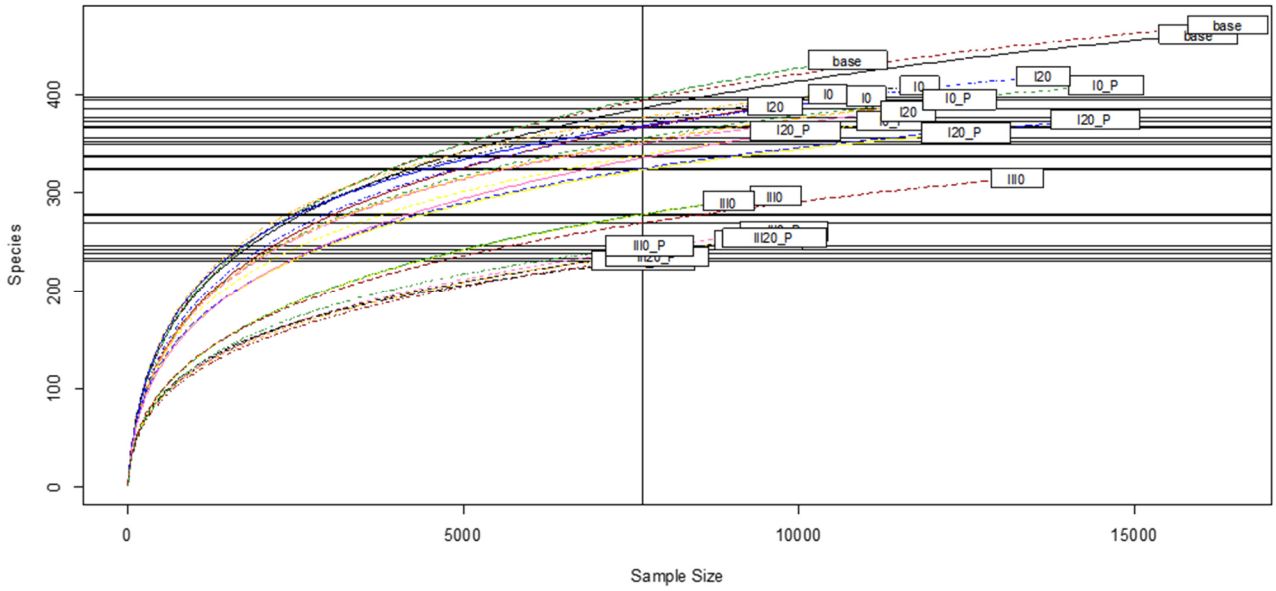

Figure 4.4. Rarefaction curves of 24 inoculum and biofilm samples based on 16S rRNA genes.
The legend on the curves indicates the corresponding sample. Curves show how many reads per sample have to be sampled to detect species on y axis. Base – inoculum samples, I – week one biofilms, III – week three biofilms, 0 – no serum in growth media, 20 – supplementation with 20% (v/v) serum, P - treatment with propidium monoazide.

All sample groups reached the asymptotes, which suggests that sample OTUs represent substantial bacterial diversity and sufficient sequencing depth. Rarefaction of 16S rRNA data showed similar trends of those observed in nonpareil curves: inoculum samples displayed the highest richness while three-week old biofilms of revealed the lowest.

4.3.6 Microbial composition of the inoculum

At the phylum level, the inoculum (comprising pooled tongue, supragingival plaque and saliva from healthy volunteers) was rich in *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Figure 4.5). The inoculum contained high proportions of species from the genera *Streptococcus* (23% – 27% of total relative abundance) and *Veillonella* (7.7% – 8.3%). Other representatives from the following genera were also prevalent: *Haemophilus* (6.7% – 7.2%), *Neisseria* (6.4 – 7%), *Actinomyces* (3%), *Prevotella* (12.3% - 14.2%) and *Alloprevotella* (7.7% - 8.5%). At the species level, *S. salivarius, S. cristatus, S. sanguinis*, *H. parainfluenzea* and *Prevotella melaninogenica* were the most abundant. In contrast, only low levels of members of genera associated with periodontal disease, such as *Porphyromonas*, *Filifactor, Tannerella* and *Treponema* were detected in the inoculum.

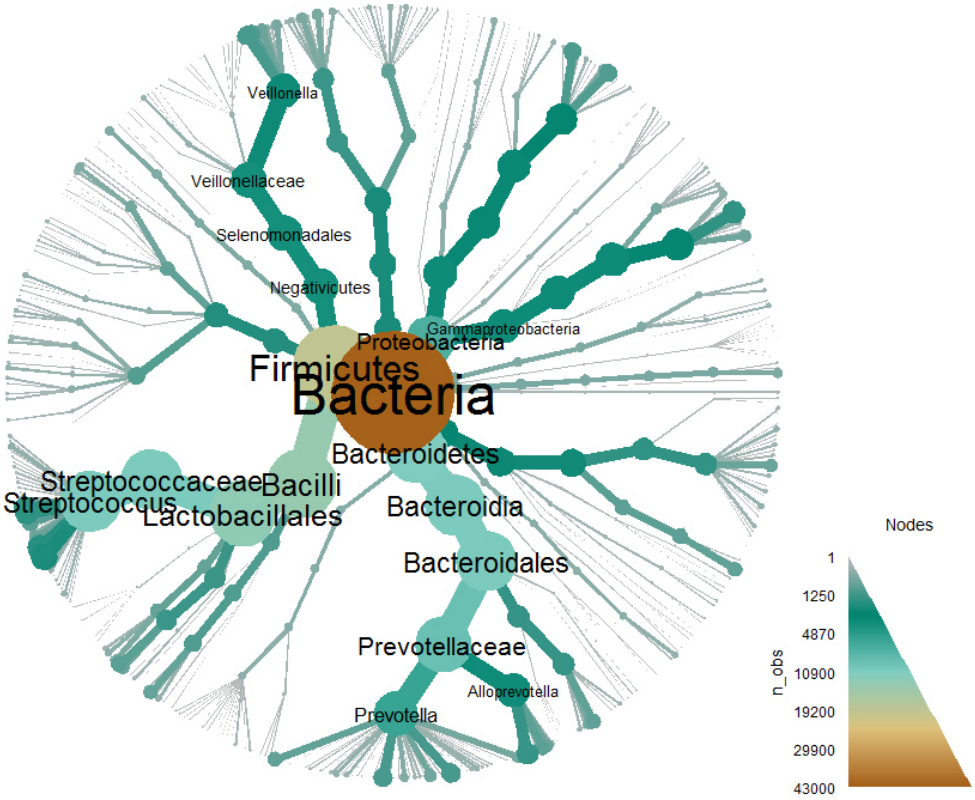

Figure 4.5. The taxonomic structure of the inoculum.
Heat tree represents all taxa as nodes while colour and size are used to represent the abundance of 16s rRNA data. Each node represents a taxon. The position in the circle indicates the level in the taxonomic hierarchy and shared branches indicate common ancestry taxon. The most abundant taxa are indicated on the branches.

4.3.7 Richness and diversity of inoculum and biofilms

Species richness and diversity were compared between inoculum and biofilms after one or three weeks of enrichment using metagenomic data, provided by MEGAN analysis. Species richness refers to the total number of observed OTU in a sample or in a sample group while species diversity describes how evenly the individual OTUs are distributed in the sample.

4.3.7.1 Differences in species richness between inoculum biofilms

Species richness, or observed number of species, was significantly higher in the inoculum than in week one or week three biofilms, $p < 0.001$, ANOVA (Figure 4.6). On average, the inoculum contained 161 ± 23 unique species, while week one and week three biofilms consisted of subsequently lower numbers of unique species. Biofilms cultured in the protein-rich medium with serum had slightly lower numbers of unique species compared to those cultured in protein-rich medium alone; however, these differences were not statistically significant, $p > 0.05$.

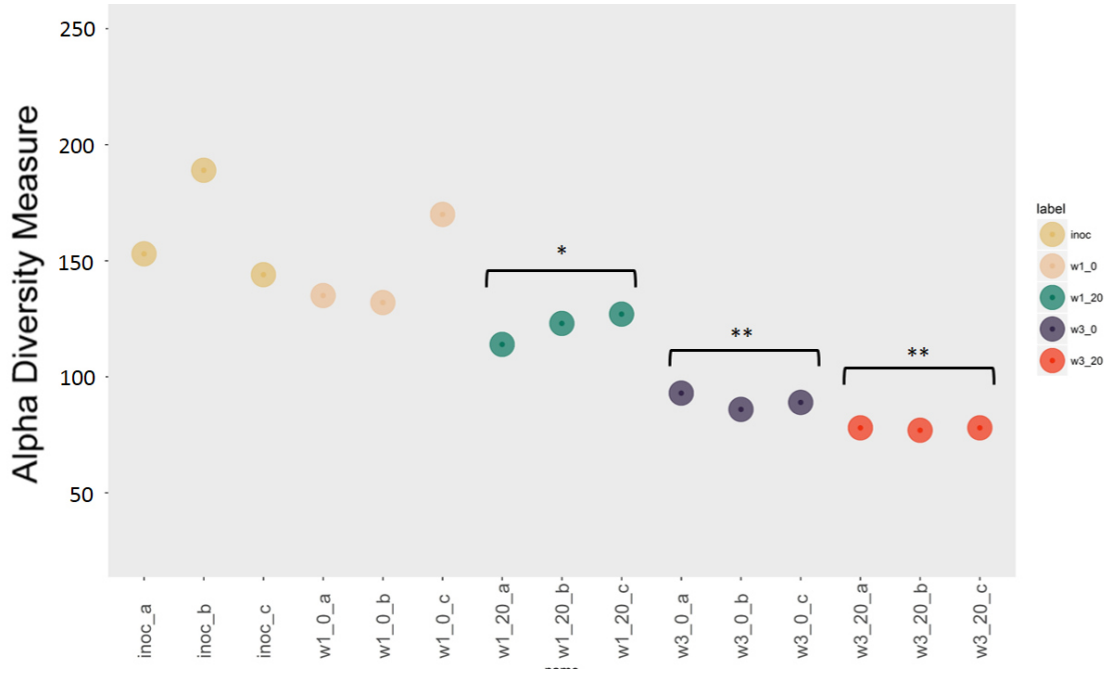

Figure 4.6. Comparison of observed number of species between inoculum and biofilms using metagenomics data.
The number of unique observed species were compared between inoculum and biofilms of week one and three. Inoc – inoculum. w1 – week one biofilms, w3 – week three biofilms, a. b, c, - biological replicates, 0 and 20 show the percentage of serum in PRM. * - $p < 0.05$, ** - $p < 0.01$, the data were analysed using HSD test. The signs mark significant differences to the inoculum.

4.3.7.2 Difference in diversity between inoculum and biofilms

The richness of bacterial communities was estimated by the Chao index and the diversities of these communities were determined using the Shannon and Inverse Simpson indexes (Table 4.1). Metagenomic and 16S rRNA data were used for the comparison. Higher diversity and richness were estimated for inoculum samples when compared with biofilms, especially if biofilms were cultured in protein-rich medium without serum. Week three biofilms had a lower species diversity than week one biofilms and inoculum samples, $p < 0.05$, ANOVA test. In summary, the analysis revealed that the inoculum consisted of many different and more equally abundant bacterial species, and over time the richness and diversity were lost. The same trends were observed between 16S rRNA and metagenomic data, however, slightly higher index values were observed for 16S rRNA data.

Table 4.1. Diversity analysis of inoculum and biofilm samples using 16S rRNA and metagenomics data.
Three diversity indexes of inoculum and biofilms were compared between 16S rRNA and metagenomes. w1 – week one, w3 – week three. Numbers (0 or 20) indicate the percentage of serum in a protein-rich medium, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, *post hoc* after Kruskal-Wallis compared to inoculum.

	16S rRNA (1.5% distance)			Metagenomics		
Sample	Chao	Shannon	Inverse Simpson	Chao	Shannon	Inverse Simpson
inoculum	555.5 ± 14.6	4.7 ± 0.02	59.4 ± 2.3	428.3 ± 49.9	3.7 ± 0.1	16.4 ± 1.2
w1_0	463.5 ± 19.7 ***	4.48 ± 0.1	39.2 ± 2.4 ***	368.3 ± 44 ***	4 ± 0.1	26.8 ± 3.1 **
w1_20	457.3 ± 48.4 ***	4.46 ± 0.1	42.9 ± 2.5 *	327 ± 15.7 ***	3.8 ± 0.1	21.5 ± 1.6 **
w3_0	324.3 ± 19.4 ***	3.90 ± 0.1	22.5 ± 2.6 ***	280.3 ± 6 ***	3.6 ± 0.1	15.7 ± 1.4
w3_20	312.3 ± 24.6 ***	3.82 ± 0.1 *	19.6 ± 1.6 ***	281.3 ± 4 ***	3.6 ± 0.1	16 ± 0.6

4.3.7.3 Phylogenetic distance comparison using Unifrac clustering ordination

UniFrac distances were used to summarise the phylogenetic variability of the samples among different culturing conditions using 16S rRNA data at 98.5% sequence similarity. Before analysing, samples were rarefied to 7672 reads per sample to minimise the impact of difference in sequencing depth. Unweighted UniFrac distances (evaluates species presence or absence) and weighted UniFrac distances (evaluates species abundance) were measured. Comparison of the bacterial community structure of the inoculum and biofilms

using UniFrac weighted distance were visualised using PCoA (principal coordinate analysis) (Figure 4.7). A small variation between experimental replicates was observed. Also, PMA treated and untreated samples clustered together showing that PMA treatment had had little effect on the detection of the bacterial composition of biofilms. For this reason we mainly concentrated on the analysis of PMA treated samples as we were interested in the DNA from intact cells. Application of a*donis* test on weighted UniFrac distances found overall significant differences between inoculum, week one and week three biofilms, $p < 0.05$.

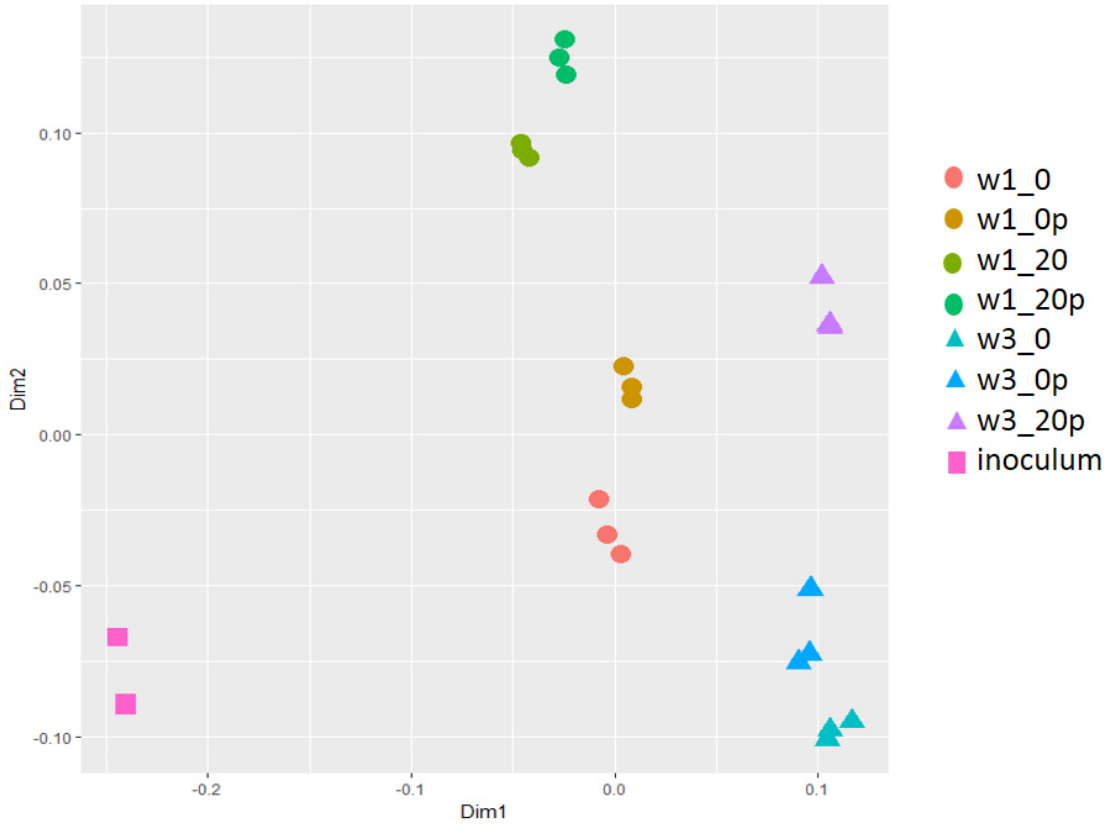

Figure 4.7. PCoA of inoculum and biofilms based on weighted UniFrac distances.
Squares represent inoculum, circles – 1st week, triangles – 3rd week samples. In a key a number means the percentage of serum in a growth medium, w1 – week 1, w3 – week 3, 0 – protein-rich medium, 20 – protein rich medium with 20% serum, p stands for PMA treatment.

4.3.7.4 Microbial composition of inoculum and biofilms

To characterize the microbial community structure of the inoculum and biofilms, a taxonomic analysis was conducted using the metagenomes of samples. Prior to the analysis the samples were rarefied to a depth of 1,827,864 reads per sample (because it was the lowest number of the reads obtained in one of the samples). *Firmicutes and Bacteroidetes* had the highest combined abundance

in both inoculum and biofilms, accounting for 58.7 ± 6.3% of the reads in the analysed samples (Figure 4.8). However, following enrichment in different media, there was a shift in the distribution of phyla. For example, the inoculum had significantly higher proportions of *Actinobacteria, Proteobacteri*a and *SR1* and lower proportion of *Synergistetes* in comparison with biofilms, $p < 0.05$, HSD Tukey test. The presence of serum enhanced the growth of representatives from the *Bacteroidetes* phylum, $p < 0.05$. In contrast, biofilms cultured in protein-rich medium without serum consisted of higher proportions of *Proteobacteria* and *Fusobacteria* compared with those cultured with serum, $p < 0.05$. Presence of serum also reduced the growth of the genus *TM7*.

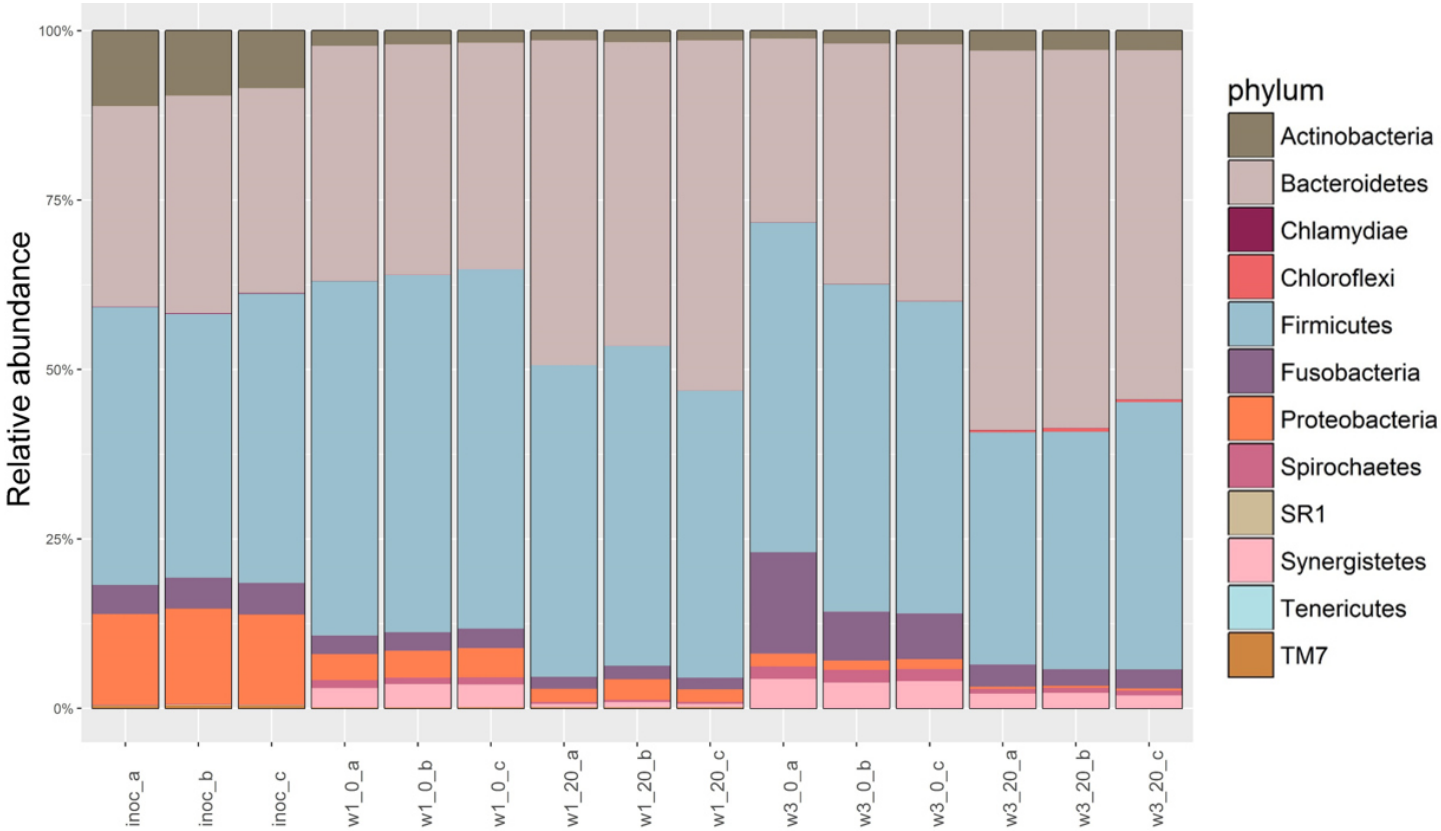

Figure 4.8. Distribution of phyla in inoculum and biofilms cultured for one and three weeks in protein-rich medium with and without serum.
The number of reads that were annotated to the *Bacteria* domain was designated as 100%. Not all phyla are presented in a visible level in all bars, so they are shown in the order in which they are laid out in the key. In the legend, w1 – week one, w3 – week three biofilms, and number indicates the percentage of foetal bovine serum in the protein-rich medium, a, b, c - biological replicates.

4.3.7.5 Differences between samples at species level

First, the effect of PMA treatment was investigated on bacterial composition of biofilms. The relative abundance between PMA-treated and -untreated biofilms were similar for the majority of the species. Only some species were less abundant in PMA-treated than PMA-untreated samples, for instance species from *Streptococcus* genus. However, these differences did not have any clear pattern and for this reason we investigated mainly PMA treated biofilms that represented DNA from intact cells at the time of DNA isolation.

As previously demonstrated in section 4.3.6, the inoculum was over-represented with Gram-positive species that are usually detected in the healthy oral cavity, while in general the cultured biofilms were over-represented with Gram-negative and fastidious species. The changes in relative abundance of species in the inoculum and biofilms are shown in Figures 4.9 and 4.10, Tables 4.2 and 4.3. DESeq2 package in R was used to determine the significance in changes in relative abundance between different conditions, p adjusted < 0.05, Wald test.

