Selection, interaction and adaptation in the oral microbiota

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy
in the Faculty of Medical and Human Sciences

2011

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List of Abbreviations

Ammonium persulphate (APS)
Autoinducer 2 (AI-2)
Base pairs (bp)
Colony forming units (cfu)
Confocal microscopy (CM)
Constant-depth film fermenter (CDFF)
Cytophaga-Flavobacterium-Bacteroides (CFB)
Days (d)
Denaturing gradient gel electrophoresis (DGGE)
Ethylenediaminetetraacetic acid (EDTA)
European Molecular Biology Laboratories (EMBL)
Extracellular polysaccharide (EPS)
Fluorescent in situ hybridization (FISH)
Gingival crevicular fluid (GCF)
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Hours (h)
Immunoglobulin (Ig)
Inflammatory bowel diseases (IBD)
Interleukin-8 (IL-8)
Intraepithelial lymphocytes (IEL)
Lipopolysaccharides (LPS)
Major outer sheath protein (MSP)
Metalloproteinases (MMPs)
Minimum inhibitory concentration (MIC)
Minutes (min)
Nicotinamide adenine dinucleotide (NAD+)
Optical density (OD)
Oscillations per minute (opm)
Pathogen-associated molecular patterns (PAMPs)
Phosphoenolpyruvate:sugar phosphotransferase system (PTS)
Polymerase chain reaction (PCR)
Polytetrafluoroethylene (PTFE)
Polyvinylmethyl ether maleic anhydride (PVM-MA)
Principal component analysis (PCA)
Revolutions per minute (rpm)
Seconds (s)
Short chain fatty acids (SCFA)
Sodium lauryl sulphate (SLS)
Temperature gradient gel electrophoresis (TGGE)
Tetramethylethylenediamine (TEMED)
Tissue inhibitors of metalloproteinases (TIMPs)
Toll-like receptors (TLRs)
Tris-acetate-ethylenediaminetetraacetic acid (TAE)
Tris(hydroxymethyl)aminomethane (TRIZMA base)
Trypticase-yeast extract-citrate-sulphite (TYCS)
Tryptone Soya Broth (TSB)
Unweighted pair group method with arithmetic mean (UPGMA)
Abstract

The mechanisms that underlie the stability and inter-individual variation of oral microbial communities are currently not well understood. The research described in this thesis represents a series of investigations with the unifying aim to better understand the processes which influence the structure, microbiological composition and temporal compositional stability of the oral microbiota. Studies of intra and inter-individual interactions between oral isolates and whole microcosms described in this thesis (Chapters 5 and 6) investigated these processes and additionally provide information which will contribute to the development of more realistic in vitro models. Bacteria were isolated from the saliva of four individuals and a modified cross-streak method used to determine positive, negative or neutral interactions among strains in all possible pair-wise combinations. Sorbarod microcosms were established with saliva from the same four volunteers which were then profiled using differential bacterial counting and PCR-DGGE to determine community relatedness, diversity and stability. The incidence of negative interactions was significantly higher than were positive interactions for both self/self and self/non-self pairings. Combining saliva in the Sorbarods resulted in unique oral microcosms, distinct from the progenitor populations, which exhibited reduced stability and decreased species diversity. Therefore oral bacteria appear to have developed mechanisms to compete against those organisms from which they derive no benefit and cooperate with a limited number of species. Compositional stability and inter-individual variation may result from stochastic events during consortial development together with various host-derived and environmental variables. Using the pooled saliva of two or more individuals as inocula for microcosm experiments may therefore result in unrepresentative modelling. Chapter 3 compares effects of exposure to a triclosan-based dentrifice (TD) and a stannous fluoride-based dentrifice (SZD) on bacterial growth, acid production and bacterial composition in salivary-derived biofilm communities. A preliminary screening method was used to assess the effects on bacterial viability and acidogenesis whilst PCR-DGGE was utilised to determine the effects on bacterial composition of the salivary consortia. Exposure to either dentrifice for 16h caused reductions in microbial diversity and resulted in the emergence of distinct community profiles, which were most marked for TD. TD more potently inhibited bacterial growth and acid production than did SZD. In Chapter 4 the frequency and strength of coaggregation between and among human oral and intestinal bacteria was assessed. Ten oral and ten enteric species were tested using a spectrophotometric coaggregation assay in all possible pair-wise combinations. Oral strains were the most polygamous coaggregators whilst strong interactions between oral and gut strains were considerably less common. Coaggregation scores were also low between members of the intestinal microbiota which indicates that the processes of coaggregation and coadhesion are not as important in the formation of intestinal biofilms as they are in the oral cavity. Heating and sugar inhibitor experiments indicated that, similar to oral microorganisms, interactions with intestinal and oral strains were lectin–carbohydrate based.
Declaration

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Acknowledgements

The completion of this thesis would not have been possible without the continued support of my supervisor Dr. Andrew McBain, who I would like to thank for all his encouragement, guidance and patience over the past few years. I am extremely grateful for the support I received after the birth of my son and extend my thanks to both Dr. McBain and Dr. David Berk whose help and advice enabled me to complete my studies.

I would like to express my gratitude to members, both past and present, of the microbiological research group in the School of Pharmacy and Pharmaceutical Sciences. Thank you to everyone for providing a source of knowledge, support and advice. Additional thanks to the late Professor Peter Gilbert who played an important role in the early stages of my studies.

I cannot thank my mum and dad enough for everything they have done for me. Without their help and support I would not have reached this point and I am enormously grateful to them.

A special thank you goes to my lovely husband Alex Glancey who has provided endless support throughout this process. Finally, a big thank you to our son Samuel to whom I dedicate this thesis.
Chapter 1

General Introduction
1.1. Introduction to prokaryote-eukaryote relationships

From the time of birth the human body plays host to a vast and complex community of microorganisms. Epithelial surfaces exposed to the external environment rapidly become heavily colonised by prokaryotic cells that eventually far outnumber our own mammalian eukaryotic cells (Backhed et al., 2005). Traditionally these microscopic companions have been associated mainly with the onset of disease and decay. However, in recent years the complex, and often mutually beneficial, relationship that exists between man and microbe has come to the forefront of public and scientific interest.

Whilst bacteria are known to have inhabited the earth for more than 2.5 billion years (Hooper & Gordon, 2001) and the very first prokaryotic life initiated over a billion years prior to that (Gould et al., 2008); fossil records indicate the emergence of eukaryotic cells approximately 760 million years ago (Cavalier-Smith, 2006). Consequently, the modern human and his evolutionary predecessors have experienced an intimate association with microorganisms that has shaped their coevolution over many millions of years (Hooper, 2004). Endosymbiotic theory proposes a union, initiated by the invasion of simple pre-eukaryotic cells by prokaryotic symbionts, which gave rise to contemporary organelles including mitochondria and plastids (Dyall et al., 2004; Margulis, 1975). Genomic and proteomic analyses support this vision of a symbiotic relationship which fundamentally impacted the metabolic and homeostatic mechanisms of the early eukaryote (Dyall et al., 2004). Defining the extent to which this, and countless other subsequent interactions, have sculpted the human genome has been hampered by the overwhelming complexity of
mammalian biology and microbial ecology (McFall-Ngai, 2002). Nevertheless, as a result of recent advances in biotechnology and bioinformatics, research in this field is flourishing. The scope of study is large and diverse; elucidating the microbial influence not only on eukaryotic evolution, but basic human physiology as we know it today.

1.1.1. Microbial ecology of the human body

Every human body is teeming with microorganisms. In fact, estimates suggest that the microbial cells of the average human microbiota outnumber human cells ten to one (Backhed et al., 2005; Hamady & Knight, 2009). The collective genome of this eclectic consortium of has been termed the microbiome (Hooper & Gordon, 2001; Lederberg, 2001). Anatomical variations between body sites result in each being colonised by a unique assortment of bacteria, fungi, protozoa, archaea and viruses (Wilson, 2005). Current research suggests that the bacterial component of the human microbiota is numerically dominant (Tlaskalová-Hogenová et al., 2004).

Mucosal surfaces, including the oral cavity, upper respiratory tract, stomach, small intestine, colon and genito-urinary system, represent the primary sites for microbial colonisation (McFarland, 2000). Important exceptions to this include: the largest organ of the human body, the skin, and the surface of the teeth, which also have extensive microbial populations (Elsner, 2006). Qualitative and quantitative differences between these microbiotas have been attributed a number of factors including: host immune response, nutrient availability, pH, oxygen potential, temperature, moisture and perhaps most importantly the availability of suitable points of attachment (McFarland, 2000). Heterogeneity
within a given anatomical site contributes to the complexity of the microbiome, the oral cavity for example, has several distinct surfaces available for colonisation and significant intra-surface variations driven by host and environmental factors (Mager et al., 2003).

1.1.2. Beneficial functions of the human microbiota

Since the work of Louis Pasteur confirmed the Germ Theory of disease over a century ago the part played by bacteria in the development of human disease has been well documented (Hooper et al., 2002). Amongst the general public the term “bacteria” frequently evokes unpleasant images of infection and disease. However, host-associated microbial communities are much more frequently involved in the maintenance of health and human physiological development (Robinson et al., 2010).

Colonisation resistance; the exclusion of allochthonous or “foreign” species by the autochthonous microbiota of a specific ecological niche, can prevent the establishment of potentially pathogenic organisms (McFarland, 2000; Stecher & Hardt, 2008). For example, high proportions of hydrogen peroxide producing lactobacilli in the vaginal microbiota have been associated with protection against the acquisition of bacterial vaginosis, as described by Hawes et al. (1996) in a cohort study of vaginal swabs taken from women attending a sexually transmitted disease clinic (Hawes et al., 1996). Similarly, lactic acid production by the normal skin microbiota results in a weakly acidic environment (pH 5 to 6) which has the potential to prevent colonisation by many pathogenic bacteria (Elsner, 2006; Wilson, 2005). Experiments with germ-free mice have shown that these animals are more susceptible to colonisation by pathogens
than those with an intact intestinal microbiota (Hazenberg *et al.*, 1981; Vollaard *et al.*, 1990). Hazenberg *et al* (1981) showed that colonisation of germ-free mice by selected intestinal anaerobes and complete human intestinal microbiotas successfully antagonised *Escherichia coli* and *Pseudomonas aeruginosa* (Hazenberg *et al*., 1981). The mechanisms that underlie colonisation resistance are numerous and will be discussed further in relation to the oral cavity (Section 1.9.); however some important processes include: the production of inhibitory substances e.g. bacteriocins, non-specific antibacterials and acids, inhibition of microbial attachment, and competition for nutrients (McFarland, 2000).

Outside of the intestinal tract, information on additional beneficial effects of the human microbiota is more limited (Bik, 2009). However, the intestinal microbiota has been shown to play a role in nutrient digestion and synthesis, energy and vitamin production, maturation of the epithelium, stimulation of intestinal transit, and immune response (Bik, 2009; McFarland, 2000) (Section 1.2.3.). In addition to those benefits afforded by the commensal microbial population it has been proposed that association with microorganisms, which is likely to include exposure to pathogens, occurring early in life may be beneficial to long term host health. Excessive hygiene measures and a corresponding decrease in exposure to microorganisms apparently increase the risk of developing allergic conditions. Allergy and autoimmune disease result from inappropriate and excessive immune responses to specific stimuli. It is thought that decreased exposure to infection in childhood adversely affects the development of the immune system and as a consequence allergy is more likely. This theory, first postulated by Strachan in 1989 is termed the ‘hygiene hypothesis’ (Macpherson & Harris, 2004).
1.1.3. The role of the normal microbiota in disease

Although most microbe-host interactions can be described as commensal (beneficial for the microbe without causing harm to the host) or mutualistic (beneficial to both microbe and host), under the right conditions; a subtle change in environment or genotype, previously symbiotic microbes can become pathogenic (Bik, 2009; Dethlefsen et al., 2007). Grice et al (2010) used a diabetic mouse model to study changes in the skin microbiota associated with environmental and immune responses in chronic wounds. After wounding the subjects skin a selective shift of Firmicutes species, frequently associated with impaired wound healing, was observed (Grice et al., 2010). In the oral cavity for example, the role of environmental acidification and a population shift towards mutans streptococci and lactobacilli in dental caries is well documented (Takahashi & Nyvad, 2011) (Section 1.11.1.)

Elements of the normally commensal human microbiota have also been implicated in systemic disease. Oral bacteria have been linked to several conditions, including infective endocarditis (Nakano et al., 2010; Seymour et al., 2007). Nakano et al (2010) describe several serotypes of Streptococcus mutans that show low levels of cariogenicity but a high virulence in blood leading to bacteraemias or infective endocarditis (Nakano et al., 2010). Immunocompromised individuals are seemingly more susceptible to the pathogenic effects of normally commensal microbes. For example, oral candidiasis is highly prevalent in HIV patients and attributed to yeasts that are frequently found in the oral microbiota of healthy individuals (Samaranayake, 1992; Yang et al., 2011).
1.2. Methodologies employed in the study of microbial ecology and the human microbiota

1.2.1. Isolation techniques

One of the first microbial biofilm communities to be observed was a human-associated one: the dental plaque community (Robinson et al., 2010). In 1683, Antonie van Leeuwenhoek observed a large number of what he termed “animalcules” in the matter he obtained from scraping his teeth. However it was not until several hundred years later that work by Koch and Pasteur introduced the concept of isolating microorganisms in pure culture. Up until the late 20th century the study of microorganisms in their natural environment was entirely based upon the need to culture the organisms in question. Culture and microscopy comprised the major tools for experimentally defining and characterising the human microbiota. Unfortunately culture of the vast majority of microorganisms is not a simple task, many are particularly fastidious requiring highly specific nutritional and environmental conditions or co-culture with other species to provide essential metabolic bi-products (Wilson et al., 1997b). The existence of these uncultured organisms resulted in a vast underestimation of the richness of the human microbiota prior to the advent of culture-independent techniques (Amann et al., 1995; Muyzer & Smalla, 1998).

1.2.2. Culture-independent techniques

Molecular microbiology has significantly advanced our understanding of the microscopic world by circumventing the need to rely wholly on culture. One of the most significant advancements of the late 20th century was the development of the polymerase chain reaction (PCR) by Kary Mullis (Mullis & Faloona, 1987)
which is fundamental to the study of microbial ecology as we know it today. This technique allows for the exponential amplification of almost any target DNA sequence without prior culture, using DNA polymerases and primers (Spratt, 2004). Once these DNA sequences are obtained nucleotide sequencing can be performed and microbes identified without the need for culture of the organisms in question. The 16S rRNA-encoding genes are the most frequently used to characterise bacterial communities, particularly those associated with the human body. This gene has proved popular for several reasons: it is present in all bacteria, the sequence is sufficiently conserved and is of an appropriate size (ca. 1500 bases) to be readily sequenced yet still large enough to contain sufficient information for identification and phylogenetic analysis (Spratt, 2004). Extensive databases now exist which allow for rapid identification of bacteria based upon 16S rRNA sequences, however these databases have inevitably suffered a certain amount of degradation. Although the use of PCR to amplify DNA represents a useful tool to analyse microbial communities, the technique can also show bias towards the amplification of specific species or groups (Pontes et al., 2007).

Other culture-independent techniques quickly followed the development of the polymerase chain reaction. One such technique, fluorescent in situ hybridisation (FISH), which utilises group-specific 16S rRNA probes was amongst several in situ hybridisation methods devised for phylogenetic analysis (Amann et al., 1990; DeLong et al., 1989; Giovannoni et al., 1988) FISH is a fluorescence microscopy-based technique that relies upon the hybridisation of fluorescently labelled oligodeoxynucleotides to complementary 16S rRNA. Specific probes can be constructed to distinguish between the primary kingdoms or even closely
related organisms in mixed community samples. Probes labelled with different fluorescent dyes can be used simultaneously to identify more than one cell type within the same sample (DeLong et al., 1989). Although FISH and other hybridisation methods such as the checkerboard DNA-DNA technique have proven to be rapid and relatively inexpensive, they suffer significantly with the problem of non-specific probe binding (Socransky & Haffajee, 2005).

Several community fingerprinting techniques that have proven significant in the study of microbial ecology were also developed on the basis of amplification of the 16S rRNA gene. One of the most useful of these techniques is denaturing gradient gel electrophoresis (DGGE). DGGE was first described by Muyzer et al. (1993) as a novel method for analysing the genetic diversity of complex microbial samples. This technique separates DNA fragments of the same length but different nucleotide sequence by electrophoresis of PCR-amplified 16S rDNA fragments in polyacrylamide gels of a set gradient. The polyacrylamide initiates melting of the DNA fragment which retards its movement through the gel. Variations in the G-C content of the sequence lead to differences in melting point, which in turn means individual fragments will migrate to different points on the gel (Muyzer et al., 1993). DGGE has been widely employed in a number of microbial ecological settings including studies of the human oral microbiota (Ledder et al., 2006; Ledder et al., 2007), the human and murine gut microbiota (Walter et al., 2000), drain microcosms (McBain et al., 2003e), freshwater ecosystems (Rickard et al., 2004) and the study of the effects of antimicrobials on drain microcosms (McBain et al., 2004). PCR-DGGE is not a quantitative technique (Ledder et al., 2007) due to the potential for heterogeneity in commonly used genes such as the 16S rRNA (Farrelly et al., 1995; Nubel et al., 2007).
and sequences falling below the threshold for detection (Muyzer & Smalla, 1998), it does however offer one of the only means of producing a reproducible visual assessment of bacterial diversity in a sample. The consortial profiles produced are also highly reproducible.

Quantitative real time PCR is another molecular method commonly used for the detection and enumeration of microbial communities (Pozhitkov et al., 2010). This technique provides a quantitative method to identify bacterial copy numbers within a given sample by using family or genus specific primers and a marker that fluoresces when intercalated into double-stranded DNA. The increase in fluorescence correlates with an increase in product that can be calculated by comparing the result to a standard curve of known copy number (Smith & Osborn, 2009). Real time PCR has been extensively used in microbial ecological studies associated with the human body, in particular the oral cavity (Asai et al., 2002; Boutaga et al., 2007; Kawada et al., 2004; Pozhitkov et al., 2010). However, the technique has one major disadvantage in that it can only be used for targeting known genes and thus is limited in its usefulness in the study of microbial communities with large numbers of unidentified components (Pozhitkov et al., 2010; Smith & Osborn, 2009).

Metagenomics is one of the more recent advances in the field of molecular microbiology. This approach analyses the entire genetic material within an environmental sample, without the need for culture and has revealed the existence of many previously unknown genes from a number of environments (Robinson et al., 2010). One of the first instruments of this type was the 454 pyrosequencer, a “sequence by synthesis” device. Briefly, sample adapter-
ligated DNA is nebulised and fixed to small beads and then amplified by PCR. The bead is placed onto a fiber optic chip and the four DNA nucleotides are added sequentially in a fixed order across the chip during a sequencing run. As the nucleotides are added, millions of copies of DNA bound to each of the beads are sequenced in parallel. Addition of nucleotides generates a chemoluminescent signal that is recorded and identifies the base sequence (Pozhitkov et al., 2011; Robinson et al., 2010). High-throughput sequencing methods such as this have already been employed in discovering the genetic diversity of the human microbiome (Gill et al., 2006; Koren et al., 2011; Lazarevic et al., 2010; Zaura et al., 2009). One of the main drawbacks to new generation sequencing techniques is cost. Recent developments currently being discussed in the literature that may circumvent cost and time constraints include nanopore technology (Pozhitkov et al., 2011; Prasongkit et al., 2011). Nanopore approaches utilise technology that electrophoretically drives a strand of nucleic acid through a nanopore, with each nucleotide base modulating the ionic current as it passes through the nanopore (Pozhitkov et al., 2011).

All analytical techniques have inherent strengths and weaknesses, to which those applied to the study of microbial ecology and the human microbiota, are no exception. The characteristics of the techniques discussed here suggest that the most prudent approach is to incorporate a number of different methods in order to obtain a true reflection of a given microbial community (Pontes et al., 2007).
1.2.3. The NIH Human Microbiome Project

The NIH Human Microbiome Project has been established in order to better understand the microbiome associated with health and disease and bring the results to the attention of the medical community. On completion the Human Microbiome Project will comprise a multi-component community resource illustrating the importance of the microbiome in human health, disease, diagnosis and treatment. The three main goals of the project are: to utilise high-throughput technologies to characterise the microbiome of multiple body sites in 250 healthy volunteers, to study the microbiome in disease states and establish links between changes in the microbiota and disease and to provide a standardised data source that can be used by the wider scientific community (Peterson *et al.*, 2009; Turnbaugh *et al.*, 2007).

1.3. The gut microbiota

1.3.1. Development and composition of the human gut microbiota

Most of the microbes inhabiting the human body are found in the intestine, however prior to birth the human gut is sterile (Hooper *et al.*, 2002). The method of delivery plays an important role in determining the initial composition of the neonatal microbiota. Several culture-based and real time PCR studies have shown that children born by caesarean section have reduced numbers of bifidobacteria, bacteroides and lactobacilli in comparison to vaginally delivered babies (Gronlund *et al.*, 1999; Penders *et al.*, 2006). Rapid changes occur in the first few days of life with the method of nutritional provision; breast or bottle, known to impact on the numbers of key species including bifidobacteria, lactobacilli, *E. coli* and *C. difficile* (Penders *et al.*, 2006). By age two the
intestinal microbiota is believed to resemble that of an adult (Macfarlane & Macfarlane, 2004). Studies utilising molecular fingerprinting techniques, including DGGE, have suggested that although the composition of the gut microbiota is normally fairly stable after weaning, it appears to be unique to each individual and heavily influenced by host genetics, in addition to environmental factors (Zoetendal EG et al., 2001). A genetically unrelated but cohabiting couple may have very different gut microbiotas, whereas identical twins that have lived separately for many years show remarkable similarity in their commensal intestinal population (Zoetendal EG et al., 2001). Within a given microbiota the predominant species do not appear to undergo significant change unless subjected to perturbations in host diet, disease, antibiotic treatment or intentional introduction of allochthonous species (Bornside, 1978; Simon & Gorbach, 1984; Tannock et al., 2000). In fact, post-antibiotic treatment the gut microbiota has been shown to revert to a pre-treatment composition: De La Cochetiere et al (2005) used temperature gradient gel electrophoresis (TGGE) to show that 60 days after a 5 day course of amoxicillin the faecal microbiota of 6 healthy volunteers exhibited an average 89% similarity to the original microbiota (De La Cochetiere et al., 2005).

In addition to harbouring the highest number of microorganisms of all the human-associated communities the large intestinal microbiota exhibits considerable biodiversity. Conservative studies put the number of bacterial species at several hundred (Bourlioux et al., 2003) with more recent molecular and metagenomic studies suggesting between 300 and 400 unique bacterial phylotypes (Eckburg et al., 2005; Gill et al., 2006). One recent study has estimated that the number may be as high as 1000 species (Qin et al., 2010).
The vast majority of these gut microbes are located in the colon where the bacterial population is around $10^{12}$/g of faeces; the strongly acidic conditions and enzymic activity of the stomach resulting in a relatively small population, approximately $10^3$/ml (Farthing, 2004). Propulsive activity in the small intestine reduces the time available for bacterial growth and numbers in this region range from $10^4$/ml to $10^6$/ml (Farthing, 2004; Guarner F, 2003; Hooper et al., 2002; Yan & Brent Polk, 2004).

The difficulties associated with the culture of many fastidious species ex vivo, had resulted in limited knowledge of the intestinal community composition until the advent of molecular techniques (Hooper et al., 2002). Culture-based studies suggested that the predominant genera included Acidaminococcus, Bacteroides, Bifidobacterium, Clostridium, Coprococcus, Enterobacter, Enterococcus Escherichia, Eubacterium, Fusobacterium, Klebsiella, Lactobacillus, Megamonas, Megasphaera, Peptostreptococcus, Proteus, Ruminococcus and Veillonella (Tannock, 1995). It is now known that the intestinal microbiota is dominated by anaerobic bacteria (Tannock, 2001) with recent metagenomic analyses suggesting over 95% of intestinal species are anaerobes (Eckburg et al., 2005; Frank et al., 2007). Qin et al (2010) describe an Illumina-based metagenomic sequencing of faecal samples from 124 individuals wherein 99.1% of the genes were bacterial, representing 1000 to 1150 prevalent species in each individual (Qin et al., 2010). Bacteroidetes and Firmicutes showed the highest abundance in this study (Qin et al., 2010). Several other researchers have suggested that the numerically dominant phylogenetic groups include Bacteroidetes and Clostridium (Suau et al., 1999; Wang et al., 2003). Suau et al (1999) studied the bacterial component of adult
faeces by PCR amplification of 16S ribosomal RNA gene and found that over 95% of the colonic microbiota consists of bacteria from these groups, whilst 76% of the retrieved cloned DNA belonged to uncultured species (Suau et al., 1999).

1.3.2. Structural components of the intestinal microbiota

Although many researchers have studied the individual components of the intestinal microbiota and their influence on host health, little is known about the structure of the microbial community in vivo. Many human-associated bacterial communities, as well as those found in other natural or man-made habitats, are known to exist as biofilm populations (Costerton et al., 1987). Biofilms have been described as “functional consortia of cells bound within exopolymer matrices and organized at interfaces” (Gilbert & McBain, 2001). Microorganisms growing in biofilms are phenotypically different from their nonadherent counterparts and existence in a multi-species biofilm can confer selective advantages over planktonic growth in many environments (Kinder & Holt, 1994). For example, the hydrated extracellular matrix of a biofilm concentrates nutrients and defensive substances as well as offering some physical protection from exogenous compounds including antibiotics (Kinder & Holt, 1994). The close physical interactions between bacteria in a biofilm may facilitate the establishment of symbiotic relationships, cross-feeding (Drago et al., 1997), cell-cell communication (Kolenbrander, 2000), genetic exchange between cells (Foster et al., 2003) and provide protection for anaerobic species against oxygen (Bradshaw et al., 1998).
Although single and multi-species biofilms have been described in the human gastrointestinal tract, particularly the heavily colonised large intestine, research in this area is limited (Macfarlane & Dillon, 2007). The intestinal epithelium experiences a high rate of cell turnover and a constant influx of food and water, yet despite this many species of bacteria are able to become resident there (Sonnenburg et al., 2004). It has been suggested that the viscoelastic mucus layer that covers the intestinal epithelium is largely responsible for the structural stability of the intestinal microbiota (Sonnenburg et al., 2004). Electron microscopy and acridine orange staining were used by Palestrant et al. (2004) to reveal the existence of biofilms in the mucus lining of the normal human gut epithelium (Palestrant et al., 2004). Intestinal mucus contains at least twenty types of mucin, high molecular weight glycoproteins, which provide points of attachment for many bacteria as well as a valuable source of nutrition (Bollinger et al., 2006; Pearson & Brownlee, 2005). The binding of microbes to mucins is believed to be advantageous to the host by preventing colonisation and therefore possible inflammation of the epithelium (McGuckin et al., 2007). However, microcolonies have been identified on the epithelial surface in rectal biopsies by MacFarlane et al. (2004) using FISH (Macfarlane et al., 2004).

Other than the fact that biofilms exist in some parts of the human gut, relatively little is known about their frequency, structure and function. The acquisition of undamaged tissue samples is one important barrier that has limited developments in this field (Macfarlane & Dillon, 2007). Further research utilising enteric isolates, *in vitro* models or molecular analysis of biopsy samples would be beneficial. A better understanding of the processes that underlie biofilm
formation in the gut would provide insight into the healthy intestine, microbially-related diseases and potential treatments.

1.4. The intestinal microbiota and host health

1.4.1. Colonisation resistance

As humans have co-evolved over many millions of years with their microbiota it seems probable that the relationship is beneficial to both parties. One of the ways in which the intestinal microbiota benefits the human host is the process of colonisation resistance. The exclusion of allochthonous or “foreign” species by the intestinal microbiota can prevent the establishment of potentially pathogenic organisms (McFarland, 2000; Stecher & Hardt, 2008). Experimentation with germ-free animals has illustrated the impact the indigenous microbiota has on host physiology and health (Guarner, 2003; Hooper et al., 2002; Yan & Brent Polk, 2004). Mice raised in an environment free of microorganisms have been shown to be more susceptible to colonisation by pathogens than those with an intact intestinal microbiota (Hazenberg et al., 1981; Vollaard et al., 1990).

1.4.2. Importance of the intestinal microbiota in host metabolism

Intestinal bacteria perform a variety of metabolic functions, liberating nutrients from foods that are indigestible to the host. This includes the anaerobic fermentation of non-digestible carbohydrates to short chain fatty acids (SCFA) namely butyrate, propionate and acetate (Chassard et al., 2008; Topping & Clifton, 2001). These molecules are an important source of nutrition to the human host and aid the absorption of essential ions such as calcium, magnesium and iron. Additionally, the intestinal microbiota plays an important
part in the synthesis of Vitamins B$_{12}$ and K which the human host cannot synthesise directly (Hill, 1997; Hooper et al., 2002). The metabolic versatility of the gut microbiota is mutually beneficial to both parties, allowing for significant diet changes without disruption to host nutrition and providing the microbiota with a nutrient-rich environment (Hooper et al., 2002).

1.4.3. Contributions of the intestinal microbiota to gut development

The human gut has a large surface area and specialised epithelial cells that are well adapted to maximise the absorption of nutrients. This efficient absorption system must be matched with a sufficient blood supply, and there is evidence to suggest that elements of the intestinal microbiota stimulate angiogenesis in intestinal villi (Hooper, 2004). Work with germ-free mice described by Stappenbeck et al (2002) showed that these animals had underdeveloped villus capillaries. The authors used confocal microscopy (CM) to compare the intestinal villi of germ-free mice, conventional mice and animals colonised solely by $B$. thetaiotaomicron. Colonisation by $B$. thetaiotaomicron was sufficient to initiate the development of the vast capillary network observed with conventional mice (Stappenbeck et al., 2002).

Proliferation and differentiation of intestinal epithelial cells are both stimulated by the presence of SCFAs produced by the gut microbiota (Frankel et al., 1994). SCFAs, specifically butyrate, are also an important nutrient for colonocytes providing 60-70% of their energy requirements (Daly et al., 2005). Experimentation with germ-free rats has shown that these animals have reduced crypt cell formation and slight hyperplasia of the villi in comparison to conventional rats (Alam et al., 1994).
1.4.4. Influences of the gut microbiota on the host immune system

Intestinal bacteria exert profound effects on the innate and adaptive immune responses in the mucosal immune system (Cebra, 1999). Numerous studies have shown that the immune system is underdeveloped in germ-free animals; an abnormality which can be reversed by the introduction of commensal bacteria (Cebra, 1999; Macpherson & Harris, 2004). In terms of the impact upon the adaptive immune response experimentation with germ-free animals has shown that they: do not appear to have payers patches or associated germinal centres, Immunoglobulin A (IgA)-producing plasma cells are reduced in number (Monteleone et al., 2006), polymeric immunoglobulin receptor (pIgR) levels are reduced (Hooper & Gordon, 2001) and intraepithelial lymphocytes (IEL) appear to be absent (Imaoka et al., 1996). The gut microbiota also impacts upon the innate immune system including regulation of mucin gene expression (Mack et al., 2003), antimicrobial peptide (AMP) secretion (Ayabe et al., 2000) and the stimulation of microfold cell proliferation (Smith et al., 1987). Bacterial degradation products are able to reach systemic circulation where they contribute to spleen and lymph node development as well as lymphoid tissue development in the gut itself (Collier-Hyams & Neish, 2005; Hooper, 2004). A delicate balance exists between the gut microbiota and the host immune system where both parties modulate one another. Perturbations in the microbiota, environmental determinants or host factors can disrupt this balance and result in the onset of disease.
1.5. Pathologies linked to the gut microbiota and potential treatments

1.5.1. Inflammatory bowel diseases (IBD)

In most instances the commensal microbiota is associated with the maintenance of host health; however there is evidence to suggest that the gut microbiota is implicated in the development of several pathologies including inflammatory bowel disease (IBD). The term IBD encompasses two conditions; Crohns disease and ulcerative colitis, both of which are chronic relapsing disorders that are characterised by inflammation of the large and/or small intestine. There is increasing evidence to suggest that IBD occurs as a result of an overly aggressive immune response to the commensal microbiota of the intestine or dysbioses in the gut microbiota which prompt immunological activation of the adaptive immune system (Sartor, 2004; Strober et al., 2007). IBD does not develop in animals raised in germ-free conditions further suggesting a link between the commensal microbiota and IBD (Guarner F, 2003; Yan & Brent Polk, 2004).

Martinez-Medina et al (2006) examined biopsy samples from the ileocolonic mucosa of Crohns disease patients and healthy volunteers using PCR-DGGE of the 16S rRNA gene. Results of the study showed that the fingerprints produced by the samples from sufferers of Crohns disease clustered separately from the healthy volunteers. Additionally, the microbiota of the Crohns disease patients showed on average a higher percentage of Clostridium spp., Ruminococcus torques and Escherichia coli, whereas Faecalibacterium numbers were less than those found in the microbiota of healthy subjects (Martinez-Medina et al., 2006). Although no particular species is over-represented in the microbiota of
patients with Crohns disease or ulcerative colitis and there is no characteristic microbiota associated with IBD, Frank et al (2007) report a reduction in the numbers of *Bacteroidetes* and *Lachnospiraceae* compared to healthy microbiotas (Frank et al., 2007). The identification of this anomaly by Q-PCR analysis of gastrointestinal tissue samples is significant because these two groups are commonly associated with intestinal health. For example, by production of SCFAs such as butyrate which stimulate the proliferation and differentiation of intestinal epithelial cells, potentially enhancing epithelial integrity and modulating immune responses (Frank et al., 2007; Frankel et al., 1994).

1.5.2. Microbial influences in gastric and colon cancer

The link between colonisation by *Helicobacter pylori* and gastric cancer is well established (Sipponen et al., 1998). Although, as an estimated 50% of the population are infected by this bacterium not all *H. pylori* infections result in the development of cancer (Ferreira et al., 2008). The exact mechanism for gastric cancer development is unclear but the role of *H. pylori* is believed to be via indirect inflammatory actions on gastric epithelial cells, and the direct action of the bacteria on epithelial cells through the induction of gene mutations (Chiba et al., 2008).

Chronic inflammation, a prominent feature of IBD and also colonisation by pathogenic bacteria, has been implicated in the development of colon cancer (Ekborn et al., 1990; Greer & O'Keefe, 2011; Guarner & Malagelada, 2003). A causative link between elements of the microbiota with colon cancer has not been established at the same level as *H. pylori* with gastric cancer, possibly due
to the huge increase in microbial richness in the colon versus the stomach. However, a recent study using mice with multiple intestinal neoplasms showed that infection with *Bacteroides fragilis*, an enterotoxin-producing human commensal organism, strongly induced colonic tumours (Wu *et al.*, 2009). Carcinogenesis in the colon is also heavily influenced by diet where the ingestion of indigestible carbohydrates and the subsequent production of SCFA by the microbiota have been shown to decrease tumour risk. The modern western diet however is low in fibre and high in meat and processed carbohydrates which result in low SCFA output with a corresponding increase in sulphur-producing bacteria whose end products are both inflammatory and genotoxic (Greer & O'Keefe, 2011).

1.5.3. **Probiotics, prebiotics and synbiotics**

Probiotics have been defined as “living microorganisms that upon ingestion in specific numbers, exert health benefits beyond those of inherent basic nutrition” (Guarner & Schaafsma, 1998). Microbial species commonly employed for this purpose include *Lactobacillus*, *Bifidobacterium* and *Saccharomyces*. Prebiotics comprise non-digestible dietary supplements, predominantly oligosaccharides, that selectively stimulate the growth of bacterial species considered beneficial to health (Hooper & Gordon, 2001). Whilst synbiotics are products that contain both probiotics and prebiotics (Sartor, 2004).

Several studies have demonstrated that probiotic treatment may be of benefit in relieving the symptoms of irritable bowel syndrome and reducing the incidence of antibiotic associated diarrhoea (Camilleri, 2006; de Morais & Jacob, 2006; Yan & Polk, 2006). Much of the research conducted in this area has focused on
infectious diarrhea, where treatment with probiotics containing strains of *Lactobacillus* and *Bifidobacterium* shortened the duration of the diarrhoea (Guarner & Schaafsma, 1998). However, more recently Martin *et al* (2009) showed that pro-, pre- and synbiotics can modulate host lipid, carbohydrate, and amino acid metabolism in multiple organs, including the liver, kidney and pancreas, suggesting a wider role for the modification of the microbiota (Martin *et al*., 2009). Evidence is also emerging to suggest that modification of the intestinal microbiota through the use of probiotics and synbiotics can improve the clinical condition of patients with IBD (Ishikawa *et al*., 2011; Kanauchi *et al*., 2009).

1.6. The Oral Microbiota

1.6.1. Anatomy and physiology of the oral cavity

Distinct habitats exist within the human mouth, including the teeth, tongue, cheeks and palate, each supporting their own unique microbial community. The teeth are of particular interest as they are the only hard, non-shedding surfaces of the body readily accessible for microbial colonisation (Marsh & Martin, 2003). Teeth are composed mainly of a bone-like material termed dentine, within which there is a pulp cavity containing a network of blood vessels, lymphatics and nerves. The upper surface of the dentine is covered by a layer of enamel and the root area covered by a material called cementum. Each tooth protrudes into the oral cavity through the alveolar bone which is covered by the gingivae. The gap between the gingival and the tooth surface is termed the gingival crevice (Figure 1.1.) (Wilson, 2005).
The oral cavity is usually at a temperature of 35 to 36°C and the resting pH between 6.5 and 7.5; suitable growth conditions for many microorganisms. In most individuals with periodontitis however, subgingival temperatures can reach 39°C. Under conditions associated with dental caries formation the pH can fall significantly below 6.5 due to bacterial acid production, whilst conversely the pH of the gingival crevice is more often raised in the range of 7.5 to 8 (Wilson, 2005).

![Figure 1.1. Structure of a tooth and adjacent structures.](image)

1.6.2. Fluid flow in the oral cavity

Fluid flow in the oral cavity impacts upon microbial ecology in various ways from the provision of nutrients and attachment mechanisms to contributions to shear forces and antimicrobial defences. Saliva is the primary fluid found within the oral cavity with the average individual producing around 1L daily (Humphrey & Williamson, 2001; Wilson, 2005). It is dilute fluid composed 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 modulate...
the pH. Calcium, phosphates and the proteins found in saliva contribute to tooth
demineralisation and remineralisation. Whilst immunoglobulins, mucins and
enzymes have a variety of functions ranging from defence to facilitating
microbial attachment and nutrition (Humphrey & Williamson, 2001; Wilson,
2005).

Gingival crevicular fluid (GCF) originates from the gingival crevice and is found
in all individuals; however the flow is markedly increased in individuals with
gingivitis. GCF is a nutrient rich fluid containing host cells, proteins,
carbohydrates, various ions and a number of immunological components
(Lamster & Ahlo, 2007). This provides an excellent source of nutrition to
bacteria inhabiting the gingival crevice (Wilson, 2005).

1.6.3. Antimicrobial and immune responses in the oral cavity

Commensal oral bacteria are believed to play an important role in the
production of defence strategies by periodontal tissue (Dixon et al., 2004). The
innate immune response to these oral microorganisms has in fact been shown
to be essential to periodontal health (Darveau, 2010). For instance, transit of
neutrophils to the gingival crevice is normally facilitated by the coordinated
expression of E-selectin, intercellular adhesion molecules and interleukin-8 (IL-
8), where they form a protective layer over the host tissue (Tonetti et al., 1998).
Yoshinari et al (1994) showed that rats treated with methotrexate, a drug that
induces neutropenia, invariably develop periodontitis and alveolar bone loss
(Yoshinari et al., 1994). Further studies in experimental animals have confirmed
the link between impairment of innate immune response and periodontal
disease (Darveau, 2010). Periodontal tissues are also documented to express
human β-defensins 1, 2 and 3, membrane bound and soluble CD14 and lipopolysaccharide-binding protein. These molecules are important in the clearance of many oral bacteria (Darveau, 2010).

Components of the adaptive immune system are also found in the oral cavity and include intra-epithelial lymphocytes and Langerhans cells (Wilson, 2005). Lymphocytes, monocytes and polymorphonuclear leukocytes are secreted via GCF and are therefore found at higher levels in the gingival crevice compared to saliva. Secretory IgA is the prominent immunoglobulin found in the oral cavity and is involved in preventing the binding of microbes to one another and to oral surfaces. IgG, which is predominantly found in GCF and thereby the gingival crevice has an important role in opsonisation, complement activation and neutralisation of bacterial toxins (Wilson, 2005). In addition to these immune components human saliva also contains numerous other antimicrobial compounds including lysozyme, lactoperoxidase, lactoferrin and transferrin which are involved in the killing and removal of microorganisms (Wilson, 2005).

The immune system of the host is constantly faced with the challenge of distinguishing between commensal and pathogenic bacteria. The significant and formidable presence of the innate and adaptive immune system in the oral cavity raises the question of how this environment is able to support such a large microbial community. Bacterial cells are believed to exhibit membrane associated molecules that identify them as targets for the innate immune system, termed pathogen-associated molecular patterns (PAMPs). However, these PAMPS are specific to a group of species or genus level and as such recent theories have suggested that a second signalling step must exist to
distinguish between species within a group to identify a pathogen. Those bacteria not expressing the relevant PAMPs are tolerated by the host immune system, whilst still stimulating it to some extent, and thus a commensal microbiota is able to exist (Sansonetti, 2011). As a consequence, in addition to being tolerated by the host immune system studies have suggested that the commensal microbiota may also contribute to immune development. Studies using germ-free mice have indicated that commensal intestinal bacteria play an important role in establishing both the adaptive and innate immune response as well as contributing to tissue development (Cebra, 1999). Although periodontal tissue has not been as fully investigated in this manner there has been suggestion that the commensal population of the oral cavity and the host tissues interact in a similar way (Darveau, 2010).

1.7. Microbial inhabitants of the oral cavity

Of all the microbial communities that thrive on the human body the oral cavity is considered to be one of the most diverse, second only to the lower gastrointestinal tract (Peterson et al., 2009). Although the oral microbiota comprises bacteria, fungi, archaea and viruses, research thus far has predominantly focused on the bacterial component of the microbiota which is numerically dominant (Kolenbrander, 2000; Marsh & Martin, 1999; Marsh, 1994; Marsh, 2005; Siqueira & Rocsas, 2009). However, one recent study of the fungal component of the oral microbiota undertaken using pyrosequencing with internal transcribed spacer primers (targeting non-functional RNA which exhibits a high degree of variation between species) found that the number of fungal
species in each individual microbiota ranged from 9 to 23 (Ghannoum et al., 2010).

1.7.1. Identifying key bacterial components of the oral microbiota

Culture and 16S rRNA-based molecular microbiology techniques have previously identified in excess of 700 bacterial species that reside in the human oral cavity (Aas et al., 2005; Paster et al., 2006). Of these species greater than 50% are yet to be cultivated and thus their role in oral microbial ecology not yet fully elucidated. It has been suggested that in reality ca. 100-200 species are likely to reside in an individual oral cavity at a given time (Siqueira & Rocas, 2010). Many species are found at multiple sites within the oral cavity; transiently within saliva and as part of dental or mucosal biofilms, whilst other taxa are site and subject specific (Aas et al., 2005; McBain et al., 2005).

More recent studies of bio-diversity in the oral cavity suggest that the number of oral phylotypes has been vastly underestimated previously. Xie et al (2010) used metagenomics to quantify unique phylotypes in human plaque samples. Using 454 pyrosequencing and Illumina sequencing technologies the authors reported 668 bacterial phylotypes from a single plaque microbiota, significantly higher than the previous estimates of 100-200 species (Xie et al., 2010). 454 pyrosequencing was also used by Keijser et al (2008) to study saliva and subgingival plaque samples from healthy adults. The study identified 3621 species level phylotypes in saliva and 6888 in subgingival plaque (n=71 and 98 respectively), far higher than previous estimates (Keijser et al., 2008). It has been suggested that the true value of bacterial diversity in the oral cavity may reside closer to 20000 phylotypes (Siqueira & Rocas, 2010).
1.7.2. Commonly detected phyla in the oral cavity

Oral bacteria identified by the currently available methodologies fall into 13 different phyla, however the majority belong to the phyla Firmicutes, Fusobacteria, Bacteroidetes, Actinobacteria, Proteobacteria, Spirochaetes and Synergistetes (Siqueira & Rocas, 2010). Distinct differences in the relative abundance of a phyla, genera or species are noted between the different sites within the oral cavity. For example, 454 pyrosequencing data obtained by Keijser et al (2008) from the saliva and subgingival plaque of healthy adults showed that the Actinobacteria, Fusobacteria and Spirochetes were overrepresented in plaque samples, whilst Bacteroides, Firmicutes and Proteobacteria were more abundant in saliva. The authors also noted that at the genus level Prevotella, Streptococcus and Veillonella constituted approximately 50% of the salivary microbiota. From the subgingival plaque samples, 50% of all sequences were members of 6 genera: Streptococcus, Veillonella, Corynebacterium, Actinomyces, Fusobacterium and Rothia (Keijser et al., 2008).

At the genus level a wide range of Gram-positive bacteria have been identified in the oral cavity, of which the streptococci constitute a large proportion (Aas et al., 2005; Burne, 1998; Marsh & Martin, 2003). Other Gram-positive cocci such as Enterococcus, Staphylococcus, Gemella and Granulicatella have been found in smaller numbers (Aas et al., 2005; Marsh & Martin, 2003). Gram-positive rods and filaments are also frequently isolated from dental plaque. Strains of Actinomyces form a major part of the oral microbiota whilst lactobacilli are
normally isolated at lower levels (Babaahmady et al., 1997) as are some strains of *Bifidobacterium* (Marsh & Martin, 2003).

Gram-negative cocci including *Neisseria* and *Veillonella* have been found at most sites within the oral cavity (Marsh & Martin, 2003). Gram-negative rods are also prominent members of the oral microbiota which includes many facultative anaerobes, the bulk of which belong to the genera *Haemophilus, Fusobacterium, Prevotella* and *Porphyromonas* constitute the majority of obligate anaerobes growing in dental plaque (Marsh & Martin, 2003).

