Interactions of Oral Bacteria with Host Tissues and Allochthonous Microorganisms

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By
RAJA M. MOMAN
B.Ph. (Hons), M.Phil.

Division of Pharmacy and Optometry
School of Health Sciences
University of Manchester
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<td>AMPs</td>
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<td>ZOI</td>
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The oral microbiome is a taxonomically diverse microbial community situated principally upon the hard and soft tissue surfaces of the mouth. It represents a readily accessible biofilm community for the investigation of bacteria-bacteria and bacteria-host interactions, which are responsible for some of the main features of oral biofilms in health and disease (colonisation resistance, antimicrobial tolerance, metabolic cross feeding, and other cooperative phenomena). In the oral cavity these relate specifically to cariogenesis and interactions with soft tissue that are responsible for periodontal disease. This doctoral thesis presents a series of investigations that consider processes for which growth in the biofilm phenotype or bacterial-bacteria or bacteria-host cell interaction are responsible. Four distinct methods were used to assess the effect of the biofilm phenotype on susceptibility of eight distinct oral hygiene actives with various modes of action. Bisphenol microbicid triclosan and the bis-biguanide chlorhexidine were most effective. All were markedly more effective against bacteria grown planktonically than the same organisms grown as biofilms illustrating antimicrobial tolerance, an important biofilm characteristic. In studies of interactions between oral isolates, bacteria previously isolated from the saliva and different oral sites of the oral cavity were tested using a modified cross streak method, in all possible pair-wise combinations. The frequency and strength of physical interactions (coaggregation) between these isolates was also assessed. The incidence of positive interactions was higher than the incidence of negative interactions (15.21% vs. 1.04%) and the incidence of coaggregation in bacteria isolated from saliva was significantly lower than for bacteria isolated from oral biofilms. Together, these data suggest that bacterial cooperation plays a greater role in oral biofilm development and maintenance than competition. With respect to putatively beneficial interactions between bacteria and host, the potential of the candidate dental probiotics *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* to protect host tissues from damage by three Gram negative periodontal pathogens were investigated using human oral cells culture and the (invertebrate) *G. mellonella* model system. All probiotics inhibited the growth of the test pathogens when applied simultaneously, and significantly decreased toxicity (*p*<0.05, in most mixtures). The rank order of cytopathic effect for the pathogens was *F. nucleatum* > *P. gingivalis* > *A. actinomycetemcomitans* in two distinct cell lines. Whilst all probiotics conferred protection against the periodontal pathogens, *L. rhamnosus* GG, had the greatest protective effect, regardless of probiotic or pathogen used, followed by *L. reuteri*. *S. salivarius* was the least effective. Prophylactic treatment with probiotics conferred greater protection than treatment concomitant with pathogen challenge. The data presented in this doctoral thesis demonstrate the functional significance of interactions between taxonomically distinct bacteria and between bacteria and host tissues. Such interactions may determine the outcome of exposure to antimicrobials and are, particularly significant in health and through further research, may be harnessed for prevention and treatment of oral disease.
Declaration

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Research contributions

Poster presentations

Society for Applied Microbiology Symposium (2017)

Moman, R., Ledder, R., O’Neill, C. and McBain, A. J.
Protection of oral epithelial cells from the effect of periodontal pathogens by three candidate probiotics.

Doctoral academy Ph.D. Conference (2017)

Moman, R., Ledder, R., O’Neill, C. and McBain, A. J.
Protection of oral epithelial cells from the effect of periodontal pathogens by three candidate probiotics.
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Dedication

I dedicate this thesis to my dear sister Elham for her love, encouragement and endless support.
CHAPTER 1

General Introduction
1.0 Introduction

1.1 Oral ecosystems

The oral ecosystem consists of oral microorganisms (the microbiota), oral cavity hard and soft tissues, and saliva. The survival of microorganisms is enhanced by their ability to attach to a surface and develop into a biofilm (Costerton, Lewandowski et al. 1995). This mode of growth provides bacteria with protection against exposure to physical and chemical hazards which in many cases would inactivate the same bacteria when growing in a dispersed, planktonic manner (Gilbert 1995). This is manifested in environments as diverse as domestic drains, implant-related infections and the oral cavity in health and disease (Bjarnsholt, Kirketerp-Moller et al. 2008, Bjarnsholt, Jensen et al. 2009, Zijnge, van Leeuwen et al. 2010). In the oral cavity, adhesion to surfaces is particularly important. Bacteria that do not adhere will be swallowed and therefore this property is likely to have been subject to strong selection pressure during the conjoint evolution of host and microbe.

A biofilm comprises microorganisms which are often but not always multi-species communities, enclosed in an intracellular matrix (EPS) which is composed of a variety of substances including polysaccharides, proteins and extracellular DNA (Costerton, Lewandowski et al. 1995, Mulcahy, Charron-Mazenod et al. 2008). The matrix supports bacteria and other microorganisms at the surface, providing adherence and profoundly influencing the physicochemical environment in a heterogeneous manner. That has relevance to bacterial physiology, facilitating metabolic collaboration (such as syntrophy) between organisms, signalling between cells, elevated resistance to antimicrobials and exchange of genetic material (Davies, Parsek et al. 1998, de Kievit 2009).
Biofilms in the oral cavity are complex communities, reportedly comprising more over 700 bacterial species or phylotypes (Paster, Boches et al. 2001, Aas, Paster et al. 2005) which are supported by the presence of many surfaces in the oral cavity. Those species inhabit the teeth, tongue, hard palate and oral mucosa et al.

In oral health and disease, the role of the oral microbiota often plays an important role, due to the presence of several hundred species as commensal or pathogenic microflora (Nyvad and Fejerskov 1987, Nyvad and Kilian 1990)

1.2 Oral habitats

The oral cavity contains hard and soft surfaces that have different physicochemical factors and maintain the growth of different microorganisms in the presence of saliva and gingival crevicular fluid. Whilst soft tissues in the oral cavity differ at different sites in the mouth, all are characterized by a continuous desquamation of surface epithelial cells, which can lead to the removal of adhering bacteria. In contrast the hard, non-shedding surfaces of the teeth provide stable surfaces for bacterial colonization (Marsh 2003). The surfaces of all oral tissues are bathed continuously by saliva and/or gingival crevicular fluid (GCF) providing hydration, nutrients, adherence factors and antimicrobial factors.

Saliva contains bacterial levels of up to $10^9$ cells/ml (Bowen 1996) and, according to Munson et al. (2004), rather than a niche hosting a characteristic microbiota, saliva is a reservoir for microorganisms derived from dental plaque (Munson, Banerjee et al. 2004). However, it seems most likely that saliva is a reservoir, where bacteria from surface-associated communities congregate, and a niche, in which these bacteria selectively proliferate.
Within the oral cavity, there are distinct microbial niches, due to the local environment provided by a particular site such as saliva, that has characteristic quantities of bacterial species due to its optimal pH that supports the growth of hard palate and gum bacteria in addition to its transportation of non-motile species to new colonization sites (Sakamoto, Umeda et al. 2000, Mager, Ximenez-Fyvie et al. 2003) or anaerobic milieu provided by periodontal pockets (Paster, Olsen et al. 2006). Additionally, oral tissues such as the buccal mucosa, tongue and gum have their own microflora (Kazor, Mitchell et al. 2003, Mager, Ximenez-Fyvie et al. 2003). According to Kolenbrander 2002 this diversity is due to the attachment, development and growth of new bacterial species that is controlled by primary colonizers (Kolenbrander, Andersen et al. 2002).

The oral microflora is of benefit to host health through for example, the competition between beneficial bacteria and virulent bacteria, a process termed colonisation resistance. This competition is complex and includes antagonistic interactions for primary colonization of tooth surfaces or later, for secondary colonizers. Such antagonism can be mediated by quorum sensing (Pirhonen, Flego et al. 1993, Parsek and Greenberg 2000), or a secretion of inhibitory substances such as bacteriocins (Tait and Sutherland 2002), hydrogen peroxide or fermentation acids, that selectively inhibit the growth of other bacteria (Kreth, Merritt et al. 2005, Kreth, Zhang et al. 2008). Moreover bacteria compete for nutrients and binding sites.
1.3 Oral diseases

Biofilms are the main cause of two of the main oral diseases, dental caries and periodontitis. Interactions between microorganisms in oral biofilm communities can effect bacterial virulence for which the interaction of bacteria with host tissues is essential (Socransky and Haffajee 2005).

Dental caries (tooth decay) is characterized by interactions that occur between oral microorganisms, products secreted by these bacteria, saliva and carbohydrates from diet on the surface of tooth leading to formation of dental plaque (biofilms) on susceptible tooth surfaces (Gibbons and Houte 1975, Selwitz, Ismail et al. 2007, Haffajee, Patel et al. 2008). Periodontal disease on the other hand, is an inflammatory disorder of tooth-supporting tissues induced by microbial infection, which in some cases leads to loss of supporting bone and ultimately, tooth loss, and had been described as a plaque-induced inflammatory disorder (Haffajee, Patel et al. 2008, Demmer and Papapanou 2010).

1.4 Biofilms

1.4.1 The incidence and architecture of biofilms

Biofilms form on essentially any surface where aqueous water can be found. With respect to the human body, tissues such as those found in the oral cavity are prime sites for colonisation (Kolenbrander and Palmer 2004) whilst the lungs of patients with cystic fibrosis (CF) are invariably colonised by bacteria and as time progresses, are normally infected with recalcitrant Pseudomonas aeruginosa populations, purportedly as biofilms (Bjarnsholt, et al. (2009). Chronic rhino-sinusitis (Sanderson, Leid et al. 2006) endocarditis (Hoiby, Doring et al. 1986), chronic
osteomyelitis (Gristina, Oga et al. 1985) and chronic wounds are also often complicated by the presence of biofilms (Bjarnsholt, Kirketerp-Moller et al. 2008, Kirketerp-Moller, Jensen et al. 2008). In terms of structure, biofilms may form mushroom-like or pyramid-like structures in their mature form and contain water channels and cavities for water sequestration and the exchange of nutrients and waste products which has encouraged some to liken them to primitive multicellular organisms (Dunne 2002).

Mature biofilms exhibit considerable tolerance to antimicrobials and additionally, biofilm growing bacteria are different from planktonic bacteria with respect to their phenotypic properties. This is due to their envelopment within the matrix including close proximity to each other, enhanced cell-cell communication and localised niches with a considerable reduced growth-rate which has been documented to limit the impact of conventional antimicrobials which are often most potent against actively metabolizing cells (Mah and O'Toole 2001).

The minimal bactericidal concentration (MBC) and minimal inhibitory concentration (MIC) of antimicrobials against bacteria growing in a biofilm are generally considerably higher than for planktonic bacteria (Anwar and Costerton 1990, Bjarnsholt, Kirketerp-Moller et al. 2007).

1.4.2 Antimicrobial resistance of biofilms

Many microorganisms have the natural tendency to attach to surfaces, to multiply and to implant themselves in a slimy matrix. This results in biofilms being able to settle in or adapt to unfavourable changes in environmental conditions with enhanced availability of nutrients and resistance to treatment with antimicrobial
agents and a reduced susceptibility to host immunological defences. These properties make biofilms important sources of bacterial resistance to antimicrobials and biofilms therefore represent an excellent example of recalcitrance physiological adaptation, rather than genetic change (Simoes 2011). New antimicrobials with enhanced anti-biofilm potency are needed to overcome the problem to help deal with biofilms but have to date, been slow to emerge. Several hypotheses have been proposed to explain the comparative reduction in the susceptibility towards antimicrobials associated with the progression from planktonic growth to sessile biofilm forms. These are as follows: i) the failure of the antimicrobial agent to penetrate the biofilm and the matrix. This is manifested in the resistance of *P. aeruginosa* biofilms to antimicrobials, which has been attributed to mucoidy (an EPS-rich mode of growth). Despite similar planktonic minimum inhibitory concentrations (MICs) the mucoid biofilms were up to 1000 times less susceptible to tobramycin than were non-mucoid biofilms (Ciofu, Mandsberg et al. 2012). Others have suggested that the matrix does not necessarily inhibit the penetration of antibiotics but may retard their penetration enough to provide a window of opportunity within which the expression of genes that mediate resistance may be induced (Jefferson, Goldmann et al. 2005). Moreover, extracellular DNA (eDNA) within the biofilm matrix has been implicated in the resistance of the biofilms and it is additionally proposed to confer physical stability on biofilm general structure, to act as a nutrient source (Mulcahy, Charron-Mazenod et al. 2010), to facilitate genetic information exchange (Molin and Tolker-Nielsen 2003) and to serve as an antimicrobial resistance factor (Tetz, Artemenko et al. 2009) through the activation of PhoP/Q and PmrA/B which are two-component regulatory systems involved in the expression of
antibiotic resistance genes in *P. aeruginosa* (Mulcahy, Charron-Mazenod et al. 2008). Another proposed mechanism for the relative recalcitrance of biofilms involves ii) slow growth within biofilms and the induction of stress response which may be biofilm-specific.

Starvation or limitation of a bacterial cell culture leading to nutrient stress can reduce specific growth-rates. This is evidenced for example, by increases in resistance to antimicrobials that accompany the transition from exponential to stationary growth phases in bacteria (Tuomanen, Cozens et al. 1986, Tuomanen, Durack et al. 1986, Brown, Allison et al. 1988, Poole 2012). Dao et al. 2011 reported that inactivation of the repression response which acts as a protective mechanism decreases the resistance of *P. aeruginosa* biofilms towards different classes of antibiotics and reported that this response is a determinant of a biofilm-specific antimicrobial resistance in *P. aeruginosa* (Dao, Joshi-Datar et al. 2011). The significance of growth rate has been studied by Gilbert and colleagues (Evans, Allison et al. 1990, Evans, Allison et al. 1991, Duguid, Evans et al. 1992, Duguid, Evans et al. 1992) under controlled growth circumstances for planktonic cultures and biofilms of *E. coli, S. epidermidis* and *P. aeruginosa*. It was observed that the sensitivity to ciprofloxacin or tobramycin increased with increasing growth rate in planktonic or biofilm mode of growth. For *P. aeruginosa* both planktonic cultures and biofilm cells were equally non-susceptible to ciprofloxacin at slow growth rate but when specific growth rates were increased by increasing the dilution rates of their chemostats, the cells became more susceptible than the biofilm cells which indicates that other biofilm properties have a role in the resistance towards antimicrobials not just the slow growth rate.
Another response which may contribute towards the reduced susceptibility of biofilms is the iii) SOS response, an inducible DNA repair system that enables bacteria to survive sudden increases in DNA damage (Michel 2005). In a comparison between mature *E. coli* biofilms and planktonic cells a significant up regulation of multiple SOS response genes was observed (Beloin, Valle et al. 2004) and this led to the observed resistance in *E. coli* biofilms through a molecular mechanism response which is still unclear (Bernier, Lebeaux et al. 2013). Physicochemical (nutrients, pH, anaerobiosis, etc.) and phenotypic heterogeneity within biofilms is often a product of gradients of nutrients and waste products which form resulting in sequential and spatial variation in cell density and gene expression. There is not a unified biofilm structure and thus a variety of models (both mathematical and laboratory-based) have been proposed for their representation. This lack of unification has delayed progress towards the understanding of biofilm formation mechanisms and has generated a huge mass of results, often conflicting, according the system used (Heydorn, Ersboll et al. 2002, Klausen, Heydorn et al. 2003) concerning quorum sensing (Davies, Parsek et al. 1998, Heydorn, Ersboll et al. 2002), stress response (Schembri, Kjaergaard et al. 2003) and the roles of flagella and motility (Prigent-Combaret, Prensier et al. 2000, Heydorn, Ersboll et al. 2002, Reisner, Haagensen et al. 2003).