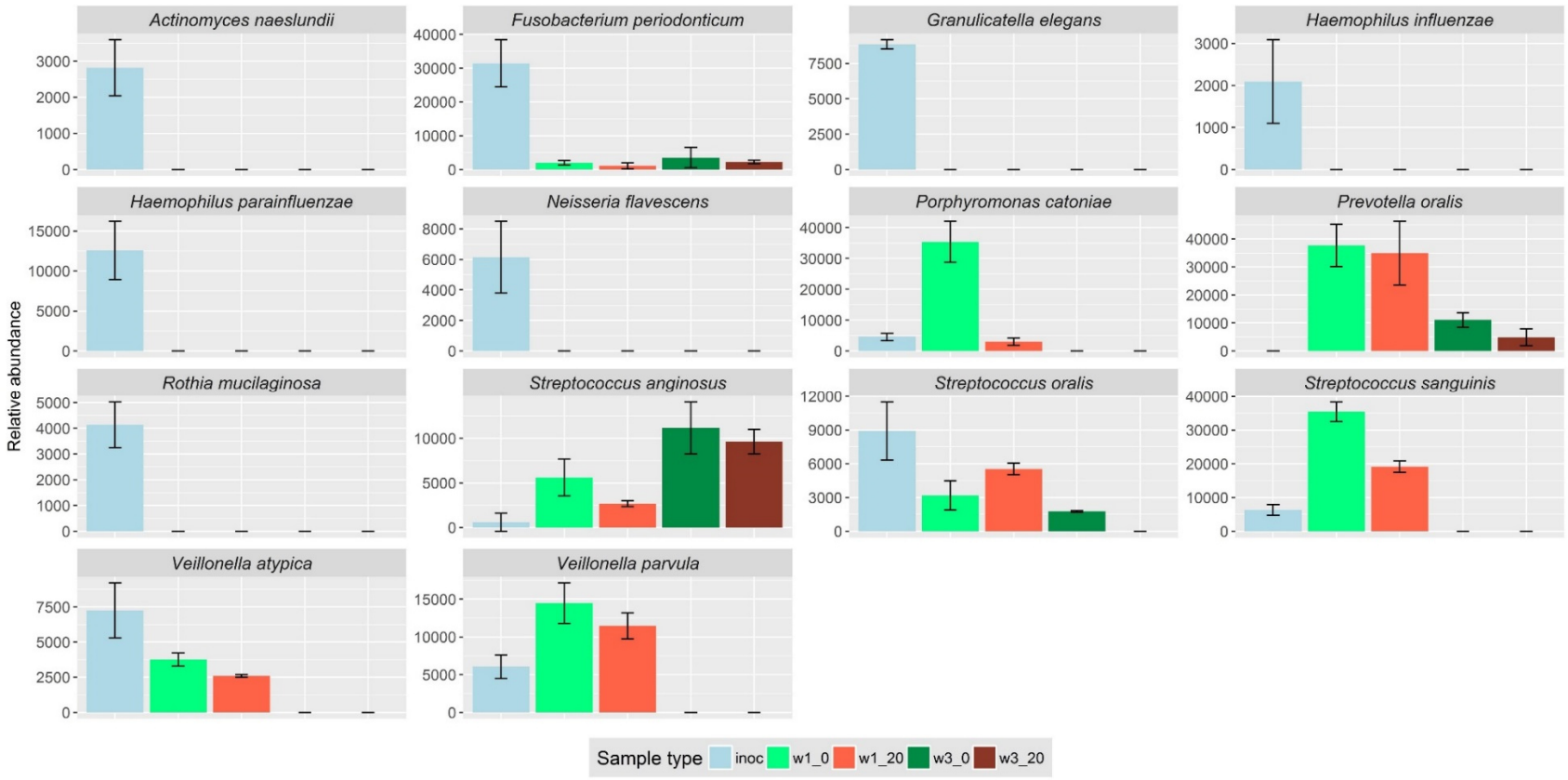

Figure 4.9. Difference in relative abundance of selected bacterial species associated with oral health following enrichment in a protein-rich medium with or without supplementation with 20% (v/v) serum.
Graphs summarise the changes in relative abundance of species between the inoculum and biofilms following enrichment on a protein-rich medium with or without serum at week one and week three. In the legend, w1 – week one biofilms, w3 – week three biofilms, and number indicates the percentage of foetal bovine serum in the protein-rich medium.

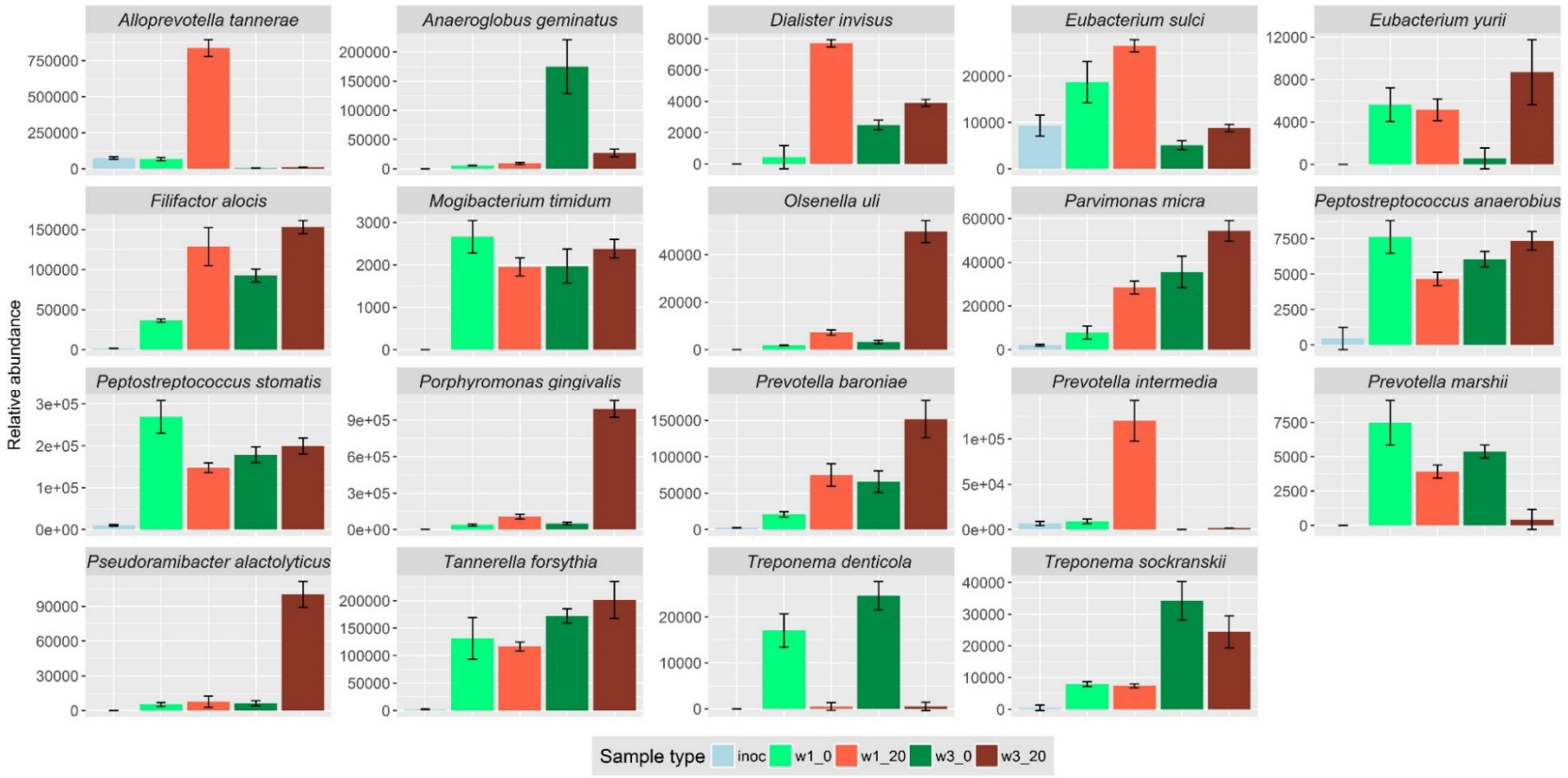

Figure 4.10. Difference in relative abundance of selected species implicated in periodontal disease following enrichment in a protein-rich medium with or without supplementation with 20% (v/v) serum.
Graphs summarise the changes in relative abundance of species between the inoculum and biofilms following the enrichment in a protein-rich medium with or without serum after week one and week three. In the legend, w1 – week one, and w3 – week three biofilms, the number indicates the percentage of foetal bovine serum in the growth medium. Other changes are listed in Table 4.2 and 4.3.

4.3.7.6 Week one biofilms

The comparison of relative abundances of biofilm species cultured in protein-rich medium and protein-rich medium supplemented with serum at week one revealed distinct bacterial profiles between those conditions. Biofilms cultured in protein-rich media were over-represented with species from *Streptococcus* (*S. sanguinis, S. anginosus, S. gordonii*), *Veillonella* (*V. parvula, V. atypica, V. dispar*), *Prevotella* (*P. salivae, P. oris, P. buccae*) genera, $p < 0.05$, Wald test (Figure 4.9). In contrast, biofilms cultured in protein-rich medium with serum were over-represented with species from genera *Dialister* (*D. invisus*), *Alloprevotella* (*A. tannerae*) and *Eubacterium* (*E. brachy, E. sulci*) together with *P. gingivalis* and *F. alocis,* $p < 0.05$, Wald test (Figure 4.10).

4.3.7.7 Week three biofilms

The same comparison was performed on biofilms cultured for three weeks. The presence of serum in the growth medium resulted in enrichment for *P. gingivalis, F. alocis, P. micra, Eubacterium infirmum* and *Prevotella baroniae* species, $p < 0.05$, Wald test (Figure 4.10). If biofilms were cultured in protein-rich medium only, there was over-abundance of *Anaeroglobus geminatus, T. denticola* and *Prevotella marshii,* $p < 0.05$, Wald test (figure 4.10).

Some species that had high abundance after one week of enrichment were found to be less prominent after three weeks growth, for example, *A. tannerae*, *E. sulci* and *P. marshii*.

4.3.7.8 Changes in OTU relative abundance over time

Many health associated species decreased in relative abundance in biofilms (Figure 4.9). Some of the species that are health associated were high in abundance in the inoculum and gradually decreased over time (*S. oralis, V. atypica*). *Porphyromonas catoniae*, *V. parvula, S. sanguinis* grew competitively at week one but their relevant abundance decreased at week three. Many species that were high in abundance in the inoculum were not detected in the biofilms (for example, species from the *Haemophilus, Neisseria, Rothia* and *Actinomyces* genera). In contrast, 86 species were found in biofilms that could not be detected in the inoculum (Appendix Table 1). Many of these species are fastidious, and included *Jonquetella anthropi*, *Desulfovibrio desulfuricans*, *D. invisus*, *Treponema maltophilum, T. denticola* and *P. marshii*.

The enrichment of species in either protein-rich medium or protein-rich medium with serum is summarised in Table 4.2. The analysis showed that the absence of serum in the growth medium was associated with the enrichment of species from *Prevotella* and *Streptococcus* genera at both time points. The presence of

serum in the growth medium was associated with the enrichment of periodontitis-associated species such as *F. alocis, P. gingivalis*, *P. micra* and species from *Prevotella* and *Eubacterium* genera.

The analysis in sections 4.3.7.1. and 4.3.7.2 showed that during culturing, the bacterial diversity decreased and small proportion of species that constituted a minor part of the inoculum became predominant in the biofilms. These data are summarised in Table 4.3 which shows how relative abundance of species changed over time in both media. In total, 19 species were over-represented in both media at week three in comparison with week one. These species included *T. sokranskii, Tannerella forsythia, P gingivalis, F. fastidiosum* and *P. baroniae* and many of them were of low abundance in the inoculum (Figure 4.10). The table also shows that serum was beneficial for the growth of some of these species, for instance *P. gingivalis, F. fastidiosum* or *J. anthropi*. At week three, 39 species were less abundant than in week one in both media. These species were from *Prevotella, Streptococcus* and *Veillonella* genera, $p < 0.05$, Wald test.

Table 4.2. Species enriched at week one and week three in protein-rich medium with and without 20% (v/v) serum.
Relative abundances of biofilm bacteria were compared between different culturing conditions. The table shows only the species that increased in abundance in the medium at both time points. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, Wald test; a – PRM – protein-rich medium.

Enriched in PRM[a]	Log_2 fold increase		Enriched in PRM + serum	Log_2 fold increase	
Species	**Week 1**	**Week 3**	**Species**	**Week 1**	**Week 3**
Oribacterium sp. OT 078	0.7*	1.3***	*Prevotella intermedia*	4***	10.8***
Catonella morbi	0.7*	1.8***	*Porphyromonas gingivalis*	1.7***	4.3***
Fusobacterium nucleatum	0.8***	2.2***	*Olsenella uli*	2.2***	4.0***
Prevotella oulorum	0.8***	12.2***	*Alloprevotella tannerae*	3.9***	1.9***
Campylobacter showae	0.8**	11.8***	*Eubacterium infirmum*	1.8***	1.4***
Peptoanaerobacter stomatis	1.3***	1.4***	*Mogibacterium sp. CM50*	0.8***	1.2***
Fretibacterium fastidiosum	2.1***	0.9***	*Prevotella baroniae*	2.1***	1.2***
Prevotella saccharolytica	3.2***	12.4***	*Eubacterium sulci*	0.8***	0.8**
Bacteroidetes bacterium OT 272	10.1***	1.6***	*Filifactor alocis*	2.1***	0.7***
Pyramidobacter sp. C12-8	10.4***	1.0***	*Parvimonas micra*	2.1***	0.6*
Jonquetella anthropi	10.8***	1.0***	*Dialister invisus*	11.7***	0.5*
Streptococcus intermedius	11.6***	0.8***			
Phocaeicola abscessus	11.6***	1.9***			

Table 4.3. Species enriched at week thee in contrast with week one in biofilms grown in protein-rich medium with and without 20% (v/v) serum.

The change in relative abundance was recorded for species that were enriched for one or three weeks. Only the species that increased or decreased in their relative abundance over time in all three replicates are provided in the table. * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, Wald test.

Increased over time (week 1 → week 3)	**Log_2 fold increase**		**Decreased over time (week 1 → week 3)**	**Log_2 fold decrease**	
Species	**PRM**	**PRM + serum**	**Species**	**PRM**	**PRM + serum**
Eggerthia catenaformis	6.3***	5.2***	*Streptococcus sanguinis*	14.8***	14.5***
Anaeroglobus geminatus	5.2***	1.4***	*Porphyromonas catoniae*	14.7***	11.8***
Eubacterium nodatum	3.5***	1.2***	*Porphyromonas sp.* OT 278	14.7***	14.5***
Phocaeicola abscessus	2.9***	13.6***	*Coriobacteriaceae bacterium* 68-1-3	14.0***	14.1***
Bacteroidetes bacterium OT 272	2.8***	12.0***	*Veillonella parvula*	13.6***	13.8***
Eubacterium infirmum	2.3***	1.9***	*Prevotella salivae*	13.4***	12.3***
Parvimonas micra	2.3***	0.8***	*Prevotella nigrescens*	13.3***	14.9***
Treponema socranskii	2.2***	1.6***	*Prevotella intermedia*	13.0***	6.4***
Prevotella baroniae	1.8***	0.8*	*Veillonella tobetsuensis*	12.5***	12.5***
Stomatobaculum longum	1.3***	1.0***	*Prevotella denticola*	12.3***	13.0***
Streptococcus anginosus	1.1**	1.7***	*Prevotella oris*	12.0***	13.3***
Olsenella uli	0.9***	2.6***	*Leptotrichia sp.* OT 215	12.0***	12.3***
Jonquetella anthropic	0.7**	11.6***	*Veillonella atypica*	11.9***	11.9***
Johnsonella ignava	0.7**	11.3***	*Candidatus Saccharibacteria* OT TM7	11.9***	13.8***
Peptostreptococcaceae bacterium OT 113	0.5***	2.1**	*Veillonella dispar*	11.7***	11.5***

Fretibacterium fastidiosum	0.5***	1.7***	*Veillonella sp*. DORA_A_3_16_22	11.6***	11.9***
Tannerella forsythia	0.5**	0.6***	*Selenomonas sp.* OT 126	11.6***	12.2***
Porphyromonas gingivalis	0.5*	3.0***	*Campylobacter curvus*	11.4***	11.8***
Pyramidobacter sp. C12-8	0.4***	10.9***	*Oribacterium sp.* OT 108	11.4***	12.3***
			Oribacterium asaccharolyticum	11.3***	12.8***
			Prevotella micans	11.1***	12.7***
			Streptococcus gordonii	11.0***	11.2***
			Prevotella falsenii	10.8***	11.8***
			Veillonella sp. DORA_B_18_19_23	10.8***	11.5***
			Alloprevotella tannerae	4.3***	6.4***
			Porphyromonas endodontalis	3.9***	3.9***
			Oribacterium parvum	3.7***	14.5***
			Prevotella saccharolytica	3.7***	13.8***
			Prevotella buccae	3.2***	1.7***
			Prevotella sp. KH2C16	2.2***	3.6***
			Eubacterium sulci	1.7***	1.8***
			Prevotella oralis	1.6***	2.9***
			Mogibacterium pumilum	1.4***	2.5***
			Bacteroidales bacterium WCE2008	1.2**	1.0***
			Desulfovibrio desulfuricans	1.2***	12.6***
			Parvimonas sp. OT 110	1.2***	3.1***
			Bulleidia extructa	0.8***	12.0***
			Streptococcus pneumoniae	0.8*	12.3***
			Streptococcus oralis	0.6*	12.9***

4.4 Functional potential analysis

In total, 4490 functions were assigned to the samples. Only 28 - 35% ± 2.5% of reads were assigned to a functional role.

4.4.1 Differences in functional potential between inoculum and biofilms

Biofilms were over-represented with genes associated with motility, protein, and phosphorus metabolism. Biofilms had relatively higher proportions of genes coding for proteins involved in methanogenesis, flagellar motility, chemotaxis, haem biosynthesis and metabolism, transport of iron, p adjusted < 0.05, Wald test. Inoculum samples had higher proportions of genes implicated in carbohydrate, secondary metabolism and stress response, and in particular were over-represented with genes associated with sucrose utilization, lactose, fructose, mannose metabolism and metabolism of Gram-positive bacteria.

The presence of serum in the growth medium was associated with the enrichment of genes coding for protein metabolism, motility and chemotaxis. Week one biofilms cultured in protein-rich medium with serum were over-represented with genes associated with conjugative transposons, DNA repair, proteolysis, iron metabolism, multidrug efflux pump, lipopolysaccharide assembly and iron acquisition when compared with biofilms cultured in protein-rich medium. Week three biofilms were over-represented with the similar genes, for instance, conjugative transposons, protein degradation, and also represented with genes associated with haem and iron metabolism, cobalamin synthesis, methanogenesis, chemotaxis (gliding), lipoic acid metabolism, lipoprotein biosynthesis and oxidative stress (Table 4.4).

In contrast, biofilms cultured in the unsupplemented protein-rich medium had higher abundance of genes associated with carbohydrate and secondary metabolism, and membrane transport compared with biofilms cultured in protein-rich medium with serum, p adjusted < 0.05. Week one biofilms cultured in protein-rich medium had a high relative abundance of genes associated with sugar utilization, lactose and galactose uptake, sialic acid metabolism, methionine synthesis, B12 biosynthesis, fructooligosaccharides and raffinose utilization, histidine biosynthesis. Week three biofilms were over-represented with lysine biosynthesis, glutamine, glutamate, aspartate and asparagine biosynthesis, fermentation of sugars, histidine biosynthesis.

Table 4.4. Summary of over-represented genes associated with functions in inoculum and week-three biofilms enriched in protein-rich medium with and without supplementation of serum.
Samples of the inoculum were compared with week-three biofilms cultured in protein-rich medium with and without serum with DESeq2 to see which genes coding for proteins were over-represented. PRM – protein-rich medium, * - $p < 0.05$, ** - $p < 0.01$ *** - $p < 0.001$, Wald test.

Comparison between inoculum and biofilms in PRM			
Over-represented in inoculum		**Over-represented in PRM**	
Functional group	**Fold change (log_2)**	**Functional group**	**Fold change (log_2)**
Secondary Metabolism	0.96***	Protein Metabolism	0.08***
Regulation and Cell signalling	0.28***	Respiration	0.12***
General Stress Response and Stationary Phase Response	0.27***	Fatty Acids, Lipids, and Isoprenoids	0.21***
Potassium metabolism	0.26**	Phosphorus Metabolism	0.22***
Nitrogen Metabolism	0.18***	Metabolism of Aromatic Compounds	0.25***
Sulphur Metabolism	0.16***	Nucleotide sugars	0.33***
Stress Response	0.14***	Motility and Chemotaxis	0.44***
Cell Wall and Capsule	0.13***	Arabinose Sensor and transport module	0.76***
Cell Division and Cell Cycle	0.11***		
Comparison between inoculum and biofilms in PRM with serum			
Over-represented in inoculum		**Over-represented in PRM with serum**	
Functional group	**Fold change (log_2)**	**Functional group**	**Fold change (log_2)**
General Stress Response and Stationary Phase Response	1.97**	Arabinose Sensor and transport module	1.19***
Secondary Metabolism	1.35***	Phages, Prophages, Transposable elements, Plasmids	0.55***
Regulation and Cell signalling	0.39***	Phosphorus Metabolism	0.38***
Central metabolism	0.21**	Metabolism of Aromatic Compounds	0.34***
Cell Wall and Capsule	0.18***	Nucleotide sugars	0.27***
Membrane Transport	0.18***	Polyamines	0.26***

Potassium metabolism	0.17***	Motility and Chemotaxis	0.22***
Nitrogen Metabolism	0.15***	Transcriptional regulation	0.19***
Sulphur Metabolism	0.15***	Thiamin	0.18***
Stress Response	0.14***	Fatty Acids, Lipids, and Isoprenoids	0.17***
Metabolite damage and its repair or mitigation	0.13***	Cofactors, Vitamins, Prosthetic Groups, Pigments	0.11***
Cell Division and Cell Cycle	0.11***	Respiration	0.11***
Carbohydrates	0.09***	Protein Metabolism	0.10***
		Dormancy and Sporulation	0.09***
		Miscellaneous	0.08***
		DNA Metabolism	0.06***
		Virulence	0.03***

4.5 Discussion

The oral microbiome exists as complex multi-species biofilms on oral surfaces. Although relatively stable in health, these biofilms can dramatically shift in their composition in disease. Experiments in Chapter 3 demonstrated the interplay between five-species microbial biofilms and different growth media in an *in vitro* biofilm model, and showed that serum could promote the selection of *P. gingivalis*. A more complex *in vitro* model was developed in Chapter 4 to culture diverse multi-species bacterial communities. This was to evaluate whether an inoculum consisting of supragingival plaque, tongue biofilms and saliva from healthy individuals could be enriched for bacteria implicated in periodontal disease in an environment that attempts to simulate aspects of the inflamed periodontal pocket (anaerobic atmosphere, high protein content and lack of sucrose in the growth environment, etc). An established metagenomic analysis pipeline was used for an in-depth characterisation and comparison of the inoculum and biofilms that developed under different conditions. The data presented here show the ability of the model to sustain complex communities derived from a natural and relevant inoculum, and to measure differential responses to culturing conditions in a longitudinal study. Subsequent changes in biofilm microbiota and potential functions over time were evaluated in two media types.

The biofilms in healthy oral areas harbour different bacteria than those from the periodontal pocket, the latter being rich in species that are barely detected in health. The reasons for changes in bacterial profile, and the source of the potential pathogens, remain unanswered. Many bacteria detected in periodontal disease are Gram-negative, obligately anaerobic and proteolytic. It has been proposed that emergence of these species in the periodontium might involve the transfer of species from the extraoral environment (Genco et al., 1988), or in response to co-infection with viruses (Slots, 2015). More acknowledged theories suggest the enrichment of low abundant constituents of plaque in a more favourable growth environment (Marsh, 2003b) followed by keystone pathogen theory, which states that low abundant keystone pathogens orchestrate inflammation leading to the enrichment of ‘inflammophilic’ species (Hajishengallis, 2014b). Our study modelled aspects of the subgingival pocket such as hydroxyapatite surface, strict anaerobic conditions, and a protein-rich

growth medium, which was supplemented with serum for subset of the samples to model an increased flow of gingival crevicular fluid during inflammation. This environment was used to culture biofilms from bacteria naturally occurring in the healthy oral cavity.

4.5.1 Composition of the inoculum

Although there are inter-subject variations in the microbiota, attempts have been made to define a core microbiome associated with oral health and, depending on the study design, this includes representatives of the genera *Streptococcus*, *Veillonella*, *Granulicatella*, *Neisseria*, *Haemophilus*, *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella* and *Fusobacterium* (Zaura et al., 2009; Dewhirst et al., 2010; Sanz et al., 2017). These organisms generally have a harmonious relationship with the host and provide important health benefits. This core health associated microbiome can be distorted by many factors: mainly frequency of tooth brushing, recent use of antibiotics, age and status of general health, etc. So, in order to obtain an inoculum of health-associated micro-organisms, some criteria were enlisted for sampling (Acharya et al., 2017). Eight systemically healthy young adult volunteers, who did not report any recent oral disease or dental treatment and brushed their teeth twice daily were sampled for saliva, tongue biofilm and supragingival plaque.

Different oral sites are inhabited by different bacterial communities, for instance, saliva, supragingival and subgingival plaque are known to display the highest species richness among other oral habitats, while mucosal and keratinized surfaces show significantly lower species richness (Zhou et al., 2013). On the other hand, the tongue is an important reservoir for periodontitis-implicated bacteria (Eick et al., 2013); it has a papillary surface which retains abundant nutrients and provides a favourable environment for the accumulation of Gram-negative anaerobic bacteria (Tyrrell et al., 2003). Moreover, these Gram-negative anaerobic species are also the constituents of the core microbiome (Diaz et al., 2016). In our study, saliva, supragingival and tongue plaque were sampled to ensure the wide selection of core bacterial species found in an oral cavity of a healthy individual. This was because we could not predict whether the enrichment cultures would be successful, and we wanted to maximise the probability of detecting putative periodontal pathogen by sampling from a range of possible reservoirs.