1.8. The dental plaque biofilm

1.8.1. Formation of dental plaque

Black (1898) was one of the first scientists to utilise the term plaque in relation to the bacterial community that develops on the surface of the teeth. He described it as "gelatinous microbial plaques" when discussing the relationship between bacteria and dental caries (Black, 1898; Listgarten, 1999). Human teeth are not uniform structures but comprised of a number of distinct surfaces that result in the development of a highly heterogeneous microbial biofilm (Wood et al., 2000). There are smooth surfaces of enamel and stagnant sites such as fissures, the gingival crevice and approximal areas which support a more dense bacterial population (Marsh & Martin, 2003). Countless microorganisms will enter the oral cavity of the average person every day and yet only a proportion of these cells are retained on the surfaces of the mouth or in saliva. Species selection and the subsequent formation of dental plaque biofilm on the tooth surface are not thought to be a product of chance, in fact quite the opposite. Dental plaque is a structurally and functionally organised
microbial community that forms as a result of the specific properties of and interactions between oral bacteria and host (Marsh, 2006a).

Immediately after the eruption of a tooth or professional cleaning, host and bacterial molecules are selectively adsorbed onto the tooth surface. These proteins, lipids and glycolipids are collectively known as the acquired pellicle and act as receptors that provide the first point of attachment for bacteria. The pellicle is less than 1µm thick and will normally take approximately 90 to 120 minutes to form (Marsh & Martin, 2003; Marsh, 2004). Histochemical staining has shown that the pellicle is largely composed of proteins of host origin (Meckel, 1965). A more recent study by Yao et al. (2001) utilised mass spectrometry to analyse in vitro and in vivo-formed pellicles and found that it is primarily made up of salivary derived proteins and protein fragments (Yao et al., 2001).

In order for the dental plaque biofilm to form bacterial cells must first make contact with the acquired pellicle. Most of these movements result from the passive diffusion process facilitated by oral secretions, as few oral bacteria are able to actively propel themselves to a surface (Marsh & Bradshaw, 1999; Scheie, 1994). Once contact is made weak, long range physicochemical interactions such as electrostatic and Van der Waals forces facilitate a reversible adhesion of cells to the pellicle (Scheie, 1994). Irreversible attachment is a result of short range stereochemical interactions between adhesins (lectins) or carbohydrates on the bacterial cell surface and complementary receptors within the pellicle (Marsh, 2004). Not all species of oral bacteria possess the necessary surface molecules to successfully attach to
the pellicle; those that do are termed early colonisers. These species are of great importance as they provide attachment substrates that facilitate the adhesion of other species of bacteria that would otherwise be unable to establish on the surface of the teeth (Li et al., 2004; Scheie, 1994).

Streptococci are known to dominate early dental plaque, in particular *Streptococcus sanguis*, *Streptococcus mitis* and *Streptococcus oralis* (Nyvad & Kilian, 1987; Scheie, 1994; Theilade et al., 1982). More recent studies including a checkerboard DNA-DNA hybridisation assay of *in vivo* dental biofilm samples by Li et al. (2004) have however revealed that the earliest colonisers of the tooth surface are *Actinomyces* species. Two hours after tooth cleaning the proportion of *Actinomyces* in the biofilm samples fell as the proportion of streptococci increased, in particular *S. mitis* and *S. oralis* (Li et al., 2004). Attachment of the late colonisers arises from an interaction with bacterial surface receptors of the pioneer species. Each interaction is specific and not all species can recognise and attach to one another (Section 1.8.2.). A mixture of specific connections and chance attachments between bacterial cells ensures colonisation of the teeth follows a similar pattern in each individual and yet produces a unique and diverse biofilm (Marsh, 2004).

Supragingival and subgingival biofilms differ in terms of composition and structure. Light and electron microscopy studies by Listgarten (1976) showed that early supragingival plaque is dominated by Gram-positive cocci that are eventually superseded by filamentous bacteria and corn cob formations of filamentous bacteria with cocci attached. Subgingival biofilm samples consisted mainly of Gram-negative and flagellated cells, as well as some spirochetes.
(Listgarten, 1976). Zijng et al (2010) have described supragingival and subgingival biofilm architecture in greater detail using FISH. The authors describe the layering of supragingival plaque: the basal layer comprising *Actinomyces* species alone, *Actinomyces* species with chains of cocci, filamentous bacteria alongside Streptococci and yeasts or streptococci growing in close proximity to *Lactobacillus* species. The second layer comprised a heterogeneous mixture of streptococci, fusobacteria, bacteroidetes, spirochaetes and *Lactobacillus* species. Subgingival plaque was markedly different: the basal layer consisting solely of *Actinomyces* species, the intermediate layer primarily of spindle-shaped cells of which *Fusobacterium nucleatum* and *Tannerella Forsythia* were most common and the top layer of the biofilm containing mainly bacteria belonging to the *Cytophaga-Flavobacterium-Bacteroides* cluster (CFB-cluster) (Zijng et al., 2010).

In the absence of any intervention, dental plaque will continue to grow until a critical size is reached and remains stable unless disrupted by cleaning of the teeth or other environmental change (Marsh & Martin, 2003). Cells are held within the biofilm as a result of both bacterial adherence and the existence of the extracellular polysaccharide matrix. Transmission electron microscopic (TEM) evaluation of the EPS produced by a six member oral biofilm by Reese and Guggenheim (2007) suggested that in vivo biofilm EPS is likely to be highly complex. The authors suggest that the composition continually evolves as a result of various anabolic and catabolic enzymes produced by different bacterial species (Reese & Guggenheim, 2007). Dental plaque is however a relatively open structure with numerous fluid filled channels that has been suggested form a rudimentary circulatory structure allowing the diffusion of nutrients,
metabolites, toxins and other host and bacterially derived substances around
the biofilm (Wood et al., 2000).

1.8.2. Coaggregation and coadhesion in dental plaque

Kolenbrander (1988) defined coaggregation as “the recognition between
surface molecules on two different bacterial cell types so that a mixed-cell
aggregate is formed” (Kolenbrander, 1988). This process of recognition and
adherence between genetically distinct bacteria is believed to play an important
role in the development of multi-species biofilms (Drago et al., 1997; Reid et al.,
1988). First described in 1970 by Gibbons and Nygaard from studies of dental
plaque bacteria (Gibbons & Nygaard, 1970); interactions of this kind have been
observed in both naturally occurring and manmade environments. However the
vast majority of research in this field has thus far focused on bacteria derived
from the human oral cavity where coaggregation is reported to be particularly
important in facilitating attachments between bacteria associated with the dental
plaque biofilm (Eke et al., 1989; Foster & Kolenbrander, 2004; Gibbons &
Nygaard, 1970; Handley et al., 1985; Rosen & Sela, 2006; Shen et al., 2005;
Umemoto et al., 1999).

Several researchers have suggested that most, if not all, species of oral
bacteria coaggregate with at least one other partner (Kolenbrander, 1993;
Kolenbrander & London, 1993; Kolenbrander et al., 2000; Whittaker et al.,
1996). These interactions can be classified as intergeneric, intrageneric or
multigeneric coaggregations (Hughes et al., 1988; Kolenbrander, 2000). Intergeneric coaggregations (those occurring between strains from different
genera) constitute the majority observed between oral bacteria. Although less
frequent; intrageneric partnerships have been reported between a number of oral species, including some streptococci and fusobacteria (Kolenbrander et al., 2000; Rickard et al., 2003b). It has been suggested that intrageneric coaggregation amongst the oral streptococci is an important determinant of the predominance of the genus in initial dental plaque structure (Kolenbrander et al., 2002), where streptococci compose 60 to 90% of the early colonisers of the tooth surface (Nyvad & Kilian, 1987). Intergeneric coaggregation is believed to be particularly important for integration of the late colonisers into the multi-species dental biofilm (Kolenbrander et al., 2002; Kolenbrander et al., 2006); i.e. those species that are unable to attach directly to the proteins, lipids and glycolipids that make up the acquired pellicle (Section 1.8.1.). Adherence of cells of the same bacterial strain; termed autoaggregation (Kinder & Holt, 1994) has been observed in many bacterial species derived from the oral cavity (Shen et al., 2005) and thus may also play a important role in the formation and structure of dental plaque.

1.8.3. Cell surface structures associated with coaggregation

The process of coaggregation is a result of highly specific lectin-carbohydrate interactions that occur between the cell surfaces molecules of aggregating partners (Cisar et al., 1979). Coaggregation was first identified as a lectin-carbohydrate based interaction by McIntire et al. (1978) whilst studying the aggregation of Actinomyces viscosus and Streptococcus sanguis. The interaction was reversed by the addition of 0.01M lactose and several other sugars. Sugar inhibition was however shown to be selective as sucrose, amongst other disaccharides had a very limited effect on the coaggregation.
The authors also noted that protease treatment destroyed the active component on the surface of *A. viscosus* but not *S. sanguis* (McIntire *et al.*, 1978). Further studies have corroborated these findings showing that coaggregation between numerous species of oral bacteria can be inhibited by the addition of specific sugars, in particular those with a structure similar to lactose (Kolenbrander & Williams, 1981; Kolenbrander & Williams, 1983; Kolenbrander & Andersen, 1989; McIntire *et al.*, 1982; Rickard *et al.*, 2000; Rosen & Sela, 2006). Evidence corroborating the existence of a lectin component in coaggregation interactions was obtained Ellen & Balcerzak-Raczkowski (1977) during a study of coaggregation between *A. naeslundii* isolates and *S. sanguis* or *Streptococcus mitis*. Exposure to heat (56°C for 30 min) or proteolytic enzymes reduced the ability of the actinomycetes, but not the streptococci, to coaggregate with untreated partner cells; indicating that *A. naeslundii* carries the protein component(s) (Ellen & Balcerzak-Raczkowski, 1977).

Coaggregation interactions have been identified as unimodal, i.e. involving just one lectin-carbohydrate receptor interaction, or bimodal, involving more than one set of cell surface receptors (Kolenbrander *et al.*, 2000). The specificity of these interactions results in some oral bacteria having just one or two partners with which they will readily aggregate and a large number of species with which they will not, whilst others have a wider set of partners (Kolenbrander, 1995). Numerous studies have identified the cell surface receptors of specific species believed to be responsible for coaggregation. For example, Maeda *et al* (2004) examined the cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity of a number of strains of oral streptococci and the ability of these strains to coaggregate with *P. gingivalis*. Although GAPDH is an enzyme
it has also been shown to have binding functions; in this study high levels of GAPDH correlated with streptococcal coaggregation with fimbriae on the cell surface of *P. gingivalis* (Maeda et al., 2004). Coaggregations between *Treponema denticola* and *Porphyromonas gingivalis* or *Fusobacterium nucleatum* have previously been characterised by Rosen *et al.* (2008). The major outer sheath protein (MSP) of *T. denticola* was analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, Periodic acid-Schiff (PAS) staining and monosaccharide analysis. Alongside protease and sugar binding studies this methodology revealed that the MSP of *T. denticola* is a glycoprotein which mediates coaggregation with *P. gingivalis* and *F. nucleatum* through its protein and carbohydrate moieties, respectively (Rosen *et al.*, 2008).

Interestingly, bacterial cell viability is not a pre-requisite to coaggregation because dead cells have frequently been observed aggregating and do so immediately when mixed with a compatible partner strain(s). These observations support the hypothesis that cell surface molecules are responsible for the recognition and aggregation process rather than an active process that would require cell viability (Kolenbrander *et al.*, 1993).

### 1.8.4. Methodologies for the study of coaggregation

A visual assay described by both Kolenbrander (1995) and Cisar *et al.*, (1979) has been frequently employed in studies of coaggregation between oral bacteria. Dense suspensions of two different species are mixed and the extent of coaggregation (i.e. the size of floccules formed) is scored on a scale of zero to four (Cisar *et al.*, 1979; Kolenbrander, 1988; Kolenbrander, 1995). This assay exploits the fact that coaggregation between two species, once washed and
suspended in buffer solution, can be observed with the naked eye (Kolenbrander, 1988). Kolenbrander (1995) described the use of the visual assay to identify coaggregation between species from the 17 most commonly isolated genera in the oral cavity. Intragenic coaggregation was only documented between streptococci and a limited number of actinomycetes, whilst intergeneric coaggregation occurred between all the genera tested (Kolenbrander, 1995). The visual assay is fairly rapid and easy to execute, particularly useful when screening large numbers of bacteria to identify coaggregating partners. However it is limited by the subjective scoring system.

A quantitative assay for measuring coaggregation has also been described, based on the spectrophotometric analysis of the supernatant phase (Ikegami et al., 2004). Ikegami et al (2004) studied the leucine-rich repeat protein LrrA, encoded by the IrrA gene, on the cell surface of Treponema denticola and found that it played a role in coaggregation with Tannerella forsythensis. As part of the study the extent of coaggregation between T. denticola (IrrA mutant and non-mutant strains) and T. forsythensis was studied by spectrophotometric analysis. This method allows the calculation of percentage coaggregation values and may provide a more comparable analysis than the visual assay.

Both the visual and spectrophotometric coaggregation assays rely on prior culture of the bacteria in question. The large proportion of uncultured species that comprise the oral microbiota cannot be studied by these methods. A recent study by Wang et al (2011) employed a membrane binding assay coupled with PCR-DGGE to study coaggregation between F. nucleatum or S. mutans and cells from human saliva samples, which would include uncultured species. F.
nucleatum or S. mutans were immobilised on nitrocellulose membranes and saliva applied, unbound cells were washed off and the bound cells analysed by PCR-DGGE. Bands excised from the DGGE gel were sequenced and the authors noted that a variety of previously uncharacterised coaggregations were identified including those with uncultured species (Wang et al., 2011).

In terms of characterising the molecular basis of coaggregation interactions several studies have used heat and protein treatment and the addition of lactose and other similar sugars (Kolenbrander & Williams, 1981; Kolenbrander & Williams, 1983; Kolenbrander & Andersen, 1989; McIntire et al., 1982; Rosen & Sela, 2006). McIntire et al (1978) utilised these methods in a study of the coaggregation mechanisms occurring between Actinomyces viscosus and Streptococcus sanguis. Coaggregation was inhibited by lactose, beta-methyl-D-galactoside, D-galactose and protease treatment of A. viscosus. The authors concluded that proteins on the fibrils of A. viscosus interacted with cell surface carbohydrates on S. sanguis to mediate coaggregation (McIntire et al., 1978).

1.9. Bacterial interactions in the oral cavity

Dental plaque is a classic example of a multi-species bacterial biofilm. As such the properties and behaviours of the species existing within it are often different to the same species growing in liquid or mono-culture (Costerton et al., 1995). Extensive interspecies interactions occur within this complex community impacting upon the localisation of species within the biofilm and thereby it’s structure and function (Kuramitsu et al., 2007).
1.9.1. Competition for nutrients in the dental biofilm

Nutrient availability is an important determining factor in the formation and maintenance of dental plaque (Kuramitsu et al., 2007). Bacteria are able to utilise carbohydrate, fatty acids, lipids and amino acids for biosynthesis and the production of energy (Moat & Foster, 1988) which are sourced from food, saliva, GCF and bi-products of other species (Kuramitsu et al., 2007).

For many species, carbohydrate metabolism is the preferred energy source used to drive the myriad of reactions occurring in each cell. Frequent intake of fermentable carbohydrates in the modern human diet means that species such as *Streptococcus mutans* that are able to metabolise sucrose more efficiently have a competitive advantage over other oral species (Hamada & Slade, 1980). Aided by the fact that *S. mutans* is highly aciduric (Loesche, 1986; Minah et al., 1985) and able to convert sucrose into molecules that facilitate attachment to the tooth surface (Gibbons & Nygaard, 1968). Saliva and GCF contain numerous proteins and glycoproteins: important sources of carbon and nitrogen for many oral bacteria (Griffiths, 2003; Van der Hoeven & Camp, 1991). Highly proteolytic organisms such as *P. gingivalis* have a competitive advantage where proteins are the limiting source of nutrients, in subgingival plaque for example (Potempa et al., 1995).

1.9.2. Metabolic cooperation and antagonism in oral biofilms

A single species may not produce the necessary proteases or glycosidases to break down these complex nutrients (Marsh & Bradshaw, 1999). However, individual strains may possess different, yet overlapping patterns of enzymatic activity that when they are grown in close contact allow for the synergistic
degradation of complex molecules. A chemostat based study by Bradshaw et al. (1994) found that adding different species of oral bacteria with novel enzyme activities to a five-member community growing on mucin (a complex glycoprotein) led to their establishment within the community and an increase in the total viable counts. From these results the authors hypothesised that metabolic co-operation can liberate additional nutrients and can therefore help to maintain diversity in the oral microbiota (Bradshaw et al., 1994). Cross-feeding has been observed between Porphyromonas gingivalis and Treponema denticola, where the growth of P. gingivalis was promoted by metabolising succinate produced by T. denticola, which was itself promoted by the presence of isobutyric acid produced by P. gingivalis (Grenier & Mayrand, 1986).

Cooperative metabolism can also benefit oral bacteria by changing the environmental conditions for the benefit of some species. Takahashi (2003) demonstrated that F. nucleatum and P. intermedia are able to generate ammonia by the fermentation of glutamic and aspartic acids found in saliva. As a result, areas of plaque in close proximity to those species are found to be less acidic and provide protection for acid-sensitive species, such as P. gingivalis, against substantial drops in pH attributed to the presence of lactic acid producing bacteria and dietary fermentable carbohydrates (Takahashi, 2003). The human mouth is largely aerobic environment and yet anaerobic bacteria make up a significant proportion of dental plaque. Studies have shown that obligate anaerobes can be protected from the toxic effects of oxygen by the presence of oxygen-consuming species such as N. subflava (Prosser, 1999).
Metabolic products of one organism often exert antagonistic effects on other organisms in the biofilm (Kuramitsu et al., 2007). For example, S. sanguinis strains can produce hydrogen peroxide, a non-specific antimicrobial, which has an antagonistic effect on the growth of S. mutans and therefore the two species are rarely found growing in close proximity (Kreth et al., 2005).

1.9.3. Bacteriocin production in oral biofilms

Many species of oral bacteria are able to exert an inhibitory effect on the growth of neighbouring species via the production of proteinaceous toxins, termed bacteriocins (Kuramitsu et al., 2007). Bacteriocins frequently target related organisms and have a narrow killing spectrum (Riley & Wertz, 2002). The streptococci, a genus which make up an estimated 60 to 90% of the early colonisers of a tooth surface (Nyvad & Kilian, 1987), are one of the most prolific producers of bacteriocins with almost every species documented to do so (Marsh & Martin, 2003; Nes et al., 2007). Streptococcus mutans for example, is known to produce at least five different bacteriocins (Hale et al., 2005). Bacteriocins produced by the streptococci are more commonly termed mutacins. Mutacins produced by S. mutans effectively inhibit the growth of Streptococcus sanguinis strains and thus S. sanguinis is unable to colonise niches occupied by large number of S. mutans (Kreth et al., 2005). Many other species of oral bacteria produce bacteriocins including late colonisers such as P. gingivalis, Prevotella intermedia, and Prevotella nigrescens. Teanpaisan et al (1998) studied bacteriocin production by 44 strains from this group of 3 species using an agar overlay technique and agar diffusion assay. A high degree of bacteriocin activity was noted between P. intermedia, and P. nigrescens and
also both species against *P. gingivalis*. Interestingly, only one strain of *P. gingivalis* exhibited bacteriocin activity against the other two species (Teanpaisan *et al.*, 1998). Bacteriocin production is believed to be regulated by the prevailing environmental conditions, impacted by pH, cell density and nutritional availability (Kreth *et al.*, 2005; Kuramitsu *et al.*, 2007; Merritt *et al.*, 2005).

### 1.9.4. Influences of bacterial signalling molecules in oral biofilm development

Bacteria growing within the dental plaque biofilm are believed to be able to communicate with one another via small signalling molecules (Prosser, 1999). Intercellular communication within biofilms, or quorum sensing, has been attributed to regulatory molecules termed autoinducers (AI) (Kuramitsu *et al.*, 2007). Quorum sensing enables a biofilm population to collectively mediate bacterial gene expression, impacting upon the production of virulence factors, antimicrobials and biofilm formation (Marsh, 2004; Yoshida *et al.*, 2005). Yoshida *et al* (2005) investigated the role of autoinducer-2 (AI-2) on *S. mutans* biofilm formation by constructing a GS-5 *luxS*-null mutant strain (*LuxS* is an enzyme involved in the production of AI-2). Biofilm formation was markedly less in the mutant strain compared to the wild type, indicating an important role for AI-2 in biofilm formation. Further studies of *LuxS* deficient mutants of *S. gordonii, S. sobrinus, S. anginosus, P. gingivalis* and *Actinobacillus actinomycetemcomitans* showed reductions in biofilm formation, however this was not observed in with several other streptococci. The authors suggest that AI-2 is involved in the regulation of sucrose dependent biofilm formation (Yoshida *et al.*, 2005).
1.10. Inter and intra-individual variations in the oral microbiota

Numerous molecular microbiology based studies have identified a high level of inter-individual variation among oral microbiotas (Diaz et al., 2006; Lazarevic et al., 2010; Ledder et al., 2006; McBain et al., 2005; Rasiah et al., 2005). However, intra-individual temporal changes in the oral microbiota as a whole are low; several studies have shown that salivary microbiotas remain stable over a period of months and even years (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005). In one such study Lazarevic et al. (2010) described the use of PCR amplification of the bacterial 16S rRNA gene and 454 pyrosequencing technology to show that the salivary microbiota of five subjects remained stable over a test period of 29 days. The authors concluded that the findings indicated the persistence of subject-specific taxa exhibiting minor fluctuations over time (Lazarevic et al., 2010). In another PCR-DGGE based study Ledder et al (2006) highlighted marked inter-individual variability in the bacterial community fingerprints of both the salivary inocula and associated multiple Sorbarod devices (Ledder et al., 2006).

Current theories suggest that the establishment of a unique individual oral microbiota is shaped by two distinct factors (He et al., 2010a): inheritance of components of the microbiota of the mother or local environment at birth (Gronlund et al., 1999; Ley et al., 2006; Mandar & Mikelsaar, 1996) and the host habitat, which includes the influences of genotype i.e. immune response and anatomical structure; and also the nutritional environment (Frank et al., 2003; Zoetendal EG et al., 2001). There is also evidence to suggest however that a core microbiome exists across all oral microbial communities. A recent study by
Zaura *et al* (2009) using 454 pyrosequencing technology to assess the microbiological profiles across several sites within the oral cavity found that 66% of the reads were common to all three individuals taking part (Zaura *et al.*, 2009).

These observations are interesting because many of the current models of oral biofilm formation, structure and function do not appear to account fully for inter-individual variation. For example, the model of species succession in dental plaque formation proposed by Ritz (1967) has remained largely unchanged to date (Ritz, 1967). Additionally the spatiotemporal schematic of dental plaque formation proposed by Kolenbrander *et al* (2002) is still one of the most prominent representations of the dental biofilm despite being based on evidence obtained from pair-wise coaggregation assays using planktonic cells (Kolenbrander *et al.*, 2002). It seems probable that a large amount of variation will exist from the formation process, suggested plaque structure and functional properties, as a direct result of inter-individual variations in the oral microbiota and is worthy of further study.

**1.11. Pathologies of the oral cavity related to the indigenous microbiota**

In a healthy mouth an ecological balance exists between the host and the numerous indigenous microorganisms (Filoche *et al.*, 2010). However, bacteria residing 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).
1.11.1. The microbiology of dental caries

Dental caries, defined as destruction of the hard tissue of the teeth by bacterial fermentation of carbohydrates, affects the majority of individuals in the industrialised world to some extent (Marsh & Martin, 2003). High frequency carbohydrate exposure increases the production of acidic bacterial fermentation products, particularly lactic acid, that demineralise enamel and result in cavitations of the tooth (Filoche et al., 2010).

The presence of cariogenic dental plaque is directly responsible for the onset and progression of dental caries (Filoche et al., 2010). What constitutes cariogenic plaque is still a matter open to some debate. One of the first theories proposed regarding the aetiology of dental caries was the specific plaque hypothesis (Loesche, 1976). This theory suggested that the formation of dental caries is directly attributable to a small number of specific species (Marsh, 1994; Marsh, 2003). Acidogenic and aciduric species, such as Streptococcus mutans and homofermentative lactobacilli, were specifically associated with dental caries because they can rapidly metabolise dietary carbohydrate to acid, which in turn demineralises the tooth enamel (Bowden, 1990; Loesche, 1986; Sissons et al., 2007). The specific plaque hypothesis was supported by numerous studies that showed that these species were amongst a limited subset of bacteria consistently isolated in higher numbers from carious lesions (Emilson & Krase, 1985; Haffajee et al., 1998; Kleinberg, 2002; Loesche, 1986; Schachtele et al., 1972; Zinner et al., 1965). Several recent clinical studies have documented a link between high levels of S. mutans in an individual’s oral microbiota and increased incidence of dental caries (Hong & Hu, 2010; Palmer
et al., 2010). However, some researchers have argued that there is evidence to suggest that the specific plaque hypothesis is incorrect or incomplete. Pathogenic species have been isolated at sites within the plaque biofilm where no disease is apparent and in addition to this analysis of the microbiota of some diseased sites shows that there are few suspected pathogens present (Bratthall, 1991; Marsh, 1994). In his review of oral bacteria in dental caries causation Kleinberg (2002) suggested that the relationship between *S. mutans* and caries should be considered associative and not causative. He argued that eliminating *S. mutans* from the dental plaque biofilm would simply enable other acidogenic species to flourish and thus a more ecological approach to the study and treatment of dental caries is appropriate (Kleinberg, 2002).

The non-specific plaque hypothesis arose as a result of these observations and attributes the formation of dental caries to interactions occurring between a heterogeneous group of bacteria found in dental plaque and the host (Marsh, 1994; Marsh, 2003; Theilade, 1986). Although this theory is not wholly different from the specific plaque hypothesis it suggests a role for a wider group of species in disease progression and the potential for influences from the host. The ecological plaque hypothesis proposed with the intention of unifying the laboratory observations and theories, suggests that there is no specific aetiology associated with dental caries: any species with the right properties can contribute to disease progression, subject to selection pressure from changes in the oral environment (Marsh, 1991). In the case of dental caries, the presence of fermentable sugars in the oral cavity exerts selection pressure for acidogenic species to metabolise the carbohydrate and where they are aciduric, also to increase in number at the expense of non-aciduric species. Thus
potential pathogens could exist within a healthy plaque biofilm at lower levels and become competitive only when the environmental conditions shift to a lower pH (Marsh, 1994). The existence of carious legions in the absence of pathogens such as the mutans streptococci and lactobacilli are accounted for by acid production, albeit at a lower rate, by other species of oral bacteria under the right conditions (Marsh, 1994). Despite many researchers now favouring the ecological plaque hypothesis over the specific plaque hypothesis a diet high in fermentable carbohydrate has been shown to result in an increase in the proportion of mutans streptococci and a concomitant fall in the numbers of other streptococci (Marsh, 1994), particularly Streptococcus sanguis (De Stoppelaar et al., 1970; Minah et al., 1985; Staat et al., 1975). However, these observations add weight to the ecological plaque hypothesis as they are frequently a result of an environmental change (increased carbohydrates resulting in a reduced pH) predisposing a site to disease which is the cornerstone of this theory.

1.11.2. The microbiology of periodontal disease

One of the first studies to demonstrate the direct relationship between the accumulation of dental plaque and periodontal disease was described by Löe et al in 1965. The authors directed the 12 participants of their study to abstain from oral hygiene measures until a clinical assessment showed inflammatory changes in the gingivae. Throughout the study the bacteriology was assessed by microscopic examination of smears taken directly from plaque. Tissues exposed to bacterial plaque exhibited significant inflammatory reactions which were reversed when oral hygiene measures were reintroduced, a strong indication for the role of plaque in periodontal disease (Loe et al., 1965).
Gingivitis, or inflammation of the gingival tissues, is now recognised as a consequence of insufficient oral hygiene. In contrast to the acidic environment associated with dental caries the inflammatory reaction that occurs in gingivitis leads to a shift in pH to more alkaline conditions and gingival crevicular fluid (GCF) flow also increases (Marsh, 2006b). The condition is characterised by swollen, red gingivae often accompanied by bleeding and halitosis (Wilson, 2005). Progression of this condition to periodontitis where inflammation causes damage to the supporting structures of the teeth is extremely common affecting 70 to 80% of the adult population. It is also the leading cause of tooth loss in people over the age of 25 (Wilson, 2005). The processes of periodontitis are characterised by destruction of the periodontal ligaments, followed formation of periodontal pockets, and alveolar bone loss (Hart et al., 2004).

The aetiiology of gingivitis and periodontitis has been associated with the incidence of high numbers of obligate anaerobic Gram-negative bacteria including *Fusobacterium*, *Porphyromonas*, *Treponema* and *Prevotella* in mature dental plaque communities (Haftajee et al., 1998; Kroes et al., 1999; Ledder et al., 2007; Mombelli et al., 1998; Moore & Moore, 1994; Socransky et al., 1998). Ledder et al (2007) analysed subgingival plaque samples from 47 individuals with healthy gingivae and active periodontitis by PCR-DGGE and multiplex PCR. DGGE analysis revealed no link between microbial complexity or specific species or groups of species and periodontal disease with a high level of inter-individual variability observed. Multiplex PCR did however show that the presence of *A. actinomycetemcomitans* was significantly linked with disease and the presence of *Treponema socranskii* and *Pseudomonas* sp. were predictors of disease (Ledder et al., 2007).
The ecological plaque hypothesis for periodontitis suggests that plaque accumulation around the gingival margin initiates a host inflammatory response and that is associated with an increase in gingival crevicular fluid (GCF) flow that provides the proteinaceous nutrients favoured by many of the obligately anaerobic oral species (Marsh, 1994). Subsequent tissue damage has been attributed to structural and secreted components of the altered bacterial microflora causing direct damage to periodontal tissue or modulating the host immune response (Madianos et al., 2005; Marsh, 1994). Implicated in this process are bacterial lipopolysaccharides (LPS), fimbriae, proteases and toxins (Madianos et al., 2005). Stimulation of the host immune system is associated with transmembrane toll-like receptors (TLRs) in conjunction with soluble and membrane bound CD14 proteins (Madianos et al., 2005) and the ensuing production of cytokines that disrupt the balance between tissue-degradating metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) (Reynolds et al., 1994). An individual’s susceptibility to periodontitis is believed to have a strong genetic component (Hart et al., 2004; Hooper & Gordon, 2001; Ledder et al., 2007). Hart et al (2004) used quantitative PCR to examine transcription of a 48 gene set in the gingiva and spleens of two different strains of mice after oral infection by Porphyromonas gingivalis. The results suggested that a molecular phenotype relating to resistance or susceptibility to alveolar bone loss exists (Hart et al., 2004).

1.11.3. Management of common dental diseases

Plaque control is key to the prevention of both dental caries and periodontal disease. Mechanical plaque control is an important part of reducing plaque
mass however is insufficient to remove all plaque, particularly from hard to reach and stagnant sites. As such the use of additional measures and oral hygiene products is essential in the prevention and management of dental diseases (Busscher et al., 2007).

1.11.4. Strategies to prevent dental caries

Considering the clinical importance of dental caries it is no surprise that a large amount of research effort has been directed towards elucidating the most effective ways to prevent and treat this condition. Frequent intake of carbohydrates and the associated drop in pH in the dental biofilm that results has been shown to increase the risk of dental caries formation (Marsh, 1994). Therefore it has been suggested that a logical course of action to reduce the incidence of caries would be to avoid sugary snacks between meals and replace sugar with substitutes such as saccharine (Marsh, 1994; Marsh & Bradshaw, 1997). The protective effects of saliva which: contains antimicrobial factors, can raise local pH and can play a role in remineralisation of enamel, can also be exploited (Marsh, 1994). Saliva flow can be stimulated by chewing of a sugar free gum which has been considered as a strategy for caries prevention (Jensen, 1986).

Fluoride has been incorporated into dentrifices, mouthwashes and even tap water for many years for the prevention of dental caries. It is widely believed that fluoride is incorporated into the tooth enamel post eruption and inhibits demineralisation whilst enhancing remineralisation of the enamel (Marsh, 1994; Rosin-Grget & Lincir, 2001). The role of fluoride as an antimicrobial has been debated, it has been suggested that it can diffuse into bacterial cells as
hydrogen fluoride. Once inside the acid dissociates and the fluoride ions interfere with the carbohydrate metabolism. Bradshaw et al (1990) showed that sub-MIC levels (1 mmol/L) of sodium fluoride prevented the selection of S. mutans into a nine-member mixed-culture system of oral species. The rate of acid production was reduced by the low fluoride levels and acid-sensitive species persisted at higher levels than in the absence of fluoride (Bradshaw et al., 1990). However many researchers question whether fluoride is present in high enough levels to exert this effect in vivo (Rosin-Grget & Lincir, 2001).

1.11.5. Strategies to prevent periodontal disease

Oral hygiene and adequate plaque control are widely regarded as essential in maintaining periodontal health (Paraskevas, 2005) and traditional methods to prevent dental disease have targeted mechanical or non-specific plaque removal (Allaker & Douglas, 2009). Another approach put forward for the prevention of periodontal disease is to raise the redox potential of the periodontal pockets and make the environment less hospitable to obligate anaerobes (Marsh, 1994). Wilson et al (1992) used the redox dye methylene blue subgingivally for 7 days at 25 different test sites and found a reduction in GCF flow, reductions in the proportion of obligately anaerobic bacteria and a concomitant increase in facultative anaerobes and cocci (Wilson et al., 1992).

1.12. Antimicrobial prophylaxis and treatment of dental disease

Antimicrobials are incorporated into oral hygiene products for the prophylaxis and treatment of both dental caries and periodontal disease. Chemotherapeutic agents are frequently used as an adjunct to mechanical plaque removal and clinical studies have indicated that many commercially available dentifrices
exhibit general anticalculus effects (Allen et al., 2002; Banoczy et al., 1995). Recent work has however shown that some antimicrobials can exhibit a more selective activity against oral biofilms with potentially beneficial outcomes for plaque control and oral health (Bradshaw et al., 1993; Kinniment et al., 1996b; Price et al., 2007). Although dentrifice-delivered antimicrobials are known to remain at concentrations above the minimum inhibitory concentration (MIC) of most oral bacteria for only a few minutes, it appears they can continue to exert selective antibacterial effects for several hours after delivery (Marsh, 2003). Several studies have shown that a number of species of bacteria specifically associated with oral health are unaffected by short contact with these antimicrobials, whilst groups associated with dental disease, such as the gram-negative anaerobes and mutans streptococci, are reduced in number (Bradshaw et al., 1993; Kinniment et al., 1996b). For example, Bradshaw et al (1993) utilised mixed culture chemostats to challenge selected oral species with triclosan and zinc citrate alone and in combination. Results from this in vitro study demonstrated that when used in combination these broad spectrum antibacterial agents showed complementary and additive inhibitory effects, and moreover acted selectively to target potentially pathogenic gram-negative anaerobes. Viable count data revealed that gram-negative anaerobes were the most sensitive group of bacteria when mixed species biofilms growing in constant-depth film fermentors (CDFF) were challenged with pulses of chlorhexidene (Kinniment et al., 1996b). Work by Price et al (2007), utilising the quantitative polymerase chain reaction (QPCR), has shown that brief contact with chlorhexidene reduced the numbers of some periodontal pathogens in in vitro plaque biofilms models (Price et al., 2007).
1.12.1. Triclosan

Triclosan, a commonly used bisphenol antibacterial, was first introduced as a topical antiseptic agent in the 1960s and since then has been widely used in dentrifice formulations (McBain et al., 2003b). Triclosan possesses broad-spectrum antibacterial activity (Regos & Hitz, 1974), and is thought to act primarily by disrupting bacterial cell membranes (Villalain et al., 2001) and thereby cell permeability and barrier function (Russell, 2004). At low concentrations triclosan is bacteriostatic, binding to the enzyme enoyl-ACP reductase (encoded by the fabI gene). This increases the enzyme’s affinity for nicotinamide adenine dinucleotide (NAD+) and results in the formation of a stable complex which is unable to participate in fatty-acid elongation (Heath et al., 1998). For oral pathogens lacking in the fabI gene, such as Streptococcus mutans, triclosan inhibits glycolysis by targeting the phospho(enol)pyruvate:sugar phosphotransferase system (PTS) and glycolytic cytoplasmic enzymes (Phan & Marquis, 2006). Phan and Marquis (2006) ascertained this by utilising proton permeability and enzyme assays to show that triclosan is a multi-target inhibitor for a number of enzymes associated with mutans streptococci; inhibiting glycolysis in dental plaque biofilms and thus reducing cariogenicity. Numerous clinical studies have highlighted the effectiveness of triclosan against oral bacteria, including an in vivo study of 15 subjects investigating a triclosan/copolymer dentrifice; where significant reductions in the number of oral anaerobes were observed six and twelve hours after brushing with the triclosan/copolymer dentrifice versus control (Fine et al., 2006). Significant inhibition of plaque regrowth has also been noted with the use of triclosan rinse in a four day clinical study (24 volunteers, twice daily rinsing with toothpaste.
slurries) (Moran et al., 2001). Furthermore, a double blind seven month clinical study reported that triclosan containing dentrifices produced a reduction in gingival bleeding of 25 percent when compared with control (Svatun et al., 1993). The added anti-inflammatory action of triclosan is thought to be mediated by the inhibition of the oxygenase/lipoxygenase pathway in arachidonic acid metabolism (Paraskevas, 2005).

1.12.2. Stannous fluoride

Stannous fluoride has been incorporated into dentrifice formulations since the early 1950’s (Paraskevas, 2005; Van Loveren, 1990). A study of the antibacterial properties of stannous fluoride using atomic absorption spectrophotometry revealed significant intracellular retention of tin, which is believed to be unique to this compound (Camosci & Tinanoff, 1984). Consequently, the anticaries properties of stannous fluoride are attributed to a reduction in acid formation caused by stannous ions adsorbing onto the bacterial cell wall and disturbing membrane transport processes, or through inhibition of enzyme systems essential in the fermentation of sugars (Svatun & Attramadal, 1978). The general antimicrobial properties of stannous fluoride are reported to be superior to those of sodium fluoride and are attributed to: binding of divalent tin cations to negatively charged plaque components thereby inhibiting bacterial coaggregation and coadhesion (Skjorland et al., 1978), and the low pH of stannous fluoride facilitating the formation of hydrogen fluoride, a more potent antimicrobial agent than the fluoride ion alone (Whitford et al., 1977). *Streptococcus mutans*, frequently associated with dental caries, appears to be particularly susceptible to stannous fluoride (Camosci & Tinanoff, 1984).
The bulk of clinical studies show that stannous fluoride produces statistically significant improvements in gingivitis when compared with control dentrifices and there is some evidence to show that it is an effective general antiplaque agent (Paraskevas, 2005).

1.12.3. Chlorhexidine

Chlorhexidine, a cationic bis-biguanide antimicrobial was first described by Davies et al in 1954 (Davies et al., 1954). This compound exhibits broad spectrum antimicrobial activity and has a long history of use in the oral cavity. The primary mechanism of action is believed to be disruption of bacterial cell membranes (Hugo & Longworth, 1966; Kuyyakanond & Quesnel, 1992). High clinical efficacy of chlorhexidine as an anti-plaque agent has been attributed in part to the substantivity of the agent which binds to tooth and mucosal surfaces (Bonesvoll, 1978). Løe et al (1972) were some of the first researchers to show that chlorhexidine has some effect in preventing the formation of dental caries. Subjects in the study rinsed their mouths with a sucrose solution nine times daily, those that also rinsed twice daily with a 0.2% chlorhexidine solution did not show any signs of caries development (Loe et al., 1972). A review of the clinical and laboratory data regarding chlorhexidine use and the prevention of caries by Emilson (1994) described it as the best documented and most effective agent (Emilson, 1994).

The effectiveness of chlorhexidine in reducing dental plaque formation led Løe and Schiott (1970) to suggest that the compound could be used in the prevention of gingivitis (Loe & Schiott, 1970). Numerous subsequent studies have supported this theory (Gunsolley, 2010; Teles & Teles, 2009) including a
recent clinical study comparing chlorhexidine to a placebo rinse. Fifty patients with gingivitis used either the antimicrobial or placebo rinse for four weeks and plaque samples were analysed by quantitative real-time PCR. The chlorhexidine group showed significant reductions in bacterial numbers in comparison to placebo (Becerik et al., 2011).

1.12.4. Additional chemical agents for plaque control

In addition to those antimicrobials already described the compound cetylpyridinium chloride (CPC) has been used in oral health care products. A recent study by Hu et al (2009) compared the effects of a CPC containing mouth rinse with a fluoride rinse. Of the 117 adults involved in the study those who used the CPC rinse had a reduced amount of anaerobic bacteria in subgingival plaque compared to the fluoride rinse (Hu et al., 2009). Essential oils have been incorporated into mouthwash formulations for plaque control since the 19th century. A systematic review of all studies comparing chlorhexidine to a standard essential oil mouthwash formulation was carried out in 2010. The results of this review indicate that with long term use essential oil based products are comparable to chlorhexidine in terms of controlling gingival inflammation (Van Leeuwen et al., 2011). Non-antibacterial agents such as sodium lauryl sulphate (SLS) have been shown to be effective in detaching co-adhesive bacteria from the tooth pellicle, beneficial for plaque control (Busscher et al., 2007).

1.12.5. Probiotics and colonisation resistance

Probiotic use and research has been largely targeted at the human digestive tract, however recent studies have investigated the role of probiotics in oral
health. The results of these studies have thus far been mixed; Kang et al (2011) showed that *Lactobacillus reuteri* can inhibit the growth of periodontopathic bacteria and inhibit the biofilm formation of *Streptococcus mutans*. The authors suggest that the results indicate that this bacterium presents a useful potential oral probiotic (Kang et al., 2011), however the study was carried out *ex vivo* and largely in pure or two species culture conditions which do not well reflect the challenges probiotics face *in vivo*. A recent study by Madhwani and McBain (2011) also looked at the probiotic potential of *L. reuteri*, in this case on salivary derived *in vitro* biofilms. Continuous culture plaques within constant depth film fermentors (CDFF) were treated with *L. reuteri* and the microbial composition of the plaques monitored by PCR-DGGE and qPCR. The results of the study indicated that the introduction of this bacterium into *in vitro* oral models altered the composition of the microbial community that persisted at least 20 days post-dosing. An increase in the numbers of both lactobacilli and gram negative anaerobes was also noted. The evidence of beneficial effects was not clear and would require further research (Madhwani & McBain, 2011).

A recent clinical study investigating a probiotic supplemented milk used in caries active adolescents showed no significant beneficial effects. The milk contained *Lactobacillus rhamnosus* and patient’s saliva and subgingival plaque samples were assessed by checkerboard DNA-DNA hybridisation. No significant differences were observed between and within the treatment and placebo groups (Lexner et al., 2010).

An earlier study by Hillman et al (2007) suggested a potential alternative to eradication of *S. mutans*: replacement therapy using a genetically modified
strain unable to generate lactic acid. Produced by deleting the open reading frame for lactate dehydrogenase, *S. mutans* A2JM readily colonised the oral cavity of rats. The authors concluded that replacement therapy would be suitable for the prevention of dental decay in humans once a strain has been produced that is not dependent upon D-alanine (Hillman *et al.*, 2007).

1.13. An overview of *in vitro* oral biofilm models

1.13.1. Selecting the appropriate inocula for an *in vitro* biofilm model

Developing and implementing effective *in vitro* model systems of the oral microbiota has been hampered by the complexity inherent to oral biofilms. No single ideal model exists and each possesses distinct advantages and disadvantages often dependent upon the aims and applications of the study (McBain, 2009). Fundamental to the choice of model is the microbiological composition of the inocula. Microcosms; where material sourced from the oral cavity is cultivated in under conditions designed to replicate the environment that exists *in vivo* (Wimpenny, 1999), have previously been successfully employed in the study of oral biofilms (*Ledder et al.*, 2006; McBain *et al.*, 2003a; McBain *et al.*, 2005; McBain, 2009; Pratten & Wilson, 1999; Pratten *et al.*, 2000). Although *in vitro* microcosms more closely mimic the prevailing physiochemical and microbiological conditions of the oral cavity, pure culture and selected consortia studies have also proven valuable (McBain, 2009). Pure culture-based studies allow for the monitoring of the response of a single organism to physiological or environmental changes that could not readily be monitored in mixed culture (*Pratten et al.*, 1998a). Selected consortia of oral bacteria have been effectively used to study ecological phenomena where the
reduction in microbial complexity in comparison to microcosm studies makes identifying variation much simpler (Dibdin & Wimpenny, 1999; Kinniment et al., 1996a; McKee et al., 1985). Pure culture and consortia-based models also provide a high degree of reproducibility that is impossible to achieve in microcosms (McBain, 2009).

1.13.2. Batch culture oral biofilm models

Whilst closed system biofilm models do not reflect the nutrient flow normally found in vivo they can form a simple and reproducible biofilm model. The simplest of all models is arguably the agar plate, where it has been suggested bacterial cells exhibit many of the traits commonly associated with biofilm growth (McBain, 2009). The high cell density associated with agar culture facilitates cell-cell, communication, cooperation and antagonism by the precipitation of nutrient and oxygen gradients and antimicrobial production (Brown & Gilbert, 1995; Liljemark et al., 1997; McBain, 2009).

Agar-based biofilm studies have been employed in the study of antimicrobial efficacy in the oral cavity. For example, Walter et al (2011) determined the susceptibility of subgingival plaque samples from patients with severe periodontitis to amoxicillin and metronidazole by viable count on antibiotic-supplemented Columbia blood agar. Both antibiotics showed antimicrobial efficacy that was enhanced when combined and the authors praised the methodology as a useful susceptibility screen prior to selecting antibiotic therapy (Walter et al., 2011). However, agar-based oral biofilm assays do experience significant limitations primarily as a result of differences in nutrient flow compared to those exhibited in vivo. Originating from the substratum the
flow of nutrients does not reflect that observed in many in vivo biofilms, in particular the dental plaque biofilm which is attached to the hard surface of the teeth (McBain, 2009).