Many studies have indicated that the effect of antimicrobials can be different and dependent upon the location of a particular cell within a biofilm community (Korber, James et al. 1994, Huang, Yu et al. 1995, Xu, McFeters et al. 2000). Specialised cells termed persisters are believed to be randomly formed dormant variants of regular cells in microbial populations characterized by high tolerance towards antibiotics.
Persisters were first reported seventy years ago by Joseph Bigger (1944) who noted that after the addition of penicillin to a *Staphylococcus aureus* culture it lysed and that the re-culturing of this clear liquid led to surviving colonies. These colonies grew into a new culture that once more lysed as a result of the addition of penicillin and another small new subpopulation was formed again.

Subpopulations of persister cells present in the biofilm have been proposed as being one of the defining properties of biofilm resistance and this has become an important paradigm in the understating to biofilm recalcitrance. Mulcahy et al. (2010) for example, observed that after treating cystic fibrosis-associated lung infections caused by *P. aeruginosa*, only persister cells survived, generating the reservoirs of viable cells that may re-grow, causing chronic infections (Mulcahy, Charron-Mazenod et al. 2010).

Persisters have been reported in candidiasis, caused by the fungus *C. albicans* (LaFleur, Qi et al. 2010). Another resistance factor that has been hypothesized to contribute to biofilm recalcitrance is efflux pumps which also play an important role in resistance towards antimicrobials in planktonic cells. Interestingly, it has been reported that specific efflux pumps are overexpressed in biofilms and that these are involved in biofilm-specific resistance (Zhang and Mah 2008). The type IV secretion system (T4SS) has been recently proposed to play a role in biofilm-specific antibiotic resistance (Abril, Brodard et al. 2010). T4SS are complexes (11-13 core proteins) spanning the cell envelope of Gram negative bacteria and are involved in the travel of DNA and/or protein from the donor cell cytoplasm to the recipient cell cytoplasm during fusion and have been implicated in diverse processes including biofilm formation, toxin delivery, virulence, inter-bacterial interaction and bacterial

There are three type IV secretion systems in *P. aeruginosa* at the loci HSI-I, HSI-II and HSI-III. It has been reported that HSI-I is responsible for the delivery of toxins to *P. aeruginosa* cells leading to the suppression of the growth of immunity lacking cells to these toxins and consequently gives the cells an important benefit for *in vivo* or *in vitro* growth (Hood, Singh et al. 2010). Zhang and colleagues demonstrated that the *tssC1* gene of HSI-I is over-expressed in biofilm growing cells which plays a significant role in the resistance of biofilms to a subset of antibiotics and showed that a *tssC1* mutation leads to a high decrease in antibiotic resistance with no effect on the formation of the biofilm (Zhang, Hinz et al. 2011). Up-regulation of the general stress response (GSR) results in the protection of the bacterial cells from physical and chemical stresses via a change in the cell physiology mediated by the expression of σ-factor, RpoS. This is induced by high cell density and evidence suggests that cells growing in a biofilm have undergone the GSR (Adams and McLean 1999, Liu, Ng et al. 2000). This is the basis of the GSR hypothesis, which has been evidenced experimentally by Prosser et al. (1987) who established that antibiotic resistance is associated with slow growth and nutrient depletion (Prosser, Taylor et al. 1987). Quorum sensing (QS), plays an important role in bacterial cell communication through the release of a diffusible signal or “autoinducer” molecules which accumulate dependent on bacterial population cell density, leading to alterations in gene expression (Bassler and Losick 2006).

Quorum sensing is believed to participate in cell attachment, detachment and resistance to antimicrobials (Burmolle, Webb et al. 2006); Cells growing within a
biofilm may communicate thus facilitating mutually beneficial cooperative behaviour (Remis, Costerton et al. 2010, van der Veen and Abee 2011).

In conclusion, it appears that no single mechanism can account for the totality of antimicrobial tolerance associated with biofilms; rather it seems likely that several mechanisms acting on concert are responsible and, since survival is strongly evolved in all organisms, the redundancy associated with the action of multiple mechanisms makes biological sense.

1.5 Biofilm control strategies

There are many strategies used and under research both for the control and eradication of biofilms. For example, some work has focussed on the attachment of the primary colonizers on the available surfaces since it is the essential step in the formation of the biofilm (Kolenbrander, Andersen et al. 1990). Others work on the inhibition of structure, development and differentiation of biofilms whilst the use of antimicrobials and dispersion of the biofilm community has also received considerable research attention (Singh, Parsek et al. 2002, Junker and Clardy 2007).

1.5.1 Strategies that reduce microbial attachment

An essential early step in biofilm formation is the microbial attachment to a surface. Recently techniques have been developed to reduce microbial attachment (Xiong and Liu 2010), including biochemical, physicochemical and biological strategies.

The biochemical approach used two different types of biocidal agents; microbicidal and non-microbicidal agents to block microbial attachment. The methods used to create antimicrobial surfaces are varied and include non-covalent binding, covalent immobilization and polymer matrix loading of antimicrobial agents.
For example, the use of antimicrobial peptides which showed effective broad spectrum antimicrobial activity although considered short-term (Kazemzadeh-Narbat, Kindrachuk et al. 2010). Novel techniques used to produce improved long-term coating of antimicrobials were described that successfully reduced the bacterial colonization and biofilm formation (Schiffman and Elimelech 2011). Among the non-microbicidal agents used were 12-mer peptide (RQERSSLSKPVV) which inhibited the adhesion of piliated Salmonella typhi with human monocytes and prevented in vivo biofilm formation (Wu, Zhang et al. 2005).

From a physiochemical point of view, some work has been done to develop modified surfaces with anti-adhesive properties such as electro-polished stainless steel (Arnold and Bailey 2000) and other trials based on peptide coating technology (Khoo, Hamilton et al. 2009) and all showed a significant decrease in microbial attachment and sessile community formation. Moreover, some researchers combined the anti-adhesive properties and microbicidal properties to design new surfaces and the strategy was reported to be effective (Yuan, Wan et al. 2011). On the other hand there is a highly evolved biological phenomenon in which active chemicals are secreted by bacterial species and inhibit others. For example, biosurfactants are synthesized by some species and inhibit attachment by their competitors. Some probiotic bacteria can secrete biosurfactants and are therefore considered to be potential biofilm control agents (Falagas and Makris 2009, Luna, Rufino et al. 2011).
1.5.2 Inhibition of growth and differentiation

To interrupt the structure, development and differentiation of a biofilm there are many aspects to be considered. Firstly the age of the biofilm is important since newly established communities are generally more susceptible towards antimicrobials than mature ones. The amount of EPS present also has a great effect on the diffusion of antimicrobials. Finally, the induction of quorum sensing in mature biofilms must be considered since this can contribute to antimicrobial susceptibility (Anderl, Franklin et al. 2000, Ito, Taniuchi et al. 2009). The role of EPS in biofilm resistance is significant, this is reflected in the large amount of studies that have been carried out attempting to chemically or enzymatically disrupt the structure and proved to be effective in affecting the biofilm structure and development (Xavier, Picioreanu et al. 2005, Longhi, Scoarughi et al. 2008, Yang, Liu et al. 2012).

Further studies dealt with metabolic interventions and showed reduction in biofilm formation (Wu, Lee et al. 2011, Wu, Wang et al. 2011) and others studied the intervention with quorum sensing which is involved in biofilm architecture and resistance by the use of quorum sensing inhibitors that were found to act by binding to the quorum sensing receptor proteins by displacing the cognate auto-inducers (Glansdorp, Thomas et al. 2004, Rasmussen, Bjarnsholt et al. 2005).

Recently many researchers recognized and identified some quorum-quenching enzymes that act by degradation of quorum sensing signal molecules. Ng et al. (2011) applied these enzymes as anti-biofilm agents after they had been tested on surfaces by other researchers and proved to reduce biofouling by bacteria in water treatment reactors (Yeon, Cheong et al. 2009, Ng, Wright et al. 2011).
1.5.3 Dispersal of extant biofilms

It is well known that some changes in environmental conditions may promote biofilm dispersion; for instance, a change in the concentration of oxygen or carbon or starvation has been shown to activate biofilm dispersion (Sauer, Cullen et al. 2004, Roy, Petrova et al. 2012).

Additionally, different genetic regulators have been found that are involved in both biofilm construction and the increased dispersion of microorganisms (Thormann, Saville et al. 2005, Gjermansen, Nilsson et al. 2010). Moreover, the investigation of these molecules has led to the identification of dispersion induction which significantly improved the anti-biofilm effects of other antimicrobials (Barraud, Hassett et al. 2006, Wu, Lee et al. 2011).

1.5.4 Eradication by antimicrobials

Attempts to eradicate biofilms using chemical treatment are often unsuccessful due to the diversity and biological redundancy in biofilm resistance mechanisms (Liu, Knapp et al. 2013). Recently new strategies have been assessed, among which is the use of agents which improve antimicrobial penetration. Several studies deal with the penetration ability of antimicrobials inside biofilms (Colvin, Gordon et al. 2011, Tseng, Zhang et al. 2013). The presence of dense microcolonies surrounded by the EPS matrix together with the efflux pump system has a significant effect on the activity of antimicrobials (Kvist, Hancock et al. 2008, Koo, Falsetta et al. 2013). Studies showed that co-administration of the enzymes alginate lyase and DNase with different antibiotics significantly increased the activity of gentamicin, tobramycin and amikacin and reduced biofilm growth and recovery of Pseudomonas aeruginosa.
in cystic fibrosis samples (Alkawash, Soothill et al. 2006, Alipour, Suntres et al. 2009). Another study used a lipopeptide biosurfactant (V9T14 biosurfactant) in combination with different antibiotics namely, ampicillin, tobramycin, ciprofloxacin, trimethoprim/sulfamethoxazole, cefazolin, ceftriaxone and piperacillin. This work showed that there was a synergistic increase in the activity of antibiotics towards *E. coli* biofilms in addition to biofilms total eradication in some instances (Rivardo, Martinotti et al. 2011).

Attempts to enhance antimicrobial penetration using micelle-encapsulated antibiotics and antibiotic-encapsulated biodegradable polymeric nanoparticles were described as very effective (Jones 2005, Cheow, Chang et al. 2010).

In addition, Liu et al. (2010) reported that the use of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) together with iron chelators inhibited *P. aeruginosa* growth and biofilm formation and suggested that these combinations are promising in enhancement of antimicrobial activity against biofilms (Liu, Yang et al. 2010). Another line of research used ‘phage as substitutes for antibiotics in the eradication of biofilms and demonstrated an improvement in the antimicrobial treatment of biofilm related infections (Carson, Gorman et al. 2010, Verma, Harjai et al. 2010).

1.6 Oral microbiota

1.6.1 Dental plaque

Dental plaque is a biofilm that firmly adheres to the acquired pellicle coating the tooth surface which allows bacteria to resist the mechanical shearing forces such as
salivary fluid flow (Russell and Mestecky 1986, Lee, Progulske-Fox et al. 1989, Bowen, Schilling et al. 1991). Bacterial adherence to this surface is the determining factor of bacterial colonization. There are several mechanisms for bacterial-host adhesion including general mechanism of microbial host interaction involving physicochemical factors such as hydrogen bonding, Van der Waals bonding, hydrophobic (Rosenberg and Kjelleberg 1986, Van Oss, Good et al. 1986) and ionic forces (Olson and Salop 1976). An additional mechanism involves interactions between bacterial adhesins and host components (Jones and Isaacson 1982). Lastly, highly specific interactions are mediated by bacterial surface proteins and receptors (protein or carbohydrate) on the host surface (Gibbons and van Houte 1980, Scannapieco 1994, Jenkinson and Demuth 1997). Following initial bacterial adhesion; coadhesion (interaction between one bacterium in solution to surface adherent bacterium) and/or coaggregation (interaction between two species in solution) leads in addition to bacterial growth and multiplication to plaque maturation (Scheie 1994, Filoche, Anderson et al. 2004). Commensal species such as streptococci and actinomycetes are early colonizers of dental plaque (Socransky, Manganiello et al. 1977, Nyvad and Fejerskov 1987, Li, Helmerhorst et al. 2004). Afterwards more actinomycetes and related organisms arrive and finally Gram-negative and spirochaetes colonize (Rickard, Gilbert et al. 2003, Periasamy and Kolenbrander 2009). These bacteria are surrounded by a matrix composed of the host and bacteria derived products (Beighton, Smith et al. 1986, Bradshaw, Homer et al. 1994, Marsh and Bowden 2000).
1.6.2 Calculus

Dental calculus is solidified dental plaque (Hillson 1986, Mandel 1990, Hillson 1996). It is composed of mineral salts of calcium phosphate between and within fragments of non-viable microorganisms covered by viable bacteria (Mandel 1990) formed either at the supragingival (Friskopp 1983) or subgingival under the gingival margin (Friskopp 1983, Hillson 1996). However, calculus is not a main cause of oral disease but the viable bacteria on the surface provoke the oral disease (Mandel 1986, White 1997).

1.6.3 Host tissue microbiota

All the epithelium covering buccal soft tissues is colonized to some extent with microbes. This sheds regularly due to epithelia desquamation. The invasion of epithelial cells may allow some bacteria including periodontal pathogens such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans to be sheltered and protected from host defence factors (Lamont, Chan et al. 1995, Rudney, Chen et al. 2001). Additionally, the tongue may provide protection to attached bacteria and aid in its colonization due to the presence of papillae on its surface (Gibbons, Spinell et al. 1976). Interestingly, it has been reported that the dorsum of the tongue was colonized by a large number of bacteria more than any other site in the oral cavity with exception to the teeth and gingival sulcus (Asikainen, Alaluusua et al. 1991, Avila, Ojius et al. 2009).

1.7 Prophylaxis and control of dental infections by antimicrobials

Antimicrobials used in the mouth are normally incorporated into oral care products (toothpastes and mouth washes), many are broad spectrum. The delivery
of these products leads to an immediate high concentration of the antimicrobial on oral surfaces but in a short time it is lost due to salivary flow and swallowing (Vanderoudera and Cummins 1989). On the other hand saliva can prolong periods of potential antimicrobial activity by transportation of released agents between different oral sites. Bradshaw et al., 1993 stated that ‘these agents can suppress potentially pathogenic organisms, especially Gram-negative anaerobes, that are more sensitive to these agents on short exposure, while not affecting some of the Gram-positive species associated with oral health’ (Bradshaw, Marsh et al. 1993) and this is the most important characteristic of a potential oral health product. There are many antimicrobials with different modes of action used as an adjunct to physical plaque removal and treatment of oral diseases (dental caries and periodontitis) amongst these the products used in this study.

1.7.1 Fluoride

Teeth cleaning agents have been used since ancient times in the form of powders, creams and pastes (Lippert, Hara et al. 2013). In the second half of the 20th century, fluoride compounds were added to toothpastes as anti-caries and cleaning agents. Fluoride compounds added to toothpastes including sodium fluoride, stannous fluoride, sodium mono fluorophosphates and amine fluoride (Paraskevas 2005, Lippert, Hara et al. 2013). The anti-caries properties of fluoride are attributed to the effects on demineralization/remineralisation at the interface between tooth and oral fluids since it is present in the aqueous phase in the matrix of the enamel (Cate 1999).

