POLYPHASIC CHARACTERISATION

OF

THE HUMAN ORAL MICROBIOME

Submitted in Partial Fulfilment of the Requirements of the

Degree of Master of Science

IN ORAL MICROBIOLOGY

By

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CONTENTS

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ABSTRACT

The oral cavity supports a complex and finely balanced consortia of microbial species, many of which co-operate within highly structured biofilm communities. Given the importance of this microbiome in oral disease, considerable scientific effort has been put into surveying its diversity, determining the nature of interactions between its members, and exploring its determinants. This dissertation addressed each of these areas; the three principal objectives were (1) to assess microbial diversity in the mouth using culture-based methods, (2) to use next-generation sequencing technologies to explore person-to-person and temporal variation in oral microbiome composition, and (3) to use an in vitro model system to analyse variation in the biofilm forming capacity of members of the oral microbiome.

Objective 1: Samples of the microbiome were collected from one individual and plated onto a range of different axenic media, incubated under a range of different conditions. The diversity of isolates obtained was assessed on the basis of classical phenotypic characteristics and by using partial 16S rDNA sequence comparison. Twelve species were identified, all of which were well-recognised members of the oral microbiota.

Objective 2: Next-generation sequencing was performed on 16S rDNA fragments amplified from plaque samples were collected from the oral cavity of three healthy adult human volunteers each month for a period of eight months. A wide diversity of OTUs was detected in all samples that could be delineated into 13 phyla and 48 families. 60 OTUs could be identified at the species level. As expected, general linear models revealed statistically significant variation among the OTUs present in different individuals and within individuals over time.

Objective 3: Fourteen different oral streptococci strains were screened for biofilm formation using the established microtitre plate biofilm assay. The results of this study were inconsistent but it appeared that most strains best formed biofilms after about four days of incubation, and by day seven, bacteria had died. Optimisation of this technique is required.

The results of this dissertation add to current knowledge about the diversity and dynamics of the human oral microbiome. This study has also obtained a set of low-passage isolates of various members of the human microbiome and has begun to optimise an in vitro biofilm assay. Together, these will provide a useful resource for future exploration of the contribution of individual bacterial species to human oral biofilm infrastructure.

CHAPTER ONE: INTRODUCTION

ANATOMY AND PHYSIOLOGY OT THE HUMAN MOUTH

Within the oral cavity comprises of hard surfaces such as the teeth and the soft surfaces of the oral mucosa, which include the tongue, cheek plate and tonsils (Zaura *et al*, 2009, Dewhirst *et al*, 2010). The oral mucosa dominates the surface of the mouth, representing about 80% of a total surface area of approximately 200 cm^2 (Wilson, 2005). The cheeks compose the lateral sides of the human mouth and at the entrance to the oral cavity they end in fleshy folds called the lips (labia) which are covered on the outside by skin (Figure 1). The hard and soft palates make up the roof of the mouth and are composed of bone and muscle respectively (Wilson, 2005). The hard palate splits the oral cavity from the nasal cavity, whereas the soft palate separates the oropharynx and nasopharynx. A muscular process known as the uvula hangs from the soft palate (Figure 1.1) (Wilson, 2005).

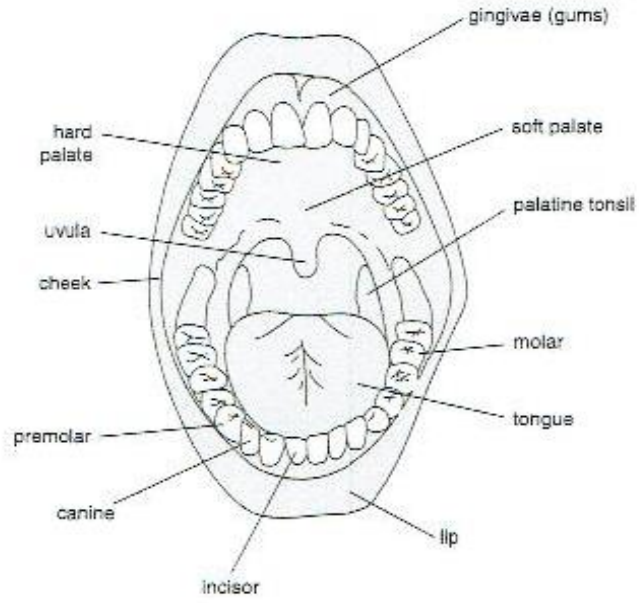


Figure 1.1: Depiction of the major anatomical structures of the oral cavity (Wilson, 2005).

The oral cavity floor is formed by the tongue which is a muscular structure. The movements of the tongue drive mastication and the formation of the bolus, and propel the bolus to the back of the mouth for swallowing (Wilson, 2005). The dorsal surface

and sides of the tongue are covered with papillae, which include foliate, filliform, fungiform and circumvallale papilla, and the lingual and plataine tonsils.
Teeth are mineralised structures which erupt into the oral cavity from sockets within alveolar bone (Figure 1.2). The gingivae (gums) cover the upper regions of alveolar bone and surround the teeth to create a seal that prevents microbial invasion into the underlining tissues (Wilson, 2005). The gingiva forms a collar around the teeth known as the gingival sulcus (Figure 1.2) which, when healthy, extends no more than 2mm (Lamont *et al*., 2006).

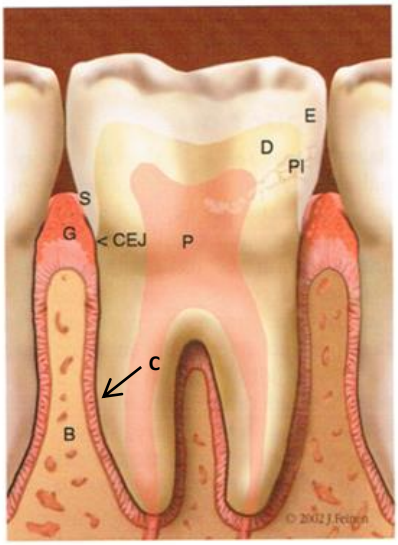


Figure 1.2: Cross-sectional diagram of a tooth, depicting the gingivae (G), the gingival sulcus (S), the cement-enamel junction (CEJ), the alveolar bone (B), and the three tissues of the tooth, namely the enamel (E), cementum (C), dentine (D), pulp (P), Plaque (Pl) is also depicted (from Lamont *et al*., 2006).

The gingivae are covered by a stratified, squamous, keratinised epithelium which merges with the non-keratinised “sulcular” epithelium in the gingival crevice. Gingival crevicular fluid (GCF) is a serum-like exudate from the gingival crevice that continually enters the oral cavity (Wilson, 2005).

The tooth itself consists of four tissues; enamel, dentine, cementum and pulp, (Figure 3). The crown of the tooth composed of enamel, which is the most richly mineralized tissue in the body, containing 96% inorganic material (Lamont *et al*., 2006). The mineral composition consists mainly of hydroxyapatite and fluorapatite (Wilson, 2005).

The major organic constituents of enamel are members of two families of proteins: amelogenins (90%) and enamelins (10%) (Wilson, 2005). Below the enamel layer is dentine (Figure 1.2). Dentine also has a high mineral content (70% minerals, primarily in the form of hydroxyapatite, 30% water and organic matrix) and makes up the bulk of the tooth. Dentine is supported by matrix proteins such as collagen, osteopontin, and acidic glycoprotein-75 (Wilson, 2005). Cementum is a very similar component, composed of hydroxyapatite (60%), organic material (23%) and water (17%), the main function which is to provide attachment points between the tooth and the periodontal ligament (Wilson, 2005). Below the dentine layer is pulp (Figure 1.2). Pulp is a soft tissue and is not mineralised, instead being made up of collagen fibrils and different type of proteins and proteoglycans. The pulp also contains nerve fibres and blood vessels which provide sensory innervation, nutrition, and a means of metabolite disposal to the tooth (Lamont *et al*., 2006). These vessels and nerves are connected to the rest of the circulatory, lymphatic, and nervous systems via the root canal that runs from the pulp cavity through each root of the tooth. Each tooth may have one or more roots (Wilson, 2005), which are the lower extremities of the tooth, attached to the underlying alveolar bone.

Fluid flow in the oral cavity

In addition to the hard and soft surfaces described above, the oral cavity has a fluid phase resulting from the production of saliva. Saliva is a dilute, aqueous fluid with a number of functions, including lubrication, digestion, temperature regulation, and host defence (Wilson, 2005). Saliva is produced by numerous glands sited around the mouth, referred to as major (parotid, submandibular, and sublingual) and minor (labial, lingual, buccal and palatal) salivary glands (Samaranayake, 2012). The average individual produces around 1 litre of saliva per day (Humphrey and Williamson, 2001; Wilson, 2005). Saliva is dilute fluid consisting of electrolytes (including sodium, potassium, calcium, magnesium, bicarbonate and phosphate), immunoglobulins, proteins, enzymes, mucins, urea and ammonia. Bicarbonates, phosphates and urea act as buffers in saliva helping to regulate the pH. Immunoglobulins, mucins and enzymes have a different functions ranging from defence to facilitating nutrition (Humphrey and

Williamson, 2001; Wilson, 2005). Calcium, phosphates and the proteins found in saliva contribute to tooth demineralisation and remineralisation processes. Saliva also buffers the oral cavity against the effect of organic acids (Garg, 2010; Hiremath, 2007). Saliva coats the enamel surface of teeth to form a pellicle (Huang *et al*., 2011). A second fluid present in the oral cavity is GCF, as introduced above. GCF is a nutrient rich fluid containing host cells, proteins, carbohydrates and various ions with a number of immunological components (Lamster and Ahlo, 2007).

BACTERIAL DIVERSITY OF THE HUMAN ORAL MICROBIOME

It is now well recognised that the oral cavity supports one of the richest and most diverse of all the microbial communities that thrive on the human body, second only to the lower gastrointestinal tract (Peterson *et al*., 2009). One of the important determinants of this diversity is the unusual tissue types that exist in the mouth; teeth are the only example within the body of a hard tissue being naturally exposed to the external environment. Furthermore, teeth are not shed or turned over in the manner of soft tissues/epithelia. Therefore, the oral microbiota has evolved mechanisms to exploit environments that are not experienced by other microbiota (Avila *et al*., 2009). The architecture of teeth and their juxtaposition with other teeth and supporting soft tissues provide various niches which are exploited by the microbiome. An overgrowth of microbiome constituents yields dental plaque, which is commonly associated with oral diseases such as caries and periodontal diseases (Figure 1.3).

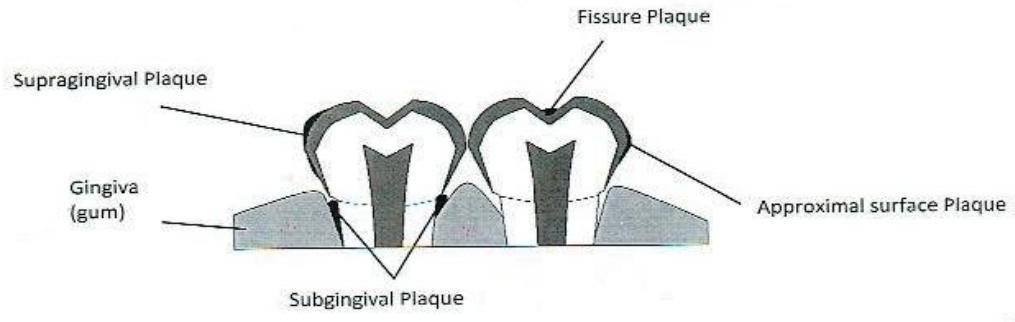


Figure 1.3: Diagram of tooth surface, plaque distributions and associated nomenclature (reproduced from Samanayake, 2012).

The oral microbiota comprises of bacteria, fungi, archaea and viruses. Most research to date has focused on the bacterial component of the microbiota (Kolenbrander, 2000; Marsh, 2005; Siqueira and Rocas, 2009), although exploration of other components including viruses and fungi have been reported (Ghannoum *et al*., 2010; Wylie *et al*., 2014).

Cultured-based exploration of the oral microbiome

Members of the human oral microbiome were among the first bacteria ever to be observed. In 1683, Antonie van Leeuwenhoek used his microscope to observe a large number of what he named "animalcules" in scrapings taken from his teeth. However it was not until over 200 years later, following the seminal work of Koch, Pasteur and their contemporaries that these animalcules were identified as microorganisms and the first isolates of cultivatable members of the oral microbiome were studied in the laboratory.

One of Koch's contemporaries, Willoughby Miller, is lauded as being the father of oral microbiology. Miller divided his time between practicing dentistry and working in Koch's laboratory studying the "germs" that were responsible for tooth decay. In his 1890 book titled "Microorganisms of the Human Mouth" (1890), Miller proposed his "chemoparasitic" theory which suggested that, in individuals who frequently consumed fermentable carbohydrates, oral microorganisms would convert these carbohydrates into acid, leading to the demineralization of teeth (tooth decay). A decade later, the second key element of the modern concept of the aetiology of dental caries was provided by Black and Williams who published the first descriptions of "gelatinous microbic plaques", now commonly known as "dental plaque", (Black, 1898; Williams, 1898). The first isolation of a member of the oral microbiota was achieved by Clarke in 1924. The organism he isolated was a Gram positive coccus that he named as *Streptococcus mutans*. Clarke explored the fermentative properties of *S. mutans* showing it to behave as Miller had predicted in 1890.

Progress in understanding the aetiology of dental caries was slow, but by 1960 the infectious and transmissible nature of dental caries had been demonstrated using animal models (Keyes, 1960). Furthermore, using similar models, the role of *S. mutans*, by now recognised as a member of the "conventional" microflora in healthy individuals, in inducing caries was also demonstrated (Fitzgerald and Keyes, 1960). Concurrent with these important studies on dental caries were studies on periodontitis, the second most common oral disease worldwide. Bacteria were implicated in periodontal disease with the observation that administration of penicillin inhibited periodontitis in laboratory animals (Mitchell and Johnson, 1956), and the infectious nature of periodontitis was demonstrated by its transmissibility in animal models (Keyes and Jordan, 1964). Other studies identified several oral isolates, including *Actinomyces* species that were suspected as being involved in the aetiology of peridontitis (Howell *et al*., 1965).

Following these discoveries, oral microbiologists have tried to isolate specific microorganisms that could be the causative agents and responsible for these disease in order to address Koch's postulates for dental caries and peridontitis. Improved cultivation techniques, in particular the introduction of anaerobic conditions, resulted in the isolation of an increasing number of new bacterial taxa from the human mouth. By 2000, more than 300 different oral bacteria species had been isolated in pure culture (Kolenbrander, 2000), including organisms taken from healthy and diseased sites. Characterisation of these isolates confirmed the abundance of diverse taxa in the mouth, including clinically relevant *Streptococcus, Actinobacillus, Actinomyces, Porphyromonas* and *Treponema* species (Kolenbrander, 2000). Although the limitations of culture-based exploration of the oral microbiota would become apparent with the publication of the results of culture-independent molecular-based biodiversity quantifications, the availability of isolates of many members of the oral microbiota has been key not only to their own characterisation but also to exploration of the biology of oral microbial communities and their role in dental disease. For example, availability of these isolates has permitted whole genome sequence determination and comparison (Table 1.1).

Species name	Microbiological characteristics	Pathogen?	Isolates for which genomic data are available	Reference
Streptococcus mitis	+* FA†	commensal	NCTC12261, IS1381, IS861, ISSpn2, ISSsu4, iSSmi1, iSSmi2, iSSmi3, iSSmi4, issmi5, ISS66	(Denapaite *et al*., 2010)
Streptococcus salivarius	+ FA	commensal	JIM8777, Ps4	Delorme *et al*., 2011 ; Matrin *et al*., 2012)
Streptococcus oligofermentas	+ FA	commensal		
Streptococcus mutans	+ AN	dental caries	UA159NG8, JH1005, BM77, GB14, CT11	(Lamont *et al*., 2006)
Veillonella parvula	- AN	endodontic infection	ATCC10790T, ATCC17745, RBV162, RBV167	(Arif *et al*., 2008)
Veillonella atypica	- AN	commensal	ATCC17744	(Marchandin, 2005)
Aggregatibacter actinomycetemcomitans	+ AN	periodontitis	HK165, D75, CCUG12396, CCUG23125, CCUG26442	(Lamont *et al*., 2006; Christensen & Bisgaard, 2004)
Fusobacter nucleatum	+ AN	periodontitis	ATCC25586	(Lamont *et al*., 2006)
Porphyromonas gingivalis	- AN	peridontitis	W83c, 33277c, TDCGOC, W50 p, W83 p	(Nation *et al*., 2008)
Prevotella dentalis	- AN	commensal	DSM688	(Dowens *et al*., 2006)
Treponema denticola	- AN	peridontitis	SK36, JF36, 0169, JFP76, JFP56	(Turner *et al*., 2009)

* + gram positive, - gram negative

† FA facultative anaerobe, AN anaerobe

Table 1.1: Abundant and/or clinically-important species of the oral microbiome for which whole genome sequence data are available (as of March 2015).

Culture-independent exploration of the oral microbiome

The frequent observation of discrepancy between direct microscopic counts and numbers of culturable bacteria from clinical and environmental samples is just one of several indications that culture-based exploration of the diversity of microorganisms is inherently restrictive (Amann *et al*., 1995). In addition, as there is a wide mixture of aerobic and anaerobic microbes in the oral cavity, for a culture-based approach to be as inclusive as possible, a huge variety of different media, incubated under different atmospheric conditions would be required. This would be extremely expensive (Paster *et al*., 2006). The advent of culture-independent molecular approaches to biodiversity assessment, introduced about 20 years ago and subsequently becoming increasingly

powerful, has addressed these shortfalls and has resulted in an entirely new perspective on the extent of microbial diversity within the oral cavity.

Comparison of 16S rRNA-encoding gene sequences

The small subunit rRNA molecule is essential for the translation of mRNA into polypeptide chain and thus is ubiquitous among bacteria and beyond. The gene encoding this molecule, 16S rDNA, has been extensively studied as is described as a mosaic molecule, comprising of regions of conservation interspersed with regions of variability (Tran *et al*., 1996). This characteristic has been exploited to permit the alignment and comparative analysis of sequence data as a basis of phylogenetic inferences between bacterial taxa and even across domains (Woese *et al*., 1990). Databases of 16S rDNA sequences are well-established and extensive (e.g. Chun *et al*., 2007) allowing the relative taxonomic/phylogenetic position of newly characterised organisms to be determined with great accuracy.

Early applications of 16S rDNA analysis to explore the diversity of the oral microbiota involved the random amplification of partial 16S rDNA fragments by means of PCRs containing conserved eubacterial primers, then cloning of amplification products followed by analysis of individual clones either by sequencing or cheaper alternatives such as restriction fragment length polymorphism analysis (RFLP). This approach was first described in 1996 (Dymock *et al*., 1996) in a study exploring the microbiota associated with dentoalveolar abscesses. Clones representative of five predominant groups of uncultured organisms were sequenced. Three were closely related to previously recognised oral taxa (*Porphyromonas gingivalis, Prevotella oris* and *Peptostreptococcus micros)*, but two did not correspond to known, previously sequenced organisms; one was related to *Zoogloea ramigera*, a species of aerobic waterborne organisms, while the other was distantly related to the genus *Prevotella*. Subsequent studies used a similar approach to assess the diversity of bacteria in other oral niches including the subgingival plaque of healthy subjects and subjects with periodontal diseases (Paster *et al*., 2001), the dental plaque in children with rampant caries (Becker *et al*., 2002) or noma (Paster *et al*., 2002), the saliva of healthy people (Sakamoto *et al*., 2000), the subgingival plaque of a subject with gingivitis (Kroes *et al*.,

1999) and the tongue dorsa (Kazor *et al*., 2003). Taken together, these and a large number of more recent 16S rDNA cloning-based studies detected well over 500 species/ phylotypes, about half of which were novel. However, the revelation of so much diversity created its own problems; it has now been recognised that as the 100s of taxa detected in the mouth but known only as 16S rRNA phylotypes cannot be formally named (naming requires growth and full phenotypic characterization), an alternative taxonomic scheme is required if the plethora of 16S rDNA data are to be managed in a logical manner, allowing investigators and the literature to refer to provisionally named taxa rather than clone sequences. Thus, in 2010 the Human Oral Microbiome Database (HOMD) was established to develop a provisional taxonomic scheme for the unnamed human oral bacterial isolates and phylotypes and to analyze the (at the time) 36,043 16S rDNA oral clone sequences available to determine the number of clones observed for each human oral taxon and to identify additional taxa not included in the initial setup of the HOMD.

After its establishment, HOMD included 619 taxa in 13 phyla, as follows: *Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes,* SR1, *Synergistetes, Tenericutes*, and TM7. Analysis of the 36,043 16S rDNA gene clones isolated from studies of the oral microbiota identified 1,179 taxa, of which 24% were named, 8% were cultivated but unnamed, and 68% were uncultivated phylotypes. As a result of this analysis, 434 novel, non-singleton taxa were added to the HOMD. The number of taxa needed to account for 90%, 95%, or 99% of the clones examined was 259, 413, and 875, respectively (Dewhirst *et al*., 2010). HOMD therefore became the first curated description of a human-associated microbiome and provided tools for use in understanding the role of the microbiome in health and disease. It remains a key resource today, but has now expanded far beyond being merely a 16S rDNA archive (see below).