Batch culture biofilm modelling can also be undertaken using multi-well plates, with the well surface providing a point of attachment for biofilm growth. The primary function of these studies is the quantification of adherent biomass which can be determined by removing planktonic phase from the well, staining the adherent cells (usually by crystal violet), dissolving the stain with ethanol and using spectrophotometric analysis to determine the extent of biofilm formation in the well. Single species, consortia or saliva samples can be used and specific environmental conditions modified to monitor their impact on biofilm formation. Sanberg et al (2008) used this methodology to evaluate novel antimicrobial products against a biofilm forming strain of Staphylococcus aureus and described it as a useful screening tool (Sandberg et al., 2008). Such systems offer a high degree of reproducibility and are fairly simple and cheap to run, however they are limited by the fact that they are batch culture models which do not reflect conditions commonly observed in nature.

The addition of hydroxyapatite discs to the wells of multi well plates has also been documented in several batch culture biofilm models (Guggenheim et al., 2001; Ledder et al., 2009). Hydroxyapatite is a major component in the makeup of both bones and teeth and is therefore a good substratum for modelling dental biofilms. Ledder et al (2009) used 24 well tissue culture plates containing hydroxyapatite discs to evaluate the effects of the enzymes amylase, lipase and protease on early plaque development. Differential bacterial counts showed that
enzyme treatment resulted in no differences in the numbers of culturable bacteria (Ledder et al., 2009).

1.13.3. Chemostat Systems

A chemostat consists of a large vessel in which organisms of interest are cultivated in continuous culture. The vessel is fed with a constant flow of nutrients whilst the overflow is simultaneously removed, and thus the volume of growth media remains constant. Immersing a surface (hydroxyapatite, glass or dental enamel for example) into a largely planktonic bacterial culture growing in a chemostat frequently results in bacterial attachment and biofilm formation on the substratum (Wimpenny, 1999). These models are fairly simple and inexpensive to run and offer the opportunity to introduce various substrata that can be removed and analysed throughout a model run (McBain, 2009).

One of the first chemostat systems developed to utilise a suspended substratum system was employed in the study oral bacteria and dental biofilm formation (Bradshaw et al., 1996). Bradshaw et al (1996) studied the deposition of 10 species of oral bacteria onto a suspended hydroxyapatite disc over a period of 21 days. The authors noted that overall bacterial numbers in the biofilm increased over time, the proportion of anaerobic species was increased in older biofilms and that glucose pulsing selected for species that were considered cariogenic (Bradshaw et al., 1996). A more recent study by Knight et al (2008) used a continuous chemostat culture system to examine the ability of Streptococcus mutans and Lactobacillus acidophilus to form biofilms on ozone treated dentine discs. Ozone infusion prevented biofilm formation in the treated
samples whilst significant biofilm formation was observed in the controls (Knight et al., 2008).

1.13.4. Flow Cells

In order for an *in vitro* model of an oral biofilm to be representative the designer seeks to replicate *in vivo* conditions as closely as possible. Batch culture models and chemostat based systems lack the shear forces and fluid flow that are associated with biofilm formation in the oral cavity. These forces are likely to have a significant effect on the composition of the microbial community composition and characteristics and as such it is important that they are taken into account (Dunsmore et al., 2002; Stoodley et al., 1999). Flow cell systems incorporate laminar or turbulent flow of culture fluid through transparent chambers or composite blocks with transparent lids or capillary tubing into which bacteria or antimicrobials can be introduced (McBain, 2009). They allow for the microscopic examination of biofilm formation by oral bacteria under fluid flow conditions.

Foster and Kolenbrander (2004) used a saliva conditioned flow cell model to examine the impact of coaggregates on biofilm formation. Their device consisted of a glass cover slip secured to a polyethylene block into which several channels had been drilled. After pre-treatment with saliva the flow cells were inoculated with four bacterial strains (*S. gordonii*, *A. naeslundii*, *V. atypical* and *F. nucleatum*) together as coaggregates or separately in sequential order. The results showed that coaggregation that occurs in the planktonic phase influences the formation of a multi-species oral biofilm. Using FISH the authors showed that biofilms produced by inoculating each species separately
developed to a greater biovolume than biofilms derived from coaggregates. *S. gordonii* grew most successfully in monoculture and in mixed culture whilst coaggregates of *A. naeslundii* and *V. atypica* appeared in higher numbers than the same species added sequentially (Foster & Kolenbrander, 2004). Stoodley *et al* (1999) used the capillary flow model to determine the impact of laminar and turbulent fluid flow on single and mixed species biofilms. High shear forces resulted in deformation and thinning of single and mixed species biofilm structure and a change in properties of the biofilm from that representative of a viscoelastic solid to a viscoelastic liquid (Stoodley *et al.*, 1999).

### 1.13.5. Constant Depth Film Fermentors (CDFF)

The CDFF is a popular model for the study of oral biofilms because it can generate a large number of replicate biofilms whilst controlling biomass accumulation to a set depth, which some researchers believe produces a dynamic steady state similar to that observed *in vivo* (McBain, 2009). The apparatus consists of polytetrafluoroethylene (PTFE) pans fitted into fifteen holes in rotating stainless steel ring. Each pan contains plugs onto which discs (made from PTFE, hydroxyapatite, dentin etc.) are placed as a substratum for biofilm growth, the depth of which is controlled by pre-set blades that scrape the surface. Dental plaque models are usually set at a depth of 200 to 300µm (McBain *et al.*, 2003b; McBain *et al.*, 2003c; Pratten & Wilson, 1999). The growth medium is added in a drip-wise manner through the top of the sealed glass unit that holds the whole system with a waste outlet at the bottom (McBain *et al.*, 2003a; Wimpenny, 1999) (Figure 1.2.).
Oral biofilms can be maintained in this system at a set depth by rotation of the turntable below the scraper blades. The model is inoculated via the sampling port and a constant flow of nutrients maintained by drip-flow onto the turntable via the growth medium inlet. A total of 15 pans can be inserted into the device and each pan can hold up to 5 PTFE plugs. Diagram adapted from Pratten and Wilson (1999).

Frequently the CDFF has been employed in the study of the effects of antimicrobials on single and mixed species biofilms (McBain et al., 2003b; McBain et al., 2003c). Pratten et al (1998) utilised a CDFF to evaluate the growth of *Streptococcus sanguis* on PTFE, enamel and hydroxyapatite and in response to challenge by three different antimicrobial mouthwashes. The results of this study showed that the choice of substrata was significant in determining the ability of an antimicrobial to inhibit biofilm growth (Pratten et al., 1998b). Kinniment et al (1996) have also successfully used a CDFF in the study of biofilm formation by oral bacteria utilising a consortium of nine oral species (Kinniment et al., 1996a). Dental plaque microcosms were established in CDFFs by McBain et al (2003) to examine the effect of pulse feeding with complex nutrients on the composition and stability of dental plaque. Data
analysis by culture and PCR-DGGE showed that the feeding regimens impacted upon population dynamics (McBain et al., 2003a).

1.13.6. Single and multiple Sorbarod devices

Sorbarod filters were initially devised for the micropropogation of plant tissue (Donkin et al., 1989) and first employed in the study of bacteriology by Hodgson et al. (1995) to establish Staphylococcus aureus and Pseudomonas aeruginosa biofilms (Hodgson et al., 1995). They have since been used to support biofilm growth in an evaluation of the dynamics (Al-Bakri et al., 2004) and antimicrobial susceptibility of Pseudomonas aeruginosa biofilms (Driffield et al., 2008; Marques et al., 2005; Parveen et al., 2001), determining changes in gene expression in Neisseria meningitidis biofilms (O'Dwyer et al., 2009) and assessing antimicrobial susceptibility in Staphylococcus aureus (Gander et al., 2005; Haddadin et al., 2009) and Streptococcus pneumoniae biofilms (Budhani & Struthers, 1997). Sorbarod filters have been successfully employed to support oral microcosms derived from salivary inocula within the format of the multiple Sorbarod device (MSD) (Ledder et al., 2006; Ledder et al., 2009; McBain et al., 2005).

Figure 1.3. shows a schematic view of the single Sorbarod device that contains one Sorbarod filter onto which a biofilm of the chosen inocula may form. Growth media is applied in a drip-flow manner to the top of the filter and the effluent collected at the bottom. Cellulose fibres within the filter bind salivary proteins and mucins from the growth medium and provide a large surface area for colonisation and biofilm formation, resulting in a relatively large yield of biomass (McBain et al., 2005; McBain, 2009). A limited
Figure 1.3. Schematic representation of a single Sorbarod device. The cellulose fibres of the filter provide a surface for biofilm formation. Growth media is fed from the top of the device via the syringe needle whilst the effluent can be readily collected and analysed from the bottom. The filter can be retrieved and analysed at the end of the model run.

planktonic phase and constant fluid flow through the model is believed to be more representative of the conditions prevailing *in vivo* than chemostat-related systems (Sissons, 1997). As the Sorbarod devices are continuous flow systems
there is the opportunity to analyse the perfusate from the model in addition to the biofilm matter both by viable count and molecular microbiological methods.

Validated for use with complex oral bacterial communities by McBain et al (2005), the multiple Sorbarod device (MSD) uses cellulose Sorbarod filters to create a fermentation system similar to the single Sorbarod device (McBain et al., 2005). The MSD comprises a stainless steel chamber into which five Sorbarod filters can be inserted and replicate biofilms produced within the same unit (McBain, 2009). Ledder et al (2006) successfully used the MSD to assess inter-individual variations in the oral microflora (Ledder et al., 2006). The advantages of both Sorbarod systems lie in their simplicity, reproducibility and the ability to generate a large amount of biomass (McBain et al., 2005). As the MSD contains five filters this device allows for sampling biofilm material at points during the model run in addition to at the end point which is not possible with the single filter device. The biofilms obtained from both systems are highly heterogeneous although this is not problematic when modelling oral biofilms which tend to be heterogeneous *in vivo* (McBain, 2009).

1.14. Aims and objectives

The aims of this PhD thesis can be divided into three distinct sections with the general theme of investigating interactions between oral bacteria. Recent technological advancements in molecular microbiology have revealed that a significantly higher level of species diversity exists in the oral microbiota than previously thought and is unique to each individual exhibiting a high level of temporal stability. These observations call into question the generic models of oral bacterial communities that predominate in the literature and also the use of
pooled saliva and plaque samples in microcosm studies. By studying inter and intra-individual interactions between salivary-derived bacteria at the species and community level, the work described in Chapters 5 and 6 aimed to better understand the mechanisms that underlie bacterial interactions occurring in the individual oral microbiota.

Coaggregation has been observed in a number of naturally occurring polymicrobial communities and is believed to be an important process in the formation of oral biofilms, particularly dental plaque. Chapter 4 aimed to identify the extent to which the processes of bacterial coaggregation occur in another human-associated microbial community by studying selected numerically important isolates from the intestinal microbiota. Additionally, the ability of these intestinal species to coaggregate with selected oral isolates was also investigated.

The response of bacteria to antimicrobials in pure culture or via clinical in vivo studies forms a significant proportion of the research involving the oral microbiota. However, the microbial ecological effects of antimicrobial containing dentrifices on salivary-derived communities have received relatively little research attention. The work described in Chapter 3 of this thesis aimed to compare the impact of two dentrifices containing: stannous fluoride and zinc lactate (SZD) or triclosan (TD) on the microbial composition of bacterial communities derived from a single individual.
Chapter 2

General Experimental Methods
2.1. General methods

2.1.1. Chemicals and bacteriological growth media

Unless otherwise stated, all chemicals used were supplied by Sigma (Poole, Dorset, UK) or BDH (London, UK) and were of analytical reagent quality. Dehydrated growth media were supplied by Oxoid (Basingstoke, UK) and were rehydrated and prepared according to the manufacturer’s instructions.

2.1.2. Sterilisation of equipment, solutions and growth media

Bacteriological growth media and glassware were sterilised in an autoclave at 121°C for 15 min (1kg/cm²) (Bridson & Brecker, 1970). Larger volumes of media (>2L) were sterilised at 121°C for 30 mins. Solutions were heat sterilised by the same protocol or filter sterilised using a 0.22µm filter.

2.1.3. Growth of bacterial cultures

A variety of growth media were employed to support organisms derived from both the oral cavity and gastrointestinal tract. Wilkins Chalgren agar and broth were utilised to grow oral and intestinal isolates in both aerobic and anaerobic conditions. Additional selective, solid media were used to select for selected major functional groups of oral bacteria (Table 2.1.).

Mixed communities of oral bacteria were also grown in tryptone soya broth (TSB) and an artificial saliva solution which comprised mucin 2.5g/L, peptone 2g/L, tryptone 2g/L, yeast extract 1g/L, sodium chloride 0.35g/L, potassium chloride 0.2g/L calcium chloride 0.2g/L, cysteine 0.1g/L, haemin 0.001g/L and vitamin k1 0.0002g/L. Half strength thioglycollate broth was used for the serial
dilution of homogenised samples prior to differential bacteriological analysis where the maintenance of a reduced environment was necessary.

Table 2.1. Summary of bacterial growth media used throughout this thesis.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilkins Chalgren agar/broth</td>
<td>Culture of oral and intestinal isolates</td>
</tr>
<tr>
<td>Tryptone soya broth (TSB)</td>
<td>Culture of salivary bacteria</td>
</tr>
<tr>
<td>Artificial saliva</td>
<td>Feeding continuous culture microcosm models and culture of salivary bacteria</td>
</tr>
<tr>
<td>Half strength thioglycollate broth</td>
<td>Serial dilution of oxygen sensitive samples for viable counts</td>
</tr>
<tr>
<td>Wilkins Chalgren agar with gram negative supplements</td>
<td>Culture of Gram negative anaerobes</td>
</tr>
<tr>
<td>Rogosa agar</td>
<td>Selective culture of lactobacilli</td>
</tr>
<tr>
<td>Typtcase-yeast extract-citrate-sulphite (TYCS) agar</td>
<td>Selective culture of streptococci</td>
</tr>
</tbody>
</table>

Anaerobic cultures were maintained at 37°C in a Mark 3 Anaerobic Work Station (Don Whitely Scientific, Shipley, UK) (Gas mix: 80% N₂, 10% CO₂ and 10% H₂). Aerobic species were also incubated at 37°C in a standard incubator (Memmert incubator, Schwabach, Germany). Bacteria were cryopreserved at -60°C in nutrient broth supplemented with 10% glycerol.

2.2. DNA extraction for PCR and sequence analysis

2.2.1. Extraction of DNA from bacterial colonies

Bacterial colonies (2-4) were aseptically transferred from the agar plate to a sterile microcentrifuge tube containing 200μl of nanopure water. After vortex mixing for 1 min, the suspension was heated at 100°C in a boiling water bath for 10 mins followed by centrifugation at 13 226xg for 2 mins in a microcentrifuge (MSE Microcentaur; Sanyo, Loughborough, UK). The pellet was discarded and the liquid supernatant containing cellular DNA retained as a template for PCR amplification. The success of the extraction was assessed using gel
electrophoresis with 1% agarose gels and the extracted DNA stored in sterile microcentrifuge tubes at -60°C.

2.2.2. Extraction of DNA from bacterial consortia

Bacterial community DNA was extracted from liquid samples using a QiaAmp DNA stool mini kit (Qiagen, Sussex, UK). Bacterial suspensions (200µl) were macerated in the presence of a cell lysis buffer using a FastPrep FP120 bead beater (Qbiogene, California, USA) and 0.5g zirconia beads (full speed for 45 s) to mechanically aid cell lysis. DNA extraction was then completed according to the manufacturer’s protocol: The samples were further incubated in lysis buffer for 5 min at 70°C to maximise DNA yield. After lysis, DNA-damaging substances and taq polymerase inhibitors present in the sample were adsorbed onto a specialised matrix (InhibitEX tablets) and separated from the DNA by centrifugation. Proteins in the sample were digested and degraded under denaturing conditions during 70°C incubation with a proteinase. The DNA was then loaded onto the spin column and adsorbed onto a silica membrane by centrifugation. DNA bound to the membrane was washed in two centrifugation steps in order to remove any residual impurities. Purified, concentrated DNA was eluted from the spin column in a low-salt buffer. Eluted DNA extracts were stored in sterile microcentrifuge tubes at -60°C. DNA yield from this process is typically 15–60µg but, may range from 5–100µg.

2.2.3. Extraction of DNA from Sorbarod filters

Sorbarod filters were aseptically removed from the Sorbarod device and cut in half using a sterile scalpel. One half was utilised in culture analysis and the second retained in a sterile microcentrifuge tube for DNA extraction. Lysis buffer
from the QiaAmp DNA stool mini kit (Qiagen, Sussex, UK) was added to the filter along with 0.5g zirconia beads. To ensure total bacterial communities were removed from the filter, three bead beating steps at full speed for 45 s (FastPrep bead beater FP120; Qbiogene, California, USA) were performed prior to extraction with the kit. DNA extraction was completed according to the manufacturer’s protocol (see Section 2.2.2.) and eluted DNA extracts were stored in sterile microcentrifuge tubes at -60°C.

2.3. PCR amplification

2.3.1. PCR Amplification of 16S rRNA for sequence analysis

Amplification of partial 16S rRNA gene sequences was performed for the identification of bacterial cultures using the primers 8FPL1 (5′- GAG TTT GAT CCT GGC TCA G -3′) and 806R (5′- GGA CTA CCA GGG TAT CTA AT -3′) (Eurofins MWG Operon, Ebersberg, Germany) (McBain et al., 2003d) at 5 μM each. The reactions were performed in 0.2ml tubes with a DNA thermal cycler (T-gradient model, Biometra, Germany). Each PCR consisted of Red Taq DNA polymerase ready mix (25μl), forward and reverse primers (2μl each), nanopure PCR water (16μl) and extracted bacterial DNA (5μl). Positive and negative controls were included for each run; extracted DNA was substituted for nanopure PCR water (5μl) for the negative control and extracted DNA (5μl) from Staphylococcus aureus colonies for the positive control. The thermal program ran as follows: 35 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min. A 15 min chain elongation step (72°C) was incorporated into the final cycle (McBain et al., 2003d). The quality and quantity of DNA obtained by extraction and PCR was assessed using gel electrophoresis with 1% agarose gels.
2.3.2. PCR Amplification of 16S rRNA for DGGE

For DGGE analysis of DNA extracted from mixed community samples the eubacterium-specific primers HDA1 (including an additional GC clamp) (5'-CGCCGGCGC GCC CCG GGC GGG GGC GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3') (Ledder et al., 2006) were chosen to amplify the V2-V3 variable region of the 16S rRNA gene. Reactions were carried out in 0.2ml tubes using a DNA thermal cycler (T-gradient model, Biometra, Germany). Each reaction mixture consisted of Red Taq DNA polymerase ready mix (25µl), forward and reverse primers (2µl each), nanopure PCR water (16µl) and extracted bacterial DNA (5µl). Positive and negative controls were included for each run; extracted DNA was substituted for nanopure PCR water (5µl) for the negative control and extracted DNA (5µl) from Staphylococcus aureus colonies for the positive control. The thermal program ran as follows: 94°C for 4 min followed by 30 thermal cycles of 94°C for 30 s, 56°C for 30 s and 68°C for 60 s. The final cycle included a 7 min chain elongation step at 68°C (Ledder et al., 2006). The quality and quantity of DNA obtained by extraction and PCR was assessed using gel electrophoresis with 1% agarose gels.

2.3.3. Purification and sequencing of PCR products

PCR amplicons were purified in order to remove primers, nucleotides, enzymes and other impurities before sequence analysis was carried out. The QIAquick PCR purification kit (Qiagen, Sussex, UK) was utilised according to the manufacturer’s protocol to purify amplicons of 800 base pairs (bp) (from the primers 8FPL1 and 806R) and 200 bp (from the primers HDA1 and HDA2) in
length. The samples were first mixed with a DNA binding buffer and then added to a specialised spin column with a silica membrane designed to bind DNA. Unwanted primers and impurities do not bind to the silica membrane but flow through the column on centrifugation. An ethanol containing buffer was then applied to the membrane to wash out unwanted salts. DNA was eluted from the membrane by a basic, low salt elution buffer.

Purified PCR products (5µl) were analysed using gel electrophoresis with 1% agarose gels. A quantitative ladder (Hyperladder IV; Bioline, London, UK), with bands ranging from 100 bp to 1000 bp, was run alongside to facilitate the determination of DNA size and concentration.

Sequencing took place at an in-house laboratory. The reaction utilised the reverse primer (806R or HDA2) and was carried out in a Perkin-Elmer ABI 377 sequencer. The sequencing reaction was as follows: 94°C for 4 min followed by 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Nucleotide sequences were visualised using CHROMAS-LITE (Technelysium, Australia) software. A search for matching sequences was carried out using the European Molecular Biology Laboratories (EMBL) prokaryote database (http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html). The closest relative identified by the database search, the percentage similarity of the sequences and the number of ambiguous bases included in the test sequence were recorded.

2.4. Agarose gel electrophoresis

Gel electrophoresis, using 1% agarose gels, was utilised to assess the quality, size and quantity of DNA obtained by extraction and PCR amplification. The
gels were constructed with 0.4g agarose (Type I) diluted in 40ml Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. The TAE was diluted with distilled water from a 50 times stock solution containing: 40mM tris(hydroxymethyl)aminomethane (TRIZMA base), 20mM glacial acetic acid and 1mM ethylenediaminetetraacetic acid (EDTA) adjusted to pH 8.0 at 25°C. The solution was heated at full power in a microwave for 1 min to ensure complete dissolution of the agarose powder. Upon cooling, 4µl of the nucleic acid dye GelRed (Biotium, CA, USA) was added to the molten agarose solution for DNA staining.

Gels were cast in Bio-Rad mini gel tanks (Bio-Rad, Hemel Hempstead, UK) with comb in place to create 16 wells. When the agarose gel had solidified the comb was removed and 5µl of DNA extract or PCR product was loaded into each well. Extracted DNA and purified PCR products were mixed with 1µl loading dye (Coloured loading buffer, blue; Bioline, London, UK) before loading. Red Taq DNA polymerase ready mix includes a loading dye and thus unpurified PCR products did not require additional loading buffer. The gel was then submerged in approximately 400ml TAE buffer. 5µl of quantitative ladder (Hyperladder IV; Bioline, London, UK), with bands ranging from 100 bp to 1000 bp, was run alongside the samples to facilitate the determination of DNA size and concentration. The gels underwent electrophoresis at 70V for 1 hour. Visualisation of stained nucleic acids occurred under UV illumination (UV transilluminator; UVP, California, USA) at 312nm and was photographed using a Canon EOS D60 digital camera (Canon, Surrey, UK).
2.5. Denaturing gradient gel electrophoresis (DGGE)

2.5.1. Preparation of DGGE solutions

DGGE solutions were prepared at two different denaturant concentrations; 30% (low) and 60% (high), in order to cast gels with a denaturing gradient. The denaturants used were formamide (40% v/v in an undiluted solution) and urea (7.0M in an undiluted solution). In addition, all gels contained 10% acrylamide prepared from 40% v/v acrylamide/bis-acrylamide (ratio of 37.5:1). The reagents were dissolved in TAE buffer (see Table 2.1.), filtered through a 0.45µm filter under a vacuum and degassed for 10 mins. Solutions were protected from light and stored at 4°C prior to use.

Table 2.2. Summary of denaturant solution composition.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>30%</th>
<th>60%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrylamide/bis-acrylamide 40% (v/v)</td>
<td>25%</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>50 x TAE buffer (v/v)</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
<tr>
<td>formamide (v/v)</td>
<td>12%</td>
<td>24%</td>
<td>40%</td>
</tr>
<tr>
<td>urea (M)</td>
<td>2.1</td>
<td>4.2</td>
<td>7.0</td>
</tr>
<tr>
<td>deionised water</td>
<td>to 100ml</td>
<td>to 100ml</td>
<td>to 100ml</td>
</tr>
</tbody>
</table>

2.5.2. Assembly of DGGE gel apparatus

DGGE analysis was carried out using the DCode Universal Mutation Detection System (Bio-Rad, Hemel Hempstead, UK). Prior to casting a gel the equipment was prepared according to the manufacturer’s protocol. Two glass plates (16cm by 20cm and 18cm by 20cm) were placed on top of one another with the larger plate at the back. Plastic 1mm spacers were placed on both sides in between the plates. The glass plates were clamped together to form the gel “sandwich”. Correct plate alignment was ensured by placing the sandwich in a casting
stand. Before casting a gel foam sealants were placed underneath the assembly to prevent leakage.

2.5.3. Casting of DGGE gels

High and low DGGE solutions containing high and low concentrations of denaturants were prepared as described in Section 2.4.1. To prepare gels 15ml of each solution was decanted into separate plastic Universal bottles. For ease of identification and gradient visualisation, 300µl of D-Code dye (bromophenol blue 0.5% w/v and xylene cyanol 0.5% w/v in 10ml TAE buffer) was added to the high solution. Gel polymerising agents were then added to each solution (50µl of tetramethylethylenediamine (TEMED) and 100µl of ammonium persulphate (APS). Each solution was drawn up into separate 30ml syringes and agitated to remove any air bubbles. The syringes were then placed into the DCode gradient delivery system. Plastic tubing attached each syringe to a sterile 19 gauge needle (Fisher Scientific, Leicestershire, UK) which was placed between the glass plates of the gel sandwich assembly. The wheel of the gradient delivery system was then turned to push the solutions from the syringes and into the sandwich. A constant turning speed was maintained to ensure the gradient formation was even and correct. Once the solutions had been delivered the needle was removed and a 16 well comb inserted between the glass plates. The gel was left to polymerise at room temperature for a minimum of 1 hour.

Polymerised DGGE gels were removed from the casting stand and the 16 well comb gently removed from the sandwich. Wells were flushed with TAE buffer to remove gel fragments and to check well integrity.
2.5.4. Loading and running DGGE gels

Polymerised gel sandwiches were attached to the core module of the DCode Universal Mutation Detection System and placed into a gel tank filled with 7L of TAE buffer. After overnight submersion which, in previous validation studies, has been shown to produce better separation of PCR amplicons, the gel tank was heated to 60°C. PCR amplicons (45μl) to be analysed by DGGE were homogenised with 10μl of loading dye (bromophenol blue 2% w/v, xylene cyanol 2% w/v and glycerol 70% v/v in deionised water). Samples were loaded into the wells, excluding the outer lanes, using gel-loading tips. At least one well on every gel was loaded with a comparator sample (comprising PCR amplicons from a standard saliva sample), or ladder, to facilitate matching of bands between independent gels. Electrophoresis was carried out for 750 volt hours at 60°C.

2.5.5. Staining and visualisation of PCR amplicons within DGGE gels

The gel sandwich was removed from the gel tank and core module following completion of 750 volt hours of electrophoresis. After disassembly, the glass plate from the top of the sandwich was removed and the lower plate, together with attached polyacrylamide gel was immersed in 200ml of TAE buffer. In order to stain the DNA 20μl of SYBR® Gold nucleic acid gel stain (Molecular Probes, Leiden, The Netherlands) was added and the gel agitated to ensure even stain distribution. After staining for 20 mins with intermittent agitation, the gel was visualised under UV light at 312nm with a UV transilluminator (UVP, California, USA). Each gel was photographed under UV light using a Canon EOS D60 digital camera (Cannon, Surrey, UK).
2.5.6. Excision of bands from DGGE gels

The polyacrylamide gel was illuminated under UV light at 312nm (UV transilluminator; UVP, California, USA) and bands of interest were identified. Each band was carefully excised from the gel using a sterile scalpel and placed in a nuclease-free tube containing 50µl of nanopure water. The excised bands were stored overnight at 4°C to ensure diffusion of DNA from the band into the nanopure water. Band position on the gel was recorded and excised bands stored at -60°C.

2.5.7. Identification of DNA from excised bands

Excised bands in 50µl nanopure water were vortexed for 30s to ensure distribution of bacterial DNA in the water and then centrifuged for 10 min at 13 000 rpm (MSE Microcentaur, Sanyo, Loughborough, UK) to pellet the gel. PCR amplification of the DNA was undertaken as outlined in Section 2.3.2. using 5µl of supernatant. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Sussex, UK) according to the manufacturer’s instructions. Agarose gel electrophoresis, sequencing and sequence analysis were performed as described in Section 2.3.3.

2.5.8. DGGE gel analysis and dendrogram construction

Alignment of DGGE gel photographs was done using Adobe Photoshop CS2 software (Adobe, California, USA). Manual alignment was facilitated by the inclusion of two marker lanes per gel, produced with DNA extracted from a single murine faecal sample. Image quality was optimised to reduce background noise and maximise horizontal alignment. Relative similarity between lanes was determined by analysis of the aligned gel image using
Bionumerics software (Applied Maths, Saint-Martens, Latem, Belgium). Band assignment was completed manually for each lane and an unweighted pair group method with arithmetic mean (UPGMA) dendrogram produced by comparison of the band profiles. The UPGMA algorithm computes the average similarity of each lane to an extant cluster (Sneath & Sokal, 1973). The results of which can be used to observe similarities and clustering patterns between samples that have undergone DGGE analysis.

2.5.9. Principal component analysis (PCA)

Principal component analysis (PCA) allows the large amount of data produced from cluster analysis to be condensed into a smaller number of principal components that describe the majority of the variability between samples. It also has an advantage over dendrogram analysis in that it is non-hierarchical in nature and the similarity of two samples can be ascertained by determining the distance between two plots. PCA was undertaken by first selecting the band matching option for gels that had undergone cluster analysis with Bionumerics software. This generated a table of similarity matrices that were exported and analysed using the statistical package SPSS (version 16.0, SPSS Inc.). The number of principal components extracted was determined by the eigenvalue of each: those contributing 10% or greater to the total variance were included in the analysis. Plots of the components explaining the greatest amount of variance were prepared using SigmaPlot software (version 10, Systat Software Inc., Chicago, USA).
Chapter 3

Comparison of the effects of a stannous fluoride/sodium hexametaphosphate dentrifice and a triclosan/sodium fluoride dentrifice on the bacterial composition of in-vitro oral microbiotas
3.1. Abstract

Dental plaque is a diverse microbial consortium which plays a role in the maintenance of oral health and also in the initiation of dental caries and periodontitis. Most oral hygiene products are designed to achieve non-specific plaque removal as determined by reductions in clinical plaque accumulation indices. The microbial ecological effects of dentrifices and the associated active ingredients have received relatively little research attention. The present chapter compared the effect of exposure to a triclosan-based dentrifice (TD) and a stannous fluoride-based dentrifice (SZD) on bacterial growth, acid production and bacterial composition in salivary-derived biofilm communities. 

**Methods:** A preliminary screening method was used to assess the effects on bacterial viability and acidogenesis and thereafter, eubacterial-specific PCR and PCR-denaturing gradient gel electrophoresis (DGGE) were used to determine the effect of the test dentrifices on bacterial composition of salivary consortia. 

**Results:** Exposure to either dentrifice for 16h caused reductions in microbial diversity and resulted in the emergence of distinct community profiles, which were most marked for TD. TD more potently inhibited bacterial growth and acid production than did SZD. **Conclusions:** TD exhibited greater antimicrobial potency than SZD which manifested as i) larger reductions in consortia acidogenesis and ii) larger decreases in bacterial diversity of exposed *in vitro* plaques.
3.2. Introduction

Bacterial succession is a naturally occurring process in all microbial communities, including the microbial community that comprises dental plaque, and is believed to influence the arrangement and species diversity of the developed ecosystem (Kolenbrander et al., 2006); playing an integral role in maintaining microbial homeostasis (Alexander, 1971). Numerous studies have shown that, despite constant microbial immigration and food intake, oral biofilms can demonstrate remarkable stability over time (Lazarevic et al., 2010; Marsh, 2003; Rasiah et al., 2005; Tannock, 1995). However, perturbations of key environmental factors such as the availability of fermentable sugars and associated reductions in pH, plaque accumulation and variations in saliva flow, may result in alteration in the composition of the oral microbiota (Loesche, 1986; Marsh, 1994). The “ecological plaque hypothesis” proposes that these environmental changes are intrinsically linked to an increase in the pathogenic effects of the community, resulting in a shift in the oral environment from a state of health to one implicated in the initiation and perpetuation of disease (Marsh, 1991; Sissons et al., 2007).

Although the aetiology of dental disease (specifically periodontitis and dental caries) is no longer believed by many researchers to be directly attributable to a small number of specific species (i.e. the specific plaque hypothesis (Marsh, 2003)), a limited subset of bacteria are consistently isolated in higher numbers from carious lesions and diseased periodontium (Haffajee et al., 1998; Tanner et al., 2007; Ximenez-Fyvie et al., 2000). Acidogenic and aciduric species, such as Streptococcus mutans and homofermentative lactobacilli, are often
associated with dental caries because they can rapidly metabolise dietary carbohydrate to acid, which in turn demineralises the tooth enamel (Bowden, 1990; Loesche, 1986; Sissons et al., 2007). The non-specific plaque hypothesis attributes the formation of dental caries to interactions occurring between all groups of bacteria found in dental plaque (Marsh, 2003). The ecological plaque hypothesis however, suggests that there is no specific aetiology: any species with the right properties can contribute to disease progression, subject to selection pressure from changes in the oral environment. In the case of dental caries, the presence of fermentable sugars in the oral cavity exerts selection pressure for acidogenic species to metabolise the carbohydrate and where they are aciduric, also to increase in number at the expense of non-aciduric species. Despite many researchers now favouring the ecological plaque hypothesis over the specific plaque hypothesis a diet high in fermentable carbohydrate has been shown to result in an increase in the proportion of mutans streptococci and a concomitant fall in the numbers of other streptococci (Marsh, 1994), particularly Streptococcus sanguis (De Stoppelaar et al., 1970; Minah et al., 1985; Staat et al., 1975).

With respect to oral disease of the soft tissue, the aetiology of gingivitis and periodontitis has been associated with the incidence of high numbers of obligate anaerobic Gram-negative bacteria including Fusobacteria, Porphyromonas, Treponema and Prevotella in mature dental plaque communities (Haffajee et al., 1998; Kroes et al., 1999; Ledder et al., 2007; Mombelli et al., 1998; Moore & Moore, 1994; Socransky et al., 1998). The ecological plaque hypothesis for periodontitis suggests that plaque accumulation around the gingival margin initiates a host inflammatory response and that is associated with an increase in
gingival crevicular fluid (GCF) flow that provides the proteinaceous nutrients favoured by many of the obligately anaerobic oral species (Marsh, 1994). Subsequent tissue damage has been attributed to structural and secreted components of the altered bacterial microflora causing direct damage to periodontal tissue or modulating the host immune response (Madianos et al., 2005; Marsh, 1994). Implicated in this process are bacterial lipopolysaccharides (LPS), fimbriae, proteases and toxins (Madianos et al., 2005). Stimulation of the host immune system is associated with transmembrane toll-like receptors (TLRs) in conjunction with soluble and membrane bound CD14 proteins (Madianos et al., 2005) and the ensuing production of cytokines that disrupt the balance between tissue-degrading metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) (Reynolds et al., 1994). Both periodontitis and dental caries represent a significant burden on the healthcare system and are largely responsible for the commercial interest in the development of more effective oral hygiene formulations because the morbidity of both diseases is extremely high in the adult population (Allaker & Douglas, 2009; Loesche, 1986; Marsh & Martin, 2003; McBain et al., 2005).

The two commercially available dentrifices selected for the current study contain different commonly utilised antimicrobials of proven oral hygiene efficacy in both human and in vitro studies: triclosan and stannous fluoride. Triclosan, a commonly used bisphenol antibacterial, was first introduced as a topical antiseptic agent in the 1960s and since then has been widely used in dentrifice formulations (McBain et al., 2003b). Triclosan possesses broad-spectrum antibacterial activity (Regos & Hitz, 1974), and is thought to act primarily by disrupting bacterial cell membranes (Villalain et al., 2001) and thereby cell
permeability and barrier function (Russell, 2004). At low concentrations triclosan is bacteriostatic, binding to the enzyme enoyl-ACP reductase (encoded by the fabI gene). This increases the enzyme's affinity for nicotinamide adenine dinucleotide (NAD\(^+\)) and results in the formation of a stable complex which is unable to participate in fatty-acid elongation (Heath et al., 1998). For oral pathogens lacking in the fabI gene, such as Streptococcus mutans, triclosan is believed to inhibit glycolysis by targeting the phosphoeneolpyruvate:sugar phosphotransferase system (PTS) and glycolytic cytoplasmic enzymes (Phan & Marquis, 2006). Phan and Marquis (2006) ascertained this by utilising enzyme assays to show that triclosan is a multi-target inhibitor for a number of enzymes associated with mutans streptococci, thereby inhibiting glycolysis. Ultimately, this could result in a decrease in acidogenesis and the incidence of caries; however the non-specific bacteriostatic/bactericidal actions of triclosan are likely to achieve the same general suppression of plaque growth and metabolism. Numerous clinical studies have highlighted the effectiveness of triclosan as an oral hygiene agent; active against oral bacteria (Fine et al., 2006; Moran et al., 2001; Svatun et al., 1993) with anti-inflammatory properties (Paraskevas, 2005). The added anti-inflammatory action of triclosan is thought to be mediated by the inhibition of the oxygenase/lipoxygenase pathway in the metabolism of arachidonic acid, a key inflammatory intermediate (Paraskevas, 2005).

Stannous fluoride has been incorporated into dentrifice formulations since the early 1950s (Paraskevas, 2005; Van Loveren, 1990). The anti-caries properties of this compound result from stannous ions adsorbing onto bacterial cell membranes and disturbing membrane transport processes, and through the inhibition of enzyme systems involved in the fermentation of sugars (Svatun &
Attramadal, 1978) In addition to this, the binding of divalent tin cations to negatively charged plaque components is believed to inhibit bacterial coaggregation and coadhesion, the process by which dental plaque communities develop (Skjorland et al., 1978). *Streptococcus mutans*, frequently associated with dental caries, appears to be particularly susceptible to stannous fluoride (Camosci & Tinanoff, 1984). The bulk of clinical studies undertaken with stannous fluoride show statistically significant anti-caries efficacy and improvements in gingivitis when compared with control dentrifices. There is some evidence to indicate that it is an effective general antiplaque agent (Paraskevas, 2005).

Molecular techniques for profiling microbial communities such as PCR denaturing gradient gel electrophoresis (DGGE) have been successfully used in many previous studies of oral microbial ecology (Diaz et al., 2006; Fujimoto et al., 2003; Li et al., 2006; Maukonen et al., 2008; McBain et al., 2003a; Muyzer & Smalla, 1998; Perea, 2004; Rasiah et al., 2005; Siqueira et al., 2005; Spratt, 2004; Zijing et al., 2003). In this chapter PCR-DGGE (using PCR primers specific for the V2-V3 region of the 16S rRNA gene) was used to compare the bacterial profiles of dentrifice treated (triclosan (TD) and stannous fluoride (SZD) based) and untreated salivary-derived bacterial communities. Analysis of partial 16S rRNA gene sequences, excised from the DGGE gel, were used to identify abundant members of each community. In addition to the qualitative assessment, PCR-DGGE provided an account of diversity in each sample (Li et al., 2006). Culture-based microbiological techniques were also utilised; the impact of dentrifice treatment on bacterial growth was studied using a spectrophotometric assay of salivary-derived cultures growing in the presence
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of varying concentrations of dentrifice. From this the minimum concentration of dentrifice required to inhibit the growth of the consortia of oral bacteria was determined. Additionally, a novel pH assay using the pH sensitive, colour indicator phenol red was developed. The pH dependent colour changes were used to gauge the effect of each dentrifice, at varying concentrations, on bacterial acid production.

Whilst a large body of evidence substantiates the role of triclosan and stannous fluoride in dental plaque control and disease prevention, the vast majority comprise clinically based studies with clinical assessment outcomes including reduction in plaque volume, reduced gingival bleeding on probing and reductions in tooth loss (Paraskevas, 2005). Although such studies have provided valuable data regarding treatment outcomes (Li et al., 2006), the impact of the composition of these antimicrobials on oral microbial ecology is yet to be fully elucidated. Key questions regarding their effect on the oral environment include, do they perturb microbial homeostasis and do they inhibit or encourage the growth of potential pathogens? The principle aim of the present chapter therefore was to compare the impact of two dentrifices containing: stannous fluoride and zinc lactate (SZD) or triclosan (TD) on the microbial composition of salivary-derived communities.
3.3. Materials and Methods

3.3.1. Preparation of dentifrice slurries

Colgate Total® (Colgate-Palmolive, 300 Park Avenue, New York, USA) and Crest Pro-Health™ (Procter & Gamble, Cincinnati, Ohio, USA) dentrifices were supplied by a representative of the Colgate-Palmolive company. Both products were available for retail sale in the USA.

Slurries of Colgate Total® (TD) (triclosan 0.3% w/w, sodium fluoride 0.32% w/w) and Crest Pro-Health™ (SZD) (stannous fluoride 0.454% w/w) toothpastes (100mg/ml) were prepared in both tryptone soya broth (TSB) and artificial saliva. Artificial saliva comprised: mucin, 2.5g/L; peptone, 2g/L; tryptone, 2g/L; yeast extract, 1g/L; sodium chloride, 0.35g/L; potassium chloride, 0.2g/L; calcium chloride, 0.2g/L; cysteine, 0.1g/L; haemin, 0.001g/L and vitamin k₁, 0.0002g/L. Toothpaste (10g) was added to a pre-sterilised 250ml glass, screw top flask containing approximately 1850 glass beads (50cm³, 3mm diameter) and 100ml of growth media. Slurries were vigorously agitated at approximately 800rpm using a Griffin Flask Shaker (Wrist action shaking machine, Griffin and George, Loughborough, UK) for 2h.

Particulate matter within the dentifrice formulation, including dental silicas, carrageenan, sodium lauryl sulphate (SLS) and titanium dioxide, inhibited the spectrophotometric assay. In order to separate the antimicrobial constituents, which were believed to be soluble, from the particulate matter 50ml of slurry was subjected to centrifugation at 14400xg for 15min (Beckman-Coulter J2-21) and the supernatant collected. The remaining 50ml of non-centrifuged slurry was retained for comparison of antimicrobial activity with the supernatant to
ensure antimicrobials had not been lost during the centrifugation step. The process was repeated to obtain eight distinct slurries (Table 3.1.). All mg/ml values given in this chapter are calculated on the basis of the weight in volume of dentrifice prior to centrifugation and thus provide an indication of the amount of dentrifice required to produce a given effect not the weight of soluble antimicrobial(s).

Table 3.1. Summary of dentrifice slurries used in the microtitre plate assay, pH assays and molecular analysis.

<table>
<thead>
<tr>
<th>Centrifuged</th>
<th>Non-centrifuged</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) TD (100mg/ml) in TSB</td>
<td>(5) TD (100mg/ml) in TSB</td>
</tr>
<tr>
<td>(2) TD (100mg/ml) in artificial saliva</td>
<td>(6) TD (100mg/ml) in artificial saliva</td>
</tr>
<tr>
<td>(3) SZD (100mg/ml) in TSB</td>
<td>(7) SZD (100mg/ml) in TSB</td>
</tr>
<tr>
<td>(4) SZD (100mg/ml) in artificial saliva</td>
<td>(8) SZD (100mg/ml) in artificial saliva</td>
</tr>
</tbody>
</table>

3.3.2. Phenol red assay of bacterial acidogenesis

The inhibition of salivary bacterial acidogenesis by dentrifice slurries under both aerobic and anaerobic conditions was investigated using a pH indicator and a microtitre plate assay (96 well, BD Falcon™, Oxford, UK). Incorporated into the methodology was a study of the effects of a centrifugation step to remove surplus particulate matter from the slurry.

A serial dilution of each of the eight slurries (prepared as detailed in Table 3.1.) was performed horizontally across microtitre plates, with concentrations ranging from 100mg/ml to 0mg/ml toothpaste. Initial tests had shown that the bacterial acidogenesis inhibition breakpoint for both dentrifrices fell between 100mg/ml and 0.39mg/ml. Each well of the 96 well plate contained 194µl of dentifrice slurry, 2µl saliva (total concentration 1% v/v) and 4µl phenol red (total concentration 2% v/v). All rows were prepared in duplicate, plate layouts are detailed in Figure 3.1. Plates were maintained anaerobically in a Mark 3
Anaerobic Work Station (Don Whitely Scientific, Shipley, UK) (Gas mix: 80 % N₂, 10 % CO₂ and 10 % H₂) or aerobically (MEMMERT incubator, Schwabach, Germany) at 37°C for 16h. The plates were photographed using a Canon EOS D60 digital camera (Cannon, Surrey, UK).

![Figure 3.1. Microtitre plate layout for the phenol red assay of bacterial acidogenesis. Microtitre plates (n=4) were prepared as detailed in this figure; utilising TSB as the growth media (n=2) and artificial saliva (n=2). Each well contained 194µl of dentifrice slurry, 2µl saliva (total concentration 1% v/v) and 4µl phenol red (total concentration 2% v/v).]

### 3.3.3. Microtitre plate assay of bacterial growth inhibition

Dentifrice slurries were prepared as detailed in Section 3.3.1. and diluted to concentrations ranging from 3mg/ml to 0mg/ml (TD) and 4.5mg/ml to 0mg/ml (SZD), as detailed in Figure 3.2. The concentration ranges were determined by an initial screen for the bacterial growth breakpoint using dentifrice at concentrations ranging from 100mg/ml to 0mg/ml. A microtitre plate (96 well, BD Falcon™, Oxford, United Kingdom) was utilised to assess the effect of the dentifrice on the growth salivary-derived microorganisms. Each well contained 198µl of dentifrice slurry with 2µl (total concentration 1% v/v) saliva. Negative controls comprised 200µl growth media without dentifrice or salivary inocula. The plate was incubated aerobically in a microtitre plate reader (PowerWave™ XS: BioTek, Bedfordshire, UK) at 37°C for 16h. Spectrophotometric readings
were taken at 470nm every 20min throughout the incubation period. The incubation protocol included a plate shaking step (at the medium setting to provide sufficient agitation to obtain a true reflection of the absorbance but without causing overspill between wells) 10s prior to each absorbance reading.