It has been proposed in several systemic reviews that antimicrobial properties vary between different fluoride compounds and some are superior among others.
(Twetman, Axelsson et al. 2003, Twetman 2009, Wright, Hanson et al. 2014). For example, stannous fluoride is more effective than sodium fluoride because it binds to the negatively charged plaque components and inhibits coadhesion and coaggregation. Additionally, stannous fluoride inhibits bacterial metabolism because it interferes with bacterial acid production. This occurs via the inhibition of metabolic enzymes which disrupts proton gradients across bacterial membranes and therefore makes bacteria more susceptible to a low pH (Skjörland, Gjermo et al. 1978, Buzalaf, Pessan et al. 2011). Moreover, toothpaste containing amine fluoride and stannous fluoride has very effective cariostatic properties and reduces both plaque and the incidence of gingivitis (Brecx, Brownsfone et al. 1992, Mengel, Wissing et al. 1996, Klimek, Ganss et al. 1998, Warrick, Miller et al. 1999).

Three important factors have to be considered for an efficient anti-caries effect when using fluoride toothpastes. Specifically, concentration (<600 ppm provides less protection than 1000 ppm or a higher concentration), regularity of brushing (at least twice daily) and rinsing post brushing to minimize fluorosis risk (Davies, Ellwood et al. 2003).

1.7.2 Triclosan

Triclosan containing toothpastes have been demonstrated to inhibit plaque and gingival inflammation (Jackson 1997). Triclosan is a bisphenol broad spectrum antibacterial and is one of the most common antibacterial compounds used in dentifrice formulations (McBain, Bartolo et al. 2003). It acts by disrupting bacterial cell membranes (Russell 2004) and blocking fatty acids synthesis at the envoy-acyl carrier protein reductase (FabI) step (McMurry, Oethinger et al. 1998, Russell 2004).
It is a highly effective antibacterial agent with low MICs values ~0.01-0.1 mg/L, bacteriostatic at low concentrations and bactericidal at higher levels (Suller and Russell 1999, Suller and Russell 2000). It is effective against *Escherichia coli*, staphylococci, some streptococci and *Proteus* spp., while *Pseudomonas aeruginosa* is highly resistant (Schweizer 2001). It was found that triclosan is retained in the oral cavity after dosing from toothpaste for 8h in bacterial plaque and 3h in oral mucosa and this presence may be responsible for its anti-plaque efficacy (Gilbert and Williams 1987). In addition anti-caries effects and effects on *Streptococcus mutans* have been attributed to its inhibition of glycolysis in dental biofilms by inhibiting a number of enzymes (Phan and Marquis 2006). The presence of other additives with triclosan in oral hygiene products such as co-polymer or zinc salts enhances its activity (Moran, Addy et al. 2001). In addition it has been reported that triclosan possess anti-inflammatory properties in the triclosan/fluoride/copolymer delivery system (Panagakos, Volpe et al. 2004). Furthermore, triclosan can inhibit IL-1β induced prostaglandin E₂ production by human gingival fibroblasts at relatively low concentrations (Gaffar, Scherl et al. 1995, Paraskevas 2005). Triclosan could maintain a low level of plaque consistent with gingival health (Xiong and Liu 2010).

### 1.7.3 Zinc salts

Zinc salts are inorganic antibacterial agents that possess antiplaque activity (Budtz-Jorgensen and Bertram 1972, Fischman, Picozzi et al. 1973). Zinc citrate and zinc chloride are frequently incorporated into toothpastes (Vlock 1990) and there are many reports regarding the plaque inhibitory effect of zinc ions. Various mechanisms of action have been proposed including the displacement of calcium ions from the pellicle and bacterial surfaces by binding of zinc ions into plaque mass (Rolla 1976, Shah 1982, Lynch 2011), interference with glucan formation and plaque formation by
inhibiting the enzyme glycosyl transferase (Scheie and Kjeilen 1987), alteration of the surface charge on the bacteria and hence adherence to surfaces, interference with bacterial protein synthesis (Jones, Stephen et al. 1988) and Inhibition of protease-induced bacterial adhesion (Li and Ellen 1990). Moreover, zinc may be retained by dental plaque after brushing with 0.5% with zinc citrate toothpaste and inhibits regrowth without disrupting the oral ecology (Gilbert and Williams 1987, Ingram, Horay et al. 1992).

1.7.4 Chlorhexidine

Chlorhexidine, is a cationic bis-biguanide antimicrobial agent and was introduced as an antiseptic in 1954 (Davies, Francis et al. 1954). In 1969 it was suggested by Schroeder as an effective anti-plaque and anti-calculus agent (Schroeder and Shanley 1969). Subsequently it has been extensively evaluated for plaque and gingivitis control (Fardai and Turnbull 1986, Mandel 1988, Gjermo 1989). Chlorhexidine is a broad spectrum antimicrobial agent which acts by disrupting bacterial cell membranes (Hugo and Longworth 1966). It does not penetrate the oral epithelium and its mode of action as an antiplaque agent is through a topical effect since it can bind to the teeth and mucosal surfaces (Lindhe, Heyden et al. 1970, Bonesvoll and Gjermo 1978). Another study demonstrated that chlorhexidine could inhibit the development of gingivitis and dental caries, even with a sucrose challenge. The individuals involved in the study rinsed 9 times daily 50% of sucrose and it was noted that the test group subjects rinsed daily with chlorhexidine in this study did not had any existence of plaques and the same applied to caries index (LÖE, FEHR et al. 1972).
Furthermore, it has been shown that effective control of gingivitis in children can be achieved using 0.1% or 0.2% chlorhexidine mouth rinses as an adjunct to daily tooth brushing routine (Lang, Hotz et al. 1982). Similarly, 0.12% chlorhexidine gluconate can provide the same effect on prevention and control of gingivitis in adults (Grossman, Reiter et al. 1986). In another long term study on the effect of chlorhexidine on bacterial populations after prolonged exposure for 2 years and 7 months it was established that there was a 30-50% reduction in the number of bacteria in the saliva detected after treatment with chlorhexidine without producing an obvious shift in the population. However, the number of *Streptococcus mutans* isolated from the saliva of some subjects in the study decreased during treatment and no change in the number of large Gram negative rods was observed (Rindom-Schiöstt, Briner et al. 1976).

1.7.5 Natural products

Based upon the belief that natural products may contain undiscovered antimicrobials they have been used to treat dental diseases since antiquity and across world cultures (Guerini 1909). There are many anti-caries agents that have been discovered from plants and food. Plant metabolites include microbial growth inhibition compounds including phenolic acids, anthraquinones, flavonoids, tannins, terpenoids and alkaloids. Mostly act by affecting the bacterial cell wall or bacterial metabolism. Essential oils are among natural products used to treat oral disease and incorporated in many mouth rinses due to their effect on pathogens and as a alternates due spread of drug resistance (Prabuseenivasan, Jayakumar et al. 2006).
Essential oils are aromatic oily liquids extracted from plants, their antimicrobial effect is generally attributed to terpenoids and phenolic compounds (Oussalah, Caillet et al. 2006). It has been reported that essential oils are effective as antiseptic solutions against oral pathogens (Thosar, Basak et al. 2013, de Camargo Smolarek, Esmerino et al. 2015).

1.8 Periodontopathogens

Periodontopathogens adhere to the gingivae via adhesins. Receptors for these bacteria are expressed on the surfaces of the host; they allow these bacteria to invade and replicate within human epithelial cells which can lead to progressive periodontal disease. This can ultimately result in the destruction of tooth supporting tissue (Williams 1990).

Currently, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum and Porphyromonas gingivalis are considered to be the predominant contributors to human periodontitis (Slots, Bragd et al. 1986, Dzink, Socransky et al. 1988, Duncan, Nakao et al. 1993, Sandros, Papapanou et al. 1994).

1.8.1 Aggregatibacter actinomycetemcomitans

A. actinomycetemcomitans is a capnophilic Gram negative coccobacillus that colonizes the human oral cavity. It requires 5-10% CO₂ for growth. Surface layers of cells have molecules that stimulate bone resorption in addition to serotype-determining polysaccharides (Fine, Kaplan et al. 2006). A. actinomycetemcomitans produces a range of virulence factors such as leukotoxin (Ltx1) which is a membrane active toxin that targets leukocytes and helps the bacterium evade the host immune response during infection. A. actinomycetemcomitans also produces collagenase,
protease and immunosuppressive factors (Herminajeng, Asmara et al. 2001, Gallant, Sedic et al. 2008, Guentsch, Puklo et al. 2009). Furthermore, it is invasive to epithelial cells and an opportunistic pathogen associated with aggressive periodontitis (Fine, Markowitz et al. 2007, Haubek, Ennibi et al. 2008).

1.8.2 Fusobacterium nucleatum

*Fusobacterium nucleatum* is a Gram negative anaerobe but can grow in the presence of up to 6% oxygen. It is a dominant species in the oral cavity and found naturally in the oral microflora in health and disease (Kolenbrander 2000). Higher serum antibody titres have been reported in patients with periodontitis than in patients with gingivitis or healthy individuals (Tolo and Schenck 1985, Vincent, Cornett et al. 1985, Danielsen, Wilton et al. 1993). Its pathogenic capability is attributed to its mucopolysaccharide capsule (Brook and Walker 1986, Brook 1994). *F. nucleatum* adheres to host tissue by a specific interaction mediated by macromolecules on the bacterial surface complemented with certain receptors on the host tissue surface (London 1991). Additionally it participates in multi generic coaggregation in the oral cavity which is related to oral diseases (Kolenbrander and London 1993).

1.8.3 Porphyromonas gingivalis

*Porphyromonas gingivalis* is a Gram negative strict anaerobe. It has been associated with the development and progression of chronic periodontitis (Lamont and Jenkinson 1998). *P. gingivalis* produces several potential virulence factors (Fim A and gingipains) involved in tissue colonization and destruction and immunity perturbation (Kadowaki, Nakayama et al. 2000). *P. gingivalis* has the ability to invade various cell lines such as epithelial cells, endothelial cells and fibroblasts (Lamont, Chan et al. 1995, Nakagawa, Amano et al. 2002). Adhesion to host cells involves
fimbriae, lipopolysaccharides (LPS), proteases and hemagglutinins (Cutler, Kalmar et al. 1995). The ability to adhere and to invade epithelial cells in the initial stages of infection is essential in the pathogenesis of periodontitis.

1.9 in vitro models of bacterial biofilms

A variety of models have been developed in order to study sessile communities ranging from simple in vitro (Guggenheim, Giertsen et al. 2001, Coenye and Nelis 2010) to complex in vivo models (Andes, Nett et al. 2004) of device-related infections or tissues. These models have been developed to better understand the specific properties of biofilms (Dalton, Dowd et al. 2011), to evaluate novel antimicrobial treatments and to elucidate the relationship between biofilms and human infections (Lebeaux, Chauhan et al. 2013). In vitro biofilm methods can be classified to three groups:

1.9.1 Static models

These are closed systems with limited nutrients and aeration which enables direct rapid quantification of biofilm mass. These include colony biofilm models and microtiter plates (Christensen, Simpson et al. 1985, Stepanović, Vuković et al. 2007). In this study the Calgary biofilm device was used to study the minimum biofilm eradication concentration (MBEC) of some antimicrobials used in oral hygiene products and a colony biofilm model was used to grow biofilms on polycarbonate membrane filters (Anderl, Franklin et al. 2000). Simple to prepare, preserve and study. Most importantly they allow for short-term exposure of the tested biofilm especially dental plaque to selected antimicrobials. Other advantages of these systems include working with small volumes of media which may be constituted from natural substrates such as saliva. Another examples of these models include the use
of glass surfaces (LeChevallier, Cawthon et al. 1988, Li and Bowden 1994), and hydroxyapatite discs (Guggenheim, Gierts et al. 2001).

1.9.2 Dynamic models
Dynamic models are open systems similar to continuous cultures. These models have been used extensively to study physical and chemical resistance of biofilms (Lebeaux, Chauhan et al. 2013). Many dental plaque multispecies dynamic models have been described and applied; consisting of flow cells (Sjollema, Busscher et al. 1989) and chemostats with modifications allowing insertion and removal of colonisable surfaces (Bradshaw, Marsh et al. 1996, Bowden 1999). These devices have helped in the understanding of many biofilm formation aspects including adhesion, growth and development; however their use has some disadvantages such as the difficulty in their use over a long period of time.

1.9.3 Microcosms
Microcosms are more sophisticated models that aim to closely mimic in situ conditions (Rudney, Chen et al. 2012).

1.10 Models of bacterial infection
1.10.1 In vitro model of bacterial infection
Infection is complex and dynamic and it is a continuous relationship between the host and microorganism and between microorganisms themselves. In vitro infection models are often used to study these interactions (Lebeaux, Chauhan et al. 2013). In this study two human oral cells namely, primary human oral keratinocytes and non-tumour-derived immortalized human oral epithelial cells were used. As in vitro models offer simplified vision of the environment, the results achieved could be used as a beginning step to test hypotheses, after which there is a necessity to
validate these results using in vivo model such as higher organisms or clinical settings.

1.10.2 Non-mammalian in vivo models

Non mammalian in vivo models have been previously used to study bacterial infection to overcome ethical, handling, housing and cost related issues (Page and Schroeder 1982, Giannobile, Finkelman et al. 1994). Non mammalian models have been demonstrated to be very effective in our understanding of infections and virulence mechanisms for the infection (O’Callaghan and Vergunst 2010). Hosts such as Acathamoeba castellani, Drosophila melanogaster and Galleria mellonella have been used previously. G. mellonella was demonstrated that its immune system display homology with mammals innate immune response (Fuchs and Mylonakis 2006, Mesa-Arango, Forastiero et al. 2013).

1.11 Aims and Objectives

Bacterial-bacterial interactions and bacterial-host tissue interactions are thought to have a significant impact on the colonization of microorganisms in the oral cavity. Consequently, the understanding of dental plaque formation may also have more general implications as a model of oral diseases (Gibbons 1989). This doctoral project seeks to investigate the various aspects of bacteria-bacteria and bacteria-host interactions in order to better understand the implications of such events, and the potential for their manipulation using antimicrobials and probiotic bacteria.
CHAPTER 2

General experimental methods
2.0 Materials and Methods

2.1 Chemicals and bacteriological growth media

Unless otherwise stated all chemicals used in this study were of the highest grade available 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.

2.2 Sterilisation of equipments, solutions and growth media

Growth media, heat stable solutions, heat stable plastic consumables and glassware were sterilised in an autoclave at 121°C for 15 min (1kg/cm²) (Dixons, Vario, Essex, UK). Some solutions sterilized by heating and heat-labile solutions were filter-sterilised using 0.22 µl nitrocellulose syringe filters (Millipore, Watford, UK).