Next-generation sequencing techniques

The 16S rDNA cloning approach described above revolutionised our understanding of the diversity of the oral microbiota. However, its reliance on Sanger sequencing of individual clones made it extremely labour-intensive and expensive. Typically, in 2005, Sanger sequencing of a single clone cost about £10, hence even well-funded studies were limited to surveying no more than a few 1000 clones. Furthermore, the bioinformatic tools for manipulation of genomic data at even this modest scale of magnitude were in their infancy, so the process was extremely labour intensive. The advent of next-generation sequencing (NGS) technologies, in which millions of sequencing reactions occur simultaneously in a hugely parallel process, and associated rapid bioinformatic developments overcame the "clumsiness" of cloning and Sanger sequencing as a means of isolating and characterising individual PCR amplicons, thereby opening the door to the exploration of microbiome diversity on a scale that was previously unimaginable. The first NGS machine to be widely adopted was the Roche/454 FLX (Margulies *et al*., 2005), which supported a technology referred to as sequencing by synthesis, or pyrosequencing (Pozhitkov *et al*., 2011; Liu *et al*., 2012). The first reported application of pyrosequencing to the exploration of the oral microbiota appeared in 2008 (Keijser *et al*., 2008). The study collected saliva and supragingival plaque from 71 and 98 healthy adults, respectively then amplicons from the V6 hypervariable region of the small-subunit ribosomal RNA gene were generated by PCR, pooled into saliva and plaque pools, and pyrosequenced. A total of 197,600 sequences were generated that fell into about 29,000 unique sequences, representing 22 taxonomic phyla. Grouping the sequences into operational taxonomic units (OTUs) (defined as clusters of 16S small subunit (SSU) rRNA genes that are used as approximations of microbial taxa; these are universally adopted in culture-independent, sequence-based ecological characterization of microbial communities) yielded 3621 and 6888 species-level phylotypes in saliva and plaque, respectively. These observations gave a radically new insight into the diversity of human oral microflora, indicating that it was considerably higher than previously reported (Keijser *et al*., 2008). Numerous other studies followed, as reviewed by Chen and Jiang (2014).

Concurrent with 454/Roche's pyrosequencing platform were other parallel sequencing technologies, from among which that developed by Solexa/Illumina would emerge

most strongly (Bentley, 2006; Korbel *et al*., 2007). Illumina sequencing exploits reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands. Although, initially at least, read length of Illumina was much shorter than that obtained by pyrosequencing, the number of reads generated by Illumina is far greater than 454 (>100x). The first Illumina-based exploration of 16S rDNA amplicons derived from oral microbiota appeared in 2009 (Lazarevic *et al*., 2009), trumpeting a much greater depth of coverage than previous 454-based oral microbiota studies. The study amplified samples from the oral cavity of three healthy individuals using 16S rDNA primers covering an approximately 82-base segment of the V5 loop, identifying 135 genera or higher taxonomic ranks from the resulting 1,373,824 sequences. While the overall findings of the study were largely comparable to previous 454-based studies, the authors emphasized their approach allowed them to identify several taxa and to assess that at least 30,000 additional reads would be required to identify only one additional phylotype. As with pyrosequencing, Illumina sequencing has now been repeatedly applied to exploration of the human oral microbiome (reviewed by Chen and Jiang, 2014).

Today, it is generally accepted that the microbiome of apparently healthy individuals can contain members of up to fourteen bacterial phyla, including Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, Fusobacteria, Euryarchaeota, Chlamydia, Chloroflexi, SR1, Synergistetes, Tenericutes, Cyanobacteria, OD2, and TM7 (Bik *et al*., 2010; Griffen *et al*., 2011). Among these phyla, the vast majority (>80% of the taxa) of oral bacteria belong to Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and Fusobacteria (Ling *et al*., 2010). At genus level, over 200 genera have been found in the oral microbiota, among which *Streptococcus, Prevotella, Neisseria, Haemophilus, Porphyromonas, Gemella, Rothia, Granulicatella, Fusobacterium, Actinomyces* and *Veillonella* are the most common. At species level, it had been estimated that the number of species-level phylotypes lies between 500 and 10000 (Keijser *et al*., 2008; Lazarevic *et al*., 2009). Studies focusing on specific niches within the mouth have suggested each supported 266 species-level phylotypes on average (Zaura *et al*., 2009).

The power of NGS has opened the possibility of conducting large population-based studies of human microbiome, providing insight into the diversity and community structure of the human microbiome in relation to health and disease. Studying diversity in this manner has allowed the formulation of the concept of a core microbiome, defined as a microbiome shared with most of individuals and comprised of the predominant species in healthy conditions of oral cavity (Zarco *et al*., 2012). At present, this core microbiome is thought to comprise of five phyla including Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria, and Bacteroidetes, and at least nine genera including *Actinomyces, Capnocytophaga, Corynebacterium, Derxia, Leptotrichia, Neisseria, Prevotella, Streptococcaceae, Streptococcus* and *Veillonella* (Chen and Jiang, 2014).

Microarrays and HOMIM

Although NGS is now the approach of choice for assessing the diversity of the human oral microbiome, 16S rDNA-based microarrays, and in particular the Human Oral Microbe Identification Microarray (HOMIM) (Chen *et al*., 2010), have been important resources for the exploration of the oral microbiome over the last decade or more. The HOMIM platform includes probes for about 300 of the most prevalent oral bacterial species and thus represents a means of identifying microbial profiles directly from oral samples that is relatively rapid and cost effective. The platform has been extensively validated and widely used over the past 6 years, primarily in the context of large population-based studies of the oral microbiome (see for a review of these studies http://homings.forsyth.org/references.html). The performance of HOMIM in comparison to pyrosequencing has been studied and resulted in the conclusion that microbiome community profiles assessed by the two approaches were highly correlated at the phylum level and, when comparing the more commonly detected taxa, also at the genus level. However, the study also suggested that pyrosequencing may provide a broader spectrum of taxa identification, a distinct sequence-read record, and greater detection sensitivity (Ahn *et al*., 2011). Studies using HOMIM continue to appear today (e.g. Lin *et al*., 2015; Moutsopoulos *et al*., 2015), demonstrating the ongoing utility of this microarray.

Metagenomics analysis of the oral microbiota

Metagenomic techniques (also called community genomics) used to explore the ecology of the oral microbiome, have revealed new species and their associations with health and disease (Filoche *et al*., 2010). However, it is important to note that even though these culture independent approaches are powerful and produce a wealth of information, they are not without limits (Zoetendal *et al*., 2004; Sakamoto *et al*., 2005; Steward and Rappe, 2007). Many new species have been encountered in metagenomic studies of dental plaque (Kumar *et al*., 2003; Aas *et al*., 2008; Preza *et al*., 2009) using 16S ribosomal DNA sequencing.

A 16S rDNA metagenomic approach involves the parallel sequencing of many millions of products generated by a single PCR. The principal advantage of this approach is that as it quantifies biodiversity in a culture-independent manner, it is free of the inherent bias that culture introduces, and it does not require labour-intensive cloning and individual analysis of clones. However, one of the limits of this technique is that cannot accommodate very long sequencing reads, so it cannot produce full-length 16S rDNA sequences that facilitate sesnsitive taxonomic studies. Furthermore a metagenomics approach is very expensive. The first reported metagenomics analysis of the oral microbiome was published by Keijser *et al*. (2008). The collection of saliva and plaque from healthy adults showed that diversity was one order of magnitude higher than has been previously described, and the authors said that there are at least 19,000 species of level phylotypes that participate to the diversity of oral microbiome (Keijser *et al*., 2008). Anther approach called shotgun sequencing or metagenome sequencing refers to sequencing DNA fragments extracted from microbial populations. Because this technique captures the complete genomes of all the organisms in the population, mosaicism and biases have little effect (Bik *et al*., 2012). The comprehensive information obtained by this method enables accurate phylogenetic inferences of close and distant relatives. However, the main substantial advantage is the information it provides about the genes present in the bacterial population, without assembling the individual bacterial genomes. Functional gene groupings can be more informative and stable than a record of bacterial species. However, a high error rate in

constructing the continuous set of overlapping sequences is one of disadvantages of this technique and it is computationally intensive as well (Zarco *et al*., 2012).

DETERMINANTS OF THE HUMAN ORAL MICROBIOME

The oral microbiome lives in a defined habitat composed of physical and chemical elements. This composition of the microbiome is influenced by changes to its habitat and thus its composition fluctuates in response to variation in the physical and chemical characteristics of the mouth. However this relationship is not unidirectional, as the metabolism of the microbial community can change the physical and chemical properties of the surrounding environment (Pflughoeft and Versalovic, 2012). The regulatory forces affecting the oral ecosystem can be classified into four major categories: physiochemical, host-related, bacteria-related (microbial related factors), and external factors (environmental factors) (Marcotte and Lavoie, 1998).

Physiochemical factors

The physicochemical factors represent the specific environmental parameters which result from the combined action of host, microbial and external factors. *In vivo* and *in vitro*, the growth of microorganisms is controlled by considerable fluctuations in these oral environmental parameters such as temperature, pH, availability of water, availability of nutrients, and oxidation-reduction potential (Badger *et al*., 2011).

Temperature

In the mouth of a normal person the temperature may vary substantially. For example, during a standardised drinking regimen of hot coffee (72.5^{o}C) followed by refrigerated orange juice, the intra-oral temperature rose to 68.0^{o}C then dropped to 15.4^{o}C (Lamont *et al*., 2006). Localized inflammatory processes may also affect the oral tissue temperature (Lamont *et al*., 2006). The temperature of the healthy gingival sulci has been recorded to range from 33.7^{o}C to 36.6^{o}C (slight variation from tooth to tooth) (Lamont *et al*., 2006). In general, subgingival temperatures can reach 39°C (Wilson, 2005), but the temperatures within a diseased periodontal pocket may have dramatic variation (*et al*., 2006).

pH

The pH conditions within the mouth vary considerable depending on the niche tested and on its health status. The pH of the mouth is influenced by the slight acidity of saliva (pH 6.7) (Samaranayake, 2012), but the pH of the oral microbiota in a healthy mouth has been estimated to be slightly alkaline (pH 7.2) and some niches, such as the gingival crevice are even more alkali (pH 7.5-8.0) (Wilson, 2005). However, in a diseased mouth, possessing severe caries, the pH may be as low as pH 5.5 as a result of acid production by pathogenic bacteria (Ling *et al*., 2010). These conditions promote the overgrowth of acidophilic bacteria such as lactobacilli that, in turn, increase the rate of fermentation (Samaranayake, 2012).

Oxidation-reduction potential

The oxidation-reduction potential of the environment (Eh) varies in different locations of the oral cavity. For example, redox potential falls during plaque development from an initial Eh of over +200mV (highly oxidized) to -141mV (highly reduced) after 7 days (Samaranayake, 2012). This reduction is probably due to rapid oxygen consumption by respiring bacteria. Regions with the lowest oxygen concentrations such as the tongue (the mucosal crypts of the tongue support anaerobic microbiota, which are the main cause of halitosis) (Scully and Greenman, 2008) and the gingival crevice, are regions where obligate anaerobes are particularly abundant. Failure to practice oral hygiene has a dramatic effect on the oxygen content of plaque accumulating on different surfaces; this is presumably because of oxygen consumption by the constituent bacteria (Wilson, 2005).

Nutrients

Members of the oral microbiome obtain nutrients from two broad sources, either remnants of the host's diet, such as starch or peptides, or components of the host itself, such as salivary constituents (Kuramitsu *et al*., 2007). The major organic constituents of saliva are proteins and glycoproteins such as amylase, mucin, immunoglobulins (mainly IgA), lysozyme, lactoferrin and sialoperoxidase. Furthermore, the gingival crevice is bathed with GCF, which provides an additional source of

nutrients such as albumins and glycoproteins (Cimasoni, 1983). The difference in the endogenous nutrients of the GCF is one of the critical factors for the dental microbial variations compared to other oral sites (Cimasoni, 1983).

Host innate immunity

Host immunity is an important determinant in both the establishment and development of the oral microbiome. The natural innate immunological defence of the supragingival environment is controlled mainly by the constant salivary flow, enhanced by muscular actions which wash bacteria from both hard and soft oral surfaces (Tenovuo *et al*., 1987). Other nonspecific defence elements involve mucins, salivary glycoproteins, lactoferrin, lysozyme and peroxidise, that all play an important role in inhibiting microbial adhesion and growth (Samaranayake, 2012). Mucins are high-molecular-weight glycoproteins which are secreted by submandibular, sublingual, and minor salivary glands. There are two types of mucins in the saliva: MG1 and MG2. MG1 is of high molecular weight and is included mainly in hard and soft tissue coatings to protect teeth from acid demineralization affect. MG2 has a low molecular mass and has been shown to acts as a barrier to streptococcal aggregation and adherence (Tenovuo *et al*., 1987). Lysozyme is a small cationic protein of saliva which can hydrolyse glycosidic linkages in some bacterial cell walls (Mandel, 1987). IgG, IgM and IgA antibodies have been identified in GCF (Samaranayake, 2012). These antibodies may affect the oral microbiome by interfering with bacterial adherence (Smith *et al*., 1994) as well as there more established role in opsonization (Samaranayake, 2012).

Hormones

The major hormonal fluctuations in humans occur during puberty and pregnancy. These chronological changes are associated with high plasma levels of steroid derivatives in the main body fluids, including GCF and saliva (Evans *et al*., 1984, Lachelin and McGarrigle, 1984). A higher prevalence of gingivitis in pregnant women was first noted almost 60 years ago (Loe, 1965), and subsequently numerous studies have explored changes in the oral microbiome and oral disease associated with puberty or pregnancy. During their transition to adolescence, children exhibit major

physiological and hormonal changes which directly affect the nature of the oral microbiome (Mombelli *et al*., 1989). In this study, the microbiome composition was assessed by microscopic analysis and culturing techniques. *Actinomyces odontolyticus* showed an increase of a significant time trend among the Gram-positive bacteria and demonstrated a direct relationship with puberty indices in both boys and girls such as testicle growth in boys and Tanner score for breast development in girls. Amongst Gram negative species, members of *Capnocytophaga* and *Bacteroidetes* were predominant. The shift to Gram-negative anaerobic bacteria (including *Bacteroides*) was found to be significant during the period of the mixed dentition (Wojcicki *et al*., 1987). For example; *P.gingivalis* has been isolated from the plaque of 80% of children during and after puberty (Watson *et al*., 1991). *V. atypica, P. denticola and P. melaninogenica* were among the species that contributed most to changes in subgingival microbial composition during puberty (Gusberti *et al*., 1990).This maturation of the oral microbiome during puberty was confirmed by high through-put sequencing (Gezani *et al*., 2009; Lazarevic *et al*., 2009; Ling *et al*., 2010; Crielaard *et al*., 2011).

In the study of Cirelaard *et al*. (2011), plaque samples from eleven adolescent subjects (ages 11 to 18 years) were analysed using PCR amplification of the 16S rDNA and high throughput 454 pyrosequencing. Results showed that this age group had an increase in the amount of species from the *Bacteroidetes* (mainly *Prevotella*, *Veillonellaceae* and *Spirochaetes*). Within recent years, further clinical follow-up studies have been performed, aiming to evaluate shifts in the microbiological status during pregnancy, to find any involvement of specific pathogens with pregnancy gingivitis, and to assess any correlations between the oral microbiota and elevated hormone levels during pregnancy. A few examples are given as follows: Yokoyama and co-workers (2005) showed in their *in vitro* studies that estradiol is able to increase the growth of *C. rectus*, which is another potential periodontal pathogen. In their cross-sectional study, a positive correlation was found between the salivary estradiol concentrations and levels of *C. rectus, P. gingivalis*, and *F. nucleatum* in pregnant women (Yokoyama *et al*., 2008). Additionally, the salivary *C. rectus* levels positively correlated with the percentages of sites with a 4 mm pocket depth without any attachment loss. According

to a pilot study, when the third molars were present, the efficacy of scaling and root planning during pregnancy proved to be limited to reduce the amount of periodontal pathogens measured from the mesiobuccal sites of first molars (Moss *et al*., 2008). Especially, increased counts of *T. forsythia* and *P. nigrescens* were significantly associated with the presence of third molars. Thus, third molars in pregnant subjects are suggested to act as a reservoir for periodontal pathogens by serving as suitable niches for their growth.

However, there are discrepancies in the current literature about the correlation between increased hormone levels and subgingival microbiota during pregnancy. In a follow-up study, no significant changes in the proportions of subgingival *P. intermedia* were found between pregnant and non-pregnant subjects (Jonsson *et al*., 1988). In addition, no correlation was found between the microbiological or clinical parameters and hormonal status. In contrast, in a recent study by Carrillo-de-Albornoz *et al*. (2010), pregnant women without periodontitis, harbouring *P. gingivalis* or, *P. intermedia,* in subgingival biofilms, presented a significantly increased gingival inflammation tendency during mid-pregnancy and the presence of *P. intermedia* and *P. gingivalis* positively correlated with the salivary female sex hormone levels. Studies have shown that, *P. intermedia* were observed in higher levels compared to *P. gingivalis*. The inconsistent results in the literature regarding the oral bacteria associated with the onset of periodontal diseases during pregnancy can be explained by the different methodology used in each study (Gürsoy *et al*., 2008; Machado *et al*., 2012). In addition, during pregnancy, some periodontal pathogens, such as *T. forsythia, P. gingivalis, A. actinomycetemcomitans*, and *T. denticola*, are also important because of the relationship between certain subgingival species and preterm birth (Hasegawa *et al*., 2003; Madianos *et al*., 2001). Higher levels of *Campylobacter rectus* and *Prevotella nigrescens* have also been reported among these women (Yokoyama *et al*., 2008; Gürsoy*et al*., 2009). Higher levels of *C. rectus* and *P. nigrescens* have also been reported among these women (Yokoyama *et al*., 2008; Gürsoy *et al*., 2009). More studies are needed to describe the etiologic factors thatmight show this relationship more clearly (Adriaens*et al*., 2009). Gingival alterations associated with the biofilm formation during pregnancy are often observed, and this may increase the severity of

gingivitis. In addition, variations in the oral microbiota and cellular metabolism have been observed, as well as an increase in estrogen levels, such as progesterone, affecting the vascular permeability, producing oedema, and increasing the gingival alteration (Markou *et al*., 2009). Results show the presence of *A. actinomycetemcomitans* among pregnant women at second and third trimester, which might be important (Borgo *et al*., 2014).

Host Genetics

The effect of ethnicity on the oral microbiome was recently assessed by an analysis of the oral microbiome in children from China and United States using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Interestingly, it was found that a species of the genus *Granulicatella* were more abundant in the plaque samples of children from the United States with a high percent of caries (Kanasi *et al*., 2010) and less abundant in a similar population from China (Ling *et al*., 2010). The composition of the subgingival microbiome in twins was surveyed by Moore *et al*. (1993). This study included a group of 10 monozygotic and 10 dizygotic twins, who were followed longitudinally and sampled during a three year period (aged 11 to 14 years). Subgingival microbiome components in monozygotic twins (11 to 14 years of age) found to be more similar than in dizygotic twins (Moore *et al*., 1993). In a further study, Corby *et al*. (2007) tested 204 pairs of twins (80 monozygotic and 124 dizygotic) aged from 24 to 36 months. Using the reverse capture checkerboard hybridization assay and genetic technique, the 10 most abundant species among caries-free twins was determined to be moderate to highly heritable; the species found were, in descending order: *S. parasanguinis, Abiotrophia defectiva, Gemella haemolysans, S.mitis/oralis,S. sanguinis, S. cristatus, Streptococcus* sp. clone CH016, *Eubacterium sp. clone DO016, Gemella morbillorum* and *S. salivarius*. It is known that tooth and root morphologies differ according to ethnic affiliation, as do innate immune responses to infectious agents, for instance, Toll-like receptor-4, mannose binding lectin and heat-shock proteins (Miller and Cappuccio,2007) and thus it is possible that ethnicity plays an important role in bacterial selection by defining the environment for bacterial colonization. The concept that the host genotype influences what will survive and thrive is particularly useful in assessing susceptibility and in developing targeted

therapies to fight against polymicrobial infections. For instance, pathogens belonging to the genera *Filifactor, Staphylococcus, Mycoplasma, and Treponema* were found to be in significantly higher levels in Chinese and Latinos, and it is possible that their presence in health may contribute to the raised disease susceptibility that has been observed in these cohorts (Albandar, 2002). Although it remains unproven that genetic factors are determinants in the ecological shift that leads to oral disease, they could participate in the development of oral disease in an indirect manner. Firstly, an individual may have a specific genetic makeup that encodes for a permanent immune system disorder that may then affect the microbiome. For instance, a person with Crohn's disease, an autoimmune disease of the gastrointestinal tract, has a lower abundance of Bacteroidetes in the intestines (Badger *et al*., 2011). A similar situation in the oral cavity could result in a decrease of biodiversity and could lead to disease. In addition, because an individual's genotype contributes to the makeup of its unique microbiome (Turnbaugh *et al*., 2007), one's genetic makeup could directly either prevent the existence of certain beneficial bacteria in the body or produce a bodily environment in which certain pathogenic species can increase. For instance, 20% of individuals are long-term carriers of *Staphylococcus aureus*. These individuals are more prone to staph infections, especially if the bacteria are not controlled. Also, certain people may lack genes that encode for specific protective proteins and antibodies in saliva and, thus, be more prone to plaque accumulation or cavities.

Host age

Different studies have suggested that the oral cavity is susceptible to colonization by various bacterial species at different times over the course of the human life span. For instance, early studies reported that anaerobic bacteria such *as Porphyromonas gingivalis* do not colonize in the mouths of children in appreciable numbers (Richard *et al*., 2006). These organisms begin to colonize in the oral cavity around the time of adolescence. This group of bacteria that includes the mutans group streptococci, need teeth or other nonshedding surfaces (for example, a denture) in order to colonize the mouth. Due to this reason these bacteria are seen only transiently in the mouth of children before tooth eruption. In older people the activity of the host defences can

become less and this why the isolation of staphylococci and enterobacteria from the oral cavities of the older person increases (Marsh *et al*., 1992).

Immune system disorders

The presence of an immune system disorder can lead to an ecological shift in the microbiome. It is known that, the immune system regulates interactions between the microbiome and the host, a compromised immune system usually disrupts mutual or commensal relationships (Badger *et al*., 2011). Even though microbial relationships during diseased states are parasitic, some pathogens can also facilitate the growth of other pathogen species. In tooth decay, *S. mutans* is responsible, in part, for creating the lactic acid rich environment in which *Veillonella* species thrive (Kanasi*et al*., 2010). In biofilm research, *Veillonella* species have also been found to increase the growth of *S. mutans* (Klutymans*et al*., 1997). Furthermore, immune systems that are compromised could inhibit the proper flow of saliva or decrease the number of nutrients present in saliva, allowing dental plaque to build up of dental plaque. For example, Sjo¨ gren's syndrome is an autoimmune deficiency that attacks the exocrine glands and inhibits the flow of any saliva through the oral cavity, leading to 'dry mouth' and more dental complications (Taubert *et al*., 2007). In fact, there is also an altered oral microflora in people with HIV but it is known that acute infection is the leading cause of morbidity and mortality in immunosuppressed patients with HIV infection (Zarco *et al*., 2012).