**Figure 3.2.** Microtitre plate layout for the assessment of growth inhibition of salivary-derived bacteria. Each well contained either TSB or artificial saliva with and without dentifrice as indicated above, and was prepared in duplicate. All wells (except controls) contained 2µl saliva.

### 3.3.4. Molecular determination of the taxonomical selectivity of TD and SZD in artificial saliva and TSB milieu

Dentifrice slurries (as detailed in Section 3.3.1.) were prepared and centrifuged at 14400xg for 15min (Beckman-Coulter J2-21). The supernatant was collected and diluted to concentrations ranging from 3mg/ml to 0.3mg/ml dentifrice in 10ml aliquots. Suspensions of growth media without dentifrice were also prepared. Each was inoculated with 100µl (1% v/v saliva) and incubated aerobically at 37°C (MEMMERT incubator, Schwabach, Germany) for 16h.

### 3.3.5. DNA extraction, PCR amplification and DGGE analysis

DNA was extracted from the dentifrice slurries and growth media inoculated with saliva using a QiaAmp DNA stool mini kit (Qiagen, Sussex, UK) as
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described in Section 2.2.2. The control sample comprised a pure culture of *Staphylococcus aureus*. The extracted DNA was then amplified by polymerase chain reaction (PCR) with primers specific for the V2-V3 region of the eubacterial 16S rRNA gene: HDA1 (including an additional GC clamp) (5’-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5’-GTA TTA CCG CGG CTG CTG GCA C-3') (Ledder et al., 2006) (Section 2.3.2.). The quality and quantity of DNA obtained from this process was ascertained using agarose gel electrophoresis as described in Section 2.4.

Extracted and amplified DNA from 12 different samples containing varying concentrations of dentrifice were chosen to undergo DGGE analysis alongside the positive control. Sample selection aimed to provide a representative concentration range of both dentifrices and include both types of media. DGGE analysis was performed as detailed in Section 2.5. The resultant gel was visualised under UV light at 312nm with a UV transilluminator (UVP, California, USA) and photographed using a Canon EOS D60 digital camera (Cannon, Surrey, UK). PCR amplicons (i.e. bands) of interest were excised from the polyacrylamide gel by the process described in Section 2.5.6. and DNA sequences identified as detailed in 2.5.7.

Gel images were optimised using Adobe Photoshop CS2 software (Adobe, California, USA) to reduce background noise and lane smiling which may have hindered analysis. A relative similarity between lanes was ascertained by analysis of the aligned gel image using Bionumerics software (Applied Maths, Saint-Martens, Latem, Belgium). See Section 2.5.8. for details. The results were
used to observe similarities in the DNA profiles and clustering patterns between the samples, and to produce a hierarchical dendrogram. Principal component analysis was performed using the SPSS statistical package (Version 16.0, SPSS Inc.) as detailed in Section 2.5.9.
3.4. Results

3.4.1. Phenol red assay of bacterial acidogenesis

Bacterial acidogenesis in the presence of varying concentrations of dentifrice was assessed with a microtitre plate assay using phenol red as a pH indicator. At a pH 6.6 or below the phenol red indicator is yellow in colour; above pH 6.6 it is red. Figure 3.3. shows an image of the microtitre plates used in the assay.

Figure 3.3. Phenol red assay of bacterial acidogenesis in the presence of varying concentrations of dentifrice. At pH 6.6 or below the phenol red indicator is yellow, above pH 6.6 it is red in colour.

Yellow colouration observed in the control wells (TSB plates only) demonstrated that the salivary-derived bacteria generated acid when growing in the absence of dentifrice. Acid production was inhibited by the presence of both TD and SZD at different concentrations. TD was a more potent inhibitor of bacterial acid production requiring a concentration end point of 0.78mg/ml to inhibit acid production compared to 3.13mg/ml for SZD. This effect was observed under aerobic incubations.
both aerobic and anaerobic environmental conditions. Centrifugation of
dentifrice slurries did not influence the pH values. The blue colour of SZD
toothpaste impeded the phenol red assay at concentrations above 12.5mg/ml.
However, the pH related colour change was at a dentifrice concentration lower
than 12.5mg/ml and therefore it did not impact on the results (when TSB was
used as the growth medium).

An unknown component of the artificial saliva solution underwent a chemical
reaction with the phenol red indicator and the artificial saliva assays were
unsuccessful. Although the wells containing TD showed inhibition of acid
production at a concentration of 100mg/ml, control wells were unreliable and
varied in colour. Discolouration and unreliable controls were also noted for SZD.
Again, no discernable differences were identified between samples subjected to
centrifugation and those that were not.

3.4.2. Microtitre plate assay of the inhibitory effects of dentifrices on
salivary-derived bacteria

The minimum concentration of TD required to prevent bacterial growth from the
salivary sample was 1.8mg/ml in TSB and 2.7mg/ml in artificial saliva. The
concentrations of SZD required were 3.3mg/ml in TSB and 3.6mg/ml in artificial
saliva.

3.4.3. Estimation of the increase in lactic acid concentration necessary to
initiate a colour change in a solution containing phenol red indicator

When dentifrice was added to the wells of the microtitre plates at concentrations
ranging from 0.78mg/ml to 1.8mg/ml and 3.13mg/ml to 3.3mg/ml (TD and SZD
respectively) bacterial growth was observed in the microtitre plates whilst the
results of the pH assay indicated that acid production by salivary bacteria was inhibited. The calculation detailed below was performed to determine whether this effect could be attributed to dentrifice-catalysed inhibition of bacterial acid production, or simply a reduction in overall bacterial numbers resulting in the surviving bacteria producing acid as normal but not at concentrations sufficient to lower the pH and produce a colour change. Solutions containing phenol red indicator change from red to yellow when the pH decreases from 6.6 to 6.5. Lactic acid is the strongest acid product of most bacteria and the primary acid produced by oral bacteria, therefore the $pK_a$ of this acid was used in the calculation (Kuramitsu et al., 2007; Muntz, 1943):

$$\text{pH} = \frac{1}{2} (pK_a - \log C_0)$$

Where $C_0$ is the concentration of lactic acid and the $pK_a$ of lactic acid is 3.86. To decrease the pH from 6.6 to 6.5 the lactic acid concentration must increase from $4.5 \times 10^{-10}$ M to $7.2 \times 10^{-10}$ M. The volume of the well in the microtitre plate was 200$\mu$l and thus the amount of lactic acid needed to produce this pH change is $5.4 \times 10^{-14}$ moles. The average concentration of lactic acid in saliva is estimated to be ca. 2.273mM and therefore in the 2$\mu$l inoculum approximately $4.54 \times 10^{-9}$ moles (Linke et al., 1997). Increases in lactic acid concentration in response to food intake have been shown to be in the region of ca. 6.5mM ($1.3 \times 10^{-8}$moles in 2$\mu$l) (Linke et al., 1997). Therefore, in order to decrease the pH of a 200$\mu$l well from 6.6 to 6.5 the number of moles of lactic acid needed is approximately 241000 times smaller than the number of moles normally produced in response to food intake.
3.4.4. Molecular determination of the selectivity of TD and SZD in artificial saliva and TSB milieu

Molecular analysis by PCR-DGGE revealed differences in the DNA profiles between bacterial communities, derived from the same saliva sample, when cultured in the presence of different dentrifice slurries and different growth media. Visual inspection of the gel shows variations in band patterns and intensity between all the samples (Figure 3.4.).

![Figure 3.4. Negative image of a parallel DGGE gel showing the profiles of selected saliva/dentrifice slurries. The diagrammatic representation of migrated bands positioned to the right of the photograph and the highlighted bands on the gel show those that were selected for excision and sequencing. The numbering system corresponds to data in Table 3.2.](image-url)
Table 3.2. Sequencing and identification of PCR amplicons derived from DGGE gels

<table>
<thead>
<tr>
<th>Band</th>
<th>Closest relative (% sequence similarity)</th>
<th>Sequence Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Streptococcus mitis.</em> EF473998 (98)</td>
<td>173 (0)</td>
</tr>
<tr>
<td>2</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (93)</td>
<td>174 (0)</td>
</tr>
<tr>
<td>3</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (91)</td>
<td>173 (0)</td>
</tr>
<tr>
<td>4</td>
<td>Unidentified oral bacterium SH25 AB028342 (92)</td>
<td>173 (0)</td>
</tr>
<tr>
<td>5</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (97)</td>
<td>177 (1)</td>
</tr>
<tr>
<td>6</td>
<td><em>Streptococcus mitis</em> strain C115 EF473998 (98)</td>
<td>174 (1)</td>
</tr>
<tr>
<td>7</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>176 (0)</td>
</tr>
<tr>
<td>8</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (98)</td>
<td>174 (1)</td>
</tr>
<tr>
<td>9</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (99)</td>
<td>173 (1)</td>
</tr>
<tr>
<td>10</td>
<td>Uncultured bacterium AB099790 (98)</td>
<td>171 (1)</td>
</tr>
<tr>
<td>11</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (98)</td>
<td>175 (1)</td>
</tr>
<tr>
<td>12</td>
<td>Uncultured <em>Streptococcus</em> sp. DQ016888 (94)</td>
<td>170 (2)</td>
</tr>
<tr>
<td>13</td>
<td><em>Bacillus cereus</em> strain EF528295 (99)</td>
<td>177 (1)</td>
</tr>
<tr>
<td>14</td>
<td><em>Bulleidia extracta</em> AF220064 (80)</td>
<td>170 (1)</td>
</tr>
<tr>
<td>15</td>
<td><em>Streptococcus mitis</em> strain C115 EF473998 (98)</td>
<td>176 (1)</td>
</tr>
<tr>
<td>16</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (96)</td>
<td>173 (1)</td>
</tr>
<tr>
<td>17</td>
<td>Uncultured <em>Veillonella</em> sp. DQ677558 (99)</td>
<td>171 (2)</td>
</tr>
<tr>
<td>18</td>
<td><em>Veillonella atypica</em> strain ATCC 17744 (97)</td>
<td>174 (0)</td>
</tr>
<tr>
<td>19</td>
<td><em>Haemophilus influenzae</em> MCCM 02082 AF224308 (98)</td>
<td>174 (0)</td>
</tr>
<tr>
<td>20</td>
<td><em>Lactobacillus fermentum</em> strain KLB 261 EF535257 (89)</td>
<td>172 (1)</td>
</tr>
<tr>
<td>21</td>
<td>Uncultured bacterium clone P1D1-526 EF511979 (93)</td>
<td>176 (0)</td>
</tr>
<tr>
<td>22</td>
<td><em>Haemophilus</em> sp. oral clone BJ021 AY005034 (98)</td>
<td>173 (0)</td>
</tr>
<tr>
<td>23</td>
<td><em>Veillonella</em> sp. oral clone BP2-48 AB121922 (98)</td>
<td>169 (0)</td>
</tr>
<tr>
<td>24</td>
<td>Uncultured <em>Veillonella</em> sp. clone A42 DQ677558 (95)</td>
<td>171 (0)</td>
</tr>
<tr>
<td>25</td>
<td><em>Streptococcus genomospor.</em> C8 16S AY278609 (93)</td>
<td>173 (2)</td>
</tr>
<tr>
<td>26</td>
<td><em>Streptococcus mitis</em> strain C115 EF473998 (99)</td>
<td>176 (1)</td>
</tr>
<tr>
<td>27</td>
<td><em>Veillonella</em> sp. oral clone BP2-49 AB121923 (98)</td>
<td>174 (0)</td>
</tr>
<tr>
<td>28</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>176 (1)</td>
</tr>
<tr>
<td>29</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>174 (0)</td>
</tr>
<tr>
<td>30</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>178 (1)</td>
</tr>
<tr>
<td>31</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>177 (1)</td>
</tr>
<tr>
<td>32</td>
<td><em>Streptococcus</em> sp. oral clone FX003 AY134901 (87)</td>
<td>171 (3)</td>
</tr>
<tr>
<td>33</td>
<td><em>Bacillus</em> sp. SDB21A DQ323748 (91)</td>
<td>180 (5)</td>
</tr>
<tr>
<td>34</td>
<td><em>Bacillus</em> sp. SDB21A DQ323748 (97)</td>
<td>178 (1)</td>
</tr>
<tr>
<td>35</td>
<td><em>Bacillus cereus</em> AJ969107 (99)</td>
<td>178 (1)</td>
</tr>
<tr>
<td>36</td>
<td><em>Streptococcus</em> sp. oral clone AB121902 (100)</td>
<td>176 (1)</td>
</tr>
<tr>
<td>37</td>
<td><em>Veillonella</em> sp. oral clone AB121922 (96)</td>
<td>175 (1)</td>
</tr>
<tr>
<td>38</td>
<td>Uncultured <em>Veillonella</em> sp. clone BL045B72 DQ188783 (98)</td>
<td>174 (2)</td>
</tr>
<tr>
<td>39</td>
<td>No significant similarity found</td>
<td>163 (4)</td>
</tr>
<tr>
<td>40</td>
<td><em>Streptococcus salivarius</em> strain C161 EF474000 (99)</td>
<td>176 (1)</td>
</tr>
</tbody>
</table>

*aBased on EMBL database searches. bThe number of ambiguous bases are given in parenthesis. See Figure 3.4. for band locations.*
Data in Table 3.2. show closest relatives based on results of EMBL searches with DNA sequences obtained from bands excised from the DGGE gel shown in Figure 3.4. The dominant genera were taxonomically related to streptococci, lactobacilli and veillonella. A significant proportion of the DNA sequences (35%) had homology (>90%) to species that are yet to be, or are not readily culturable. Bands 2 and 8 were most closely related to uncultured species and apparently selected for by higher concentrations of both dentrifices. Band 21 is found with higher concentrations of TD but conversely only at lower concentrations of SZD.

*Streptococcus mitis* was the most commonly isolated species overall and the bacterium found in all the sample communities. Band 24 (uncultured *Veillonella* sp.) and band 14 (*Bulleidia extructa*, a gram positive bacilli associated with endodontic infection) were only detectable in those samples cultured in the presence of dentrifice. At higher dentrifice concentrations bands 18, 23, 37 and 38 (all *Veillonella* sp.) were less intense or not detectable.

Greater diversity was noted in the DGGE profiles from artificial saliva based communities when compared with those grown in TSB (52 bands versus 45). Samples from TSB based communities had a greater number of darker, more intense bands. In all cases, increasing the concentration of dentrifice in the sample reduced bacterial diversity according to the overall number of bands in the DGGE profile (Table 3.2.). In addition, visual examination reveals that band intensity appears to be reduced in a number of cases as the dentrifice concentration was increased.


Table 3.3. Values indicate the number of bands counted in each corresponding DGGE profile as shown in Figure 3.4.

<table>
<thead>
<tr>
<th>TD(mg/ml)</th>
<th>SZD(mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>TSB</td>
<td>37</td>
</tr>
<tr>
<td>Artificial saliva</td>
<td>44</td>
</tr>
</tbody>
</table>

Cluster analysis, to produce a UPGMA dendrogram calculated using the Dice coefficient, was performed with Bionumerics software (Applied Maths, Saint-Martens, Latem, Belgium). Each dentrifice-media combination clustered separately with percentage similarities ranging from 55 to 68 % (average 57.2 %) between groups (Figure 3.5.). Within the groups the percentage similarity between DNA profiles ranged from 69 to 91 % (average 76.8 %).

Figure 3.5. A UPGMA dendrogram showing the percentage matching of samples from dentrifice slurries inoculated with saliva.

The data was further analysed by principal component analysis (PCA) as described in Section 2.5.9. and the results displayed graphically in Figure 3.6.
Figure 3.6. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of bacterial communities growing with and without dentrifice. Samples without dentrifice: TSB (♦) and artificial saliva (♦). Samples with dentrifice: TD in TSB (■), TD in artificial saliva (■), SZD in TSB (■) or SZD in artificial saliva (■); concentration 0.09% (●), 0.18% (■) and 0.30% (▲).

Bacterial communities grown in the presence of TD (front left of Figure 3.6.) cluster away from those grown in the presence of SZD. Those communities grown in the presence of SZD show greater similarity both to one another and to communities grown in the absence of any dentrifice.

3.4.5. Comparison of growth media

In order to determine whether the artificial saliva formula described earlier in this chapter would produce bacterial communities more representative of those occurring in the salivary inocula than TSB, the DGGE profiles of each were compared using Bionumerics software and a UPGMA dendrogram constructed. Figure 3.7. shows the results of this analysis, where it can be seen that the community established in the artificial saliva showed much greater similarity to
the pure saliva sample than the one established in TSB (84% and 59% respectively).

**Figure 3.7.** A UPGMA dendrogram and corresponding DGGE gel showing the similarity between bacterial communities found: in a pure saliva sample, in TSB 16h post inoculation with saliva and in artificial saliva 16h post inoculation with saliva from the same individual.
3.5. Discussion
Dental plaque is a taxonomically diverse microbial consortium that has an established role in both the maintenance of oral health and the initiation of disease. Microorganisms were first implicated in the formation of dental caries as early as 1881 (Miller, 1883) and the relationship between the accumulation of dental plaque and onset of gingivitis was first described in 1965 (Loe et al., 1965). Since these initial investigations, concerted research effort has led investigators to propose several hypotheses regarding the aetiology of the dental diseases. The specific plaque hypothesis suggests that the initiation and perpetuation of disease is directly attributable to a small number of specific species (Marsh, 2003) whilst the non-specific plaque hypothesis implies that disease is a result of the overall interaction between all groups of bacteria found in dental plaque. The ecological plaque hypothesis however, suggests that there is no specific aetiology: any species with the right traits can contribute to disease progression, subject to selection pressure from changes in the oral environment (Kleinberg, 2002; Ledder et al., 2007; Marsh, 1994). The principal aims of this study were therefore to investigate the effect of two distinct dentrifices, marketed for the prevention of dental diseases, on microbial ecology, overall bacterial growth, acid production, and species composition within salivary-derived communities. To this end both culture and molecular microbiology techniques were utilised.

3.5.1. Spectrophotometric analysis of microbial growth inhibition in the presence of dentrifice and assessment of acid production using a novel pH assay
Spectrophotometric analyses showed that the concentration of dentrifice required to completely inhibit microbial growth was higher for SZD in
comparison to TD. Although the concentration values themselves are relatively small and thus both dentrifices are potent, the difference represented a ca. 50% increase in dentrifice concentration required of SZD when compared with TD. Concentrations required for bacterial inactivation will differ in vivo depending upon the time spent brushing, the substantivity of the toothpaste (Zero et al.) and intra-individual variation. However, greater antimicrobial potency in an oral care formulation may be of benefit, particularly as the antimicrobial concentration that can be delivered to oral tissues will fall rapidly between brushings.

In vivo, dietary carbohydrate is rapidly metabolised to acid by acidogenic and aciduric bacteria and this results in the demineralisation of tooth enamel (Bowden, 1990; Loesche, 1986; Sissons et al., 2007). Thus in the present chapter the effect of varying concentrations of dentrifice on bacterial acid production was analysed using a novel visual pH assay. TD proved to be a more potent inhibitor of acid production showing efficacy at lower concentrations than the SZD dentrifice, with a fourfold concentration increase needed to maintain the pH above 6.6 with SZD. As dentrifice concentrations in vivo are likely to vary between individuals and fall considerably between use, comparative potency represents an important advantage for oral healthcare products.

An important factor in the anti-acidogenic properties observed for each dentrifice is likely to be a result of the general bactericidal effects of the antimicrobials in the formulation. However, at low concentrations (0.78 to 1.8mg/ml TD and 3.13 to 3.3mg/ml SZD respectively) acid production was not detected whilst limited bacterial growth was observed. This could be because
the surviving bacteria were producing acid normally but the reduction in overall numbers resulted in an acid concentration that was not sufficient to initiate a colour change of the pH indicator or that acidogenic metabolic pathways were inhibited by the formulation. In this respect, a calculation of the moles of lactic acid required to reduce the pH of a 200µl solution from 6.6 to 6.5 and initiate a colour change of the phenol red indicator showed that this number was approximately 241000 times smaller than the number of moles of lactic acid normally produced by salivary bacteria in response to exposure to fermentable carbohydrates (Linke et al., 1997). Furthermore, buffering of saliva occurs in vivo when acids are neutralised by bicarbonate, the production of which is determined by saliva flow. Bicarbonate is not produced in vitro and thus could not account for the lack of acidogenesis. It therefore seems likely that within the concentration ranges described the dentrifices are in fact inhibiting bacterial acid production by some oral species. Similarly, Phan and Marquis (2006) have shown that the levels of triclosan, the antimicrobial component of TD, required for inhibiting glycolysis in S. mutans and S. sanguis biofilms were lower than those required for killing (Phan & Marquis, 2006). The reason for these differences is not clear; particularly as S. mutans relies on glycolysis for ATP production. In this chapter, the inhibition of bacterial acid production observed at low dentrifice concentrations could be due to a stress response by the surviving bacteria to the antimicrobial exposure. It is also possible that in aerobic conditions only the aerobes and facultative species survived dentrifice treatment and were producing ATP by oxidative phosphorylation as opposed to anaerobic fermentation. Although fermentation and thereby lactic acid production can occur in aerobic conditions if sufficient sugars are present.
3.5.2. Rationale for the utilisation of PCR amplification of 16S rDNA and characterisation by DGGE

Numerous authors have suggested the usefulness of molecular profiling techniques in understanding the microbiology of the oral cavity (Diaz et al., 2006; Fujimoto et al., 2003; Li et al., 2006; Muyzer & Smalla, 1998; Perea, 2004; Rasiah et al., 2005; Siqueira et al., 2005; Spratt, 2004; Zijinge et al., 2003). However, molecular-based studies tracking the bacteriological effects of biocide use in the oral cavity are limited. Of the existing studies relevant to this chapter, Li et al (2006) employed PCR-DGGE (V2-V3 region of the 16S ribosomal DNA) in a clinical study of a fluoride-based toothpaste (Colgate Cavity Protection, Colgate-Palmolive) and the authors stated that the technique was useful in terms of both accuracy and speed when tracking microbial changes in the oral cavity. PCR-DGGE has also been utilised by McBain et al (2003) to assess microbial diversity in oral microcosms growing in constant-depth film fermentors (CDFF) exposed to a chlorhexidine-containing mouthwash (McBain et al., 2003c). PCR-DGGE is not a quantitative technique (Ledder et al., 2007) due to the potential for heterogeneity in commonly used genes such as the 16S rRNA (Farrelly et al., 1995; Nubel et al., 1996) and sequences falling below the threshold for detection (Muyzer & Smalla, 1998), it does however offer one of the only means of producing a reproducible visual assessment of bacterial diversity in a sample. The consortial profiles produced are also highly reproducible.

3.5.3. Assessment of bacterial species diversity in salivary-derived communities in the presence of dentrifice

Data from the present study is in agreement with the observations of Li et al (2006) and McBain et al (2003); where exposure of in vitro saliva-derived
communities to antimicrobial oral health care products, in this case TD (triclosan) or SZD (stannous fluoride), at concentrations below those required to completely inhibit microbial growth, leads to a reduction in bacterial species diversity. In the current study this was detected by reductions in the number of PCR amplicons obtained. Importantly, the resultant altered bacterial community profiles differed with changes in the dentrifice concentration and also between products; samples derived from each dentrifice clustering separately (Figure 3.5.). This observation suggests that the differences observed between the dentrifice-treated and untreated samples were not solely due to a decrease in the overall number of bands in the DGGE profiles, but to a shift in the composition of the microbial community. Principal component analysis (PCA) of the data further supports this hypothesis (where decreasing distance between two points on a plot of the principal components corresponds to an increase in similarity); with the communities exposed to TD clustering on the opposing side of the graph to both the SZD and dentrifice-free samples (Figure 3.6.). Additionally, the positioning of these data points suggests that the presence of TD caused a shift away from the normal bacterial composition of the salivary-derived community that is significantly greater in magnitude than that observed with SZD. This provides evidence of differential susceptibility between the two dentrifrices.

3.5.4. Molecular analysis of species specific changes in dentrifice treated salivary-derived communities

Sequence analysis of bands excised from DGGE gels revealed the dominant genera across all the treated and untreated samples were taxonomically related to streptococci, lactobacilli and veillonella. This is not an unexpected outcome
as these genera are normally important in the oral cavity (Aas et al., 2005; Dalwai et al., 2006). Close relatives of *Streptococcus mitis* were the most commonly identified species overall. This observation correlates with the data reported by Aas et al. (2005) in a 16S rRNA PCR sequencing-based study of the oral cavity of healthy human subjects. The authors documented *S. mitis* as the most frequently detected species across all oral sites, representing 79% of the clones identified from one subject. Data in the current chapter suggests that dominant species such as *S. mitis* are more likely to survive dentrifice treatment. The persistence of this species in the dental biofilm may be beneficial because *S. mitis* may produce bactericidal levels of hydrogen peroxide to which some periodontopathic bacteria are susceptible (Li et al., 2004; Vernazza & Melville, 1979; Willcox & Drucker, 1988).

Periasamy and Kolenbrander (2010) have shown the importance of *Veillonella* sp. in the attachment of middle and late colonising dental plaque bacteria commonly associated with dental diseases such as gingivitis (Periasamy & Kolenbrander, 2010). In this chapter, a number of bands identified as veillonellae in the untreated sample fingerprints were absent following exposure to the higher concentrations of dentrifice. This was particularly noticeable in those cultured in artificial saliva and exposed to SZD. A reduction in the overall number of veillonellae could impede the attachment of potential pathogens and prove to be advantageous *in vivo*.

### 3.5.5. Effect of dentrifice treatment on potential oral pathogens in salivary-derived bacterial communities

*Solobacterium moorei* (formerly *Bulleidia extracta*) a gram positive bacillus associated with endodontic infection (Downes et al., 2000; Schirrmeister et al.,
2009) and systemic complications (Detry et al., 2006; Lau et al., 2006) was found only in the samples that had been exposed to dentrifice, and thus had probably clonally expanded under the selective conditions produced by the presence of dentrifice. Bands attributable to this bacterium were most prominent in the samples which had been exposed to TD. Schirrmiester et al (2009) found S. moorei in 33% of the periradicular (around the root of a tooth) lesions they tested using 16S rRNA gene sequencing. Moreover, the presence of Fusobacterium nucleatum was associated with the presence S. moorei in five out of seven cases. F. nucleatum is frequently implicated in periodontitis (Haffajee et al., 1998; Kroes et al., 1999; Ledder et al., 2007; Mombelli et al., 1998; Moore & Moore, 1994; Socransky et al., 1998) and although it was not detected in this study the link between increase in S. moorei, F. nucleatum and dentrifice treatment could be indicative of a potential negative impact on oral health. Although the high level of inter-individual variability between oral microbiotas should be considered (Ledder et al., 2006; Rasiah et al., 2005), numerical increases in certain taxa as a result of dentrifice use could have both positive and potentially negative implications and deserves further investigation.

Several studies have suggested that triclosan and stannous fluoride exhibit antimicrobial efficacy against Gram negative anaerobes and members of other functional groups of oral bacteria, including Streptococcus mutans (Bradshaw et al., 1993; Camosci & Tinanoff, 1984; Phan & Marquis, 2006). S. mutans or any of the gram negative anaerobes associated with dental disease were not detected in samples analysed in this study. Although it is possible that both dentrifices inhibited the growth of this group of bacteria, it is also possible that
these taxa were amongst the bands not selected for sequencing, or present in low enough levels to produce only a faint band on the DGGE gel.

3.5.6. Detection of uncultured species in salivary-derived communities using PCR-DGGE

In addition to the data already described, sequence analysis of the DGGE bands revealed that 35% of those excised were taxonomically related to uncultured species. One of the forty bands produced a sequence for which no significant similarity was found; an uncharacterised bacterium that may represent a novel phylotype. Estimates suggest that over half of the bacterial species inhabiting the oral cavity have not been cultivated (Aas et al., 2005) and hence this result is not unexpected. As these yet uncultured species constitute a considerable proportion of the oral microbiota it seems likely that their contribution to microbial homeostasis is significant.

3.5.7. Assessment of artificial saliva and TSB as growth media for the reproduction of salivary bacterial diversity

The secondary objective of this study was to validate the use of an artificial saliva solution for this chapter and future work. Successful modelling of the oral cavity is intrinsically linked with closely matching the growth conditions that predominate in vivo (Marsh et al., 1983) and a representative growth media is an important part of this. Saliva is a very dilute fluid containing varying concentrations of immunoglobulins, proteins, enzymes, mucins, nitrogenous products and electrolytes (Humphrey & Williamson, 2001). Here, the artificial saliva solution used was formulated by McBain et al. (2005) and comprised of mucin, peptone, tryptone, yeast extract, sodium chloride, potassium chloride, calcium chloride, cysteine, haemin and vitamin k₁ (McBain et al., 2005).
Although saliva composition varies markedly within and between individuals and thus the exact physicochemical conditions cannot easily be replicated, DGGE and dendrogram analysis revealed that the community established in artificial saliva more closely matched the DNA profile directly obtained from a saliva sample than the community established in TSB (84 % and 59 % respectively). This result suggests that the artificial saliva solution devised by McBain et al (2005) would be an appropriate growth medium for future models of the oral cavity.

3.6. Conclusions

Whilst oral hygiene products have been extensively studied and tested in the clinical setting, to our knowledge the methodology adopted in this chapter, whereby PCR-DGGE was used in conjunction with culture to directly compare two dentrifices is relatively uncommon. TD and SZD have both demonstrated characteristic effects on salivary-derived biofilm microcosms that lead to a reduction in microbial diversity and species-specific compositional changes. One of the most striking differences was observed by dendrogram and principal component analysis of the PCR-DGGE data where TD exhibited better overall potency, reducing the number of bacterial strains in the samples and resulting in a shift away from the normal bacterial composition of the salivary-derived community that was greater in magnitude than SZD. Significant perturbations in the in vivo oral microbiota could result in a breakdown of bacterial homeostasis; the implications of this warrant further investigation. Exposure to TD was also associated with the putative clonal expansion of the oral bacterium Solobacterium moorei, frequently found alongside Fusobacterium nucleatum (Schirrmeister et al., 2009). SZD however was linked to a decrease in the
number of veillonellae, a group that facilitate the attachment of late colonising bacteria frequently associated with gingivitis (Periasamy & Kolenbrander, 2010). Both dentrifices had little impact on *S. mitis*, a species associated with oral health (Aas *et al.*, 2005; Teughels *et al.*, 2007) the persistence of which is potentially beneficial.

Data obtained from the culture-based studies confirmed the results obtained by PCR-DGGE, showing that TD had greater potency than SZD. Antimicrobial activity at lower concentrations confers a significant advantage to TD as dentrifice concentrations *in vivo* are likely to vary between individuals and fall considerably between use. At specific low concentrations both dentrifices were observed to inhibit bacterial acid production whilst not completely inhibiting growth. This effect was observed within a wider concentration range of TD, another potential advantage for this dentrifice. PCR-DGGE and culture techniques played an important role in this study and the divergence in the results illustrates the potential benefits of using both methodologies together. The strength of the PCR-DGGE approach in particular lies in the production of visual community fingerprints that are reproducible, readily analysed by both hierarchical dendrograms and non-hierarchical principal component analysis and largely free from detection bias. Culture and molecular analysis collectively have shown that the suggested artificial saliva solution is superior to the simple sugar based growth medium, producing a microbial community with a greater similarity to saliva.
Chapter 4

Coaggregation between and among human intestinal and oral bacteria
4.1. Abstract

Coaggregation has been observed in a number of naturally occurring polymicrobial communities and is believed to be an important process during the formation of multi-species biofilms, particularly dental plaque. Although biofilms have been described in the human gastrointestinal tract, the occurrence and significance of coaggregation in the gut has rarely been investigated. The aim of this chapter therefore was to measure the frequency and strength of coaggregation between and among human oral and intestinal bacteria. Stationary phase cultures of 10 oral and 10 enteric species, chosen on the basis of numerical and ecological significance in their respective environments, together with their ease of cultivation, were tested using a spectrophotometric coaggregation assay in all possible pairwise combinations to provide quantitative coaggregation scores. Oral strains were the most polygamous coaggregators (40% of possible partnerships coaggregated strongly), whilst strong interactions between oral and gut strains were considerably less common (8% incidence). Coaggregation scores were also low between members of the intestinal microbiota (4% incidence), apart from Bacteroides fragilis with Clostridium perfringens, and Bifidobacterium adolescentis with C. perfringens. Oral and intestinal bacteria did not strongly interact, apart from B. adolescentis with Fusobacterium nucleatum, Actinomyces naeslundii with C. perfringens and F. nucleatum with Lactobacillus paracasei. Coaggregation was characterised using a semi-quantitative, visual assay which confirmed the occurrence of the coaggregation interactions already described, with the exception of B. fragilis with C. perfringens, and B. adolescentis with C. perfringens, which were not observed by eye. Heating and sugar inhibitor experiments indicated that, similar to oral microorganisms, interactions with intestinal and oral strains were lectin–carbohydrate based.
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4.2. Introduction

Coaggregation has been defined as “the recognition between surface molecules on two different bacterial cell types so that a mixed-cell aggregate is formed” (Kolenbrander, 1988). This process of recognition and adherence between genetically distinct bacteria is believed to play an important role in the development of multi-species biofilms. First described in 1970 by Gibbons and Nygaard from studies of dental plaque bacteria (Gibbons & Nygaard, 1970); interactions of this kind have been observed in both naturally occurring and manmade environments. Isolates derived from aquatic ecosystems (Rickard et al., 2000; Rickard et al., 2003b) and from biofilms found on and within the human body, such as the gut and oral cavity (Drago et al., 1997; Reid et al., 1988) have been observed to coaggregate. Coaggregation is reported as being particularly important in facilitating attachments between bacteria associated with dental plaque (Gibbons & Nygaard, 1970) and the vast majority of research in this field has thus far concentrated on bacteria derived from the human oral cavity (Eke et al., 1989; Foster & Kolenbrander, 2004; Handley et al., 1985; Rosen & Sela, 2006; Shen et al., 2005; Umemoto et al., 1999).

It has been suggested that most, if not all, species of oral bacteria coaggregate with at least one other partner (Kolenbrander, 1993; Kolenbrander & London, 1993; Kolenbrander et al., 2000; Whittaker et al., 1996). Coaggregation has been defined as intergeneric, intrageneric or multigeneric (Hughes et al., 1988; Kolenbrander, 2000). Intergeneric coaggregations (those occurring between strains from different genera) make up the majority occurring in the oral cavity. Although less common; intrageneric partnerships have been reported between
a number of species, including some streptococci and fusobacteria (Kolenbrander et al., 2000; Rickard et al., 2003b). Streptococci compose 60 to 90% of the earliest colonisers of the tooth surface (Nyvad & Kilian, 1987) and it has been suggested that coaggregation within the genus is an important determinant of the predominance of streptococci in initial dental plaque structure (Kolenbrander et al., 2002). For species that are unable to attach directly to the proteins, lipids and glycolipids that make up the acquired pellicle (see Section 1.8.1.); termed late colonisers, intergeneric coaggregation is believed to be particularly important for integration into the multi-species biofilm (Kolenbrander et al., 2002; Kolenbrander et al., 2006). In addition autoaggregation, defined as adherence of cells of the same bacterial strain (Kinder & Holt, 1994), has been observed in many species derived from the oral cavity (Shen et al., 2005) and thus may also play a significant role in the formation of dental plaque.

Frequently observed in the oral cavity, coaggregation is dependent on highly specific lectin-carbohydrate interactions that occur between the aggregating partners (Cisar et al., 1979). Coaggregation was first characterised as lectin-carbohydrate based when the aggregation of Actinomyces viscosus and Streptococcus sanguis was shown to be reversed by the addition of 0.01M lactose (McIntire et al., 1978). Further studies have shown that coaggregation between many species of oral bacteria can be inhibited by the addition of sugars, in particular those with a structure similar to lactose (Kolenbrander & Williams, 1981; Kolenbrander & Williams, 1983; Kolenbrander & Andersen, 1989; McIntire et al., 1982; Rickard et al., 2000; Rosen & Sela, 2006). Early studies of coaggregation between A. naeslundii isolates and S. sanguis or
Streptococcus mitis strains revealed that exposure to heat or proteolytic enzymes reduced the ability of the actinomycetes, but not the streptococci, to interact with untreated partner cells; indicating that A. naeslundii carries the protein component(s) (Ellen & Balcerzak-Raczkowski, 1977).

Coaggregation can be unimodal, i.e. involving just one lectin-carbohydrate receptor interaction, or bimodal, involving more than one set of cell surface receptors (Kolenbrander et al., 2000). Oral bacteria usually have a partner, or set of partners, with which they will readily aggregate and a large number of species with which they will not (Kolenbrander, 1995). Cell viability is not a prerequisite to coaggregation because dead cells are able to aggregate and do so immediately when mixed with a compatible partner strain(s). Such observations support the hypothesis that cell surface molecules are responsible for the recognition and aggregation process rather than an active process that would require cell viability (Kolenbrander et al., 1993).

Oral bacteria are believed to derive a number of benefits from coaggregation and the subsequent formation of complex dental plaque biofilms. Existence in a multi-species biofilm can confer selective advantages over planktonic growth in many environments (Kinder & Holt, 1994). For example, the hydrated extracellular matrix of a biofilm concentrates nutrients and defensive substances as well as offering some physical protection from exogenous compounds including antibiotics (Kinder & Holt, 1994). The close physical interactions between coaggregating bacteria may facilitate the establishment of symbiotic relationships, cross-feeding (Drago et al., 1997), cell-cell communication (Kolenbrander, 2000), genetic exchange between cells (Foster
et al., 2003) and provide protection for anaerobes against oxygen (Bradshaw et al., 1998). In addition, it has been suggested that oral bacteria may have evolved the ability to coaggregate as a protective mechanism to resist removal by the high shear forces found in the oral cavity and subsequent swallowing (Handley et al., 2001).

Coaggregation can be observed with the naked eye within paired coaggregation assays (Kolenbrander, 1988). Thus, bacteria of any origin can be studied in vitro by observing the flocculation of washed and resuspended test strains. A visual assay described by both Kolenbrander (1995) and Cisar et al., (1979) has been frequently employed in studies of oral bacteria whereby dense suspensions of two different species are mixed and the extent of coaggregation (i.e. the size of floccules formed) is scored on a scale of zero to four (Cisar et al., 1979; Kolenbrander, 1988; Kolenbrander, 1995). This assay is particularly useful when screening large numbers of bacteria to identify coaggregating partners. A quantitative assay for measuring coaggregation has also been described, based on the spectrophotometric analysis of the supernatant phase (Ikegami et al., 2004). This method allows the calculation of percentage coaggregation values and may provide a more comparable analysis than the visual assay. The molecular basis of coaggregation interactions have been investigated using heat and protein treatment and the addition of lactose and similar sugars.

Whilst coaggregation studies between bacteria derived from the oral cavity are common in the literature, the study of bacteria isolated from other ecosystems such as the gut, are very limited in number. Currently most of the literature has
focused on the genus *Lactobacillus* in the gastrointestinal (GI) tract of chickens (Vandevoorde *et al.*, 1992), pigs (Kmet *et al.*, 1995), and with human enteropathogens (Drago *et al.*, 1997). Although it seems logical to suggest that cellular interactions which occur in the oral cavity may occur similarly in other multi-species biofilms, the incidence and mechanisms of coaggregation between members of the human intestinal flora have yet to be fully elucidated. It is also postulated that some oral bacteria will coaggregate with members of the intestinal microbiota, as oral species may associate with gut bacteria when swallowed with food and saliva.

In view of the paucity of coaggregation research beyond the oral cavity, particularly in multi-species biofilms associated with humans, the intestinal microbiota was identified as an area for further investigation. The gut harbour the largest bacterial population in the human body (Hooper, 2004), plays an important role in host health and disease (Guarner & Malagelada, 2003) and due to the physical proximity to the mouth could be expected to receive a continual influx of oral bacteria; yet little is known about the bacterial attachments that occur there. Vast biodiversity; with some studies suggesting between 300 and 15 000 unique species (Eckburg *et al.*, 2005; Frank *et al.*, 2007) and the difficulties associated with the culture of many fastidious species *ex vivo*, has resulted in limited knowledge of bacterial attachment in the gut thus far (Hooper *et al.*, 2002). However, analyses using culture-independent molecular tools such as PCR amplification and sequencing of 16S rDNA, and the cloning of large fragments of DNA isolated directly from environmental samples (metagenomics) (Rondon *et al.*, 2000) has identified several numerically dominant phylogenetic groups, including the Bacteroidetes and
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*Clostridium* groups which encompass many readily culturable anaerobes (Suau *et al.*, 1999; Wang *et al.*, 2003). One study has suggested that over 95% of the colonic microbiota consists of bacteria from these groups (Suau *et al.*, 1999), and in his 2005 review paper Fredrik Bäckhed suggested that the divisions Cytophaga-Flavobacterium-Bacteroides constitute greater than 90% of bacteria in faeces. Study of such isolates therefore will provide insight into the attachment mechanisms of bacteria belonging to the numerically important genera. The enteric isolates selected for this study were chosen on the basis of numerical and ecological importance in their respective environments and fall into the divisions Cytophaga-Flavobacterium-Bacteroides, Firmicutes, Proteobacteria and Actinobacteria.

This chapter aims to identify the extent to which the processes of bacterial coaggregation occur in selected numerically important isolates from the intestinal microbiota. Additionally, the ability of these intestinal species to coaggregate with selected oral isolates was also investigated. Assessment of coaggregation between the oral isolates served as internal controls. A semi-quantitative, visual coaggregation assay was used, together with a novel previously validated quantitative spectrophotometric assay to investigate these interactions. Characterisation of the mechanistic basis of the interactions observed was done using heat and sugar-addition experiments. If the same lectin-carbohydrate interactions occur between enteric isolates that are responsible for the coaggregation of oral isolates, then subjecting cells to heat treatment would denature the protein component and prevent coaggregation. Addition of sugars, such as lactose, to one of the partner strains in suspension would inhibit coaggregation by binding to the cell surface lectins thereby
blocking the coaggregation partner from doing so. The selection of sugars for the study was based upon those previously used to characterise coaggregations occurring in other multi-species biofilms including the oral cavity (Kolenbrander & Williams, 1981; Rickard et al., 2000; Rosen & Sela, 2006).
4.3. Materials and Methods

4.3.1. Bacterial strains and culture conditions

The oral bacteria used in this study were *Actinomyces naeslundii* WVU 627, *Fusobacterium nucleatum* NCTC 10562, *Lactobacillus rhamnosus* AC 413, *Neisseria subflava* A1078, *Prevotella oralis* NCTC 11459, *Porphyromonas gingivalis* NCTC 11834, *Streptococcus mutans* NCTC 10832, *Streptococcus oralis* NCTC 11427, *Streptococcus sanguis* NCTC 7863 and *Veillonella dispar* ATCC 17745. Enteric isolates comprised *Bacteroides fragilis* NCTC 9343, *Bacteroides vulgatus* M40.2 (AM990169), *Bifidobacterium adolescentis* NCIMB 702231, *Bifidobacterium infantis* M40.6 (AM990173), *Clostridium perfringens* NCTC 8346, *Enterococcus faecalis* ATCC 51299, *Enterococcus faecium* M40.1 (AM990168), *Escherichia coli* NCTC 9001, *Enterobacter hormaechei* M40.7 (AM990174) and *Lactobacillus paracasei* M40.9 (AM990176). Non-culture collection strains were kindly donated by S. Macfarlane (Gut Group, University of Dundee, Ninewells Hospital Medical School, Dundee, UK) and the identities confirmed by a process of DNA extraction (Section 2.2.1.), amplification of the 16S rRNA gene by PCR (Section 2.3.1.), purification of the PCR products and DNA sequencing (Section 2.3.3.). Numbers in parentheses indicate the accession number of these clinical strains in the EMBL database.

*Neisseria subflava* was grown on Wilkins Chalgren agar or broth and incubated aerobically at 37°C. Other bacteria were grown on Wilkins Chalgren anaerobe agar or broth in a Mark 3 Anaerobic Work Station (Don Whitely Scientific, Shipley, UK) at 37°C (gas mix: 80% N₂, 10% CO₂ and 10% H₂).
4.3.2. Preparation of inocula for coaggregation assays

Cultures of test bacteria were grown under conditions previously validated to produce stationary phase cells by inoculating each of the strains into 500 ml of pre-reduced Wilkins Chalgren broth and incubating in an anaerobic environment for 7 d (Bradshaw et al., 1998). *Neisseria subflava* was grown in Wilkins Chalgren broth aerobically without mixing for 3 d.

4.3.3. Spectrophotometric coaggregation assays

A modified quantitative spectrophotometric assay described by Ikegami et al., (2004) was initially used to determine the coaggregation activity between all test species. Bacteria were harvested by centrifugation at 24 000 xg (Beckman-Coulter J2-21) for 20 min and resuspended in coaggregation buffer (Budu et al., 2003). This buffer comprised 1 mM CaCl$_2$, 0.1 mM MgCl$_2$ and 0.15 M NaCl dissolved in 20 mM Tris buffer adjusted to pH 7.0. The strains were then washed three times by centrifugation (10 000 rpm, 20 min) in coaggregation buffer and resuspended to give an optical density (OD) reading of 1.5 at 600 nm. Equal volumes (1 ml) of each suspension were then mixed in sterile cuvettes and the optical densities immediately recorded. Autoaggregation was determined using an identical method, by combining two equal volumes of the same bacterial suspension. In all cases the mixtures were left for 1 h at room temperature to allow coaggregation to occur, after which OD of the supernatant was once again recorded. Each assay was repeated to give a total of three values to facilitate the calculation of means. Percentage coaggregation was calculated by the following equation:
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Coaggregation = \frac{(\text{preincubation value [OD}_{600} \text{]} - \text{test value [OD}_{600} \text{]}) \times 100}{\text{preincubation value [OD}_{600} \text{]}}

Strongly coaggregating pairs that included one or more intestinal isolate (identified by the spectrophotometric assay) and oral isolate control pairs were chosen for further analysis.