2.3 Bacterial strains used in the course of this study

Acenitobacter sp. IK103 (SBb1), Aceinetobacter Iwoffii (GBc3), Enterococcus faecalis E (GBb8), Enterococcus faecium L703 (LBF2) A04 (GBb8*), Enterococcus sp. 34-2 (SBb10), Lactobacillus fermentum A011 (LBA1), Lactobacillus fermentum SM38 (KJ690753), Lactobacillus fermentum FQ022 (LBB2), Lactobacillus fermentum KDLLL2-1 (GBc1), Lactobacillus oris F0423 (LBA10), Lactobacillus sp. oral clone CX036 (LBC8), Paenibacillus motobuensis (GBA6), Staphylococcus aureus Z1588 (NBC2), Staphylococcus aureus (NBC3), Staphylococcus aureus 1CCIP2_Nondiabetic (GBc3), Staphylococcus aureus DF8TA (GBA5), Staphylococcus epidermidis P8 (GBc9), Streptococcus sp. ACC21 (SBA1), Streptococcus anginosus SA1 (GBb1), Streptococcus salivarius 7073 (ABB4),
Streptococcus salivarius 7YE (Aba5), Streptococcus salivarius SAM3 (NBc1), Streptococcus sp. SR5 (Aba2), Streptococcus sp. ChDC B363 (NBA1), Streptococcus salivarius Gt2 (GBc10), Streptococcus sp. ChDCB266 (GBb4), uncultured bacterium clone nbw 1070h04c1 (GBb2), some have closest match as Uncultured bacterium clone070058_178 (Aba10), uncultured bacilli bacterium clone MS064A-F08 (NBA2), uncultured bacterium clone sp2-lib12-3H (GBb2) and uncultured bacterium clone ncd 1405g02c1 (GBb2) were all isolated from healthy human saliva; Enterobacter ludwignii (Tga12), Enterobacter sp. KK1 (Tga32), Streptococcus salivarius M_Sw_oHS_10/11_8_2 (1) (Tga22) and Uncultured streptococcus species clone SC004B32 (Tgn32) were isolated from the surface of tongue; Enterobacter gergoviae TyB1 (Gma22), Pseudomonas putida NCB0308-456 (AB294558), Streptococcus sp. ChDC B364 (Gmn12”) and Streptococcus mutans P11 (Gmn12) were isolated from gum; Enterobacter mori CB2B2 (Tha12), Enterobacter sp. Cd20b (Tha21) and Staphylococcus sp. O-10 (Thn21) were isolated from supra-gingival plaques from surface of teeth; Enterobacter sp. CF-S19(Upa32) and Streptococcus sp. SR5 (Upn12) were isolated from upper palate; Micrococcus luteus OS-139 (Cka12) and Streptococcus sp. JCM 5703 (Ckn12) were isolated from cheeks inner mucosa. E. coli k12 C600 (MBRG), Staphylococcus aureus ATCC 6538 (MBRG), Staphylococcus epidermidis ATCC 14990 (MBRG), Streptococcus mutans NCTC 10832 and Lactobacillus rhamnosus Goldin and Gorbach (L. rhamnosus GG, ATCC 53103) (MBRG) were provided kindly by Professor Andrew McBain, The University of Manchester; Fusobacterium nucleatum ATCC 10953 and Porphyromonas gingivalis ATCC 33277 were obtained from Culti-Loops™, (Thermo Fisher Scientific, UK) Aggregatibacter actinomycetemcomitans ATCC 33384 obtained from (NCTC, Public Health England, Salisbury, UK);
Lactobacillus reuteri ATCC 55730 and Streptococcus salivarius K-12 provided kindly by Dr. Gavin Humphreys, The University of Manchester.

2.4 Bacterial culture maintenance

Strains were preserved at -80°C (Glacier NU-9668 Upright Large Capacity -86°C Ultra Low Freezer, NUaire, Japan) in freezer protect vials consisting of porous ceramic beads suspended in a cryopreservative (Technical Service Consultants™, Fisher Scientific UK LTD). Organisms were recovered on agar and broth on a range of culture media as follows: Thioglycolate broth (TGB), Nutrient agar and broth (NA &NB) for aerobic bacteria incubated aerobically at 37°C, Wilkins Chalgren agar (WC) (incubated aerobically or anaerobically) for total aerobes and total anaerobes respectively, Wilkins Chalgren agar with Gram-negative (GN) supplement [containing (mgL-1); 5.0, haem in; 0.5, menadione; 10, nalidixic acid; 10, vancomycin; and 2.5, sodium succinate (incubated anaerobically)] for total Gram negative anaerobes, and Rogosa agar (RA) for total lactobacilli. For cultivation of anaerobes, agar plates were pre-reduced in an anaerobic chamber for 24h prior to use. After plating, media were transferred immediately to MG-1000-anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) (Gas mix 80% N₂, 10% CO₂ and 10% H₂) and incubated at 37°C from 1-5 days, aerobic plates were incubated in a bench-top incubator (Memmert incubator, Schwabach, Germany) at 37°C for up to 3 d after which bacteria were preserved at -80°C. Trypticase yeast extract cysteine sucrose agar (TYCS) (Van Palenstein Helderman, Ijsseldijk et al. 1983; Schaeken, Vanderhoeven et al. 1986) [containing (mgL-1); yeast extract; 5.0, casein hydrolysate; 15, L-cysteine; 0.2, sodium hydrogen carbonate; 2.0, di-sodium hydrogen orthophosphate anhydrous; 2.0, sodium sulfite; 0.1, sodium chloride; 1.0, sodium acetate; 20, sucrose; 50 and
agar-agar; 15 (incubated aerobically and anaerobically)] was used for cultivation of *S. mutans*; incubated at 37°C MG-1000-anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) (Gas mix 80% N₂, 10% CO₂ and 10% H₂) and Tryptic soya agar and broth supplemented with 0.6% yeast extract (TSA & TSB) was used for *A. actinomycetemcomitans* and incubated in 5% CO₂ atmosphere at 37°C. Strains identity checked regularly by Gram staining, morphology of colonies and biochemical testing.

2.5 Collection of samples

2.5.1 Different oral sites samples

Oral bacteria isolates for use in subsequent analysis were obtained from a healthy subject who had received no recent antibiotic therapy or invasive dental treatment for at least one year before collecting the samples. Before sampling, the oral cavity was flushed with drinking water. The following five sites were analysed: dorsum of the tongue, hard palate, cheeks inner mucosa, gum, and supra-gingival plaques from teeth surfaces. Microbiological samples were collected with sterile swabs and plated on different culture media plates as described above.

2.5.2 Saliva samples

Saliva samples used in this study were obtained from a healthy donor with no extant periodontal disease who had not recently used antibiotics before donating saliva. Unstimulated saliva was collected into a sterile Universal bottle. The time between sampling and processing was less than 30 min. immediately after collection, the samples were centrifuged at 3220 xg for 5 min at room temperature using Universal 320 centrifuge (Andreas Hettich GmbH, Germany) to remove particulate matter. Aliquots were stored in Eppendorf tubes at -80 °C for subsequent
analysis. The average pH of all samples before processing and analysis was 6.90 ± 0.31 (normal range 6.5 - 7.5). For ethics statement, Advice was taken from the Chair of the University of Manchester Research Ethics Committee regarding the correct procedures associated with the use of human saliva samples. The committee granted exemption from formal ethics approval due to the nature of the work.

2.5.3 **Isolation and enumeration of functional bacterial groups of saliva and oral samples**

For bacteriological enumeration, samples of human saliva and human different buccal sites were homogenised by vortexing for 1 min. Samples were then serially diluted in sterile pre-reduced, half strength thioglycolate medium (USP). Thioglycolate broth was used to protect anaerobic species from oxygen exposure. To differentially isolate and enumerate various functional groups of oral bacteria, appropriate dilutions (100μl) were plated in triplicate onto a variety of proprietary agar media mentioned in Section 2.2.

2.6 **Washed cell suspension preparation**

Cells were grown overnight either aerobically or anaerobically according to the strain used. A 100-fold dilution of overnight cultures was made in sterile broth which was incubated until the desired growth phase was reached according to the constructed growth curve for each strain used in this study. Cells were harvested by centrifugation at 3220 xg for 15 min. The supernatant discarded and the pelleted cells were washed and re-suspended in sterile phosphate buffer saline (PBS) (0.01M PBS, pH 7.4). These steps repeated twice and the cell suspension adjusted to a final optical density at 600 nm of 0.8 (3.5x10⁶ - 7.2x10⁸ CFU/ml as confirmed by colony
counts on agar plates) using a Spectronic Helios UV-Vis spectrophotometer (Thermo Electron Corporation, USA).

2.7 Spent culture fluid preparation
Overnight cultures (10 ml) of test bacteria were centrifuged at 3220 xg for 15 minutes at room temperature. The supernatant was collected and filtered using a 0.22 µm pore filter (Millipore, Billerica, USA). For confirmation that this spent culture fluid was free from any residual viable bacteria, 100 µl was distributed on the surface of an agar plate and incubated for 48 h at 37°C either aerobically, anaerobically or at 10% CO₂ atmosphere according to the microorganism used to produce the spent fluid.

2.8 Bacterial lysate preparation
Overnight cultures (50ml) of test bacteria were centrifuged at 3220 xg for 15 minutes at room temperature and were then washed once and re-suspended in 25 ml of phosphate buffered saline (0.01M PBS, pH=7.4). The suspension was kept on ice during sonication using a SONOPULS Ultrasonic homogenizer (Sonoplus mini 20, BANDELIN electronic GmbH & Co., Germany) at 16 kHz for three minutes and filtered using sterile syringe filters (0.22 µm) to remove any viable bacteria from the solution. Sonicated culture fluid (100 µl) was distributed on the surface of an agar plate and incubated for 48 h at 37°C aerobically, anaerobically or at 10% CO₂ atmosphere according to the microorganism used to produce the lysate to confirm the removal of any viable bacteria.
2.9 Growth kinetics observation by optical density

Overnight cultures of all studied strains were set to 0.1 at OD600 nm (5.0\times10^5 to 8.0\times10^5 cfu/ml as confirmed by colony counts on agar plates) and then resuspended in fresh media. Inoculated media was transferred to a 96 well plate in triplicate and incubated in an automated Power wave XS plate reader (Biotek, Bedfordshire, UK). There were six replicates per sample and sterile media served as a negative control. The absorbance of each well was measured automatically every 1h at OD600 nm after a 10 s vigorous shake cycle for a 24h at 37°C. At the end of the experiment the results were saved digitally using the plate reader software. The average absorbance for each time point was calculated and plotted allowing the time period required to reach specific growth phases to be determined. Construction and analysis of the growth kinetics were done using the Gen 5 software program (Biotek, Bedfordshire, UK) and Microsoft Excel 2010.

2.10 Identification of oral bacterial isolates using partial 16S rRNA gene sequencing

2.10.1 DNA extraction

To a tube containing 50 µl of nuclease-free water (Qiagen, west Sussex, UK) one bacterial colony from axenic culture was added, mixed well and placed in a water bath at 100°C for 5 min to lyse the cells and obtain the DNA. The lysate was centrifuged at 14,000 rpm for 10 min (Sigma Microcentrifuge 1-14, SIGMA Laborzentrifugen GmbH, Germany) to separate the cell lysate. A 100 µl aliquot of the supernatant was transferred to a sterile tube and used directly as a template in PCR.

2.10.2 PCR amplification

The amplification of the extracted DNA was carried out using primers directed to the V2-V3 region of eubacterial 16S rDNA namely 8FPL1 (5’-GAG TTT GAT CCT
GGC TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') (McBain, Bartolo et al. 2003) purchased from (Eurofins Genomics GmbH, Germany). Each PCR reaction mixture consisted of: 25 µl Red Taq DNA polymerase ready mixture, 1 µl of 8FPL1 and 1 µl 806R at 5 µM, 18 µl nanopure nuclease-free water and 5 µl of extracted DNA. Thermocycling conditions were performed using a DNA thermal cycler (T-gradient, Biometra, Germany) as follows: 35 cycles of: 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, ending with a 15 min chain elongation step at 72°C. In each reaction set negative control included by replacing template DNA with 5 µl of nanopure water. Amplicons were resolved using 1% agarose gel electrophoresis. 0.3g of powdered agarose was added to 30ml of TBE Buffer (diluted in distilled water from a 50 x stock solution: 890 mM Tris, 890 mM Boric acid, 20 mM EDTA). The agarose solution was heated in microwave oven until fully dissolved and become clear solution. Left to cool then add 2 µl of a fluorescent nucleic acid stain GelRed (Biotium, Middlesex, UK).

2.10.3 PCR analysis
Gels were cast using suitably spaced combs placed in a gel tank (Bio-Rad, Hemel Hamstead, UK). Once set the combs were removed and the gel covered with 1 x TBE buffer (400ml), 5 µl of each amplicon was loaded in each well. The DNA ladder used was Quick-Load 1 kb DNA Ladder (New England Biolabs, Hertfordshire, UK). Electrophoresis was performed at 70 V for 30 min. gels were visualised by T 2201 UV transilluminator, wavelength operating at 312 nm (Sigma, Dorset, UK).

2.10.4 Preparation of PCR products to sequencing
Purification of PCR products was carried out using Qiaquick PCR purification kits (Qiagen, West Sussex, UK) in accordance with the manufacturer’s instructions and products were analysed using Nanodrop™ spectrophotometer (Labtech
to quantify DNA. A final concentration of 50 ng of PCR product per reaction and 4 pmol of 806R primer in a final volume of 10 µl was required for partial 16S rRNA gene sequencing at the University of Manchester DNA Sequencing Facility (www.manchester.ac.uk/dnasequencing).

2.10.5 Sequence analysis

Sequence data was entered into the BLAST (Basic Alignment Search Tool) online sequence analysis tool (http://www.ncbi.nlm.nih.gov/blast) to search the European Molecular Biology Laboratories’ (EMBL) prokaryote database for matches to the submitted 16S rRNA amplicon sequences. The closest matching relatives, EMBL accession numbers and degree of similarity were recorded for each organism. Species was assigned if the sequence yielded query coverage of 98 to 100% (spanning a minimum of 500 bp) with an identity of 96 % to 100% to a single organism.

2.11 Minimum inhibitory concentration determination (MIC)

Test bacteria were inoculated on tryptic soy agar plates and incubated aerobically at 37°C for 24 h and anaerobic strains and saliva on Wilkins Chalgren agar or TYCS agar plates anaerobically in a MG-1000-anaerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) at 37°C (Gas mix 80% N2, 10% CO2 and 10% H2) for 24-72 h. Bacteria were then harvested and suspended either in tryptic soy broth or brain-heart infusion broth respectively at a concentration of $10^8$ cfu/ml. The MICs and MBCs of the different test products were determined following recommendations of the BSAC using broth microdilutions (Andrews 2001) using 100 µl of an overnight bacterial suspension adjusted to $10^6$ cfu/ml plus 100 µl of tested product dilution in each well. Each plate containing 200µl of broth and inoculum served as a positive control and
200μl of broth and test agent as a negative control. Plates were sealed with plastic film to prevent evaporation and the MIC was determined as the lowest concentration that inhibited growth visually (the appearance of turbidity) after an overnight incubation either aerobically or anaerobically at 37°C for 48 h. All the plates were prepared in triplicate with one strain in each plate.

2.12 Minimum bactericidal concentration determination (MBC)

Aliquots (10μl) from each challenge plate well (Section 2.11) were removed after 24 h of incubation for each strain and spotted on the surface of Muller-Hinton agar (Oxoid) plates or WC agar plates according tested strain in triplicate. The MBC was read as the lowest concentration with no growth after 48 h of incubation.