Results from studies on the subgingival microbiota in HIV-infected patients are quite diverse. There are some studies showing the similarity between the microbiota in HIV-positive and HIV-negative patients with periodontitis (Teanpaisan *et al*., 2001; Tsang and Samaranayake, 2001). While some other studies show a higher prevalence of putative periodontal pathogens such as *A.actinomycetemcomitans, F. nucleatum, P. phyromonasgingivalis, P. intermedia, T. forsythia* and *T. denticola* in HIV-positive patients, in comparison to HIV-negative patients (Cross and Smith, 1995; Scully *et al*., 1999; Alpagot *et al*., 2004). In contrast there are studies that present the exact opposite, for instance the studies claim that putative pathogens are less prevalent in HIV-positive patients. (Paster *et al*., 2002; Patel *et al*., 2003; Botero *et al*., 2007;

Goncalves de Souza *et al*., 2007). As shown in most of the studies (Goncalves de Souza *et al*., 2004, 2007, 2009; Aas *et al*., 2007), bacteria that are not usually linked with periodontal disease, such *as Enterococcus faecalis, Acinetobacter baumanii, Pseudomonas aeruginosa* and *Campylobacter pylori*, which were frequently detected in HIV-infected patients. In addition the recognition of different microbial profiles in the subgingival area of these patients may be significant. More complex microbial profiles were described in diseased sites than in the healthy periodontium in HIV-infected patients (Paster *et al*., 2002), whereas certain combinations of microbes were detected exclusively in HIV-infected individuals. These specific complexes' may lead to chronic periodontitis in this group of patients (Patel *et al*., 2003) since it is known that changes in the humoral and cellular immunity can affect the establishment and growth of pathogens and the resultant combination of microbes in the subgingival pockets of HIV- infected patients(Mataftsi *et al*., 2011).

Cancers

Most patients who have oral cancer do not practice good hygiene (Meurman, 2010).In general, several studies conducted around the world have shown that poor oral health and tooth loss raise the risk of gastric, pancreatic and other cancers. Inflammation is usually the first symptom of compromised oral health and it gets worse as health declines. It has been suggested that up to 20% of human tumours contain pathogenic agents which derived from inflammatory infections. Proper oral hygiene will maintain control of such inflammatory agents (Meurman, 2010). Conversely, cancer can lead to poor oral health. Carcinogens can introduce toxic agents into the salivary fluid which then can damage DNA, cause mutations, and damage the integrity of oral cavity (Meurman, 2010).

Diabetes

Diabetes and periodontal disease have a strong bidirectional relationship (Pihlstrom*et al*, 2005; Kuo *et al*, 2008; Williams *et al*., 2008). In one direction, the bacteria involved in periodontal disease jeopardize the body's control of glycaemic levels (Kuo *et al*, 2008) .*P. gingivalis*, a guide agent in periodontal disease, produces a lipopolysaccharide (LPS) which is toxic to certain cytokine proteins that regulate insulin

activity under normal conditions. Other bacterial infections can also inhibit the ability of skeletal muscles to take up insulin-mediated glucose. This can produce whole body insulin resistance (Kuo *et al*, 2008). Fortunately, periodontal treatments can benefit patients with diabetes by decrease pathogen secretions of LPS and improving the body's glycaemic control (Pihlstrom *et al*., 2005). Poorly controlled diabetes raises the risk of periodontal disease activation and severity, and the rate of periodontal bone loss (Pihlstrom *et al*., 2005; Preshaw, 2009; Filoche *et al*., 2010). Diabetes can also lead to other oral complications such as burning mouth syndrome, fungal infections, dental caries, and salivary functional disorders (Kuo *et al*., 2008).

Cardiovascular disease

Peridontal pathogens signal excessive amounts of antigens, endotoxins, cytokines, and C-reactive proteins that also contribute to cardiovascular complications such as lipid deposition, smooth muscle proliferation, and platelet aggregation (Kuo *et al*, 2008). Pathogens such *as P. gingivalis* and *S.sanguis* have abilities to increase platelet aggregation and accumulate as arterial plaque (Williams *et al*., 2008). *A. actinomycetemcomitans* in the periodontal pockets has also been detected in the atherosclerotic plaque (Bahekar *et al*., 2007). The organism accesses the circulatory system through oral tissue and makes its way to the arteries where it secretes LPS and inflammatory-response mediators, leading to ather othrombogenesis. As with diabetes, periodontal treatments may also alleviate cardiovascular diseases (Tonetti *et al*., 2007; Kuo *et al*., 2008). The exact pathway from cardiovascular disease to periodontal disease has yet to be established.

Lifestyle choices

Inadequate oral hygiene practices combined with other factors mentioned above such as aging processes, genetic factors and immune changes in the host affect ehe oral environment and encourage a disease-associated state (Marsh *et al*., 2003). Poor oral hygiene is largely responsible for the accumulation of bacteria within biofilms. Failure to detach accumulating plaque will lead to overgrowth of bacteria that may become pathogenic, reduce biodiversity of the oral cavity, and ultimately cause diseases such as dental caries or periodontal disease (Zaura *et al*., 2009). Anaerobic microflora in the

crypts of the tongue can also grow out of proportion and develop halitosis, or consistent bad breath (Zaura *et al*., 2009). Proper oral hygiene practice is crucial because it is the only voluntary way to prevent oral disease. Moreover the microbial environment can easily be affected by good oral hygiene practices (iatrogenic factors), such as dental scaling which can radically alter the composition of the periodontal pocket flora of disease sites which will alter the balance in favour of colonization of such sites by flora which are associated with health (Samaranayake, 2012).

Modern lifestyles involve the use of medicines (including antibiotics), Oral or systemic antibiotics may reach the oral cavity via saliva and GCF and alter the microbiome balance and have a negative effect on oral flora, for example broad-spectrum antibiotics such as tetracycline can wipe out most of the endogenous flora and assist the emergence of yeast species (Samaranayake, 2012). Diet is another external factor which has an affect on oral microorganisms. For example fermentable carbohydrates are the main class of compounds which alter the oral ecology; they act as a major source of nutrient and encourage the growth of acidogenic flora. The production of extracellar polyscharides promotes adherence of organisms to surfaces, whereas the intracellar polysacharides serve as food resources (Samaranayake, 2012). Other factors, for instance gum chewing, amalgam fillings, socio-economic-status in particularly in case of dental caries, by access to fluoridated water that might affect the oral microbiota (Mignogna and Fedele, 2006).

It is well known that the two most important risk factors for oral cancer are tobacco and alcohol (Johnson, 2001). Certain pathogenic strains of oral microorganisms are inclined to increase carcinogenic acetaldehyde concentrations in saliva when metabolizing ethanol and tobacco smoke (Meurman, 2010; Yang *et al*., 2011). However, not all who drink alcohol or smoke are subject to oral cancer. But those individuals are at higher risk. Also, each microbiome differs in the rate at which it metabolizes the ethanol and tobacco compounds (Meurman, 2010). Other extra-oral factors such as wearing dentures (Marsh *et al*., 1992) and the use of oral contraceptives (Zachariasen, 1993) may affect the oral microbiome.

Microbial factors

In the oral environment, microorganisms interact with each other both in promoting and suppressing neighbouring microbial species. This is accomplished through a combination of physical, metabolic and chemical interactions between different microbial species.

Antagonistic mechanisms include (1) competition for receptors that mediate adhesion to oral surfaces. Prior occupation of the colonizing site can prevent attachment of competing species (Samaranayake, 2012). (2) The production of antimicrobial toxins, for example; bacteriocins which kill cells of the same or other bacterial species. Bacteriocin production plays an important role in the establishment of many streptococcal species in oral communities. *S. mutans* produce the bacteriocins mutacin I and mutacin IV, whilst *Streptococcus gordonii* and *S. sanguinis* produce streptocins and sanguicin respectively (Kreth *et al*., 2005). (3) The production of metabolic end products can inhibit the growth of competitors. For example, short chain carboxylic acids, such as lactic acid, can lower the pH of gingival crevicular fluid, and also act as noxious, antagonistic agent. Another example is the production hydrogen peroxide (H_2O_2) by many oral streptococcal species that inhibits the growth of the cariogenic species *S. mutans* (Kreth *et al*., 2005).

By contrast, co-operative inter-species relationships include (1) physical interactions (co-aggregation) between different members of oral microbial communities that have been well documented and are largely governed by surface expressed adhesins (Nobbs *et al*., 2011). Over 1000 pair-wise co-aggregation interactions have been demonstrated between and among different strains of oral bacteria (Kolenbrander *et al*., 2006). (2) Metabolic end-products are also responsible for several co-operative interactions, being of use to other bacteria for nutritional purposes. For example, the growth of *Veillonella* species is enhanced in the presence of lactate produced by oral streptococci (Samaranayake, 2012). (3) Chemical communication, via the quorum sensing signal auto-inducer-2, can influence physical interactions between different species. For

example, quorum sensing signals have been shown to affect co-operation between *S. gordonii* and *P. gingivalis* (McNab *et al*., 2003).

Oral microbial communities exist in complex polymicrobial biofilms, the composition of which is driven by these antagonistic and co-operative inter-species interactions. A closer look at the mechanisms behind these interactions is needed to understand how oral biofilms develop and evolve in the transition from oral health to disease.

ORAL BIOFILMS

A biofilm is a complex structure that can be found almost everywhere associated with water. In the natural environment, biofilms are commonly found as slimy layers that grow in on the surface of rocks in shallow waters or coating the inside of water pipes (Samaranayake, 2012). The oral cavity, in which saliva constantly flows over hard, enamel surfaces, represents an ideal environment for biofilm development. Biofilms are usually defined as surface-associated microbial communities, surrounded by an extracellular polymeric substance (EPS) matrix (Flemming and Wingender, 2010). Biofilm formation is a feature of wide-ranging bacterial species and this mode of growth is very important for survival within microbial communities. These complex structures can comprise multiple microbial species and have been associated with many important biological processes, such as nutrient re-cycling and inhibition of pathogen colonisation. By contrast; many bacterial pathogens typically form biofilms during chronic infections, such as osteomyelitis (Brady *et al*., 2008), rhinosinusitis (Perloff and Palmer, 2004), urinary tract infections (Connell *et al*., 1997), Crohn's disease (Badger *et al*., 2011) and infections associated with foreign bodies inserted into the human body, such as artificial joints or catheters (Trampuz and Zimmerli, 2008). In all these situations, the existence of biofilms is thought to underlie the ability of the constituent bacteria to resist host immune responses and antibiotic treatment (Hall-Stoodley and Stoodley, 2009).

Overview of biofilm structure

A biofilm community consists of bacterial microcolonies, in which bacteria are surrounded by an extracellular matrix with fluid channels that enable distribution of nutrients and removal of waste (Nield-Gehrig, 2005). When the bacteria attach to a surface and to each other, they cluster together to form sessile, mushroom-shaped microcolonies which are attached to the surface at a narrow base (Figure1. 4). Polymicrobial biofilms often support the survival of many different species with diverse optimal growth conditions. For example; oxygen concentrations can vary in different areas of the biofilm enabling the survival of strict anaerobes deep within the structure, whereas other bacteria at the edges of the fluid channels may live in an aerobic environment (Nield-Gehrig, 2005). Thus, the biofilm structure provides multiple customized living environments (with differing pHs, nutrient availability, and oxygen concentrations) within which bacteria with different physiological needs can survive. Within and between each micro-colony, chemical signals are important mediators of bacterial communication and greatly influence biofilm development (Nield-Gehrig, 2005).

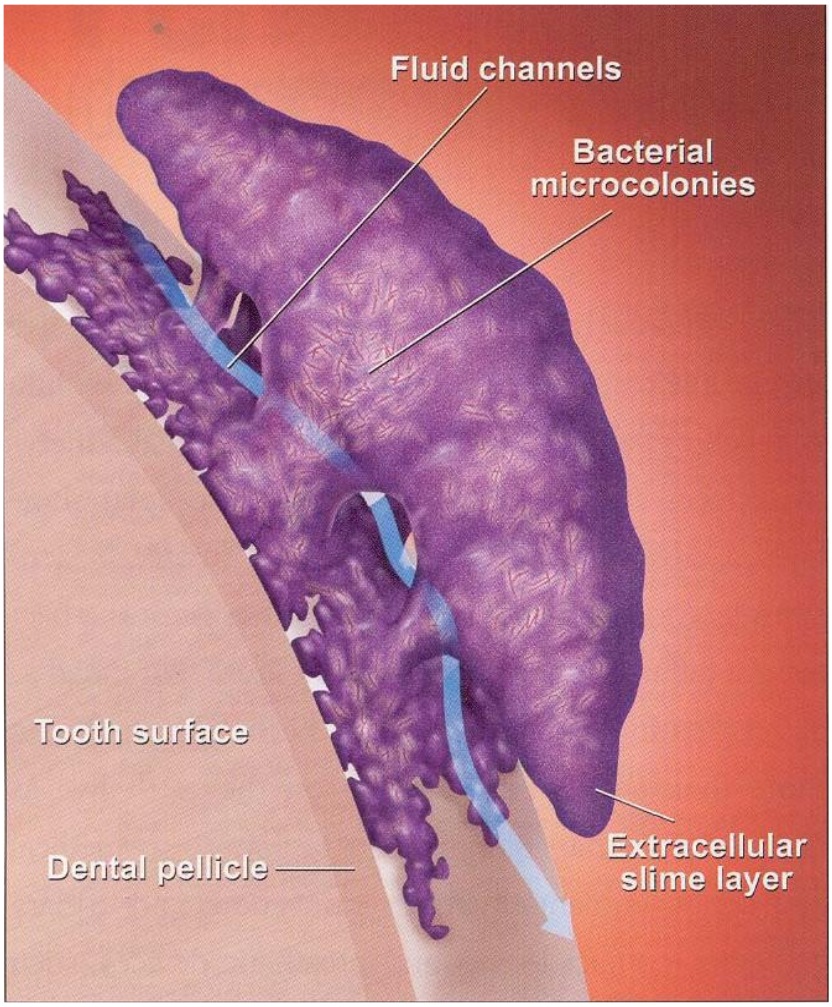


Figure 1.4: Gross structure of a biofilm (Reproduced from Nield-Gehrig, 2005)

Formation of oral biofilms

The formation of biofilms in the oral cavity has been the subject of a large body of research, involving different constituent micro-organisms. There is now a general consensus as to how oral biofilms form, and this process is classically divided into four stages, pellicle formations, adhesion, maturation and dispersion (Figure 1.5).

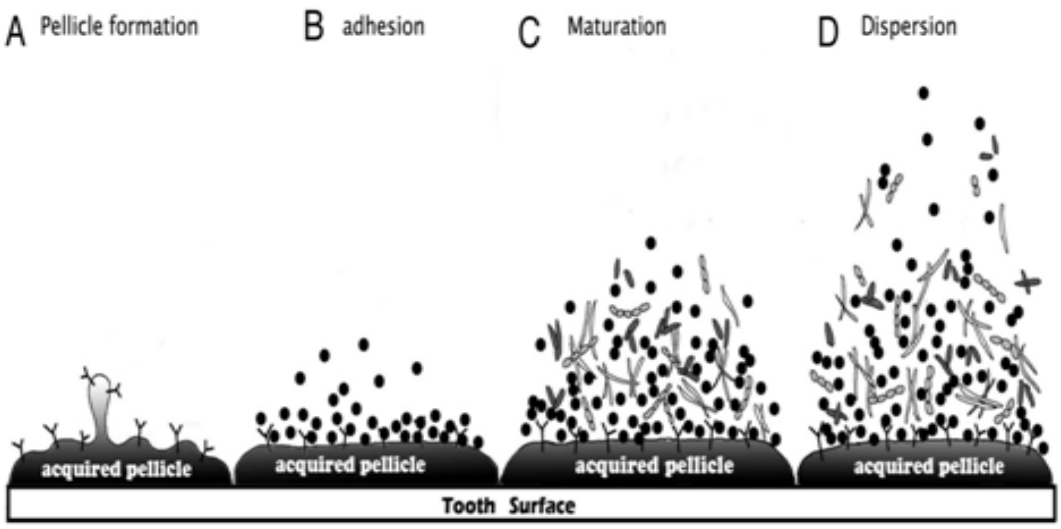


Figure 1.5: Schematic overview of oral biofilm formation. (A) Pellicle formation: The pellicle is a thin film derived from salivary glycoproteins attached directly to clean tooth surfaces. (B) Adhesion: Bacteria in saliva recognise binding proteins in the acquired pellicle and attach to them. (C) Maturation: Different bacterial species co-aggregate and a mature biofilm forms. (D) Dispersion: Bacteria detach from the biofilm surface and spread to colonize elsewhere (Reproduced from Huang *et al*., 2011).

The first step of oral biofilm formation is the attachment of an acquired pellicle, which is a thin protein-containing film that comes originally from salivary glycoproteins. These have been shown to attach to the tooth surface immediately after cleaning (Nield-Gehrig, 2005). This pellicle adheres to the tooth surface on one side and on the other side it offers an adhesive surface which provides a means of bacterial attachment to the tooth surface (Huang *et al*., 2011). The second step of biofilm formation is bacterial adhesion to the acquired pellicle. Initially, bacterial attachment is reversible and is mediated by pili and fimbriae (Bendtsen *et al*., 2005; Telford *et al*., 2006). The role of fimbriae has been explored for numerous bacterial species associated with the oral biofilm, for example the type I fimbriae of *Actinomyces naeslundii* (renamed *A. oris*) promote adhesion to proline-rich proteins within the salivary pellicle (Yeung, 2000; Mishra *et al*., 2010). Other species that adhere via fibrial proteins include *P. gingivalis* (Morten Enersen *et al*., 2013) and several *Streptococcus*

species including *S. salivarius*, *S. parasanguis,* members of the *S. mitis* group (Handley *et al*., 1990) and *S. agalactiae* (group B *streptococci*) (Lauer *et al*., 2005). The first bacteria to adhere to the salivary pellicle are referred to as primary colonisers, or pioneer species. Members of the genera *Actinomyces*, *Streptococcus*, *Haemophilus*, *Capnocytophaga*, *Veillonella* and *Neisseria* are considered the main pioneer bacterial genera (Kreth *et al*., 2005).

Adhesion of bacteria to the acquired pellicle is strengthened by interactions between bacterial adhesins and specific receptors located in the acquired pellicle. The adhesion–receptor interactions are strong and irreversible (Whittaker *et al*., 1996). Examples of these receptors that are found in the superagingival plaque are acidic proline-rich proteins that bind *S. gordonii* (Gibbons *et al*., 1991; Hsu *et al*., 1994) and *A. naeslundii* (*A. oris*) (Sigmund and Anned, 2001) via type 1 fimbriae (Nesbitt *et al*., 1992). *Fusobacteria* and *Veillonella* species have been shown to bind to the pellicle via statherin (McCabe and Donkersloot, 1977). *S. mutans* further promotes adhesion by converting sucrose into glucan, a reaction mediated by glucosyltransferase enzymes (Takahashi and Nyvad, 2008; Tanzer *et al*., 2001).

The maturation stage of oral biofilm development is initiated when secondary and late bacterial colonizers adhere to already attached bacteria (Kolenbrander *et al*., 2000). This process results in an increase in microbial diversity within the developing biofilm (Kolenbrander *et al*., 2006). As the biofilm matures other taxa co-colonise including *Actinomyces* and *Veillonella* species (Al-Ahmad *et al*., 2009; Diaz *et al*., 2006). The most common late colonisers are *F. nucleatum*, *T. denticola*, *T. forsythensis*, *P. gingivalis* and *A. actinomycetemcomitans* (Foster and Kolenbrander, 2004; Kolenbrander *et al*., 2002). Several late colonisers are strict anaerobes and are strongly associated with the progression of periodontal disease. Whilst *P. gingivalis* has been shown to co-aggregate with other species via fimbrial proteins MfaI and FimA; *T. denticola* attaches via the major surface protein (Msp) (Nobbs *et al*., 2011).

The maturation stage is also characterised by the synthesis of exopolymers to form a biofilm matrix (Burgess, 2005). An extracellular matrix is a common feature of all

biofilms and makes a significant contribution to the structural composition by retaining water, nutrients and enzymes within the biofilm. The chemistry of the matrix may also prevent the penetration of other molecules, such as some charged antimicrobials, for example chlorhexidine and quaternary ammonium compounds (Hata and Mayanagi, 2003). As the number of species in the biofilm increases, the structure becomes more complex and this complexity also bestows important properties on the biofilm, such as an increased resistance to antibiotics (Costerton, 1999). Indeed, the antibiotic resistance of bacterial cells in biofilms has been reported to be 1,000 to 1,500 times greater than the resistance of planktonic cells (Levy, 1998). The close proximity of cells to one another in a biofilm facilitates numerous synergistic and antagonistic interactions between adjacent species, and helps the development of food chains (Kuramitsu *et al*., 2007).

As the biofilm matures further, bacteria begin to leave the biofilm by single cell detachment. Two important drivers for detachment have been proposed. The first is nutrient limitation, with bacteria leaving to find more nutrient-rich niches, and the second is that bacteria are constantly lost as a result of the sheering forces applied to oral biofilms by the passage of saliva and other fluids (Ruijie Huang *et al*., 2011).

BACTERIAL INTERACTIONS IN ORAL BIOFILMS

As summarised above, close contact and specific interactions between micro-organisms is a characteristic of biofilms. The natures of the antagonistic and synergistic interactions between bacteria and the underlying mechanisms have been studied in some detail, some of which are summarised in Figure 6.