4.3.4. Visual coaggregation assays

A commonly used visual assay (Kolenbrander, 1995) was employed to characterise the composition of the interactions between coaggregating strains identified by the spectrophotometric assay. To facilitate a comparison, the selected pairings were first reassessed by visual assay prior to heat treatment or the addition of sugars. For this, cells were resuspended at an OD 600 nm of 1.0 after the washing step. Equal volumes of each suspension (1 mL) were thoroughly mixed together in sterile cuvettes and left to stand at room temperature for 15 min. Coaggregation was assessed by a visual assay using the following scoring system (Kolenbrander, 1995): rapid and complete settling of large coaggregates leaving a water-clear supernatant (+4); coaggregates that settled rapidly but with a supernatant that remained slightly cloudy (+3); coaggregates that formed immediately, but remained suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1); suspensions with no evidence of aggregation or coaggregation (0).

4.3.5. Effect of sugars on the strength of coaggregation

The ability of specific sugars to reverse each coaggregation interaction was determined by the following method: the sugars (lactose, N-acetyl-D-galactosamine, D-galactose, D-galactosamine, D-fucose, L-fucose, a-methyl-D-
galactoside, b-methyl-D-galactoside, L-rhamnose or N-acetyl glucosamine), at a final concentration of 100 mM, were added individually to 1 ml of a suspension containing one of the coaggregating strains and mixed. After standing at room temperature for 15 min, 1 ml of a suspension containing the partner strain was added and mixed again. Coaggregation was then assessed using the visual assay described in Section 4.3.3.

4.3.6. Influence of heating on coaggregation

The effect of heat on each coaggregation interaction was determined by heating one of the two partner strains in a water bath at 85°C for 30 min (Kolenbrander, 1995). An equal volume (1 ml) of suspension containing the partner was added, and coaggregation was again gauged using the visual assay (Section 4.3.3). This process was repeated for the second partner, i.e. each of the two partners heated in separate coaggregation assays.
4.4. Results

4.4.1. Spectrophotometric coaggregation assays

Spectrophotometric analyses (Table 4.1.) showed that interspecies coaggregation between oral isolates was relatively common (frequency: 40%) with strong interactions (strength of coaggregation >60%) occurring between the oral isolates (1) *F. nucleatum* with *A. naeslundii*, (2) *A. naeslundii* with *P. gingivalis*, (3) *F. nucleatum* with *P. gingivalis*, (4) *L. rhamnosus* with *P. gingivalis* and (5) *P. gingivalis* with *N. subflava*. Cultures of *S. sanguis* scored the highest for strength of autoaggregation (37%), and *A. naeslundii* was the most promiscuous coaggregating oral species overall, associating markedly (strength of coaggregation >60%) with six of the nine oral species tested and moderately (strength of coaggregation >20%) with two of the nine species tested.

Data in Table 4.2. show that coaggregation between members of the intestinal microbiota was infrequent, occurring in just 8% of pairings. Coaggregations that were observed were weak (strength of coaggregation <12%) apart from *B. fragilis* with *C. perfringens* and *B. adolescentis* with *C. perfringens*, which coaggregated with strengths of 39% and 58%, respectively when assessed spectrophotometrically. *Clostridium perfringens* was the most polygamous intestinal bacterium, scoring the highest overall for both frequency and amplitude of coaggregation among the gut species tested.
Table 4.1. Coaggregation scores of pairs of selected oral bacterial species.

<table>
<thead>
<tr>
<th>Species</th>
<th>A. naeslundii</th>
<th>F. nucleatum</th>
<th>L. rhamnosus</th>
<th>N. subflava</th>
<th>P. gingivalis</th>
<th>P. oralis</th>
<th>S. mutans</th>
<th>S. oralis</th>
<th>S. sanguis</th>
<th>V. dispar</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. naeslundii</td>
<td>30.4 (8)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F. nucleatum</td>
<td>71.2 (6.4)</td>
<td>4.06 (3)*</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>L. rhamnosus</td>
<td>63.7 (5.7)</td>
<td>42.9 (12)</td>
<td>35.3 (5.2)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>N. subflava</td>
<td>63.1 (1.4)</td>
<td>62.3 (5.8)</td>
<td>34.8 (7.9)</td>
<td>2.5 (0.5)*</td>
<td></td>
<td></td>
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<tr>
<td>P. gingivalis</td>
<td>86.3 (1.3)</td>
<td>73.6 (11)</td>
<td>89 (2.4)</td>
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<td>S. mutans</td>
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<td>33.6 (8)</td>
<td>3 (0.03)</td>
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<tr>
<td>S. oralis</td>
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<td>32.6 (16)</td>
<td>45.4 (4)</td>
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<td>7.7 (5.8)*</td>
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<tr>
<td>S. sanguis</td>
<td>61.5 (1.2)</td>
<td>59.4 (3.2)</td>
<td>26.4 (0.1)</td>
<td>1.9 (1.6)</td>
<td>1.87 (2.7)</td>
<td>2.4 (1.5)</td>
<td>3.7 (3)</td>
<td>1.5 (0)</td>
<td>36.8 (8)*</td>
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</tr>
<tr>
<td>V. dispar</td>
<td>20.4 (14)</td>
<td>2.7 (2.8)</td>
<td>14.1 (2.5)</td>
<td>2.5 (1.5)</td>
<td>2 (3.3)</td>
<td>2.3 (0.4)</td>
<td>2.8 (0.01)</td>
<td>5.1 (2)</td>
<td>3.5 (1)</td>
<td>7.1 (7.2)*</td>
</tr>
</tbody>
</table>

Percent coaggregation as measured by OD (600nm) change over 1 hour (Section 4.3.3.). Data are means from three separate experiments (standard deviations are given in parenthesis). Coaggregative pairs scoring between 20 % and 60 % are shaded light grey, and >60 % dark grey.

*Autoaggregation scores representative of interaction between cells from the same culture.
Table 4.2. Coaggregation scores of pairs of the 10 human intestinal bacterial species.

<table>
<thead>
<tr>
<th>Species</th>
<th>B. fragilis</th>
<th>B. vulgatus</th>
<th>B. adolescentis</th>
<th>B. longum</th>
<th>C. perfringens</th>
<th>E. faecalis (i)</th>
<th>E. faecalis (ii)</th>
<th>E. coli (i)</th>
<th>E. coli (ii)</th>
<th>L. paracasei</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. fragilis</td>
<td>2.9 (1.4)*</td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>3.5 (1.6)</td>
<td>4.6 (0.7)*</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. adolescentis</td>
<td>3.4 (1)</td>
<td>3.6 (1.6)</td>
<td>4.1 (1.9)*</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>B. longum</td>
<td>2 (0.9)</td>
<td>2 (1.7)</td>
<td>2.8 (2)</td>
<td>2.7 (2.8)*</td>
<td></td>
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</tr>
<tr>
<td>C. perfringens</td>
<td>39.2 (0.1)</td>
<td>10.9 (1)</td>
<td>58.4 (2.5)</td>
<td>7.4 (0.9)</td>
<td>1.5 (3.2)*</td>
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<tr>
<td>E. faecalis (i)</td>
<td>4.2 (0.02)</td>
<td>4.7 (1.7)</td>
<td>6.1 (1.1)</td>
<td>3.8 (1.8)</td>
<td>1.2 (3.1)</td>
<td>6.1 (0.8)*</td>
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<tr>
<td>E. faecalis (ii)</td>
<td>3.6 (2.4)</td>
<td>4.7 (1.7)</td>
<td>4.8 (0.7)</td>
<td>6.2 (1.8)</td>
<td>1.2 (0.6)</td>
<td>8 (1.6)</td>
<td>4.1 (0)*</td>
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</tr>
<tr>
<td>E. coli (i)</td>
<td>2.6 (1.9)</td>
<td>2.6 (1.6)</td>
<td>3.7 (1.4)</td>
<td>2.3 (1.4)</td>
<td>1.5 (0.5)</td>
<td>4 (0)</td>
<td>4.6 (2)</td>
<td>1.3 (1.9)*</td>
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<tr>
<td>E. coli (ii)</td>
<td>1.6 (2.3)</td>
<td>1.4 (2)</td>
<td>3.2 (0)</td>
<td>1.6 (1.4)</td>
<td>1.6 (0.4)</td>
<td>3.6 (2.9)</td>
<td>2.3 (0)</td>
<td>3.2 (1.8)</td>
<td>1.6 (1.3)*</td>
<td></td>
</tr>
<tr>
<td>L. paracasei</td>
<td>4.1 (0)</td>
<td>6.4 (1)</td>
<td>7.1 (2.3)</td>
<td>5.1 (1.4)</td>
<td>1.7 (6.2)</td>
<td>8 (1.3)</td>
<td>11.4 (2.9)</td>
<td>6.3 (0.4)</td>
<td>6.3 (1.5)</td>
<td>7.8 (3.3)*</td>
</tr>
</tbody>
</table>

Percent coaggregation as measured by OD (600nm) change over 1 hour (Section 4.3.3.). Data are means from three separate experiments (standard deviations are given in parenthesis). Coaggregative pairs scoring between 20% and 60% are shaded light grey.

*Autoaggregation scores representative of interaction between cells from the same culture.
Data in Table 4.3. indicates that the frequency of coaggregation interactions between members of the oral and intestinal microbiotas was low overall (8% incidence). Of the interactions observed all were described as weak or moderate (strength of coaggregation <30%) apart from (1) *B. adolescentis* with *F. nucleatum* (81%), (2) *A. naeslundii* with *C. perfringens* (43%), (3) *F. nucleatum* with *L. paracasei* (51%) and (4) *A. naeslundii* with *L. paracasei* (51%). *Actinomyces naeslundii* interacted most strongly with five of the 10 strains, giving coaggregation scores of over 20%.
Table 4.3. Coaggregation scores of pairs of the 10 oral and 10 intestinal bacterial species.

<table>
<thead>
<tr>
<th>Species</th>
<th>B. fragilis</th>
<th>B. vulgatus</th>
<th>B. adolescentis</th>
<th>B. longum</th>
<th>C. perfringens</th>
<th>E. faecalis (i)</th>
<th>E. faecalis (ii)</th>
<th>E. coli (i)</th>
<th>E. coli (ii)</th>
<th>L. paracasei</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. naeslundii</td>
<td>23.9 (3.1)</td>
<td>28 (5.9)</td>
<td>26 (4.2)</td>
<td>16.3 (2.5)</td>
<td>43 (25)</td>
<td>24.2 (2.6)</td>
<td>16.1 (5.3)</td>
<td>14.4 (9.4)</td>
<td>19.2 (3)</td>
<td>22.5 (7.3)</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>5.3 (3.3)</td>
<td>14.5 (4.4)</td>
<td>81 (1.4)</td>
<td>12.5 (2.4)</td>
<td>6.4 (1.6)</td>
<td>1.8 (2.6)</td>
<td>11.3 (5.6)</td>
<td>3.3 (2.8)</td>
<td>9.1 (1.6)</td>
<td>51.2 (12)</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>0.7 (0.7)</td>
<td>8.5 (2.3)</td>
<td>3.5 (1.4)</td>
<td>1.3 (1.3)</td>
<td>2.2 (1)</td>
<td>3.1 (0.4)</td>
<td>4.4 (2.4)</td>
<td>5.2 (4.4)</td>
<td>2.6 (1.5)</td>
<td>3.6 (1.4)</td>
</tr>
<tr>
<td>N. subflava</td>
<td>1.6 (0.8)</td>
<td>2.2 (0.4)</td>
<td>2.2 (0.4)</td>
<td>0.9 (0.4)</td>
<td>2.1 (0.4)</td>
<td>1.6 (1)</td>
<td>4.1 (3.3)</td>
<td>2.7 (0.7)</td>
<td>1.3 (0)</td>
<td>2.4 (0.4)</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>6.9 (1.2)</td>
<td>6.3 (1.3)</td>
<td>12.7 (0.4)</td>
<td>5.1 (1.1)</td>
<td>5.1 (2.6)</td>
<td>7.6 (5.8)</td>
<td>4 (3.5)</td>
<td>3.9 (0.8)</td>
<td>6.6 (0.6)</td>
<td>9.9 (1.5)</td>
</tr>
<tr>
<td>P. oralis</td>
<td>0.9 (0.4)</td>
<td>1.1 (0.4)</td>
<td>1.4 (0.7)</td>
<td>1.7 (1.5)</td>
<td>2 (0.9)</td>
<td>1.6 (0.4)</td>
<td>2.2 (0.8)</td>
<td>1.8 (0.4)</td>
<td>1.4 (0.7)</td>
<td>1.9 (1.2)</td>
</tr>
<tr>
<td>S. mutans</td>
<td>6.9 (1.7)</td>
<td>1.8 (0.4)</td>
<td>7.6 (2.8)</td>
<td>2.2 (0.3)</td>
<td>9.1 (7.6)</td>
<td>3.5 (2.1)</td>
<td>5.4 (2.4)</td>
<td>5.1 (0.4)</td>
<td>3.9 (1.6)</td>
<td>2.1 (1.9)</td>
</tr>
<tr>
<td>S. oralis</td>
<td>2 (0.4)</td>
<td>1.3 (0.4)</td>
<td>3.3 (3)</td>
<td>1.6 (0.4)</td>
<td>1.8 (0.8)</td>
<td>1.8 (1.7)</td>
<td>1.7 (0.4)</td>
<td>1.2 (0.4)</td>
<td>1.2 (0.4)</td>
<td>2 (0.4)</td>
</tr>
<tr>
<td>S. sanguis</td>
<td>1.4 (1.4)</td>
<td>1.3 (0.1)</td>
<td>8.1 (4)</td>
<td>0.9 (0.8)</td>
<td>1.3 (0.7)</td>
<td>1.2 (0.8)</td>
<td>1.1 (0.4)</td>
<td>4.9 (4.9)</td>
<td>1.1 (1)</td>
<td>1.4 (0.7)</td>
</tr>
<tr>
<td>V. dispar</td>
<td>1.39 (0.7)</td>
<td>0.8 (0.4)</td>
<td>1.6 (0.4)</td>
<td>0.4 (0.2)</td>
<td>1.7 (0.4)</td>
<td>1.6 (0.4)</td>
<td>1.3 (0.7)</td>
<td>1.4 (1.1)</td>
<td>0.7 (0.6)</td>
<td>0.7 (0.7)</td>
</tr>
</tbody>
</table>

Percent coaggregation as measured by OD (600nm) change over 1 hour (Section 4.3.3.). Data are means from three separate experiments (standard deviations are given in parenthesis). Coaggregative pairs scoring between 20 % and 60 % are shaded light grey, and >60% dark grey.
4.4.2. Visual coaggregation assays

Data in Table 4.4. gives visual coaggregation assay scores which are analogous to those observed in the spectrophotometric assay. From the oral isolates selected for further analysis, A. naeslundii again proved to be the most prolific coaggregator and the following pairings the most substantial (scoring +4 or +3): (1) F. nucleatum with A. naeslundii, (2) F. nucleatum with L. rhamnosus and (3) F. nucleatum with P. gingivalis. The highest scores for strength of autoaggregation (scoring +3) occurred with A. naeslundii and L. rhamnosus. Of the pairings involving an intestinal and oral isolate; B. adolescentis with F. nucleatum showed the most substantial coaggregation with a score of +4 (see illustration in Figure 4.1.). Coaggregation between A. naeslundii and the following four isolates was noted (all scoring +1): C. perfringens, B. fragilis, B. vulgatus and B. adolescentis.

![Figure 4.1. Coaggregation between B. adolescentis and F. nucleatum. Cuvettes contain suspensions of (left to right): (1) B. adolescentis, (2) F. nucleatum, (3) B. adolescentis and F. nucleatum immediately after mixing, (4) B. adolescentis and F. nucleatum 15 min after mixing. Discernible levels of coaggregation between the intestinal isolates B. fragilis and C. perfringens or B. adolescentis and C. perfringens were not observed with the visual assay. Thus sugar addition and heating tests were not carried out for these two pairings.](image)
Table 4.4. Visual assay; coaggregation scores of pairs of the 4 oral and 4 intestinal bacterial species.

<table>
<thead>
<tr>
<th>Species</th>
<th>A. naeslundii</th>
<th>F. nucleatum</th>
<th>L. rhamnosus</th>
<th>P. gingivalis</th>
<th>C. perfringens</th>
<th>B. fragilis</th>
<th>B. vulgatus</th>
<th>B. adolescentis</th>
</tr>
</thead>
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<tr>
<td>A. naeslundii</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>F. nucleatum</td>
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<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>+2</td>
<td>+4</td>
<td>+3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>+2</td>
<td>+3</td>
<td>+3</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>C. perfringens</td>
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<td>0</td>
<td>0</td>
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<td></td>
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</tr>
<tr>
<td>B. fragilis</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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</tr>
<tr>
<td>B. vulgatus</td>
<td>+1</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>B. adolescentis</td>
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<td>+4</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Coaggregation scores were assessed by visual assay. Rapid and complete settling of large coaggregates leaving a water-clear supernatant (+4); coaggregates that settle rapidly but supernatant remains slightly cloudy (+3); coaggregates form immediately but remain suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1) no evidence of aggregation or coaggregation (0). Coaggregations scored +4 or +3 are shaded dark grey, those scored +2 or +1 are shaded light grey.
### Table 4.5. Coaggregation scores of pairs of the oral and intestinal bacterial species before and after the addition of sugars

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lactose</th>
<th>N-acetyl-D-Galactosamine</th>
<th>D-Galactose</th>
<th>D-Galactosamine</th>
<th>D-Fucose</th>
<th>L-Fucose</th>
<th>α-methyl-D-galactoside</th>
<th>β-methyl-D-galactoside</th>
<th>L-Rhamnose</th>
<th>N-acetyl-D-Glucosamine</th>
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</thead>
<tbody>
<tr>
<td>AN/FN</td>
<td>+3</td>
<td>+3</td>
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<td>+3</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
</tr>
<tr>
<td>AN/LR</td>
<td>+2</td>
<td>+2</td>
<td>+2</td>
<td>+2</td>
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</tr>
<tr>
<td>AN/PG</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>AN/CP</td>
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<td>+1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AN/BV</td>
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<td>+1</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
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<td>+1</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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<td>0</td>
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</tr>
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<td>+4</td>
<td>+4</td>
<td>+4</td>
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<tr>
<td>FN/PG</td>
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<tr>
<td>FN/BA</td>
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<td>+3</td>
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<td>+4</td>
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<td>+4</td>
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<td>+4</td>
<td>+4</td>
<td>+4</td>
<td>+4</td>
</tr>
<tr>
<td>LR/PG</td>
<td>+3</td>
<td>+1</td>
<td>0</td>
<td>+1</td>
<td>+1</td>
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<td>+1</td>
<td>+1</td>
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</table>

Coaggregation scores were assessed by a visual assay. Rapid and complete settling of large coaggregates leaving a clear-water supernatant (+4); coaggregates that settle rapidly but supernatant remains slightly cloudy (+3); coaggregates form immediately but remain suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1) no evidence of aggregation or coaggregation (0). Coaggregative interactions inhibited by a sugar are shaded light grey (a decrease of 1) or dark grey (a decrease of 2 or more). AN, *Actinomyces naeslundii*; FN, *Fusobacterium nucleatum*; LR, *Lactobacillus rhamnosus*; PG, *Porphyromonas gingivalis*; CP, *Clostridium perfringens*; BF, *Bacteroides fragilis*; BV, *Bacteroides vulgatus*; BA, *Bifidobacterium adolescentis*. 
4.4.3. Inhibition assays

Data in Table 4.5. show that the majority of coaggregations were partially or completely inhibited by at least one of the sugars used in this study. Only the following pairings were unaffected: (1) *A. naeslundii* and *F. nucleatum*, (2) *A. naeslundii* and *L. rhamnosus* and (3) *F. nucleatum* and *L. rhamnosus*. Table 4.6. shows that all but three of the coaggregating pairs tested were affected by heat treatment. Unaffected pairings included (1) *A. naeslundii* and *L. rhamnosus*, (2) *A. naeslundii* and *C. perfringens* and (3) *F. nucleatum* and *L. rhamnosus*. In most cases, heating one of the partner strains inhibited coaggregation, while heating the other had no effect on the interaction.

<table>
<thead>
<tr>
<th>Partner 1</th>
<th>Partner 2</th>
<th>Control</th>
<th>Partner 1 Heated</th>
<th>Partner 2 Heated</th>
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</thead>
<tbody>
<tr>
<td>AN</td>
<td>FN</td>
<td>+3</td>
<td>+3</td>
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</tr>
<tr>
<td>AN</td>
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<td>PG</td>
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<tr>
<td>AN</td>
<td>CP</td>
<td>+1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>AN</td>
<td>BF</td>
<td>+1</td>
<td>+1</td>
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<tr>
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<td>BV</td>
<td>+1</td>
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<td>AN</td>
<td>BA</td>
<td>+1</td>
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<td>LR</td>
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</tbody>
</table>

Coaggregation scores assessed by visual assay. Rapid and complete settling of large coaggregates leaving a water-clear supernatant (+4); coaggregates that settle rapidly but supernatant remains slightly cloudy (+3); coaggregates form immediately but remain suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1) no evidence of aggregation or coaggregation (0). Coaggregative interactions completely inhibited by a sugar are shaded dark grey and partial inhibitions are shaded light grey. (AN; *A. naeslundii*, FN; *F. nucleatum*, LR; *L. rhamnosus*, PG; *P. gingivalis*, CP; *C. perfringens*, BF; *B. fragilis*, BV; *B. vulgatus*, BA; *Bif. adolescentis*.)
4.5. Discussion

Coaggregation is considered by some to be fundamental to the formation and maintenance of dental plaque (Kolenbrander & London, 1993; Palmer et al., 2003); facilitating bacterial attachment (Whittaker et al., 1996), metabolic exchange and allowing interactions between distinct bacterial genomes (Foster et al., 2003; Kolenbrander, 2000). Although single and multi-species biofilms have been described in the human gastrointestinal tract, particularly the heavily colonised large intestine (Macfarlane & Dillon, 2007), information concerning the coaggregation interactions and the formation of these communities is limited. According to a search of the PubMed database, this is the first study to have investigated coaggregation among numerically and ecologically important intestinal bacteria, and between intestinal bacteria and oral isolates.

Previous studies of coaggregation between oral bacteria have mainly utilised the semi-quantitative visual assay (Bradshaw et al., 1998; Cisar et al., 1979; Kolenbrander, 1995; Shen et al., 2005; Weiss et al., 2000). In the current study, a reproducible quantitative spectrophotometric assay (Ikegami et al., 2004) was used to measure the extent of coaggregation interactions; with the visual assay being used to verify these results and for inhibition studies intended to elucidate adherence mechanisms. According to data generated by the current study, the quantitative assay provided data that are broadly congruent with the commonly used semi-quantitative, visual assay. Both methods have inherent strengths for particular applications; the visual assay enables a more rapid screening of a large amount of pairings. It is particularly useful for inhibition assays where a quantitative result is not essential and has been successfully utilised in studies
of isolates from freshwater biofilms (Rickard et al., 2003b). The spectrophotometric assay provides a quantitative measurement.

Coaggregation among organisms originating from the intestinal tract was limited in both frequency (a 4% incidence) and strength (Table 4.2.), indicating that this phenomenon may not be as important for the development of multi-species biofilms in the human gut as it has been suggested to be in the oral cavity. This could be because hydrodynamic and mechanical shear forces in the large intestine, where the majority of gastrointestinal microorganisms are located, are generally much lower than those that prevail in the mouth (Wilson, 2005). Therefore the selection pressure for expression of the appropriate proteinaceous adhesins and receptors mediating coaggregation is less marked. The viscous mucus produced by goblet cells of the intestinal epithelium may also limit the selection pressure for coaggregation because it often acts as the primary interface for bacterial colonisation. Mucins; high molecular weight glycoproteins, provide points of attachment for bacteria (Leitch et al., 2007) that may render coaggregation between some enteric isolates unnecessary. In fact, studies have implicated bacterial-mucin interactions in the formation of intestinal biofilms (Bollinger et al., 2006). The hard, non-shedding nature of teeth provides an ideal surface for the accumulation of large polymicrobial communities (Marsh & Martin, 2003). Coaggregation is believed to be important in the formation and maintenance of oral biofilms as many species are unable to attach directly to the acquired pellicle and the high shear forces that predominate in the mouth are likely to carry away the majority of unattached cells. Conversely, there are no hard surfaces in the intestinal epithelium and host cell turnover is rapid (Creamer, 1967). It seems likely therefore, that enteric bacterial cells have a
more transient existence, forming biofilms within the slow-moving viscous mucus of the intestine, with limited capabilities to coaggregate. However, spectrophotometric analysis did show that *C. perfringens* coaggregated with both *B. fragilis* and *B. adolescentis* (strength of coaggregation 39% and 58% respectively), indicating that if these Gram-positive species could survive the prevailing environmental conditions of the gut they may interact *in vivo*, possibly for co-operative feeding or gene exchange. This result is significant as bacteroidetes and clostridium have been proposed as two of the most numerically important taxonomic groups in the colonic microbiota (*Suau et al.*, 1999).

The frequency of coaggregation between oral and enteric bacteria was also low overall (an 8% incidence) (Tables 4.3. and 4.4.), suggesting that ecological interactions between these two groups may be limited *in vivo*, although exceptions to this were *F. nucleatum* and *B. adolescentis*, and *A. naeslundai*, together with both of the bacteroides tested, *B. adolescentis, C. perfringens* and *L. paracasei*. This could be attributed to the expression of receptors and adhesins that are similar to those formed by taxonomically related oral bacteria such as *Prevotella* spp. and *L. rhamnosus*. Contact between oral and intestinal isolates is most likely to occur when oral species are swallowed and subsequently transferred through the gastrointestinal tract. The stomach has traditionally been viewed as a barrier to microorganisms due to its strongly acidic environment. However, a recent study utilising 16S rDNA sequencing isolated 128 different phylotypes from the gastric biopsies of 23 human volunteers, which included approximately 33% that had previously been described in the mouth (*Bik et al.*, 2006). It is likely that some oral species may
survive transit to the colon and interact with the intestinal microbiota. Interestingly, strains of *Fusobacterium* normally associated with the oral cavity were amongst isolates highlighted in the study by Bik *et al* (2006) as oral bacteria found in the stomach; the strongest coaggregation interaction between an oral and intestinal isolate in our study involved *F. nucleatum* and *B. adolescentis*.

Coaggregation assays conducted for a range of oral bacteria effectively served as positive controls. In agreement with the literature, of the species on test *A. naeslundii*, *F. nucleatum*, *P. gingivalis* and the streptococci were shown to be the most promiscuous oral coaggregators. *Actinomyces* represent a significant proportion of the primary colonizers of tooth enamel (Palmer *et al.*, 2003), and are regarded as being fundamental in the development of dental plaque (Nyvad & Kilian, 1987). Their ability to coaggregate widely with other oral bacteria is well recognized (Hamada *et al.*, 1998; Li *et al.*, 2001) and they are known to exhibit multiple adherence mechanisms, including fimbriae-associated adhesins and protease-resistant receptors (Whittaker *et al.*, 1996). *A. naeslundii* itself has two kinds of fimbriae, of which the type 2 bears a coaggregation adhesin for oral streptococci (Rickard *et al.*, 2003a). As the streptococci constitute the majority of primary colonizers of dental plaque (Kolenbrander, 2000; Nyvad & Kilian, 1987) their inter- and intrageneric coaggregative ability is essential in maintaining community structure as plaque matures. Fusobacteria reach considerable numbers in mature dental plaque (Moore & Moore, 1994) and have previously been shown to coaggregate extensively with representatives of virtually every oral genus tested to date (Anderson & Sinclair, 1998; Kolenbrander & Andersen, 1989; Kolenbrander, 1995; Whittaker *et al.*, 1996).
The ability of species such as *F. nucleatum* to act as a bridging organism between early and late colonisers makes them fundamental to the formation of mature dental plaque (Bachrach et al., 2005). Results from the present study showed that *F. nucleatum* coaggregated considerably (> 30 %) with six of the nine oral strains tested; however, significant coaggregation between *F. nucleatum* and the intestinal isolates was limited to *B. adolescentis* (81 %). *P. gingivalis* and other obligate anaerobes such as *Treponema denticola* and *F. nucleatum* are commonly associated with the development of periodontitis. Coaggregation between *P. gingivalis* and other oral bacteria has been shown to be important in the development of subgingival biofilms that precipitate the development of gingivitis and periodontitis (Metzger et al., 2001; Yamada et al., 2005). In this study *P. gingivalis* coaggregated substantially (> 60 %) with four of the nine oral strains on test.

Inhibition studies revealed that, as with the interactions between oral strains, coaggregations between oral and gut bacteria are mediated by lectin–carbohydrate interactions. Previous studies have shown that coaggregation of oral bacteria is inhibited by specific sugars, often with a structure similar to that of lactose (Kolenbrander & Williams, 1981; Kolenbrander & Williams, 1983; Kolenbrander & Andersen, 1989; McIntire et al., 1982; Rickard et al., 2000; Rosen & Sela, 2006). The sugars chosen for this study have previously been used in the characterisation of coaggregation between strains derived from the oral cavity (Kolenbrander & Andersen, 1989). The results showed that all of the interactions occurring between oral and intestinal strains were inhibited to some extent by the addition of individual sugars. For the oral strains all coaggregations except (1) *F. nucleatum* and *L. rhamnosus*, (2) *A. naeslundii*
and *F. nucleatum* and (3) *A. naeslundii* and *L. rhamnosus* were inhibited by one or more of the sugars chosen for this study. It seems logical to conclude that it is the unique tertiary structure of cell surface proteins that means only specific sugars with complementary stereochemistry are able to bind and inhibit coaggregation. Thus even two diastereoisomers may not be able to inhibit the same coaggregation. This is illustrated in the current study as L-fucose inhibited the following interactions whilst D-fucose did not: (1) *A. naeslundii* and *C. perfringens* and (2) *A. naeslundii* and *Bacteroides vulgatus*.

Interestingly, for the pairing of *A. naeslundii* and *L. rhamnosus*, six of the ten sugars tested marginally increased the levels of coaggregation observed. It is possible that, in some cases, the sugar molecules act as a bridge between two species expressing lectins with complementary affinities. Application of these data in conjunction with existing knowledge of the cell surface structure of a particular strain may assist in identifying the carbohydrate receptors responsible for each coaggregation.

Heat treatment of coaggregating isolates can indicate whether one or both partners carry a proteinaceous adhesin that mediates coaggregation. *F. nucleatum*, one of the most prolific coaggregators in the mouth, appeared to carry the protein component in the majority of pairings used in this investigation. The effect of heating on this strain was most noticeable when studying the interaction with *B. adolescentis*, where a strong coaggregation was completely inhibited. Pairings that exhibited inhibition, when either partner was heated, may be mediated by a number of interactions where lectins and proteins are present on the cell surface of both strains.
4.6. Conclusions

This chapter aimed to identify whether the processes of bacterial coaggregation and coadhesion occur between selected species of the human intestinal microbiota. Bacteria chosen for the study included those from the genera *Bacteroides, Clostridium* and *Eubacterium* as these groups comprise over 95% of the bacteria found in faeces and intestinal mucus (Backhed *et al.*, 2005; Suau *et al.*, 1999). The results have demonstrated that strong coaggregation interactions between gut isolates and between oral and gut bacteria are markedly less common than among species belonging to the oral microbiota, suggesting that the role of coaggregation in community development in the intestinal tract, and in interactions between oral and gut populations *in vivo*, may be less important. Host cell turnover is rapid in the intestinal epithelium and shear forces are generally low, thus the selection pressure for expression of the appropriate proteinaceous adhesins and receptors mediating coaggregation may be less marked. It seems likely that biofilm architecture is more transient in the gut, with enteric bacteria held within slow moving viscous mucus and predominantly binding to host molecules. Additionally, these results suggest that, as with the coaggregation of oral bacteria, the coaggregations between oral and gut bacteria that were identified are mediated by lectin–carbohydrate interactions. Consequently they can be inhibited by specific sugars and by heat treatment of one or both partners.

The study also enabled a comparison of the visual and spectrophotometric coaggregation assays to be done. The visual assay provided a comparatively rapid, semi-quantitative assessment that has been shown in this study to
produce results congruent with those from the quantitative assay. It is particularly useful when performing inhibition assays with a large number of pairings and sugars. The fully quantitative spectrophotometric assay appears to provide a more sensitive analysis and is thus useful for screening isolates and comparing pairings.

Further work could include an assessment of coaggregation between a larger number of intestinal isolates, perhaps focusing on strains related to those that have been shown to coaggregate in this study. It may also be useful to look at other multi-species biofilms associated with the human body, such as the skin. The methods utilised in this study would be appropriate to assess isolates from any body site.
Chapter 5

Analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas
5.1. Abstract

Whilst the processes which underlie the formation and maintenance of the oral and other microbiotas are likely to be highly conserved, individual microbiotas are both compositionally unique and highly stable. The mechanisms that underlie stability and inter-individual variation at species and community level are however not well understood but are likely to involve the interplay of antagonistic and positive interactions between bacteria. The current chapter tests the hypothesis that should antagonism be a major determinant of consortial composition, antagonistic bacterial interactions would occur at higher frequency between rather than within individual microbiotas. **Methods:** Bacteria were isolated from the saliva of four individuals using enrichment techniques to maximise recovery. Interaction outcomes were then determined using a modified cross-streak method which determines positive, negative or neutral interactions among paired strains. Quantitative coaggregation assays were additionally performed on selected pairings. **Results:** From a total of 1176 aerobic and 136 anaerobic pairings the incidence of negative interactions was significantly higher than the incidence of positive interactions (41.5% vs. 5.4%). No significant difference was apparent in the frequency of positive or negative interactions between self/self and self/non-self pairings amongst aerobic isolates (mean 6.7% and 36.7% vs. 5.2% and 38.6%, respectively). Intragenera interactions, in particular amongst the streptococci, exhibited a higher than average level of negative outcomes. The incidence of coaggregation amongst selected isolates was low with no obvious correlation to the cross-streak results. **Conclusions:** The majority of interactions observed were antagonistic regardless of whether the pairing was classified as self/self or self/non-self. Oral bacteria therefore have apparently developed mechanisms to compete against those organisms from which they derive no benefit and cooperate with a limited number of species. This compositional stability and inter-individual variation in the oral microbiota may result from stochastic events during consortial development together with various host-derived and environmental variables.
5.2. Introduction

With over 700 bacterial species identified thus far the oral microbiota is one of the most diverse and widely studied human-associated bacterial communities (Aas et al., 2005; Paster et al., 2006). Many species are found at multiple sites within the oral cavity; transiently within saliva and as part of dental or mucosal biofilms, whilst other taxa are site and subject specific (Aas et al., 2005; McBain et al., 2005). Numerous studies have described the processes by which oral bacteria interact to integrate into functionally organised oral biofilms as dependent upon the prevailing physical and biological properties of the anatomical site (Aas et al., 2005; Bowden et al., 1975; Mager et al., 2003; Marsh & Bradshaw, 1999; Marsh, 2004). Whilst the key features that underpin this process appear to be universal, the oral microbiota of each individual is unique and exhibits considerable microbial stability over time (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005). The mechanisms that underlie significant inter-individual variation and stability at both the species and community level are currently not well understood.

He et al (2010) proposed that the establishment of unique individual oral microbiotas, akin to other human-associated microbial communities, are shaped by two distinct factors (He et al., 2010a): i) a legacy effect, whereby components of the microbiota of the mother or local environment at birth are inherited (Gronlund et al., 1999; Ley et al., 2006; Mandar & Mikelsaar, 1996) and ii) the host habitat, which includes selection pressures from genotype i.e. immune response and anatomical structure; and also the nutritional environment (Frank et al., 2003; Zoetendal EG et al., 2001). The authors
suggest that these unique communities are then maintained by a process termed community selection, whereby the established microbiota excludes the integration of foreign species ensuring its own continuity and stability (He et al., 2010a; He et al., 2010b). This hypothesis has been established on the basis of several studies including some that investigated community antagonism between oral and gut microbiotas using PCR-DGGE (He et al., 2010a) and which demonstrated the exclusion of a “foreign” strain of *Escherichia coli* by an established oral microbiota (He et al., 2010b).

Oral bacteria are believed to derive a number of benefits from existence as part of a stable biofilm that may in turn, drive the selection pressure for the exclusion of adventitious species. For example, the hydrated extracellular matrix of a biofilm offers physical protection from exogenous compounds, whilst concentrating nutrients, metabolic bi-products and defensive substances by limiting their diffusion out of the biofilm (Kinder & Holt, 1994). The close physical interactions, including coaggregations, between bacteria may facilitate the establishment of cross-feeding (Drago et al., 1997), cell-cell communication (Kolenbrander, 2000), genetic exchange between cells (Foster et al., 2003) and provide protection for anaerobes against oxygen (Bradshaw et al., 1998). However, these complex consortia are likely to be established and maintained not only by the numerous synergistic interactions described but by the complex interplay of both positive and antagonistic bacterial interactions, including the production of bacteriocins and inhibitory metabolic products (Kuramitsu et al., 2007; Marsh & Bradshaw, 1999).
Chapter 5, Ecological analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas

Numerous studies have identified synergistic inter-species interactions occurring between inhabitants of the oral cavity. For example, cross-feeding has been observed between *Porphyromonas gingivalis* and *Treponema denticola*, where the growth of *P. gingivalis* was promoted by metabolising succinate produced by *T. denticola*, which was itself promoted by the presence of isobutyric acid produced by *P. gingivalis* (Grenier & Mayrand, 1986). Cooperative metabolism has also been described between dental plaque isolates. Takahashi (2003) demonstrated that *F. nucleatum* and *P. intermedia* are able to generate ammonia by the fermentation of glutamic and aspartic acids found in saliva. As a result areas of plaque in close proximity to those species are found to be less acidic and provide protection for acid-sensitive species, such as *P. gingivalis*, against substantial drops in pH attributed to the presence of lactic acid producing bacteria and dietary fermentable carbohydrates (Takahashi, 2003). Signalling molecules such as autoinducer 2 (AI-2) are also important for mutualistic inter-species interactions. For example, Rickard et al. (2006) have shown that AI-2 concentration is critical for the formation of biofilms composed of two oral species; *A. naeslundii* and *S. oralis* (Rickard et al., 2006).

Many species of oral bacteria are also able to exert an inhibitory effect on the growth of neighbouring species (Kuramitsu et al., 2007). One of the ways in which this occurs is via the production of proteinaceous toxins, termed bacteriocins. The streptococci, a genus which make up an estimated 60 to 90% of the early colonisers of a tooth surface and a significant proportion of dental plaque as a whole (Nydad & Kilian, 1987), are one of the most prolific producers of bacteriocins (Nes et al., 2007). *Streptococcus mutans* for example, is known
to produce at least five different bacteriocins, commonly termed mutacins (Hale et al., 2005). Mutacins produced by *S. mutans* effectively inhibit the growth of *Streptococcus sanguinis* strains and thus *S. sanguinis* is unable to colonise niches occupied by large number of *S. mutans* (Kreth et al., 2005). Bacteriocin production is believed to be regulated by the prevailing environmental conditions, impacted by pH, cell density and nutritional availability (Kreth et al., 2005; Kuramitsu et al., 2007; Merritt et al., 2005). Many species of oral bacteria are also known to produce non-specific antimicrobial components that shape the biofilm community (Kuramitsu et al., 2007). *S. sanguinis* strains can produce hydrogen peroxide, which has an antagonistic effect on the growth of *S. mutans* and therefore the two species are rarely found growing in close proximity (Kreth et al., 2005).

Antagonistic and synergistic bacterial interactions are fundamental to the formation of oral biofilms, impacting upon the localisation of species within the biofilm and thereby it’s structure and function (Kolenbrander, 2000; Kuramitsu et al., 2007). Although many inter-species interactions have been documented it seems likely that there are far more still to be characterised. In particular there has been, as yet, limited investigation of bacterial interactions within and between microcosms. By studying inter and intra-individual interactions between species, and later at community level (Chapter 6), work in this chapter aimed to elucidate the microbial phenomena responsible for individual variation and community stability within oral biofilms. Initial work has focused on individual oral isolates and the nature of interactions between them. It was hypothesised that in order to facilitate a stable and resilient biofilm the frequency of positive or synergistic interactions would be much greater between
isolates derived from the same mouth compared to those from different mouths. As several studies have indicated that the oral microbiota is able to effectively exclude “foreign” species (He et al., 2010a; He et al., 2010b), antagonistic interactions were expected to increase in frequency between isolates derived from different salivary microcosms.

A rapid screening method capable of dealing with a large number of interactions between salivary isolates was required for this study and a modified cross-streak method was adopted. This assay was based on previous studies of interactions between human-associated bacteria including a drop-plate method proposed by Rosebury et al. (1954), which utilised overlapping drops of liquid bacterial cultures on different agars to identify growth promotion and inhibition (Rosebury et al., 1954). The majority of studies in this area however have looked primarily for growth inhibition and/or bacteriocin production (Krausse et al., 2005; Scrivener et al., 1950; Wang & Kuramitsu, 2005). Frequently the methodology has included the cultivation and killing of one species suspected of producing bacteriocins prior to the introduction of the second species and thus limits the ability of the study to identify synergistic interactions (Kekessy & Piguet, 1970; Krausse et al., 2005). The method developed for this chapter was designed to elucidate both growth promotion and inhibition. It was also anticipated that the high cell density achieved in this assay and the enforced proximity of the two species would facilitate the conditions necessary for cross-feeding, cooperative metabolism or inhibition by antimicrobial components.

The specificity and strength of coaggregation has been previously used to examine inter-species interactions in early plaque development. The ability of
two species to coaggregate is also likely to affect the localisation of species throughout a biofilm (Cisar et al., 1979; Kolenbrander, 1997). Rickard et al (2006) have shown that coaggregation between Actinomyces naeslundii and Streptococcus oralis facilitates the intimate contact required between the two species to result in a mutualistic interaction involving increased production of autoinducer 2 (AI-2) (Rickard et al., 2006). It was hypothesised that the frequency of coaggregation, assessed by quantitative coaggregation assay (Ikegami et al., 2004), would be higher between pairings that resulted in growth promotion of one or both species in the cross-streak assay than those that resulted in inhibition or no interaction.

The current chapter aimed to test the hypothesis that should antagonism be a major determinant of consortial composition in the oral cavity, antagonistic bacterial interactions would occur at higher frequency between, rather than within, individual microbiotas. In addition to investigating the frequency of antagonistic and positive interactions the frequency and characteristics of coaggregation between selected isolates was assessed.
5.3. Materials and Methods

5.3.1. Isolation of salivary bacteria

Fresh saliva samples were obtained from four healthy subjects who had received no recent antibiotic therapy or invasive dental treatment. Volunteers denoted A and B are white males of UK origin aged 24 and 25 respectively, volunteer C an Asian female of Indian origin aged 23 and volunteer D a white female of UK origin aged 26. Enrichment techniques were utilised to maximise diversity in recovery of bacterial isolates. 100μl of fresh saliva from volunteer A was added to five different growth media comprising Wilkins-Chalgren broth supplemented with either: 1) 1g/L glucose, 2) 1g/L sucrose, 3) 1g/L casein hydrolysate, 4) 1g/L mucin (hog gastric) or 5) 0.25mg/ml triclosan. Each sample was incubated aerobically at 37°C for 48h. A duplicate set of samples was incubated anaerobically (Gas mix: 80% N₂, 10% CO₂ and 10% H₂) at 37°C for 48 h in a Mark 3 Anaerobic Work Station (Don Whitely Scientific, Shipley, U.K.). This process was repeated with saliva samples from volunteers B, C and D.

0.1ml aliquots of homogenised suspension from each culture were plated in duplicate onto Wilkins-Chalgren agar and incubated aerobically or anaerobically at 37°C for 24h. Unique single colonies were identified and subcultured, incubated for a further 24h and then archived at -70°C in a solution of nutrient broth with 10% glycerol.

5.3.2. Identification of salivary isolates

In order to identify the isolates bacterial DNA was extracted by boiling a suspension of the samples for 10min then centrifuging for 10min at 13226 xg (Section 2.2.1.). DNA from the supernatant was amplified using PCR primers
specific for the 16S rDNA region of the eubacterial genome, 8FPL1 (5’- GAG TTT GAT CCT GGC TCA G -3’) and 806R (5’- GGA CTA CCA GGG TAT CTA AT -3’) (Eurofins MWG Operon, Ebersberg, Germany) (McBain et al., 2003d) (Section 2.3.1.). The quality and quantity of DNA obtained from the extraction and PCR process was assessed by agarose gel electrophoresis (Section 2.4.). Isolates from which an insufficient amount of DNA was extracted by the boiling and centrifuging method were extracted using the QiaAmp DNA stool mini kit (Qiagen, Sussex, UK) (Section 2.2.2.). Sequence analysis was carried out on the purified PCR products and identification took place using the EMBL-EBI FASTA nucleotide similarity online database (Section 2.3.3.).

5.3.3. Cross-streak method

Pair-wise interactions of: 49 unique isolates cultured aerobically and 17 unique isolates cultured anaerobically, were tested using a modified cross-streak method (Krausse et al., 2005; Scrivener et al., 1950). Test organisms were grown overnight in broth culture (Wilkins-Chalgren, 10mL) at 37°C in either an aerobic or anaerobic environment. 20μL of each suspension was pipetted onto Wilkins-Chalgren agar to form a vertical line approximately 30mm in length. After drying 20μL of a second suspension containing a different isolate was pipetted horizontally across the first streak to form a cross shape. This process was repeated for every possible pair-wise interaction resulting in 1176 cross-streaks of aerobic isolates and 136 cross-streaks of anaerobic isolates. Plates were incubated aerobically and anaerobically as appropriate at 37°C for 24 hours. Interactions were analysed visually and classified as positive (growth promotion of one or both species), negative (growth inhibition of one or both species), mixed (growth inhibition of one species and promotion of the second)
or neutral (no reaction). Each plate was photographed using a Canon EOS D60 digital camera (Cannon, Surrey, UK).