2.13 Minimum biofilm eradication concentration determination (MBEC)

In order for minimum biofilm eradication concentration to be determined for the tested products in this study, the Calgary Biofilm Device (CBD) (Innovotech Inc., Calgary, Canada) was used to cultivate biofilms. The device consist of a plastic lid with 96 pegs that fit into a flat-bottom 96-well microtiter plate (M9410 - Nunc-Immuno™ MicroWell™ 96-Well Microplates, Sigma, UK). Biofilms were grown as follows: frozen stocks of bacteria were streaked out on tryptic soy agar plates or Wilkins Chalgren agar and incubated aerobically at 37°C for 24 h to obtain a first-subculture, respectively. A single colony was picked from the first subculture and again streaked out on agar to obtain a second sub-culture. Colonies were collected from the second-subculture and suspended in broth medium to a concentration of 10^8 cfu /ml. This suspension was diluted 1:100 in broth and used to inoculate the MBEC assay plate as 150 μl in each well. Covered with the lid with attached pegs
and incubated aerobically or anaerobically at 37°C for 48-72 h. For fresh saliva the plates were filled with 150μl fresh saliva, covered and incubated anaerobically at 37°C for 48-72 h. After this time, challenge plates were prepared containing 170μl of test product dilutions in broth (the dilutions used are the same as the one used in MIC test) including positive and negative controls. The lid with attached pegs was removed from the growth plates and used to cover the challenge plates (Section 2.13) and incubated as usual for 24 h at 37°C. After incubation the treated biofilms were rinsed twice in 200μl PBS to remove excess planktonic organisms and transferred to new recovery plates containing 170μl of broth and incubated for 72 h at 37°C either aerobically or anaerobically as appropriate. The MBEC was determined as the lowest concentration of the test solution that inhibited growth visually after an incubation. All the plates were prepared in triplicate with one strain in each plate.

2.14 Live/Dead staining

LIVE/DEAD bacterial-viability stain (BacLight; Molecular Probes, Leiden, The Netherlands) was performed for antimicrobial treated bacterial biofilms formed on cyclopore polycarbonate black membrane Whatman ™ (Sigma-Aldrich, Dorest, UK) in accordance to manufacturer’s instructions. The slides were then incubated in dark for 15 minutes before examination using the 10X objective lens of epifluorescence microscope (Axioskop 2, Zeiss, Hertfordshire, UK) using ImageJ 64 software (obtained from http://imagej.nih.gov). Cells were scored as live (green) or dead (red) in ten random fields and the average calculated for three individual experiments with triplicate samples within each individual experiment.

2.15 Mammalian cell culture
Two different human oral cell lines were used namely, Human oral keratinocytes (HOKs, Sciencell Research Laboratories, USA) and Non-tumour-derived, immortalized human oral epithelial cells (GMSM-K) (Gilchrist, Moyer et al. 2000) were provided by Dr. Krystyna Konopka, University of the Pacific (California, USA). GMSM-K cells were maintained in keratinocyte growth medium 2 (PromoCell, Heidelberg, Germany) supplemented with bovine pituitary extract (0.004ml/ml), epidermal growth factor (recombinant human) 0.125ng/ml, insulin (recombinant human 5μg/ml), hydrocortisone (0.33μg/m), epinephrine (0.39μg/ml), transferrin, (holo human 10μg/ml) and 0.06mM CaCl2 and HOKs were maintained in oral keratinocyte medium (OKM, Sciencell Research Laboratories, USA) supplemented with oral keratinocyte growth supplement (OKGS) and penicillin/streptomycin solution (P/S). Medium was substituted twice weekly and cells were incubated in a humid atmosphere with 5% CO₂ at 37°C. Cells were cultured in T-25 or T-75 vented culture flasks and 24 well plates (Corning, sigma, USA). Both the flasks and the well plates used for HOKs were coated with Poly-L-Lysine (PLL) (Sciencell Research Laboratories, USA). PLL is a nonspecific attachment factor for cells useful in promoting cell adhesion to solid substrates by enhancing electrostatic interaction between negatively charged ions of the cell membrane and the cell culture surface, when it is absorbed to the cell culture surface; it increases the number of positively charged sites available for cell binding. All experiments were performed on third to fifth passage cultures for HOKs. The cells were plated, at density 10 × 10⁴ cells per well, in 1 ml of the appropriate medium either in 12 or 24 well plates according to the experiment and used after 24 h incubation at 37 °C at approximately 90-100% confluence.
2.16 Passaging of mammalian cell culture

Cells were passaged at regular intervals depending on their growth characteristics (80-90% confluency). Media was aspirated from the cell culture dish and cells were washed with 100 μl Hanks Buffered Salt Solution (HBSS) per cm² of vessel surface for 15 seconds. Aspirate the Hepes BSS from culture vessel and add 100 μl Trypsin/EDTA Solution (0.3% / 0.4%) per cm² of vessel surface cells and incubate for 2–10 min at 37°C until the cells are rounded up and detached from the cell culture dish followed by adding the same volume of 0.1% Trypsin Neutraliser solution (TNS). Cells were then pelleted by subjecting them to 3 minutes of centrifugation at 1000 rpm. The supernatant was discarded and the cells were re-suspended in keratinocyte medium.

2.17 Cells cryopreservation

To cryopreserve human oral keratinocytes (HOKs), a 500 μl suspension consisting of 2x10⁵ cells/ml was prepared. A similar volume of the solution of freezing medium (Gibco™ Recovery™ Cell Culture Freezing Medium, Thermo Fisher Scientific, UK) added slowly drop by drop. 1000 μl of the freezing medium/cell solution added to each cryovial and the cells were frozen at -80°C overnight, followed by long-term storage in liquid nitrogen.

2.18 Trypan blue exclusion assay

Trypan blue exclusion assay was used to determine the viability of cells. For cells grown in 24 well plates, media was aspirated and cells were washed twice with PBS. The cells were then detached using 450 μl of Trypsin/EDTA Solution (0.3% / 0.4%) per well following the protocol explained in section 2.16 then 50 μl of Trypan
blue solution (0.4% w/v) (Invitrogen, Life Technologies Ltd, Paisley, UK) was added to each well for 30 seconds. Cells were counted under phase contrast inverted research live cell microscope using a haemocytometer. Non-viable cells retained their blue colour whilst viable ones remained unstained. Technical and biological replica applied as triplicate for each sample. The percentage viability of the keratinocytes was calculated using the following equation:

\[
\text{Percentage viability} = \frac{\text{Viable count}}{\text{Total count}} \times 100/1.
\]

2.19 Statistical Analyses

All experiments were performed at least 3 times biologically, which included three technical repeats. All data are shown as mean values plus/minus standard deviations. For experiments comparing two treatments, a paired student’s \( t \)-test was used. Results were considered statistically significant if \( P\lt0.05 \) (Microsoft Excel 2010).
CHAPTER 3

Exposure of multi-species communities to antimicrobial formulations
3.0 Abstract

In the oral cavity, antimicrobial agents are usually combined with physical removal methods (e.g. brushing and flossing) for hygienic control and the prevention of oral diseases. Oral health formulations including toothpastes, gels, sprays and mouthwashes commonly contain various antimicrobial agents with different modes of action to inactivate microorganisms or reduce the rate of regrowth following physical removal. These compounds include quaternary ammonium compounds such as cetylpyridinium chloride, biguanides such as chlorhexidine, fluorinated compounds such as stannous fluoride and other fluoride salts, zinc salts and essential oils, e.g. thymol. The antibacterial effects of various formulations used for oral hygiene were investigated in this chapter against different bacterial strains as planktonic and biofilm mode of growth along with abstracted oral bacterial communities. In order to determine the effects of physical apposition of bacteria on bacterial susceptibility to 8 different antimicrobial formulations with diverse modes of action, the susceptibility of four model organisms (E. coli, S. aureus, S. epidemidis and S. mutans) and abstracted oral bacterial communities were tested to derive minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC), minimum biofilm eradication concentrations (MBEC) and whole plaque viability (viability mapping) where the inactivation of plaques was assessed by fluorescence microscopy. In the majority of cases bacteria were significantly more susceptible in planktonic culture than in colonies or biofilms, supporting the commonly observed phenomenon of biofilm recalcitrance. The rank order of effectiveness of the test formulations held true in most cases for MIC, MBC and MBEC with respect to the least and most effective agent, regardless of test bacterium. However the rank order according to viability mapping was markedly different in relation to antimicrobials used and may represent a more realistic indicator of in situ effectiveness. In general, formulations containing the bisphenol microbicide triclosan or the bis-biguanide chlorhexidine were most effective.
3.1 Introduction

3.1.1 Oral biofilms

The composition of mature oral microbial communities often remains relatively stable through a process that has been termed microbial homeostasis (Marsh 2006). Certain shifts in this homeostasis may promote dental caries and/or periodontitis. The oral microbiota contributes to host health directly and indirectly by supporting the innate and adaptive host defences in removing pathogenic microorganisms, in addition to its responsibility for the natural development of the host physiology (Marsh 2009). Like other habitats in the body the oral cavity possesses a distinctive microbial community because it is a warm and humid environment with good nutrient availability (Marsh 2009). Whilst the oral microbiota includes viruses, archaea, fungi, protozoa and mycoplasma, bacteria are by far the most numerous form of microorganism.

According to William Wade, less than 50% of the residential oral microbiota can be cultivated by ordinary laboratory techniques (Wade 1999; Wade 2002). This might seem low but the oral microbiota is actually one of the most culturable of human microbial communities. Recently, the availability of information about the diversity and richness of the oral microbiota has increased significantly as a result of significant advances in the application of culture-independent molecular techniques (Ling, Kong et al. 2010; Jiang, Ling et al. 2014). Novel techniques based on amplification, cloning and direct 16S rRNA gene sequencing have led to nearly 700 different species being identified on mucosal and dental surfaces with approximately 20-30 predominant species (Aas, Paster et al. 2005).
3.1.2 Progression of multi-species oral biofilms (Dental plaque)

Oral biofilms have been described as microbial communities that develop on the tooth surface, surrounded by a matrix of polymers of bacterial and salivary origin (Marsh 2009). There are several distinct stages for the oral biofilm formation resulting in a structurally and functionally organized species rich community. These start with the formation of a conditioning film on the tooth surface, which changes the surface properties and is known as the acquired pellicle (Teughels, Van Assche et al. 2006; Hannig and Hannig 2009). This film consists of molecules derived from the saliva and from gingival crevicular fluid in the subgingival region; after it has been formed bacteria interact directly with these molecules through reversible and irreversible adhesion events (Teughels, Van Assche et al. 2006; Hannig and Hannig 2009). The conditioning film is believed to produce a charge on the tooth surface which leads to an interaction with the microbial cell surface charges described as a weak and long range physicochemical interaction (Busscher, Engels et al. 2008), after which the interaction between adhesins on the bacterial cell surface and the receptors present in the acquired pellicle become strong and tend to be irreversible (Whittaker et al. 1996). After the establishment of the primary colonizers, secondary colonizers adhere via their surface adhesins to the receptors of these primary colonizers in a co-adhesion chemotaxis and attached to the eDNA leading to an increase in the microbial diversity of the developing biofilm (Kolenbrander 2000; Barken, Pamp et al. 2008; Yang, Nilsson et al. 2009; Yang, Rybtke et al. 2009).

Later on the attached cell multiplication increases the biomass and forms the biofilm matrix, which is an important structural component of dental plaque and contributes towards insusceptibility to antimicrobials (Billings, Millan et al. 2013).
Once the biofilm has been formed there is a degree of stability amongst the microbial species. This is due to microbial interactions which can be either synergistic or antagonistic and unless any environmental stress takes place such as oral hygiene measures or food intake which may lead to a breakdown in this microbial homeostasis and result in an oral disease. Detachment and new niche colonization occurs as a consequence to an environmental shift taking place. Such changes may result in bacteria up-regulating enzymes that cleave their adhesins, allowing the cells to detach and colonize new places (Kaplan, Meyenhofer et al. 2003).

The relationship between bacteria, plaque and oral diseases is clearly recognized as indicated by the main emphasis for the treatment of oral diseases being on the development and use of various antimicrobial agents such as triclosan/copolymer (Volpe, Petrone et al. 2002; Cullinan, Westerman et al. 2003), bis-biguanide as chlorhexidine (Clavero, Baca et al. 2003; Hope and Wilson 2003; Santos, Herrera et al. 2004) and essential oil-containing mouth rinses which contains for example; thymol, menthol and eucalyptol (Charles, Mostler et al. 2004; Sharma, Charles et al. 2004; Fine, Markowitz et al. 2007; Cortelli, Cortelli et al. 2013) and many other compounds.

### 3.1.3 Dental plaque and antimicrobials

Several oral antimicrobial tested products clinically and in vitro showed an effect in reducing plaque formation, improvements in indices of gingival health and to some extent control of oral diseases which were attributed to the antimicrobial effect of the different compounds used (Rosling, Dahlen et al. 1997; Adams, Theobald et al. 2003; Tong, Guo et al. 2008; Yimcharoen, Rirattanapong et al. 2011; Pires dos
Santos, Nadanovsky et al. 2013). However, oral biofilm is not completely removed by oral hygiene of either physical or chemical measures. For example, after powered brushing only up to 60% of biofilm from oral sites was removed (Adams, Theobald et al. 2003) and consequently exposed several times to antimicrobials from toothpastes and mouthwashes. This could make subsequent removal challenging, since this residual biofilm may become a reservoir for antimicrobials and fluoride (Buzalaf, Pessan et al. 2011; Otten, Busscher et al. 2012).

3.2 Aims and objectives

In order to determine the effects of physical apposition of bacteria on bacterial susceptibility to a range of antimicrobial formulation with diverse modes of action the susceptibility of four model organisms (*E. coli, S. aureus, S. epidemidis and S. mutans*) and abstracted oral bacterial communities were tested using four distinct methods to derive minimum inhibitory concentrations, minimum bactericidal concentrations, minimum biofilm eradication concentrations and whole plaque viability (viability mapping). Viability mapping was used to demonstrate and evaluate the effect of these formulations on oral plaques after short term exposure (2 minutes) using a fluorescence microscopic method by applying LIVE/DEAD bacterial viability staining (BacLight; Molecular Probes, Leiden, The Netherlands) by evaluation of the intact bacterial biomass on the black membranes.
3.3 Materials and methods

3.3.1 The polycarbonate membrane filter biofilm model
This method was used to assess the effect of five antimicrobials on the biofilm cells viability and attachment to the substrate. The biofilm growth system consists of a polycarbonate filter membrane (Whatman® Nuclepore™ React-Etched Membranes) of 25mm diameter and 0.2μm pore size were (Bhardwaj, Moore et al. 2013; Saloni Singla 2014)

3.3.2 Microorganisms for validation studies of polycarbonate membrane Model
Polycarbonate membranes have been used previously as substrata for development of biofilms (Saloni Singla 2014) but their validation for the growth and attachment of salivary biofilm microcosms has not yet been reported. Oral microbiomes may be composed of several hundred bacterial species (Marsh 1999) so E. coli C600 was selected as a simple paradigm to be used in validation studies as a mono species biofilm. The results achieved led to the further validation of the black membranes by growing salivary microcosm biofilms using fresh human whole saliva.

The antimicrobial efficacy of eight antimicrobial formulations intended as oral hygiene control products with diverse modes of action on intact bacterial biomass of human saliva derived plaque grown on polycarbonate membrane were tested. The comparative experiments were performed using four distinct methods to derive minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum biofilm eradication concentrations (MBEC) and a whole plaque viability mapping technique using LIVE/DEAD bacterial-viability staining. Different bacterial strains were selected as a simple paradigm to be used in studies of
antibacterial efficacy of different compounds and the outcome of these results correlated with that of the whole saliva plaque.

3.3.3 Strains used in this study

*E. coli* K12 C600, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 14990, *Streptococcus mutans* NCTC 10832 and whole saliva derived plaque. Cells were maintained as per Section 2.4.

3.3.4 Saliva samples

Sampling and processing of saliva as explained in Section 2.5.2.