The pooled inoculum used in these studies underwent an in-depth characterisation which confirmed the presence of high proportions of species from the genera *Streptococcus, Veillonella, Haemophilus, Actinomyces* and *Gemella.* The majority of the dominant bacteria were considered early-colonisers, that are abundant in 2 – 4 h old supragingival plaque (Diaz et al., 2006; Sanz et al., 2017). Metagenomic analysis and qPCR showed that only small proportions of some species implicated in periodontal disease, such as *Porphyromonas, Treponema, Fretibacterium* and *Tannerella*, were detected in the inoculum. This is in agreement with previous reports of the detection of these species in low proportions in healthy plaque (Salminen et al., 2015; Diaz et al., 2006).

PMA treatment was applied to the samples. This treatment disrupts extracellular DNA, so that only DNA from intact cells was analysed (Exterkate et al., 2014). PMA can improve the detection of shifts in *in vitro* polymicrobial biofilms, as potentially only the viable fraction of bacteria is included in the analysis. The comparison between PMA treated and untreated samples revealed some minor differences in species abundance. For instance, PMA treated samples had slightly higher numbers of *Bacteroidetes* OT 272*, Bacteroidetes* OT 274 or *Anaeroglobus geminatus,* while PMA untreated samples had higher numbers of *Streptococcus* species, *F. nucleatum spp. vincentii* and *F. alocis*. However, clear patterns in changes could not be observed, so analyses concentrated on the PMA treated samples, as they potentially reflect only a viable fraction of bacteria.

4.5.2 Changes in pH over time

During the experiment described in Chapter 4, the pH values of supernatants were monitored at certain time points. The small increase in pH was detected up to week 2.5 followed by a small decline at week three. The optimum growth conditions for various periodontal pathogens are around pH 7.0 – 8.0 (McDermid et al., 1988; Rogers et al., 1991). These values have been recorded during inflammation in periodontal pockets (Eggert et al., 1991). The rise in pH observed in the experiment in Chapter 4 might reflect the activity of a proteolytic microbial community, as protein, peptide, and amino acid deamination by proteolytic bacteria is associated with a rise in pH (Barros et al.,

2016). Interestingly, the enrichment of *P. gingivalis* was observed by qPCR at week one and two, especially if serum was used in a growth medium. At week three, slightly lower values of *P. gingivalis* were detected, corresponding with a decline in the pH of supernatants. However, a limitation of our study was that we did not measure the continuous change of pH but recorded the pH of supernatants every 3.5 days. It is possible, that fluctuation was greater, and varied especially in the biofilms. A relatively stable pH was observed throughout the biofilm cultures described in Chapter 4, which suggests that fast growing species did not become enriched as was seen in Chapter 3. In Chapter 3, decline in pH correlated with the high numbers of *Streptococcus* and *Actinomyces*, and low numbers of fastidious species in the biofilms.

4.5.3 Advantage of metagenomics approach

For many years, the knowledge of bacterial species implicated in periodontal health and disease was based on low throughput techniques such as culturing, bright field microscopy or targeted detection, mainly PCR, DNA-DNA hybridization or qPCR. The targeted detection of only a small fraction of opportunistic species emphasised the prevalence of Gram-negative, obligate anaerobes in plaque associated with periodontitis. Later, Sanger sequencing and especially high-throughput sequencing substantially enhanced the knowledge about the species implicated in periodontal disease and revealed a more diverse and heterogeneous microbiota. Open-end sequencing technologies benefited from high sensitivity being able to identify species regardless of their abundance and culturability in plaque samples. Contemporary studies report that about 30% of species detected in the oral cavity are currently unculturable; with higher proportions of unculturable and fastidious species being detected in plaque from periodontitis-implicated areas (Griffen et al., 2012). Whole metagenome shotgun analysis, used in this Chapter, was accomplished by unrestricted sequencing of the genomes of all microorganisms present in the sample. Some of the genes were good indicators of the species, while others allow a certain function to be assigned to a detected sequence and relate gene functions to a community. Established analysis pipelines were used to investigate the bacterial composition of biofilms cultured in the modelled periodontal environment, aiming to detect changes in the whole bacterial community.

In our experiment, bacterial composition was evaluated using either filtered 16S rRNA genes from metagenomes or whole metagenomes. The analysis based on 16S rRNA can be performed more quickly and offers a reliable identification up to family or genus level. Therefore, it was used for initial screening of the data, for example to evaluate the clustering of the samples based on taxonomical similarity (Figure 4.7). In contrast, metagenomics offers an increased resolution but is more computationally challenging. In our experiment, similar trends in microbial composition of samples were found when the outputs from applying these two approaches were compared. The MEGAN pipeline employs a robust lowest common ancestor algorithm, which bins reads in low levels of taxonomical hierarchy (species level) only if specific parameters are met. For example, in order to detect a species in a sample, at least 0.005% of reads have to be to attributed to that species and in our experiment that accounted for 1827 – 6117 reads per sample. This method rejects many false positives; however, some low abundant species might be excluded from the analysis. It should be noted that unreliable identification of species by the majority of the current methods that are in use has been reported in some instances (Jovel et al., 2016).

4.5.4 Changes in community measures show decreasing diversity over time, especially in a medium supplemented with serum

The length of culture had the greatest impact on the diversity of enriched biofilms, while medium composition had a smaller effect. This finding was consistent with other studies that cultured oral plaque and investigated the effects of the growth medium on bacterial diversity, reporting a decrease in alpha diversity over time and the highest alpha diversity in the inoculum (Kistler et al., 2015; Thompson et al., 2015; Fernandez et al., 2017). The pooled inoculum used in our study comprised 144 - 170 OTUs based on MEGAN, while biofilms grown for three weeks contained around 77 - 93 OTUs assigned to different taxa at species level. This high complexity of the inoculum was associated with three different ecological sampling sites and high volunteer number. The decrease in species numbers over time was caused mainly by the loss of health-associated species in biofilms after one and three weeks enrichment (for example, species from genera *Streptococcus, Actinomyces* and *Veillonella*) (Figure 4.9). The decrease in alpha diversity and Shannon index

over time shows that only a small proportion the inoculum bacteria were able to establish in the biofilm community under the environments provided. This shift might be attributed to an ability of a small proportion of bacteria to benefit from the provided environment and become more competitive than other species (Marsh, 2003b; Wake et al., 2016).

It remains contentious as to whether the plaque microbial community differs in terms of alpha and beta diversity between periodontal health and disease. For example, Langfeldt et al. (2014) performed an *in situ* study to investigate bacterial shifts in healthy adults' plaque over two weeks and concluded that any time-related patterns in terms of alpha or beta diversity could not be observed due to high inter-individual variability. Other *in vivo* studies report that diversity in plaque from heathy or periodontitis-implicated areas is similar (Galimanas et al., 2014), or even decreases (Ai et al., 2017; Jorth et al., 2014), while the majority *in vivo* studies suggest that more complex communities can be detected in periodontal disease than in health (Dabdoub et al., 2016; Diaz et al., 2016). The studies described in this Chapter showed a decrease in diversity over time in a closed *in vitro* system, which was caused by overgrowth of anaerobic periodontitis-associated species and disappearance of health-related bacteria. In the mouth, a constant supply of bacteria is provided by the flow of saliva, while our model was inoculated only once. Interestingly, some species appeared in the samples that were not detected in the inoculum. This must mean that some disease-associated species were low in abundance in the inoculum, and thus below the detection limit (< 0.005% species detection threshold in the sequencing analysis) but became enriched in biofilms throughout culture.

4.5.5 Time effect on biofilms at species level

One of the experimental aims was to investigate whether periodontitis-associated species could be detected after culturing the inoculum derived from healthy subjects in an enrichment model. The analysis identified species that were negatively or positively associated with older biofilms. A number of species demonstrated a significant change in relative abundance after one and three weeks of biofilm culture, irrespective of the composition of the growth media. Species that were enriched over time in protein-rich medium with and

without serum belonged to genera *Peptostreptococcus, Porphyromonas, Fusobacterium, Prevotella, Fretibacterium* and *Eubacterium*.

Some of the most abundant species in week one biofilms belonged to metabolically-dependant genera *Streptococcus* and *Veillonella*. Streptococci, as early coloniser, benefit from surface receptors and adhesion proteins that enable their direct attachment to salivary pellicle. *Veillonella* promote metabolic cooperation between early, middle and late colonisers by utilising lactic acid, produced by Streptococci, setting up a food chain in plaque (Periasamy and Kolenbrander, 2010). Week one biofilms also consisted of high proportions of species from genera *Prevotella, Alloprevotella, Catonella* and *Peptostreptococcus.* These subsequent colonisers are asaccharolytic and proteolytic. Some of them, for example *P. intermedia,* can alter its metabolic characteristics depending on nutrients available in the environment; it can utilise glucose as well as nitrogenous compounds. Moreover, it can grow in a slightly acidic pH and increase the pH to suitable levels for late colonisers through amino acid metabolism (Takahashi et al., 1997).

The further maturation of the biofilms was related to the enrichment of Gram-negative, fastidious species. Week three biofilms were dominated by established perio-pathogens from genera *Porphyromonas, Prevotella, Peptostreptococcus, Tannerella, Parvimonas* and *Filifactor*. Many of these species were among newly identified pathogens described by Perez-Chaparro et al. in a systematic review which showed that these species have moderate or mild associations with periodontal disease (Perez-Chaparro et al., 2014). Moreover, some of these species were detected in high proportions in biofilms but barely detected in the inoculum.

Other studies also report the sequential change in dominant bacterial species in biofilms over time. For example, Wake et al. (2016) in an *in situ* study observed plaque maturation in periodontally healthy areas. Biofilms at a baseline were colonised by mainly Gram-positive species and populations subsequently converted to Gram-negative anaerobes. In their study, the maturation was associated with the increased proportions in species from *Bacteroidetes* and *Fusobacterium* and decrease in *Firmicutes* and *Actinobacteria* phyla (Wake et al., 2016)(Wake et al., 2016)(Wake et al., 2016)(Wake et al., 2016)(Wake et al., 2016)(Wake et al., 2016) In the study

described here, there was a shift in the composition of the biofilms over three weeks of enrichment. Health-associated Gram-positive species declined over time, with only low proportions detected by week three, while some other species (for example *P. oralis, Fusobacterium OT 370, S. intermedius*) did not change in relevant abundance. However, enrichment after three weeks led to an increased abundance of Gram-negative, obligately anaerobic organisms, most likely detected due to an increasingly anaerobic environment following maturation of the biofilm and the high protein content of the growth medium. The findings demonstrated a sequential change of species over time initiated by aerotolerant species providing the attachment and later replaced by anaerobic and less aerotolerant bacteria.

4.5.6 Serum effect on biofilms at species level

The inflammatory response to plaque accumulation that occurs in periodontal disease ensures the rapid delivery of an array of host defence factors to counter the microbial insult; however, GCF also contains proteins and glycoproteins that can be exploited as nutrients by many of the fastidious and 'inflammophilic' bacteria associated with these pro-inflammatory biofilms (Hajishengallis, 2014b). Plaque accumulation at the gingival margin will trigger the host's inflammatory response. This response manifests in activation of inflammatory, innate and adaptive immune responses and triggers an increased flow of GCF. In our study serum was used as a surrogate to model an increased flow of GCF during periodontal inflammation, as has been done previously (ter Steeg et al., 1987b). During the experiment, the species that increased over time and were positively associated with serum in the growth medium were mainly Gram-negative, obligate anaerobic, proteolytic and fastidious to culture, including members of *Dialister, Mogibacterium, Bacteroidetes, Prevotella, Porphyromonas, Peptostreptococcus* and *Granulicatella* genera. Some Gram-positive species, such as *P. micra*, *F. alocis* and members of *Eubacterium* and *Gemella* genus, were also enriched over time in biofilms, especially if serum was used in a medium. All these species are usually reported to be implicated in periodontal disease (Downes et al., 2005; Downes and Wade, 2006; Perez-Chaparro et al., 2014).

It is known, that serum contains proteins and glycoproteins but the effect of serum on the growth of complex microbial communities have only been investigated to a limited extent. For instance, an early study performed batch-wise enrichment cultures using three subgingival plaque samples from patients with untreated pockets (4-7 mm deep) in a growth medium supplemented with human serum, and looked for the presence of *Bacteroides intermedius* (now classified as *P. intermedia* and *P. nigrescens*) (ter Steeg et al., 1987b). This organism could not be detected in two of the inocula, and constituted less than 1% of the total bacterial community of the third. After four step-wise enrichments in this medium, which was intended to mimic GCF, *B. intermedius* was detected in all three samples, ranging between 4 – 15% of the total bacteria present. Further enrichment studies were performed using continuous culture and the same serum-based medium. Communities that were capable of degrading molecules involved in the host defences (e.g. immunoglobulins, complement, haptoglobin, transferrin, etc) were selected, and spirochaetes and obligately anaerobic Gram-negative bacteria predominated (ter Steeg et al., 1988).

The studies described above cultured plaque collected from periodontal pockets, which was not characterised, and the identification was performed using conventional culture methods. In the studies described here, these limitations were excluded by using a characterised inoculum from healthy volunteers (and which did not include subgingival plaque), and used the advanced methods for identification of bacterial communities. Protein-rich medium with and without supplementation with serum was used to model the inflammatory environment, and was predicted to serve the nutritional needs of certain asaccharolytic and proteolytic bacteria to obtain essential amino acids, peptides and iron. The ‘Ecological plaque hypothesis’ states that perio-pathogenic bacteria could be minor constituents of normal plaque and under balanced conditions exist in a homeostasis with the host. Changes in environmental conditions can favour the growth of these low abundant species beyond the threshold compatible with health (Marsh, 2003b). This is also in accordance with key-stone pathogen hypothesis proposed by Hajishengallis, which states that low-abundant species are able to manipulate the host inflammatory response and modulate the ecological conditions of a periodontal

pocket and favour the growth of ‘inflammophylic’ bacteria (Hajishengallis, 2014b). The experiment performed here revealed that cultured biofilms consisted of newly-dominant community members that only accounted for a small proportion of an initial inoculum. The majority of enriched species were proteolytic anaerobes that are usually detected in the plaque of a periodontal pocket. The experiment showed that the nutritional environment was an important factor in shaping the biofilm community and raised a question as to whether modulation of the environment could reverse the deleterious change in the composition of the biofilm, and restore the microbiota to a profile that resembles that associated with oral health.

4.5.7 Functional potential

Functional potential analysis showed clear differences between the inoculum and biofilms cultured in media with or without serum. The inoculum was over-represented with genes responsible for carbohydrate metabolism, including di- and oligo-saccharide utilization, while the enriched biofilms had an abundance of genes associated with proteolysis, methanogenesis, virulence, motility and chemotaxis. These findings are consistent with results from functional studies of biofilms taken from healthy individuals and from patients with generalised chronic periodontitis (Dabdoub et al., 2016). Although this metagenomics approach does not reveal what functions were carried out by biofilm microbiota, as this analysis should be carried out by metatranscriptomics, it can shed some light on the functional potential of the community. The analysis using SEED annotation showed that communities were enriched with genes capable of metabolising proteins, which is consistent with the communities growing in media with a high protein content. Functional potential analysis showed that established biofilm communities were over-represented with genes associated with B12 and transposon and ABC transporter synthesis. This and other examples provided in section 4.4.1. indicated a biofilm with a greater virulence potential.

4.5.8 Conclusion and Future Work

These findings support the concepts behind the original ‘Ecological Plaque Hypothesis’ (Marsh, 1994a; Marsh, 2003a) and the more recent ‘Polymicrobial Synergy and Dysbiosis’ model of periodontal disease (Hajishengallis and

Lamont, 2012), in which the microbiota isolated from pockets gains benefit from the substrates derived from inflammation and tissue breakdown, and this drives community restructuring. Disease is a consequence of a dysbiotic shift in the microbiota driven by a change in the local environment. Implicit in these concepts is that disease can be managed or prevented by interfering with the drivers of dysbiosis. When the inflammatory environment was controlled in a *P. gingivalis*-induced model of periodontitis in rabbits using Resolvin E1, there was tissue regeneration and a decrease in Gram-negative anaerobic species (Hasturk et al., 2007). Now we have succeeded in developing a model of 'pathogen' enrichment, this approach could be applied to see if decrease in periodontitis-associated species can be achieved by modifying the environment.

Chapter 5 The influence of nutrient source on the development of complex oral biofilms

5.1 Introduction

Nutrition plays a major role in determining the composition of the oral microbiome at a site. The previous chapter described the use of the CBD to grow complex oral biofilms using a protein-rich growth medium, with and without supplementation with bovine serum. The inoculum comprised pooled samples of saliva, supragingival plaque and tongue scrapings from dentally-healthy young adults. The regular supply of fresh medium maintained the viability of biofilms during these enrichment studies, and a wide range of species that had previously only been isolated from periodontal pockets increased in abundance over the three weeks period of culture. In contrast, many species that were associated with health either declined or were lost from biofilms during enrichment, while many taxa were found in the biofilms that had not been detected in the inoculum. The conclusion was that these taxa must have been present in these health-associated samples but at levels too low to be detected by the metagenomics approaches used, and that these organisms were able to respond to the altered nutritional condition, and increase in abundance.

Differences were observed in the composition of the biofilms depending on whether the enrichment cultures had been performed in the presence of absence of serum. In light of these results, it was decided to refine the experimental design so as to further investigate the influence of nutrients on the selection process, and to increase the biological relevance of the model system. In the initial experiment, for simplicity, foetal bovine serum was used as it is readily available. In order to determine if the source of the serum had any impact on the growth of the low abundance fastidious bacteria in the inoculum, heat-inactivated human serum was compared with FBS. This also has the potential advantage of increasing the relevance of the model by more closely simulating the composition of human GCF. In addition, for comparative purposes, the inoculum was also cultured on sterile human saliva and human saliva supplemented with human serum as these are the primary endogenous

nutrients that sustain the growth of the resident oral microbiota (Marsh et al., 2016a).

Finally, the possibility of reversing the dysbiotic enrichment by serum of putative periodontal pathogens at the expense of health-associated species was investigated. After growth for three weeks in the presence of different media supplemented with serum, biofilms were cultured for a further two weeks in the presence of human saliva without serum supplementation.

5.2 Aims of the experiment

The aims of the experiments described in this chapter were to:

1) assess the impact of different nutritional conditions on the growth of complex oral biofilms and the selection of putative periodontal pathogens derived from an inoculum from healthy adult volunteers, and then to

2) determine whether such deleterious changes in the microbiota could be reversed by returning the dysbiotic biofilms to a health-associated nutritional environment, i.e. human saliva.

5.3 Results

The same eight volunteers were recruited as in Chapter 4 for plaque collection described in section 2.3.1.1. Different media, aiming to mimic the periodontal environment more accurately than in Chapter 4, were used to culture the pooled plaque for three weeks; the composition of these media are detailed in Table 2.5. The comparisons were made between:

- the communities following the growth on foetal bovine or human serum,
- the communities following the growth on sterile human saliva or sterile human saliva supplemented with human serum
- the communities following the growth on protein-rich medium with human saliva and human serum or human saliva with human serum

Subsequently, the possibility of reversing the dysbiotic community at the expense of enrichment of health-associated species, by changing the growth environment to human saliva for two weeks was also investigated (Table 5.1).

Table 5.1. The main comparisons of the impact of nutrient source on biofilm composition.
Comparisons were made between groups to investigate the effects of media components on the composition of biofilms.

Comparisons and investigations		
1	Protein-rich medium +human saliva + FBS	Protein-rich medium + human saliva + human serum
2	Human saliva	Human saliva + human serum
3	Protein-rich medium + human saliva + human serum	Human saliva + human serum
4	week three biofilms	week five biofilms (growth in human saliva)

5.3.1 Sequencing quality

On average, sequencing yielded 13.5 ± 2.5 million sequences per sample. After quality control, the samples consisted of 12.7 ± 2.3 million sequences per sample. After filtering the samples against SILVA (not an acronym) for 16S rRNA genes, 0.43% ± 0.001 of reads were selected for the subsequent analysis. Of those filtered, 49.38% ± 0.025 clustered within the provided identity

to HOMD. Sequences presented 64620 ± 8007 taxonomically annotated OTUs in inoculum samples, 37705 ± 8193 in three-week cultured biofilms and 38806 ± 6105 in five-week cultured biofilms of bacterial origin. Analysis of metagenomes detected 13.3 ± 3.3 million assigned reads of bacterial origin in merged paired-end samples per sample. At the species level, the inoculum and biofilms had 310 OTUs between 24 different samples. Two samples that were prepared with the kit for low DNA yield samples did not differ from others in terms of read quality and community structure (samples inoculum_b and human saliva + human serum_a). SEED annotation yielded 3322 genes encoding for proteins assigned to the samples.

5.3.2 Validation of the sample quality

Rarefaction curves showed that diversity was higher in inoculum samples than in cultured biofilms. Figure 5.1 shows rarefaction curves that approach the asymptote, which confirms that substantial sequencing depth was acquired for 16S rRNA datasets. The three curves showing the higher species richness (around 500 unique OTUs per sample) represent inoculum samples.

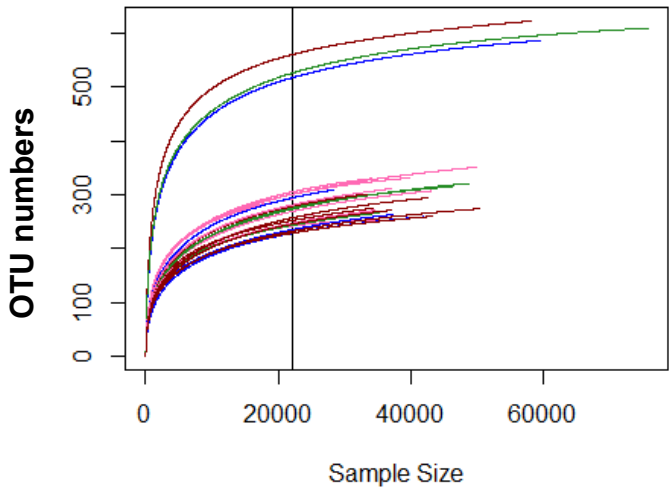

Figure 5.1 Sample-based rarefaction curves.
Samples were scaled by the detected number of OTUs based on 98.5% similarity of the 16S rRNA gene. The curves represent species richness. Number of different OTUs per sampled 16S rRNA genes.

The Nonpareil program in R was used to investigate if adequate coverage of metagenomes was acquired during sequencing (Rodriguez and Konstantinidis, 2014). All samples had coverage higher than 95% (Figure 5.2) and average coverage per sample group ranged from 97.3% to 99.8%. The curves clustered in two separate groups indicating different levels of diversity. A lower group on

the right (red-pink samples) represents inoculum samples and shows the highest diversity.

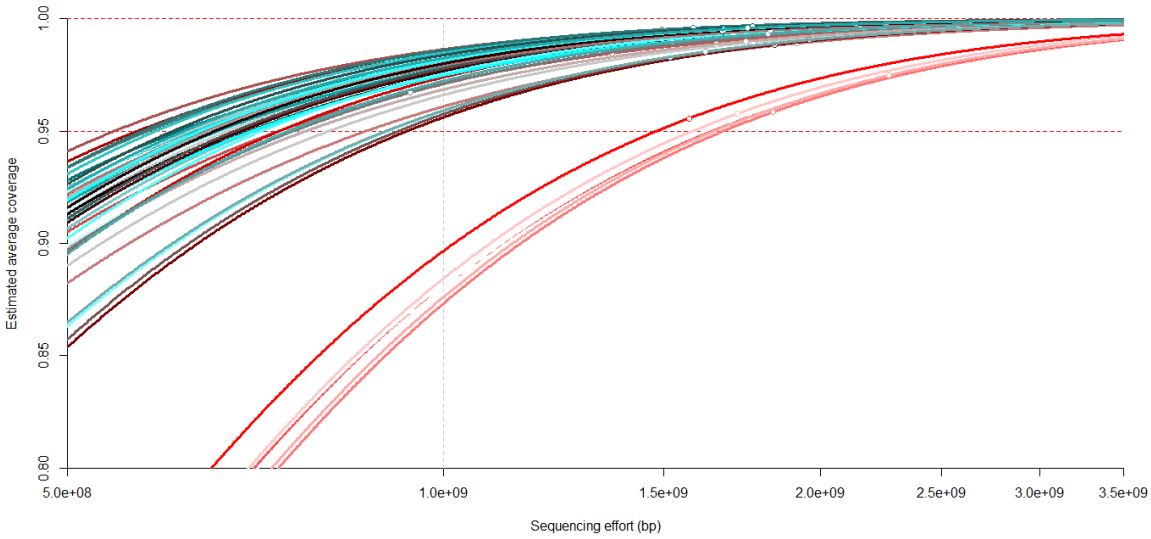

Figure 5.2 Comparison of nonpareil curves for the metagenomes of the inoculum and biofilm samples.
The plot displays the fitted models of the nonpareil curves which represents estimated average coverage at a given sequencing effort. The dashed horizontal line indicates 95% coverage.