**5.3.4. Fisher’s Exact Test**

Results of the cross-streak interactions were analysed statistically using the Fisher’s Exact Test in the SPSS statistical package (version 16.0, SPSS Inc.). Fisher’s exact test is used to examine the significance of the association (contingency) between two variables, and although similar to a chi-square test it can be used regardless of the sample size or characteristics. The test was utilised in this chapter by establishing a null hypothesis and presenting the data in a contingency table as illustrated by the example in Figure 5.1.

**Null Hypothesis (H₀):** There is no significant difference in the likelihood of a negative interaction occurring between species isolated from volunteers of the same sex compared with those isolated from volunteers of different sexes.

<table>
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<tr>
<th></th>
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</tr>
<tr>
<td>Other</td>
<td>c</td>
<td>d</td>
<td>c + d</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
<td>b + d</td>
<td>n</td>
</tr>
</tbody>
</table>

**Figure 5.1.** Example of a null hypothesis and 2x2 contingency table with the numerical values substituted for letters.

The SPSS software calculated the probability (p) of obtaining this set of values using the hyper geometric distribution (a discrete probability distribution that describes the number of times an event happens in a fixed number of trials):

\[
p = \frac{(a+b)!(c+d)!(a+c)!(b+d)!}{n!a!b!c!d!}
\]

Where a, b, c, d and n were found in the contingency table (Figure 5.1.). A p value below the chosen significance level (in this case below the 1% significance level (p < 0.01)) led to the null hypothesis being rejected.
5.3.5. Preparation of inocula for coaggregation assays

Selections of pairings were subjected to coaggregation assays including those that were found to have resulted in a positive, negative and neutral interaction in the cross streak analysis. Cultures of test bacteria were grown under conditions previously validated to produce stationary phase cells by inoculating each of the strains into 500ml of Wilkins Chalgren broth and incubating in an aerobic environment at 37°C for 3d (Bradshaw et al., 1998).

5.3.6. Spectrophotometric coaggregation assays

A modified quantitative spectrophotometric assay described by Ikegami et al., (2004) was initially used to determine the coaggregation activity between all test species. Bacteria were harvested by centrifugation at 24 000 xg (Beckman-Coulter J2-21) for 20min and resuspended in coaggregation buffer (Budu et al., 2003). This buffer comprised 1mM CaCl₂, 0.1mM MgCl₂ and 0.15M NaCl dissolved in 20mM Tris buffer adjusted to pH7.0. The strains were then washed three times by centrifugation (24 000 xg, 20min) in coaggregation buffer and resuspended to give an optical density (OD) reading of 1.0 at 600nm. Equal volumes (1ml) of each suspension were then mixed in sterile cuvettes and the optical densities immediately recorded. Autoaggregation was determined using an identical method, by combining two equal volumes of the same bacterial suspension. In all cases the mixtures were left for 1h at room temperature to allow coaggregation to occur, after which OD of the supernatant was once again recorded. Each assay was repeated to give a total of three values to facilitate the calculation of means. Percentage coaggregation was calculated by the following equation:
Chapter 5, Ecological analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas

\[ \text{Coaggregation} = \frac{(\text{preincubation value [OD}_{600}]) - \text{test value [OD}_{600}])}{(\text{preincubation value [OD}_{600}])} \times 100 \]

Pairings that showed significant levels of coaggregation (>10%) were chosen for further analysis by heat and sugar inhibition assays.

5.3.7. Effect of lactose on the strength of coaggregation

The ability of lactose to reverse selected coaggregation interactions was determined by the following method: lactose, at a final concentration of 100mM, was added to 1ml of a suspension containing one of the coaggregating strains and mixed. After standing at room temperature for 15min, 1ml of a suspension containing the partner strain was added and mixed again. Optical density readings were taken immediately and after 60min and percentage coaggregation values calculated. Each assay was undertaken in triplicate.

5.3.8. Influence of heating on coaggregation

The effect of heat on the selected coaggregation interactions was determined by heating one of the two partner strains in a water bath at 85°C for 30min (Kolenbrander, 1995). An equal volume (1ml) of suspension containing the partner was added, optical density readings were taken immediately and after 60min and percentage coaggregation values calculated. Each assay was undertaken in triplicate. This process was repeated for the second partner, i.e. each of the two partners heated in separate coaggregation assays.

The degree of inhibition of the coaggregation by the addition of lactose or heat treatment was calculated using the following equation:

\[ \% \text{ inhibition} = \frac{\% \text{ coaggregation without treatment} - \% \text{ coaggregation with treatment}}{\% \text{ coaggregation without treatment}} \]
5.4. Results

5.4.1. Identities of isolates cultured under aerobic conditions

Data in Table 5.1. show the closest relatives (based on results of EMBL searches) of the salivary isolates cultured under aerobic conditions.

Table 5.1. Sequencing and identification of salivary isolates grown under aerobic conditions.

<table>
<thead>
<tr>
<th>ID</th>
<th>Closest relative (% sequence similarity)</th>
<th>Sequence Length Base pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1*</td>
<td><em>Streptococcus thermophilus</em> L (98.2) EM_PRO:CP000023</td>
<td>776 (11)</td>
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<tr>
<td>A2*</td>
<td><em>Streptococcus salivarius</em> (98.2) EM_PRO:AY188352</td>
<td>774 (9)</td>
</tr>
<tr>
<td>A3*</td>
<td><em>Streptococcus vestibularis</em> (97.0) EM_PRO:AY188353</td>
<td>781 (13)</td>
</tr>
<tr>
<td>A4*</td>
<td><em>Streptococcus thermophilus</em> C (97.4) EM_PRO:CP000024</td>
<td>777 (6)</td>
</tr>
<tr>
<td>A5</td>
<td><em>Streptococcus salivarius</em> (97.4) EM_PRO:AY188352</td>
<td>785 (18)</td>
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<td>A6*</td>
<td><em>Enterococcus faecium</em> (97.3) EM_PRO:AB232954</td>
<td>777 (15)</td>
</tr>
<tr>
<td>A7</td>
<td><em>Streptococcus vestibularis</em> (97.7) EM_PRO:AY188353</td>
<td>777 (11)</td>
</tr>
<tr>
<td>A8*</td>
<td><em>Streptococcus salivarius</em> (98.7) EM_PRO:M58839</td>
<td>772 (9)</td>
</tr>
<tr>
<td>A9*</td>
<td><em>Streptococcus mitis</em> ATCC 903 (98.0) EM_PRO:AY281078</td>
<td>779 (12)</td>
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<td>A19</td>
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*aBased on EMBL database searches. *The number of ambiguous bases are given in parenthesis. Letters in ID column refer to volunteers A, B, C and D. *Isolates selected for cross streak study.
<table>
<thead>
<tr>
<th>ID</th>
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<th>Base pairs</th>
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<td>768</td>
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</tbody>
</table>

*aBased on EMBL database searches. bThe number of ambiguous bases are given in parenthesis.

Letters in ID column refer to volunteers A, B, C and D.

*Isolates selected for cross streak study.
5.4.2. Identities of isolates cultured under anaerobic conditions

Data in Table 5.2. show the closest relatives (based on results of EMBL searches) of the salivary isolates cultured under anaerobic conditions.

<table>
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<tr>
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<th>Sequence Length Base pairs b</th>
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<td>*Neisseria flavescens (96.4) EM_PRO:L06168</td>
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<tr>
<td>D5*</td>
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*aBased on EMBL database searches. bThe number of ambiguous bases are given in parenthesis.
Letters in ID column refer to volunteers A, B, C and D.
*Isolates selected for cross streak study.
Table 5.2. Sequencing and identification of salivary isolates grown under anaerobic conditions.

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<tr>
<th>ID</th>
<th>Closest relative (% sequence similarity)</th>
<th>Sequence Length</th>
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</tr>
<tr>
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<td>752 (6)</td>
</tr>
<tr>
<td>B2a</td>
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</tr>
<tr>
<td>B3a</td>
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<td>803 (7)</td>
</tr>
<tr>
<td>B4a</td>
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</tr>
<tr>
<td>B5a*</td>
<td>Lactobacillus gasseri ATCC 3 (98.5) EM_PRO:CP000413</td>
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</tr>
<tr>
<td>B6a</td>
<td>Streptococcus thermophilus L (98.2) EM_PRO:CP000023</td>
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</tr>
<tr>
<td>B7a</td>
<td>Streptococcus anginosus (98.3) EM_PRO:AY986762</td>
<td>784 (7)</td>
</tr>
<tr>
<td>B8a</td>
<td>Staphylococcus epidermidis R (99.6) EM_PRO:CP000029</td>
<td>777 (4)</td>
</tr>
</tbody>
</table>

*Based on EMBL database searches. The number of ambiguous bases are given in parenthesis.
Letters in ID column refer to volunteers A, B, C and D.
Isolates selected for cross streak study.
## Chapter 5, Ecological analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas

<table>
<thead>
<tr>
<th>ID</th>
<th>Closest relative (% sequence similarity)</th>
<th>Sequence Length Base pairs</th>
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<tbody>
<tr>
<td>B9a</td>
<td><em>Lactobacillus fermentum</em> (98.4) EM_PRO:AB362610</td>
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</tr>
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<td>B13a</td>
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</tr>
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<tr>
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<tr>
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<td><em>Streptococcus pneumoniae</em> D39 (86.6) EM_PRO:CP0000410</td>
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</tr>
<tr>
<td>B19a</td>
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</tr>
<tr>
<td>B20a</td>
<td><em>Peptostreptococcus stomatis</em> (98.4) EM_PRO:DQ160208</td>
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</tr>
<tr>
<td>C1a</td>
<td><em>Veillonella parvula</em> 16S rRNA gene (98.7) EM_PRO:X84005</td>
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</tr>
<tr>
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</tr>
<tr>
<td>C3a</td>
<td><em>Streptococcus thermophilus</em> L (99.0) EM_PRO:CP000023</td>
<td>770 (2)</td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td><em>Streptococcus agalactiae</em> 260 (98.8) EM_PRO:AE014207</td>
<td>771 (9)</td>
</tr>
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<td>C16a</td>
<td><em>Streptococcus gordonii</em> strain (99.1) EM_PRO:CP000725</td>
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</tr>
<tr>
<td>C17a</td>
<td><em>Veillonella atypica</em> 16S rRNA gene (99.2) EM_PRO:X84007</td>
<td>785 (0)</td>
</tr>
<tr>
<td>C18a</td>
<td><em>Veillonella atypica</em> 16S rRNA gene (99.0) EM_PRO:X84007</td>
<td>784 (1)</td>
</tr>
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<td>C19a</td>
<td><em>Streptococcus parasanguis</em> 16 (98.2) EM_PRO:AF003933</td>
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<td>C20a</td>
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<td>793 (4)</td>
</tr>
<tr>
<td>C21a</td>
<td><em>Streptococcus anginous</em> strain (98.6) EM_PRO:AY691536</td>
<td>789 (11)</td>
</tr>
</tbody>
</table>

*Based on EMBL database searches. The number of ambiguous bases are given in parenthesis. Letters in ID column refer to volunteers A, B, C and D. *Isolates selected for cross streak study.
**5.4.3. Analysis of results of the cross-streak assay undertaken using isolates cultured under aerobic and anaerobic conditions**

49 species isolated under aerobic conditions were selected for the cross-streak study, with representatives from the salivary microbiota of all four volunteers. Of the 1176 cross-streaks performed 581 (49.4%) resulted in an interaction of some kind, positive, negative or mixed. The remaining 50.6% resulted in neither
growth inhibition nor promotion of either species. The frequency of negative interactions was much higher than positive interactions for all subjects (Table 5.3).

**Table 5.3.** Overview of the interactions occurring between the 49 aerobically cultured isolates selected for cross-streak analysis.

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
<th>Mixed&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Number of interactions of that type</td>
<td>71</td>
<td>12.2%</td>
</tr>
<tr>
<td>Both isolates affected</td>
<td>5</td>
<td>7.0%</td>
</tr>
<tr>
<td>% of total cross-streaks performed</td>
<td>6.0%</td>
<td>42.1%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mixed indicates that the interaction resulted in the growth of one species and the inhibition of a second species.

17 species isolated under anaerobic conditions were selected for the cross-streak study, with representatives from the salivary microbiota of all four volunteers. Of the 136 cross-streaks performed 49 (36%) resulted in an interaction of some kind. The remaining 64% resulted in neither growth inhibition nor promotion of either species. No positive or mixed interactions were observed amongst the anaerobically cultured isolates (Table 5.4).

**Table 5.4.** Overview of the interactions occurring between the 17 anaerobically cultured isolates selected for cross-streak analysis.

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
<th>Mixed&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>Number of interactions of that type</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Both isolates affected</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>% of total cross-streaks performed</td>
<td>0%</td>
<td>36%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mixed indicates that the interaction resulted in the growth of one species and the inhibition of a second species.

The frequency of interactions of any type appeared to increase in those species cultivated under aerobic conditions compared to anaerobic isolates, but this difference was not considered statistically significant (Fisher’s Exact Test p=0.086). However, the likelihood of an interaction resulting in the growth
inhibition of one or both species was statistically higher than the likelihood of
growth promotion ($p=0.0038$) regardless of whether the isolates were cultivated
under aerobic and anaerobic conditions.

Examples of a positive interaction, a negative interaction and no interaction are
shown in Figure 5.2.

![Figure 5.2](images)

**Figure 5.2.** Digital photographs showing example interactions.
a. growth promotion of *Streptococcus thermophilus* C isolated from subject B by *Enterococcus faecium* isolated from subject A; b. inhibition of *Enterococcus faecium* isolated from subject A by *Streptococcus sanguinis* isolated from subject C; c. no interaction occurring between *Streptococcus thermophilus* L isolated from subject A and *Neisseria flavescens* isolated from subject C.

### 5.4.4. Analysis of the frequency and characteristics of self/self and self/non-self interactions between isolates

The term self/self is used to describe interactions occurring between two
species isolated from the salivary microbiota of one volunteer. Self/non-self
refers to those interactions occurring between two species isolated from
different volunteers. For species isolated under aerobic conditions analysis of
the cross streak data using Fisher’s exact test showed that there was no
statistically significant difference ($p=0.787$) in the frequency of interactions
(either positive or negative) between self/self and self/non-self pairings (mean
positive: 6.7% and negative: 36.7% vs. positive: 5.2% and negative: 38.6%
respectively). For species isolated under anaerobic conditions analysis using
Fisher’s exact test showed that there was no statistically significant difference in the frequency of interactions between self/self and self/non-self pairings (mean positive: 0% and negative: 16.7% vs. positive: 0% and negative: 41.5% respectively). However, the likelihood of a negative outcome for one or both species appeared to be higher for the self/non-self pairings (p=0.0168), and had the threshold for rejecting the null hypothesis been set at the 5% significance level as opposed to 1% the difference would have been considered statistically significant.

5.4.5. Analysis of growth inhibition and promotion patterns in cross streak pairings within and between individuals

Variations in the number of cross streak pairings that resulted in bacterial growth promotion or inhibition exhibited inter-individual variation as demonstrated in Figure 5.3. Interestingly, a correlation was observed between the aerobic and anaerobic isolates from each individual: isolates from volunteer D exhibiting the highest percentage of interactions and volunteer B the least.
Figure 5.3. Cross streak data comparing the percentage of pairings for each volunteer that resulted in an interaction, positive or negative, for one or both species. Closed bars, aerobes; open bars, anaerobes.

Data in Figure 5.4. shows the proportion of interactions involving each of the four individuals that were classified as positive or negative: the general trends were remarkably similar across all volunteers. Aerobic isolates from volunteers B were more likely to be inhibited by isolates from other volunteers than to cause growth inhibition (p=0.0022). However, aerobic isolates from volunteer C were more likely to inhibit the growth of isolates from other volunteers than to be inhibited (p=0.003). Aerobic isolates from volunteer A were much more likely to promote the growth of isolates from other volunteers (p<0.001), whilst growth of isolates from volunteer D were more likely to be promoted by interactions with “foreign” isolates (p=0.003).
Figure 5.4. Data in this figure shows the distribution of positive and negative interactions across isolates from all four volunteers in the cross streak study. O₂ denotes isolates grown under aerobic conditions and AnO₂ those grown under anaerobic conditions. +ve indicates those interactions classified as positive, where the growth of one isolate was promoted. -ve indicates those interactions classified as negative, where the growth of one isolate was inhibited. The open bars and those shaded dark grey show the proportion of the interactions where the isolate that was affected (positively or negatively) was derived from the salivary microbiota of the volunteer in question e.g. far left, A. The closed bars and those shaded light grey show the proportion of interactions where the isolates from the volunteer in question inhibited or promoted the growth of one of the other three volunteers e.g. far left, B, C or D.

*Indicates results that were found to be statistically significant, detailed in Section 5.4.5.
5.4.6. The impact of volunteer gender on the outcome of cross streaks between salivary isolates

Data in Figure 5.5. shows that the gender of the volunteer from which the salivary isolates were obtained impacted upon the outcome of the cross streak analysis. Intra-gender positive interactions occurred at a similar frequency to the overall average. However, growth promotion was significantly less frequent between isolates from a different gender ($p < 0.001$), particularly growth promotion of isolates from male volunteers. The incidence of growth inhibition between isolates from two different male volunteers was significantly lower than any other gender combination ($p < 0.001$).

![Figure 5.5](image)

**Figure 5.5.** Data to show the proportion of cross streaks that resulted in promotion or inhibition of the growth of aerobic isolates, within and between the two genders.  

*+ve* indicates those interactions classified as positive, where the growth of one isolate was promoted.  

*-ve* indicates those interactions classified as negative, where the growth of one isolate was inhibited.  

*male/male* indicates that the cross streak was performed with isolates derived from the saliva of two different individuals who were both male.  

*female/female* indicates that the cross streak was performed with isolates derived from the saliva of two different individuals who were both female.  

*male/female* indicates that the cross streak was performed with isolates derived from the saliva of two different individuals one female one male. The hatched bars represent the proportion of interactions where the female isolate was affected from the cross streak of male/female isolates.  

*Indicates results that were found to be statistically significant, detailed in Section 5.4.6.
5.4.7. Interactions between the same species isolated from different volunteers and the characteristics of intra-genera cross streaks

Twenty-five of the self/non-self cross-streaks involved the same species isolated aerobically from two different volunteers; 40% of these were neutral, 52% were negative, 4% were positive and 4% were mixed. The oral panel included aerobic isolates from ten different genera; dermococci (n=1), enterobacteria (n=1), enterococci (n=3), klebsiellae (n=1), lactobacilli (n=2), neisseriae (n=7), pseudomonades (n=1), rothia (n=1), staphylococci (n=3) and streptococci (n=29). Of the 412 intra-genera cross-streaks 45% were neutral, 49% were negative, 4% were positive and 2% were mixed. Most of the isolates were either streptococci or neisseriae and these results were also analysed independently. Cross-streaks between streptococci produced the following results: 46% were neutral, 49% were negative, 4% were positive and 1% were mixed. For Neisseriae: 39% were neutral, 43% were negative, 14% were positive and 4% were mixed.

The oral panel included anaerobic isolates from ten different genera; prevotellaceae (n=2), veillonella (n=3), peptostreptococci (n=1), megasphaera (n=1), lactobacilli (n=3), selenomonad (n=1), acinetobacteraceae (n=1), atopobium (n=1), clostridium (n=1) and streptococci (n=3). Of the 126 intra-genera cross-streaks 58% were neutral, 40% were negative, 0% were positive and 0% were mixed. Data in Figure 5.6 shows the distribution of interactions amongst the different genera isolated for this study, both aerobically and anaerobically.
Figure 5.6. Data shows the distribution of positive (bars shaded black), negative (bars shaded dark grey), mixed (bars shaded light grey) and no interactions (white bars), between the genera isolated aerobically and anaerobically for this chapter.
5.4.8. Spectrophotometric coaggregation assays between selected oral isolates

*S. thermophilus* and *S. salivarius* isolated from the saliva of volunteer A showed very high levels of autoaggregation in the spectrophotometric coaggregation assay (82 and 76% respectively). Coaggregation assays were not completed with these isolates as they could not be dispersed sufficiently to reach an optical density of 1. Autoaggregation scores of 19% for *S. parasanguis* isolated from volunteer A and 17% for *S. salivarius* isolated from volunteer C were also recorded.

Data in Table 5.3. show that the frequency of coaggregation amongst the selected isolates was low. Of the four coaggregation interactions observed the strength of the coaggregations ranged from 11 to 33%. Three of these interactions occurred between *S. parasanguis* from volunteer A’s salivary microbiota and isolates from volunteer C and D. *S. parasanguis* exhibited autoaggregation of 19% which may account for at least part of the coaggregations observed. A coaggregation interaction was identified between *L. fermentum* from volunteer D and *S. salivarius* from volunteer C and was measured at 13%, although again this could be due to the high levels of autoaggregation of *S. salivarius*.

*S. thermophilus* was isolated from three different volunteers and each isolate behaved differently in the coaggregation assays. *S. salivarius* was isolated from two different volunteers and these two isolates also showed different behaviours in the coaggregation assay.
### Table 5.5. Coaggregation scores of pairs of selected oral isolates from the salivary microbiotas of four different individuals.

<table>
<thead>
<tr>
<th>Species (Volunteer)</th>
<th>S. thermophilus</th>
<th>S. salivarius</th>
<th>N. meningitides</th>
<th>S. aureus</th>
<th>S. parasanguis</th>
<th>S. thermophilus</th>
<th>S. salivarius</th>
<th>S. agalactiae</th>
<th>E. hormaechei</th>
<th>L. fermentum</th>
<th>R. dentocariosa</th>
<th>S. thermophilus</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. salivarius (A)</td>
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<td>76(4)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. meningitides (A)</td>
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<td>*</td>
<td>7(0)</td>
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<td>*</td>
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<td></td>
</tr>
<tr>
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<td>*</td>
<td>3(0)</td>
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<td>*</td>
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<td>5(2)</td>
<td>33(5)</td>
<td>3(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>S. agalactiae (C)</td>
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<td>*</td>
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<td>1(0)</td>
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<td></td>
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<td>*</td>
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<td>6(1)</td>
<td>6(1)</td>
<td>3(2)</td>
<td>3(0)</td>
<td>2(1)</td>
<td>2(1)</td>
<td></td>
<td></td>
<td>6(1)</td>
</tr>
<tr>
<td>L. fermentum (D)</td>
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<td>*</td>
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<td>2(0)</td>
<td>1(2)</td>
<td>3(0)</td>
<td></td>
<td>13(3)</td>
<td>1(0)</td>
<td>3(0)</td>
<td>6(1)</td>
<td></td>
</tr>
<tr>
<td>R. dentocariosa (D)</td>
<td>*</td>
<td>*</td>
<td>6(1)</td>
<td>6(1)</td>
<td>5(1)</td>
<td>3(1)</td>
<td>4(0)</td>
<td>3(1)</td>
<td>4(0)</td>
<td>2(1)</td>
<td>4(0)</td>
<td></td>
</tr>
<tr>
<td>S. thermophilus (D)</td>
<td>*</td>
<td>*</td>
<td>3(1)</td>
<td>2(1)</td>
<td>11(3)</td>
<td>2(1)</td>
<td>7(2)</td>
<td>1(2)</td>
<td>2(0)</td>
<td>5(1)</td>
<td>2(1)</td>
<td>3(1)</td>
</tr>
</tbody>
</table>

Percent coaggregation as measured by OD (600nm) change over 60min (Section 5.3.6.). Data are means from three separate experiments (standard deviations are given in parenthesis). Coaggregative pairs scoring above 10% are shaded light grey. Autoaggregation scores representative of interaction between cells from the same culture.

*Denotes species exhibiting an exceptionally high level of autoaggregation. As a result of heavy clumping an OD of 1.0 could not readily be achieved and thus reliable coaggregation assays could not be performed. Autoaggregation scores in this case were taken immediately after vortex mixing.
Chapter 5, Ecological analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas

Of the four coaggregation interactions that were observed, two of the pairings had produced no interaction in the cross streak study (*S. parasanguis* (A) with *S. salivarius* (C) and *S. salivarius* (C) with *L. fermentum* (D)) and two had resulted in the growth inhibition of one or both species (*S. parasanguis* (A) with *S. thermophilus* (C) and *S. thermophilus* (D)). Pairings that had resulted in growth promotion in the cross-streak assay did not show any significant coaggregation.

Inhibition assays showed that the addition of lactose to one, or in one case both partners, resulted in significant inhibition of coaggregation (Table 5.4.). Heating one of the partners (both in the case of *S. salivarius* and *L. fermentum* coaggregation) also resulted in the inhibition of coaggregation (Table 5.5).

**Table 5.6.** The effect of the addition of lactose on coaggregation interactions observed between selected oral isolates.

<table>
<thead>
<tr>
<th>Partner 1</th>
<th>Partner 2</th>
<th>Control</th>
<th>Lactose Partner 1</th>
<th>Lactose Partner 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. thermophilus</em> (C)</td>
<td>15(2)</td>
<td>81(8)</td>
<td>5(1)</td>
</tr>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. salivarius</em> (C)</td>
<td>33(5)</td>
<td>94(3)</td>
<td>14(7)</td>
</tr>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. thermophilus</em> (D)</td>
<td>11(3)</td>
<td>92(4)</td>
<td>26(2)</td>
</tr>
<tr>
<td><em>S. salivarius</em> (C)</td>
<td><em>L. fermentum</em> (D)</td>
<td>13(3)</td>
<td>96(28)</td>
<td>92(6)</td>
</tr>
</tbody>
</table>

Percent inhibition of coaggregation as measured by OD (600nm) change over 60min (Section 5.3.6.). Data are means from three separate experiments (standard deviations are given in parenthesis). Lactose was added at a concentration of 100mM.

**Table 5.7.** The effect of heat treatment on coaggregation interactions observed between selected oral isolates.

<table>
<thead>
<tr>
<th>Partner 1</th>
<th>Partner 2</th>
<th>Control</th>
<th>Partner 1 Heated</th>
<th>Partner 2 Heated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. thermophilus</em> (C)</td>
<td>15(2)</td>
<td>95(1)</td>
<td>0(1)</td>
</tr>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. salivarius</em> (C)</td>
<td>33(5)</td>
<td>96(1)</td>
<td>0(17)</td>
</tr>
<tr>
<td><em>S. parasanguis</em> (A)</td>
<td><em>S. thermophilus</em> (D)</td>
<td>11(3)</td>
<td>92(1)</td>
<td>0(32)</td>
</tr>
<tr>
<td><em>S. salivarius</em> (C)</td>
<td><em>L. fermentum</em> (D)</td>
<td>13(3)</td>
<td>85(3)</td>
<td>91(6)</td>
</tr>
</tbody>
</table>

Percent inhibition of coaggregation as measured by OD (600nm) change over 60min (Section 5.3.6.). Data are means from three separate experiments (standard deviations are given in parenthesis). Heat treatment consisted of 30min at 85°C.
5.5. Discussion

Oral biofilms are complex microbial consortia which are believed to be established and maintained by the interplay of numerous synergistic and antagonistic interactions (Kuramitsu et al., 2007; Marsh & Bradshaw, 1999). Although the oral microbiota has been the focus of a large amount of research effort a general model of the formation of the dental plaque biofilm proposed by Ritz (1967) describing the succession of species from pioneer streptococci to the domination of mature dental plaque by gram-negative anaerobes has remained largely unchanged to date (Ritz, 1967). The physical and biological properties of different anatomical sites within the human mouth and the dentine itself are known to impact on biofilm composition and structure (Aas et al., 2005; Bowden et al., 1975; Mager et al., 2003; Marsh & Bradshaw, 1999; Marsh, 2004). However, a generic spatiotemporal schematic of dental plaque structure proposed by Kolenbrander et al (2002) is still one of the most prominent representations of the dental biofilm (Kolenbrander et al., 2002). It is likely that many of the key features of dental biofilm formation are universal; however the oral microbiota of each individual has been shown to be both unique and highly stable (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005). The mechanisms that underlie inter-individual variation and community stability within oral biofilms are not well understood at present.

Many inter-species interactions have been documented in vitro, however the focus of the majority of studies has been to characterise a specific interaction between two reference strains. Synergistic interactions such as cross-feeding (Grenier & Mayrand, 1986), cooperative metabolism (Takahashi, 2003) and
antagonistic interactions such as bacteriocin production (Hale et al., 2005; Nes et al., 2007) or neutralisation of virulence factors (Tong et al., 2007) have frequently been identified (Kuramitsu et al., 2007). As yet, there has been limited investigation of the interactions that can occur within and between microcosms (Kuramitsu et al., 2007). By studying inter and intra-individual microbial interactions between isolates derived directly from the salivary microbiotas of four individuals this study aimed to elucidate some of the microbial phenomena responsible for the unique and stable oral microbiota.

5.5.1. Rationale for the development and use of the modified cross-streak assay in the context of this study

A major component of this study was the determination of the frequency of positive and negative interactions between large numbers of salivary isolates. In order to achieve this aim a rapid, reliable and reproducible screening method was sought. A number of methods used previously by other researchers to identify interactions between large numbers of isolates were deemed unsuitable as they are only capable of detecting antagonistic interactions (Kekessy & Piguet, 1970; Krausse et al., 2005; Scrivener et al., 1950; Wang & Kuramitsu, 2005). For example, Wang and Kuramitsu (2005) cultured a selection of oral and intestinal bacteria on chocolate agar, killed them by exposure to chloroform, before applying a culture of Helicobacter pylori and observing its growth and the existence of zones of inhibition. This method identified antagonistic effects by bacteriocins or lysis products of the selected bacteria on the growth of H. pylori, but not the existence of growth promotion (Wang & Kuramitsu, 2005).
The modified cross streak method was selected because it presented a number of advantages for the study of a large amount of oral isolates. The technique allowed for the identification of bacterial growth promotion in addition to growth inhibition, proved more reliable and easier to execute than an overlapping drop method also tested and was easily reproduced. Both aerobic and anaerobic isolates were cultured in Wilkins Chalgren broth and on Wilkins Chalgren agar and thus there were no inherent differences in nutrient availability resulting from the growth media. The high cell density obtained with this method was believed to be important in reflecting the cell density associated with in vivo biofilms (Liljemark et al., 1997). Bacterial cell density is also significant as inhibitory substances or signalling molecules must be present in sufficient levels to produce an observable effect.

All in vitro models experience some limitations and the cross-streak assay is no exception. The methodology is unable to detect interactions that occur or are dependent upon the presence of more than two species, which may be common within multi-species biofilms. It also does not take into account the large number of uncultured species present in the oral cavity.

5.5.2. General observations on the frequency of interactions between oral isolates

Data from this study showed that the incidence of negative or inhibitory interactions was significantly higher than the incidence of positive interactions (41.5% vs. 5.4%). The predominance of negative or antagonistic interactions is perhaps not surprising because, like many ecosystems, the oral cavity has a limited supply of both nutrients and surface area and a large amount of
organisms competing to survive within it (Bowden & Li, 1997; Rupf et al., 2008). However, numerous cooperative and synergistic interactions have been reported between species commonly found in the oral cavity (Grenier & Mayrand, 1986; Palmer et al., 2001; Takahashi, 2003) and thus the low incidence of positive interactions in this study was unexpected. It seems likely therefore that some of the synergistic interactions documented by other researchers are a result of specific nutritional environments. For example, non-proteolytic organisms have been shown to co-inhabit areas occupied by highly proteolytic organisms such as *P. gingivalis* and take advantage of this protein digestion for their own growth (Darenfed et al., 1999). However, in order to document this effect *in vivo* the appropriate nutritional conditions must be replicated, in this case protein as a limiting source of nutrients (Kuramitsu et al., 2007).

5.5.3. Frequency and characteristics of inter and intra-individual interactions

Initially it was hypothesised that in order to facilitate a stable and resilient biofilm the frequency of positive interactions would be much greater between isolates derived from the same mouth compared to those from different mouths, with a corresponding decrease in antagonistic interactions. Interestingly this did not appear to be the case; no statistically significant difference in the frequency of positive or negative interactions between isolates from the same mouth and those from different mouths was observed, apart from a slight increase in negative interactions amongst self/non-self pairings amongst anaerobic isolates that was not considered statistically significant. Intra-individual interactions were on the whole no more likely to be harmonious than inter-individual interactions.
Seemingly, the general bias towards negative interactions and the congruence between data for self/self and self/non-self pairings does not support the theory proposed by He et al (2010) of “community selection” effect; whereby an established oral microbiota preferentially antagonises and excludes the integration of foreign species ensuring its own continuity and stability (He et al., 2010a; He et al., 2010b). However, as evidenced by the data in this chapter, intra and inter-individual interactions are not simply defined by cooperation amongst isolates from the same mouth and competition with “foreign” isolates. In fact, the individual microbiota should be considered a complex ecosystem in a constant state of “war and peace” with numerous antagonistic and synergistic interactions taking place at any one time (Kuramitsu et al., 2007). It seems probable that a specific combination of positive and negative interactions, and the adaptation of individual species to these conditions, create individuality and stability in the oral microbiota and facilitate the exclusion of “foreign” species.

5.5.4. Analysis of specific differences in the frequency of interactions between individuals and the impact of host gender

Outcomes of the cross-streak assay were analysed to identify differences and/or trends in the frequency or characteristics of interactions occurring between isolates from the four different salivary microbiotas. Statistical analysis of the data showed that isolates from the microbiota of volunteer C were more likely to inhibit the growth of isolates from other volunteers than be inhibited by others. This observation is particularly interesting because volunteer C was born and raised in Asia whilst all the other participants were born and raised in the UK. Genetic dissimilarity between the oral isolates as a result of the geographical differences in volunteer origin (Igboin et al., 2009; Xu & Mitchell,
2003) could be an important factor in this phenomenon. Species or strains that have historically had little opportunity to interact with one another are unlikely to have developed mechanisms to neutralise virulence factors/bacteriocins or cooperate with one another. However, this research area would benefit from further study. To date, there has been little investigation of inter-individual interactions and no data published on interactions between isolates from the oral microbiotas of people from different countries or ethnic backgrounds. Interestingly in one of the few relevant published studies, Nasidze et al (2009) analysed 16S rRNA sequences found in saliva samples of 120 individuals from 12 different worldwide locations and found high levels of inter-individual variation with no obvious geographical structure (Nasidze et al., 2009a).

Volunteer gender also appeared to have an impact upon the outcome of the cross-streaks. The frequency of negative interactions was significantly lower between isolates from the salivary microbiotas of the two UK males and positive interactions were less frequent between isolates from different genders. Gender has previously been recognised as a determining factor influencing the development of the oral microbiota, although the mechanisms that underlie this sexual dimorphism are not fully understood (Lazarevic et al., 2010). Gender dependent responses to microbial infection by both the innate and adaptive immune system have been well documented (Marriott & Huet-Hudson, 2006; Shiau & Reynolds, 2010), and in many instances have been attributed to oestrogen and androgen levels (Marriott & Huet-Hudson, 2006). In general, females have been shown to generate a more vigorous and protective humoral and cell-mediated immune response following microbial challenge whilst their male counterparts mount a more aggressive inflammatory immune response.
Chapter 5, Ecological analyses of pair-wise interactions amongst bacteria derived from individual oral microbiotas

(Marriott & Huet-Hudson, 2006). Kent et al (1992) have shown that S. mutans antibody levels are consistently higher in adult males than females, amongst those classified as at risk of root-surface caries (Kent et al., 1992). The prevalence and severity of periodontitis has also been shown to be at an increased level in men (Shiau & Reynolds, 2010). It therefore seems likely that the impact of host gender on immune response will in turn impact upon the response to both pathogenic and commensal bacteria in the oral cavity. These differences may be reflected in bacterial phenotype and consequently expression of bacteriocins or antimicrobials and thus the outcome of interactions observed in the cross-streak study.

5.5.5. Analysis of intra and inter-genera interactions and the characteristics of intra-species interactions in the cross-streak study

Twenty-five of the cross-streaks involved species that were isolated from different volunteers but had been identified as close relatives of the same species by 16S rDNA sequencing. Interestingly 52% of these interactions were described as negative or antagonistic to one or both species. Phenotypic differences within a particular species have been documented both between and within the oral cavities of individuals (Kamiya et al., 2005; Rupf et al., 2008). These differences are driven by the prevailing anatomical and environmental conditions of the host oral cavity and can be responsible for changes in the expression of bacteriocins (Kreth et al., 2005; Kuramitsu et al., 2007; Lazarevic et al., 2010; Liljemark et al., 1997; Rupf et al., 2008). Kamiya et al (2005) used arbitrarily primed polymerase chain reaction (AP-PCR) to study the relationship between genotype and mutacin production by S. mutans in individuals with and without dental caries. The authors noted that none of 16
individuals studied showed a phenotypic grouping identical to the genotypic grouping. Genotypes that were identical did not present the same mutacin production profile (Kamiya et al., 2005; Rupf et al., 2008). These observations and the data obtained from the cross-streak study emphasise the significance of bacterial phenotype and the resultant behavioural characteristics of a given species. Further studies of oral isolates would benefit from considering bacterial behaviours and antimicrobial products in the context of the environment from which the isolates are derived and not in isolation.

Negative interactions occurred at an increased frequency with intra-genera pairings in comparison to the general overall trends. The highest percentage of negative interactions was observed amongst the streptococci, perhaps not surprising as they are one of the most prolific bacteriocin producers (Nes et al., 2007). Streptococci also make up a significant proportion of the early colonisers of dental surfaces (Li et al., 2004). In order to achieve successful adherence it is essential that bacteria develop mechanisms to avoid clearance by the high shear forces of the oral cavity or inhibit secretory IgA (sIgA) which can prevent adhesion (Li et al., 2004). High numbers of streptococci in the early stages of dental plaque formation means they experience significant intra-genera competition for both nutrients and space, which may explain the increased incidence of inhibitory interactions in the cross streak study. Proteases produced by early colonisers such as Streptococcus gordonii have previously been shown to interfere with the subsequent colonization by S. mutans in vitro (Wang et al., 2010). Studies have also demonstrated the effectiveness of streptococcal mutacins on other members of the genus (Hale et al., 2005; Kreth et al., 2005). Streptococci were the dominant species isolated from the salivary
microbiotas of all four individuals, most likely because many strains are readily culturable and found in high numbers in the oral cavity (Aas et al., 2005; Nyvad & Kilian, 1987).

5.5.6. Coaggregation interactions amongst selected pairings

Coaggregation is believed to be important in the formation of oral biofilms (Cisar et al., 1979; Kolenbrander, 1997). It was hypothesised that those pairings which resulted in a positive interaction i.e. growth promotion of one or both species would be more likely to coaggregate and pairings which resulted in a negative interaction would be less likely to coaggregate. However, no coaggregations were observed between isolates described to have a positive interaction in the cross-streak study, in fact of the four coaggregations observed two of the pairings had previously been described as having a negative interaction in the cross streak study. Coaggregations that were identified were shown to be mediated by lectin-carbohydrate interactions akin to many that have previously been described between oral isolates (Kolenbrander & Williams, 1981; Kolenbrander & Williams, 1983; Ledder et al., 2008; McIntire et al., 1982; Rosen & Sela, 2006).

The overall incidence of coaggregation was low amongst the isolates selected whilst a high level of autoaggregation was observed amongst some of the isolates. This result is unexpected as coaggregation is commonly reported amongst oral species (Kolenbrander et al., 2000). However, the majority of the pairings selected involved species from the genus Streptococcus and intra-generic coaggregations are markedly less common than inter-generic coaggregation interactions (Kolenbrander et al., 2000). A larger scale study of
coaggregation between isolates from the salivary microbiota of different individuals may prove beneficial.

5.5.7. Proposals for improvement on the methodology and further study

The majority of synergistic and antagonistic interactions occurring in vivo between oral bacteria are regulated in some way by the nutritional environment (Dalwai et al., 2006; Kamiya et al., 2005; Kreth et al., 2005; Kuramitsu et al., 2007; Lazarevic et al., 2010; Liljemark et al., 1997; Rupf et al., 2008). It would be of interest to re-evaluate the interactions between the oral isolates identified in this chapter under different nutritional conditions to see if congruence existed between the results, perhaps by using a variety of different supplemented agars. Should sufficient time and resources allow characterising the interactions identified by the cross-streak assay could be of interest. Bacteriocins have previously been characterised by purifying and analysing the culture supernatant of a species which has exhibited specific antimicrobial properties (Bhunia et al., 1987; Kaewsrichan et al., 2004). Kaewsrichan et al (2004) have used ammonium sulphate precipitation followed by anion-exchange and gel filtration chromatography to purify a bacteriocin from Prevotella nigrescens. SDS-PAGE was then used to identify and determine the molecular weight of the protein (Kaewsrichan et al., 2004).

5.6. Conclusions

Communication and cooperation are fundamental to the development of multispecies biofilms such as those found in the oral cavity. Thus it was hypothesised that bacterial isolates from the same mouth would exhibit many more positive interactions than those isolated from different volunteers and
potentially a decrease in antagonistic interactions. Results from this study show that this is not the case. The vast majority of interactions had a negative outcome for one or both species regardless of whether they were classified self/self or self/non-self. It seems likely that within the highly populated microbial ecosystem of the human mouth bacterial competition and antagonism play an integral role in biofilm development and maintenance. Oral bacteria appear to cooperate with a limited number of species and have developed mechanisms to out compete those from which they derive no benefit. The high level of inter-individual variation in the oral microbiota is most likely a result of the unique combination of positive and negative interactions interlinked with numerous host-derived environmental factors.

Bacterial phenotype therefore appears to be integral to the variation observed between oral microbiotas. For example, several species were isolated from the saliva of two or more volunteers and yet exhibited markedly different characteristics in the cross-streak assay. In addition, the data also showed that the frequency of positive and negative interaction amongst the isolates was dependent not only on the individual from which the sample was obtained but also the volunteer’s gender, which could be attributed to hormonal and immune differences. Intra-genera interactions showed a higher rate of antagonism, potentially as a result of being in direct competition for an ecological niche in vivo. Although it seems possible that high proportion of streptococci may have influenced the results as they are well known for their prolific mutacin production.
The formation of the oral microbiota is well studied with documented changes in structure and function in varying disease states. In addition, several studies have indicated that each person’s salivary microbiota is unique and highly stable. There is however a significant gap in the research regarding the underlying mechanisms that precipitate these features. Data presented in this chapter goes some way towards addressing these questions and has determined that intra-individual interactions between oral isolates are not as harmonious as would have perhaps been expected.
Chapter 6

Ecological analyses of Sorbarod-based oral microcosms derived from pooled and individual salivary oral microbiotas
6.1. Abstract

Representative *in vitro* modelling of the oral microbiota relies upon closely reproducing aspects of the physical and nutrient conditions that prevail *in vivo*. An additional consideration is the fact that, since all oral microbiotas are unique, the maintenance of individualised microbiotas should be considered. This calls into question the use of pooled saliva or other inocula. The aim of this chapter was to evaluate the impact on microcosm stability, composition and species diversity of using pooled saliva as inocula for Sorbarod-based microcosms. Additionally, the study aimed to observe whether the characteristics of the biofilm derived from combined saliva would resemble that of one, both or neither individual. **Methods:** Eleven Sorbarod microcosms were established by inoculation with fresh saliva from four separate volunteers; alone, in pair-wise combination and all four microbiotas combined. Sample analysis comprised differential bacterial counts of the main functional bacterial groups and PCR-denaturing gradient gel electrophoresis (DGGE) of 16S rDNA with excision and sequence analysis of prominent bands from the gels. Hierarchical dendrograms were constructed and principal component analysis utilised to determine community relatedness and diversity. **Results:** Viable counts in biofilm and perfusate indicated that the proportion of Gram-negative anaerobes increased over time with a concomitant fall in the number of streptococci. Although these general trends were maintained across all microcosms, those derived from combined saliva exhibited a loss of stability; illustrated by increased intra-microcosm variability in the viable count data in both perfusate and biofilms. PCR-DGGE analysis revealed that combining saliva resulted in unique oral microcosms distinct from the parent populations. Species diversity was increased in the perfusate of the combined microcosms. However, species diversity in the biofilm population was comparatively low. DGGE band sequencing revealed a high numbers of streptococci, veillonella, prevotella and lactobacilli, alongside a significant proportion (25%) of uncultured species. Many of the prominent bands identified from the microcosms derived from a single saliva sample were also present in the profiles of the combined saliva-based microcosms. **Conclusions:** Using the pooled saliva of two or more individuals as inocula for *in vitro* Sorbarod models appeared to compromise the stability of the microcosms. The bacterial communities developed from the combined saliva were distinct from the microbiotas from which they were derived and no evidence of significant similarity to, or domination by, one microbiota was obtained. This data suggests that the use of pooled saliva in *in vitro* modelling compromises the integrity of the resultant microcosm and impacts on the relevance of the model.
6.2. Introduction

The oral cavity harbours one of the most complex microbial communities associated with the human body; thought to be comprised of upwards of 700 bacterial species, the majority of which are yet to be cultured (Aas et al., 2005; Dewhirst et al., 2010). Several distinct habitats exist within the oral cavity including: the teeth, the only hard non-shedding surface available for microbial colonisation; and the soft surfaces of the tongue, palate and buccal mucosa (Marsh & Martin, 2003; McBain et al., 2005). Heterogeneity within and between the microbial consortia populating each surface is high as a result of key environmental differences between anatomical sites (Aas et al., 2005; Dewhirst et al., 2010; Mager et al., 2003; Paster et al., 2006). Inter-individual variation among microbiotas is also high, identified by numerous molecular microbiology based studies (Diaz et al., 2006; Lazarevic et al., 2010; Ledder et al., 2006; McBain et al., 2005; Rasiah et al., 2005). However, intra-individual temporal changes in the oral microbiota as a whole are low; several studies have shown that salivary microbiotas remain stable over a period of months and even years (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005).