3.3.5 Antimicrobials containing products used in this study

The products selected for the current study are detailed in Table 3.1 chosen because they contain different commonly used antimicrobial agents of confirmed oral hygiene efficacy *in vivo* and *in vitro.*
Table 3.1 Test products used in the study

<table>
<thead>
<tr>
<th>Test Product</th>
<th>Code</th>
<th>Content</th>
<th>Concentration range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toothpastes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colgate Total®</td>
<td>CT</td>
<td>SLS, Sodium fluoride (0.243% w/w) and Triclosan (0.3% w/w)</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td>Colgate Cavity Protection®</td>
<td>CP</td>
<td>SLS, Sodium monofluoro phosphate (0.76% w/w) = fluoride ion (0.15% w/v)</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td>Crest Pro Health®</td>
<td>PH</td>
<td>SLS, Stannous fluoride (0.454% w/w) = fluoride ion (0.16% w/v) and (1% w/w zinc lactate)</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td>Aqua fresh Extreme clean®</td>
<td>AEC</td>
<td>SLS, Zinc chloride and Sodium fluoride (0.306% w/w) = 1400ppm fluoride</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td>Mentadent P®</td>
<td>MP</td>
<td>SLS, Zinc citrate and sodium fluoride =1450ppm fluoride</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td>Zinc containing toothpaste</td>
<td>SZ</td>
<td>SLS, fluoride, Zn citrate and Zn oxide</td>
<td>1% to 0.002%</td>
</tr>
<tr>
<td><strong>Mouth washes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Listerine®</td>
<td>LIS</td>
<td>Thymol, 0.064%; eucalyptol, 0.092%; methyl salicylate, 0.060% and menthol, 0.042%</td>
<td>100% to 0.2%</td>
</tr>
<tr>
<td>Corsodyl®</td>
<td>CHX</td>
<td>Chlorhexidine 0.2% w/w</td>
<td>40% to 0.078%</td>
</tr>
</tbody>
</table>

3.3.6 MIC, MBC, MBEC and Live/ Dead staining of biofilms
The procedures explained in details in Sections from 2.11 to 2.14

3.3.7 Growing of biofilms on polycarbonate membranes
Sterile membranes (n=3) were placed aseptically (Figure 3.1a) on a surface of Wilkins Chalgren agar (WC) plates. Aliquots of 10µl of fresh human saliva were dispensed on the surface of resting membranes on agar culture medium (three spots per membrane) as shown in (Figure 3.1b). Plates were then incubated either aerobically (48 h at 37°C) or anaerobically in a MG-1000-an aerobic cabinet (Don Whitley Scientific, West Yorkshire, UK) at 37°C (Gas mix 80% N₂, 10% CO₂ and 10%
H₂) for 48 h. after incubation each membrane cut into three parts containing growing biofilm each which are then vortexed for 20 seconds in 9ml pre-reduced half strength sterile thioglycolate broth contained in a 25 ml universal bottle before serially diluted in half strength broth and plating out on a range of different selective agar plates as described previously in Section 2.4. Each validation assay was carried out in triplicate using a different membrane in each trial, in triplicate.

The same procedure was repeated after the exposure of the membranes to 10% slurries of different tooth paste formulations, 40% of Corsodyl® and 100% of Listerine® for two minutes after which the membranes were washed for 30 sec with sterile distilled water and treated as mentioned above and plated and incubated as appropriate.

**3.3.8 Microscopic evaluation of bacterial biomass**

Biofilms formed on membranes were prepared as described above (Section 3.3.5). The antibacterial efficacy of different oral hygiene products was evaluated using *E. coli* C600 biofilms and salivary–derived microcosms. Each membrane has been cut into three parts, each containing one of the three biofilms grown as shown (Figure 3.1d) and transferred aseptically onto the surface of a clean microscopic slide using sterile forceps (Figure 3.1e) and then treated with 10% slurries of designated tooth paste or 100% mouth wash product for two minutes before washing with sterile distilled water (30 seconds) for the removal of excessive antimicrobial agent, stained with Live / Dead stain as described in section 2.14 and observed under an epifluorescent microscope.
Figure 3.1 Steps of preparation of membrane biofilm for microscopic evaluation of bacterial biomass. (a) Membranes placed on agar surface, (b) inoculation of saliva on membranes, (c) Growing of salivary biofilms, (d, e and f) Preparation of growing biofilms for staining.
3.3.9 Statistical analyses

All data are shown as mean values plus/minus standard deviations. For viable count analysis a paired student’s t test was used to determine whether the use of polycarbonate membranes for biofilm formation were significant for validation studies. Additionally student t test was used to determine whether the effects of various oral hygiene products on formed biofilms viability and attachment were significant. In all cases p≤0.01 was considered to be statistically significant except for one case p was <0.05 and considered significant too (Microsoft Excel 2010).

3.4 Results

3.4.1 MIC, MBC and MBEC

The current investigation demonstrated that in all tested bacterial strains in addition to the whole salivary microcosms, the minimum biofilm eradication concentrations were more than the minimum inhibitory concentrations which is in agreement with published data regarding the recalcitrance of bacteria growing in biofilms (Brown, Allison et al. 1988; Costerton, Stewart et al. 1999).

Triclosan is a broad spectrum antimicrobial agent acting on various cytoplasmic and membrane targets. The MICs of triclosan for Gram positive strains (S. aureus and S. epidermidis) were lowest followed by E. coli and S. mutans which had the same MIC values. Whole saliva was the least susceptible. Moreover, these results applied to both planktonic growth inhibition and biofilm modes of growth as shown in Figure 3.2a. The only one difference noted was that the MBEC for S. mutans was higher than E. coli whilst the MIC readings were the same. Likewise, the MBC readings were lower for Gram positive strains except S. mutans again which was higher than E. coli. The highest MBC value was again for salivary microcosms as shown in Table 3.2. According to many studies the antibacterial action of fluoride for
oral bacteria is due to effects on bacterial metabolism (Marquis 1995; Barboza-Silva, Castro et al. 2005). In our study this formulation was more effective on *E. coli* as indicated by MIC and MBC values. For biofilm modes of growth (MBEC), *S. mutans* was less susceptible followed by *E. coli* as shown in Figure 3.2b.

It has been previously reported that stannous fluoride and zinc salts damage the process of carbohydrate fermentation and glycolysis which leads to the inhibition of acid production by bacteria and can protect from dental caries (Forbes, Latimer et al. 2016). The highest MIC and MBEC values that were obtained for stannous fluoride was for *S. mutans*. For *E. coli* MIC cannot be determined by used concentrations and if higher concentrations used the turbidity of slurries makes it impossible to detect the MIC point but the most interesting observation was that salivary biofilms were most susceptible to fluoride and the value of MBEC was about 1/8 the concentration needed to inhibit biofilms of *E. coli*, *S. aureus* and *S. mutans* and 1/4 the MBEC value needed to inhibit *S. epidermidis* biofilms as shown in Figure 3.2c. The MIC, MBC and MBEC were not determined for both *E. coli* and *S. aureus* by the applied concentrations and again the fresh saliva biofilm was susceptible to this formulation as shown by the low MBEC value as shown in Table 3.2 and Figure 3.2d.

It has been shown that zinc can reduce sugar uptake in bacteria by inhibiting the phosphate transferase transport system and sulphahydril enzymes (OPPERMANN, RØLLA et al. 1980; Phan, Buckner et al. 2004). For MP the MIC results were the same concentration for *S. aureus*, *S. epidermidis* and *S. mutans*. For salivary microcosms the concentration of MP was the double and no result determined by used concentrations due to turbidity of slurries if higher concentrations used. For MBC and MBEC *S. aureus* has the highest concentration in comparison with other strains and the saliva biofilm as shown in Figure 3.3a.
For zinc-containing formula as shown in Figure 3.3b, salivary biofilms was the most susceptible to the used concentrations in comparison to other strains biofilms as well as MIC but not for MBC. The MIC for *E. coli* was the lowest followed by *S. mutans* and saliva and the least susceptible were *S. aureus* and *S. epidermidis*. When strains treated with Corsodyl, *E. coli* and the salivary biofilm MBEC values indicated that both were more susceptible than other Gram positive strains as shown in Figure 3.3c. The MIC for saliva when treated with Listerine was the highest and *S. epidermidis* the least as shown in Figure 3.3d. The MBEC results again the same manner as MIC where saliva and *S. mutans* more susceptible than other strains and *S. epidermidis* the least and the same results applied to the MBC.

The lowest and highest MIC values (% W/V) for each formulation were as follows: CT, 0.002 and 0.5; CP, 0.0625 and 1.0; PH, 1.25 and 1.0; AEC, .5 and 1.0; MP, 0.5 and 1.0; SZ, 0.5 and 1.0; CRO, 0.625 and 20; LIS, 12.5 and 50. The lowest and highest MBC values (% W/V) for each formulation was as follows: CT, 0.625 and 1.0; CP, 0.25 and 2.5; PH, 2.5 and 5.0; AEC, 1.0 and 5.0; MP, 1.0 and 10.0; SZ, 1.0 and 5.0; CRO, 1.25 and 20; LIS, 25.0 and 100. The lowest and highest MBEC values (% W/V) for each formulation was as follows: CT, 0.125 and 1.25; CP, 1.25 and 5.0; PH, 1.25 and 10.0; AEC, 1.0 and 5.0; MP, 1.0 and 5.0; SZ, 1.25 and 5.0; CRO, 1.25 and 20; LIS, 50.0 and 100. (Tables 3.3 and 3.4). In the majority of cases bacteria were more susceptible in planktonic culture than in colonies or biofilms supporting the commonly observed phenomenon of biofilm recalcitrance. The rank order of effectiveness of the test formulations held true in most cases for MICs (planktonic growth inhibition), MBCs (planktonic bactericidal concentrations) and MBECs (the determination of biofilm eradication by cultivation) with respect to the least and most effective agent, regardless of test bacterium.
Table 3.2 Minimum Inhibitory Concentrations (MIC), Minimum bactericidal Concentrations (MBC) and Minimum Biofilm Eradication Concentrations MBEC) of different tested tooth paste products against tested strains and fresh saliva.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Colgate Total®</th>
<th>Colgate Cavity Protection®</th>
<th>Crest Pro-Health®</th>
<th>Aqua Fresh Extreme Clean®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MBEC</td>
<td>MIC</td>
</tr>
<tr>
<td>E. coli C600</td>
<td>0.0625</td>
<td>0.25</td>
<td>0.5</td>
<td>0.0625</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.002</td>
<td>0.0625</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>0.002</td>
<td>0.0625</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Strep. mutans</td>
<td>0.0625</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fresh saliva</td>
<td>0.5</td>
<td>1.0</td>
<td>1.25</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Data were determined by broth dilution endpoint (doubling dilutions). The units are percentage (%) w/v. The data show means from replicate experiments (n = 3). nd = not determinable by the used concentration.
Figure 3.2 MIC (green bar) and MBEC (red bar) values of (a) Colgate Total®, (b) Colgate Cavity Protection®, (c) Crest Pro-Health® and (d) Aqua Fresh Extreme Clean® against E. coli k12 C600, Staphylococcus aureus ATCC 6538, Staphylococcus epidermidis ATCC 14990, Streptococcus mutans NCTC 10832 and Fresh human saliva. Data are means ± SD.
Table 3.3 Minimum Inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum biofilm eradication concentrations (MBEC) of two tested tooth paste products and two mouth washes against tested strains and fresh saliva.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Mentadent P®</th>
<th>Zinc containing toothpaste</th>
<th>Corsodyl®</th>
<th>Listerine®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MBEC</td>
<td>MIC</td>
</tr>
<tr>
<td>E. coli C600</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>S. aureus</td>
<td>0.5</td>
<td>2.5</td>
<td>5.0</td>
<td>0.5</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>0.5</td>
<td>1.0</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Strep. mutans</td>
<td>0.5</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Fresh saliva</td>
<td>1.0</td>
<td>10</td>
<td>2.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Data were determined by broth dilution endpoint (doubling dilutions). The units are percentage (%) w/v. The data show means from replicate experiments (n=3). nd = not determinable by the used concentration.
Figure 3.3 MIC (green bar) and MBEC (red bar) values of (a) Mentadent P®, (b) Superior Zinc Citrate, (c) Corsodyl® and (d) Listerine® against E. coli k12 C600, Staphylococcus aureus ATCC 6538, Staphylococcus epidermidis ATCC 14990, Streptococcus mutans NCTC 10832 and Fresh human saliva. Data are means ± SD.
3.4.2 Polycarbonate membranes validation

Polycarbonate membranes have been tested for their ability to be used as substrata for successfully reproducing biofilms firstly by using *E. coli* C600 as a simple paradigm of a mono-species biofilm that was successful and then applied for growing salivary microcosm biofilms using human whole saliva. This validation was considered to be important for additional microscopic investigations of the effects of previous tested formulations on biofilm attachment. Figure 3.4 illustrates the cells attachment following incubation of saliva on polycarbonate membranes substrata, as measured by viable counts of the functional groups of the salivary microcosms on three different membranes for each.

In general, the data represented in Figure 3.4 shows the ability to produce reproducible biofilms of salivary microcosms on polycarbonate membranes. Additionally an insignificant difference (p > 0.05) of the viable counts of different functional groups was also detected.

3.4.3 Antimicrobial efficacy of different formulations on whole saliva

The polycarbonate membranes model was used to evaluate and compare the efficacy of different oral antimicrobial formulations against mono-species biofilm (*E. coli* C600) and multi-species biofilm (fresh human whole saliva). Figure 3.9 illustrates the effects of all tested formulations after two minutes exposure and staining with Live/Dead stain according to the method explained in Section 2.14.

Generally with both biofilms there are significant effects of the formulations on the viability of formed biofilms on polycarbonate membranes.
Figure 3.4 The effect of microorganisms’ attachment of whole saliva on polycarbonate membranes substrata on total counts; M1, M2 and M3 represent viable counts after incubation on the substrata. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (p>0.05, n=3).
3.4.4 Antimicrobial efficacy of different formulations on different bacterial functional groups of whole saliva

The antimicrobial activities of different tested formulations against total facultative anaerobes derived from salivary microcosms maintained on polycarbonate membranes after two min exposures are showed in Figure (3.5) there was a significant reduction of bacterial viable counts after the two min exposure of the salivary biofilm to different tooth paste slurries and chlorhexidine containing mouth wash. However for Listerine® there was a non-significant reduction in counts compared to the control viable counts. The antimicrobial efficacies against other bacterial functional groups in the salivary-derived biofilm were also tested.