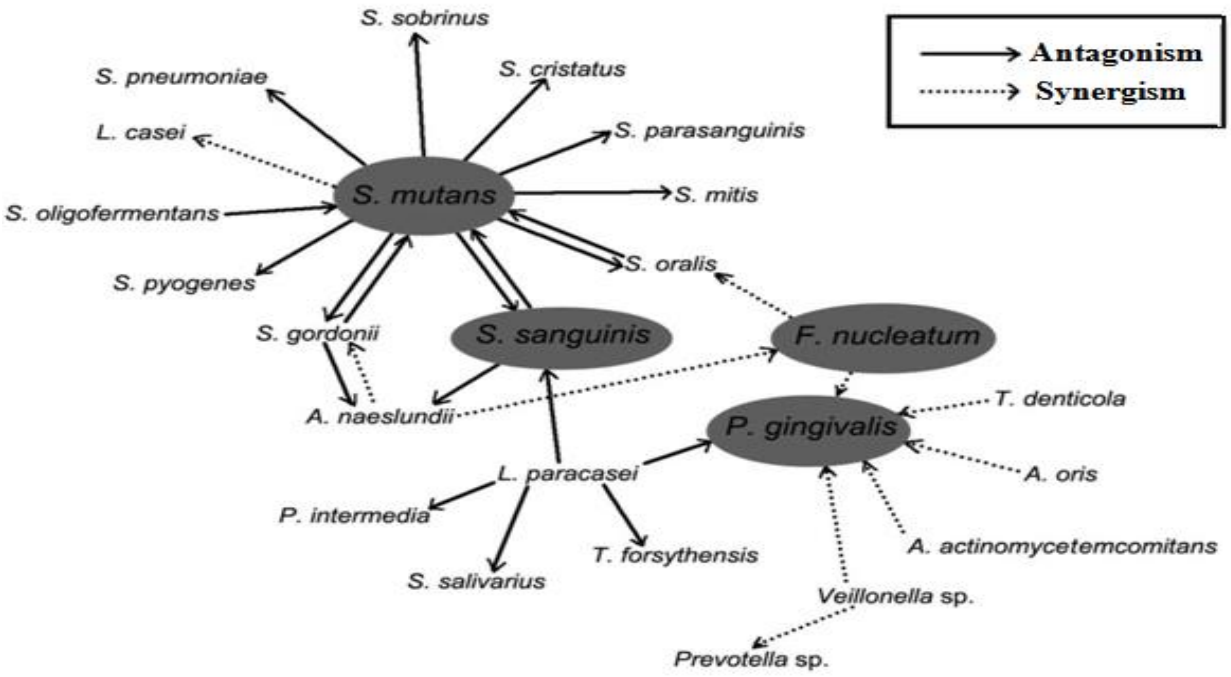


Figure 1.6: Interactions between selected members of the oral microbiome. Solid lines show antagonistic interactions while dashed lines show synergetic interactions. The direction of the arrow indicates the direction of antagonism or synergism (reproduced from Huang *et al*., 2011).

Infighting and collaborations between primary colonisers

One of the most studied antagonistic interactions within the oral microbiome is that between *S. mutans* and *S. sanguinis*. This antagonism possibly explains why *S. sanguinis* is less abundant in the mouths of individuals with ongoing caries than in those of caries-free subjects (Huang *et al*., 2011). The principle mechanism by which *S. mutans* inhibits the growth of *S. sanguinis* is by the production of a large amount of organic acid (Huang *et al*., 2011). Both species metabolize glucose to produce lactate, but *S. mutans* has a greater capacity to produce acid due to its greater ATP-glucose phosphotransferase activity (Komiyama and Kleinberg, 1974). *S. mutans* excretes lactic acid into the environment, which inhibits the growth of *S. sanguinis* (Iwami and Yamada, 1980). Kreth and colleagues (2005) co-cultivated *S. mutans* and *S. sanguinis*

under acidic conditions and observed that *S. mutans* grew better than *S. sanguinis.* Acidic conditions can repress or damage the ATP-glucose phosphotransferase activity in both species, but repression on *S. mutans* is less than that on *S. sanguinis* (Figure 1.7) (Iwani and Yamada, 1980).

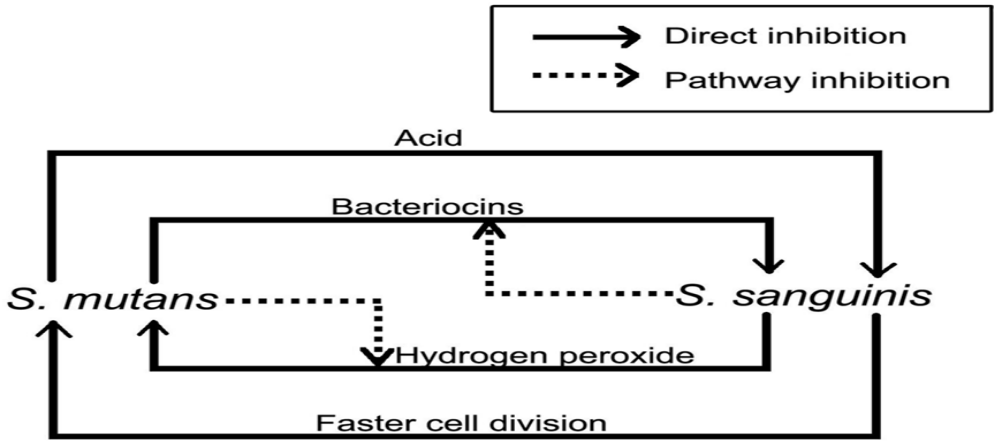


Figure 1.7: Illustration of the interactions between *S. mutans and S. sanguinis*. Lines show inhibition pathways, and the direction of the arrow presents the direction of inhibition or facilitation (Huang *et al*., 2011).

The relative intolerance of *S. sanguinis* to acid in the presence of *S. mutans* appears to result from *S. mutans* inhibiting the ability of *S. sanguinis* to excrete hydrogen peroxide. Hydrogen peroxide excretion by *S. sanguinis* was reduced by 66% when co-cultivated with *S. mutans* compared to when cultivated alone (Kreth *et al*., 2005). *S. sanguinis* is one of the early colonizers of oral surfaces and is considered a beneficial species in the oral biofilm ecosystem. In contrast, *S. mutans* is a major cause of dental caries (tooth decay). The production of hydrogen peroxide by early pioneer species acts as a source of oxygen and a non-specific antimicrobial agent inhibiting the growth of *S. mutans* and other anaerobic peridontal pathogens. The inhibition of *S. mutans* by *S. sanguinis* is greater under aerobic conditions than anaerobic conditions (Kreth *et al*., 2008). *S. sanguinis* also inhibits the production of the bacteriocin mutacin by *S. mutans* (Hale *et al*., 2005). Mutacin produced by *S. mutans* effectively inhibits the growth of *S. sanguinis*, which is therefore unable to colonise niches dominated by *S. mutans* (Kreth *et al*., 2005). Kreth and colleagues (2005) showed that *in vitro* mutacin production by *S. mutans* was reduced by 80% when it was co- cultivated with *S. sanguinis* (Figure

1.7).Finally, the doubling time of *S. sanguinis* is shorter than that of *S. mutans* enhancing its competitiveness with *S. mutans* (Wen, 2010) (Figure 1.7).

S. gordonii is another key pioneer species of oral biofilms, for which multiple interactions with other species have been well characterised. Like *S. sanguinis*, *S. gordonii* demonstrates antagonistic interaction with *S. mutans* and, again similar to *S. sanguinis*, this interaction is more apparent under aerobic conditions than under anaerobic conditions (Kreth *et al*., 2008). Interestingly, inhibition of *S. mutans* growth by *S. gordonii* was found to be reduced in the presence of glucose, which represses pyruvate oxidase, thereby reducing hydrogen peroxide production (Wen, 2010). This decrease in inhibition was more significant in *S. gordonii* than in *S. sanguinis* (Wen, 2010). The production of hydrogen peroxide by *S. gordonii* has also been shown to inhibit *Actinomyces naeslundii* growth (Bradshaw, 1998). *S. gordonii* appears to demonstrate synergistic interaction with *Veillonella* species, and the two taxa are commonly co-localised in oral biofilms (Bradshaw, 1998). This interaction could involve interspecies metabolic signalling as suggested by studies with *V. atypica* (Egland *et al*., 2004). Analysis of *V. atypica* and *S. gordonii* co-aggregation in a saliva-conditioned flow-cells detected enhanced expression of the amylase-encoding gene in *S. gordonii.* As amylase degrades starch to glucose, the authors hypothesised that induction of amylase gene expression in *S. gordonii* would benefit growth of *V. atypica*, an efficient glucose fermenter (Egland *et al*., 2004).

Several key adhesins that mediate co-aggregation of oral bacteria have been identified. The streptococcal Antigen I/II family polypeptides play an important role in the attachment of oral streptococci to the salivary pellicle; but also to other bacterial species. For example, SspB of *S. gordonii* binds to several oral surfaces; but also to the short fimbriae of the late coloniser*, P. gingivalis* (Forsgren *et al*., 2010)

The pivotal role of bridging species

A. naeslundii (renamed *A. oris*) is one of the primary bacteria in dental biofilm formation and it can cause peridontal disease and root caries. Co-aggregation of other

biofilm members is thought to offer protection from H_2O_2 as *A. naeslundii* is able to metabolise this molecule via protein oxidation (Periasamy *et al*., 2009). Furthermore, the resulting reduction in oxygen concentration is thought to lead to a more permissive environment for the growth of several anaerobes (Stadtman and Levine, 2003). This species can therefore be considered to act as a "bridge" between primarily commensal early colonisers and later colonisers that are more associated with disease. *A. naeslundii* is thought to provide *S. gordonii* with arginine, one of the products of protein oxidation, thus the growth of *S. gordonii* is increased when co-localised with *A. naeslundii*. In the presence of *A. naeslundii, S. gordonii* can grow under arginine-deficient conditions, conditions in which *S. gordonii* alone cannot survive (Jakubovics *et al*., 2008).

F. nucleatum is one of several *Fusobacterium* species that are the predominant Gram-negative bacterial species in mature subgingival dental plaque ((Moore *et al*., 1982; Kolenbrander *et al*., 2002). The co-aggregation of *Fusobacterium* species and other bacteria is also considered a bridge connection between initial and late dental plaque colonization (Kolenbrander *et al*., 2002). *F. nucleatum* co-aggregates with several oral streptococci via the arginine-sensitive adhesion, RadD (Kaplan *et al* 2009); and with *P. gingivalis* via the lectin-like adhesin, FomA (Kinder and Holt, 1993). *F. nucleatum* also supports *P. gingivalis* growth by providing a capnophilic environment (increased levels of CO_2) when growing in a low oxygen concentrations (Diaz *et al*., 2002).

F. nucleatum and the yeast *C. albicans* have each been shown to co-aggregate with *Streptococcus* species in supragingival plaque to form distinctive corncob structures (Figure 1.8). These formations are composed of a central filamentous cell (*F. nucleatum* or *C. albicans*) surrounded by multiple adherent streptococci (Lancy *et al*., 1983; Zijne *et al*., 2010). These interactions could act as a connecting link between the transformations of supra- to subgingival plaque.

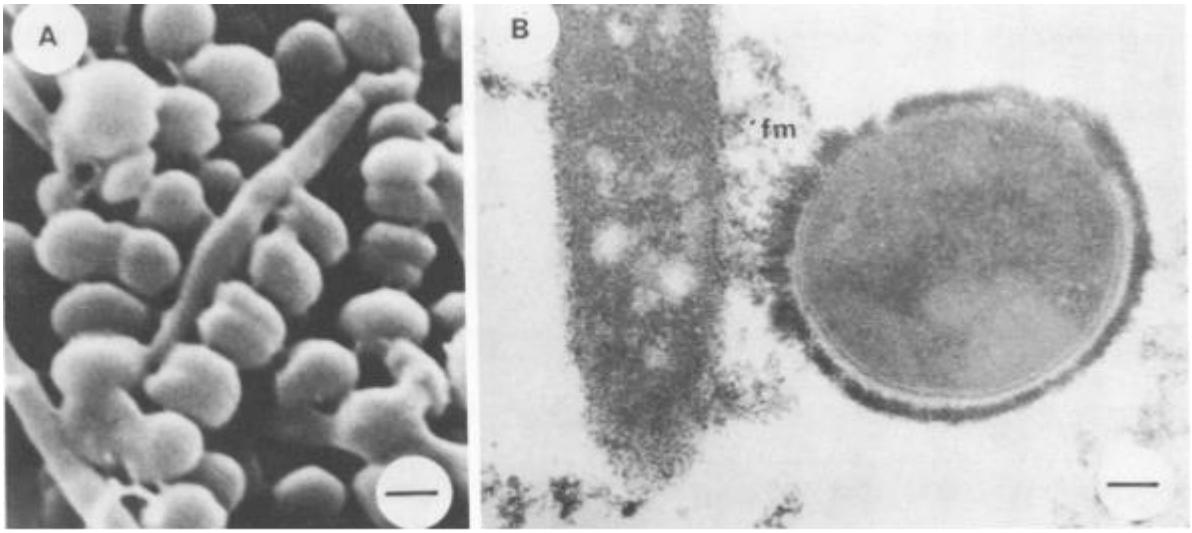


Figure 1.8: Electron microscopy of bacterial corncob forms *in vitro*. (A) Scanning electron micrograph of *F. nucleatum* - *S. sanguis* CC5A corncobs, bar = 1 µm. (B) thin section of *F.nucleatum* - *S. sanguis* CC5A corncobs. Fm = fimbriae, bar = 0.1 µm (from Lancy *et al*., 1983).

Shifting conditions to welcome the late colonizers

The bridging species play a pivotal role in the reduction of oxygen to create anaerobic microenvironments that are optimal for the growth of oral anaerobes (Bradshaw *et al*., 1998; Diaz *et al*., 2002). *F. nucleatum* also produces ammonia from glutamic and aspartic acids, which results in increased pH to levels that enhance the growth of *P. gingivalis* (Takahashi, 2003). Oral anaerobes have been shown to co-operate in a similar way in supragingival plaque. For example, *T. denticola* produces succinate, which is used by *P. gingivalis*. In turn, *P. gingivalis* enhances the growth of *T. denticola* by generating proteinaceous substrates and isobutyric acid as a metabolic end product (Grenier and Mayrand, 1986; Grenier, 1992; Mahajan*et al*., 2013; Kuboniwa and Lamont 2010).

Metabolic cross-feeding has been suggested to enhance persistence and pathogenicity of *Aggregatibacter actinomycetemcomitans* by streptococci producing L-lactate in a polymicrobial infection model. (Ramsey *et al*., 2011). Finally, *Veillonella* species are thought to metabolise organic acids produced by *S. mutans* (Huang *et al*., 2011) and

produce vitamin K, which is utilised by *Prevotella* and *Porphyromonas* species for growth (Marcotte and Lavoie, 1998). The increased abundance of any of these late colonisers in oral communities has been implicated in the development of oral diseases (Li *et al*., 2014; Ge *et al*., 2013; Wang *et al*., 2013).

THE ROLE OF THE ORAL MICROBIOME IN ORAL DISEASE

In a healthy oral cavity an ecological balance exists between the host and the numerous indigenous microorganisms (Filoche *et al*., 2010). However, bacteria that live in the dental plaque biofilm are generally believed to be responsible for the two most common oral diseases, dental caries and periodontitis (Papaioannou *et al*., 2009). These oral diseases are the most prevalent diseases in the world (Horz and Conrads, 2007; Selwitz *et al*., 2007), affecting all ages and geographic populations. Therefore, studying the microbial communities responsible for healthy status and disease activation and progression is important for combating human and oral diseases (Jenkinson and Lamont, 2005).

The role of the oral microbiome in dental caries

Dental caries is a worldwide chronic infectious disease (Petersen *et al*., 2005). Many studies have reported that *S.mutans* is the major pathogen of dental caries, because it is the most frequently detected bacteria in the caries lesions (Loesche, 1986; Matee *et al*., 1992). However, culture-independent methods for bacterial identification and enumeration such as array-based DNA hybridization (Corby *et al*., 2007; Aas *et al*., 2008; Kanasi *et al*., 2010) or Sanger sequencing of 16S rRNA gene clones (Aas *et al*., 2005) have revealed a wide array of bacteria correlated with caries progression. In addition as new pyrosequencing techniques are applied in oral microbiology, ever greater numbers of bacteria have been identified as being correlated with caries progression. A recent study, applying high- throughput barcoded pyrosequencing combined with PCR-denaturing gradient gel electrophoresis, found 120 genera in saliva and supragingival plaque from children aged 3–6 years with and without tooth decay. A review by Chen and Jiang (2014) found that the oral microbiota in children was far more diverse than previous studies reported and more than 200 genera belonging to

10 phyla were found in the oral cavity. The abundance of six genera (*Streptococcus*, *Veillonella*, *Actinomyces*, *Granulicatella*, *Leptotrichia,* and *Thiomonas*) has been shown to be significantly different between subjects with dental caries and caries-free subjects (Ling *et al*., 2010). Others have shown that *Streptococcus*, *Granulicatella* and *Actinomyces* genera exhibit a relative higher abundance in children with severe early caries, while *Aestuarimicrobium* was more abundant in caries free subjects. These findings suggest that there might be no specific pathogens but rather pathogenic population structure shifting would lead to the occurrence of dental caries (Jiang *et al*., 2013). Yang *et al*. (2012) reported that caries microbiomes were significantly more variable then the healthy ones. They found 147 OTUs associated with adult dental caries (Yang *et al*., 2012). Further research also found that the oral microbiota was specific at different stages of caries development. Gomar-Vercher *et al*. (2014) found that *Porphyromonas* and *Prevotella* species showed increasing abundance in the oral cavity of children with caries compared to healthy children and that bacterial diversity diminished as the severity of the disease increased. Chen & Jiang (2014) analysed the microbial composition of plaque from caries-active subjects in different caries stages including intact enamel, white spot lesions and carious dentine lesions by pyrosequencing technique. In accordance with Gomar-Vercher's study, their data indicated that the diversity of the total plaque bacterial community in the health subjects were more complex than caries subjects. Furthermore thirteen genera including (*Capnocytophaga*, *Fusobacterium*, *Porphyromonas*, *Abiotrophia*, *Comamonas*, *Tannerella*, *Eikenella*, *Paludibacter*, *Treponema*, *Actinobaculum*, *Stenotrophomonas*, *Aestuariimicrobium,* and *Peptococcus*) were associated with dental health. Eight genera (*Cryptobacterium*, *Lactobacillus*, *Megasphaera*, *Olsenella*, *Scardovia*, *Shuttleworthia*, *Cryptobacterium*, and *Streptococcus*) increased significantly in cavitated dentine lesions, whereas *Actinomyces* and *Corynebacterium* were present at significantly higher levels in white spot lesions. *Flavobacterium*, *Neisseria*, *Bergeyella,* and *Derxia* were found to be enriched in the intact surfaces of caries sites (Jiang *et al*., 2014). Others have shown that high proportions of *Atopobium Prevotella*, or *Propionibacterium* with *Streptococcus* or *Actinomyces* dominated in carious dentine lesions (Obata *et al*., 2014).

The oral microbiome in peridontitis

Periodontitis is an infectious and inflammatory disease in which oral bacteria play an important role in the progress of disease. This condition has been associated with a polymicrobial etiology, and comprehensive studies have been performed to elucidate the dynamics of these complex communities between healthy and periodontally affected patients (Ashimoto *et al*., 1996). There are significant differences in abundance of many species in periodontally healthy individuals compared to subjects with chronic periodontitis. Griffen *et al*. (2012) found that community diversity was higher in health than in disease. They identified 123 species that were significantly more abundant in individuals with chronic periodontitis and 53 species to be associated with healthly individuals. Among them, *Spirochaetes*, Synergistetes, and Bacteroidetes were health-associated, while *Proteobacteria*, *Clostridia*, *Negativicutes* and *Erysipelotrichia* were associated with chronic peridontitis (Griffen *et al*., 2012). Severe periodontitis is characterised by extensive tissue destruction and bone re-sorption, leading to the development of deep periodontal pockets (Kim *et al*.,2006; Elter *et al*., 2004). By comparing the oral microbiome in deep (diseased) and shallow (healthy) sites using sequencing 16SrRNA genes the abundance of 14 genus-level OTUs, including *Streptococcus*, *Actinomyces* and *Veillonella*, was decreased in deep pockets. In contrast, 37 genus-level OTUs (including *Prevotella*, *Porphyromonas*, *Treponema,* and *Fusobacterium*) were present in increased abundance compared to shallow sites (Ge *et al*., 2013).Others, utilizing the same pyrosequencing technique, have shown that the Gram-negative genera *Selenomonas*, *Prevotella*, *Treponema*, *Tannerella*, *Haemophilus* and *Catonella* are significantly enriched during periodontal disease, while Gram-positive genera are significantly enriched in healthy subjects: *Streptococcus*, *Actinomyces,* and *Granulicatella* (Liu *et al*., 2012). Bacteroidetes has been suggested as the most abundant phylum in samples of periodontal disease, whereas Actinobacteria and Proteobacteria were significantly increased in plaque of periodontally healthy subjects in another metagenomics sequencing analysis. At the genus level, the microbiota of periodontally healthly individuals was dominated by *Streptococcus*, *Haemophilus*, *Rothia,* and *Capnocytophaga*, whereas microbial

communities in periodontal disease exhibited high levels of *Prevotella* (Wang *et al*., 2013). Another 16S rRNA gene sequencing analysis reported that *Fusobacterium*, *Porphyromonas*, *Treponema*, *Filifactor*, *Eubacterium*, *Tannerella*, *Hallella*, *Parvimonas*, *Peptostreptococcus* and *Catonella* showed higher relative abundances in periodontitis patients (Li *et al*., 2014).

Metagenomics and 16S pyrosequencing studies of healthy and diseased mouths highlight the complexity of this polymicrobial aetiology. Although there are common findings throughout, such as the association of *Prevotella spp* with disease, there are many conflicting reports. Further studies that link other host and environmental factors to the oral microbiota are required.

AIMS OF THIS STUDY

The aims of this study were; (1) to quantify microbial diversity in the mouth using culture-based and culture-independent methods, (2) to use next-generation sequencing technologies to explore variation in microbiome composition in different individuals over different months of the year, and (3) to use an *in vitro* model system to analyse variation in the biofilm forming capacity of members of the oral microbiome.

CHAPTER TWO: MATERIALS AND METHODS

BIODIVERSITY ASSESSMENTS

Subjects of the study

Samples were collected from three subjects during this study. The first subject (OBA) was a 37 year old woman, the second (OBB) a 39 year old man and the third (OBC) a 38 year old woman. All three subjects lived in the North West of England throughout the study. Subjects A and B were husband and wife of Arabic descent, so cohabitated and shared a very similar lifestyle and diet. Subject OBA became pregnant in January 2013 and gave birth in September 2013. Subject OBC was married and of Caucasian descent. Approval for this study was obtained from the College of Science and Technology Research Ethics Panel at University of Salford (REP REFERENCE CST 14/39).