5.3.3 Communities measures – alpha diversity

The observed number of unique OTUs was significantly higher in the inoculum than in cultured biofilms, $p < 0.001$. Differences in alpha diversity measures (observed number of unique OTUs and Chao1 index) were similar between three- and five-week cultured biofilms, but significantly different between the inoculum and cultured biofilms, Tukey HSD test, $p < 0.05$. Alpha community measures indicated a higher diversity and evenness in inoculum samples (Figure 5.3). The Chao1 index, which bases richness evaluation on rare OTUs, was higher for the inoculum, $p < 0.05$, and was not significantly different for biofilm samples. The Shannon index, which describes community evenness by combining richness and diversity data, was significantly higher for the inoculum (Figure 5.3). This shows that cultured biofilms were dominated by a few OTUs while samples of the inoculum were comprised of more equally distributed communities.

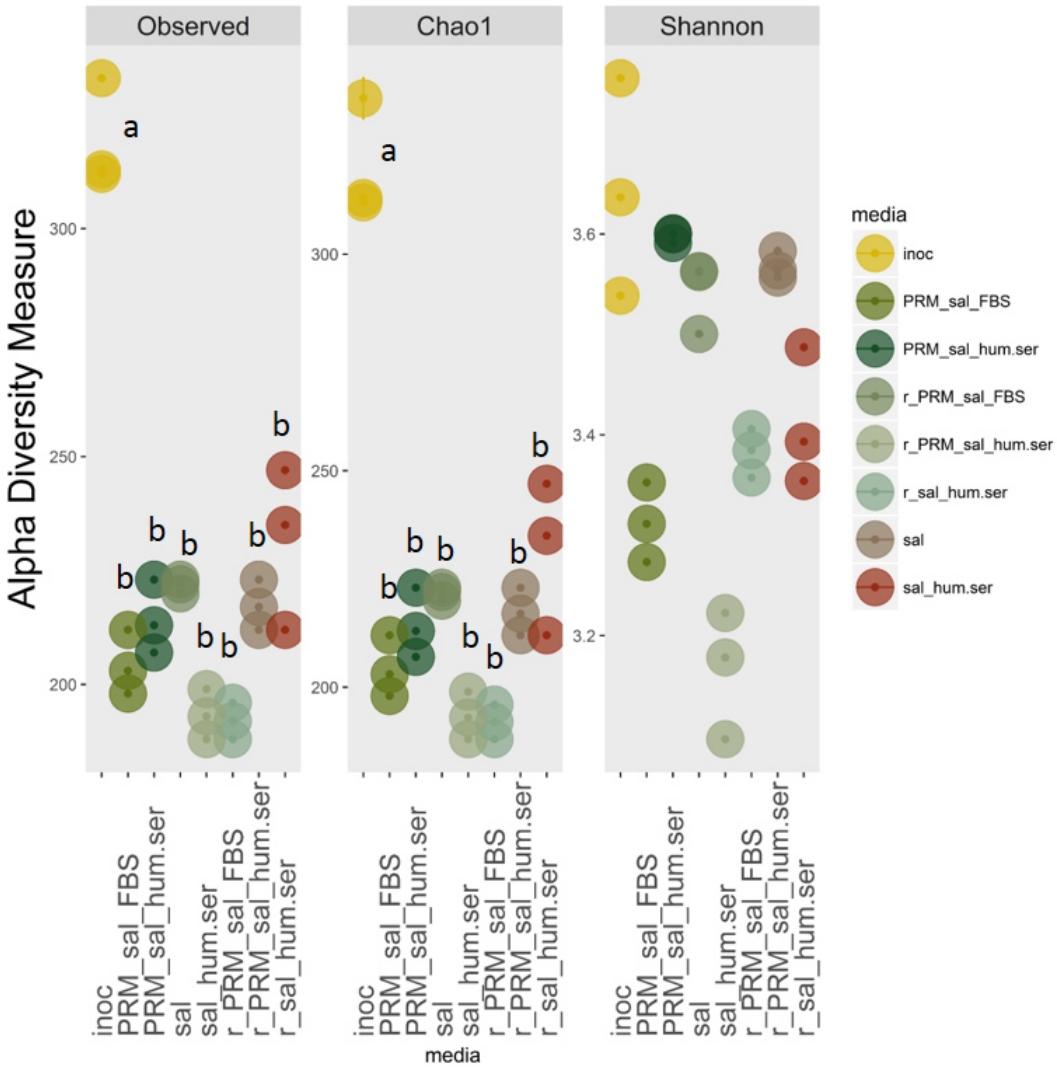

Figure 5.3 Alpha diversity measures of inoculum and biofilm samples cultured for different times under different nutritional conditions.
FBS –supplementation with 20% (v/v) foetal bovine serum, PRM – protein-rich medium, sal – sterile human saliva, r – samples additionally cultured for 2 weeks in sterile human saliva (5 weeks total). a and b represent sample groups that are significantly different after the multiple comparison Tukey HSD test, $p<0.05$. Inoculum, week 3 and week 5 samples are showed in the graph.

5.3.4 Beta diversity – phylogeny-based analysis

Beta diversity measures are routinely used for the comparison among microbial communities to explore sample relatedness for 16S rRNA gene datasets. UniFrac distance metrics are based on branches on a phylogenetic tree that are either shared or unique to each sample. Analysis showed that inoculum samples clustered separately from biofilms (Figure 5.4). Small inter-sample variation was observed. Biofilms cultured for three weeks generally clustered together, although biofilms cultured solely in human saliva had a different phylogeny. Two groups of five-week samples (saliva +human serum and PRM

+ saliva + human serum) clustered separately from biofilms cultured in FBS. This suggests that the type of serum but not the presence of protein-rich medium in a growth environment was a more critical component contributing to the changes in the community membership for five-week old biofilms.

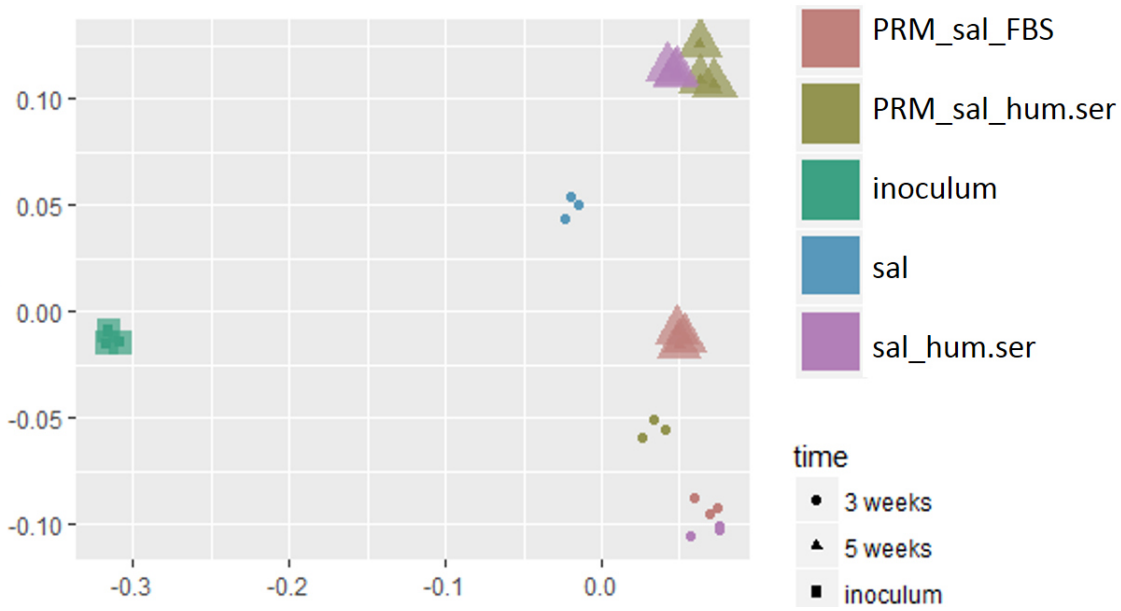

Figure 5.4. Inoculum and biofilm samples plotted on PCoA based on UniFrac weighted diversity distances.
PRM – protein-rich medium, sal – sterile human saliva, hum.ser – human serum. Colours represent sample type and culturing medium, while shape and size distinguish samples by time: the rectangles represent inoculum, small circles represent biofilms after three weeks and large triangles represent biofilms after five weeks of growth.

A permutation test was performed to explore if there was a statistical significance in weighted UniFrac distances (assessment of community structure that takes into account OTU abundance) between groups. After performing 5000 permutations on the means of distances, it was confirmed that sample groups have different mean probability distributions, $p=0.038$. Then, a permutational multivariate analysis of variances using weighted and unweighted UniFrac (assessment of community membership by considering only OTU presence and absence) distance matrices was performed from GUniFrac package, PERMANOVA test. Significant differences ($p<0.001$) were found in these distances between groups.

5.3.5 Changes in abundance in hierarchies levels (OTU-based approach)

The distribution of phyla between the inocula and biofilms was explored by plotting relative abundances of each phylum (Figure 5.5). Many differences were detected between inoculum and biofilm samples. Inoculum samples mainly consisted of *Firmicutes* (38% - 42.1%), *Bacteroidetes* (24.9% - 25.7%), *Actinobacteria* (17% - 19.1%), *Proteobacteria* (9.3% - 11.1%), *Spirochaetes* (0.3% - 0.9%), *SR1* (0.6% - 0.9%) and *Synergistetes* (0.04% - 0.2%). These samples had higher proportions of *Firmicutes, Proteobacteria, Fusobacteria* and *SR1,* $p < 0.001$ with Tukey HSD test, than enriched biofilms. In contrast, biofilm samples had significantly higher proportions of *Bacteroidetes, Spirochaetes* and *Synergistetes*, $p < 0.001$ than the inoculum. Biofilm communities enriched for 3 weeks were dominated by *Bacteroidetes* (36.4% - 62.4%), *Firmicutes* (20.5% - 41.4%) and *Synergistetes* (4.7% - 8.2%) phyla. A similar trend was observed for biofilms that had been enriched for 5 weeks. The latter biofilms were dominated by *Bacteroidetes* (36.7% - 51.4%), *Firmicutes* (25.2% - 36.7%) and *Synergistetes* (5.8% - 12.8%). Week three samples cultured in protein-rich medium with FBS had significantly more *Bacteroidetes* than any other biofilm type ($p < 0.001$) and less *Actinobacteria* and *Firmicutes* than other biofilms ($p < 0.05$). The relative abundance of *Spirochaetes* depended the most on the serum type (FBS vs. human) and was significantly higher in biofilms grown in the presence of human serum ($p < 0.05$). This was true for both three- and five-week old biofilms. The highest proportions of *Actinobacteria* were associated with growth in whole saliva (relative abundance 11.8% - 17.7%), and also increased when the growth medium was changed from serum to sole human saliva in five-week biofilms ($p < 0.05$). The biofilms growing on sterile human saliva at week five also had higher proportions of *Firmicutes* and lower proportions of *Synergistetes.* The unculturable *SR1* was not detected in any biofilms, irrespective of culture conditions.

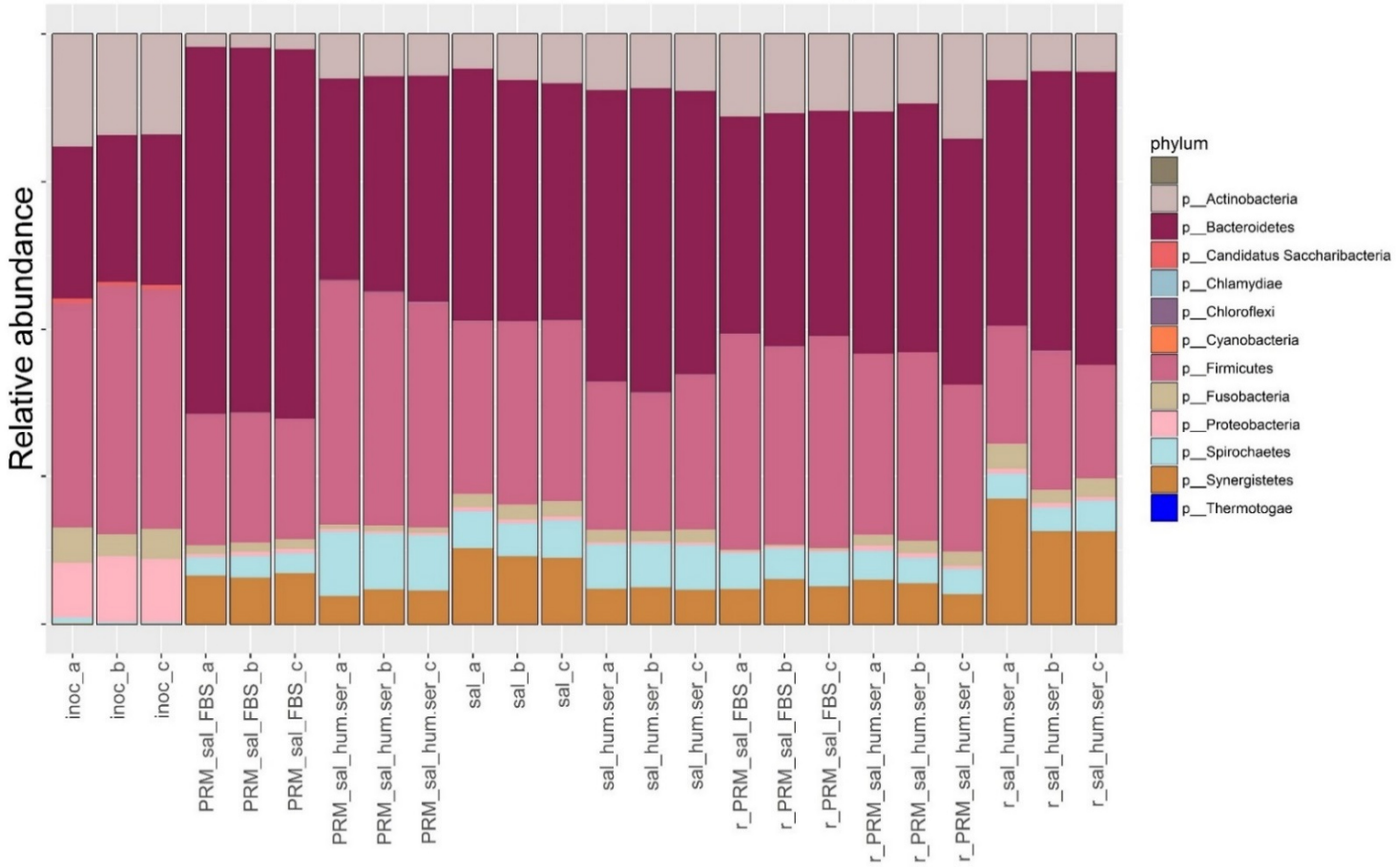

Figure 5.5 Distribution of phyla between the inoculum and biofilms cultured under different nutritional conditions.
The growth media contained PRM – protein rich medium, FBS – foetal bovine serum, sal – sterile human saliva, hum.ser – human serum, inoc – inoculum samples, r- biofilms additionally cultured for 2 weeks in sterile human saliva (5 weeks total) , a, b, c – replicate samples.

5.3.5.1 Changes in relative abundance at the species level (OTU-based approach)

Metacoder was used to produce heat trees and investigate the taxonomical structure of the inoculum. Figure 5.6 shows the composition of one of the samples of the inoculum; however, there were no significant differences, $p > 0.05$ in term of community membership when two other samples were analysed. The heat tree demonstrates that the most abundant phylum was *Firmicutes* which contained many representatives from *Bacilli* class. Samples were abundant with species from the following genera: *Streptococcus, Actinomyces, Prevotella, Veillonella* and *Haemophilus*. The most abundant species were *S. salivarius, S. sanguinis* and *S. lactarius*.

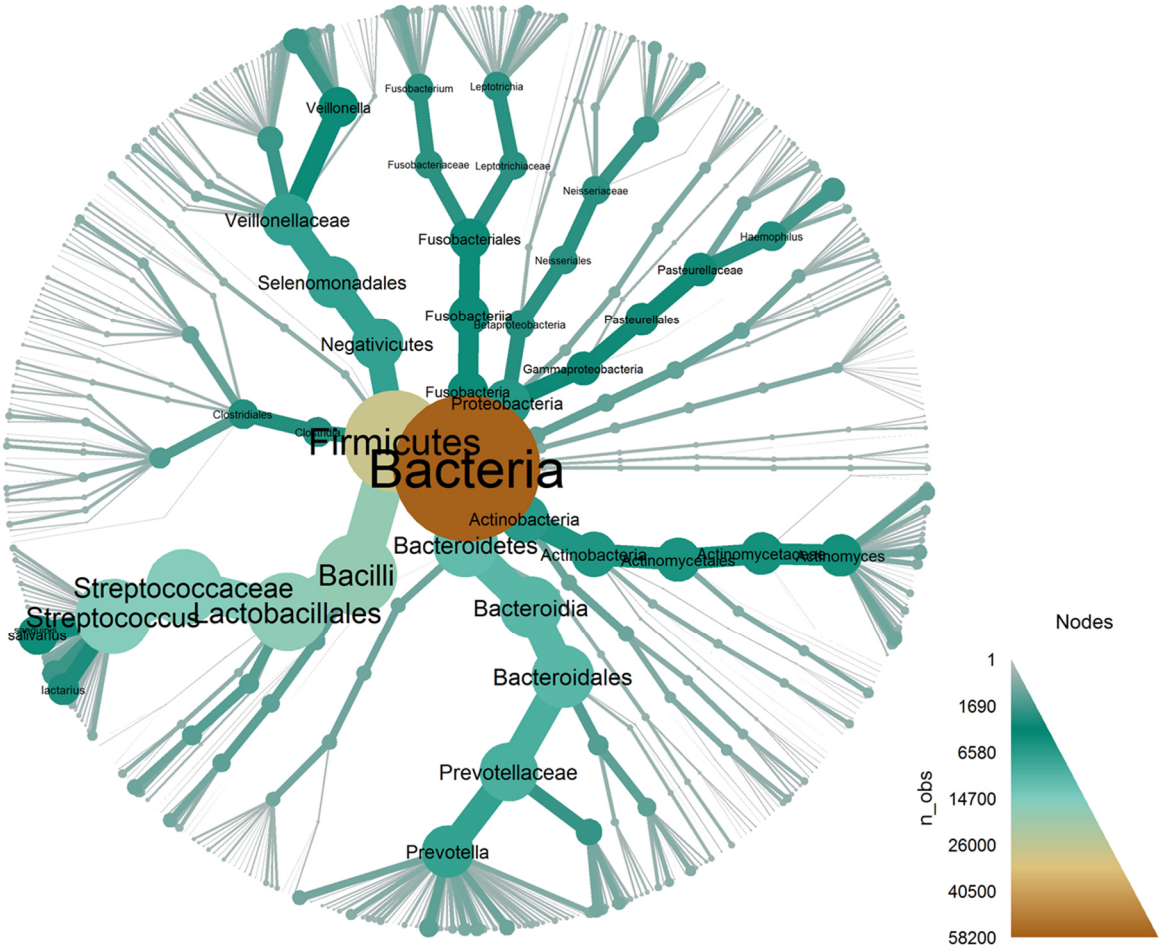

Figure 5.6 The community structure of a sample of the inoculum in a taxonomic context based on 16S rRNA gene analysis.
The heat tree represents all taxa as nodes while colour and size are used to represent the abundance. Each node represents a taxon. The position in the circle indicates the level in the taxonomic hierarchy. The most abundant taxa are indicated on the branches. Shared branches indicate that taxa share the same taxon classification.

The samples of the inoculum had high abundance of species from *Streptococcus, Prevotella, Actinomyces* and *Veillonella* genera (Table 5.2). Bacteria from these genera were relatively common in the inocula, but declined following the enrichment. The biofilms at week three were enriched with certain genera such as *Bacteroidetes, Porphyromonas, Treponema* and *Peptostreptococcus*.

Table 5.2. The relative abundance of most prevalent genera in the inoculum and week 3 biofilm samples. Relative abundance is presented as means of percentages of different biological replicates, N=3. Tukey HSD test was used to determine significant differences between samples groups and is presented in the superscript. Samples with the same letter were not statistically different from each other. Significance levels was set at $p < 0.05$.

Genus	Inoculum % ± std	Protein-rich medium + human saliva + foetal bovine serum % ± std	Protein-rich medium + human saliva + human serum % ± std	Human saliva % ± std	Human saliva + human serum % ± std
Porphyromonas	2.40 ± 0.50 ^{d}	49.47 ± 1.24 ^{a}	21.74 ± 0.42 ^{c}	19.39 ± 1.34 ^{c}	34.61 ± 4.71 ^{b}
Tannerella	0.85 ± 0.13 ^{c}	5.54 ± 0.22 ^{a}	4.13 ± 1.35 ^{a,b}	2.66 ± 0.24 ^{b}	3.60 ± 0.53 ^{b}
Fusobacterium	3.53 ± 0.81 ^{a,b}	2.15 ± 0.14 ^{b,c}	1.16 ± 0.14 ^{c}	3.39 ± 0.41 ^{a,b}	4.27 ± 1.31 ^{a}
Treponema	0.67 ± 0.48 ^{d}	2.51 ± 0.24 ^{c}	13.22 ± 0.80 ^{a}	5.70 ± 0.74 ^{b}	5.72 ± 0.77 ^{b}
Fretibacterium	0.08 ± 0.06 ^{c}	9.13 ± 0.38 ^{b}	6.02 ± 0.87 ^{b}	8.20 ± 1.85 ^{b}	19.43 ± 3.54 ^{a}
Actinomyces	11.19 ± 2.22 ^{a}	0.25 ± 0.03 ^{b}	0.11 ± 0.06 ^{b}	0.11 ± 0.03 ^{b}	0.15 ± 0.10 ^{b}
Bifidobacterium	0.03 ± 0.01 ^{b}	0.20 ± 0.02 ^{b}	1.29 ± 0.01 ^{a}	0.03 ± 0.00 ^{b}	1.62 ± 0.33 ^{a}
Slackia	0.04 ± 0.01 ^{b}	1.03 ± 0.31 ^{b}	2.10 ± 1.10 ^{b}	15.53 ± 3.97 ^{a}	3.09 ± 0.33 ^{b}
Streptococcus	28.70 ± 4.10 ^{a}	1.77 ± 0.18 ^{b}	2.61 ± 0.61 ^{b}	5.21 ± 0.71 ^{b}	1.41 ± 0.20 ^{b}
Mogibacterium	0.34 ± 0.06 ^{c}	1.23 ± 0.06 ^{b,c}	3.62 ± 0.96 ^{a}	0.49 ± 0.08 ^{b,c}	1.78 ± 0.58 ^{b}
Filifactor	0.01 ± 0.01 ^{d}	2.58 ± 0.15 ^{c}	8.83 ± 1.05 ^{a}	0.60 ± 0.08 ^{d}	4.50 ± 0.41 ^{b}
Peptostreptococcus	0.32 ± 0.06 ^{d}	3.52 ± 0.33 ^{c}	9.61 ± 0.83 ^{a}	1.90 ± 0.10 ^{c,d}	5.88 ± 1.01 ^{b}
Selenomonas	1.44 ± 0.80 ^{a}	0.04 ± 0.01 ^{b}	0.02 ± 0.00 ^{b}	0.07 ± 0.01 ^{b}	0.04 ± 0.00 ^{b}
Anaeroglobus	0.04 ± 0.02 ^{b}	0.42 ± 0.22 ^{b}	0.72 ± 0.07 ^{b}	9.35 ± 0.98 ^{a}	0.20 ± 0.16 ^{b}
Dialister	0.28 ± 0.23 ^{c}	1.30 ± 0.09 ^{a,b}	1.17 ± 0.32 ^{a,b}	0.75 ± 0.06 ^{b,c}	1.33 ± 0.24 ^{a}
Veillonella	8.87 ± 1.08 ^{a}	0.10 ± 0.01 ^{b}	0.03 ± 0.02 ^{b}	0.38 ± 0.06 ^{b}	0.04 ± 0.01 ^{b}
Parvimonas	0.10 ± 0.02 ^{b}	0.98 ± 0.19 ^{a}	4.46 ± 0.81 ^{b}	0.37 ± 0.09 ^{b}	0.23 ± 0.10 ^{b}
Prevotella	19.97 ± 0.75 ^{a}	3.66 ± 0.64 ^{c}	2.58 ± 0.21 ^{c}	9.67 ± 2.20 ^{b}	2.05 ± 0.27 ^{c}

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5.3.5.2 The effect of source of serum on biofilm composition

One of the aims of this experiment was to investigate the changes in bacterial composition following enrichment on different sources of serum (Table 5.1). The species abundance was compared with DESeq2 package in R between two sample groups. The comparison of relative abundances of taxa within these biofilms showed that the levels of individual species varied depending on whether human or bovine serum was used for the enrichment cultures. Figures 5.7 and 5.8 show changes in some species abundance between two media types. Figure 5.7 shows that *P. anaerobius, S. constelatus* and *T. vincentii* enriched more in the biofilms of human serum, compared to foetal serum.