Figure 3.5 Effects of different formulations on total facultative anaerobic viable bacterial counts of saliva-derived oral microcosms maintained on polycarbonate membranes after two min. exposure; (NTC) no treatment control, (MP) Mentadent P®, (PH) Crest pro health®, (SZ) Superior Zinc Citrate (Colgate recipe), (AEC) Aqua fresh extreme clean®, (CT) Colgate total®, (CP) Colgate cavity protection®, (COR) Corsodyl®, (LIS) Listerine®). The red bars represent counts (untreated microcosms) while green bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). * Above bars represents a significant change with respect to control counts (p < 0.01).
Figure 3.6 illustrates the antimicrobial activity of different formulations on the viable counts of total aerobic bacterial groups derived from salivary microcosms grown on polycarbonate membranes after two min exposures. In contrast with what was previously shown with total facultative anaerobes, Listerine® demonstrated a highly significant (p<0.001) reduction in the viable counts of the total aerobes compared to the control. Moreover the superior zinc toothpaste formula caused a highly significant reduction (p<0.001) in counts comparable to Listerine®. All other formulations significantly reduced the counts (p<0.1) too. For total Gram-negative anaerobes again Listerine® showed a highly significant (p<0.001) reduction in viable counts. Triclosan, chlorhexidine and advanced zinc tooth paste formula showed a significant reduction in counts also, while all the rest of the compounds showed non-significant reduction of counts of Gram negatives as shown in Figure 3.7. With regards to the streptococci, all the formulations caused a highly significant reduction (p<0.001) in viable counts that was not observed in any of the other bacterial functional group that were quantified as illustrated in Figure 3.8.
Figure 3.6 Effects of different formulations on total aerobic viable bacterial counts of saliva-derived oral microcosms maintained on polycarbonate membranes after two min. exposure; (NTC) no treatment control, (MP) Mentadent P®, (PH) Crest pro health®, (SZ) Superior Zinc Citrate (Colgate recipe), (AEC) Aqua fresh extreme clean®, (CT) Colgate total®, (CP) Colgate cavity protection®, (COR) Corsodyl®, (LIS) Listerine®). The red bars represent counts (untreated microcosms) while green bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). */** above bars represents a significant change with respect to control counts (p < 0.01/ P<0.001).
Figure 3.7 Effects of different formulations on **total Gram-negative anaerobic** viable bacterial counts of saliva-derived oral microcosms maintained on polycarbonate membranes after two min. exposure; (NTC) no treatment control, (MP) Mentadent P®, (PH) Crest pro health®, (SZ) Superior Zinc Citrate (Colgate recipe), (AEC) Aqua fresh extreme clean®, (CT) Colgate total®, (CP) Colgate cavity protection®, (COR) Corsodyl®, (LIS) Listerine®). The red bars represent counts (untreated microcosms) while green bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). */** above bars represents a significant change with respect to control counts (p < 0.01/ p <0.001).
Figure 3.8 Effects of different formulations on total streptococci viable bacterial counts of saliva-derived oral microcosms maintained on polycarbonate membranes after two min. exposure; (NTC) no treatment control, (MP) Mentadent P®, (PH) Crest Pro Health®, (SZ) Zinc containing formulation, (AEC) Aqua fresh extreme clean®, (CT) Colgate total®, (CP) Colgate cavity protection®, (COR) Corsodyl®, (LIS) Listerine®. The red bars represent counts (untreated microcosms) while green bars represent the treated biofilm. Data represent mean values of triplicate experiments; error bars represent standard errors of the mean (n=3). ** Above bars represents a significant change with respect to control counts (p <0.001).
3.4.5 Microscopic evaluation of different oral hygiene formulations on *E.coli* biofilms and artificial plaque of whole saliva formed on polycarbonate membranes substrata

Firstly, the capability of all tested oral hygiene formulations to affect the viability of formed *E. coli* C600 and whole saliva biofilms on polycarbonate membranes after a brief exposure (2 minutes) was evaluated using a viability mapping technique. This short exposure time shows a reduction in the viability of biofilms formed for all tested products determined by viable staining caused significant reduction in biofilm viability (p <0.01) compared to a non-treated control as shown in Figure 3.9. For salivary microcosm’s plaque, a chlorhexidine-containing formulation (COR) was the most efficacious product and a combined stannous fluoride and zinc lactate containing formulation (PH) was the least effective. Secondly the effect of the tested products to penetrate and / or detach the biofilms in addition to any antibacterial action was determined by using a Live / Dead stain. Data generated shows numbers of individual live and dead cells developed in control and formulations-exposed environments with either *E. coli* or whole saliva biofilms grown on polycarbonate membranes substrata. Most formulations significantly (p<0.01) reduced the number of live cells compared to non-treated controls (Figures 3.11-3.26). Additionally the number of dead cells was significantly increased after exposure to selected products compared to non-treated controls. The percentage of fields of view in most cases was observed to be less than 100% because it was correlated to both the antimicrobial effect of formulations and to the detachment of the biofilms and therefore there may be a washing effect observed after exposure to the test products. The efficacy of different antimicrobial formulations against *E. coli* C600 and saliva microcosms tested by viability mapping was for *E. coli* as follows:
CP > CT > AEC > LIS > SZ > COR > PH > MP and for saliva microcosms as follows: COR > SZ > MP > CT > CP > AEC > LIS > PH.

**Figure 3.9** Effects of different antimicrobial formulations on attachment and viability of; (a) Saliva-derived oral microcosms and (b) *E. coli* K12 C600 as determined by viability mapping. Red bar, control (untreated biofilm); green bars, treated biofilms using products label each bar. (NTC) no treatment control, (MP) Mentadent P®, (PH) Crest Pro Health®, (SZ) Zinc containing Toothpaste, (AEC) Aqua Fresh Extreme Clean®, (CT) Colgate Total®, (CP) Colgate Cavity Protection®, (COR) Corsodyl®, (LIS) Listerine®. Data are means ± SD from 3 polycarbonate membranes, each of which counted ten fields of view. (p < 0.05).
Figure 3.10 (a) Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Colgate Total® (live); (c) Control (dead); (d) treated with Colgate Total® (dead). Total magnification: x1000.

(b) Effects of Colgate Total® tooth paste on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Colgate Total® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.11 (a) Epifluorescence microscopy images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Colgate ® cavity protection (live); (c) Control (dead); (d) treated with Colgate Cavity Protection® (dead). Total magnification: x1000.  

(b) Effects of Colgate Cavity Protection ® tooth paste on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Colgate ® Cavity Protection treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
**Figure 3.12(a)** Epifluorescence microscopy images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Crest Pro Health® (live); (c) Control (dead); (d) treated with Crest Pro Health® (dead). Total magnification: x1000.

**(b)** Effects of Crest Pro Health® tooth paste on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Crest pro health® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.13 (a) Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated Mentadent P® (live); (c) Control (dead); (d) treated with Mentadent P® (dead). Total magnification: x1000.

(b) Effects of Mentadent P® tooth paste on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Mentadent P® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.14 (a) Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Aqua Fresh Extreme Clean® (live); (c) Control (dead); (d) treated with Aqua Fresh Extreme Clean® (dead). Total magnification: x1000. (b) Effects of Aqua Fresh Extreme Clean® tooth paste on attachment and viability of *E. coli* k12 C600. Dark green bars, control (untreated biofilm); green bars, Aqua Fresh Extreme Clean® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
**Figure 3.15 (a)** Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Zinc containing Toothpaste (live); (c) Control (dead); (d) treated with Zinc containing Toothpaste (dead). Total magnification: x1000. (b) Effects of Zinc containing Toothpaste on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Zinc containing Toothpaste treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.16 (a) Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Corsodyl® (live); (c) Control (dead); (d) treated with Corsodyl® (dead). Total magnification: x1000. (b) Effects of Corsodyl® on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Corsodyl® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.17 (a) Epifluorescence microscope images showing LIVE/DEAD-stained *E. coli* K12 C600 biofilm formed on polycarbonate membrane. (a) Control (live); (b) treated with Listerine® (live); (c) Control (dead); (d) treated with Listerine® (dead). Total magnification: x1000. 

**Figure 3.17 (b)** Effects of Listerine® on attachment and viability of *E. coli* K12 C600. Dark green bars, control (untreated biofilm); green bars, Listerine® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.18 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Colgate Total® (live); (c) Control (dead); (d) treated with Colgate Total® (dead). Total magnification: x1000. (b) Effects of Colgate Total® tooth paste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Colgate Total® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.19 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Colgate Cavity Protection® (live); (c) Control (dead); (d) treated with Colgate Cavity Protection® (dead). Total magnification: x1000. (b) Effects of Colgate Cavity Protection® tooth paste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Colgate Cavity Protection® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.20 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Crest Pro Health® (live); (c) Control (dead); (d) treated with Crest Pro Health® (dead). Total magnification: x1000.

(b) Effects of Crest Pro Health® tooth paste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Crest Pro Health® treated biofilm (p<0.05). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.21 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Mentadent P® (live); (c) Control (dead); (d) treated with Mentadent P® (dead). Total magnification: x1000. (b) Effects of Mentadent P® tooth paste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Mentadent P® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.22 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Aqua Fresh Extreme Clean® (live); (c) Control (dead); (d) treated with Aqua Fresh Extreme Clean® (dead). Total magnification: x1000. (b) Effects of Aqua Fresh Extreme Clean® tooth paste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Aqua Fresh Extreme Clean® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.23 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Zinc containing Toothpaste (live); (c) Control (dead); (d) treated with Zinc containing formula (dead). Total magnification: x1000. (b) Effects of Zinc containing Toothpaste on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Zinc containing Toothpaste treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
Figure 3.24 (a) Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Listerine® (live); (c) Control (dead); (d) treated with Listerine® (dead). Total magnification: x1000. (b) Effects of Listerine® on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Listerine® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
**Figure 3.25 (a)** Epifluorescence microscope images showing LIVE/DEAD-stained saliva-derived plaque formed on polycarbonate membrane. (a) Control (live); (b) treated with Corsodyl® (live); (c) Control (dead); (d) treated with Corsodyl® (dead). Total magnification: x1000. **(b)** Effects of Corsodyl® on attachment and viability of saliva-derived oral microcosm. Dark green bars, control (untreated biofilm); green bars, Corsodyl® treated biofilm (p<0.01). Data are means ± SD from 3 biofilms on polycarbonate membranes, each of which counted ten fields of view.
3.5 Discussion

This chapter compared the antibacterial efficacy of different antimicrobial formulations intended as oral hygiene control products with diverse modes of action such as disrupting the cell membrane of bacteria (chlorhexidine), acting on cytoplasm by inhibiting fatty acid synthesis (triclosan) or topical effects on teeth such as mineralization (fluoride) and membrane perforation (thymol). Products used including triclosan-containing toothpaste, fluoride-containing toothpastes, chlorhexidine-containing and essential oil-containing mouth washes. The experiments were performed using four distinct methods to derive minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum biofilm eradication concentrations (MBEC) and whole plaque viability mapping. Four model organisms as planktonic cells and as biofilms were used (E. coli C600, S. aureus, S. epidermidis and S. mutans) and abstracted oral bacterial communities. Whole biofilm viability of E. coli C600 and saliva-derived plaque formed on polycarbonate membrane after short-term exposures (2 min) to the test agent was determined using epiflourescent microscope. In agreement with what is published (Bradshaw, Marsh et al. 1993; Addy and Moran 1997; Ledder, Latimer et al. 2014; Forbes, Latimer et al. 2016), the results suggested that triclosan-containing toothpaste was the most efficacious against pure cultures and to saliva-derived microcosms in terms of the MIC, MBC and MBEC values.

For the saliva-derived microcosms, triclosan-containing toothpaste was the most effective applied agent in all experiments, whilst the all the other formulations tested were comparable in terms of the MIC, MBC and MBEC data. In terms of mouthwash, the chlorhexidine-containing formulation was more effective than the essential oil formulation in all experimental approaches. In most cases bacteria were more susceptible in planktonic culture than in colonies or biofilms, in agreement with
observed phenomenon of biofilm recalcitrance (Lewis 2008; Percival, Hill et al. 2011).

**Table 3.5** Conclusion of the efficacy of different antimicrobial formulations against all strains and saliva microcosms tested by MIC, MBC and MBEC

<table>
<thead>
<tr>
<th>Organism tested</th>
<th>MIC</th>
<th>MBC</th>
<th>MBEC</th>
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<tbody>
<tr>
<td><em>E. coli</em> C600</td>
<td>CT, CP&gt; COR&gt; LIS</td>
<td>CT, CP&gt; COR&gt; PH&gt; LIS</td>
<td>CT&gt; CP&gt; COR&gt; PH&gt; LIS</td>
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<tr>
<td><em>S. aureus</em></td>
<td>CT&gt; CP&gt; PH, MP&gt; SZ&gt; COR&gt; LIS</td>
<td>CT&gt; PH&gt; CP, SZ&gt; COR&gt; MP&gt; LIS</td>
<td>CT&gt; CP&gt; MP, SZ, COR&gt; PH&gt; LIS</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>CT&gt; COR&gt; CP, PH, AEC, MP, SZ&gt; LIS</td>
<td>CT&gt; COR&gt; CP, AEC, MP, SZ&gt; PH&gt; LIS</td>
<td>CT&gt; CP&gt; COR&gt; PH, AEC&gt; MP&gt; SZ&gt; LIS</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>CT&gt; AEC, MP, SZ&gt; CP&gt; PH&gt; COR&gt; LIS</td>
<td>CT&gt; AEC, MP, SZ&gt; CP&gt; PH&gt; COR&gt; LIS</td>
<td>CT, AEC, MP&gt; SZ&gt; CP&gt; PH&gt; COR&gt; LIS</td>
</tr>
<tr>
<td>Fresh saliva</td>
<td>CT&gt; CP, PH, AEC, MP, SZ&gt; COR&gt; LIS</td>
<td>CT&gt; CP&gt; PH&gt; AEC, SZ&gt; MP&gt; COR&gt; LIS</td>
<td>CT, CP, PH, AEC, SZ&gt; MP&gt; COR&gt; LIS</td>
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(MIC) minimum inhibitory concentration, (MBC) minimum bactericidal concentration, (MBEC) minimum biofilm eradication concentration.

Microscopic evaluation experiments using viability mapping were conducted to investigate the lethality of all formulations and showed clearly that for *E. coli* C600 fluoride-containing toothpaste was able to rapidly kill the microorganisms that were maintained on the polycarbonate membranes. The data showed that ca. 60% of this formulation-treated *E. coli* C600 biofilm was killed, ca. 30% live and ca. 10% absent or detached from the membranes (Figure 3.11 a, b).

For the triclosan-containing formula, ca. 55% of treated *E. coli* C600 biofilm were killed, ca. 20% live and ca.25% absent or detached from the membrane (Figure 3.10 a, b) and it was clearly observed that there were fewer viable cells following
exposure to a triclosan-containing formula than for the other test products. Treatment of biofilms with a zinc chloride and sodium fluoride-containing formula resulted ca. 50% cells killed, ca. 30% cells live and ca. 20% absent or detached cells from the membrane (Figure 3.14 a, b). A similar result was observed for essential oils-containing mouthwash with ca. 50% killed ca. 10% cells live and ca. 40% cells absent or detached from the membrane (Figure 3.17 a, b). This was confirmed visually because the biofilm was detached from the membrane.

Exposure of biofilms to the chlorhexidine-containing mouthwash resulted in 48% inactivation *E. coli*, with ca. 17% live cells and ca. 35% cells absent or detached from the membrane (Figure 3.16 a, b); zinc citrate and sodium fluoride-containing formula was the less effective agent and data showed that ca. 42% cells killed, ca. 33% live cells and ca. 25% cells absent or detached from the membrane as shown in Figure 3.13 a, b. Superior zinc formula (containing fluoride, zinc citrate and zinc oxide) killed ca. 50%, ca. 20% live and ca. 30% absent or detached from the membrane as shown in (Figure 3.15 a, b) and finally fluoride-containing tooth paste data showed that ca. 47% killed, ca. 20% live and ca. 30% absent or detached from the membrane as shown in (Figure 3.12 a, b). The figures for salivary bacterial consortia that were maintained on polycarbonate membranes showed that the most effective in killing was chlorhexidine-containing mouthwash as it killed ca. 65% of the treated consortia, ca. 25% live and ca. 10% absent or detached from the membrane (Figure 3.25 a, b); followed by superior zinc formula (containing fluoride, zinc citrate and zinc oxide) which killed ca. 60%, ca. 015% live and ca. 25% absent or detached from the membrane (Figure 3.23 a, b); fluoride and zinc citrate formula had nearly the same result with superior zinc citrate formula because it killed ca. 60%, but ca. 25% live and ca. 15% absent or detached from the membrane which
pointed out the effect of zinc oxide addition as the percentage of live consortia was 11% less than that of zinc citrate and fluoride formula only (Figure 3.21 a, b).