Collection of isolates for culture-based diversity assessments

All samples were collected from individual OBA in March 2013. Samples were collected from the mouth firstly by rolling a sterile cotton swab (CLASSIQSwabs, COPAN) across the gingival region and the roof and floor of the oral cavity and secondly by using dental floss to sample from the sub-gingival region. Swabs and floss were submerged in 1 ml sterile brain heart infusion broth (BHI) (Oxoid CM135) and mixed rigorously to generate a suspension of microbiome-associated bacteria.

Aliquots of this suspension were inoculated onto two types of bacteriological agar, firstly Columbia agar (Oxoid CM0331) containing 5% defibrillated horse blood (TCS, HB034), and secondly the same medium as above supplemented with 10mg/ml of vancomycin (VWR) (to inhibit Gram-postive organisms thereby allowing better survey of Gram negative organisms). Fifteen replicates of each medium were inoculated and incubated under different conditions; (i) 37°C in a 5% CO_2 atmosphere, (ii) 37°C in an anaerobic atmosphere, (iii) 37°C in an aerobic atmosphere, and (iv) room temperature in an aerobic atmosphere. All cultures were checked every day for up to 4 days for the presence of bacterial colonies.

If present, colonies were picked from primary isolation plates and passaged onto new media and re-incubated under the same conditions. Second passage isolates obtained in this manner were transferred to cryovials containing BHI + 10% (v/v) glycerol (Sigma) and stored at -80°C until required.

Phenotypic characterisation of isolates

Isolates were provisionally identified on the basis of their colonial morphology and Gram staining. For Gram staining, individual bacterial colonies were smeared on clean glass slides with a drop of sterile distilled water, air dried, and heat fixed by gently passing through a flame. The slides were flooded with crystal violet solution (Fisher) for one minute then flooded with Gram's iodine (Fisher) for one minute. The slides were washed with water and colour removed with 100 % ethanol (BDH) until no more violet colour was visible in the drain off solution. The slides were washed with water and counter stained with safranin stain (Fisher) for 30 sec and washed again with water. The slides were blotted dry and examined under a microscope using a 100x objective lens and oil immersion.

Genotypic characterisation of isolates

Bacterial isolates collected from the oral cavity during this study and oral streptococcal isolates obtained from Liverpool University were characterised using partial 16S rDNA sequencing. Crude DNA extracts were prepared by suspending fresh bacterial colonies in sterile dH_2O (50 µl). Samples were heated at 100°C in a heat block for 5 min to lyse the bacterial cells and release the DNA. Each extract (2 µl) was incorporated into a PCR mix containing 2µl of a 10 pmol/µl solution of forward primer EUB530 (5' CAGCAGCCGCGGTAATAC 3'), 2 µl of a 10 pmol/µl solution of reverse primer EUB790 (5' CTACCAGGGTATCTAAT 3') (Hunt *et al*., 2013), 25 µl of 2xMyTaq Red master mix (Bioline) and 19 µl of sterile dH_2O. PCR mixes were subjected to a thermal programme of 96°C for 3 min, then 30 cycles of 96°C for 10 sec, 55°C for 10 sec and 72°C for 50 sec, completed with a final step of 72°C for 5 min.

The presence and size of amplicons was determined by UV visualisation of 1% (w/v) agarose gels made with Tris borate EDTA (TBE) buffer (Biotech) containing 1.7 µg/ml of

Gel Red (Cambridge Biosciences) on which post-reaction PCR mixes (5 µl) had been electrophoretically resolved (100 volts for 60 min). Amplicon sizes were estimated by comparison with a Hyperladder I molecular weight marker (Bioline). Amplicons of the expected size (approximately 300 base pairs) were purified for sequencing using a QIAquick PCR purification kit (Qiagen).

Sequencing of amplicons was performed commercially (Source Bioscience). Both strands of each amplicon were sequenced using the same primers as used for their initial amplification, described above. Sequence data from each strand were verified then combined with one another using Chromas Pro (Technelysium Ltd). Primer sequences were removed from the extremities, leaving an unambiguous sequence of about 260 base pairs to be used for genotypic comparison. This was achieved using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/Blast/).

Collection of samples for analysis of biodiversity using culture-independent studies.
Longitudinal samples were collected from the oral cavities of all three subjects on the same day every month between May and December 2013. Sampling involved thorough brushing of teeth for two min using a sterile dental brush, an interdental brush and dental floss. Dental plaque was obtained by suspending the used tooth brush, the interdental brush and the dental floss in 30 ml of phosphate buffered saline Sigma(PBS) () and vortexing for 1 min. All dental hygiene tools were removed and dental plaque was harvested from each suspension by centrifugation at 7000g for 15 min. Pellets were resuspended in 1ml of sterile PBS and transferred to 1.7ml Eppendorf microfuge tubes. These tubes were centrifuged at 13000g for 2 min then stored at -80^{o}C until required.

Extraction of DNA
Oral samples were thawed and re-suspend in 500 ul Tris-EDTA (TE) buffer (10 mM Tris-HCl; 1 mM EDTA, pH 7.4) and transferred to Pathogen Lysis L tubes (Qiagen). Bacterial cells were lysed using Epicentre Ready-Lyse lysozyme (1000 U) and incubated with agitation (300 rpm) at 37°C for 2 h. To ensure thorough lysis of all cells, samples were further processed using a mechanical bead beater (Fastprep24, MPBiomedicals);

samples were pulsed at 6.5 m/s for 2 x 45 sec with 5 min rest between bursts (on ice). Total DNA was extracted from lysed oral samples using the QIAamp mini DNA extraction kit (Qiagen) according to the manufacturer's instructions. DNA was eluted from each column using 10 mM Tris•Cl; 0.5 mM EDTA; pH 9.0. DNA was quantified using a Nanodrop 2000 spectrophotometer. All samples yielded between 17 and 190 ng/µl DNA with a 260/280 ratio of between 1.8 and 2.0.

Preparation of sequencing libraries

Standard 16S primers (F515/R806) with Illumina adapters were used to amplify the V4 region of the 16S rDNA. The same forward primer was used for all reactions. However, the reverse primer included 25 different barcodes (one for each sample, plus negative control) with a 12-base error-correcting Golay code. Both PCR primers contained sequencer adapter regions, designed to amplify region V4 (291 bp) (Caporaso *et al* 2011).

Reactions were set up as follows: 10 µl NEBNext® High-Fidelity 2X PCR Master Mix (New England Biolabs) 0.05 µM F515 primer; 0.05 µM 806R primer with unique barcode; 1 µl DNA. Cycling conditions were as follows: 94°C for 3 min to denature the DNA, with amplification proceeding for 20 cycles at 94 °C for 45 sec, 50 °C for 60 sec, and 72 °C for 90 sec; a final extension of 10 min at 72 °C was added to ensure complete amplification.

Post-amplification clean-up, quality control and sequencing

PCR amplicons (400 bp; 291 bp V4 region + barcodes) were visualised on a 1.5 % TBE agarose gel and purified using solid-phase paramagnetic AGENCOURT® AMPure® XP beads (Beckman Coulter). Briefly, an equal volume of PCR amplicon and AMPure beads were mixed thoroughly by vortexing. DNA-bound AMPure beads were separated from suspension by placing sample tubes in a Dynamag™ magnetic stand (Life Technologies). Beads were washed twice using 70% ethanol. All traces of ethanol were removed and DNA was eluted from beads, using nuclease-free water (20 µl) (Life Technologies).

Purified amplicons were quantified using a Qubit®2.0 Fluorometer with QuantiT™ dsDNA HS Assay reagents (Life Technologies) according to the manufacturer's instructions. DNA concentrations were then normalised and pooled. An equal volume (16 µl) of pooled sample and Ampure beads were mixed together for further purification as described above.

Automatic targeted size selection (400 bp) was performed on the purified 16S rRNA library using a Pippin prep capillary based gel system (Sage Science). The purified library was analysed for quality using a DNA High Sensitivity chip on the 2100 Bioanalyser (Agilent Technologies). Paired end sequencing (2x250 bp) was performed on the pooled amplicon sample using the illumina MiSeq platform generating data off in excess of 12M clusters.

Next generation sequencing sample preparation and processing was performed by Dr Chloe James in the Centre for Genomic Research at the University of Liverpool. Briefly, a total of 24 pools of amplicons were subject to paired-end sequencing using the MiSeq platform. Paired-end reads were trimmed and aligned using PANDAseq (Masella *et al* 2012). Concatenated reads were further processed by clustering sequences with at least 97% similarity using USEARCH (Edgar, 2010). Operational taxonomic units (OTUs) were assigned to each sequence using Qiime (Caporaso *et al* 2010) and searching the Greengenes database (McDonald *et al* 2012).

Different relative abundances of OTUs were assessed at the phylum and family levels. The significance of temporal and person-to-person variation in the relative abundance of specific OTUs was assessed using ANCOVA (analysis of covariance) in MINITAB 16. Correlations with P values of equal to or less than 0.05 were considered as being significant.

ASSESSMENT OF BIOFILM-FORMING ABILITIES OF *STREPTOCOCCUS* ISOLATES

Description of isolates

Oral streptococci isolates used in the biofilm assays included those obtained in this study and two panels of isolates, the first kindly donated by Professor Howard Jenkinson at the University of Bristol and the second from The Biomedical Research Centre at the University of Liverpool. Details of the identities of the isolates in these panels are provided in Table 2.1.

Species name	**Strain**	**Lab identifier**	**Source**	**Reference**
Streptococcus parasanguinis	FW 213	UB2535(B1)	Bristol	Chen *et al*., *2013*
Streptococcus infantis	SK959	UB1619 (B2)	Bristol	Hoshino *et al*., 2005
Streptococcus oralis	NCTC11427	UB385(B3)	Bristol	Do *et al*., 2009
Streptococcus cristatus	CR311	UB602(B4)	Bristol	Wang *et al*., 2009
Streptococcus oralis	34	UB2148(B5)	Bristol	Tawse-Smith *et al*., 2014
Streptococcus salivarius	HB	UB608(B6)	Bristol	Wessel *et al*., 2014
Streptococcus sanguinis	SK36	UB1330(B7)	Bristol	Turner *et al*., 2009
Streptococcus intermedias	ATCC 27335	UB601(B8)	Bristol	Aguirre-Arzola *et al*., 2013
Streptococcus salivarius	NCTC 8606	UB384 (B9)	Bristol	Gamboa & Chaves 2012
Streptococcus pectoris	N/A	L108752(13)	Liverpool	This study
Streptococcus parasanguinis	N/A	L108764(33)	Liverpool	This study
Streptococcus spp.	N/A	L 108814(115)	Liverpool	This study
Streptococcus salivarius	N/A	L 108811(125)	Liverpool	This study
Streptococcus species	N/A	L 108816(117)	Liverpool	This study

Table 2.1: *Streptococus* strains used in this study obtained from Professor Howard Jenkinson at the University of Bristol and The Biomedical Research Centre, University of Liverpool. Isolates from the University of Liverpool were obtained during an

endoscope study of healthy subjects at the University of Liverpool and had been identified by mass spectrometry.

Biofilm assays

Bacterial biofilm studies were conducted using a simple *in vitro* biofilm assay modified from Lemos *et al*., 2010.

Saliva collection and standardisation

Volunteers who kindly provided saliva for this study were asked to (i) avoid drinking alcohol one day before sample collection, (i) not to exercise any oral hygiene after 10pm on the day prior to collection, and (iii) not to eat for at least 60 min prior to sample collection. Each volunteer firstly rinsed his/her mouth with water to remove food residue then repeatedly swallowed to increase saliva production. Saliva was spat into a sterile collection beaker and the volunteer was asked to continue until 25ml of saliva was in the beaker. The volunteer then took a 30 minute break and drank copious water before repeating the saliva collection process.

Harvested saliva was pooled and mixed (1:1) with an adsorption buffer (50 mM KCl, 1 mM potassium phosphate (0.35 mM K_2HPO_4 plus 0.65 mM KH_2PO_4), 1mM $CaCl_2$, 0.1 mM $MgCl_2$. Adjust pH to 6.5. Store at room temperature, then treated with the protease inhibitor Pefabloc SC (Sigma) 1 mM/ml by have it down at 500 µl of 0.1M Pefabloc per litre of saliva to prevent protein degradation (manufacturer). The saliva is then subsequently aliquoted and centrifuged to pellet food debris, saliva-borne bacteria and human cells. The supernatant (now referred to as *clarified whole saliva*) was then filter-sterilised via a low protein binding 0.22µm filter at 4°C to remove all remaining bacterial and human contaminants and this will include live cells (Wei and Bobek, 2005) and directly frozen.

This clarified saliva was obtained from Dave Greenwood (PhD student in School of Environment & Life Sciences at Salford University).

Biofilm Medium (BM)

BM comprised of base medium (58mM K_2HPO_4(BDH), 15mM KH_2PO_4 (BDH), 10mM$(NH_4)_2SO_4$, 35mM NaCl; 1mM MnCl $4H_2O$ (Sigma), 0.003mM $FeSO_4$ $7H_2O$ (BDH),2% (w/v) casamino acids (Difco), amino acids (4mM L-glutamic acid (BDH), 1.4mM L-arginine (VWR), 6.5mM L-cysteine HCL (SLS) and 1mM l-tryptophan (Sigma)), vitamins (1.4mM pyridoxine HCL (Fisher Scientfic), 0.3mM nicotinic acid (SLS), 0.1mM pantothenic acid (SLS), 0.01mM riboflavin (Sigma), 0.003mM thiamine HCL (Sigma) and 0.0005mM D-biotin (Sigma), $MgSO_4$ $7H_2O$ (0.8mM)(Sigma), 0.2mM $CaCl_2$ $2H_2O$ (Sigma) and 1000mM glucose(Sigma). Media was made fresh, filter sterilised and stored at 4^oC for up to 1 week.

The microtitre plate assay

Different streptococcal strains were screened to assess their abilities to form biofilms by using a slight modification of a previously described microtiter plate assay (Lemos *et al*., 2010).

Each strain was streaked onto a 5% Columbia blood agar then incubated in the presence of 5% CO_2 at 37°C for 24 hours. Colonies from this plate was used to seed a liquid starter culture consisting of 5ml brain heart infusion (BHI) broth, which was incubated in the presence of 5% CO_2 at 37°C. An overnight broth culture was sub-cultured by transferring a 100 µl aliquot into 5ml of fresh BHI broth, which was then incubated at 37°C in the presence of 5 % CO_2 until it reached mid-exponential growth phase. This was assessed by measuring the optical density of the culture at a wavelength of 600 nm (OD_{600}). An OD_{600} of 0.5 was considered to indicate mid-exponential phase.

BM was freshly prepared and pre-warmed to 37°C for 1 h. Individual wells of sterile, flat-bottomed 96 well microtitre plates (BD Falcon) were filled with 50 µl of clarified saliva and incubated for 1 h at 37°C. Unbound saliva was removed by blotting the plate on clean absorbent paper. Each well was then filled with 200 µl aliquots of the mid-exponential phase cultures (diluted 1:100). BM without culture was used as a negative control.

Microtitre plates were incubated for 1-7 days at 37°C in the presence of 5% CO_2. Planktonic and biofilm growth were measured for each strain at 24 hour time intervals for up to 7 days. Planktonic growth was measured by removing liquid culture (planktonic cells) from the first well of each row and measuring OD_{600}. BM was used as a negative control. Biofilm formation was measured by removing culture media from each well and blotting the plate on a paper towel to remove all liquid. Loosely bound cells were removed by carefully immersing the microtiter plate, three times, in a large sterile dish containing sterile distilled water. Each washed microtitre plate was blotted on a paper towel then each well on the plate was flooded with 0.1% (w/v) crystal violet solution and incubated for 15 min at room temperature. Unbound crystal violet was removed by again immersing the microtiter plate in a large dish with distilled water three times. All liquid was removed from each well and microtitre plates were air dried. Bound crystal violet stain was solubilised with 70% ethanol for 10 min at room temperature. Optical density (OD) of the wells was determined using a micro ELISA auto reader (ThermoFisher Scientific, UK) at a wavelength of 570 nm. These OD values were considered as an indicator of attachment to surface and biofilm formation. The OD value of the negative control was subtracted from those of each of the test samples and the resulting values were plotted against time interval to determine planktonic and biofilm growth of the streptococcal strains. Each isolate was tested five times and for each, mean values and standard deviations of values about these means were calculated using Excel.

CHAPTER THREE: RESULTS

Isolation and phenotypic characterisation of oral bacteria

Inoculated plates, incubated under different conditions, were examined for the presence of bacterial colonies. For each plate, the variety of colonial morphologies was noted and colonies of each morphology were individually sub-cultured onto appropriate media. Plates supported growth of up to seven distinct colonial morphological variants, giving a total of 29 different variants (Table 3.1).

Incubation conditions	Sample	Colony morphology	Gram Stain
Anaerobic, 37°C, non-selective	A3	white, large, irregular shape	Gram positive rod
Anaerobic, 37°C, vancomycin	A6	yellow, irregular shape	Gram negative cocci
	A8	white, small, irregular shape, raised	Gram negative rods
	A12	brown, punctiform, raised	Gram negative cocci
	A15	white, large, irregular shape	Gram negative rods
5% CO_2, 37°C, non-selective	A23	dark brown, smooth, irregular shape	Gram positive cocci
	A1	white, large, granular, circular shape	Gram positive cocci
	A7	white/transparent, large, circular shape with raised edge	Gram positive cocci
	A16	white, punctiform	Gram positive cocci
	A19	white, irregular in shape with fimbriate edge	Gram positive rod
	A21	grey, effuse, circular with entire edge	Gram positive cocci
	A22	brown circular with entire edge and smooth texture	Gram negative diplococci
5% CO_2, 37°C, vancomycin	A4	grey/white, large, circular with entire edge	Gram negative rods
	A5	light grey, tiny satellite colonies, small	Gram negative rods
	A13	white colour, irregular shape	Gram negative rods
	A17	light grey, circular, smooth	Gram negative cocci
Aerobic, room temp, non-selective	A2	white, circular with entire edge	Gram positive cocci
	A9	brown, circular and fimbriate edge	Gram positive rods
	A10	dull grey, irregular form and granular surface	Gram positive rods
	A11	yellow smooth and irregular shape	Gram positive rods
	A14	yellow, punctiform and convex	Gram positive cocci
	A18	small circular colonies with entire edge	Gram negative rods
	A20	filamentous form with granular texture	Gram positive cocci
	A24	white, punctiform	Gram positive cocci
	A26	brown, raised, irregular shape	Gram positive cocci
	A27	punctiform	Gram positive rods
	A28	punctiform, smooth	Gram negative cocci
	A29	punctiform, and raised	Gram positive cocci
Aerobic, room temp, vancomycin	A25	yellow, circular with entire edge and raised	Gram negative cocci

Table 3.1: Details of the incubation conditions, colonial and microscopic appearance of isolates obtained from oral swabs in this study

In total, 29 colonies were sub-cultured and characterised (Table 3.1). The range of colony morphologies, obtained under different incubation conditions, suggested a broad diversity of bacteria had been isolated. The greatest number of different colonial morphologies were obtained under aerobic incubation conditions (13 morphologies) compared to elevated CO_2 (10 morphologies) and anaerobic conditions (6 morphologies). Similarly, more morphological variation was observed among isolates obtained on non-selective blood agar compared to blood agar containing vancomycin (Table 3.1). Microscopic observation of Gram stained smears of each isolate also revealed diversity.

Molecular identification of bacterial isolates

An approximately 260 base pair amplification product was amplified from 29 isolates. All amplicons were submitted for Sanger sequencing, and unambiguous sequence data were obtained for 15 of these isolates. For the other 10 isolates, chromatograms with superimposed peaks were obtained suggesting isolates may not have been pure cultures. BLAST analysis of each unambiguous sequence yielded very high sequence similarities (>99%) with GenBank submissions (Table 3.2) and allowed most of the isolates to be characterised to species level with confidence. However for others, species or even genus-level identification was not possible as their partial 16S rDNA sequences were indistinguishable from those of multiple taxa (e.g. sample A11, Table 3.2). A broad diversity of bacteria were characterised, including representatives of three different bacterial phyla; Firmicutes such as *Streptococcus, Bacillus* and *Granulicatella* species, Proteobacteria such as *Kingella, Neissera* and *Haemophilus* species and Actinobacteria such as *Rothia, Microbacter, Leifsonia* and/or *Actinobacterium* species.

Comparison of 16S rDNA and phenotypic data revealed a good correlation, with 13 of 15 isolates possessing a colonial morphology and microscopic appearance that agreed with that predicted from the sequence data. However A28, an apparently Gram

negative coccus, did not match with the result from16S rDNA sequence data which suggested *Streptococcus salivaris*.

Sample number	**Taxa sharing highest sequence similarity**	**% sequence similarity**	**GenBank accession number**
A1	*Rothia dentocariosa*	99	NR044712
A2	*Granulicatella paraadiacensans* *Granulicatella adiacens*	100	JF803551 FR822389
A4	*Kingella denititrificans* *Neisseria elongata*	100	KC632208 AY167422
A5	*Haemophilus parainfluenzae*	100	JF506652
A7	*Streptococcus mutans*	100	NR074983
A10	*Bacillus ceruns* *Bacillus thuringiensis*	100	KJ399985 KJ206079
A11	*Microbacter spp.* *Leifsonia spp.* *Actinobacterium spp.*	100	HQ256839 JX517243 JQ229622
A13	*Bacillus licheniformis*	100	KJ469794
A17	*Neisseria bacilliformis*	100	GU397594
A18	*Haemophilus parainfluenzae*	100	JF506652
A19	*Bacillus licheniformis*	100	KJ469794
A20	*Rothia dentocariosa*	100	KC632226
A22	*Neisseria oralis*	100	JN986584
A23	*Streptococcus gordonii*	100	KJ170416
A28	*Streptococcus salivaris*	99	KF193931

Table 3.2: 16S rDNA sequence similarities of 15 oral isolates obtained in this study

Using next generation sequencing to assess person-to-person and temporal variation in the human oral microbiota.

Miseq sequence data from the 24 oral samples collected during the study were delineated into operational taxonomic units (OTUs) belonging to 13 phyla, 48 families and 60 species.

In total, 207 OTUs were identified. However, 14 of these were also present in negative control samples, so were considered as contamination and removed from further analysis (Table 3.3).