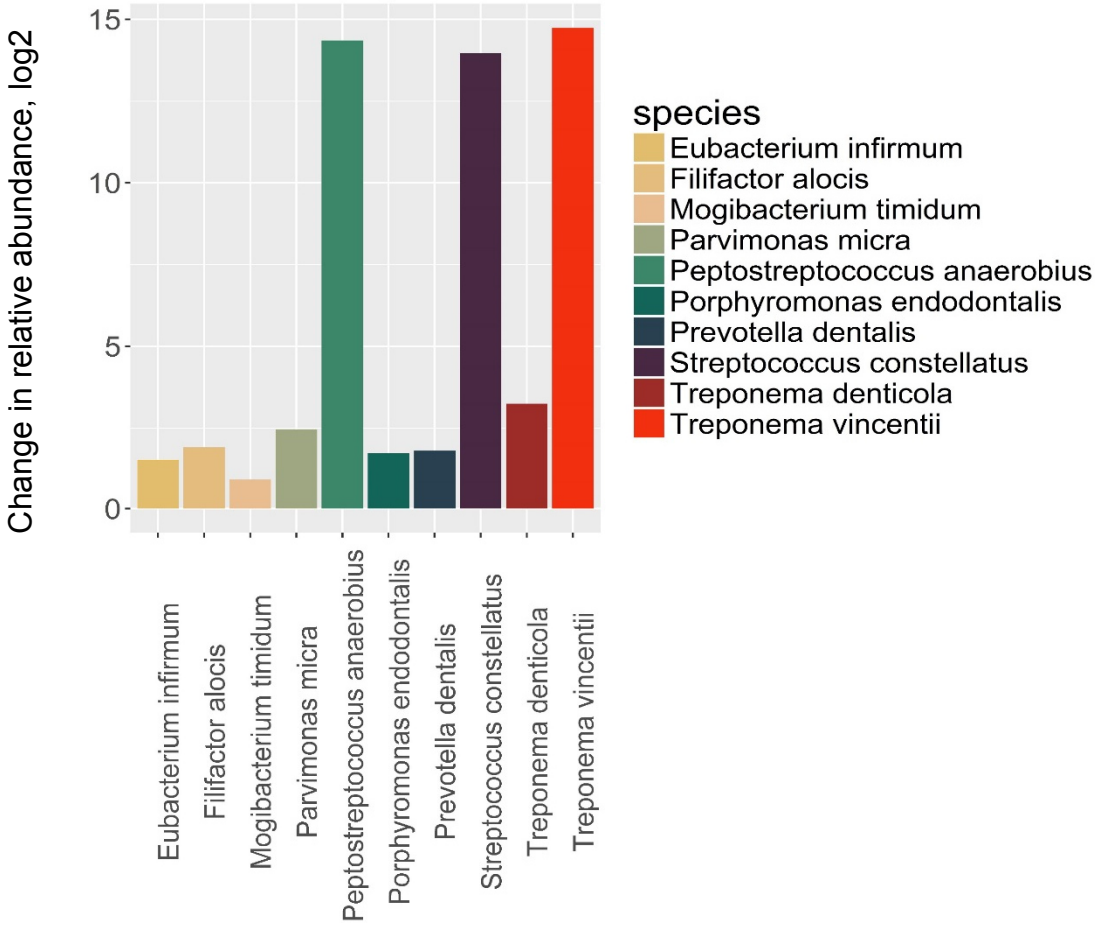

Figure 5.7. Species enriched in protein-rich medium + saliva + human serum compared to protein-rich medium + saliva + Foetal Bovine Serum. The figure summarises change in relative abundance of selection of species that became enriched in protein-rich medium supplemented with human serum compared to foetal bovine serum.

In contrast, protein-rich media supplemented with foetal bovine serum cultured communities were enriched for *P. marshii, P. intermedia* and *P. gingivalis* compared to media with human serum (Figure 5.8). At the genus level, biofilms cultured in the presence of foetal bovine serum had higher proportions of *Porphyromonas*, while in human serum –*Treponema* and *Peptostreptococcus* predominated, p < 0.05, HSD Tukey test (Table 5.2).

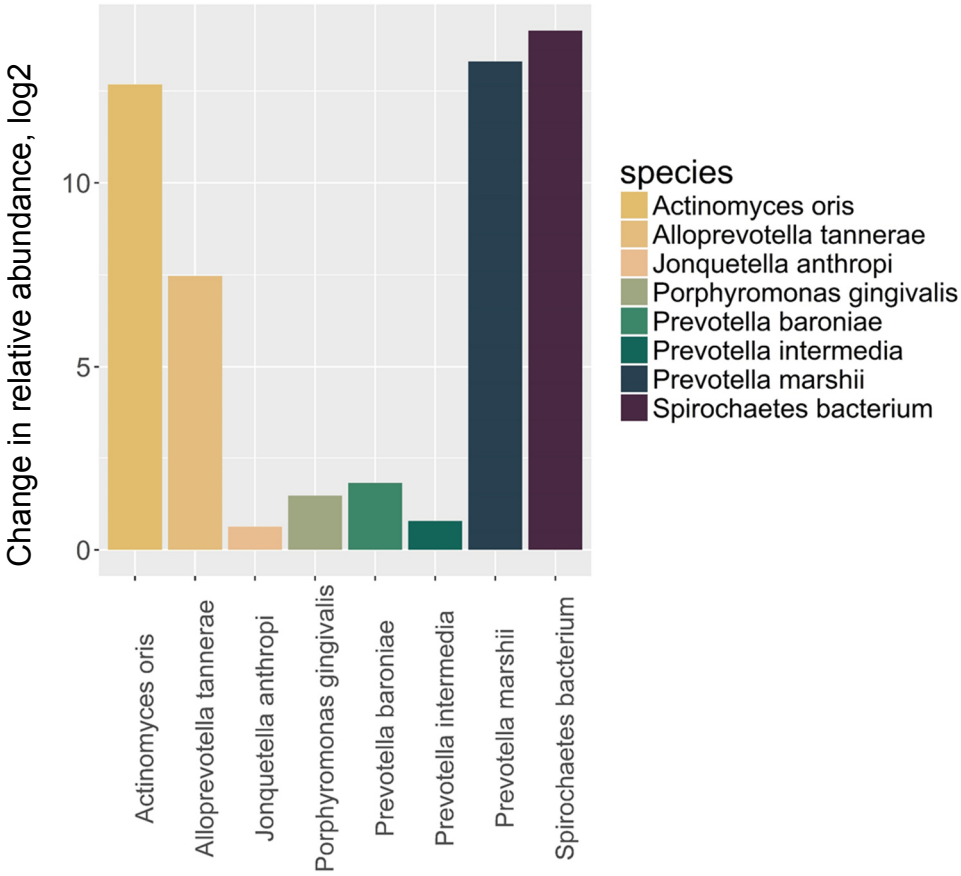

Figure 5.8. Species enriched in protein-rich medium + saliva +Foetal Bovine Serum compared to protein-rich medium + saliva + human serum, p adjusted < 0.001.
The figure summarises change in relative abundance of selection of species that became enriched in protein-rich medium supplemented with foetal bovine serum compared to human serum.

5.3.5.3 The effect of human serum on the enrichment of biofilms

In order to confirm the effect of human serum on biofilm enrichment, the abundance of species in biofilms cultured only in sterile human saliva were compared with those grown in sterile human saliva with human serum. (Figure 5.9).

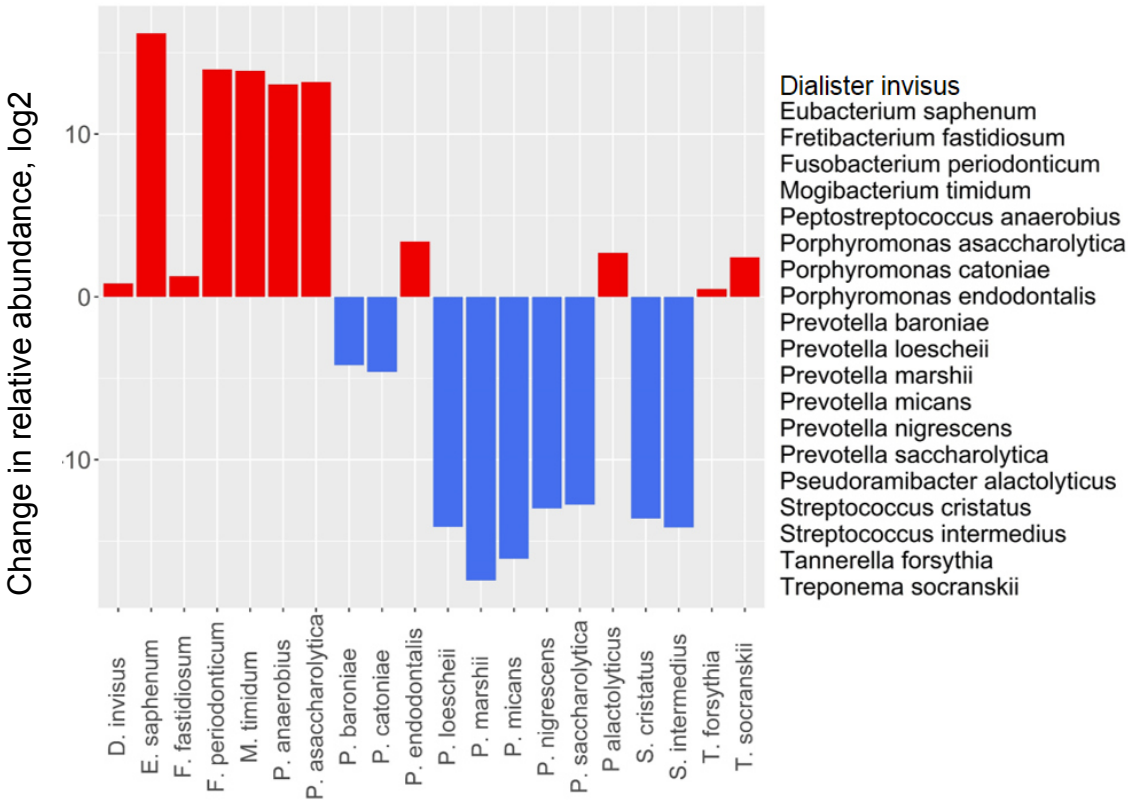

Figure 5.9. Changes in species relative abundance of biofilms enriched in sterile human saliva with human serum vs sterile human saliva.
Positive values (coloured red) indicate which species enriched in medium with human serum, while negative values (coloured blue) indicate which species enriched in the absence of serum (only sterile human saliva).

Species from the genera *Treponema, Tannerella, Porphyromonas* and *Fretibacterium* were more abundant in biofilms cultured in saliva supplemented with human serum than in biofilms cultured solely in saliva, p adjusted < 0.001. In contrast, species *P. marshii*, *P. baroniae*, *P. nigrescens*, *P. catoniae* and *S. constelatus* grew better in biofilms cultured in unsupplemented saliva, p adjusted < 0.001. Analysis of the distribution of genera showed that biofilms cultured in sterile saliva for three weeks had the highest percentage of *Streptococcus, Actinomyces, Veillonella* and *Slackia* genera among all three-week biofilms, p < 0.05, HSD Tukey test (Table 5.2).

5.3.5.4 Effect of protein-rich medium on enriched biofilms

In order to investigate the effects of protein rich medium (comprising of proteose peptone, tryptone and yeast extract) on biofilm composition, the abundance of species in biofilms cultured in PRM + saliva + human serum were compared with saliva + human serum (Figure 5.10).

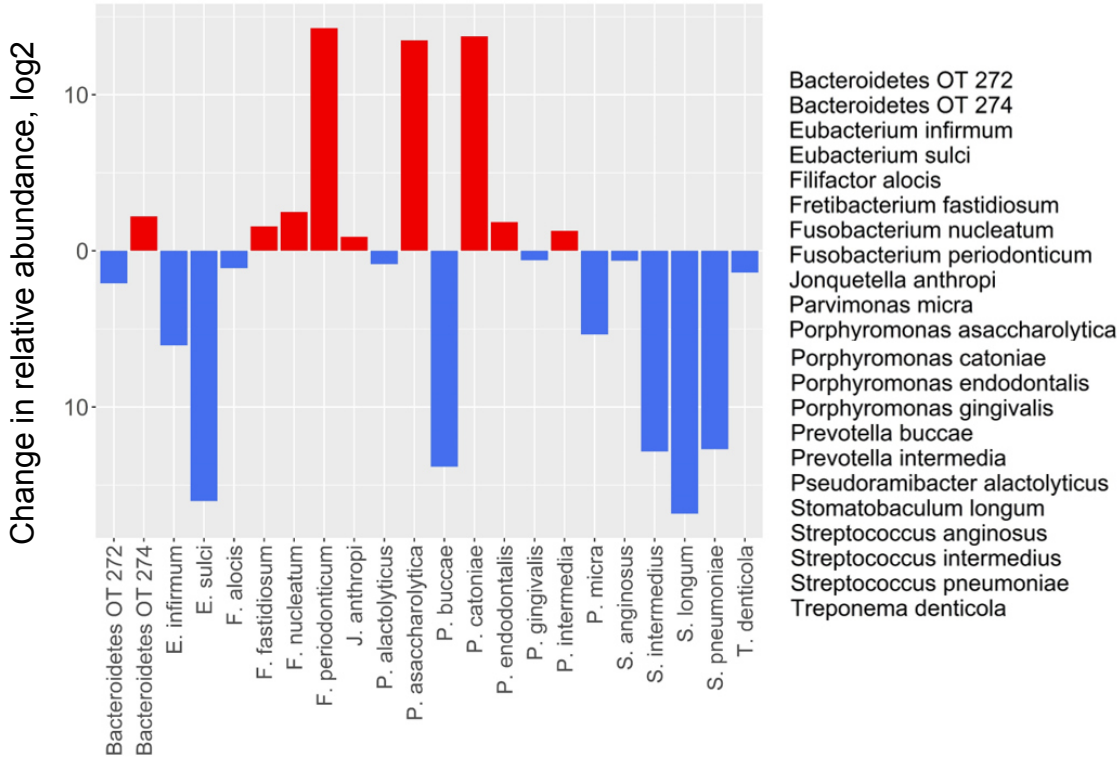

Figure 5.10 Change in species abundance of biofilms enriched in saliva with human serum vs protein-rich medium with saliva and human serum. Positive (red) values indicate which species enriched in saliva + human serum medium, while negative (blue) values indicate which species enriched in protein-rich medium + saliva + human serum medium.

The comparison in DESeq2 package showed that biofilms cultured in PRM with saliva and human serum had higher proportions of *Eubacterium infirmum, E. sulci*, *P. buccae, P. micra*, while biofilms cultured in saliva and human serum had higher proportions of *Porphyromonas asaccharolytica, P. catoniae, Fusobacterium periodonticum* (Figure 5.10). Table 5.2 shows that biofilms cultured in PRM with saliva and human serum had higher proportions of the genera *Prevotella, Peptostreptococcus* and *Treponema* than biofilms cultured in saliva and human serum only. Human saliva + human serum medium is already protein-rich the addition of PRP medium (consisting of proteose peptone, tryptone and yeast extract) might not show the effect of PRM. For a more precise investigation into the effects of a PRM (consisting of proteose peptone, tryptone and yeast extract) on biofilm composition, other experimental designs would be optimal. The medium consisting of PRM and human saliva should to be compared against those grown solely on saliva. In Chapter 5 this type of medium (PRM + saliva) was not used.

5.3.5.5 Reversal stage – biofilm composition after 5 weeks following switch to growth on human saliva

After three weeks of growth in four different media, all biofilms were transferred to the medium consisting only of sterile human saliva. The relative abundance of species cultured for three and five weeks were compared to determine which species increased in abundance at week five. At the genus level, *Catonella, Slackia, Fretibacterium* and *Treponema* were more abundant in week five biofilms, while *Streptococcus* and *Veillonella* were rarely detected (Table 5.3). Species that were enriched the most in week five biofilms compared to week three were *P. oris, Prevotella bivia T. putidum* and *T. denticola* (Figure 5.11).

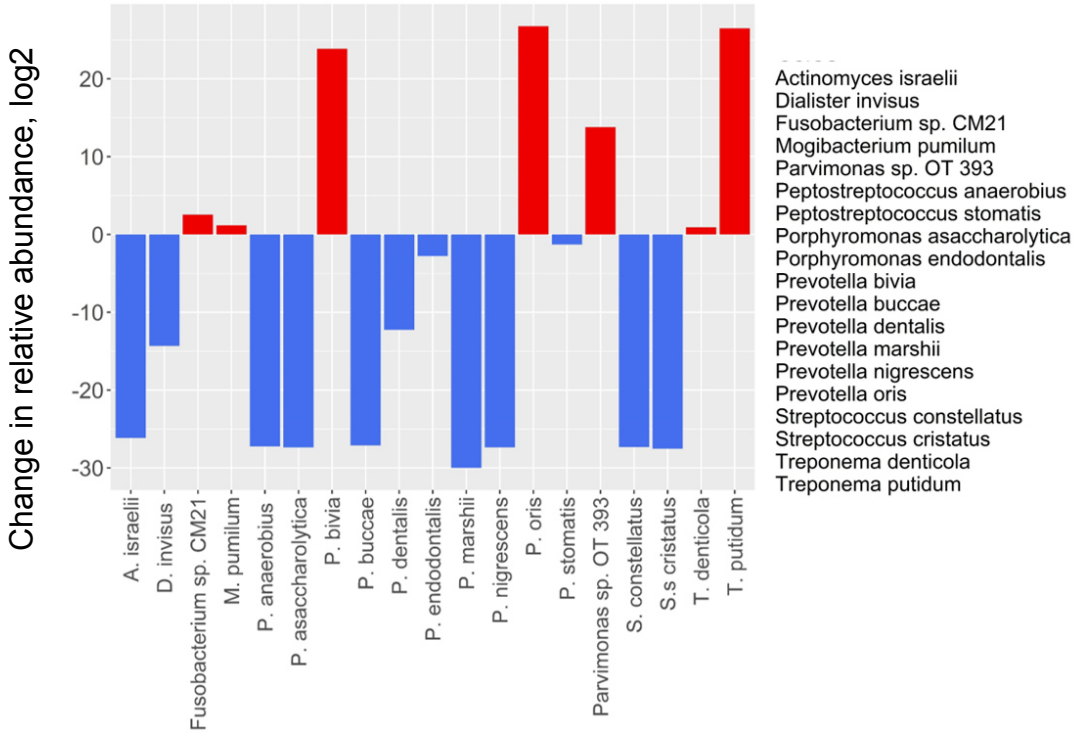

Figure 5.11. Change in relative abundance between week five and week three biofilms.
Positive (red) values indicate which species were more abundant in week five biofilms, while negative values (blue) show which species were more abundant in week three biofilms.

Table 5.2. The relative abundance of different genera in week 5 biofilms. R – biofilms additionally cultured for two weeks in sterile human saliva (five weeks total).

	R + protein-rich medium + saliva + foetal bovine serum (%)	**R + protein-rich medium + saliva + human serum (%)**	**R + saliva + human serum (%)**
Porphyromonas	16.6 – 20.7	16.8 – 21.8	16.2 – 17.1
Tannerella	9.7 – 10	2.2 – 2.6	2.1 – 2.9
Treponema	4.8 – 6.1	12.4 – 14.1	7.9 – 8.8
Fretibacterium	14.6 – 17.2	8.4 – 10	8.1 – 10.7
Slackia	5.4 – 8.4	10.3 – 10.6	10.3 - 13
Catonella	8.2 – 9	11.5 – 12.9	16.9 – 17.8
Streptococcus	1.9 – 2.3	1.9 – 2.1	1.9 – 2.6
Veillonella	0.12 – 0.13	0.3	0.3

5.3.6 Functional potential - inoculum and three-weeks samples

DESeq2 was used to evaluate which functional units were significantly over-represented in the inoculum and in the three-week biofilm samples. More unassigned reads belonged to biofilm samples: 18.5% of biofilm reads and 25% of inoculum reads were assigned to functional units. Out of 3322 functional units identified in the samples, 213 were solely observed in biofilms while 369 were detected only in the inoculum, while 2740 functional units were common to both the inoculum and biofilms. The common genes predominantly encoded for central functions such as carbohydrate and protein metabolism, and protein and amino acid synthesis. However, the abundance of these common genes was significantly different between the groups, p adjusted < 0.05. The genes unique to biofilm samples encoded for flagellar proteins and iron metabolism (Appendix Table 2). Biofilms cultured in human serum compared to those cultured in sterile saliva were overrepresented with genes associated with resistance to antibiotics, cobalamin synthesis, ABC

transporters, biofilm formation, haem, haemin and iron metabolism, motility, and lipoprotein synthesis (Appendix Table 3).

5.3.6.1 Five-week and three-week samples

More biofilm reads (18.5%) from week three biofilms were assigned to functional units, while only 16.5% of reads from week five were assigned to genes associated with the protein function. 3134 functional units were assigned to three- and five-week samples in total and no unique functional units were detected in five-week biofilms compared to three-week biofilms or *vice versa*.

In total, 107 functional groups were under-represented and 110 over-represented in all biofilms after the media were changed from protein-rich and containing sera to sterile human saliva (Figures 5.12 and 5.13). All five-week biofilms samples decreased in genes that were associated with cobalamin synthesis and metabolism, haem metabolism, motility (gliding), and virulence (Appendix Table 4).

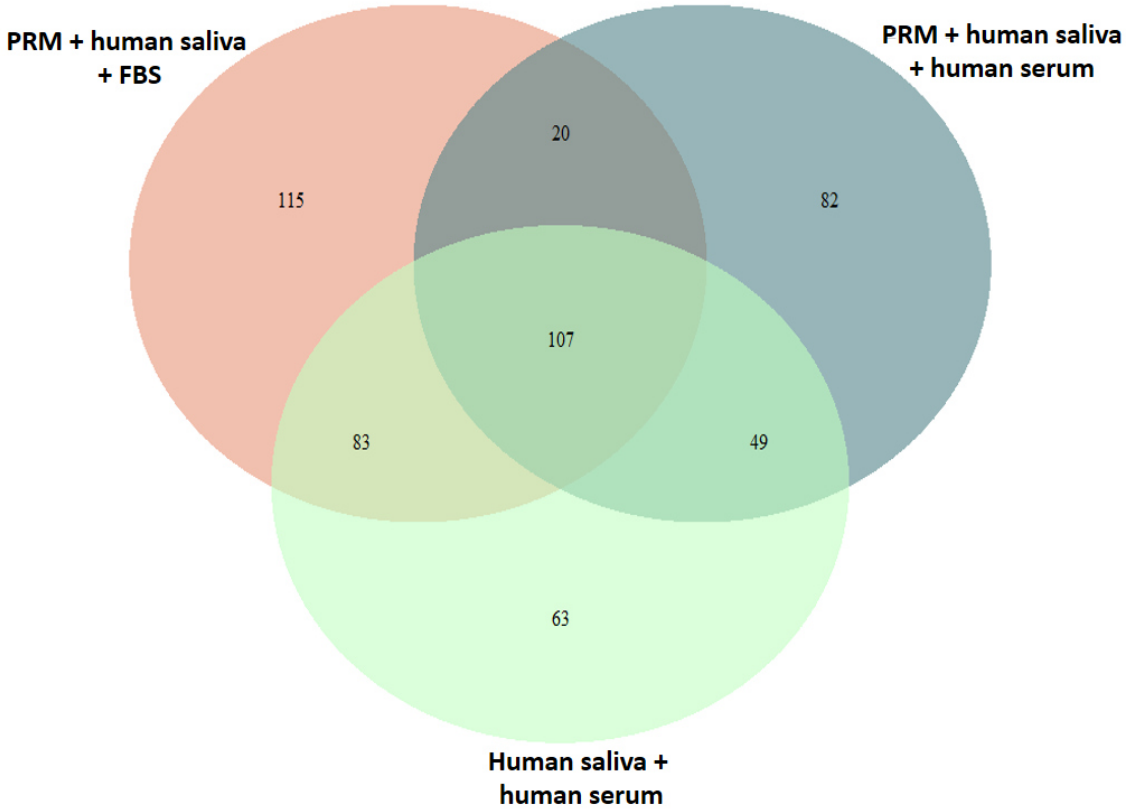

Figure 5.12 VENN diagram summarises how many gene groups were under-represented in five-week biofilms compared to three-week biofilms.
PRM – protein-rich medium, FBS - foetal bovine serum.