Of the studies relevant to this chapter, Lazarevic et al. (2010) have described the use of PCR amplification of the bacterial 16S rRNA gene and 454 pyrosequencing technology to show that the salivary microbiota of five subjects remained stable over a test period of 29 days. Interestingly, samples taken at closer time intervals did not necessarily show greater similarity to one another than those taken over longer time intervals. The authors concluded that these findings indicate the persistence of subject-specific taxa exhibiting minor fluctuations over time (Lazarevic et al., 2010). Rasiah et al. (2005) utilised PCR-
DGGE to analyse saliva samples and found that inter-individual variability of relative species abundance was significantly greater than the variability observed within a single individual over time. A relatively stable bacterial community (>87% concordance of DGGE fingerprints) was observed in the saliva of one individual over a time period of 7 years, whilst concordance between the DGGE fingerprints of the saliva of 10 volunteers proved to be just 66% (Rasiah et al., 2005). In another PCR-DGGE based study Ledder et al. (2006) highlighted marked inter-individual variability in the bacterial community fingerprints of both the salivary inocula and associated multiple Sorbarod devices (Ledder et al., 2006). As a result of these, and other molecular microbiology based studies (McBain et al., 2005; Nasidze et al., 2009a; Nasidze et al., 2009b), the oral microbiota is now considered by many researchers to be a relatively stable microbial community with a subject-specific species composition.

It has been proposed that the establishment of a unique individual oral microbiota, and other human-associated microbial communities, is shaped by two distinct factors (He et al., 2010a): inheritance of components of the microbiota of the mother or local environment at birth (Gronlund et al., 1999; Ley et al., 2006; Mandar & Mikelsaar, 1996) and the host habitat, which includes the influences of genotype i.e. immune response and anatomical structure; and also the nutritional environment (Frank et al., 2003; Zoetendal EG et al., 2001). Despite the evidence of inter-individual variation between oral microbial communities, including dental plaque, most models depict a generic structure and formation process for oral biofilms. The view of plaque formation as a succession of species beginning with the pioneer streptococci, increasing
numbers of actinomyces and resulting in a community dominated by gram-negative anaerobes, proposed by Ritz (1967) has remained largely unchanged to date (Ritz, 1967). A spatiotemporal schematic of dental plaque formation proposed by Kolenbrander et al (2002) is one of the most prominent representations of the dental biofilm. However, this model was largely based on evidence obtained from pair-wise coaggregation assays using planktonic cells (Kolenbrander et al., 2002). Bacterial cells growing in a biofilm are often phenotypically distinct from their planktonic counterparts (Sauer et al., 2002) and thus the attachment processes that determine the structure of dental plaque in vivo may differ from those described by the model. It seems probable that a large amount of variation will exist from the formation process, suggested plaque structure and functional properties, as a direct result of inter-individual variations in the oral microbiota. In their 2010 review of the formation of disease-associated oral biofilms Jakubovics and Kolenbrander suggest that there is likely to be a certain amount of functional redundancy between cell-surface structures mediating coaggregation and a better understanding, at the molecular level, of the processes that underpin the formation of dental plaque is needed (Jakubovics & Kolenbrander, 2010).

One of the most valuable means available for investigating dental biofilm formation, composition, structure and response to external stimuli i.e. environmental changes or antimicrobial challenge, is in vitro modelling. Commonly used models include: chemostat-based systems with removable colonisable surfaces (Bowden, 1999; Bradshaw et al., 1996; Kinniment et al., 1996a), simple culture on hydroxyapatite discs (Guggenheim et al., 2001), constant depth film fermentors (CDFF) (McBain et al., 2003b; McBain et al.,
Chapter 6. Ecological analyses of Sorbarod-based oral microcosms derived from pooled and individual salivary oral microbiotas

2003c; Pratten & Wilson, 1999; Vroom et al., 1999), saliva-conditioned flow cells (Decker et al., 2005; Foster & Kolenbrander, 2004), drip-flow reactors (Adams et al., 2002), single and multiple Sorbarod devices (Ledder et al., 2006; Ledder et al., 2009; McBain et al., 2005) and artificial mouth systems involving the drip feeding of growth media onto a variety of solid substrata supporting microbial growth (Sissons et al., 1991; Sissons et al., 1992; Sissons, 1997).

The model system used in this chapter was the Single Sorbarod device, inoculated with saliva. Sorbarod filters were initially devised for the micropropogation of plant tissue (Donkin et al., 1989) and first employed in the study of bacteriology by Hodgson et al (1995) to establish Staphylococcus aureus and Pseudomonas aeruginosa biofilms (Hodgson et al., 1995). They have since been used to support biofilm growth in an evaluation of the dynamics (Al-Bakri et al., 2004) and antimicrobial susceptibility of Pseudomonas aeruginosa biofilms (Driffield et al., 2008; Marques et al., 2005; Parveen et al., 2001), determining changes in gene expression in Neisseria meningitidis biofilms (O'Dwyer et al., 2009) and assessing antimicrobial susceptibility in Staphylococcus aureus (Gander et al., 2005; Haddadin et al., 2009) and Streptococcus pneumoniae biofilms (Budhani & Struthers, 1997).

Sorbarod filters have been successfully employed to support oral microcosms derived from salivary inocula within the format of the multiple Sorbarod device (MSD) (Ledder et al., 2006; Ledder et al., 2009; McBain et al., 2005). Cellulose fibres within the filter bind salivary proteins and mucins from the growth medium and provide a large surface area for colonisation and biofilm formation (McBain et al., 2005). A limited planktonic phase and constant fluid flow through the model is believed to be more representative of the conditions prevailing in vivo.
than chemostat-related systems (Sissons, 1997). As the Sorbarod devices are continuous flow systems there is the opportunity to analyse the perfusate from the model in addition to the biofilm matter. Additionally, a relatively small amount of salivary inocula is needed to establish the microcosm, which lends itself well to this study.

Successful modelling of the oral microbial community is reliant upon closely matching the conditions that prevail in vivo. It could therefore be argued that studies of the oral microbiota must account for and/or reproduce the significant inter-individual variability that exists in the oral cavity in order to be considered accurate and relevant (Ledder et al., 2006). Most in vitro models lack many host habitat factors that contribute to the formation and stability of the oral microbiota, including numerous immune factors (Ledder et al., 2006). Therefore, the microbial composition of the inoculum is of particular importance when preparing an in vitro model. In light of the evidence suggesting that the composition of the oral microbiota is both unique to every individual and inherently stable, the use of pooled saliva as inoculum in many in vitro studies is of concern. It seems logical to assume that the compositional variation extends to structural and metabolic activities and therefore the response to nutrients or antimicrobial challenge (Ledder et al., 2006). Consequently, pooling salivary inocula may compromise the integrity of oral microcosms and impede representative in vitro modelling. Despite these limitations numerous studies have employed pooled saliva (Dalwai et al., 2006; Harper et al., 2000; Pratten et al., 2000; Pratten et al., 2003; Wilson et al., 1997a; Wilson, 1999) in efforts to increase bacterial diversity in the microcosms or to obtain sufficient material to set up the model.
Although disadvantages are associated with pooling, the salivary microbiota itself comprises a diverse consortia of bacteria from most of the distinct oral niches (Ledder et al., 2006) and thus with the use of an appropriate in vitro model, salivary inocula can generate representative microcosms. In contrast to the complexity of salivary inocula, many studies have employed single species (Pigman et al., 1957) or selected consortia in models of the oral cavity (Bradshaw et al., 1989; Bradshaw et al., 1990; Bradshaw et al., 1993; Bradshaw et al., 1994; Bradshaw et al., 2001; Foster & Kolenbrander, 2004; Guggenheim et al., 2001; Kinniment et al., 1996a; Kinniment et al., 1996b; McKee et al., 1985; Russell & Coulter, 1977). These methods, although reproducible, are limited in their capacity to mimic oral biofilms due to significantly reduced bacterial diversity.

This chapter describes the study of in vitro oral microcosms established within single Sorbarod devices, derived from the saliva of four individuals alone, in pair-wise combinations and all four salivae combined. Perfusate samples from each microcosm and the end biofilm population were analysed by differential bacterial count of the main functional bacterial groups and PCR-DGGE analysis of the whole community. The aim of this work was to evaluate the outcome of pooling saliva on microcosm stability, composition and species diversity. Although several studies have evaluated the potential for integrating new species or communities into a stable oral microcosm (He et al., 2010a; He et al., 2010b), the work described in this chapter is believed to be the first investigation where two or more oral communities have been combined simultaneously with the intention of studying the resultant microcosm.
A previous study of the multiple Sorbarod device (MSD) showed that selective pressures present in the Sorbarod environment result in a bacterial population with only a fraction of the genetic variability of the parent saliva population, which is comprised of species from all of the oral niches (Ledder et al., 2006). Therefore it was hypothesised that the microcosms derived from combined saliva would exhibit a small increase in bacterial diversity when compared to those derived from a single saliva sample, although this may not result in a more representative microcosm. It also seemed likely that the stability exhibited by salivary communities and their resultant microcosms in previous studies (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005) would be compromised by combining inocula, as a result of perturbations of the complex structural and metabolic relationships that normally exist between species from the same mouth. The possibility also exists that many of the characteristics and predominant species of one individual’s salivary microbiota may prove to be dominant over another.
6.3. Materials and Methods

6.3.1. Chemicals and growth media

An artificial saliva solution was used to support the salivary derived microcosms growing within the single Sorbarod models. Artificial saliva comprised; mucin 2.5g/L, peptone 2g/L, tryptone 2g/L, yeast extract 1g/L, sodium chloride 0.35g/L, potassium chloride 0.2g/L calcium chloride 0.2g/L, cysteine 0.1g/L, haemin 0.001g/L and vitamin k, 0.0002g/L (McBain et al., 2005). A variety of selective and non-selective agars were used for viable count determination including: Wilkins–Chalgren agar (total aerobes and anaerobes); Wilkins-Chalgren agar with Gram-negative supplements of haemin (5mg/L), menadione (0.5mg/L), nalidixic acid (10mg/L), vancomycin (10mg/L), sodium succinate (2.5g/L) (Gram-negative anaerobes) (Oxoid Manual, 8th edition); Rogosa agar (total lactobacilli) and tryp ticase yeast extract, cysteine, sucrose agar (TYCS) comprising casein hydrolysate 15g/L, yeast extract 5g/L, L-cysteine 0.2g/L, sodium sulphite 0.1g/L, sodium chloride 1g/L, disodium hydrogen phosphate 2g/L, sodium bicarbonate 2g/L, sodium acetate 20g/L, agar agar 15g/L and sucrose 50g/L (Van Palenstein Helderman et al., 1983) (Streptococcus spp.). Half strength thioglycollate broth was used for the serial dilution of homogenised samples in order to maintain a reduced environment.

6.3.2. Single Sorbarod device

Single Sorbarod devices were used to grow the salivary derived bacterial biofilms (Figure 6.1.). The Sorbarod filter itself consists of compacted cellulose fibres encased in a cylindrical paper sleeve (llacon Ltd, Kent, UK).
Here, Sorbarod filters were inserted into lengths of clear PVC tubing (10mm diameter) which in turn were pushed into plastic funnels. A 2ml disposable syringe (‘Monoject’, Sherwood Medical, Crawley, UK) (plunger removed but rubber seal left in place) was inserted into the top of each PVC tube and then the device was sterilised by autoclaving at 121°C for 30min. Using aseptic
technique, each Sorbarod was removed from the autoclave, attached to a pre-
sterilised 2L glass collection vessel and a sterile, disposable needle (21 Gauge)
inserted through the top of the rubber seal. Sterilised media inlet tubing (121°C
for 30min) was attached via the needle and sterile artificial saliva was delivered
by a peristaltic pump at a controlled rate of 16.7ml/h. Each device was
maintained in a 37°C incubator.

The artificial saliva growth medium was allowed to run through the model for
two hours prior to inoculation in order to pre-wet the Sorbarod filter. A sample of
this perfusate was taken by placing a sterile universal tube directly underneath
the Sorbarod device. In order to ascertain the sterility of the model 0.1ml of
perfusate was plated out onto Wilkins-Chalgren agar and incubated aerobically
at 37°C for 16h.

After pre-wetting the filter each model was inoculated with 1ml of freshly
donated saliva. The flow of growth media was suspended whilst saliva was
added drop wise onto the Sorbarod filter via a sterile needle and syringe pushed
through the rubber seal. In total eleven unique models were prepared by this
method. Four healthy volunteers with no history of periodontal disease aged 23
to 26 (two females, two males) donated samples and their microcosms were
designated letters A to D. Four of the models were inoculated with 1ml of saliva
from one (different) volunteer; six models were inoculated with homogenised
saliva samples from a different pair-wise combination of two volunteers (0.5ml
from each) and one model was inoculated with homogenised saliva samples
from all four volunteers combined (0.25ml from each). Post inoculation the flow
of artificial saliva was restarted and ran continuously for 10d.
6.3.3. Perfusate and biofilm sample collection

Inoculation of the model was denoted day 0 and once the microcosm was established (ca 2d) perfusate samples were taken (days 2, 3, 4, 5, 6 and 10). Approximately 5ml of perfusate was collected at each sampling point by placing a sterile universal tube underneath the outlet of the Sorbarod device. 1ml of this fluid was then taken for bacteriological analysis and 2ml archived at -60°C for subsequent molecular analysis. The model was sacrificed on day 10 and the filter aseptically removed from the PVC tubing. Sterile scalpels were used to split each filter longitudinally, with one half used immediately for bacteriological analysis and the second archived at -60°C.

6.3.4. Differential bacteriological analysis of perfusate and biofilm samples

Selective and non-selective agars were used to culture and enumerate the major segments of the bacterial populations in each microcosm. Samples of perfusate (1ml) were homogenized by vortex mixing for 1min. Sorbarod filters were macerated by vortex mixing in 9ml of pre-reduced half-strength thioglycollate broth, along with 1.5g of sterile glass beads (3.5 – 5.5mm diameter) (BDH, Pool, UK) for 1min. Perfusate and filter samples were then serially diluted using pre-reduced, half strength thioglycollate broth. Appropriate dilutions (0.1ml), ascertained on a trial and error basis, and were then plated in triplicate onto a variety of selective and non-selective agar using disposable sterile L-shaped spreaders. As detailed in Section 6.3.1. the media used included: Wilkins–Chalgren agar (total aerobes and anaerobes); Wilkins-Chalgren agar with Gram-negative supplement (Gram-negative anaerobes); Rogosa agar (total lactobacilli) and TYCS agar (Streptococcus spp.) These
agars were transferred immediately to an anaerobic cabinet (Gas mix: 80% N₂, 10% CO₂ and 10% H₂) (Mark 3 Anaerobic Work Station, Don Whitely Scientific, Shipley, UK) and were maintained for 5d; except for one set of Wilkins-Chalgren agar which was incubated aerobically at 37°C (MEMMERT incubator, Schwabach, Germany). All colonies were counted and average colony forming units per ml (cfu/ml) calculated using at least three counts over two or more dilution factors where possible. Data was analysed using the two sample t test method (Microsoft Office Excel 2007 software); a direct analysis of the mean and variance values of two sample sets, to identify statistically significant differences.

6.3.5. PCR-DGGE analysis of perfusate and biofilm samples

Bacterial community DNA was extracted from the perfusate and saliva samples using the QiaAmp DNA stool mini kit (Qiagen, Sussex, UK) as described in Section 2.2.2. 200μl of bacterial suspension was mixed with lysis buffer using a FastPrep FP120 bead beater (Qbiogene, California, USA) and 0.5g zirconia beads (full speed for 45s) to mechanically aid cell lysis. DNA extraction was then completed according to the manufacturer's protocol. To ensure total bacterial communities were removed from the Sorbarod filters, three bead beating steps at full speed for 45s were performed prior to extraction with the kit.

Extracted DNA was amplified by polymerase chain reaction (PCR) with primers specific for the V2-V3 region of the bacterial 16S rRNA gene: HDA1 (including an additional GC clamp) (5'-CGC CCG GGG CGC GCC GGC GCC GCC GGG GGG GCA CGG GGG GAC TCC TAC GGG AGG AGG CAG CAG T-3') and HDA2
(5′-GTA TTA CCG CGG CTG CTG GCA C-3′) (Ledder et al., 2006) (as detailed in Section 2.3.2.). The quality and quantity of DNA obtained from this process was ascertained by agarose gel electrophoresis as described in Section 2.4.

DGGE analysis was performed as detailed in Section 2.5. The resultant gel was visualised under UV light at 312nm with a UV transilluminator (UVP, California, USA) and photographed using a Canon EOS D60 digital camera (Cannon, Surrey, UK). Dendrogram and principal component analysis were completed as described in Sections 2.5.8. and 2.5.9. respectively. Bands of interest were excised from the DGGE gel as described in Section 2.5.6. and the DNA amplified and purified as detailed in Section 2.5.7. Sequence analysis and identification was completed as outlined in Section 2.3.3 using the European Molecular Biology Laboratories (EMBL) prokaryote database (http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html).
6.4. Results

6.4.1. Differential bacterial counts from microcosm perfusate samples

Differential bacterial counts of both total culturable aerobes and anaerobes; obtained from perfusate samples from the Sorbarod microcosms, showed broadly similar trends across all four volunteers alone and in combination. Numeric variability was greater within and between the functional groups of bacteria, in particular the lactobacilli. In all cases, anaerobic species were numerically dominant with perfusate population’s ca 9 Log_{10} cfu/ml by day 10. Gram negative anaerobes constituted the majority of culturable anaerobic species with most counts ranging from 7 to 9 Log_{10} cfu/ml by day 10. Total aerobic populations, inclusive of facultative anaerobic species, numbered between 7 and 9 Log_{10} cfu/ml. Culturable streptococci also formed a large part of the perfusate population numbering between 6 and 8 Log_{10} cfu/ml in the majority of cases. Lactobacilli counts showed the greatest inter-individual variability; ranging from undetectable to ca 7 Log_{10} cfu/ml.

Visual analysis of the differential count data in individual graph format (Figures 6.2. to 6.7.) indicates that in the majority of cases the total number of culturable aerobes and anaerobes remained relatively stable in the perfusate of Sorbarod microcosms derived from combined saliva of two volunteers. In most cases a dynamic steady state was achieved by day 2 and maintained throughout the test period. However, within the other main functional bacterial groups tested, microcosms derived from combined saliva samples did exhibit a loss of stability, determined by the differential counts from model perfusate. Although inter-microcosm variability was not always numerically significant, intra-individual changes in the number of gram negative anaerobes and streptococci shed in
perfusate over the 10 day period indicated that the combined saliva microcosms were less stable. Lactobacilli counts proved to be particularly unstable also.

Data in Figure 6.2. illustrates the differential bacterial counts from microcosm perfusate of the Sorbarod models inoculated with saliva from volunteers A (male, Caucasian, aged 24) and B (male, Caucasian, aged 25) alone and combined. The colony forming units shed from each microcosm over the ten day period showed congruence in both the trend, and overall number of: total culturable aerobes, anaerobes, gram negative anaerobes and streptococci. Analysis by the two sample t test identified no statistically significant differences in the mean viable count data at day 10 within these groups, for any of the three microcosms. Statistically significant differences were observed however, in the numbers of lactobacilli shed in perfusate, within and between the microcosms.

Figure 6.3. shows the differential counts from perfusate of the models inoculated with saliva from volunteers A and C (female, Asian, 23 years) alone and combined. Visual analysis indicates that more inter-microcosm variability occurred between these three populations compared to the data obtained for volunteers A and B. Furthermore, the perfusate of the microcosm derived from combined saliva contained significantly more gram negative anaerobes than either A or C alone (two sample t test at 5% significance level). Lactobacillus counts were the most unstable.

Data in Figure 6.4. indicates that, apart from the lactobacilli, the differential bacterial counts from the combined saliva model of volunteers A and D (female, Caucasian, 26 years) followed the same general trends already discussed for the previous models. The profile of the combined saliva microcosm shows
Figure 6.2. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are log_{10} colony forming units per ml of perfusate (log_{10} cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject A alone (O), subject B alone (●) or subjects A and B combined (○).

* Viable counts for subject B significantly greater than A alone and A and B combined (p<0.01).
** Viable counts for B significantly greater than A and B combined (p<0.01).
*** Viable counts for B significantly greater than A alone but no significant difference between B alone and A and B combined (p<0.01).
Figure 6.3. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are log₁₀ colony forming units per ml of perfusate (log₁₀ cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject A alone (O), subject C alone (●) or subjects A and C combined (○).

* Viable counts for subject A and C combined significantly greater than A and C alone (p<0.05).
** Viable counts for A and C combined significantly greater than A and C alone (p<0.01).
Figure 6.4. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are log_{10} colony forming units per ml of perfusate (log_{10} cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject A alone (○), subject D alone (●) or subjects A and D combined (●). * Viable counts for subject D significantly greater than A and D combined and A alone (p<0.01). ** Viable counts for D alone and A and D combined significantly greater than A alone (p<0.01).
distinct similarities to the profile of the microcosm derived from the saliva of volunteer A. Statistical analysis (two sample t-tests, 1% significance level) confirms that by day 10, there were no significant differences in the number of gram negative anaerobes and streptococci shed from the filter of the combined saliva model compared to volunteer A’s microcosm. However, the viable counts from the model inoculated with saliva from volunteer D were significantly lower.

Data in Figure 6.5. shows the differential bacterial counts from the perfusate of the model inoculated with saliva from volunteers B and C alone and in combination. Numbers of total aerobes, total anaerobes, gram negative anaerobes and streptococci were in keeping with the general trends observed in previous comparisons. The counts obtained from the perfusate of the combined model showed no consistent resemblance to either B or C alone. Again the majority of variation occurred between the lactobacilli populations.

Visual analysis of the graphical data for the model inoculated with the combined saliva of volunteers B and D appears to show that the differential bacterial counts were predominantly similar to those of volunteer B (Figure 6.6.). Statistical analysis confirms that there were no significant differences between any of the counts from the combined model and the model from the saliva of volunteer B at day 10. The numbers of gram negative anaerobes and streptococci isolated from the perfusate of volunteer D’s model however, were significantly higher than both the combined model and volunteer B.
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Figure 6.5. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are log_{10} colony forming units per ml of perfusate (log_{10} cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject B alone (●), subject C alone (○) or subjects B and C combined (○). * Viable counts for subject B and C combined significantly greater than and C alone (p<0.01).
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Figure 6.6. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are $\log_{10}$ colony forming units per ml of perfusate ($\log_{10}$ cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject B alone (O), subject D alone (●) or subjects B and D combined (Θ).

* Viable counts for subject D significantly greater than and B and D combined and B alone ($p<0.01$).
Data in Figure 6.7. shows that the total aerobic and anaerobic populations of the model perfusate of volunteers C and D alone and in combination were not significantly different by day 10. The number of gram negative anaerobes and streptococci found in the perfusate of the combined model closely resembled those from the model inoculated with the saliva of volunteer C. Statistical analysis confirmed that at day 10 the number of gram negative anaerobes shed from the microcosm of volunteer D was significantly higher than those from volunteer C and the combined saliva. In contrast, the lactobacilli population in the perfusate of the combined saliva model was numerically similar to the model derived from volunteer D’s saliva. No lactobacilli were detected in the perfusate from volunteer C’s microcosm and thus the difference between this and the combined model was significant throughout days 2 to 10.
Figure 6.7. Viable counts of selected groups of oral bacteria from the perfusate of single Sorbarod models. Data are means with ± standard deviation from a single device analysed in triplicate. Units are log_{10} colony forming units per ml of perfusate (log_{10} cfu/ml) and time in days (d). Models were inoculated with fresh saliva from either subject C alone (○), subject D alone (●) or subjects C and D combined (○).

* Viable counts for subject D significantly greater than and C and D combined and C alone (p<0.01).
** Viable counts for subject D alone and C and D combined significantly greater than C alone (p<0.01).
6.4.2. Differential bacterial counts from Sorbarod biofilm samples

In addition to the data obtained from the collection of microcosm perfusate, differential bacterial counts were taken directly from the Sorbarod filter at the end of each model run. Data in Figure 6.8. shows the number of culturable aerobes derived from all of the filters allocated into two groups; those from a model inoculated with one saliva sample (open boxes) and those inoculated with combined saliva samples (closed boxes). Viable count data from the perfusate is also included in the figure, in the same format, to facilitate a comparison of the perturbations in the average number of aerobes, between combined and single saliva models, over the test period.

![Box plot showing differential bacterial counts](image)

**Figure 6.8.** Differential bacterial count data comparing the bacterial composition (aerobes and facultative anaerobes) of model perfusate and biofilms; inoculated with the saliva of a single volunteer (open boxes) or mixed saliva from two volunteers (closed boxes). Median, 5th, 25th, 75th and 95th percentile values shown; outliers denoted by ●.

As anticipated, the number of aerobes found to be colonising the Sorbarod filters were significantly higher than the numbers shed in microcosm perfusate:
ca 9 to 10 Log$_{10}$ cfu/filter. Inter-microcosm variability in the number of aerobes per filter was greater between the mixed saliva microcosms when compared with the microcosms derived from a single saliva sample. Perfusate counts of total aerobes generated from each model were combined for days 2, 6 and 10 to obtain median, 5$^{th}$, 25$^{th}$, 75$^{th}$ and 95$^{th}$ percentile values (Figure 6.8.). This method of analysis revealed that the microcosms derived from single saliva samples exhibited a steady increase in number of aerobes shed from the filter over time. Microcosms derived from combined saliva samples exhibited less stability, with total aerobic counts rising to day 6 and then dipping at day 10.

![Figure 6.9.](image)

**Figure 6.9.** Differential bacterial count data comparing the bacterial composition (anaerobes) of model perfusate and biofilms; inoculated with the saliva of a single volunteer (open boxes) or mixed saliva from two volunteers (closed boxes). Median, 5$^{th}$, 25$^{th}$, 75$^{th}$ and 95$^{th}$ percentile values shown; outliers denoted by ●.

Data presented in Figure 6.9. shows that the number of anaerobes found to be colonising the Sorbarod filters were significantly higher than the numbers shed in microcosm perfusate: ca 9 to 11 Log$_{10}$ cfu/filter. Inter-microcosm variability
was again greater between the mixed saliva filter microcosms compared to those derived from a single saliva sample. The patterns in anaerobic counts over the test period from the combined microcosms resembled those observed for the aerobes: a rise in numbers at day 6 and a dip at day 10, in contrast to the steady rise in over days 2 to 10 that was observed in the single saliva models.

![Graph showing differential bacterial count data]

**Figure 6.10.** Differential bacterial count data comparing the bacterial composition (Gram negative anaerobes) of model perfusate and biofilms; inoculated with the saliva of a single volunteer (open boxes) or mixed saliva from two volunteers (closed boxes). Median, 5th, 25th, 75th and 95th percentile values shown; outliers denoted by ●.

Data in Figure 6.10. illustrates that the average number of gram negative anaerobes found to be colonising the Sorbarod filters were significantly higher than the average numbers shed in microcosm perfusate: ca 7 to 9 Log_{10} cfu/filter. Differential counts of the gram negative anaerobes also proved to be less stable in the perfusate of microcosms derived from combined saliva. The pattern identified from analysis of both aerobes and anaerobes in perfusate: with numbers rising to day 6 and dipping at day 10 was repeated when average
values were obtained for the gram negative anaerobes. The number of gram negative anaerobes colonising the Sorbarod filter appeared, on average, to be higher in the microcosms associated with combined saliva. However the amplitude of inter-microcosm variability between combined and single saliva was similar.

![Figure 6.11](image)

**Figure 6.11.** Differential bacterial count data comparing the bacterial composition (Streptococci) of model perfusate and biofilms; inoculated with the saliva of a single volunteer (open boxes) or mixed saliva from two volunteers (closed boxes). Median, 5th, 25th, 75th and 95th percentile values shown; outliers denoted by ●.

Figure 6.11. shows that the number of culturable streptococci colonising the Sorbarod filters was significantly higher than the number shed in microcosm perfusate: ca 8 to 9 Log_{10} cfu/filter. The amplitude of inter-microcosm variability in the number of streptococci isolated from the Sorbarod filters was greater in the microcosms derived from combined saliva. Microcosm stability with respect to the number of streptococci also appeared to be compromised by combining saliva samples.
Interestingly, the combined viable count data for the lactobacilli (Figure 6.12.) differed significantly from the general trends observed for the other functional bacterial groups tested. Overall biofilm populations did not appear to be higher than perfusate populations and no discernable patterns could be identified within the data over the time period. The amplitude of inter-microcosm variability was, in general, greater between the microcosms derived from single saliva samples.
6.4.3. PCR-DGGE analysis of perfusate and biofilm samples

Analysis of salivary inocula, perfusates and the formed biofilms using PCR-DGGE, revealed clear differences in the community composition within and between the microcosms. Figure 6.13. shows the DGGE fingerprints of all the samples analysed alongside a single repeated ladder (bacterial DNA derived from murine faeces).

Species diversity within the samples was assessed by enumerating the dominant PCR amplicons in each DGGE fingerprint. Data in Figure 6.14. shows the average number of visible PCR amplicons in the DGGE fingerprints of the perfusate, biofilm and saliva from the single, combined (two volunteers) and combined (four volunteers) microcosms. The average number of PCR amplicons per lane on day 2 was higher in the perfusate of the microcosms derived from the combined saliva, compared to the single saliva microcosms (28 bands versus 25). Diversity steadily increased over the test period in all of the models and at day 10 the average number of PCR amplicons from the model perfusate of the combined and single saliva microcosms was 45.2 versus 43.5. Interestingly, the number of amplicons from the perfusate of the model derived from the combined saliva of all four volunteers was significantly higher than the combined saliva (two volunteers) and the single saliva throughout the 10 day test period (day 2: 40 bands, day 10: 52 bands).

The number of PCR amplicons present in the DGGE fingerprints of the samples obtained directly from the Sorbarod filters was higher than the perfusate in all cases. However, in contrast to the perfusate data, average
Figure 6.13. DGGE profiles of selected single Sorbarod device perfusate (P: day 2, 3, 4, 5, 6 and 10) and biofilm (BF) samples together with salivary inocula (S).
Volunteer A ( ), volunteer B ( ), volunteer C ( ), volunteer D ( ), volunteers A and B combined ( ), volunteers A and C combined ( ), volunteers A and D combined ( ), volunteers B and C combined ( ), volunteers B and D combined ( ), volunteers C and D combined ( ) and volunteers A, B, C, and D combined ( ).
number of PCR amplicons was highest in the samples derived from the filters inoculated with a single saliva sample, and lowest in the filter inoculated with the combined saliva of four volunteers. The average number of PCR amplicons in the saliva samples was broadly similar to that observed from the combined saliva models (two and four volunteers).

6.4.4. Dendrogram and PCA analysis of the DGGE fingerprints associated with microcosm perfusate and biofilms

Bionumerics software (Applied Maths, Saint-Martens, Latem, Belgium) was utilised to determine the relative similarity between DGGE fingerprints and produce UPGMA dendrograms. Data in Figure 6.15. shows the percentage
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Figure 6.15. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer A (■), volunteer B (▲) or volunteers A and B combined (◆). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva.
* Clusters that are found in both dendrogram and PCA analysis.

Figure 6.16. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer A (■), volunteer B (▲) or volunteers A and B combined (◆); perfusate samples from day 2 (●), day 6 (▲), day 10 (■) and biofilm samples (◆).
* Clusters that are found in both dendrogram and PCA analysis.
matching and clustering of the DNA fingerprints from the models inoculated with saliva from volunteers A and B alone and in combination. Perfusate samples from the models inoculated with a single saliva sample (volunteer A: days 2 to 6 and B: days 2 to 5) both cluster separately. These two clusters score just 47% concordance with the other fingerprints. Within the largest group; the perfusate from day 10, biofilm and saliva samples of volunteer A’s microcosm formed a distinct cluster, as did the perfusate samples from the combined saliva model (days 2 to 4). Perfusate samples: days 6 and 10, and the biofilm fingerprints from the combined model showed a greater similarity to the day 10 perfusate and biofilm samples derived from volunteer B’s microcosm than volunteer A (71% versus 62% concordance). Principal component analysis (PCA) of the data (Figure 6.16.) confirms that the early perfusate (day 2) from all three microcosms was significantly different to the biofilm fingerprints. There was also congruence between the dendrogram and PC analyses confirming the clustering of perfusate(days 6 and 10) and biofilm samples derived from the combined saliva model. However, the 3D plot of the PCA shows that this particular cluster sits quite far away from the biofilm fingerprints of the single saliva models, which is not obvious from the dendrogram analysis. This result suggests that the microcosms derived from single saliva samples of volunteer A or B are more similar to one another and the combined model significantly different from both.

Distinct clusters were observed when the DGGE fingerprints of perfusate samples from the microcosms of volunteers A and C alone and in combination were analysed (Figure 6.17.). The greatest variation observed was inter-microcosm with intra-microcosm concordance being upwards of
Figure 6.17. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer A (■), volunteer C (▲) or volunteers A and C combined (★). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva.
* Clusters that are found in both dendrogram and PCA analysis.

Figure 6.18. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer A (■), volunteer C (▲) or volunteers A and C combined (★); perfusate samples from day 2 (●), day 6 (▲), day 10 (■) and biofilm samples (◆).
* Clusters that are found in both dendrogram and PCA analysis.
80% for the perfusate samples from days 2 to 6. The three biofilm fingerprints fell within one cluster (>70% concordance) along with volunteer A’s saliva sample. PCA of the DGGE banding patterns supports the results of the dendrogram analysis, with the three biofilm fingerprints falling in close proximity to one another on the 3D plot (Figure 6.18.). The early perfusate samples are spread out over the plot indicating significant differences between the DGGE profiles from all the model perfusates on days 2 to 6.

Data obtained for the models inoculated with the saliva of volunteers A and D alone and in combination shows much the same general trends as observed for A and C. Figure 6.19. shows most of the early perfusate samples forming separate clusters with intra-microcosm concordance ca 70% and above in the early stages. The three biofilm fingerprints again cluster separately from the majority of perfusate samples, showing just 52% concordance with them. The biofilm profiles of volunteer D alone and A and D in combination are the most similar at 82% concordant. PCA supports these findings with the biofilm fingerprints forming a cluster on the left of the plot away from the early perfusate samples. It can also be seen in Figure 6.20. that the perfusate samples from volunteer D alone (days 6 and 10) cluster very closely to the biofilm samples. Indicating that the combined microcosm bears more similarity to that derived from volunteer D’s saliva alone.
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Figure 6.19. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer A ( ), volunteer D ( ) or volunteers A and D combined ( ). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva.

* Clusters that are found in both dendrogram and PCA analysis.

Figure 6.20. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer A ( ), volunteer D ( ) or volunteers A and D combined ( ); perfusate samples from day 2 ( ), day 6 ( ), day 10 ( ) and biofilm samples ( ).

* Clusters that are found in both dendrogram and PCA analysis.
Data in Figure 6.21. shows that the microcosm derived from the combined saliva of volunteer B and C shows a greater similarity to volunteer B than C. The perfusate and saliva samples from volunteer C cluster together with just 45% concordance with the other samples. The biofilm fingerprint clusters separately showing 55% concordance to the combined saliva model. Perfusate fingerprints from volunteer B show 45% (days 2 to 5) and 60% concordance (days 6, 10 and biofilm) with the combined saliva samples. DGGE fingerprints of the perfusate and biofilm samples taken from the model derived from the combined saliva form two distinct clusters. The first containing the perfusate samples from days 2 and 3 and the second containing the remaining samples. Fingerprints within this second, larger cluster show between 67% and 82% concordance to one another.

Principal component analysis of the data (Figure 6.22.) confirms the conclusions drawn from the dendrogram data. Perfusate samples from volunteer C alone cluster on the left of the graph away from volunteer B and the combined saliva samples. The biofilm samples of all three models cluster together however. Visualisation of the PCA data on the 3D plot indicates that the inter-microcosm variability is much greater than the intra-microcosm differences over the test period. The distances between the values from the same microcosm are less than those between the different microcosms.
Chapter 6, Ecological analyses of Sorbarod-based oral microcosms derived from pooled and individual salivary oral microbiotas

Figure 6.21. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer B (■), volunteer C (■) or volunteers B and C combined (■). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva.
* Clusters that are found in both dendrogram and PCA analysis.

Figure 6.22. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer B (■), volunteer C (■) or volunteers B and C combined (■); perfusate samples from day 2 (■), day 6 (▲), day 10 (■) and biofilm samples (◆).
* Clusters that are found in both dendrogram and PCA analysis.
Data from the microcosms derived from the saliva of volunteers B and D alone and in combination is outlined in Figures 6.23 and 6.24. Dendrogram analysis shows three distinct clusters containing the perfusate samples from days 2 through 5 of each of the three microcosms, with ca 50% concordance between them. Two further clusters are observed: the first containing the day 10 perfusate, biofilm and saliva fingerprints of volunteer B and the second the days 6 and 10 perfusate and biofilm samples of volunteer D. The similarity between these two clusters is 65%. The remaining samples follow no obvious cluster patterns and share between 50 and 60% concordance.

PCA of the data shows that in contrast to most of the previous analyses the data points are quite evenly spread with few obvious clusters. The biofilm samples from volunteer D and B and D combined are closely positioned on the plot, indicating they are similar. Volunteer B’s samples broadly cluster on the right hand side of the plot.

Data in Figure 6.25. shows that the samples from volunteers C and D alone and in combination show the same general trends observed for the previous models. The early perfusate samples (days 2 to 6) form 3 separate clusters with intra-microcosm concordance ca 70% to 95% between these samples. The biofilm fingerprints for volunteer D alone and C and D combined fall within the same cluster that includes both perfusate day 10 samples and perfusate day 6 from volunteer D. Indicating that these two microcosms show a greater similarity to one another than the microcosm of volunteer C. PCA supports these findings with the biofilm fingerprints from volunteer D and C and D combined forming a cluster on the right of the plot. Figure 6.26.
Chapter 6. Ecological analyses of Sorbarod-based oral microcosms derived from pooled and individual salivary oral microbiotas

Figure 6.23. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer B (■), volunteer D (■) or volunteers B and D combined (■). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva.

Figure 6.24. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer B (■), volunteer D (■) or volunteers B and D combined (■); perfusate samples from day 2 (■), day 6 (▲), day 10 (■) and biofilm samples (◆).
Chapter 6, Ecological analyses of Sorbarod-based oral microcosms derived from pooled and individual salivary oral microbías

Figure 6.25. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from three models inoculated simultaneously. With saliva from volunteer C ( ), volunteer D ( ) or volunteers C and D combined ( ). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10), BF denotes biofilm samples obtained from the Sorbarod filters and S denotes pure saliva. * Clusters that are found in both dendrogram and PCA analysis.

Figure 6.26. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer C ( ), volunteer D ( ) or volunteers C and D combined ( ); perfusate samples from day 2 ( ), day 6 ( ), day 10 ( ) and biofilm samples ( ). * Clusters that are found in both dendrogram and PCA analysis.
also shows that the distribution of the other points on the 3D plot is a fairly even spread indicating no other significant similarities or clusters, in contrast to the dendrogram analysis.

Figure 6.27. shows a dendrogram comprised of all the volunteers individually and then the model derived from all four volunteer’s combined saliva. Four distinct clusters can be seen that contain the fingerprints of perfusate from days 2 to 5 or 6 from the models inoculated with a single saliva sample showing intra-microcosm variability is significantly less than inter-microcsm variability during this time. A large cluster can also be observed containing all of the biofilm fingerprints from the four of the individual models (concordance 65 to 75%). Interestingly, all of the perfusate samples and the biofilm sample from the model inoculated with the combined saliva of the four volunteers forms a single distinct cluster with intra-microcosm concordance of 72 to 98%. The similarity between this cluster and the other samples is 38 to 60%.

Principal component analysis of the data is illustrated in Figure 6.28. and clearly shows two distinct clusters: one containing all of the plots for the combined model and the other containing all of the biofilm fingerprints from the single saliva models. The early perfusate samples (day 2) for volunteers A, C and D alone are all positioned on the left of the plot some distance from the rest of the samples, indicating they are significantly different.
Figure 6.27. A UPGMA dendrogram showing percentage matching of single Sorbarod device samples from models inoculated with saliva. From volunteer A (■), volunteer B (■), volunteer C (■), volunteer D (■) or volunteers A, B, C and D combined (■). P denotes perfusate samples and day of sampling (2, 3, 4, 5, 6 or 10) and BF denotes biofilm samples obtained from the Sorbarod filters. * Clusters that are found in both dendrogram and PCA analysis.
Figure 6.28. Plot of principal components (PC) 1, 2 and 3 of the similarity profiles generated from a selection of single Sorbarod model samples. Volunteer A ( ), volunteer B ( ), volunteer C ( ), volunteer D ( ) or volunteers A, B, C and D combined ( ); perfusate samples from day 2 ( ), day 6 ( ), day 10 ( ) and biofilm samples ( * ).

* Clusters that are found in both dendrogram and PCA analysis.

6.4.5. DGGE band excision and sequence analysis

Selected bands were excised from four of the DGGE gels and the DNA sequenced to identify the species or the closest phylogenetic relative. Figure 6.29. shows a negative image of these gels with a diagrammatic representation of the excised bands to the right hand side. The numbering system corresponds to the data in Table 6.1.; showing the closest relatives based on the results of EMBL searches. Bands were selected on the premise of identifying important similarities and differences within and between the microcosms over time. Thus bands repeatedly appearing in many of the lanes, those that appeared and disappeared over time were amongst those excised.
Figure 6.29. Negative image of 4 parallel DGGE gels showing the profiles of selected perfusate (P: number denotes day of sampling), biofilm (BF) and saliva (S) samples. The diagrammatic representation of migrated bands positioned to the right of the photographs shows those that were selected for excision and sequencing. The numbering system corresponds to data in Table 6.1.

Data in Table 6.1. shows that, of the selected bands excised from the DGGE gels, the dominant genera were those related to the streptococci (36%), veillonella (14%), prevotella (9%) and lactobacilli (7%). A significant proportion of the DNA sequences (25%) were closely related to species that are yet to be, or are not readily culturable.
Table 6.1. Sequencing and identification of PCR amplicons derived from DGGE gels.

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<th>Base pairs^b</th>
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*aBased on EMBL database searches. ^bThe number of ambiguous bases are given in parenthesis.
Data from the DGGE gels shows that many of bands found in the fingerprints from the samples taken from the models inoculated with a single saliva sample are maintained in the communities established from combined saliva. Examples include band 22 (uncultured bacterium) which was present in all the samples from the microcosms derived from the saliva of volunteer C alone and A and C combined. This particular PCR amplicon was not present in the profiles of A alone, D alone and C and D combined. Band 40 (a close relative of *Fusobacterium nucleatum*) only appeared in the perfusate (day 10) and biofilm samples derived from the saliva of volunteer D alone and in combination with C. Two of the major bands (3 and 23) present in all the early perfusate samples across all microcosms were identified as streptococci.
6.5. Discussion

Marked inter-individual variation in species composition has been observed in the oral microbiota (Lazarevic et al., 2010; Ledder et al., 2006; Rasiah et al., 2005), with significant heterogeneity occurring between different anatomical sites (Aas et al., 2005; Dewhirst et al., 2010; Mager et al., 2003; Paster et al., 2006). However, intra-individual temporal changes in the oral microbiota are less evident, with a subject-specific taxa persisting for months or even years exhibiting only minor fluctuations (Lazarevic et al., 2010; Rasiah et al., 2005). These observations call into question the use of inocula comprised of pooled saliva samples or selected bacterial consortia for representative in vitro modelling of the oral microbiota. Utilising culture and molecular microbiology techniques including differential bacterial counts, PCR-DGGE and sequence analysis the study described in this chapter seeked to examine the impact on microcosm stability, composition and species diversity of using the combined saliva samples of two or more individuals as inocula for Sorbarod-based microcosms.

6.5.1. Rationale for the use of culture-based and culture-independent molecular microbiology techniques in this study

Sorbarod filters have previously been used successfully to support oral microcosms derived from salivary inocula (Ledder et al., 2006; Ledder et al., 2009; McBain et al., 2005). Particular advantages of this system include, the opportunity for continued monitoring of population dynamics via sampling of model perfusate, the small amount of material needed for inoculation and the generation of a relatively large amount of biofilm for analysis. Sorbarod model systems have also been shown to maintain key oral species at levels similar to
those found in vivo (McBain et al., 2005). Ledder et al (2006) have shown that Sorbarods are capable of supporting stable, individualised oral microcosms (Ledder et al., 2006). In common with most in vitro models the Sorbarod device cannot reproduce the precise conditions that prevail in vivo, however the advantages associated with this system outweighed the limitations for the purposes of this study.

Subgingival plaque is frequently dominated by gram-negative anaerobes and Lactobacillus sp. (Listgarten, 1976). Supragingival and early dental plaque however, comprise a higher proportion of streptococci and Actinomyces sp. (Palmer et al., 2003). It has been suggested that the salivary-derived Sorbarod biofilm more closely resembles subgingival plaque or a soft tissue microbial community than supragingival plaque, due in part to the presence of limited hydrodynamic shear forces (Ledder et al., 2006; McBain et al., 2005). Therefore, in order to best characterise the nature of, and the changes occurring within each microcosm in this study, the enumeration of the streptococci, lactobacilli and gram-negative anaerobes in both perfusate and biofilm, were an appropriate choice. Enumeration of the total culturable aerobes and anaerobes also proved useful in establishing the overall stability of the microcosms.

PCR-DGGE has a well established role in profiling oral microbial communities (Diaz et al., 2006; Fujimoto et al., 2003; Li et al., 2005; Li et al., 2006; Maukonen et al., 2008; McBain et al., 2003a; Muyzer & Smalla, 1998; Perea, 2004; Rasiah et al., 2005; Siqueira et al., 2005; Spratt, 2004; Zijnge et al., 2003), including those established within Sorbarod filters (Ledder et al., 2006).
The technique is one of the only means of achieving reproducible, consortial fingerprints. In addition to this, further analysis of the DGGE fingerprints allows the construction of hierarchical dendrograms and principal component analysis of the data. The phylogenetic origin of selected bands from the gel can also be identified by excision and sequence analysis (Ledder et al., 2006). PCR-DGGE therefore provided an appropriate and useful means of establishing any changes in community stability and composition between microcosms derived from single and combined saliva samples.