OTU	**Relative abundance (%) in negative control**	**Relative abundance (%) in samples**
Veillonella	3.13	5.75 - 22.73
Actinomyces	3.13	0.45 - 3.49
Parascardovia	3.13	2.06 - 7.65
Bacteroides	28.13	0.00 - 0.07
Prevotella	15.63	1.83 - 14.80
Fibrobacter	3.13	0.00 - 0.01
Streptococcus	6.25	2.56 - 14.05
Ruminococcus	3.13	0.00 - 0.01
Megasphaera	3.13	0.05 - 2.15
Fusobacterium	9.38	1.34 - 13.13
Novosphingobium	6.25	0.00 - 0.00
Escherichia	6.25	0.00 - 0.00
Acinetobacter	3.13	0.00 - 0.00
Treponema	6.25	0.05 - 5.06

Table 3.3: The 14 OTUs, and their relative abundances, present in the negative control sample.

A further 76 OTUs were removed from further analysis because they accounted for less than 0.01% of the reads in all samples. Thus, 117 OTUs remained for comparative analysis. These data were used to explore person-to-person and temporal variation in human oral microbiota.

Estimates of biodiversity at the phylum level

In general, the microbiota in all samples was dominated by members of two phyla, the Proteobacteria and the Firmicutes. Together these two phyla accounted for between about 35% and 70% of the total abundance of bacteria in all samples. Three other phyla, Fusobacteria, Bacteroidetes and Actinobacteria were also relatively abundant in all 24 samples tested. All other phyla represented less than 20% of the relative abundance of bacteria in all samples (Figure 3.1).

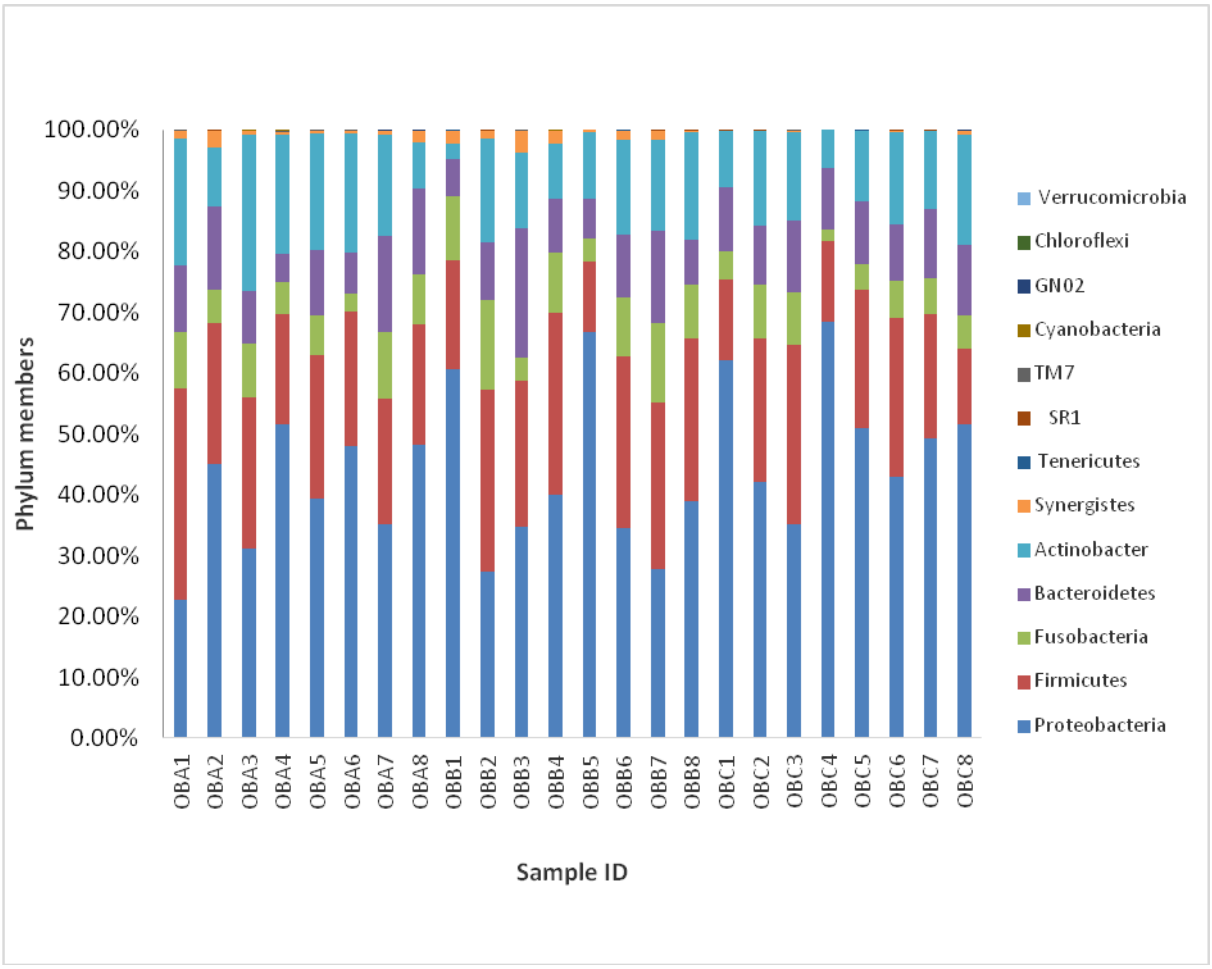


Figure 3.1: Relative abundance of phylum members of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

Estimates of biodiversity at the family level

As expected, a general summary of microbiota diversity at the family level was far more difficult than at the phylum level, as variation between samples was far more apparent. However, numerous families were present in all samples and some were consistently among the most abundant encountered. *Pasteurellaceae* members accounted for between 10% and 40% of the relative abundance of bacteria in all samples. Each of *Neisseriaceae* and *Veillonellaceae* accounted for between about 5%

and 30% of the total abundance of bacteria in all samples. Six other families, *Actinomycetaceae, Leptotrichiaceae*, *Micrococcaceae*, *Streptococcaceae*, *Corynebacteriaceae* and *Paraprevotellaceae* were also relatively abundant in all 24 samples tested. No other families represented more than 5% of the relative abundance of bacteria in all samples (Figure 3.2).

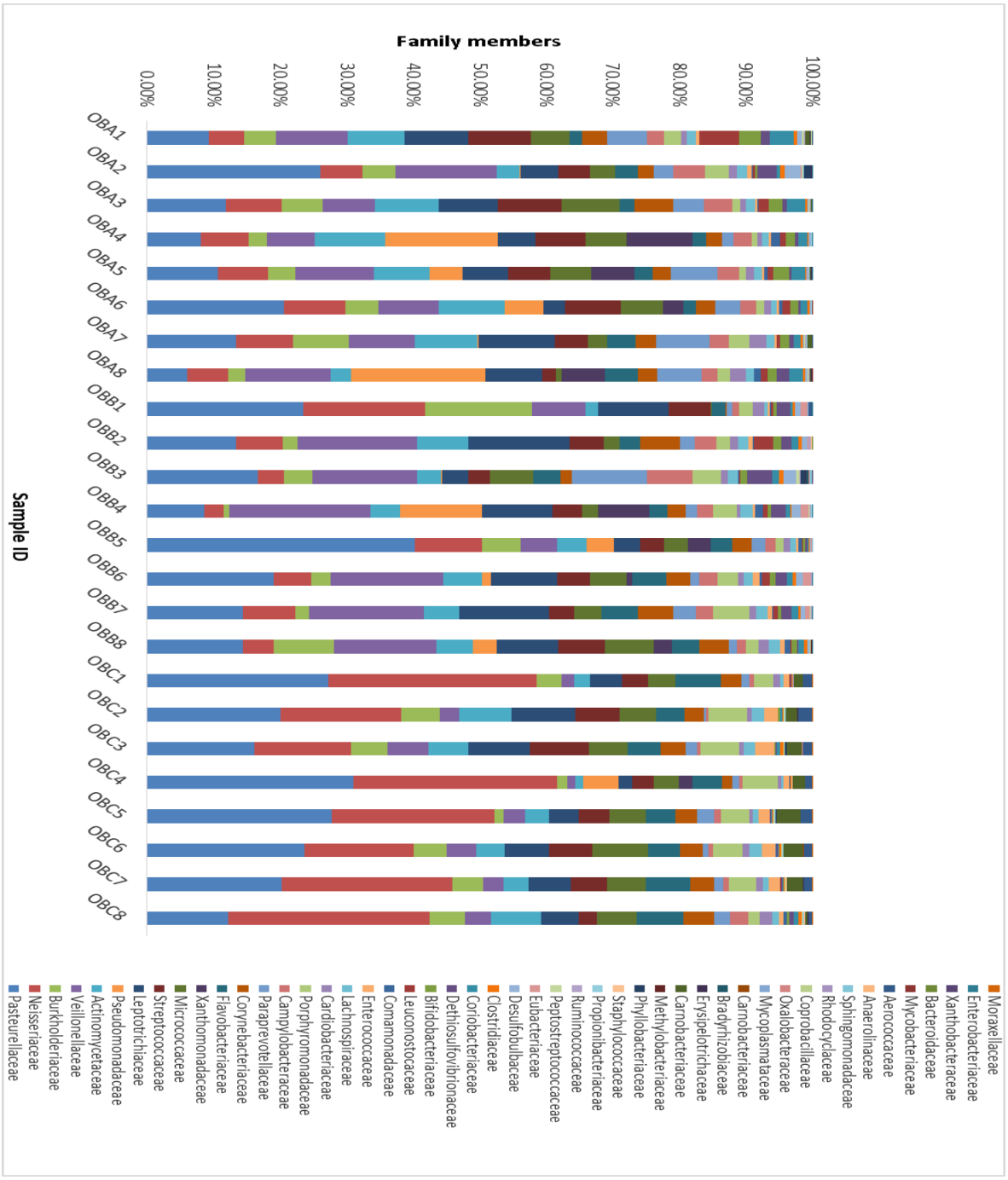
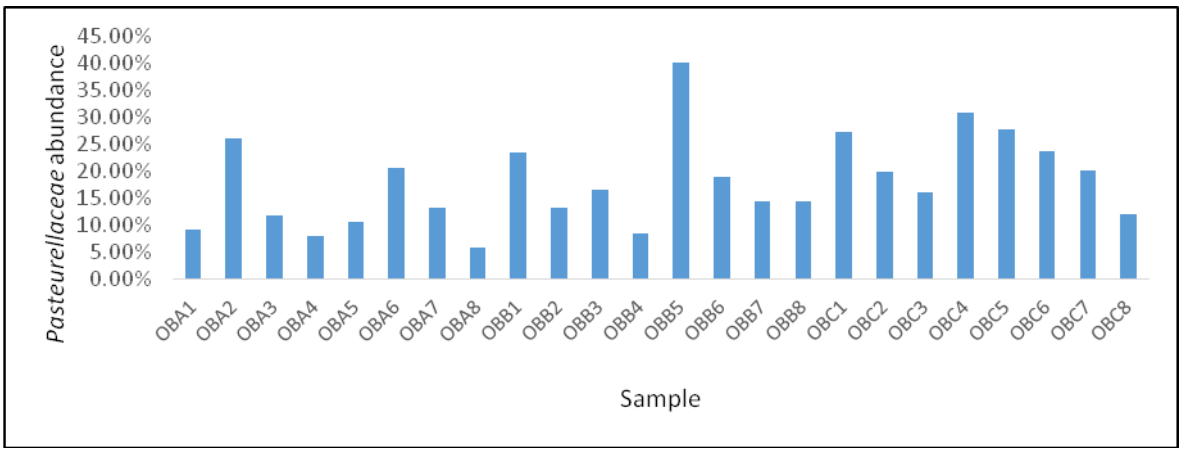
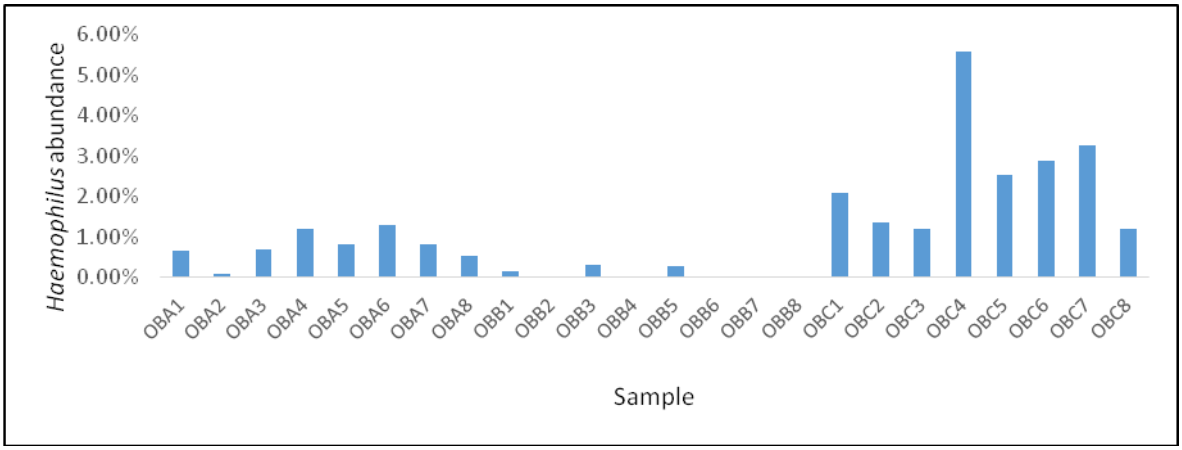


Figure 3.2: Relative abundance of family members of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

Person-to-person variation in oral microbiota

Three people, OBA, OBB and OBC, provided samples for the study. All were in their late 30s. OBA and OBC were females, OBB was male. OBA and OBB were of Arabic decent whereas OBC was Caucasian. Comparison of the relative abundance of OTUs in samples from different individuals revealed some significant differences. OBC possessed a significantly higher abundance of numerous OTUs than OBA and OBB including *Pasteurellaceae* ($p = 0.05$), *Aggregatibacter* ($p < 0.01$), *Prophyromonas* ($p < 0.001$), *Granulicatella* ($p < 0.001$), *Haemophilus* ($p < 0.001$), *Streptococcaceae* ($p = 0.001$), *Neisseria* ($p < 0.001$), *Abiotrophia* ($p < 0.001$), *Lactobacillales* ($p < 0.001$) and *Gemellales* ($P < 0.001$) (Figure 3.4a-c).





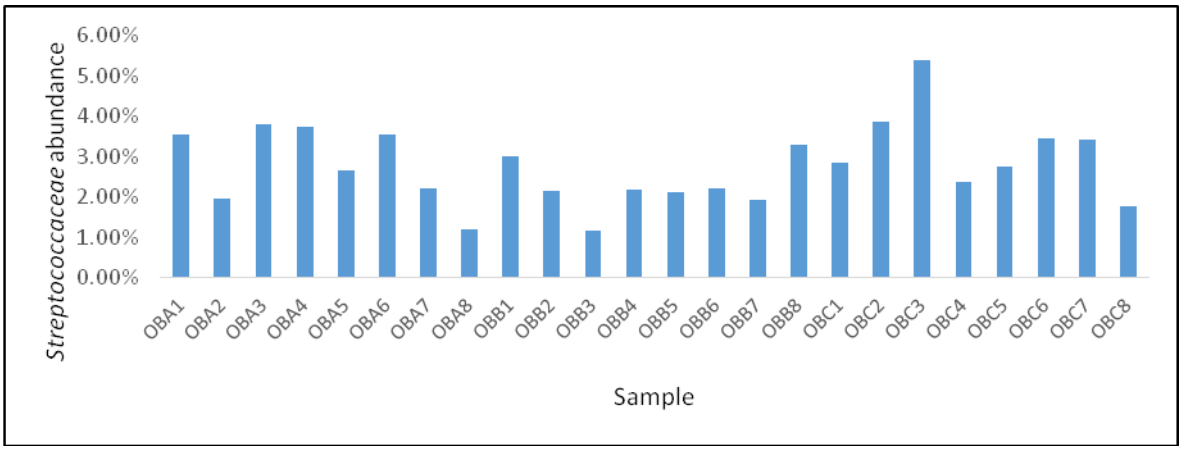
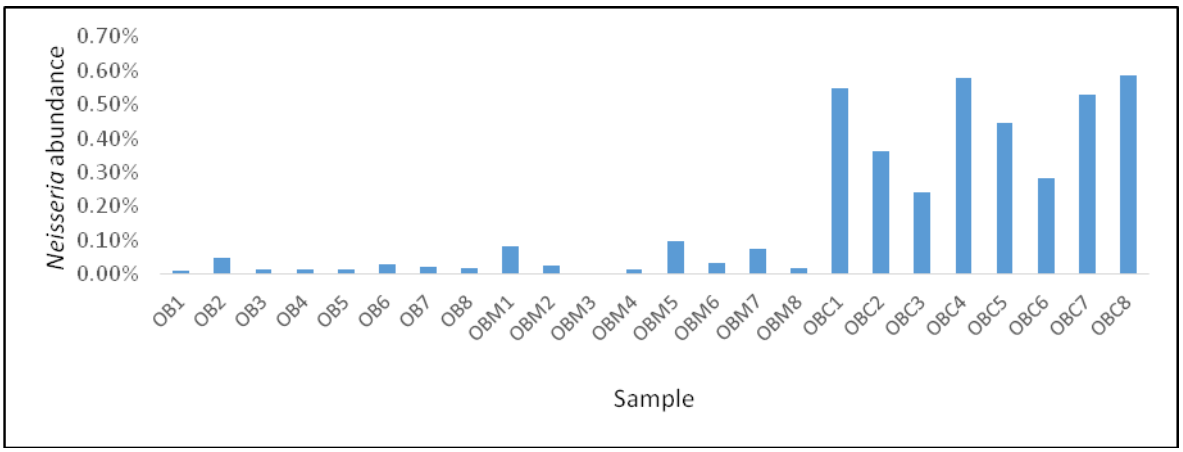
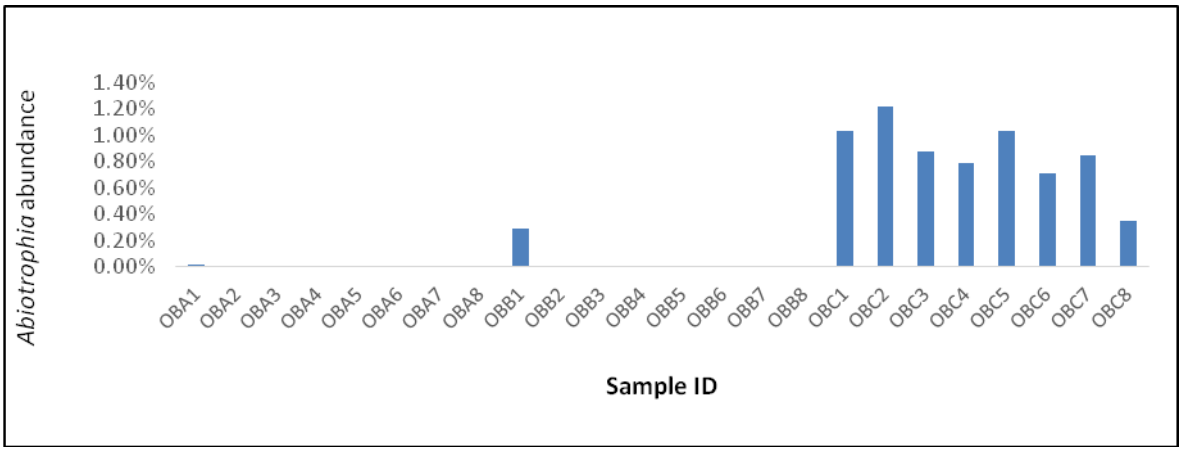


Figure 3.4a: Relative abundance of *Pasteurellaceae, Haemophilus* and *Streptococcaceae* OTUs of the oral communities in the 24 samples analysed in this

study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).