Genes that were overrepresented in five-week biofilms were associated with central functions such as carbohydrate metabolism, respiration, metabolite damage and its repair, and metabolism of aromatic compounds. The functional units that were upregulated in all five week-biofilm types were responsible for carbohydrate utilization, fermentation, amino acid utilization (arginine, lysine, glutamine) or synthesis (lysine, proline), urea cycle (Appendix Table 5).

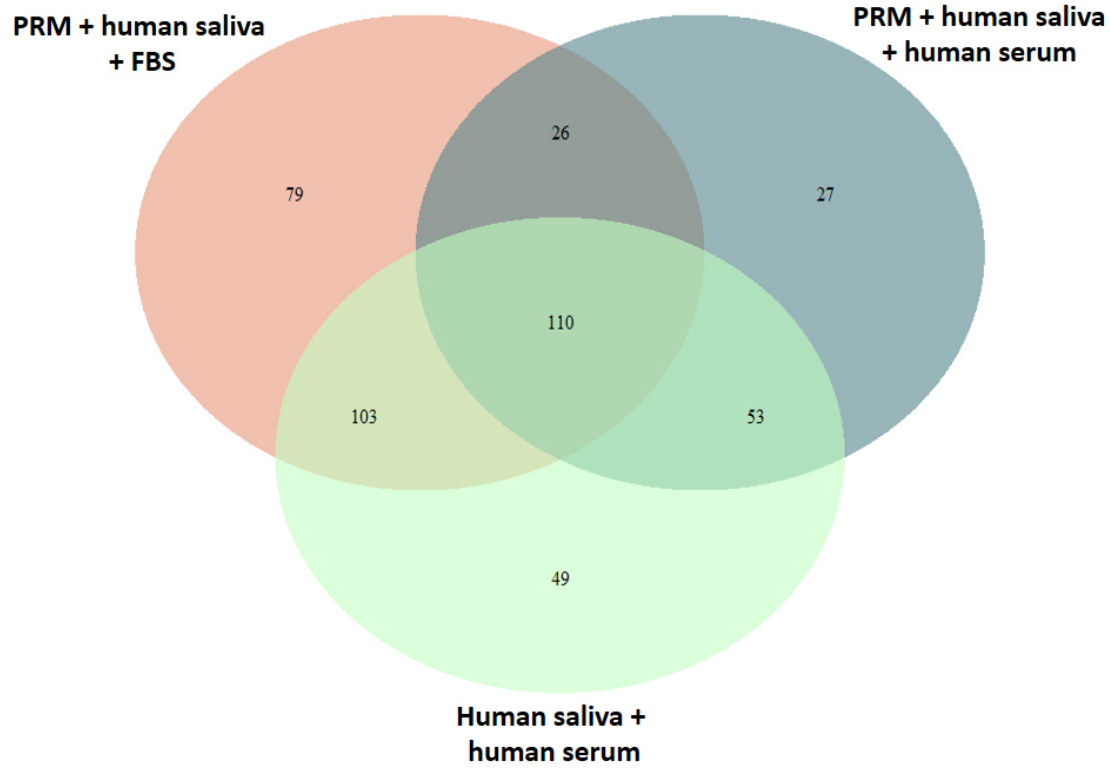

Figure 5.13 VENN diagram shows how many functional units were over-represented in five-week biofilms.
PRM – protein-rich medium, FBS - foetal bovine serum.

5.5 Discussion

The study in this chapter aimed to investigate the effects of the growth medium on the composition of biofilms derived from an inoculum of pooled supragingival and tongue plaque and saliva from healthy volunteers and cultured for several weeks. Initially, the studies compared the effects of human and bovine serum on the selection of putative periodontal pathogens. Subsequently, the media were changed to whole sterile human saliva to determine whether it was feasible to reverse the enrichment of these pathogens and restore some of the species associated with oral health.

Complex bacterial communities were successfully established on the pegs in the CBD system after culture for three and five weeks. The composition of the biofilms changed slightly at week five when media were changed to be composed solely of saliva. After PMA treatment, which eliminates genomic material of cells with damaged membranes, adequate levels of DNA for library preparation were obtained for three- and five-week cultured biofilms. The diverse communities with low inter-sample variation were maintained in the model biofilms throughout the whole experiment. The diversity and number of unique species decreased in cultured samples, but did not change significantly between three-week and five-week biofilms or between biofilm groups cultured in different media. The sequencing coverage and rarefaction curve analysis of 16S rRNA showed that qualitative and quantitative parameters of sample reads were adequate for the comparison of taxonomy and functional potential.

5.5.1 Composition of the inoculum

The inoculum samples were dominated by *Firmicutes* phyla, *Bacilli* family, and species from the genera: *Streptococcus, Veillonella, Haemophilus* and *Actinomyces*. Species that have previously been reported as health- or caries associated, including *S. parasanguinis, H. parainfluenzae, S. sanguinis, A. dentalis* were the most abundant in the inoculum (Aas et al., 2005). These species are found in high abundance in supragingival plaque. Previous studies also reported high levels of anaerobic species such as *Veillonella* and *Prevotella* being detected on the highly papillate surfaces of the tongue, so high abundance of these species in the inoculum might be associated with sampling from the dorsum of the tongue (Mager et al., 2003). Very few species of

periodontitis-associated bacteria were detected in the inoculum: these included representatives from *SR1, Porphyromonas* and *Treponema* genera, and low levels of these organisms can be found in plaque samples from healthy subjects (Aas et al., 2005).

During the experiment, salivary and plaque bacteria were used as inoculum. Its culturing in four different environments yielded biofilms of significantly different taxonomy and functional potential. Statistical analysis showed that biofilm groups varied one from another and from inoculum in terms of beta diversity and consisted of differently abundant functional units. The aim of experiment in Chapter 5 was to determine the effect of more physiologically-relevant medium components in the media on biofilm development. The enrichment studies in Chapter 4 showed that diluted protein-rich medium in artificial saliva with or without serum enhanced the growth of species implicated in periodontal disease. It was decided to extend the studies and try to replicate the oral environment more accurately. Consequently, sterile whole saliva and human serum were used as nutrients and compared their effects with those of foetal serum.

5.5.2 The comparison between foetal bovine serum against human serum

One of the aims of Chapter 5 was to evaluate the effects of different medium components for the development and enrichment of complex microbial communities. First, foetal bovine serum was compared against human serum. The rationale to use human serum was to reflect the more precise inflammatory environment of the periodontal pocket than in Chapter 4. Although in microbial research foetal bovine serum is a routinely used medium component, some authors claim that human serum is a better substrate to investigate the behaviour of bacterial community than human serum substitutes (Runci et al., 2017; ter Steeg et al., 1987a). In Chapter 5 it was found that medium supplemented with foetal serum significantly enhanced the growth of species from *Porphyromonas, Fretibacterium* and *Fusobacterium* genera, while medium with human serum enriched species from genera *Peptostreptococcus, Treponema* and *Mogibacterium*. Moreover, studies investigating foetal serum stresses high variation of iron and ferritin levels in foetal serum (Patrikoski et al., 2017). Foetal bovine serum is known to contain high levels of iron and its

ferritin levels are reported to be higher in bovine than in human foetuses (Kakuta et al., 1997). In a recent study, the favoured growth of *P. gingivalis* was observed in the medium that contained bovine serum, although the bacterium enriched in all biofilms. The enrichment of *P. gingivalis* and black-pigmented, Gram-negative species was also observed in FBS containing media in Chapter 4. This enrichment might be associated with high levels of iron in the environment. However, a very few studies described the use of human serum as a growth medium component for oral plaque culture. Presumably, this is due to high cost of such experiments. However, if used human serum was shown to enrich *Bacteroidetes* enrich in batch culture (ter Steeg et al., 1987a; Ammann et al., 2012; Guggenheim et al., 2011). Interestingly, it is also reported that human serum acts as an iron-poor environment for bacterial strains (Runci et al., 2017). So, the surplus of iron and its compounds' in FBS might have a selective effect for the enrichment on complex communities and should be considered when choosing the serum for an *in vitro* model.

5.5.3 Comparison between biofilms enriched in sterile human saliva and sterile human saliva with human serum

Biofilms cultured in sterile human saliva for three weeks were colonised by both periodontitis- and health-associated species. These biofilms contained higher proportions of some health-associated species, such as *Streptococcus, Slackia, Catonella* and *Prevotella* than biofilms enriched in sterile saliva and human sera. However, biofilms cultured in sterile saliva for three weeks were still low in many health-associated species, that were more abundant in inoculum. For instance, very low proportions of *Actinomyces, Neisseria, Kingella, Rothia* were detected on undetected in these biofilms. Other *in vitro* models also reported a poor survival of fast growing Gram-positive bacteria in sterile human saliva after prolonged periods (Tong et al., 2011). For example, De Jong et al. showed that dental plaque cultured in whole sterile saliva under anaerobic conditions enriched with *Streptococcus sanguinis* and gram-negative anaerobic species, such as *P. micra* and *F. nucleatum* (De Jong and Van der Hoeven, 1987). The latter study evaluated planktonic growth and used culture on selective media for detection, while experiment in Chapter 5 employed metagenomics to analyse biofilm bacteria. However, the enrichment of perio-pathogenic species in saliva as a sole nutrient in anaerobic conditions was

detected in both studies. For example, Roy et. al, 2010 proposed that salivary mucins, or their terminal sugars (sialic acid) can be used as a key nutrient source by fastidious *T. forsythia* (Roy et al., 2010). So, human saliva contains the necessary compounds for complex communities and fastidious microorganisms to thrive (Marsh et al., 2016a; Rafay et al., 1996). On the other hand, biofilms cultured in sterile saliva with human serum had significantly higher proportions of established perio-pathogens, such as *Porphyromonas, Fretibacterium* and *Peptostreptococcus,* showing the enrichment of perio-pathogenic species in media containing serum, as demonstrated in Chapter 4 and section 5.3.5.3 in Chapter 5.

5.5.4 Comparison between three-week and five-week biofilms

The subversion to dysbiosis due to environmental stress (pH or temperature change, nutrient availability) is discussed in detail in The Ecological Plaque Hypothesis (Marsh et al., 2016a). It proposes that once the resilience of biofilm community to maintain homeostasis exceeds the capacity to cope with extrinsic stress, the community becomes dysbiotic. In Chapter 5 the media were changed to sterile human saliva alone to investigate if medium without serum and additional proteins can be beneficial for the growth of health-associated species.

The biofilms cultured in human saliva with human serum enriched with *Mogibacterium, Porphyromonas, Treponema, Fretibacterium* species while corresponding samples cultured in sole saliva had higher numbers of *Prevotella*, *Streptococcus* and *Slackia* species at week three. However, after the subsequent growth in sterile saliva for two more weeks, biofilm in general enriched with *Catonella, Fretibacterium* and *Treponema* species, while such health-associated species as *Streptococcus, Actinomyces, Neisseria* were low in abundance of even undetected in these biofilms.

In terms of functional potential changes between week three and week five biofilm, older biofilms had fewer genes associated with iron and haem metabolism. This may reflect the fact that serum containing media was no longer in use and therefore genes associated with its metabolism would no longer be of benefit. These biofilms had higher proportions of genes associated with carbohydrate metabolism, for example, suggesting that biofilms consisted

to a greater extent bacteria adapted to use compounds of salivary origin (such as glycosylated terminal branches of salivary mucins) as nutrients than biofilms at week three.

Lately, the adapted model of the dysbiosis development of the gut was proposed to be valid for the dysbiosis development in oral environment. Mira et at. 2017, proposed that due to environmental stimuli on healthy community, when the threshold for protection is exceeded, the community transfers to semi-dysbiotic stage. It can be reversed to either homeostatic stage or, after continual environmental pressure, can reorganise into complete dysbiosis that cannot be reversed by variation in the environment. This implies that periodontal disease in a sequential and dysbiosis development is a multi-stage process, but once developed, it cannot be reversed to health. Review by P. Diaz suggests that a particular bacterial profile is detected in initial gingivitis that is different from advanced periodontitis or healthy plaque – so called “transfer bacterial profile” (Diaz et al., 2016). The semi-dysbiotic stage being different form advanced periodontitis is also documented in Yost et al. (2015) study. Belstrom et al. (2017) in his study showed that after the discontinuation of the oral hygiene, biofilms enriched with *Tannerella* and *TM7* species in dental plaque which could not be restored after the introduction of oral hygiene (Belstrom et al., 2017a). The experiment described in Chapter 5 also shows that media changes introduced after three weeks did not restore the abundance of health-associated species as hypothesised. We propose, that at the stage when serum containing medium was replaced with sole saliva the community was dysbiotic and could not be reversed to an initial profile with more health-associated species and diversity. The data presented above is from *in vitro* study, so its relevance to *in vivo* situations should be evaluated with caution. However, it might suggest that biofilms colonised by periodontitis-associated species cannot reduce its pathogenic potential by the modulation of the environment so the mechanical elimination of the biofilms should be the primary treatment option for the periodontal disease.

Chapter 6 General Discussion

Everything is everywhere, but, the environment selects, is a statement of a Dutch microbiologist Professor Lourens Gerhard Marinus Baas Becking published in 1934. In the context of biogeography of microorganisms it suggested that all microbial life is distributed worldwide, but the majority is below our detection limit. Only an appropriate environment can resuscitate and enrich a specific microbial community (de Wit and Bouvier, 2006). In recent years the citation is quoted increasingly frequently in publications regarding community assembly and the development of dysbiosis (Rosier et al., 2014; Sharma et al., 2018). Highly sensitive culture-independent sequencing technologies showed a great diversity of species residing in the human body, reporting around 700 species capable of inhabiting the oral cavity, however around 100-300 species may be harboured a single mouth (Chen et al., 2018). These technologies have also revealed that some of the disease-associated species can be detected in health in very low abundance, while some disease-associated species have never been found in healthy sites. This PhD project investigated whether an appropriate environment could enrich disease-associated species from plaque of systemically and orally healthy individuals.

6.1 Model development

An *in vitro* pathogen enrichment model was developed using the CBD, a protein-rich medium and an anaerobic growth environment. Initially, five-species biofilm communities were developed using a defined inoculum. Supplementation with different concentrations of bovine serum were compared in terms of their effect on the growth and stability of these simple microbial communities. The choice of species for these simple communities was found to be critical. Initially, *S. oralis* ULCP86 was used in the community, but it proved difficult to establish a stable community of all five species. Following investigation, it was eventually realised that this strain was inhibitory to the growth of the other members of the community. A stable community was only established when *S. oralis* strain ULCP86 was replaced with *S. salivarius* ULCP97. The possible explanation for inhibition was a drastic decline in pH and production of H_2O_2 when *S. oralis* ULCP86 was used in culture. The change of

the species to *S. salivarius* prevented a dramatic decline the pH of supernatants. The frequency of inoculation was also investigated. Early colonisers were inoculated first, and the later colonisers introduced afterwards on the basis that conditions might be more suitable for their growth. However, as discussed above, some of the early colonisers could produce inhibitory compounds, and so this strategy did not always succeed. Once a stable growth of five-species biofilms was achieved, the effect of serum in the growth medium was investigated. It was shown that 20% (v/v) of serum benefitted the growth of *P. gingivalis*. Then, the model was developed further to culture complex oral biofilms.

6.2 Enrichment cultures using a natural and complex inoculum

The findings described above led to the move to use a more complex and natural inoculum on the basis that these organisms were already co-existing in biofilms that had evolved naturally. The model was to be used to explore whether a change in growth conditions that reflected more closely the nutritional environment of the inflamed periodontal pocket would enrich for species associated with disease. Although some putative periodontal pathogens have been detected in low numbers at healthy sites (Abusleme et al., 2013), not all of the organisms that have been implicated in disease have been found, and their origin remain a subject of speculation. As we wanted to have as rich mixture of species as possible in the inoculum, samples were taken from multiple sites (tongue, supragingival plaque and saliva) from eight periodontally-healthy young adults, and pooled to create the inoculum to increase the probability of introducing these low abundance species into the model system. Many of these putative pathogens are fastidious in their nutritional requirements, and often grow in close association with key partner organisms, and so the choice of medium was critically important (Vartoukian et al., 2016). In the pilot studies with the five-membered community, it was found that the addition of serum boosted the growth of later colonisers such as *P. gingivalis*, with 20% being optimum, and so this medium was adopted for the enrichment cultures with the natural inoculum. Serum has been used successfully as a surrogate for GCF in a number of early enrichment culture

studies, and organisms were shown to function collectively to break down the complex molecules present (ter Steeg et al, 1988, 1989). Communities were allowed to develop as biofilms in the CBD model for one and three weeks, with frequent changes of medium. The biofilms were removed and an established metagenomic analysis pipeline was used for in-depth characterisation of these complex biofilms. Samples projected for sequencing were treated with PMA, which eliminated genomic material of cells with damaged membranes. This concentrated the analysis on intact components of biofilm, possibly able to metabolise and reproduce. The same young adult volunteers were recruited for two separate plaque and saliva culture experiments. Similar bacterial profiles were discovered in the pooled inocula on each occasion and similar patterns of enrichment were observed in each experiment.

The CBD enabled the simultaneous culture of multiple biofilms on hydroxyapatite-coated surfaces. The system was inexpensive compared to some model systems, simple to set up, and allowed regular media changes without disrupting the biofilms. The results showed that CBD was a reliable system to culture multi-species and complex biofilms for prolonged periods. Established biofilms demonstrated low inter-sample variation and were composed of highly diverse communities. A similar *in vitro* model had been previously used to culture salivary bacteria of healthy individuals and was demonstrated to produce very consistent biofilms (Kistler et al., 2015). Biofilms cultured in different media were composed of diverse communities allowing the investigation of different environments on biofilm composition.

Culture time, but not medium type had the greatest impact on the composition of biofilms, as seen in Chapters 4 and 5. The majority of species enriched in Chapter 4 in week three biofilms were fastidious, proteolytic, anaerobic and associated with periodontal disease (Perez-Chaparro et al., 2014), while week one biofilms had more health-associated species (for example, belonging to genera *Streptococcus, Veillonella* and *Prevotella*). The enrichment experiments described in Chapters 4 and 5 demonstrated a decrease in diversity with culture time. The possible explanation may be that a small fraction of species was able to thrive in the given environment and subsequently participate in the assembly of the community and subsequent enrichment. As opposed to a natural environment (i.e. mouth), the model was a closed system, with no

possibility of addition of species once inoculation had taken place, so it was not feasible for the biofilms to increase in diversity. The biofilms did not appear to have a richer composition than inoculum, due to the new environment permitting increases in abundance of a wider range of organisms. Species that were competitive enough in the provided environment overtook some inoculum species in terms of abundance (for example, aerobic, saccharolytic, fast-growing species, that were high in abundance in inocula but barely detectable after culture). Moreover, some species, that were high in abundance in biofilm cultures, were not detected in the initial inoculum.

A significant observation in this project was the major shift in the community composition during enrichment culture, leading to the appearance of many species that have been implicated in periodontal disease and a concomitant decrease in health-associated organisms (Diaz et al., 2016). Perez-Chaparro et al. (2014) identified 31 species as having a moderate or strong association with periodontal disease (Table 1.1). Out of these newly identified perio-pathogens, 10 significantly enriched during the culture, 16 of these species did not change their relative abundance and only 2 species associated with periodontal disease decreased in abundance in Chapter 4. Many periodontitis-associated species were more abundant in biofilms, if serum was used in a medium, such as *P. gingivalis, F. alocis, M. timidum, P. baroniae, E. infirmum* and *D. invisus*, showing its importance for perio-pathogen enrichment. Although changes in microbiota during culture were to be expected, the striking enrichment of so many of the species detected in periodontitis, while growing in anaerobic protein-rich environment, was dramatic and proved that healthy plaque has the potential to become ‘pathogenic’ if the environment changes in a way that promotes their growth if they are given sufficient time.

6.3 Impact of nutrition on enrichment of periodontal pathogens

The initial enrichment experiments were carried out using a medium used successfully in early chemostat studies to culture multi-species communities from a defined inoculum (Bradshaw et al., 1994). In Chapter 5, attempts were made to improve the composition of the growth medium, and increase its relevance, by selecting more natural components. Host-derived saliva and

human serum were included in the growth medium to create a more realistic bacterial growth environment, but these changes yielded relatively minor changes in species composition. For example, biofilms cultured solely in sterile human saliva in general had more *Streptococcus, Prevotella,* and *Veillonella* species but fewer *Porphyromonas* and *Peptostreptococcus* species than those cultured with serum in the medium . When human serum was used instead of foetal bovine serum, *P. gingivalis* and *P. marshii* were in lower abundance, while members of the genera *Treponema* (including *T. vincentii, T. denticola*) and *Peptostreptococcus* grew preferentially in the medium with human serum. All of the above mentioned species were barely detectable in the inoculum but were enriched with culture, however, to a different extent in different media. Majority of these species are important indicators of periodontal disease and are frequently isolated from active periodontal lesions (Socransky et al., 1998). For the accuracy of *in vitro* experiments, human serum may be a better choice, as it is more relevant and does enrich different bacteria than foetal bovine serum.

Irrespective of the type of serum used, a striking finding was the enrichment of species that had not been detected in the inoculum, even with the use of sensitive contemporary metagenomic approaches. Sixty four species were found in the three week biofilms (see Appendix Table 1) that were not apparent in the inoculum, though the presence of some were influenced by the presence or absence of serum. This confirms that periodontal pathogens can be found and persist in low numbers at sites in the healthy mouth.

6.4 Functional profiles of enriched microbial communities

The metagenomic approach enabled an investigation of both the taxonomical composition of samples and the potential functions of the biofilms by virtue of the genes detected. Functional potential analysis showed that the cultured samples had more genes associated with protein metabolism, motility and virulence than the inoculum. However, it did not show which bacterial functions were active at the time of sample collection. For that, analysis of transcriptomics, proteomics or metabolomics will be required; the metagenomics data only show the potential of transcription based on the collected DNA. Also, the analysis of functional potential from DNA may reveal

only a part of the picture. Some metatranscriptomics studies show discrepancies between taxonomical and expression data. For example, a very small taxonomical component can account for a high proportion of expressed rRNA (Nascimento et al., 2017). In a study by Benitez-Paez et al. (2014), the genus *Actinomyces* constituted a small fraction of identified bacteria from supragingival plaque, but this was the most active member in the community. In contrast, the genera *Streptococcus* and *Neisseria* were abundant, but demonstrated only low expression activity (Benitez-Paez et al., 2014; Nascimento et al., 2017). Moreover, in our study, only one third of reads were assigned to function. A more comprehensive analysis of actual functions could complement this *in vitro* study by analysing metatranscriptomes to reveal in-depth differences in bacterial expression depending on the different stages of dysbiosis and the impact of changes in the growth environment.

6.5 Is dysbiosis reversible or permanent?

One of the aims of the experiments detailed in Chapter 5 was to investigate whether elimination of serum and other proteinaceous components from the growth medium would affect the composition of the three week biofilms that had already undergone dysbiosis. Some changes were documented, such as a decrease in the genus *Porphyromonas*, and an increase in *Fretibacterium* and *Slackia* after two weeks of growth solely on natural saliva. However, no enrichment of health-associated species belonging to genera such as *Streptococcus* and *Actinomyces* was observed. This implies that it was not possible to reverse the changes simply by removing the nutritional driver, and that the newly developed dysbiotic community was stable. A stable dysbiosis concept was first proposed for the gut microbiome by Lozupone et al. (2012) and later adapted by Mira et al. (2017) for oral diseases. They proposed that up to a certain point, dysbiosis is a reversible process, and minor perturbations in biofilm composition can be reversed to re-establish a healthy community (Figure 6.1). However, after a threshold is passed, a dysbiotic community cannot be reversed and is resistant to change and colonisation and re-establishment by healthy species. It is possible that stable dysbiotic biofilm communities established in the CBD model during the first three weeks of growth that were resistant to subsequent attempts to induce changes in composition. In order to fully understand if this might be the case, an

introduction of saliva during earlier stages of biofilm development would be necessary.

The concept that '*Everything is everywhere, but, the environment selects'* was stated at the start of this Chapter. In the studies described here, numerous bacterial species were found in biofilms after enrichment culture that could not be detected in the inoculum. These results indicate that the source and reservoir of the periodontal pathogens that have been identified only recently (e.g. Perez-Chaparro et al. (2014)) is the healthy human mouth. Thus, it appears that this ecological tenet holds true in the oral cavity, and the studies reported here provide further support for the Ecological Plaque Hypothesis (Marsh, 1994, 2003).