6.5.2. Analysis of inter and intra-individual variations in the functional bacterial groups between microcosm perfusate using differential bacterial counts

Species composition of the perfusate from a Sorbarod model system inoculated with saliva has been shown to broadly reflect that of the respective biofilm (McBain et al., 2005). Here, the results of the differential bacterial counts from the perfusates showed that for all the functional bacterial groups tested key trends in the data were maintained regardless of whether the microcosm was established from the saliva of a single volunteer or a pooled sample from two individuals. In all cases the total number of culturable aerobes remained steady over the ten day period whilst the total number of culturable anaerobes exhibited on average, a tenfold increase in number. These observations suggest that as the biofilm and perfusate population grew in number the obligately aerobic and facultatively anaerobic species represented a decreasing proportion of the total community, reflecting the trends commonly associated with in vivo dental plaque biofilms where a reduction in redox potential facilitates the proliferation of obligately anaerobic organisms (Marsh & Martin, 2003).
Recent work by Zijnge et al (2010) utilising fluorescent in situ hybridization (FISH) in vivo has shown that dental plaque biofilms are initially dominated by aerobes and facultative anaerobes including Actinomyces sp., Streptococcus sp., Lactobacillus sp. and Candida sp. which eventually become outnumbered by anaerobic filamentous bacteria including F. nucleatum and T. forsythia (Zijnge et al., 2010). Subgingival biofilms are particularly associated with an increased proportion of gram-negative anaerobes (Zijnge et al., 2010). FISH has also been used by Al-Ahmad et al (2007) where the authors noted that biofilms formed in vivo exhibit a reduction in the proportion of streptococci after seven days (Al-Ahmad et al., 2007). Data obtained in this chapter shows that some of the patterns in population dynamics observed in vivo by Al-Ahmad et al (2007) and Zijnge et al (2010) are reproduced in the Sorbarod microcosm. Here, the number of culturable gram-negative anaerobes increased markedly over the test period and represented the majority of the culturable anaerobic species in all of the microcosms. A concomitant fall in the proportion of Streptococci in the biofilm over time was also observed, again in microcosms derived from both single and combined saliva samples.

Inter-microcosm variability was most notable in the numbers of Lactobacilli found in perfusate. This result is analogous to that observed by McBain et al (2005) in their validation of the MSD (McBain et al., 2005). Lactobacilli have been shown to be central to the formation of many subgingival bacterial aggregates (Zijnge et al., 2010). It seems likely that the inter-microcosm variability observed here is a result of differences in biofilm architecture wherein some salivary-derived biofilms are more heavily reliant on Lactobacilli-based aggregates for their stability and structure than others. The predominant
location of a species within the biofilm is significant in terms of the likelihood of colony forming units being sloughed away and identified in the perfusate.

Despite continuity across all microcosms in terms of the general trends observed in the main functional bacterial groups isolated from perfusate, intra-microcosm changes within the functional bacterial groups were more noticeable in the perfusate of microcosms derived from combined saliva. Interestingly, numerically the counts from the combined saliva based-microcosms did not represent an average of the two individual salivary communities in the vast majority of cases. There was no strong evidence to indicate that pooling of saliva leads to the development of a microcosm similar to one “dominant” salivary microbiota. Statistical analysis of the data showed that only two of the microcosms derived from combined saliva showed any significant similarity to one particular individual’s microcosm over another: the combined saliva of volunteers A and D which showed some statistically significant similarities to volunteer A alone, and B and D where similarities to volunteer B’s microcosm were observed. In general, data obtained from the microcosm perfusate appears to show that pooled saliva produces a novel bacterial community that exhibits characteristics of the two microbiotas it is derived from, with differential bacterial counts following similar trends over the ten days. However, intra-individual temporal variability within the functional bacterial groups indicated that the microcosm is not as stable as the communities derived from the saliva of a single individual.
6.5.3. Analysis of inter and intra-individual variations in the functional bacterial groups between Sorbarod biofilms using differential bacterial counts

Viable counts from the biofilm samples showed that overall inter-microcosm variability within the total culturable aerobes, anaerobes and streptococci was higher in those derived from combined saliva. Because all of the microcosms were derived from the same four oral microbiotas, when the data were combined (as detailed in Figures 6.8. to 6.12.) the overall variability of the combined salivary microcosms was anticipated to be within the limits set by the microcosms derived from the individual saliva. The fact that the variability exceeded these limits adds strength to the argument that the microcosms derived from the pooled saliva were less stable.

6.5.4. Effects of combining salivary inocula on species diversity in Sorbarod microcosms assessed by PCR-DGGE

One of the most interesting aspects of the data obtained from the PCR-DGGE analysis was the inter-microcosm variations in species diversity identified by enumeration of PCR amplicons in each DGGE fingerprint. The perfusate populations from the microcosm derived from the salivae of all four donors exhibited the greatest diversity throughout the ten day collection period, followed by the pair-wise combinations of saliva, with the single donor microcosms showing the least diversity. This result was not unexpected because logic suggests that the innate diversity of the perfusate population should increase with the number of oral microbiotas that contribute to it. However, data obtained from the biofilm samples showed that species diversity was highest in the single saliva donor-based microcosms and lowest in the
microcosm derived from the combined saliva of all four volunteers. It seems likely that combining salivary microbiotas increased the number of species present in both the inocula and therefore the transient perfusate population, but that this level of diversity was not incorporated into the biofilm structure Diaz et al (2006) have suggested that members of a specific oral community adapt to one another and to their environment and that this results in interrelationships between community participants that ensure spatiotemporal persistence and stability in the microbial community (Diaz et al., 2006). Applying this concept to the data obtained in this chapter suggests that combining four established salivary microbiotas resulted in a high level of competition between the communities, each trying to exclude “foreign” species that eventually resulted in an established biofilm with a decreased level of species diversity. As a direct result of this competition and exclusion the innate stability and structure of the biofilm were compromised. Recent work by He et al (2010a and 2010b) has shown that established oral microbial communities can recognise potential intruders and prevent their establishment into the biofilm (He et al., 2010a; He et al., 2010b). This theory would explain the differences observed between the perfusate and biofilm populations and although perfusate data does reflect the biofilm community it is not truly representative. This is in agreement with data obtained by Ledder et al (2006) from MSD microcosms where inter-species differences in retention within biofilms were observed and the authors concluded that the species found in perfusate do not always accurately reflect the biofilm population (Ledder et al., 2006).
6.5.5. Dendrogram and PCA analysis of the DGGE fingerprints associated with microcosm perfusate and biofilms

Hierarchical dendrogram construction and PCA analysis of the DGGE fingerprints from the perfusate and biofilm populations clearly showed that the microcosms derived from pooled saliva were distinct from both the parent populations. There was no indication in any of the microcosms of the existence of a “dominant” microbiota, i.e. where a combined saliva-based microcosm showed similarities to one individual microbiota over another.

Samples taken from the same microcosms preferentially clustered together in both the dendrogram and PCA plots. The microcosm derived from the saliva of all four volunteers appeared to cluster further away from most of the single saliva microcosm samples (38 to 60% concordance). The fact that the DGGE profile of the microcosm derived from pooling four salivae is so different from the individual microcosms, more so than the pair-wise combinations of two salivae, is further evidence that combining saliva could compromise the “normal” development and structure of an oral microcosm.

Preferential clustering between early perfusate samples, both intra and inter-microcosm was observed. This could be attributed to the limited diversity (i.e. fewer bands) in the early perfusate samples. DGGE fingerprints containing a similar number of bands appeared to show some preferential clustering, a potential limitation of this technique.

DGGE band excision and sequencing revealed high numbers of streptococci, veillonella, prevotella and lactobacilli, alongside a significant proportion (25%) of uncultured species in all microcosms, not an unexpected result for salivary
derived microcosms (Aas et al., 2005; Ledder et al., 2006). Many of the prominent bands identified from the microcosms derived from a single saliva sample were also present in the profiles of the combined saliva-based microcosms, illustrating that although unique communities are produced from pooling saliva some characteristics of the parent populations are retained.

### 6.6. Conclusions

Successful modelling of an oral microbial community is reliant upon closely matching the conditions that prevail in vivo. It was therefore argued that in order to be considered accurate and relevant studies of the oral microbiota must account for and/or reproduce the significant inter-individual variability that exists in the oral cavity (Ledder et al., 2006). Many previous studies have utilised pooled saliva or selected bacterial consortia which fall short of this specification. The aim of this chapter was to evaluate the impact on microcosm stability, composition and species diversity of using pooled saliva versus saliva from a single individual as inocula for Sorbarod-based microcosms. Additionally, the study aimed to identify whether the main characteristics of the biofilm derived from combined saliva would resemble that of one, both or neither individual.

Data described in this chapter has shown that although pooled saliva-based microcosms retain many of the characteristics of their single saliva-based counterparts they lack an equivalent inherent stability. Pooling saliva did not increase bacterial diversity in the Sorbarod biofilms, surprisingly a reduction in species diversity was observed, possibly as a result of competition between communities with pre-existing stable inter-relationships and adaptations endeavouring to exclude “foreign” species or communities. Hierarchical
dendrogram and PCA also showed that by increasing the number of salivary microbiotas in the pool, in this case from two to four, the resultant biofilm and perfusate populations become even more distinct from the “normal” or single saliva-based microcosms. There was no evidence of significant similarity to, or domination by, one salivary microbiota over another; the community produced was unique with some elements of both parent communities.

From the data produced in this chapter it can be concluded that pooled saliva-based microcosms are limited in their capacity as representative *in vitro* models. Inter-individual variations must be taken into account when modelling the oral microbiota and would be of particular relevance when studying the response of a community to external stimuli, such as changes in nutrient availability or antimicrobial challenge. Sorbarod devices have proven here to be useful for the *in vitro* modelling of oral microcosms. The apparatus would lend itself well to incorporating additional nutrients, antimicrobials or other challenges to an established microcosm.
Chapter 7

Conclusions
7.1. General Conclusions

Studies involving the human oral microbiota date back as far as the 17th century, with a large amount of research interest occurring in the late 20th century. As a result there is a wealth of data available on the community composition, structure, functions, interactions and clinical implications of the resident oral microbiota. A significant proportion of this historical research was dependent upon culture of the microorganisms in question. Recent technological advancements in molecular microbiology have suggested that the majority of microbial inhabitants of the mouth are yet to be cultured and species diversity is much greater than previously thought. Numerous studies have also identified that the oral microbiota is unique to each individual and exhibits a high level of temporal stability. These observations call into question the many generic models of oral bacterial communities that predominate in the literature and also the use of pooled saliva and plaque samples in microcosm studies.

A gap in the literature exists linking the many valid observations of oral microbial interactions, community structure and function with the emerging evidence of a highly individualised oral microbiota. These differences are significant because bacterial interactions underpin the formation and maintenance of oral biofilms which in turn impacts upon the health status of the oral cavity. A better understanding of these processes would benefit those seeking to study dental diseases and devise prophylaxis or treatment options. This thesis includes two studies devised to expand upon existing knowledge of the mechanisms that underlie bacterial interactions occurring in the individual oral microbiota. The first looking at synergistic and antagonistic interactions
within and between isolates from four different salivary microbiotas and the second studying the interactions of the whole microbiotas via Sorbarod microcosms.

As communication and cooperation are believed to be fundamental processes in the development of multispecies biofilms it was hypothesised that bacterial isolates from the same mouth would exhibit many more positive interactions than those isolated from different volunteers and a decrease in antagonistic interactions (Chapter 5). Interestingly, the majority of interactions had a negative outcome for one or both species regardless of whether they were from the same mouth. It could be concluded that within the highly populated oral microbial ecosystem competition and antagonism play an equal or greater role in biofilm development and maintenance than synergy or tolerance to other species. Oral bacteria appear to cooperate with a limited number of species and have developed mechanisms to outcompete those from which they derive no benefit; however, this does not appear to compromise the stability of the microbial community in vivo. High levels of inter-individual variation observed between oral microbiotas are most likely a result of the unique combination of positive and negative interactions interlinked with numerous host-derived environmental factors. Data described in Chapter 5 indicates the importance of host factors influencing the bacterial phenotype, where the same strain was isolated from the saliva of two or more volunteers and exhibited markedly different interactions. There was some evidence to indicate a gender contribution which could be attributed to immunological or hormonal influences.
Using the saliva of the same four individuals to study bacterial interactions at the community level, it was hypothesised that mixing saliva would impact negatively on the stability and function of oral microcosms due to the highly complex and individual nature of the oral microbiota. Data described in Chapter 6 of this thesis shows that although pooled saliva-based microcosms retain many of the characteristics of their single saliva-based counterparts they lack an equivalent inherent stability. Pooling saliva did not increase bacterial diversity in the Sorbarod biofilms, surprisingly a reduction in species diversity was observed, possibly as a result of increased competition between communities with pre-existing stable inter-relationships and adaptations endeavouring to exclude “foreign” species or communities. By increasing the number of salivary microbiotas in the pool the biofilm and perfusate populations become even more distinct from the “normal” or single saliva-based microcosms. There was no evidence of significant similarity to, or domination by, one salivary microbiota over another; the community produced was unique with some elements of both parent communities.

Successful modelling of an oral microbial community is reliant upon closely matching the conditions that prevail in vivo. It was therefore argued that in order to be considered accurate and relevant studies of the oral microbiota must account for and/or reproduce the significant inter-individual variability that exists in the oral cavity (Ledder et al., 2006). Many previous studies have utilised pooled saliva or selected bacterial consortia. From the data described in this thesis it can be concluded that pooled saliva-based microcosms are limited in their capacity as representative in vitro models. Inter-individual variations must be taken into account when modelling the oral microbiota and would be of
particular relevance when studying the response of a community to external stimuli, such as changes in nutrient availability or antimicrobial challenge.

A further gap in the literature was identified when evaluating the effect of two antimicrobial containing dentrifices (TD and SZD) on the microbial ecology of salivary-derived communities. Whilst oral hygiene products have been extensively studied and tested in the clinical setting, to our knowledge the methodology presented in Chapter 3, whereby PCR-DGGE was used in conjunction with culture to directly compare two dentrifices is relatively uncommon. In keeping with the aims of this thesis the antimicrobials were tested on salivary-derived bacteria taken from a single volunteer in an attempt to limit the impact of inter-individual variation. TD and SZD both demonstrated characteristic effects on salivary-derived biofilm microcosms that lead to a reduction in microbial diversity and species-specific compositional changes. Significant perturbations in the in vivo oral microbiota could result in a breakdown of bacterial homeostasis; the implications of this may warrant further investigation.

The final distinct objective of this thesis was to identify whether the processes of bacterial coaggregation and coadhesion that are believed to be important in the formation of oral biofilms also occur between selected species of the human intestinal microbiota. Coaggregation has been described extensively in the oral cavity; however the phenomenon is rarely reported in other human-associated biofilms. The results obtained in Chapter 4 of this thesis demonstrated that strong coaggregation interactions between gut isolates and between oral and gut bacteria are markedly less common than among species belonging to the
oral microbiota, suggesting that the role of coaggregation in community development in the intestinal tract, and in interactions between oral and gut populations in vivo, may be less important. Host cell turnover is rapid in the intestinal epithelium and shear forces are generally low, thus the selection pressure for expression of the appropriate proteinaceous adhesins and receptors mediating coaggregation may be less marked. It seems likely that biofilm architecture is more transient in the gut, with enteric bacteria held within slow moving viscous mucus and predominantly binding to host molecules. It seems possible that coaggregation is either an important adherence mechanism developed by oral bacteria as a result of specific environmental conditions in the mouth or in fact just an artefact observed between many oral species.

7.2. Suggestions for further work

In terms of the study of bacterial interactions as described in Chapter 5 of this thesis, had time allowed the basis of the interactions could have been further characterised to identify if bacteriocins were involved in the antagonism. However, as the study aimed to obtain an overall picture of the nature of inter and intra-individual interactions rather than identify the specific processes involved an expansion of the number of subjects and/or species may be more relevant. The microcosm studies could be expanded upon by challenging the single and combined salivary communities with different nutritional conditions or antimicrobials. It would be interesting to compare and contrast how individual microbiotas respond to antimicrobial challenge.
An interesting addition to the study detailed in Chapter 3 of this thesis, examining the response of salivary-derived communities to two dentrifice formulations, would be to utilise the saliva of two or more individuals. Comparing the differences in response to antimicrobial challenge of bacterial communities could be important in developing a better understanding of inter-individual variation in oral microbial ecology and treatment outcomes.

Further work to expand upon the coaggregation assays described in Chapter 4 could include an assessment of a larger number of intestinal isolates, perhaps focusing on strains related to those that have been shown to coaggregate in this study. It may also be useful to look at other multi-species biofilms associated with the human body, such as the skin. The methods utilised in this study would be appropriate to assess isolates from any body site.
Appendix
**Coaggregation between and among human intestinal and oral bacteria**

Ruth G. Ledder, Andrea S. Timperley, Melissa K. Friswell, Sandra Macfarlane, and Andrew J. McInnes

**Abstract**

Coaggregation is believed to facilitate the integration of new bacterial species into polymicrobial communities. The aim of this study was to investigate coaggregation between and among human oral and intestinal bacteria. Stationary phase cultures of 10 oral and 10 intestinal species, chosen on the basis of numerical and ecological significance in their respective environments together with their ease of cultivation, were tested using a quantitative spectrophotometric coaggregation assay in all possible pairwise combinations to provide quantitative coaggregation scores. While 40% of possible partnerships coaggregated strongly for oral strains, strong interactions between oral and gut strains were considerably less common (4% incidence). Coaggregation scores were also weak between members of the intestinal microbiota (7% incidence), apart from Bacteroides fragilis with Clostridium pefuriens, and Bifidobacterium adolescentis with C. pefuriens. Oral and intestinal bacteria did not strongly interact, apart from B. adolescentis with Pseudomonas aeruginosa, Actinomyces naeslundii with C. pefuriens and P. aeruginosa with Lactobacillus casei. Heating and sugar-addition experiments indicated that similar to oral microorganisms, interactions with intestinal bacteria and between intestinal and oral strains were mediated by lectin-carbohydrate interactions.

**Introduction**

Coaggregation has been defined as the adherence of genetically distinct bacteria, and is regarded as an important process in the development of multispecies biofilms (Kolenbrander, 1988), particularly in bacteria associated with dental plaque (Gibbons & Wyward, 1970). Physical interactions between coaggregating bacteria facilitate metabolic interactions (Drago et al., 1997), such as oxygen protection (Brandt et al., 1998), cell-cell communication (Kolenbrander, 2000) and genetic exchange between cells (Foster et al., 2003). Coaggregation usually depends on highly specific lectin-carbohydrate interactions occurring between the aggregating partners (Ciss et al., 1979), and can be defined as intergenic, intragenic or multigenic (Heggeseth et al., 1988; Kolenbrander, 2000). It is generally detected in vitro by observing flocculation of washed and resuspended test strains.

The vast majority of coaggregation research has concentrated on bacteria derived from the human oral cavity (Handley et al., 1988; Eke et al., 1989; Unemoto et al., 1999; Foster & Kolenbrander, 2004; Shen et al., 2006; Rosen & Sola, 2006). It has been postulated that coaggregative ability of oral bacteria has evolved as a protective mechanism against high shear forces that prevail in the oral cavity (Handley et al., 2004). Coaggregation studies with bacteria derived from ecosystems such as the gut have largely focused on species belonging to the genus Bacillus coaggregating with potential opportunistic pathogens as an anti-infection mechanism, for example, in the gastrointestional tract of chickens (Vandevoorde et al., 1992) and pigs (Knaat et al., 1995), as well as with human enteropathogens (Drago et al., 1997) and enteropathogens (Field et al., 1998). In addition, coaggregation has also been shown to occur between bacteria derived from aquatic ecosystems (Richard et al., 2000, 2002).

While it seems likely that many of the fundamental mechanisms of cellular interaction observed in the mouth might also occur in the gut, coaggregation between members of the human intestinal microbiota has yet to be reported. It is also possible that oral bacteria will physically associate with members of the intestinal microbiota because...
oral species swallowed with food and saliva pass though the digestive tract.

This study applies a quantitative spectrophotometric assay together with confocal microscopy to investigate coaggregative interactions within and between members of the oral and gastrointestinal microbiota, chosen as a paradigm of their respective environments. Characterization of these interactions was undertaken with heating and sugar-addition experiments.

**Materials and methods**

**Bacterial strains and culture conditions**

The oral bacteria used in this study were Actinomyces naeslundii WVU 627, Fusobacterium nucleatum NCTC 10562, Lactobacillus rhamnosus NC 413, Neisseria subflava A1078, Prevotella oralis NCTC 11639, Porphyromonas gingivalis NCTC 11834, Streptococcus mutans NCTC 10832, Streptococcus oralis NCTC 11427, Streptococcus sanguis NCTC 7863 and Veillonella dispar ATCC 17749. Enteric isolates used in this study were Bacteroides fragilis NCTC 9543, Bacteroides vulgatus M40.2 (AM990169), Bifidobacterium adolescentis NCIMB 702231, Bifidobacterium infantis M40.6 (AM990173), Clostridium perfringens NCTC 8546, Enterococcus faecalis ATCC 51299, Enterococcus faecium M40.1 (AM990168), Escherichia coli NCTC 9001, Enterobacter hormaechei M40.7 (AM990174) and Lactobacillus paracasei M40.9 (AM990176). Numbers in parentheses indicate the accession number of clinical strains in the EMBL database.

Neisseria subflava was grown on Wilkins–Chalgren agar and broth and incubated aerobically at 37 °C. Other bacteria were grown on Wilkins–Chalgren anaerobic agar or broth in a Mark 3 Anaerobic Work Station (Don Whitley Scientific, Shipley, West Yorkshire, UK) at 37 °C (atmosphere: 80% N₂, 10% CO₂, 10% H₂).

**Preparation of inocula for coaggregation assays**

 Cultures of test bacteria were grown under conditions previously shown to produce stationary phase cells by inoculating the strains into 500 mL of preduced Wilkins–Chalgren broth and incubating in an anaerobic environment for 7 days (Bradshaw et al., 1998). Neisseria subflava was grown in Wilkins–Chalgren broth aerobically without mixing for 3 days.

**Coaggregation assays and characterization of coaggregation mechanisms**

A modified quantitative spectrophotometric assay described by Begam et al. (2004) was used to determine the coaggregation activity between test species. Briefly, bacteria were harvested by centrifugation at 10,000 g (Beckman–Görter J2-21) for 20 min and resuspended in coaggregation buffer (Gisar et al., 1979). This buffer comprised 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.15 M NaCl and 3.1 mM NaN₃ dissolved in 1 mM Tris buffer adjusted to pH 7.0. The strains were washed three times in coaggregation buffer and then resuspended to give an OD₅₀₀ of 1.5. Equal volumes of each suspension were mixed in sterile cuvettes and the optical densities recorded. Autoaggregation was determined using an identical method, by combining two equal volumes of the same bacterial suspension. The mixtures were left for 1 h at room temperature to allow coaggregation to occur, after which OD was once again recorded. The percent coaggregation was calculated by the following equation:

\[
\text{coaggregation} = \left[\frac{\text{[preincubation value]}}{\text{[preincubation value]}} \times 100\right]
\]

To characterize the mechanisms of coaggregation among a selection of the strongly coaggregating species, selected strains were resuspended at an OD₅₀₀ of 1.0 after the washing step. Volumes of each suspension (500 µL) were thoroughly mixed together and left to stand at room temperature for 2 min. Coaggregation was assessed by the following scoring system (Kolenbrander, 1995): rapid and complete settling of large coaggregates leaving a water-clear supernatant (+4); coaggregates that settled rapidly but with a supernatant that remained slightly cloudy (+3); coaggregates that formed immediately, but remained suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1); suspensions with no evidence of aggregation or coaggregation (0).

**Effect of sugars on the strength of coaggregation**

The ability of specific sugars to reverse each coaggregation was determined by the following method: the sugars (lactose, N-acetyl-l-galactosamine, 8-glactose, 8-galactosamine, 8-fucose, 8-fucose, 8-methyl-l-galactoside, 8-methyl-l-galactoside, 8-rhamnose or N-acetyl glucosamine), at a final concentration of 100 mM, were added individually to 500 µL of a suspension containing one of the coaggregating strains and mixed. After standing at room temperature for 10 min, 500 µL of a suspension containing the partner strain was added and mixed again. Coaggregation was then assessed using the assay described above.

**Influence of heating on coaggregation**

The effect of heat on each coaggregative interaction was determined by heating one of the two partner strains at 85 °C for 30 min (Kolenbrander, 1995). An equal volume of suspension containing the partner was added, and coaggregation was again gauged using the visual assay.
Confocal scanning laser microscopy analysis

Completed coaggregation assays were stained for 10 min in the dark at room temperature using 2 μl of BacLight™ LIVE/DEAD stain (Molecular Probes, Leiden, The Netherlands), prepared according to the manufacturer’s instructions in phosphate-buffered saline (0.01 M, pH 7.0). The mixtures were then examined using a Zeiss Combi LSM 510 META/Confocor II inverted microscope (Jena, Germany). Excitation wavelengths at 488 and 633 nm were used to image live and dead bacteria, respectively. Confocal images were obtained using 40 x 1.3 NA DIC oil immersion objectives. Each mixture was scanned at randomly selected positions. Z-series were generated by vertical optical sectioning at every position with the slice thickness set to 0.4 μm. Image acquisition and analysis was performed with the software coas (Zeiss, version 3.2).

Chemicals

Unless otherwise stated, chemicals were obtained from Sigma (Poole, Dorset, UK). Formulated bacteriological media were purchased from Oxoid (Basingstoke, Hampshire, UK).

| Table 1. Coaggregation scores of pairs of the 10 human oral bacterial strains used in this study |
|--------------------------------------------------|----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Species                                      | A. naeuli       | F. nucleatum    | L. rhamnovus    | N. subflava     | P. gingivalis   | R. oralis       | S. mutans       | S. sanguis      | V. parahominis  |
| A. naeuli                                    | 30 (4.6)*       |                  | 40 (6.3)*       | 35 (5.2)*       | 25 (5.9)*       | 22 (4.1)*       | 22 (4.1)*       | 28 (5.2)*       | 20 (3.4)*       |
| F. nucleatum                                 | 70 (6.2)        | 100 (12.2)      | 95 (14.7)       | 90 (14.2)       | 75 (12.2)       | 70 (12.2)       | 65 (10.5)       | 75 (12.2)       | 55 (10.5)       |
| L. rhamnovus                                 | 40 (6.3)        | 100 (12.2)      | 95 (14.7)       | 90 (14.2)       | 75 (12.2)       | 70 (12.2)       | 65 (10.5)       | 75 (12.2)       | 55 (10.5)       |
| N. subflava                                  | 35 (5.2)        | 95 (14.7)       | 90 (14.2)       | 85 (12.2)       | 70 (12.2)       | 65 (12.2)       | 60 (10.5)       | 70 (12.2)       | 55 (10.5)       |
| P. gingivalis                                | 25 (5.9)        | 70 (12.2)       | 65 (12.2)       | 60 (10.5)       | 50 (10.0)       | 45 (10.0)       | 40 (9.0)        | 50 (10.0)       | 40 (9.0)        |
| R. oralis                                    | 22 (4.1)        | 70 (12.2)       | 65 (12.2)       | 60 (10.5)       | 50 (10.0)       | 45 (10.0)       | 40 (9.0)        | 50 (10.0)       | 40 (9.0)        |
| S. mutans                                    | 22 (4.1)        | 70 (12.2)       | 65 (12.2)       | 60 (10.5)       | 50 (10.0)       | 45 (10.0)       | 40 (9.0)        | 50 (10.0)       | 40 (9.0)        |
| S. sanguis                                   | 65 (10.5)       | 75 (12.2)       | 70 (12.2)       | 65 (10.5)       | 60 (10.5)       | 55 (10.5)       | 55 (10.5)       | 60 (10.5)       | 55 (10.5)       |
| V. parahominis                               | 25 (5.9)        | 70 (12.2)       | 65 (12.2)       | 60 (10.5)       | 50 (10.0)       | 45 (10.0)       | 40 (9.0)        | 50 (10.0)       | 40 (9.0)        |

Results

Coaggregation between oral isolates

Data in Table 1 show that substantial levels (>70%) of interspecies coaggregation occurred between (1) F. nucleatum and A. naeuli, (2) A. naeuli and P. gingivalis, (3) F. nucleatum and B. adolescentis, (4) L. rhamnovus and P. gingivalis, and (5) P. gingivalis and N. subflava. Cultures of S. sanguis scored the highest for autoaggregation (37%), and A. naeuli was the most promiscuous coaggregating oral species overall, associating markedly (>20%) with eight of the nine species tested.

Coaggregation between intestinal isolates

Data in Table 2 show that coaggregation scores between members of the intestinal microbiota were low (<12%) apart from (1) B. fragilis and C. perfringens and (2) B. adolescentis and C. perfringens, which coaggregated at 39% and 38%, respectively. Clostridium perfringens was the most polygamous intestinal bacterium, scoring the highest overall for coaggregation among the gut species tested.

Table 2. Coaggregation scores of pairs of the 10 human intestinal bacterial strains used in this study

<table>
<thead>
<tr>
<th>Species</th>
<th>B. fragilis</th>
<th>B. adolescentis</th>
<th>B. infantis</th>
<th>C. perfringens</th>
<th>E. coli</th>
<th>E. faecium</th>
<th>E. faecalis</th>
<th>E. hormaechi</th>
<th>I. paracasei</th>
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<tr>
<td>B. fragilis</td>
<td>2.9 (1.4)*</td>
<td>3.5 (1.6)</td>
<td>2.4 (1.6)</td>
<td>3.8 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>3.6 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>2.5 (1.6)</td>
<td>3.6 (1.6)</td>
</tr>
<tr>
<td>B. adolescentis</td>
<td>3.5 (1.6)</td>
<td>3.4 (1.6)</td>
<td>2.9 (1.6)</td>
<td>3.6 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>3.6 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>2.5 (1.6)</td>
<td>3.6 (1.6)</td>
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<tr>
<td>B. infantis</td>
<td>2.6 (1.4)</td>
<td>2.8 (1.6)</td>
<td>2.2 (1.4)</td>
<td>3.7 (1.4)</td>
<td>4.2 (1.6)*</td>
<td>3.6 (1.6)</td>
<td>4.2 (1.6)*</td>
<td>2.5 (1.6)</td>
<td>3.6 (1.6)</td>
</tr>
<tr>
<td>C. perfringens</td>
<td>3.2 (1.4)</td>
<td>3.5 (1.6)</td>
<td>2.3 (1.4)</td>
<td>3.8 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>3.6 (1.6)</td>
<td>4.1 (1.9)*</td>
<td>2.5 (1.6)</td>
<td>3.6 (1.6)</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.6 (1.3)</td>
<td>1.4 (1.2)</td>
<td>1.2 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
<td>1.6 (1.3)</td>
<td>2.3 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
</tr>
<tr>
<td>E. faecium</td>
<td>1.6 (1.3)</td>
<td>1.4 (1.2)</td>
<td>1.2 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
<td>1.6 (1.3)</td>
<td>2.3 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>1.6 (1.3)</td>
<td>1.4 (1.2)</td>
<td>1.2 (1.3)</td>
<td>1.7 (1.3)</td>
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<td>2.3 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
</tr>
<tr>
<td>E. hormaechi</td>
<td>1.6 (1.3)</td>
<td>1.4 (1.2)</td>
<td>1.2 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
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<td>2.3 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
</tr>
<tr>
<td>I. paracasei</td>
<td>1.6 (1.3)</td>
<td>1.4 (1.2)</td>
<td>1.2 (1.3)</td>
<td>1.7 (1.3)</td>
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<td>2.3 (1.3)</td>
<td>1.7 (1.3)</td>
<td>2.3 (1.3)</td>
</tr>
</tbody>
</table>
Coaggregation between members of the oral and gut microbiota

Table 3 shows that coaggregation scores between members of the oral and intestinal microbiota were low overall (<30%) apart from (1) B. adolescentis and F. nucleatum (81%), (2) A. naeslundii and C. perfringens (43%), (3) F. nucleatum and L. paracasei (51%) and (4) A. naeslundii and L. paracasei (51%). Arineomyces naeslundii intersected most strongly with five of the 10 strains, giving coaggregation scores of over 20%.

Inhibition assays

Results in Table 4 show that the majority of coaggregations were partially or completely inhibited by at least one of the sugars used in this study. Only the following oral pairings were unaffected: (1) A. naeslundii and F. nucleatum, (2) A. naeslundii and L. rhamnosus and (3) F. nucleatum and L. rhamnosus. Table 5 shows that all but three of the coaggregating pairs tested were affected by heat treatment. Unaffected pairings included (1) A. naeslundii and L. rhamnosus, (2) A. naeslundii and C. perfringens and (3) F. nucleatum and L. rhamnosus. In most cases, heating one of the partner strains inhibited coaggregation, while heating the other had no effect on the interaction.

Confocal microscopy analysis

Figure 1 shows two confocal micrographs of (a) A. naeslundii and B. vulgatus and (b) A. naeslundii and L. rhamnosus visualized using the LIVE/DEAD stain. Bacterial cells in each pairing can be seen to exist in an intimate relationship.

Discussion

Coaggregation has been shown to be an important step in the formation of dental plaque, facilitating the formation of multispecies biofilms (Kolenbrander & London, 1993; Palmer et al., 2003), and allowing the development of complex feeding webs (Divitini et al., 1999; Kolenbrander, 2000). To our knowledge, this is the first study to have investigated coaggregation among numerically and ecologically important intestinal bacteria, and between intestinal bacteria and oral isolates. Coaggregation among organisms originating from the intestinal tract was limited in both frequency and extent (Table 2), indicating that this phenomenon may not be as important for the development of multispecies biofilms in the human gut as it is in the oral cavity. This could be because shear forces in the gastrointestinal tract are generally much lower than those that prevail in the mouth, and therefore the selection pressure for expression of the appropriate adhesins and receptors is absent. However, C. perfringens coaggregated substantially with both B. fragilis and B. adolescentis, indicating that these Gram-positive species could be involved in the exchange of metabolites or co-operative feeding. Coaggregation between oral and enteric bacteria was also low overall (Table 3), suggesting that ecological interactions between these two groups may be limited in vivo, although exceptions to this were F. nucleatum and B. adolescentis, and A. naeslundii together with both of the bacteroides tested, B. adolescentis, C. perfringens and L. paracasei. This could be because of the expression of receptors and adhesins that are similar to those formed by closely related oral bacteria such as Prevotella spp. and L. rhamnosus.

Coaggregation assays conducted for a range of oral bacteria for validation purposes served as internal controls. The most promiscuous oral coaggregators tested were A. naeslundii, F. nucleatum, B. gingivalis and the streptococci. Arineomyces are primary colonizers of tooth enamel (Palmer et al., 2003), and are regarded as being fundamental in the development of dental plaque (Nyuad & Kilian, 1987). Their ability to coaggregate widely with other oral bacteria is well recognized (Hamada et al., 1998; Li et al., 2001). Fusobacteria reach considerable numbers in mature dental plaque (Moore & Moore, 1994) and have previously been shown to coaggregate extensively with representatives of practically

Table 3. Coaggregation scores of pairs of the 10 oral and 10 intestinal bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>B. fragilis</th>
<th>B. vulgatus</th>
<th>B. adolescentis</th>
<th>C. perfringens</th>
<th>F. nucleatum</th>
<th>L. rhamnosus</th>
<th>L. paracasei</th>
<th>L. casei</th>
<th>L. helveticus</th>
<th>L. hamiltonii</th>
<th>E. coli</th>
<th>L. casei</th>
<th>L. paracasei</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. naeslundii</td>
<td>23.9(10)</td>
<td>20.5(9)</td>
<td>25(12)</td>
<td>16.3(5.8)</td>
<td>23.3(13)</td>
<td>24.2(12)</td>
<td>16.1(5.6)</td>
<td>14.9(4.6)</td>
<td>19.2(6.8)</td>
<td>22.3(7.7)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>5.3(13)</td>
<td>14.9(4.4)</td>
<td>15.8(4.4)</td>
<td>12.5(2.4)</td>
<td>6.4(1.6)</td>
<td>1.8(2.6)</td>
<td>11.3(5.6)</td>
<td>3.3(2.8)</td>
<td>9.1(1.4)</td>
<td>5.3(13)</td>
<td>31.9(3)</td>
<td>5.3(13)</td>
<td>5.3(13)</td>
</tr>
<tr>
<td>L. rhamnosus</td>
<td>0.7(7)</td>
<td>8.5(2.3)</td>
<td>3.5(1.4)</td>
<td>1.3(1.3)</td>
<td>2.2(1.1)</td>
<td>3.1(0.4)</td>
<td>4.4(2.4)</td>
<td>5.2(4.4)</td>
<td>2.6(1.5)</td>
<td>3.6(1.4)</td>
<td>4.4(2.4)</td>
<td>4.4(2.4)</td>
<td>4.4(2.4)</td>
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<td>N. subflava</td>
<td>1.6(10.8)</td>
<td>2.2(0.4)</td>
<td>1.2(0.4)</td>
<td>1.0(0.4)</td>
<td>1.6(1)</td>
<td>1.4(0.4)</td>
<td>2.2(0.8)</td>
<td>1.8(0.4)</td>
<td>1.4(0.7)</td>
<td>1.8(0.4)</td>
<td>1.4(0.7)</td>
<td>1.4(0.7)</td>
<td>1.4(0.7)</td>
</tr>
<tr>
<td>F. gingivalis</td>
<td>6.9(12)</td>
<td>6.3(1.3)</td>
<td>12.7(0.4)</td>
<td>5.1(1.1)</td>
<td>5.1(0.6)</td>
<td>7.6(5.6)</td>
<td>3.3(0.6)</td>
<td>6.6(0.6)</td>
<td>9.3(1.5)</td>
<td>9.3(1.5)</td>
<td>5.3(13)</td>
<td>9.3(1.5)</td>
<td>9.3(1.5)</td>
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<td>P. oris</td>
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<td>1.1(0.4)</td>
<td>1.4(0.4)</td>
<td>1.7(1.0)</td>
<td>2.0(0.5)</td>
<td>1.8(0.4)</td>
<td>2.2(0.8)</td>
<td>1.8(0.4)</td>
<td>1.4(0.7)</td>
<td>1.8(0.4)</td>
<td>1.4(0.7)</td>
<td>1.8(0.4)</td>
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<td>S. mutans</td>
<td>6.9(17.7)</td>
<td>1.8(0.4)</td>
<td>7.6(2.8)</td>
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<td>S. oralis</td>
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<td>3.3(0.4)</td>
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<td>1.8(1.7)</td>
<td>1.7(0.4)</td>
<td>1.2(0.4)</td>
<td>1.2(0.4)</td>
<td>1.2(0.4)</td>
<td>2.0(4)</td>
<td>1.2(0.4)</td>
<td>1.2(0.4)</td>
<td>1.2(0.4)</td>
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<tr>
<td>S. sanguinis</td>
<td>1.4(1)</td>
<td>1.3(0.4)</td>
<td>0.1(0.4)</td>
<td>0.9(0.4)</td>
<td>1.3(0.7)</td>
<td>1.2(0.6)</td>
<td>1.1(0.4)</td>
<td>4.9(4.9)</td>
<td>1.1(1)</td>
<td>1.4(0.7)</td>
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<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>V. dispar</td>
<td>1.4(10.7)</td>
<td>0.8(0.4)</td>
<td>1.6(0.4)</td>
<td>0.4(0.2)</td>
<td>1.7(0.4)</td>
<td>1.8(0.4)</td>
<td>1.3(0.7)</td>
<td>1.4(0.7)</td>
<td>0.2(1)</td>
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See legend to Table 1.
Table 4. Coaggregation scores of pairs of oral and intestinal bacterial strains after addition of sugars.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lactose</th>
<th>N-Acetyl-o-galactosamine</th>
<th>P-Galactose</th>
<th>D-Galactose</th>
<th>Glucose</th>
<th>L-Fucose</th>
<th>L-Rhamnose</th>
<th>β-Methyl-o-galactoside</th>
<th>β-Methyl-o-glucoside</th>
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<td>AN/VN</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
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<td>+3</td>
<td>+3</td>
<td>+3</td>
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<td>+3</td>
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<tr>
<td>AN/UR</td>
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<td>+2</td>
<td>+2</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
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<tr>
<td>AN/PG</td>
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<td>+1</td>
<td>+2</td>
<td>+2</td>
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<td>+3</td>
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<tr>
<td>AN/CP</td>
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<td>0</td>
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<td>0</td>
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<tr>
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<tr>
<td>FM/UR</td>
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<td>+4</td>
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<td>+4</td>
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<tr>
<td>FM/PG</td>
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<td>+2</td>
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<tr>
<td>FM/BA</td>
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<td>+4</td>
<td>+4</td>
<td>+4</td>
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</table>

Coaggregation scores assessed by visual assay: rapid and complete settling of large aggregates leaving a clear-water supernatant (+4); coaggregates that settle rapidly but supernatant remains slightly cloudy (+3); coaggregates form immediately but remain suspended in a turbid background (+2); detectable but finely dispersed coaggregates (+1); no evidence of aggregation or coaggregation (0). Coaggregative interactions inhibited by a sugar are shaded light grey (a decrease of 1) or dark grey (a decrease of ≥2).

AN, Actinomyces naeslundii; FN, Fusobacterium nucleatum; LI, Lactobacillus reuteri; PG, Porphyromonas gingivalis; CP, Clostridium perfringens; BF, Bacteroides fragilis; BA, Bacteroides vulgatus; LA, Bifidobacterium adolescentis.

Table 5. Coaggregation scores of pairs of oral and intestinal bacterial strains after heat treatment at 85°C for 30 min.

<table>
<thead>
<tr>
<th>Partner 1</th>
<th>Partner 2</th>
<th>Control</th>
<th>Partner 1 heated</th>
<th>Partner 2 heated</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN</td>
<td>TN</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
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<tr>
<td>AN</td>
<td>LR</td>
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<td>+2</td>
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<tr>
<td>AN</td>
<td>PG</td>
<td>+2</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>AN</td>
<td>CP</td>
<td>+1</td>
<td>+1</td>
<td>+1</td>
</tr>
<tr>
<td>AN</td>
<td>BF</td>
<td>+1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AN</td>
<td>BV</td>
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<tr>
<td>AN</td>
<td>BA</td>
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<tr>
<td>FN</td>
<td>LR</td>
<td>+4</td>
<td>+4</td>
<td>+4</td>
</tr>
<tr>
<td>FN</td>
<td>PG</td>
<td>+3</td>
<td>+2</td>
<td>+2</td>
</tr>
<tr>
<td>FN</td>
<td>BA</td>
<td>+4</td>
<td>+4</td>
<td>+4</td>
</tr>
<tr>
<td>LR</td>
<td>PG</td>
<td>+3</td>
<td>+3</td>
<td>+3</td>
</tr>
</tbody>
</table>

See legend to Table 4.

Previous studies on coaggregation of oral bacteria have frequently utilized a semi-quantitative visual assay (Cisar et al., 1979). In this study, a reproducible quantitative spectrophotometric assay was used to measure the speed and extent of coaggregation interactions, and a semi-quantitative method was used for inhibition studies intended to elucidate adherence processes. High levels of coaggregation observed in this investigation between members of the oral microbiota showed that the quantitative assay provides data that are broadly congruent with the commonly used semi-quantitative, visual assay (Kolenbrander, 1988; Bradshaw et al., 1998; Wells et al., 2000).

Confocal microscopy of coaggregating pairs allowed visualization of the vitality and the close cellular interactions of these structures. Figure 1 indicates that the cells involved in forming the coaggregates were largely viable, suggesting that coaggregation may enhance the survival of the bacteria involved; indeed Bradshaw et al. (1998) demonstrated that coaggregation confers protection from oxygen to obligately anaerobic species.

Inhibition studies revealed that coaggregation interactions between oral strains, and between oral and gut bacteria, are mediated by lectin–carbohydrate interactions. Previous studies have shown that coaggregation of oral bacteria is inhibited by specific sugars, often with a structure similar to that of lactose (Kolenbrander & Williams, 1988, 1983; McIntire et al., 1982; Kolenbrander & Andersen, 1989; Rickard et al., 2000; Rosen & Sela, 2006). The sugars chosen for this study have previously been used in the characterization of coaggregation between strains derived from the oral cavity (Kolenbrander & Andersen, 1988). The results showed that all of the interactions occurring between oral

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every oral genus tested to date (Kolenbrander & Andersen, 1989; Kolenbrander, 1995; Anderson & Sinclair, 1998). This was substantiated in the present study in that F. nucleatum coaggregated considerably (>30%) with six of the nine oral strains tested; however, significant coaggregation (>30%) between F. nucleatum and the intestinal isolates was limited to B. adolescentis. The ability of P. gingivalis to coaggregate with other oral bacteria has been shown to be important in the development of subgingival biofilms, particularly in synergy with other periodontal pathogens such as Treponema denticola and F. nucleatum (Metzger et al., 2001; Yamada et al., 2005). Streptococcus constitute the majority of primary colonizers of dental plaque (Kolenbrander, 2000), and as such their coaggregative ability is essential in maintaining community structure as plaque matures.
Heat treatment of oral and enteric isolates indicates whether one or both partners carry a proteaceous adhesin that mediates coaggregation. *Fusobacterium nucleatum*, one of the most prolific coaggregators in the mouth, appeared to carry the protein component in the majority of pairings used in this investigation. The effect of heating on this strain was most noticeable when studying the interaction with *A. naeslundii*, where a strong coaggregation was completely inhibited. Pairings that exhibited inhibition, when either partner was heated, may be mediated by a number of cell surface interactions where lectins are present on both strains.

In summary, this study has demonstrated that strong coaggregation interactions between gut isolates and between oral and gut bacteria are markedly less common than among species belonging to the oral microbiota, suggesting that its role in community development in the intestinal tract, and in interactions between oral and gut populations in vivo, may be less important. In addition, the results suggest that as with the coaggregation of oral bacteria, the majority of these coaggregations are mediated by lectin-carbohydrate interactions.

References


References
References


References


References


References


Sansone, P. J. (2011). To be or not to be a pathogen: that is the mucosally relevant question. *Mucosal Immunol* 4, 8-14.