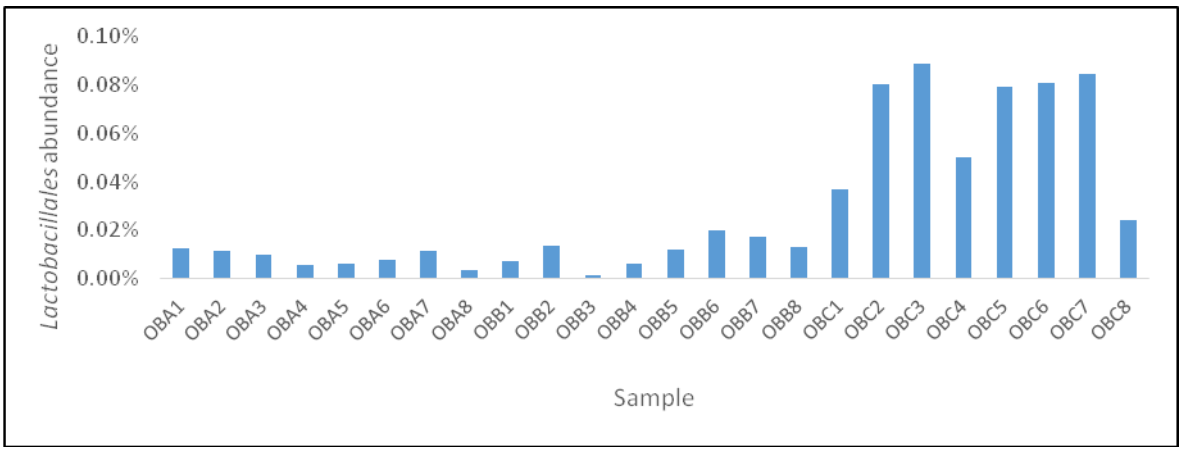


Figure 3.4b: Relative abundance of *Neisseria*, *Abiotrophia* and *Lactobacillales* OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

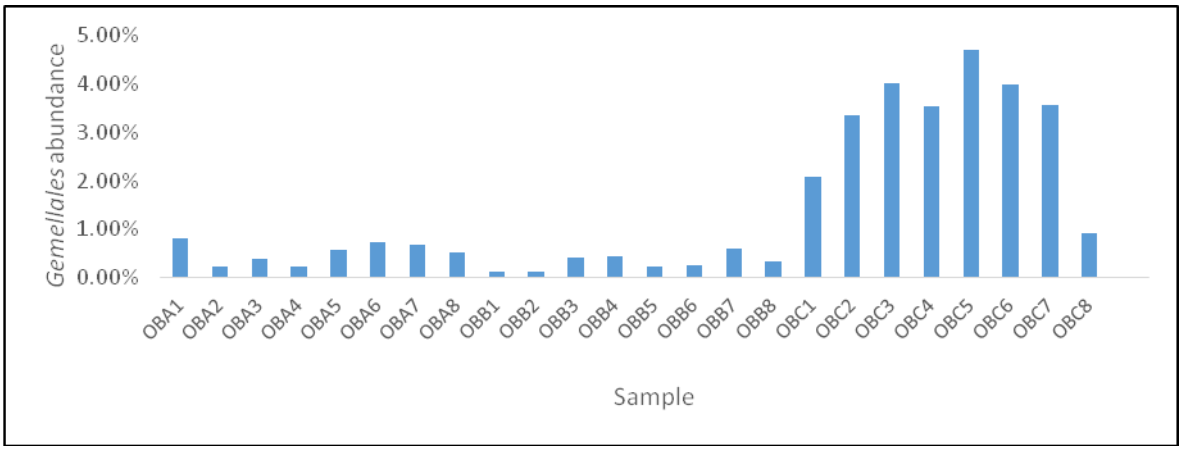
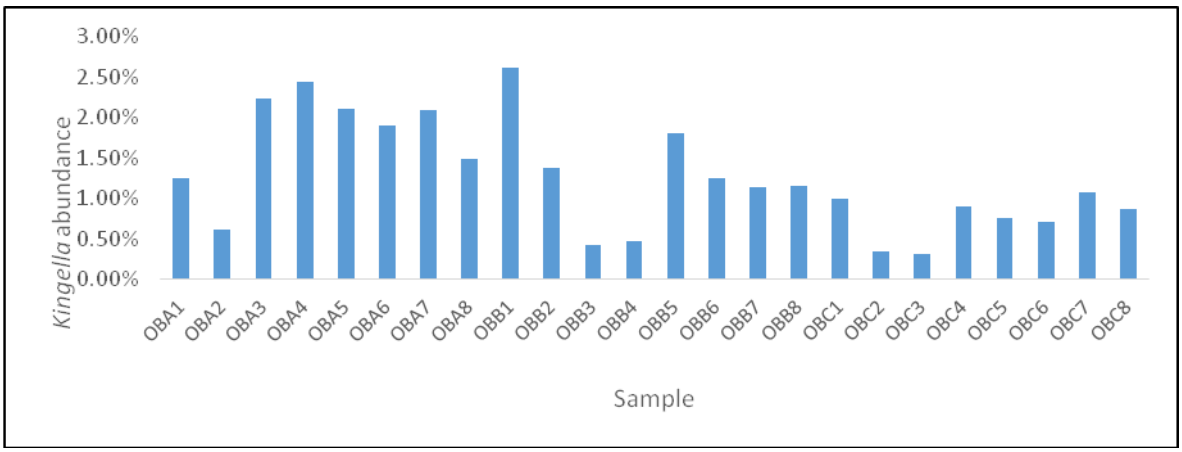


Figure 3.4c: Relative abundance of the *Gemellales* OTU of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

Conversely, OBA and OBB possessed significantly higher relative abundances of several OTUs than OBC, including *Kingella* ($p = 0.008$), *Veillonellaceae* ($p < 0.001$), *Prevotella* ($p = 0.031$), and TM7 ($p < 0.05$) (Figure 3.5a-b).



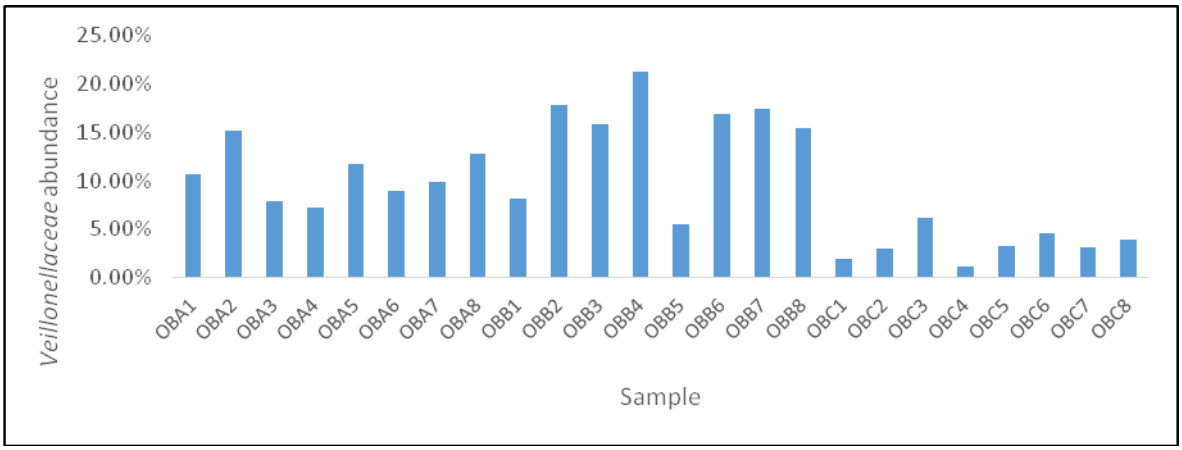
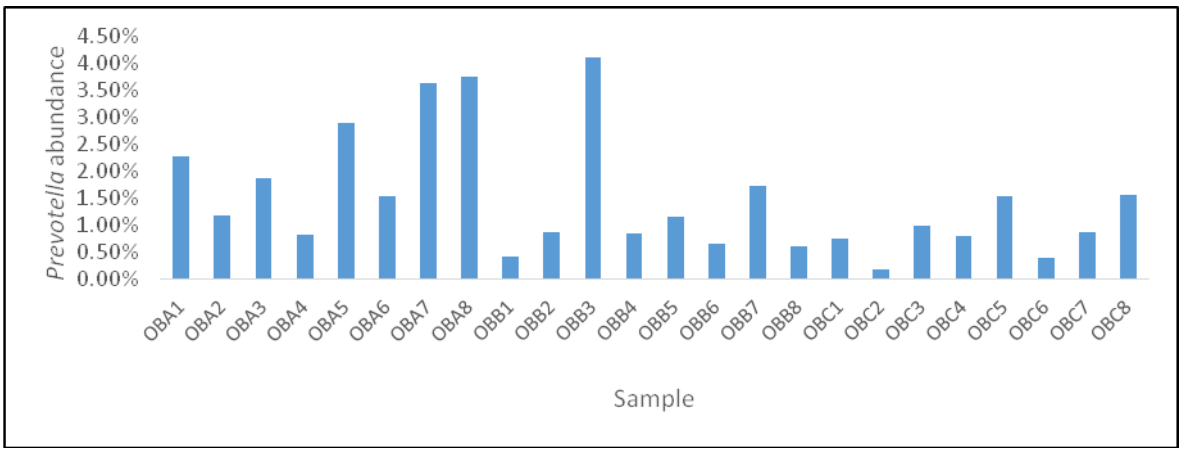


Figure 3.5a: Relative abundance of *Kingella* and *Veillonellaceae* OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).



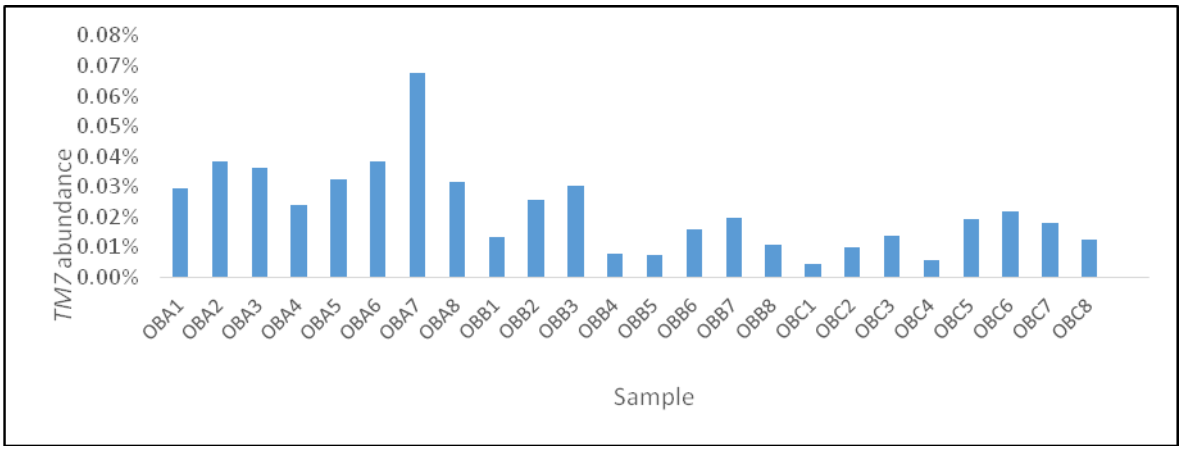
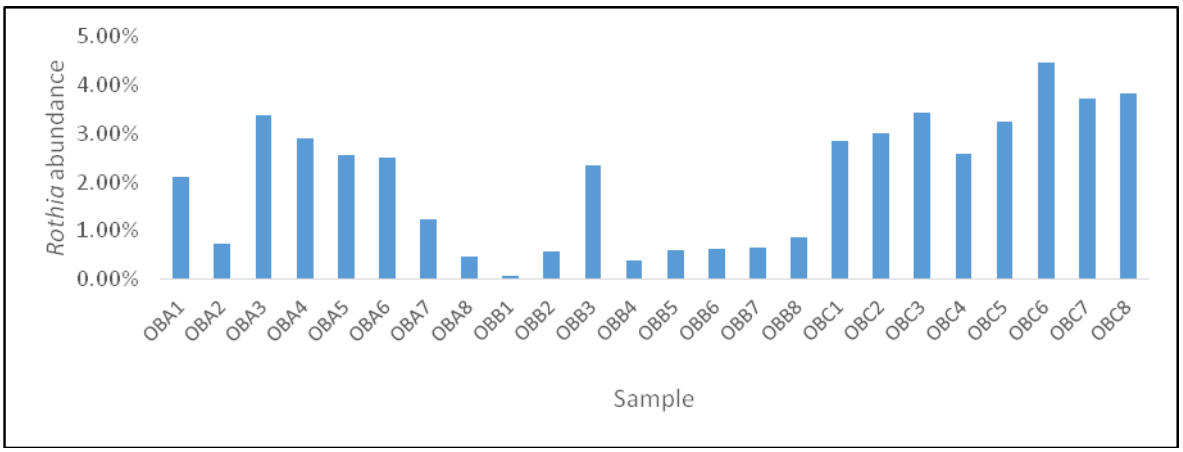


Figure 3.5b: Relative abundance of *Prevotella* and TM7 OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

OBA and OBC possessed a significantly higher relative abundance of *Rothia* than OBB ($p < 0.001$) and OBB possessed a significantly higher relative abundance of *Selenomonas* than OBA and OBC (Figure 3.6).



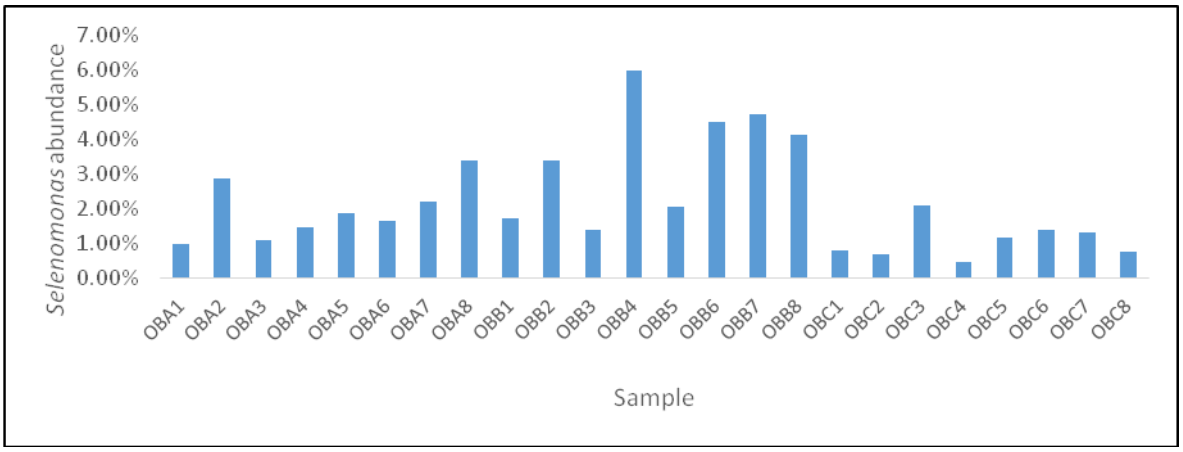


Figure 3.6: Relative abundance of different OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

OBA possessed a significantly higher abundance of *Atopobium* species than OBB and OBC ($p < 0.001$) (Figure 3.7).

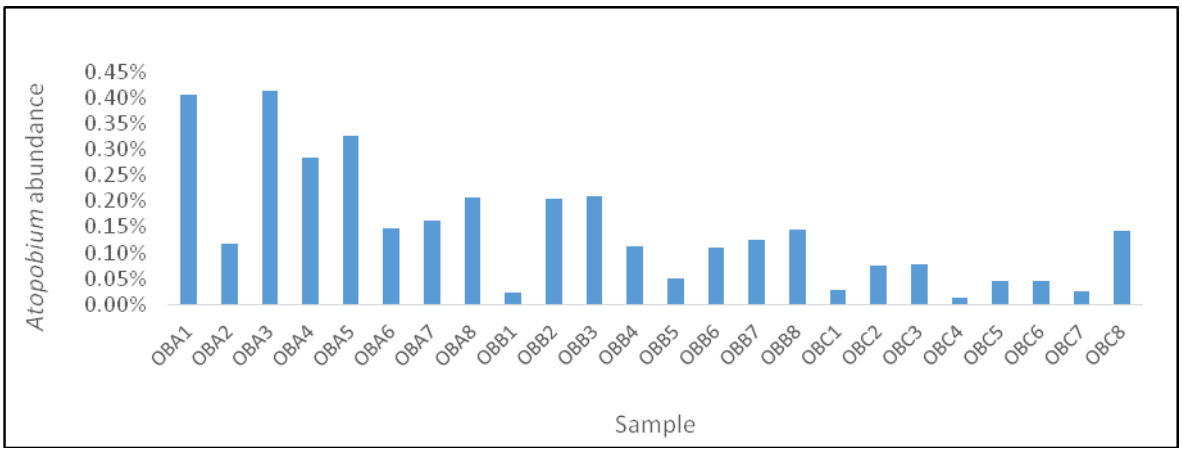


Figure 3.7: Relative abundance of different OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

Temporal variation in oral microbiota

Significant temporal variation was observed in two OTUs in the microbiota of all three subjects, namely *Capnocytophaga* ($p = 0.023$) and *Flavobacteriaceae* ($p = 0.01$). For both OTUs, their relative abundance increased significantly during the course of the study (Figure 3.8).

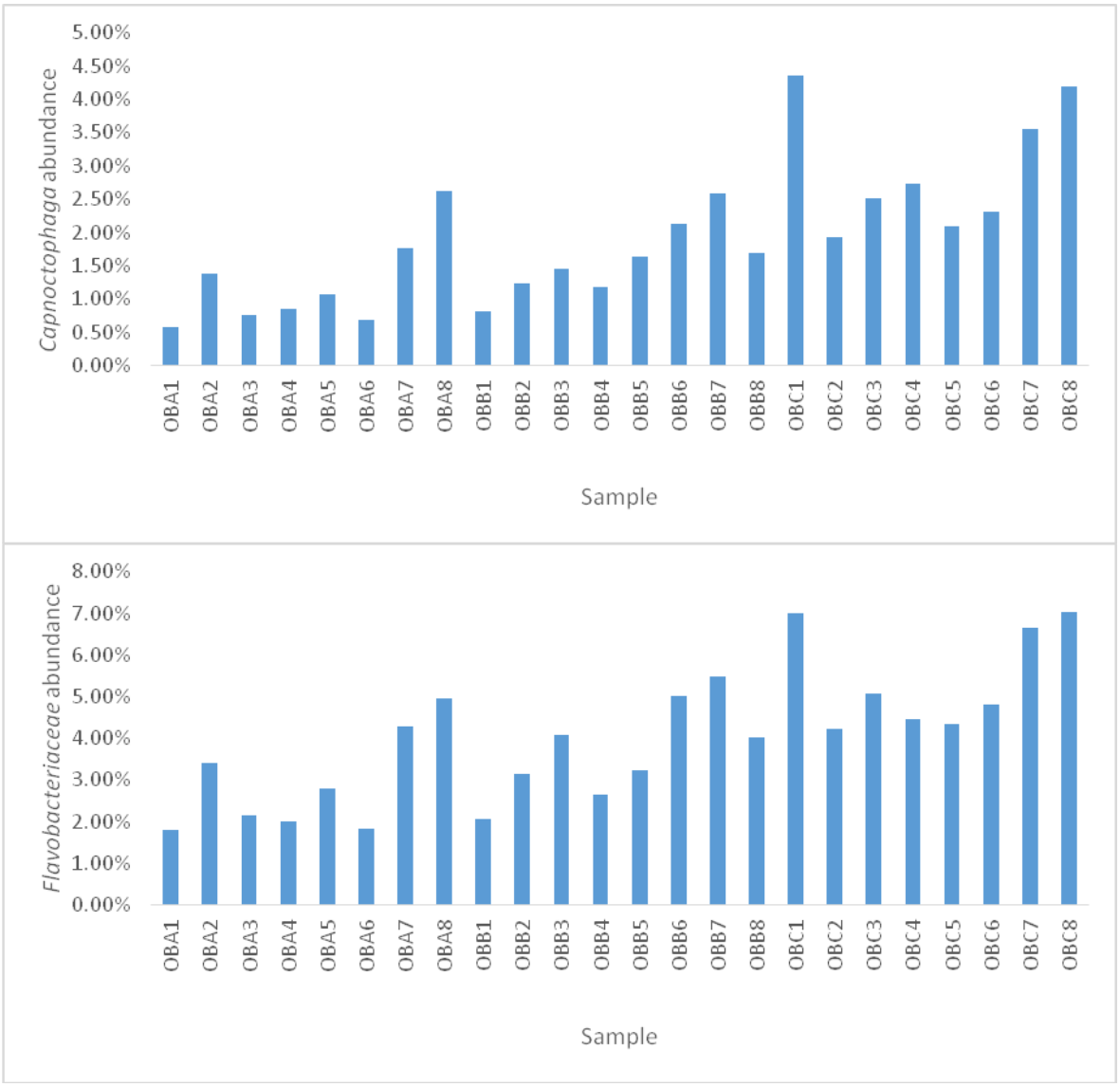


Figure 3.8: Relative abundance of different OTUs of the oral communities in the 24 samples analysed in this study. The three different individuals are identified as OBA, OBB and OBC. The numbers associated with these letter indicate which month of the study the sample was collected (1-8 = May-December 2013).

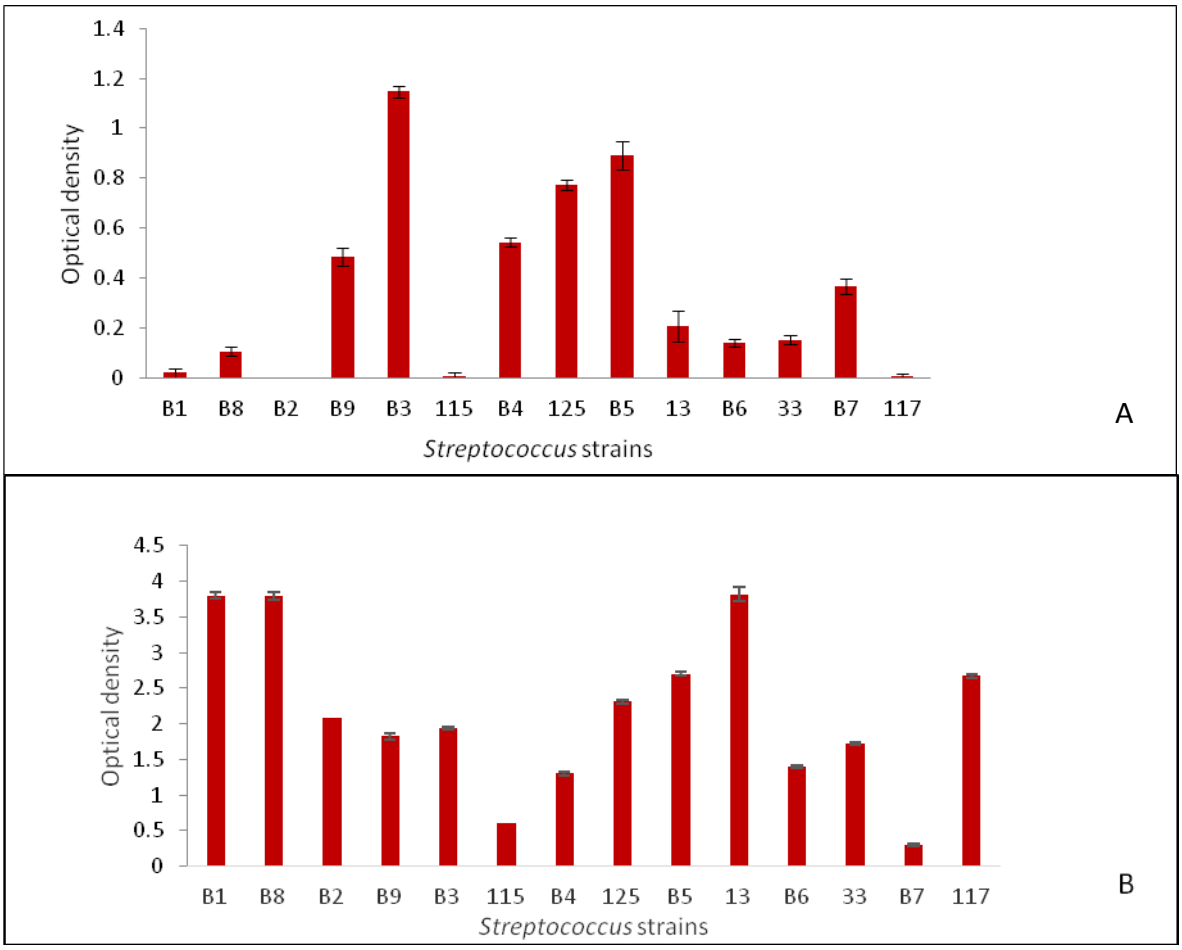
Biofilm formation by oral streptococci

This study used a simple *in vitro* biofilm assay to investigate biofilm formation by different oral streptococci. A total of 14 different oral streptococci strains were used.