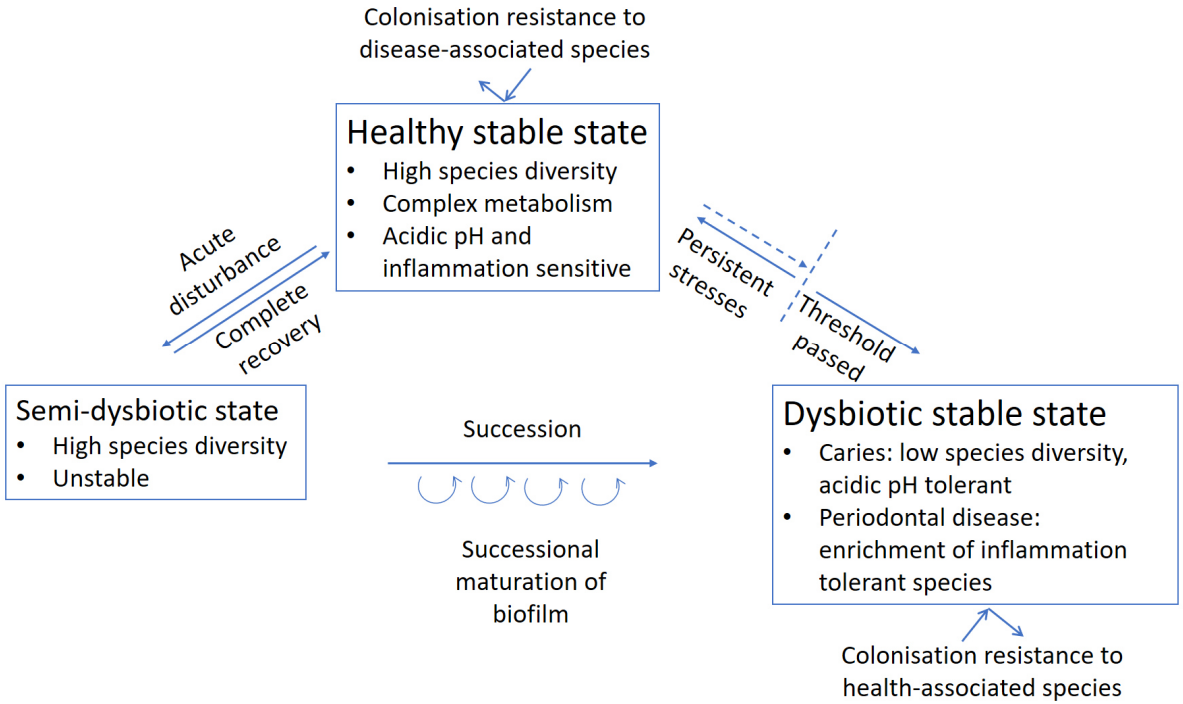

Figure 6.1 Proposed mechanism for the transition to dysbiosis in the oral cavity.
The healthy microbiome is relatively stable. In the case of oral disease, the threshold in the resilience mechanisms is overcome by, for example, intake of rapidly-fermentable carbohydrates and the subsequent fall in pH in caries. In periodontal disease, the triggers for dysbiosis may be host factors, leading to the enrichment of proteolytic and inflammophilic species. Mira et al. proposed that a stable dysbiotic stage exists, the same as for the healthy stable state, and both are resistant to compositional changes. Acute changes may also occur in health leading to an unstable semi-dysbiotic state which can be reversed to the original healthy community or alternatively undergo further compositional change to a stable dysbiotic state. Successional changes during biofilm maturation are discusses in the Introduction in section 1.6.2. Figure adapted from Mira et al. (2017).

Future work

Studies in Chapter 5 attempted to determine whether the dysbiotic changes induced by growth in a protein rich medium, with or without serum supplementation, could be reversed by exposing the biofilm to human saliva. Although a few moderate changes in the microbiota were observed, there was no enrichment of health-associated species such as streptococci or *Actinomyces*. Cultures could be repeated in future work, but with the re-introduction of saliva made earlier in the time-line, or some health-associated species added to the cultures simultaneously with saliva.

Chapters 3,4 and 5 described the application of an *in vitro* model of dysbiosis which used the CBD to culture oral biofilms. CBD is a closed culture system, so persistent metabolite accumulation may affect the formation of biofilms, and there is little ability to control or deliberately influence certain environmental factors, such as pH. *In vitro* models are unable to provide a natural habitat for bacterial communities, however continuous culture systems, although highly labour intensive, have certain advantages because they ensure constant nutrient supply and metabolite removal. Chemostats also permit the monitoring of important parameters such as pH and redox potential, and the opportunity to control or vary these and other factors as well. However, chemostats lack the surface for biofilm formation, but modifications introducing more stages, thus allowing biofilm formation and sampling could be used (Bradshaw et al., 1996a). The group of P. Diaz (University of Connecticut, USA) performed continuous culture experiments in singe-stage chemostat and showed that serum can enrich certain periodontal pathogens (unpublished data; Hoare et al., 2018, IADR abstract). A defined multi-species inoculum of around 20 species was cultured in the chemostat system. After a stable community was achieved, serum was introduced in the growth environment which significantly enriched *P. gingivalis.* In the future, a similar experiment could be performed with a natural inoculum from healthy volunteers and using continuous culture methods rather than the CBD. Novel sequencing techniques for community characterisation could identify species that benefit from the increase of serum

in the growth environment, and permit repeated sampling for time-course studies, and provide sufficient material for detailed metabolomic and transcriptomic analyses. This would provide more information on the process of dysbiosis established in the periodontal pocket during inflammation.

Only a proportion of the human population is susceptible to periodontal disease. Some individuals can have poor oral hygiene, and extensive accumulation of plaque without inflammation and loss of periodontal tissues. Zaura et al. (2017) suggested that only certain individuals are susceptible to periodontal disease based on their ecotypes. Now we have succeeded in developing a model of ‘pathogen’ enrichment, this approach could be applied to individual samples of biofilm to see whether disease-associated organisms are present at all sites and in all individuals, or whether their distribution is highly localised or they colonise only a subset of people. If the latter was the case then it might be possible to identify individuals at risk of periodontitis in advance of the development of disease and focus remedial therapy on this group.

Corrections

6.6 Strain identification in metagenomics

Metagenomics is a powerful tool providing an access to gene composition of the entire microbial community directly from the environment. Currently metagenomics projects widely use direct random shotgun sequencing: large fragments of DNA are broken down into many smaller pieces called ‘reads’, which are later mapped to reference genomes, annotated and analysed. Instead of using large pieces of DNA for the analysis, which can be slow, inaccurate and expensive, shotgun sequencing is comparatively quick and cheap. Processed gene libraries are later screened for dedicated marker genes (sequence-driven approach) or metabolic functions (function-driven approach).

The main drawbacks of metagenomics approach are that reference genomes are required and quality of the analysis will depend significantly on the quality of the reference database. Moreover, reads need to be unique in order to map with species or even strains. While DNA mainly consists of repeated sequences that do not provide discrimination between taxonomical units, only a small fraction of reads empowers the discrimination between lower levels of taxonomic ranks such as genera, species or strains.

Massive strain-level heterogeneity in the human microbiome has been observed. Recent investigations into bacterial communities showed that discrimination of strains might shed more light on pathogenesis of diseases such as peptic ulcer disease, gastric cancer and mucosa-associated lymphoid tissue lymphoma as certain pathogens can be strain specific (Espinoza et al., 2018; Segata, 2018). Different strains interact differently with host tissues (Bron et al., 2011) and modulate host immune response in different ways (Needham et al., 2013). For example, *Helicobacter pylori* or *Escherichia coli* strains can be gut commensals as well as highly pathogenic and carcinogenic (Cuevas-Ramos et al., 2010; Frank et al., 2011).

Strain is defined as a genomic variation consisting of acquisition/loss of genomic elements accumulating over microbial generations in pure cultures. However, it is possible that synonymous mutations do not provide any (dis)advantages in the same microbiome sample. The strain can also be defined as microbial entity despite some microbial heterogeneity and have the same phenotype under different conditions. Identification of strains is usually

performed using laborious isolation and cultivation techniques of species of interest, along with DNA extraction, PCR amplification, purification and sequencing of target loci.

Metagenomics enable the exploration of complex communities in environmental and human-associated ecosystems without the need for *in vitro* cultivation and utilizes computational methods to profile microbial communities and metabolic pathways in a microbiome. Although important differences between microbes occur between strains, it has been difficult to achieve accurate metagenomic profiling beyond the species level. However, some recent approaches can extract strain-level sequence fingerprints of microbial taxa from metagenomic data, using a cultivation-free metagenomics approach.

One method uses multilocus sequence typing (MLST) on metagenomics assemblies, where the assembled metagenomics contigs are mapped against the MLST databases. Databases of thousands of MLST profiles and sequences are available for large number of bacterial species most of which are opportunistic pathogens (Zolfo et al., 2017). However, this approach is computationally demanding and can only uncover abundant strains. Another method – StrainPhlAn – allows the characterisation of genomic structure of thousands of strains from hundreds of species (Truong et al., 2017). This method relies on per-sample dominant sequence variant reconstruction within species-specific marker genes and using them to estimate strain-level phylogenies. PanPhlAn is another tool that provides profiling of strains within complex communities of unknown composition (Scholz et al., 2016). PanPhlAn identifies which genes are present or absent within different strains of a species, based on the entire gene set of the species' pangenome. However, several challenges still need to be addressed while analysing strains, including profiling nondominant strains of low abundance.

Recent study used the combinations of these and novel methods (MILST, MetaPhlan) on oral metagenomes and profiled *Neisseria* species and strains. Authors found that different species and strains of *Neisseria* colonize different sites within the oral cavity and oropharynx, showing close associations between species and tissues (Donati et al., 2016).

One of the possible future works of this PhD project could be to investigate which strains were enriched in the presence and absence of human and foetal bovine serum in the growth media during both sequencing experiments.

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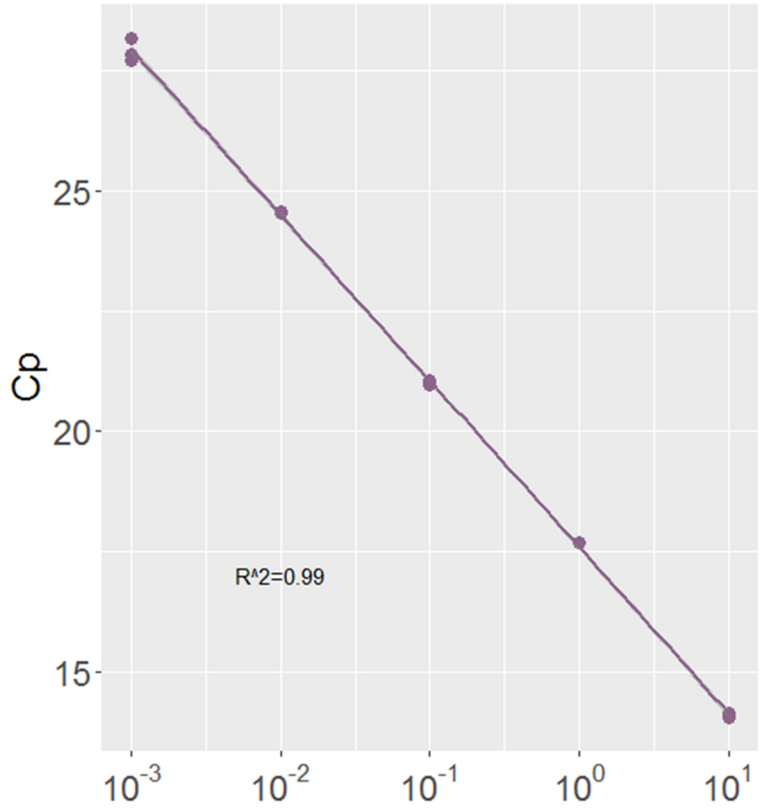
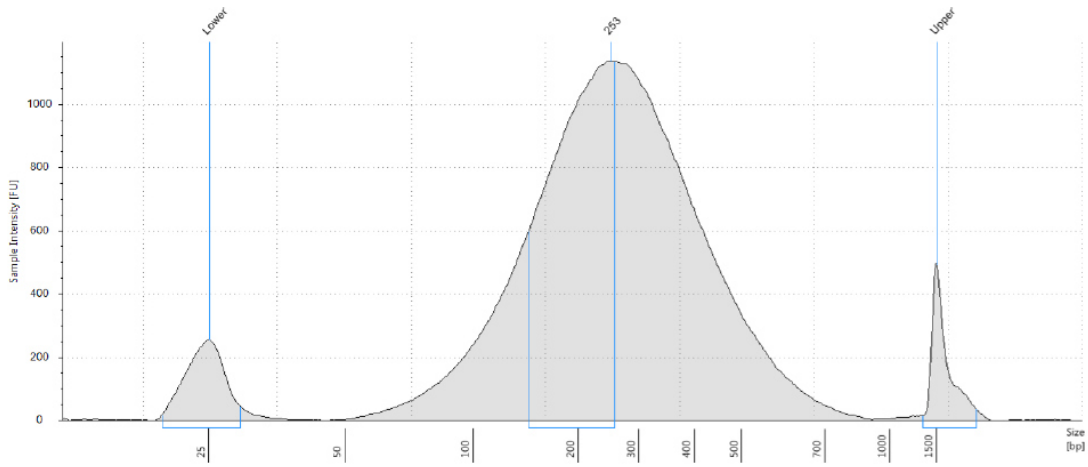
Appendix



Appendix Figure 1. Standard curve generated from P. gingivalis W83 waaA gene.
X axis shows *P. gingivalis* concentration in ng on a log10 scale and y axis shows crossing point values.

C1: I 20-3

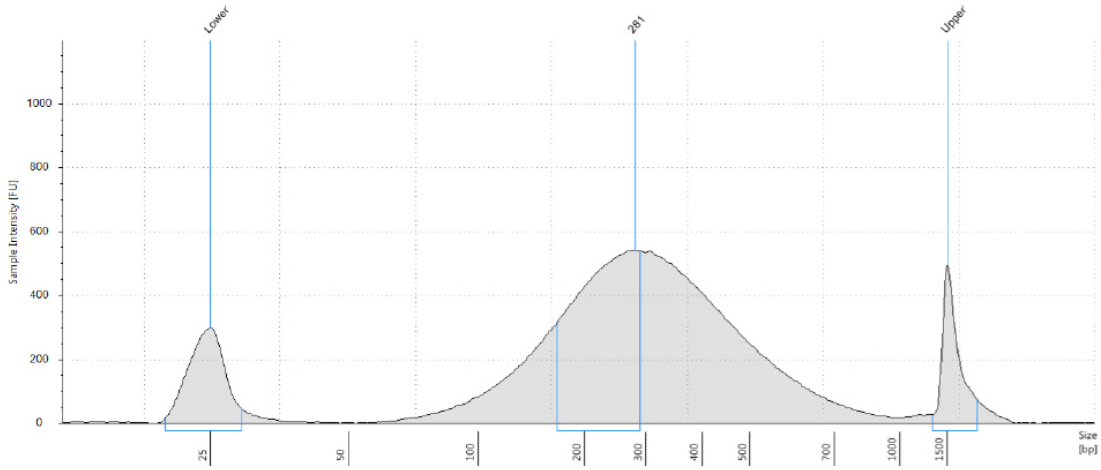


Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
C1	2320	I 20-3		

Appendix Figure 2. Example of the output obtained after quality assessment on Agilent D1000 Screen Tape.
The majority of the fragments for this sample are 253 bp long.

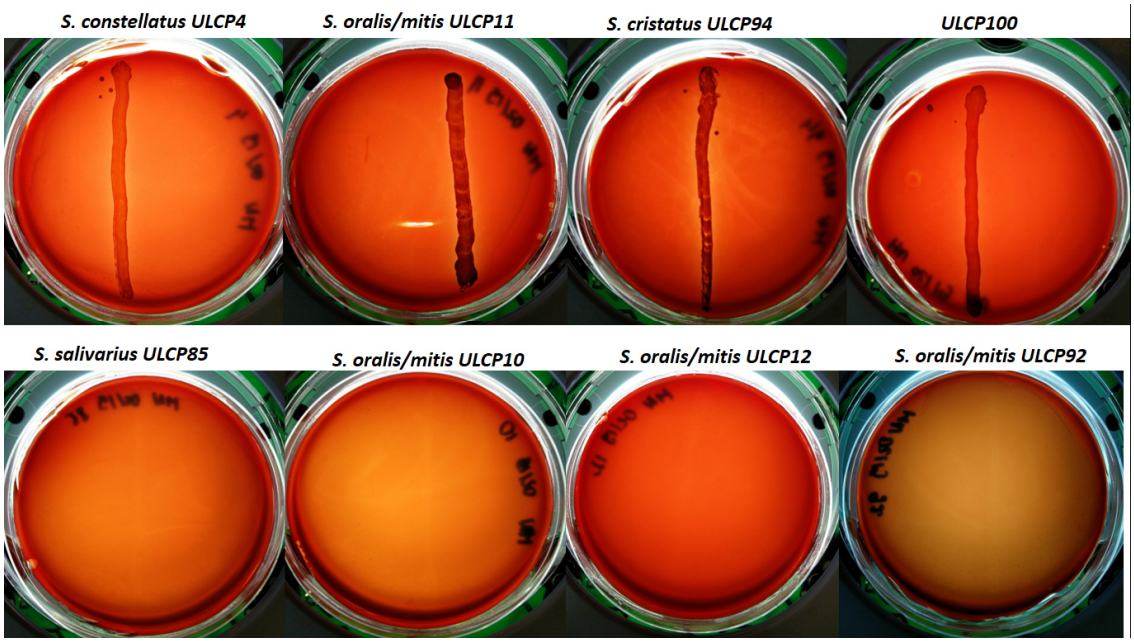
B1: III 0-1p



Sample Table

Well	Conc. [pg/µl]	Sample Description	Alert	Observations
B1	1050	III 0-1p		

Appendix Figure 3. Example of the output obtained after quality assessment on Agilent D1000 Screen Tape.
The majority of the fragments for this sample are 281 bp long.



Appendix Figure 4. *Streptococcus* species cross-streaked with *P. gingivalis*.
Columbia Blood Agar plates were plated with different *Streptococcus* species, cross-streaked with *P. gingivalis* W83 and incubated anaerobically. The growth of *P. gingivalis* was evaluated after 72h.

Appendix Table 1 Species present in biofilms enriched for three weeks in a protein-rich medium supplemented with or without serum but which were not detected in the inoculum.
The MEtaGenome ANalizer (MEGAN) was used to provide taxonomic annotations for reads using weighted lowest common ancestor (LCA) algorithm and recommended parameters. FBS – foetal bovine serum.

Detected in biofilms cultured in protein-rich medium	**Detected in biofilms cultured in protein-rich medium + FBS**	**Detected in biofilms enriched under both conditions**
Oribacterium sp. OT 108	*Actinomyces cardiffensis*	*Pseudoramibacter alactolyticus*
Fusobacterium sp. CM22	*Bacteroides sp.* 2 1 33B	*Parvimonas sp.* KA00067
Desulfovibrio sp. 31 syn3	*Bacteroides sp.* 3 1 33FAA	*Prevotella sp.* MSX73
Clostridium sp. CAG:81	*Clostridium sp.* CAG:413	*Shuttleworthia sp.* MSX8B
Oscillibacter valericigenes	*Coprobacillus sp.* CAG:235	*Anaerotruncus sp.* CAG:390
Prevotella loescheii	*Megasphaera genomosp.*	*Holdemania filiformis*
Treponema lecithinolyticum	*Eubacterium yurii*	*Prevotella sp.* OT 472
Megasphaera sp. BV3C16-1	*Porphyromonas gingivicanis*	*Clostridium sp.* SY8519
Bacteroides sp. CAG:598	*Porphyromonas gulae*	*Clostridium sp.* C105KSO13
Campylobacter rectus	*Sphaerochaeta globosa*	*Synergistes sp.* 3 1 syn1
Streptococcus sp. AS20	*Sphaerochaeta coccoides*	*Parvimonas sp.* OT 393
Streptococcus sp. DD04	*Streptococcus sp.* CM7	*Clostridium clostridioforme*
Prevotella enoeca	*Atopobium rimae*	*Clostridioides difficile*
Streptococcus sinensis		*Bacteroides sp.* CAG:770
		Prevotella buccae
		Peptostreptococcus anaerobius CAG:621
		Dethiosulfovibrio peptidovorans
		Synergistes jonesii
		Bacteroides sp. CAG:545
		Dialister invisus CAG:218
		Mogibacterium timidum
		Olsenella uli
		Jonquetella anthropi
		Dialister invisus
		Fusobacterium necrophorum

		Fusobacterium sp. CM21
		Prevotella marshii
		Bacteroides sp. CAG:709
		Mogibacterium sp. CM50
		Fusobacterium sp. OT 370
		Streptococcus constellatus
		Slackia exigua
		Desulfovibrio desulfuricans
		Slackia sp. CM382
		Treponema maltophilum
		Bacteroides sp. CAG:1060
		Anaeroglobus geminatus

Appendix Table 2. Relative abundance of functional groups than were unique to biofilms.