In the microtiter plate assay, the ability of each strain to adhere to the bottom of saliva-coated microtitre plate wells was assessed spectrophotometrically following

crystal violet staining. Initial optimisation of this assay involved testing for biofilm formation at numerous different time-points between 1 and 7 days post-inoculation.

Following optimisation, the adherence of each strain at day 1, 4 and 7 post-inoculation was assessed (Figure 3.9). There was marked variation between the adherences of different strains at each of these time points. Furthermore, in general, there was little consistency between the amounts of adherence demonstrated by each strain at different time-points. However, all strains demonstrated most adherence 4 days after inoculation (Figure 3.9).The highest amount of biofilm mass formed by strain (13) at day 4. Whereas strain (B7) failed to establish a biofilms



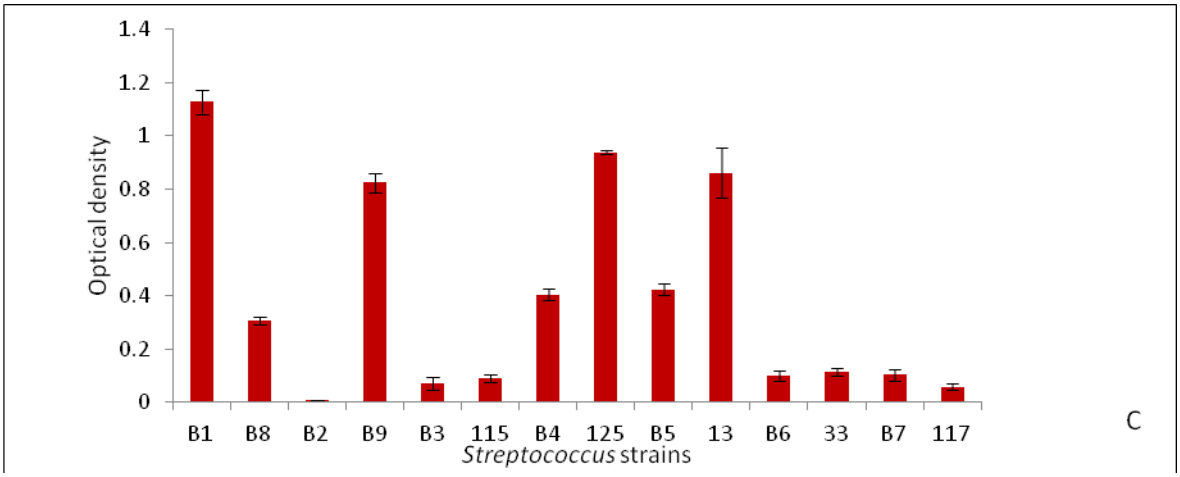


Figure 3.9: Biofilm density at days 1 (A), 4 (B) and 7 (C) after inoculation with the 14 streptococcal strains used in this study. Each isolate was tested 5 times; the Figure shows mean value and standard deviation for each replicate.

Given the nature of the variation described above, it was suspected that the patterns observed may have reflected variation in the growth rate of each strain rather than variation in their adherence. Thus, for all strains, at all time points, the amount of adherence recorded was compared to the number of planktonic (non-adherent) bacteria present. This analysis (Figure 3.10) did suggest a possible correlation between the two variables, but a statistically-significant correlation was not present (ANCOVA, p =0.1).

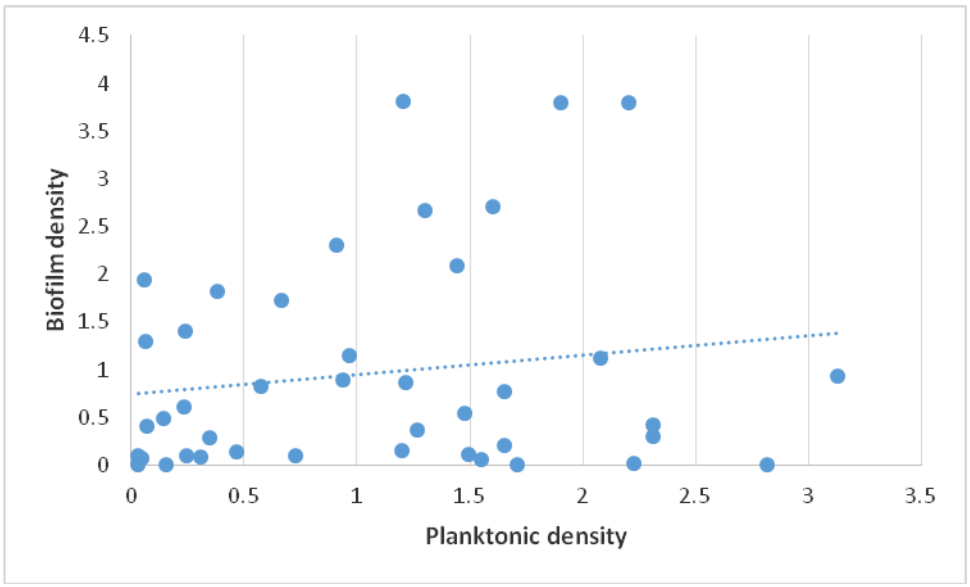


Figure 3.10: Extent of adherence in relation to extent planktonic growth recorded for 14 streptococcal strains at 1, 4 and 7 days post inoculation.

CHAPTER FOUR: DISCUSSION

IDENTIFICATION OF MICROBIAL DIVERSITY IN THE MOUTH BY USING CULTURE-BASED AND CULTURE-INDEPENDENT METHODS

It is now widely acknowledged that the vast majority of microorganisms have yet to be isolated. Furthermore, achieving many of these isolations presents a complex challenge as many as yet uncultured species are particularly fastidious and thus require highly special nutritional and environmental conditions or even co-culture with other cells to provide essential metabolic requirements (Wilson *et al*., 1997). The existence of these as yet uncultured organisms has led to a vast underestimation of the richness of the human oral microbiota, the diversity of which has only become apparent with the advent of molecular techniques (Aas *et al*., 2005 Paster *et al*., 2006). This dissertation aimed to use polyphasic (culture dependent and culture independent approaches) to analyse the diversity of bacterial flora of the human oral cavity, to track person to person and temporal changes in this diversity.

A culture-based appraoch

The range of colony morphologies obtained in this study provided a simple but nonetheless immediate indication that the oral microbiome includes a good diversity of cultivatable micro-organisms. The use of selective media and different culture conditions helped to increase the diversity of colonial morphologies obtained. However, compared to the culture-independent approach, this diversity was clearly limited and a huge underestimation. Nonetheless, these efforts did result in the acquisition of wild-type, low-passage isolates useful for the experimental studies which formed part of this dissertation.

The isolates obtained oral microbiota were characterised on the basis of colonial morphology, microscopic appearance of Gram-stained smears, and comparative analysis of partial 16S rDNA sequences obtained using Sanger sequencing of PCR products. In general, the results of these three approaches concurred, although A28 Gram negative cocci did not match with the result from16S rDNA sequence data which

is *Streptococcus salivaris*.This may be due to contamination of the sample during isolation.

The identities of the organisms isolated in this study were generally in line with what has been encountered in previous studies. Three genera were encountered most frequently (3 of 15 sequences each). Comparison of these data with the phenotypic data for the isolates for which I did not obtain unambiguous 16S rDNA data suggests that *Streptococcus* species were the most common taxon isolated (3 confirmed and 4 putative isolates among the 29 isolates obtained). *Streptococcus* species have been commonly identified from healthy subjects (Paster *et al*., 2006). *S. mutans,* which is often referred to as an oral "pathogen" has also been commonly found as part of the normal flora (Aas *et al*., 2005), although it is also the major cause of dental caries worldwide and is considered to be the most important cariogenic species of all of the oral streptococci (Ajdic *et al*., 2002). The relative abundance of *S. mutans* in oral biofilms is thought to be a crucial determinant of caries (Marsh, 2003), with an increase in the relative abundance of *S. mutans* considered as a pre-requisite for the formation of caries (Marsh, 2003). Other *Streptococcus* species, including *S. gordonii* and *S. salivarius* are also associated with dental plaque formation (Loo *et al*., 2000) but are thought to occupy different niches in the mouth, with *S. gordonii* preferentially colonizing the teeth and *S. salivarius* being found mostly on the dorsal surface of the tongue (Dewhirst *et al*., 2010, Aas *et al*., 2005). The sampling protocol used in my study involved swabbing both these areas along with other surfaces inside the mouth, so it is not surprising that both species were encountered. However, as so few isolates were characterised, I had no means of accurately determining the relative abundance of either of these, or any other species in the microbiota.

The present study also identified *Neisseria* species as being amongst the most abundant taxa present. Neisseria species have been frequently isolated from the oral microbiota previously (e.g. Aas *et al*., 2005). They are not specifically associated with subgingival plaque but are present in most other sites within the mouth, including hard and soft tissues (Aas *et al*., 2005). *Neisseria oralis* is particularly abundant, being frequently detected in the oral microbiomes of healthy individuals (Wolfgang *et al*., 2013).The third most abundant genus was *Bacillus* (3 confirmed and 1 putative

isolate). This finding was perhaps the most unexpected, particularly as two isolates where assigned to *B. licheniformis*, a soil-dwelling species associated with infection of bird feathers (Williams *et al*., 1990) rather than the human oral microbiome. However, this result must be treated with care as the sharing of an indistinguishable partial 16S rDNA sequence is not definitive confirmation that the isolate obtained was a strain of *B. licheniformis*. Dewhirst *et al*., (2010), on the basis of partial 16S rDNA sequences (c500 bp), characterised numerous members of the genus *Bacillus* in the mouth including *Bacillus anthracis*, *Bacillus subtilis*, *Bacillus fusiformis* and *Bacillus clausii* (Dewhirst *et al*., 2010). This study used a shorter length of 16S rDNA data so may have failed to differentiate *Bacillus* species as sensitively as Dewhirst and colleagues did.

The remaining isolates characterised were all well-established members of the human oral microbiome. *Rothia* species are generally considered as commensals that preferentially colonize the teeth and have also been identified in dental plaque samples (Paster *et al*., 2001). *R. dentiocariosa* has been detected in the subgingival microbiota of patients with aggressive periodontitis after treatment (Laksmana *et al*., 2012). *R. dentocariosa* was described in studies by both Aas and colleagues (2005) and Colombo and colleagues (2009) as being more predominant in healthy people than those with oral disease, whereas Paster and colleagues (2001) encountered the species more commonly in diseased individuals then in healthy people. *Granulicatella* species are considered part of the normal microbiota of the oral cavity and also are found in the urogenital and intestinal tracts (Collins and Lawson, 2000; Asa *et al*., 2005). Other studies have implicated them as pathogens (Colombo *et al*., 2009. *G. adiacens* has been frequently detected in samples from tongue dorsum scrapings of both healthy subjects without halitosis and subjects with halitosis (Kazor *et al*., 2003; Aas *et al*., 2005) and in the soft palate (Aas *et al*., 2005, Siqueira *et al*., 2006). *Haemophilus parainfuenzae* is well-recognised as a commensal of the human respiratory tract, rather than the oral microbiota, but as the two environments overlap, it is not too surprising that it was recovered in this study. Other studies focused on the oral microbiome have also encountered *H. parainfluenzae* (Paster *et al*.,2001). The final isolate characterised by 16S rDNA sequence comparison was identified as belonging to one of the genera *Microbacter, Leifsonia* or *Actinobacterium*. The ubiquity of members

of these genera in the human mouth microbiota has been previously noted (Hung *et al*., 2011).

Culture-independent methods: overall biodiversity

There have been many studies which have analysed the diversity of microbes within the oral cavity. These studies have mainly focused on sampling the periodontal pockets of the oral cavity (Haffajee *et al*., 1998; Sakamoto *et al*., 2005; Paster *et al*., 2006), however others have sampled more widely, for example Aas and colleagues (2005) explored nine different oral sites including the dorsum and lateral sides of the tongue, the buccal fold, the hard and soft palate, the labial gingiva and tonsils (of the soft tissue surfaces). Both supragingival and subgingival plaque from tooth surfaces was also sampled to discover the similarities between them. The sampling strategy in the present study was akin to that used by Aas and colleagues and thus it seems reasonable to compare their findings with those of this study. However, combination of a wider range of studies is also sensible to provide a balanced context for my work. Thus, overall, the results in the present study appear to be consistent with the work of others, who found the genera with the largest representation in healthy oral cavities including: *Streptococcus, Veillonella, Granulicatella, Gamella, Actinomyces, Corynebacterium, Rothia, Fusobacterium, Porphyromonas, Prevotella, Capnocytophaga, Nisseria, Haemophilis, Treponema, Lactobacterium, Eikenella, Leptotrichia, Peptostreptococcus, Staphylococcus*, *Eubacteria* and *Propionibacterium* (Aas *et al*., 2005; Jenkinson and Lamont, 2005; Wilson, 2005; Zaura *et al*., 2009; Bik *et al*., 2010).

Overall, the Miseq sequence data suggested that the microbiota in all samples was dominated by members of two phyla, the Proteobacteria and the Firmicutes. Together these two phyla accounted for between about 35% and 70% of the total abundance of bacteria in all samples tested. This finding is in agreement with Wang and colleagues (2013) who reported that Firmicutes (14.8–58.3%) and Proteobacteria (9.2–46.5%) were the most abundant phyla in swabs of periodontally-healthy individuals.

The results of this study were also in line with the findings of other previous studies in which the oral microbiome of healthy adult individuals were surveyed (Munson *et al*.,

2002; Aas *et al*., 2005; Preza *et al*., 2008; Keijser *et al*., 2008). The consensus from these was that Firmicutes (genera *Streptococcus* and *Veillonella*) and Bacteroidetes (genus *Prevotella*) were the predominant phyla in saliva, while Firmicutes and Actinobacteria (genera *Corynebacterium* and *Actinomyces*) dominated supragingival areas. Our results also compare well with a study by Liu and colleagues (2012) in which the taxonomic profiling of the samples, whether derived from targeted 16S rRNA sequencing or from whole-metagenomic data revealed a community dominated by the bacterial phyla Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria and Proteobacteria. These data are also consistent with other studies too (Bik *et al*., 2010; Ahn *et al*., 2011).

One possible discrepancy observed was the relative abundance of *Atopobium* species in one of the subjects of this study (OBA), who was pregnant, was greater than that in the non-pregnant female (OBC). In a previous study exploring the vaginal microbiome (Romero *et al*., 2014) it was found that the abundance of the OTU CST IV-B, which is most often dominated by *Atopobium*, was significantly lower in pregnant women compared to non-pregnant women. However, the present survey included only one pregnant and one non-pregnant woman, whereas that by Romero and colleagues included over 50 subjects and the sites surveyed in each study were different.

Culture-independent methods: person-to-person and temporal trends

Although some components of the microbiota of the human body are shared by entire populations and perhaps the entire species, significant differences between close relatives, such as members of the same family have been recorded. Although this variation has been described in many studies focused on different microbiomes of the body such as those colonising the intestinal tract, the skin and the female reproductive tract (Eckburg *et al*., 2005; Dethlefsen *et al*., 2006; Ley *et al*., 2006; Palmer *et al*., 2007; Grice *et al*., 2009; Hamady and Knight, 2009; Chaban *et al*., 2014), very little is known about person-to-person differences in the oral microbiome. Furthermore, to date, most of the NGS-based surveys of the human oral microbiota have focused on single time points, primarily as they are focused comparison of healthy versus diseased states rather than temporal variation (Bik, 2010). There are very few longitudinal studies to

assess the dynamics of these microbial communities in multiple subjects over a long period of time. One of the aims of this study was to explore both person-to-person and temporal variation in microbiome composition using pilot data derived from three subjects surveyed once a month for eight months. This generated 24 samples for analysis to determine whether distinct patterns in oral microbiome dynamics could be detected.

The combination of the use of NGS has impacted on our present view of oral microbiology and a much greater diversity of species than was previously thought has now been uncovered. One of the reasons for this is the capacity of the technology to analyse a large number of samples in great detail, thereby permitting clearer pictures of the core human oral microbiome (James *et al*., 2014).

The result from the present study showed that the individual of Caucasian descent possessed a significantly higher abundance of 10 OTUs than the two individuals of Arabic descent. In contrast, those of Arabic descent possessed significantly higher relative abundances of four other OTUs than the individual of Caucasian descent. This finding suggests that ethnicity may be an important determinat of the oral microbiome. Such a suggestion is in line with previous studies. Mason and colleagues (2014) demonstrated that ethnicity exerts an affect on the oral microbiome, and this important affect is genetic rather than environmental, since the two ethnicities surveyed (Caucasians and African Americans) despite sharing a common food, nutritional and lifestyle heritage, possessed significant differences in their microbiomes (Mason *et al*., 2014). However, perhaps of particular significance in my small-scale study, Flores *et al*. (2014) encountered a high-degree of inter-individual variability in the microbiome of the human gut, tongue, forehead, and palm. As a result, they suggested a 'personal microbiome' concept (Flores *et al*., 2014). More researchs need in this area, perhaps focused on Arabic communities, as there are currently no reports of studies in this area.

My study also revealed significant temporal variation in the microbiomes of all three individuals included in the study. For example, the relative abundance of

Capnocytophaga and *Flavobacteriaceae* OTUs increased significantly during the course of the study in all subjects. This finding of temporal variation is consistent with other research. For example, Flores and colleagues (2014) reported high levels of temporal variability in both diversity and community structure in microbiomes at four different sites in the body. These findings suggest that temporal dynamics may need to be considered when attempting to link changes in microbiome structure to changes in health status. Furthermore, these findings showed that, not only is the composition of an individual's microbiome highly personalized, but their degree of temporal variability is also a personalized feature.

Although drawing meaningful conclusions from the results of this pilot study would be somewhat foolhardy, it should serve to give impetus for further wider studies.

Use of an *in vitro* model system to analyse variation in biofilm forming capacity of members of the oral microbiome

This study set out with the aim of establishing an *in vitro* setup for mono-species cultures to analyse variation in the biofilm forming capacity of members of the oral microbiome using both well characterised strains and recently isolated strains from healthy individuals. The saliva-coated plastic surfaces of the used 96-well plates clearly did not support some of *Streptococcus* strains. Prior studies demonstrated a dependence of *S. mitis* biofilm formation on the presence of acquired pellicle and lectins (Oliveira *et al*., 2007). Similarly, the *S. oralis* strain C104 formed only low biofilm density, suggesting that this species lacks effective colonization factors for binding to abiotic surfaces but can participate in complex biofilms by binding to more successful earlier colonizers (Loo *et al*., 2000). However, I sought the best suited medium for biofilm formation of the chosen bacterial species, biofilm Medium (BM). *S. oralis* C104 grew very well in BM but could not form biofilm on abiotic surfaces. These results show a wide variation in the abilities of oral streptococci grown in BM to form biofilms on polystyrene. These variations in the ability to form biofilms were also observed by others (Loo *et al*., 2000).

Interspecies variation was seen in the ability of oral streptococci to form biofilm on polystyrene surfaces that may reflect differences in the mechanisms of colonization by

different streptococcal species. Many human viridans streptococci participate in intrageneric coaggregation, the cell-to-cell adherence among genetically distinct streptococci, and these interactions may foster the initial colonization of the tooth surfaces (Kolenbrander *et al*., 1990). This finding could explain why some strains have poor biofilm formation in this experiment, such as *Streptococcus parasanguis* FW213 (B1) and *Streptococcus intermedias* ATCC 27335 (B8). However, these two strains show the same result as Loo and colleagues (2000) who claimed that OD values below 1.0 were categorized into low or non-biofilm producers). In addition *S. oralis* NCTC11427 (B3) showed moderate biofilm growth again similar to Loo *et al*. (2000) who considered that O.D values between 1.0- 2.0 were assessed as moderate biofilm producers.

The most important advantage of using the microtitre plate assay is that it is easy and inexpensive. Numerous other studies used the same model (Loo *et al*., 2000; Lemos *et al*., 2010; Standar *et al*., 2010). Microtiter plates made of polystyrene provide an appropriate and sterile abiotic surface for observing bacterial biofilm formation. Microtitre multi-well plates were identified for use in biofilm systems because they facilitate the use of a high number of replicates. One of the limitations of this experiment was the use of one wall as planktonic growth instead of five replicates as used for the biofilm formation walls. Due to this limitation it is impossible to compare and prove that a strain had poor biofilm formation because it had poor planktonic growth.

For future studies there should be more focus on choosing strains that form good biofilms. There should be the same number of replicates for planktonic and biofilm walls and also improve the biofilm formation on good microtitre surfaces by using low nutrient concentrations, and hydroxyapatite (the main component of tooth enamel) as a removable substratum. Surfaces should be pre-treated with processed saliva which mimics the salivary pellicle, thus giving the correct receptors for the adhesion of pioneer species, as has been described previously (Standar *et al*., 2010). Alternatively, a mixed culture model which has a well-like format permits the simultaneous screening of the combinations of many species for cooperative interactions, and shows

the effect of changes in media composition and the comparison of different strains (Tribble *et al*., 2013).

It would be interesting to revisit this oral diversity analysis again as a separate study, in order to analyse many more samples and obtain a data set that is more statistically sound, as well as gaining more of an insight into the stability of the oral microflora and observe temporal changes. It would also be interesting to investigate whether, given further time and attention to the diversity analyses, an increased number of genera would be identified, or whether the same number and types of genera would be identified in higher amounts. Given the small scale of the diversity study carried out in this work, it is reasonable to presume that a larger diversity study would reveal an increased number of genera, although it must be presumed that the genera already identified would also be seen in future analyses. One option for a more in-depth study would be to work with clinical samples from patients with caries or periodontal disease attending Tripoli dental hospital in Libya, and compare these data with UK patients as few studies had been done exploring inter-racial variation, particularly involving people of Arabic descent.

In summary, this study has been set out to establish an *in vitro* setup for mono-species cultures to analyse variation in the biofilm forming capacity of members of the oral microbiome using both well characterised strains and recently isolated strains from healthy individuals.Further work is required to optimise this in vitro assay and use it to study mixed-species cultures to determine the biofilm behaviour of oral bacteria.

CHAPTER FIVE: REFERENCES

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