Functional Group	inoculum	Protein-rich medium + human saliva + foetal bovine serum	Protein-rich medium + human saliva + human serum	saliva	Human saliva + human serum
hydrolase, haloacid dehalogenase-like family protein BCZK2594	0	111	358	21	191
L-Aspartate dehydrogenase (EC 1.4.1.21)	0	89	271	20	156
UDP-glucuronic acid oxidase (UDP-4-keto-hexauronic acid decarboxylating) (EC 1.1.1.305)	0	68	48	61	154
DUF324 domain containing Cmr2-like protein	0	64	36	48	114
UDP-4-amino-4-deoxy-L-arabinose formyltransferase (EC 2.1.2.13)	0	45	30	35	101
Substrate-specific component TrpP of tryptophan ECF transporter	0	55	183	12	90
CRISPR-associated protein Csx11	0	29	19	21	72
Phosphoglucomutase (EC 5.4.2.2)	0	33	27	28	68
Serine-protein kinase rsbW (EC 2.7.11.1)	0	36	97	6	61
Polymyxin resistance protein ArnC, glycosyl transferase (EC 2.4.-.-)	0	33	23	27	59
Energy-conserving hydrogenase (ferredoxin), subunit A	0	20	29	194	51
Protein AraJ precursor	0	4	33	0	51
Predicted amino-acid acetyltransferase (EC 2.3.1.1) complementing ArgA function in Arginine Biosynthesis pathway	0	18	13	13	39

Thiosulfate reductase electron transport protein phsB	0	13	23	186	35
Spore maturation protein B	0	22	12	22	32
Spore maturation protein A	0	19	12	21	29
D-arabino-3-hexulose 6-phosphate formaldehyde-lyase (EC 4.1.2.43)	0	5	19	0	22
Fe-S-cluster-containing hydrogenase components 1	0	6	18	125	21
Xylanase	0	34	22	8	19
Heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating P-type ATPase:Heavy metal translocating P-type ATPase	0	12	20	10	18
Propanediol utilization transcriptional activator	0	76	30	3	18
Glycerol dehydratase reactivation factor large subunit	0	4	4	3	16
4-hydroxyphenylacetate 3-monooxygenase (EC 1.14.13.3)	0	63	32	1	16
Substrate-specific component TTE1586 of predicted methylthioadenosine ECF transporter	0	6	3	8	12
Tagatose-6-phosphate kinase GatZ (EC 2.7.1.144)	0	31	11	25	11
Succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B (EC 2.8.3.5)	0	9	14	159	10
Duplicated ATPase component TTE1589 of energizing module of predicted methylthioadenosine ECF transporter	0	5	4	9	9
Duplicated ATPase component of energizing module of predicted pyridoxine-related ECF transporter	0	2	14	0	9
ATP-dependent DNA helicase UvrD/PcrA, proteobacterial paralog	0	16	8	17	8
Sugar phosphate isomerases/epimerases family protein YcjR	0	16	18	7	8
virulence cluster protein B VclB	0	4	18	1	8
tRNA(U54)-2-thioribothymidine synthetase	0	14	6	15	7
Alpha-arabinosides ABC transport system, permease protein 1	0	5	5	2	7

Betaine reductase component B beta subunit (EC 1.21.4.4)	0	12	11	20	7
Osmotically activated L-carnitine/choline ABC transporter, substrate-binding protein OpuCC	0	2	8	4	7
ATP-dependent DNA helicase UvrD/PcrA/Rep, cyanobacterial paralog	0	8	16	2	7
heterodisulfide reductase, subunit E, putative	0	12	11	23	6
Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease component 1	0	19	6	11	6
tRNA (adenine57/58-N1)-methyltransferase (EC 2.1.1.36)	0	7	6	9	6
Carbon monoxide-induced hydrogenase small subunit CooL	0	4	1	4	6
cytosolic long-chain acyl-CoA thioester hydrolase family protein	0	10	5	1	6
Heme transporter analogous to IsdDEF, ATP-binding protein	0	1	3	1	6
2,3-bisphosphoglycerate-independent phosphoglycerate mutase, archaeal type (EC 5.4.2.1)	0	2	4	0	6
Hydrogenase-4 component I	0	1	3	26	5
Thiosulfate reductase precursor (EC 1.-.-.-)	0	3	5	22	5
DNA-directed RNA polymerase gamma subunit (EC 2.7.7.6)	0	2	1	3	5
Oligogalacturonate lyase (EC 4.2.2.6)	0	5	1	2	5
Catalyzes the cleavage of p-aminobenzoyl-glutamate to p-aminobenzoate and glutamate, subunit A	0	2	2	1	5
NADP-reducing hydrogenase, subunit C	0	4	25	1	5
Heme transporter IsdDEF, permease component IsdF	0	0	1	0	5
Flagellar P-ring protein FlgI	0	4	3	2	4
CoB--CoM heterodisulfide reductase 2 iron-sulfur subunit D (EC 1.8.98.1)	0	1	2	21	4
Carbon monoxide-induced hydrogenase iron-sulfur protein CooX	0	5	7	6	4

Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease component 2	0	9	4	6	4
Two-component response regulator YvcP	0	5	13	5	4
Predicted beta-glucoside transporter, GPH family	0	11	2	2	4
Propanediol utilization polyhedral body protein PduN	0	11	3	0	4
FIG018171: hypothetical protein of Cupin superfamily	0	0	2	0	4
tRNA nucleotidyltransferase, A-adding (EC 2.7.7.25)	0	7	2	11	3
Polysulfide reductase, subunit B, putative	0	1	1	5	3
Galactonate dehydratase (EC 4.2.1.6)	0	11	5	5	3
Sulfite reduction-associated complex DsrMKJOP multiheme protein DsrJ (=HmeF)	0	2	1	4	3
Phytochrome, two-component sensor histidine kinase (EC 2.7.3.-)	0	0	1	2	3
Zinc-type alcohol dehydrogenase YcjQ	0	8	3	1	3
Dipeptidyl peptidase IV in 4-hydroxyproline catabolic gene cluster	0	2	4	1	3
Urea ABC transporter, ATPase protein UrtE	0	0	0	0	3
Carbon monoxide-induced hydrogenase NuoC-like protein CooU	0	6	3	11	3
Coenzyme F420-reducing hydrogenase, beta subunit	0	2	1	6	3
Uncharacterized sugar kinase YdjH	0	12	2	3	3
Predicted cobalt transporter in sulfate-reducing delta-proteobacteria	0	1	2	3	3
CRISPR-associated protein, Cas5e family	0	0	1	1	3
[Ni/Fe] hydrogenase, group 1, small subunit	0	2	0	1	3
2-hydroxyhepta-2,4-diene-1,7-dioate isomerase (EC 5.3.3.-)	0	1	1	1	3
alternative Ribulokinase (EC 2.7.1.16)	0	1	4	0	3

Heterodisulfide reductase, cytochrome reductase subunit	0	7	4	14	2
Ni,Fe-hydrogenase III large subunit	0	5	2	5	2
D-xylose proton-symporter XylT	0	0	2	0	2
Alpha-arabinosides ABC transport system, permease protein 2	0	0	1	0	2
B12 binding domain of Methylmalonyl-CoA mutase (EC 5.4.99.2)	0	2	0	0	2
CO dehydrogenase accessory protein CooC (nickel insertion)	0	1	0	0	2
4-hydroxycinnamoyl CoA hydratase/lyase (Enoyl-CoA hydratase/lyase) (EC 4.2.1.17)	0	0	0	0	2
Ni,Fe-hydrogenase I cytochrome b subunit	0	0	0	0	2
Adenylylsulfate reductase-associated complex QmoABC, protein QmoB	0	10	7	12	2
Flagellar basal-body P-ring formation protein FlgA	0	5	3	6	2
Glutathione-dependent formaldehyde-activating enzyme (EC 4.4.1.22)	0	1	1	3	2
Transcriptional repressor, BlaI/MecI family	0	4	1	2	2
Nickel responsive regulator NikR	0	1	0	2	2
Dissimilatory sulfite reductase D	0	3	1	1	2
Hypothetical sugar kinase in cluster with indigoidine synthase indA , PfkB family of kinases	0	2	1	1	2
Putative glutathione transporter, ATP-binding component	0	0	1	1	2
D-tagatose 3-epimerase (EC 5.3.1.-)	0	1	1	1	2
Homogentisate 1,2-dioxygenase (EC 1.13.11.5)	0	0	1	1	2
NADPH-dependent methylglyoxal reductase (D-lactaldehyde dehydrogenase)	0	0	0	1	2
Ferredoxin reductase	0	1	1	0	2

Urea carboxylase-related ABC transporter, permease protein	0	0	0	0	2
2-oxoglutarate/L-arginine monooxygenase/decarboxylase (succinate-forming) (EC 1.14.11.34)	0	0	0	0	2
Arsenic resistance protein ArsH	0	0	0	0	2
UDP-Bac2Ac4Ac hydrolyzing 2-epimerase NeuC homolog	0	2	1	10	1
3-oxoadipate CoA-transferase subunit B (EC 2.8.3.6)	0	4	6	8	1
Novel D-mannonate dehydrogenase (EC 1.1.1.57)	0	6	4	7	1
High-affnity carbon uptake protein Hat/HatR	0	1	1	5	1
Glycine cleavage system transcriptional antiactivator GcvR	0	3	3	4	1
Phosphatidylinositol-specific phospholipase C (EC 4.6.1.13)	0	14	6	4	1
Nicotinate dehydrogenase, large molybdopterin subunit (EC 1.17.1.5)	0	2	3	3	1
Flagellar biosynthesis protein FliO	0	3	1	2	1
Glycolate permease	0	2	0	2	1
Formate hydrogenlyase transcriptional activator	0	1	2	1	1
NADH dehydrogenase subunit 2	0	0	2	1	1
Protoporphyrin IX Mg-chelatase subunit I (EC 6.6.1.1)	0	0	0	1	1
Putative diheme cytochrome c-553	0	0	0	1	1
(S)-2-(hydroxymethyl)glutarate dehydratase	0	1	4	0	1
Propanediol utilization polyhedral body protein PduA	0	6	2	0	1
GDP-mannose 4,6 dehydratase Gmd (EC 4.2.1.47)	0	1	2	0	1
Putative HTH-type transcriptional regulator YdjF	0	1	1	0	1
Dimethylmaleate hydratase, large subunit (EC 4.2.1.85)	0	1	1	0	1
Transcriptional activator of acetoin dehydrogenase operon AcoR	0	1	1	0	1

Predicted 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase	0	0	0	0	1
Glucose dehydrogenase, PQQ-dependent (EC 1.1.5.2)	0	0	0	0	1
related to 6-phospho-3-hexuloisomerase	0	0	0	0	1
L-fuconate dehydratase (EC 4.2.1.68)	0	0	0	0	1
Light-independent protochlorophyllide reductase subunit N (EC 1.18.-.-)	0	0	0	0	1
Hypothetical protein (associated with DNA helicase - Rad25 homolog)	0	0	0	0	1
Heme ABC transporter (Streptococcus), permease protein	0	0	0	0	1
Catechol 1,2-dioxygenase (EC 1.13.11.1)	0	0	0	0	1
HemX protein, negative effector of steady-state concentration of glutamyl-tRNA reductase	0	2	1	3	1
Sensor protein of zinc sigma-54-dependent two-component system	0	3	1	2	1
Citrate lyase, subunit 1 (2.3.3.8)	0	0	0	2	1
Acetoin catabolism protein X	0	1	2	1	1
NAD-dependent formate dehydrogenase gamma subunit	0	1	1	1	1
Carbon monoxide dehydrogenase small chain (EC 1.2.99.2)	0	1	1	1	1
Cytoplasmic trehalase (EC 3.2.1.28)	0	0	0	1	1
RNA polymerase sigma factor RpoS	0	0	4	0	1
Transmembrane component of energizing module of predicted pyridoxine-related ECF transporter	0	0	1	0	1
Predicted nitrite reductase [NAD(P)H] small subunit, associated with glutamine/glutamate metabolism	0	0	1	0	1
Glycine betaine ABC transport system, glycine betaine-binding protein OpuAC	0	2	1	0	1

D-allose-6-phosphate isomerase (EC 5.3.1.-)	0	0	1	0	1
Predicted rhamnose oligosaccharide ABC transport system, permease component	0	0	1	0	1
Duplicated ATPase component of energizing module of riboflavin ECF transporter	0	1	0	0	1
glutamine synthetase family protein	0	0	0	0	1
Monoamine oxidase (1.4.3.4)	0	0	0	0	1
5-aminolevulinate synthase (EC 2.3.1.37)	0	0	0	0	1
Urea ABC transporter, ATPase protein UrtD	0	0	0	0	1
Formaldehyde activating enzyme	0	0	0	0	1
Predicted L-arabinose ABC transport system, periplasmic arabinose-binding protein	0	0	0	0	1
L-ribulose-5-phosphate 4-epimerase UlaF (EC 5.1.3.4) (L-ascorbate utilization protein F)	0	0	0	0	1
Predicted rhamnogalacturonan lyase in rhamnose utilization cluster	0	0	0	0	1
Triacylglycerol lipase (EC 3.1.1.3)	0	0	0	0	1
Nitrite reductase probable electron transfer 4Fe-S subunit (EC 1.7.1.4)	0	0	0	0	1
Hydrogenase-4 component H	0	0	0	0	1
GTP-binding protein related to HflX	0	0	0	0	1
Adenylylsulfate reductase-associated complex QmoABC, protein QmoA	0	4	4	7	0
Inorganic pyrophospatase PpaX (EC 3.1.3.18)	0	0	0	7	0
Alpha-arabinosides ABC transport system, substrate-binding protein	0	5	3	5	0
Kynureninase (EC 3.7.1.3)	0	0	1	4	0
Hypothetical oxidoreductase YqhD (EC 1.1.-.-)	0	0	0	3	0

Propionate catabolism operon regulatory protein PrpR	0	0	0	3	0
Predicted alpha-L-rhamnosidase	0	0	1	2	0
Cysteine desulfurase (EC 2.8.1.7), NifS subfamily	0	1	1	2	0
Homocitrate synthase (EC 2.3.3.14)	0	0	0	2	0
Inositol oxygenase (EC 1.13.99.1)	0	0	0	2	0
Benzoate 1,2-dioxygenase alpha subunit (EC 1.14.12.10)	0	0	0	2	0
Peptidyl-prolyl cis-trans isomerase ppiC (EC 5.2.1.8)	0	1	1	1	0
Not a Proline racemase, nor 4-hydroxyproline epimerase [missing catalytic residues]	0	0	0	1	0
2-Methylcitrate dehydratase AcnD	0	0	0	1	0
Transmembrane component CbrV of energizing module of predicted cobalamin ECF transporter	0	0	0	1	0
Antirepressor [Bacteriophage A118]	0	0	0	1	0
Putative two-component system response regulator YedW	0	0	0	1	0
Heme ABC transporter (Streptococcus), heme and hemoglobin-binding protein SiaA/HtsA	0	0	2	1	0
TTE0858 replicon stabilization protein (antitoxin to TTE0859)	0	1	1	1	0
2-ketoaldonate reductase, broad specificity (EC 1.1.1.215) (EC 1.1.1.-)	0	1	0	1	0
Protein of unknown function DUF1446	0	1	0	1	0
3-hydroxyisobutyryl-CoA hydrolase (EC 3.1.2.4)	0	0	0	1	0
Saccharopine dehydrogenase (EC 1.5.1.9)	0	0	0	1	0
Ureidoglycolate/malate/sulfolactate dehydrogenase family (EC 1.1.1.-)	0	0	0	1	0
Alpha-1,4-digalacturonate ABC transporter, permease protein 2	0	0	0	1	0
Geranyl-CoA carboxylase biotin-containing subunit (EC 6.4.1.5)	0	0	0	1	0

7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase subunit 2	0	0	0	1	0
DNA polymerase-like protein PA0670	0	0	0	1	0
2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductases	0	0	0	1	0
Nitric oxide reductase FlRd-NAD(+) reductase (EC 1.18.1.-)	0	0	0	1	0
Glutathione S-transferase family protein	0	0	0	1	0
Putative Heme-regulated two-component response regulator	0	0	0	1	0
RNA polymerase sigma factor RpoH-related protein	0	0	0	1	0
RNA polymerase sigma factor SigW	0	0	0	1	0
L-lysine aminomutase regulator	0	4	10	0	0
CDP-glycerol: N-acetyl-beta-D-mannosaminyl-1,4-N-acetyl-D-glucosaminyldiphosphoundecaprenyl glycerophosphotransferase	0	3	4	0	0
NADH-dependent butanol dehydrogenase B (EC 1.1.1.-)	0	0	3	0	0
Nitrogenase (molybdenum-iron)-specific transcriptional regulator NifA	0	0	2	0	0
Cys-Xaa-Xaa-Xaa system radical SAM maturase	0	0	2	0	0
Alpha-glucoside transport system permease protein AglG	0	0	1	0	0
N-methylhydantoinase B (EC 3.5.2.14)	0	2	1	0	0
D-allulose-6-phosphate 3-epimerase (EC 5.1.3.-), row_id 846	0	1	1	0	0
crotonyl-CoA reductase	0	1	1	0	0
NADH-dependent butanol dehydrogenase (EC 1.1.1.-)	0	0	1	0	0
Heme transporter IsdDEF, lipoprotein IsdE	0	0	1	0	0
Potassium-transporting ATPase B chain (EC 3.6.3.12) (TC 3.A.3.7.1)	0	0	1	0	0
Bacitracin export ATP-binding protein BceA	0	0	1	0	0

Two-component sensor kinase YvcQ	0	0	1	0	0
Candidate gene for the hypothesized phosphomevalonate decarboxylase	0	2	0	0	0
CRISPR-associated protein Csx16	0	1	0	0	0
Nitrogenase (iron-iron) transcriptional regulator	0	1	0	0	0
Indolepyruvate ferredoxin oxidoreductase, alpha and beta subunits	0	1	0	0	0
Urea ABC transporter, urea binding protein	0	1	0	0	0
PTS system, diacetylchitobiose-specific IIB component (EC 2.7.1.69)	0	1	0	0	0
PTS system, mannitol-specific cryptic IIB component (EC 2.7.1.69)	0	1	0	0	0
Putative cytoplasmic protein clustered with trehalase	0	1	0	0	0
Capsular polysaccharide transcription antitermination protein, UpxY family	0	1	0	0	0
Lipoteichoic acid synthase LtaS Type IVa	0	1	0	0	0
Cobalamin biosynthesis protein BluB	0	1	0	0	0
Integron integrase IntI2	0	1	0	0	0
Two-component oxygen-sensor histidine kinase FixL	0	1	0	0	0
Streptolysin S biosynthesis protein D (SagD)	0	1	0	0	0

Appendix Table 3 The comparison of the enriched functional groups between two types of biofilms.
The relative abundance of functional groups were compared between week three biofilms cultured in sole human saliva and human saliva + human serum to investigate which functional groups were overrepresented in biofilms. * - p <0.05, ** - p < 0.01, *** - p < 0.001, DESeq2.

Functional groups enriched in biofilms cultured in sole human saliva	log2 fold change	Functional groups enriched in biofilms cultured in human saliva + human serum	log2 fold change
Secondary Metabolism	0.88***	RNA Metabolism	0.05*
Plant Glucosinolates	0.61***	Cell Division and Cell Cycle	0.07*
Central metabolism	0.61***	Virulence, Disease and Defence	0.09*
Metabolite damage and its repair or mitigation	0.40***	Virulence	0.11***
Metabolism of Aromatic Compounds	0.26***	Regulation and Cell signalling	0.12***
Respiration	0.25***	Dormancy and Sporulation	0.12***
Amino Acids and Derivatives	0.20***	Protein Metabolism	0.13***
Sulfur Metabolism	0.19***	Nucleosides and Nucleotides	0.14***
Fatty Acids, Lipids, and Isoprenoids	0.15***	Polyamines	0.22**
Carbohydrates	0.10**	Motility and Chemotaxis	0.24***
Miscellaneous	0.09***	DNA Metabolism	0.27***
Unclassified	0.07*	Phosphorus Metabolism	0.43***
Cell Wall and Capsule	0.06*	Transcriptional regulation	0.51***
		Arabinose Sensor and transport module	1.26***

Appendix Table 4. List of functional groups that decreased in abundance in week five samples compared to week 3 in all three biofilm types.

Functional Group
Cobalamin synthesis
Biotin biosynthesis in plants
B12 Biosynthesis (Tavares copy1)
Coenzyme B12 biosynthesis
B12 Cobalamin HMP
P uptake (cyanobacteria)
Biotin HMP
Mycobacterial MmpL2 membrane protein cluster
B12 duf71
CbiZ Main
CbiZ Main I
Cobalamin
Cobalamin Dh
Cobalt-zinc-cadmium resistance
EC 3.4.13.- Dipeptidases
CRISPRs
DNA replication, archaeal
Recycling of Peptidoglycan Amino Acids
Mycobacterium virulence operon possibly involved in quinolinate biosynthesis
Lysine degradation
Respiratory Complex I
YcfH
Nitric oxide synthase
DNA repair, bacterial MutL-MutS system
Unknown carbohydrate utilization (cluster Yeg)
Protection from Reactive Oxygen Species
Thiamin HMP
Raj MurE
Cellulosome
Methicillin resistance in Staphylococci
Monika MRSA

Folate HMP
Experimental ThMP RZ
Thiamin biosynthesis -ThMP phosphatase
LB thiamin
Thiamin biosynthesis in plants
Folate biosynthesis in plants
Mycobacterium virulence operon involved in an unknown function with a Jag Protein and YidC and YidD
Family 24 FUPA24 P-type ATPase
Family 24 FUPA24 P-type ATPase ver.1
Lysine fermentation MCB 432
Experimental tye
DNA structural proteins, bacterial
Pyrimidine utilization
Purine Utilization
Niacin, NAD and NADP biosynthesis in plants
rRNA modification bacteria
Bacterial motility:Gliding
Riboflavin, FMN and FAD biosynthesis in plants
Deoxyribose and Deoxynucleoside Catabolism
RCJ pfr
Thiamin biosynthesis
CLO thiaminPP biosynthesis
Pantothenate and CoA biosynthesis in plants
Thiamin biosynthesis LDP
NADH-dependent reduced ferredoxin: NADDP+ oxidoreductase
Ribosome biogenesis bacterial
Campylobacter Iron Metabolism
Lysine fermentation
CoA Pantothenate HMP
Murein hydrolase regulation and cell death
Fatty acid biosynthesis in plants (mitochondrial)
CarD
Methylthiotransferases
coA-FolK

Q RZ
NAD Niacin HMP
Biotin biosynthesis
NAD regulation
Propanediol utilization
Peptidoglycan Biosynthesis
Adenosyl nucleosidases
Plasmid replication
YgfZ-Fe-S
ThiD ThiE fusions
Glutathione-regulated potassium-efflux system and associated functions
tRNA aminoacylation, Arg
Haem LB
Haem and Sirohaem Biosynthesis
Thiamin Copy RZ
pyrimidine conversions
NAD and NADP cofactor biosynthesis global
Coenzyme A Biosynthesis
dcernst CoA Salvage
dcernst CoA Salvage2
Folate Biosynthesis
Test – Folate
DNA repair, bacterial
ATP-dependent RNA helicases, bacterial
Polyhydroxybutyrate metabolism
Translation initiation factors bacterial
Nitrosative stress
Ribosomal protein S12p Asp methylthiotransferase
Isoprenoid biosynthesis in plants, nonmevalonate branch
Nonmevalonate Branch of Isoprenoid Biosynthesis
Ribonucleotide reduction
Isoprenoid Biosynthesis
tRNA processing
Test – Thiamin
riboflavin to FAD

Phage cyanophage
Acetyl-CoA fermentation to Butyrate
RuvABC plus a hypothetical
Biotin synthesis & utilization
NAD and NADP - test subsystem
Riboflavin, FMN and FAD metabolism Extended
CoA Test

Appendix Table 5. List of functional groups that increased in abundance in week five samples compared to week 3 in all three biofilm types.

Functional group
Chorismate Synthesis
Sugar utilization in Thermotogales
Glycolysis and Gluconeogenesis
Evolution of Proline Biosynthesis (for review by Fichman et al)
Chorismate biosynthesis in *Streptococci*
Methanofuran
Methanofuran erick jmorales
TN RidA: all subgroups, 981 genomes carbamoyl-P
Arginine metabolism and urea cycle
Test – DHFR
RidA proteins copy with new groups
Lysine and threonine metabolism
Quinate degradation
RidA family in 981 representative prokaryotes
D-galactarate, D-glucarate and D-glycerate catabolism
D-galactarate, D-glucarate and D-glycerate catabolism - gjo
Glutamine, glutamate, aspartate, asparagine metabolism in plants
Arginine and Ornithine Degradation
Menaquinone and Phylloquinone Biosynthesis

Omega-amidase KE2
Succinate dehydrogenase
Lysine biosynthesis in Streptococci
Glutamine, Glutamate, Aspartate and Asparagine Biosynthesis
Family 23 FUPA23 P-type ATPase
Lacto-N-Biose I and Galacto-N-Biose Metabolic Pathway
AdoMet Repair
AdoMet Repair LMTB
Ammonia assimilation
RidA2 subgroup (YoaB-like)
Starch biosynthesis
Quinone disambuiguation
Threonine and Homoserine Biosynthesis
Methionine Salvage
Homomethionine biosynthesis and methionine chain elongation pathway for glucosinolates in plants
Fermentations: Lactate
Vibrio Polysaccharide (VPS) Biosynthesis
Acetyl-CoA biosynthesis in plants
Respiratory dehydrogenases 1
Arginine deiminase and agmatine deiminase pathways in *Streptococci*
EC 4. 1. 1.- Carboxy-lyases
Methionine Biosynthesis
Methionine in *Streptococci*
Quinones HGM
Omega-amidase KE
Fructooligosaccharides(FOS) and Raffinose Utilization
Leucine Biosynthesis
Histidine Biosynthesis in plants

Khodge314 Isoleucine Biosynthesis
Glycine reductase, sarcosine reductase and betaine reductase
Histidine Biosynthesis in *Streptococci*
D-gluconate and ketogluconates metabolism
Tryptophan metabolism in plants
Xylose utilization
D-Galacturonate and D-Glucuronate Utilization
Auxin biosynthesis
Glutamine synthetases
Arginine Biosynthesis extended
Xylan degradation in plants
Legionaminic Acid Biosynthesis
Toxin-antitoxin replicon stabilization systems
Flagellar motility
Xylose utilization
Mercuric reductase
Branched-Chain Amino Acid Biosynthesis
Branched-chain amino acid metabolism in plants
Inositol catabolism
D-Alanyl Lipoteichoic Acid Biosynthesis
Streptococcus agalactiae virulome
Phosphatidylethanolamine biosynthesis in plants
Two-component regulatory systems in Campylobacter
HMG CoA Synthesis
Phenylalanine and Tyrosine Branches from Chorismate
Acetoin, butanediol metabolism
CMP-N-acetylneuraminate Biosynthesis
Arginine Deiminase Pathway
Tyrosine and phenylalanine metabolism in plants

L-Arabinose CS
VC0266
Aromatic amino acid degradation
Steroid sulfates
L-fucose utilization temp
Sucrose utilization *Shewanella*
Streptococcus pyogenes Virulome
Histidine Biosynthesis
Rrf2 family transcriptional regulators
L-Arabinose utilization
Bacterial Chemotaxis
CRISPR-associated cluster
NiFe hydrogenase maturation
Sucrose utilization
RidA: all subgroups, 8100 genomes
Serotype determining Capsular polysaccharide biosynthesis in *Staphylococcus*
Toxin-antitoxin systems (other than RelBE and MazEF)
Dihydroxyacetone kinases
Choline Transport
Uptake of selenate and selenite
An Arabinose Sensor
CFE Sulfur Oxidation
L-fucose utilization
Alpha-Amylase locus in *Streptococococcus*
Osmotic stress cluster
Mycobacterium virulence operon involved in fatty acids biosynthesis
Universal stress protein family
Tetrathionate respiration
beta-glucuronide utilization

Transport of Molybdenum
Carotenoid biosynthesis
2-O-alpha-mannosyl-D-glycerate utilization