Triclosan-containing and sodium monoflouro-phosphate-containing formulas equally killed ca. 60% but for the triclosan-containing formula ca. 12% live, ca. 28% absent or detached from the membrane (Figure 3.18 a, b). While for sodium monoflouro-phosphate -containing formula data showed more live consortia ca. 30% and ca. 10% absent or detached from the membrane (Figure 3.20 a, b). The data for zinc chloride and sodium fluoride-containing formula presented that ca. 56% killed, ca. 25% live and ca. 19% absent or detached from the membrane (Figure 3.22 a, b) while the effect of stannous fluoride-containing formula was the least as it killed ca. 47% and ca. 30% live and ca. 23% absent or detached from the membrane (Figure 3.20 a, b). Finally the result of essential oils-containing mouth wash showed that it killed ca. 55% and ca. 30% live and ca. 15% absent or detached from the membrane (Figure 3.24 a, b). Additionally the significance grids done for all the formulations against both the salivary derived plaque biofilms and *E. coli* C600 mono species biofilms clearly show the significance differences in the effect of each of the studied formulations towards the other as shown in table 3.5 for *E. coli* C600 viability mapping test and Table 3.6 for saliva plaques viability mapping results.

### 3.6 Conclusions

According to viable mapping data after a brief exposure (2 min) to different antimicrobial agents there was a significant reduction in the number of live cells compared to untreated controls for most tested formulations and the increase in the number of dead microorganisms was statistically significant in most cases after exposure to different formulations compared to untreated biofilms as shown in
Figures 3.10-3.25. Measurements of the live/dead cells, however, do not necessarily correlate only with lethality of the tested product, because the data represented in some figures show that the sum of the treated live and dead cells was still lower than 100% field of view. This observation is in agreement with the images presented alongside with the figures.

The number and the aggregations of the scored live and dead microorganisms after exposure to most formulations were lower per field of view compared to control and this is proposed to be because of the detachment of the cells and the washing effect. *In vitro* studies showed that superior zinc formula was the most effective tooth paste formula against salivary-derived biofilms and chlorhexidine-containing mouth wash was more effective than essential oils-containing formula while for mono species (*E. coli* C600) biofilms, sodium fluoride-containing tooth paste was the most effective among other tested tooth pastes.
Table 3.5 Significance grids for viability mapping of *E. coli* C600 dosed with eight different antimicrobial formulations.

<table>
<thead>
<tr>
<th></th>
<th>NTC</th>
<th>CT</th>
<th>CP</th>
<th>PH</th>
<th>MP</th>
<th>AEC</th>
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- Green indicates statistically significant difference in viability following exposure and yellow indicates no significant difference (p < 0.05)

Table 3.6 Significance grids for viability mapping of saliva plaques dosed with eight different antimicrobial formulations.

<table>
<thead>
<tr>
<th></th>
<th>NTC</th>
<th>CT</th>
<th>CP</th>
<th>PH</th>
<th>MP</th>
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- Green indicates statistically significant difference in viability following exposure and yellow indicates no significant difference (p < 0.05)

CHAPTER 4

Positive and negative interactions between bacteria derived from the human oral microbiota
4.0 Abstract

Microorganisms that colonise the oral cavity exist in close association. Physical and metabolic interactions are common in the dental plaque biofilm. These interactions include synergistic or promotional interactions which may stimulate the growth of one or more conjoiners, including adhesive and co-operative interactions and antagonistic interactions. Such processes influence the biological activities and pathogenic potential of oral biofilm communities. The current chapter investigates the hypothesis that synergistic interactions between species will be more common than those marked by antagonistic, within bacteria isolated from a human volunteer. Bacteria were isolated from the saliva and different oral sites of the oral cavity namely the tongue, teeth, cheeks inner mucosa, upper palate and gum from a healthy donor and identified by partial 16S rRNA gene sequencing. A modified cross streak method was used to investigate interactions between bacterial species scored as promotional, antagonistic or neutral interactions among paired strains. Furthermore, the coaggregation ability of selected species which resulted in promotional growth interactions was tested using a semi-qualitative coaggregation assay. From a total of 1729 cross-streaks under aerobic and anaerobic conditions, 281 (16%) resulted in either growth promotion 263 (93.6%) or inhibition 18 (6.4%), whereas the remaining 1448 (84%) were neutral. The incidence of positive interactions was significantly higher than the incidence of negative interactions (15.21% vs. 1.04%). Furthermore, the frequency of both promotional interactions and antagonistic interactions were more common between non salivary isolates. Additionally, the incidence of coaggregation among oral site isolates was higher between pairings that had promotional interactions in cross assays. The percentage of coaggregation interactions examined for oral sites isolates showed the strongest degree of coaggregation reactions (52.5%). The frequency of coaggregation among salivary isolates was low with no evidence of correlation with cross-streak results. Mixtures of saliva and oral sites isolates demonstrated positive coaggregation reactions for 14.17% of pairings. In conclusion, promotional interactions between oral isolates from the same mouth dominated. Additionally, the majority of bacteria had the ability to coaggregate with each other and with some fresh saliva isolates however, salivary isolates almost failed to coaggregate with each other or with different oral sites isolates.
4.1 Introduction

The oral cavity has a variety of unique habitats for microbial colonization (Dewhirst, Chen et al. 2010) and it is the only part of the body where hard tissues (the teeth) are exposed to the external environment and facilitate highly diverse surface-adherent communities of microorganisms (biofilms) to adhere and grow on teeth forming dental plaque. Furthermore other surfaces are available for microbial colonization namely the tongue; buccal mucosa; vestibular mucosa; gum and hard palate. All these tissues are immersed in saliva which has a great influence on the microbial ecology of this niche (Mager, Ximenez-Fyvie et al. 2003). In addition, this mode of growth affects both the colonizing species ecology and oral disease development.

Microorganisms colonizing the oral cavity exist in very close association with one another allow them to interact while forming the biofilm structures, carrying out physiological functions, and inducing microbial pathogenesis. These interactions include synergistic interactions, which may stimulate the growth of one or more residents such as adhesive and cooperative interactions that control community composition and stability, which is driven by production of surface adhesins that allows colonization to host tissues and adherence of different bacterial species to one another with high affinity (Breznak, Cooksey et al. 1984; Jenkinson and Lamont 2005). Antagonistic interactions reportedly influence composition, biological activities of microorganisms and pathogenic potential of the community (Kreth, Zhang et al. 2008) such as competition between residents for nutrients, production of antagonistic compounds by one species which may inhibit the growth of another, neutralization of a virulence factor produced by one organism by other bacteria and interference in
the growth-dependent signalling mechanisms of one species by another (Kreth, Merritt et al. 2005; Kuramitsu, He et al. 2007; Xiao, Klein et al. 2012). It has been reported that high levels of *Streptococcus mutans* in the oral cavity are related significantly to low levels of *Streptococcus sanguinis* due to antagonism (Loesche, Rowan et al. 1975; Mikx, van der Hoeven et al. 1976; Caufield, Dasanayake et al. 2000).

### 4.2 Diversity of the oral microbiota

One of the most complex bacterial communities in the human body is the oral microbiome including from 500 - 700 prevalent taxa at species level (Paster and Dewhirst 2009; Dewhirst, Chen et al. 2010), with different subsets predominating at different niches (Dewhirst, Chen et al. 2010). According to current estimates, approximately half of these species are uncultured. Bacterial community composition varies from one person to another and may be influenced by many factors (Li, Ge et al. 2007; Bik, Long et al. 2010). It has been reported that 200 to 300 species may reside in any individual but, depending on the local environment, the profile varies between oral sites (Fabian, Fejerdy et al. 2008; Wade 2013). The composition of the community can be different between individuals with functionally similar but genetically different species colonizing a given niche (Avila, Ojcius et al. 2009; Nasidze, Li et al. 2009; Ling, Kong et al. 2010).

### 4.3 Bacteria-bacteria interactions in the oral cavity

#### 4.3.1 Synergistic interactions

The most important phenomenon to keep the attachment of microorganisms and later colonization of the oral cavity is adhesion between cells of different species which is called coaggregation and it is the first step in development of mixed species biofilms on available oral surfaces (Rickard, Gilbert et al. 2003). This process
involves physicochemical aspects depending mostly on surface charge and hydrophobicity as well as presence of adhesins on bacterial cell surfaces (fimbriae, or major outer membrane proteins in Gram negative bacteria and fibrils, fimbriae, pili or prominent cell-wall linked surface proteins in Gram positive bacteria) and distinct binding receptors on host surfaces which result in strong binding (Telford, Barocchi et al. 2006). Another factor affecting the bacterial adhesion and colonization is the host surface. As teeth are highly mineralized structures that mostly reside permanently in the oral cavity, coating of newly erupted or clean teeth with salivary proteins and glycoproteins occurs within minutes, bacteria adhere and establish the primary colonization of the saliva coated surface and account for up to 80% of early plaque. These primary colonizers together with their products provide adhesion opportunities for secondary colonizers and a build-up of the community (Gibbons 1989). On the other hand, epithelial surfaces turn over continuously and bacteria attached to these cells are lost from the mouth. This accounts for the differences observed between the oral epithelia and tooth surface microbiontas both qualitatively and quantitatively (Beachey 1981; Gibbons, Hay et al. 1990; Gibbons 1996).

### 4.3.1.1 Coaggregation

The colonization of pathogenic bacteria in the human oral cavity is most likely dependent on the contact and interaction of commensal organisms and pathogens and these interactions are essential to the growth of plaque. These interactions are initiated by physical contact which is followed by metabolic exchange, quorum sensing and genetic material exchange. A salient feature of human oral bacteria is their ability to interact with other oral bacteria by coaggregation (Kolenbrander 1988) and it is now considered that most isolated oral bacteria have the ability to aggregate to at least one other bacteria from the oral cavity (Kolenbrander 2000).
Coaggregation or inter-bacterial aggregation was firstly reported in the seventies by Gibbons and Nygaard and defined as the specific cell-to-cell recognition and adhesion between genetically distinct bacteria. It was shown that there is specificity in this phenomenon since bacteria are selective in terms of coaggregation. Bacterial cells coaggregate with some partners, but not with others (Gibbons and Nygaard 1970). Additionally, the understanding of the coaggregation profile of each strain is shedding light on the role of this ability in terms of colonisation ability (Shen, Samaranayake et al. 2005; Kolenbrander, Palmer et al. 2010).

4.3.1.2 Specificity of coaggregation

Pairwise coaggregation tests of many bacterial strains have previously been carried out and showed a variety of coaggregation profiles. For example, bacterial strains can coaggregate with specific sets of partner strains. This specificity is not related to the Gram reaction since it was found that some Gram-positive bacteria coaggregate with both Gram-positive and Gram-negative strains.

Additionally, it has been observed that coaggregation is not universal amongst bacteria and is restricted to some genera for example Fusobacterium; and upon this selectivity a number of set of partner strains are produced (Kolenbrander, Andersen et al. 1985). A good example of a highly specific coaggregation partnership involves Streptococcus spp. and Actinomyces spp. both of which are primary colonizers in the human oral cavity as shown in Figure 4.1 (Kolenbrander, Palmer et al. 2006). It has been confirmed that each coaggregation interaction is mediated by one or more complementary sets of adhesion-receptor pairs (Kolenbrander and London 1992; Kolenbrander, Palmer et al. 2006). Moreover, these interactions are not specific at strain level and may be inter-generic.
4.3.1.3 Coaggregation competition

In certain cases multiple cell types recognize similar receptors on the common coaggregation partner and in this state both compete for binding (Kolenbrander, Andersen et al. 1985) which may explain why the use of probiotic bacteria in oral cavity is arising (Mukai, Asasaka et al. 2002).
Fig 4.1 Diagrammatic representation of the coaggregation between members of the six streptococcal coaggregation groups (numbered circles) and the six actinomycetes coaggregation groups (lettered oblong shapes). Adapted from Kolenbrander et al. (2006)
4.3.1.4 The inhibition of Coaggregation

It has been reported that attachment in oral and aquatic bacteria is mediated by lectin-O-Saccharide interactions. During coaggregation one aggregated partner should bear the carbohydrate and the other bear the complementary lectin which creates a lectin-saccharide interaction. This can be blocked by the addition of simple sugars (Ledder, Madhwani et al. 2009; Jacobs and Chenia 2011) and can be inactivated by heating at 85°C for 30 minutes. However there are other non–lectin adhesins involved in some interactions, namely protein-protein interactions (Kolenbrander and London 1992; Rickard, Gilbert et al. 2003).

4.3.2 Antagonistic interactions

One of the main factors that play a role in defining the construction and activity of the oral biofilm is competition between species of the community. The presence of bacterial species in close proximity results in a limitation of resources and as a result members of the biofilm may compete for resources. Occasionally competition will involve one species actively inhibiting the growth of others either by (i) the end product of one species directly inhibits the growth of another species or organism in the community (plaque) by production of organic acids (lactic acid and citric acids) that inhibit the growth of acid-sensitive organisms (Donoghue and Tyler 1975; Tong, Zhou et al. 2012), (ii) production of hydrogen peroxide ($H_2O_2$) which is toxic to number of bacteria lacking some enzymes to detoxify oxygen (Kreth, Merritt et al. 2005; Kreth, Zhang et al. 2008) or (iii) production of some antibiotics (bacteriocins) (Riley and Wertz 2002; Chatterjee, Paul et al. 2005; Nes, Diep et al. 2007).

Species producing the inhibitory agents will dominate within a mixed biofilm and subsequently the architecture of the community (Rao, Webb et al. 2005).
4.4 Bacteria-surface interactions in the oral cavity

There are different bacterial ecosystems in the oral cavity according to the ecological niches including bacteria in the saliva, bacteria on the tongue, bacteria on the buccal mucosa, supragingival plaque and subgingival plaque (Morhart and Fitzgerald 1976; Quirynen, Gizani et al. 1999). It has been shown that most of the bacteria attached to the tooth exist in biofilms which contains more than 30 categories of microorganisms (Whittaker, Klier et al. 1996). Moreover, the maintenance of homeostasis within the oral cavity is largely mediated by the host salivary immune system and the serum immune system (Ebersole, Taubman et al. 1987; Tenovuo 1998; Mathews, Jia et al. 1999).

The oral cavity is lined with a mucous membrane which consists of mucosa (epithelia and lamina propia) and submucosa (Squier and Hill 1989). These tissues line the oral cavity and work to protect the deeper tissue, protect the body from infection and produce secretions such as mucus. There are different types of tissues according to the site for example, around the tongue, cheeks and hard palate the tissues may be keratinized or non-keratinized (Roedpete and Renstrup 1969). In addition, a third type of tissue exists which consists of highly specialized cells called the taste buds. The epithelium is a non-keratinized stratified squamous epithelium with variability in the thickness according to the location in the mouth. The lamina propia is separated from the epithelium by a basement membrane and the most common cell type in the lamina and submucosa is the fibroblast in addition to macrophages and mast cells. Plasma cells, lymphocytes and neutrophils may be present under specific conditions such as inflammation (Douglas 2009). Furthermore, in contribution to regional variations in the oral cavity there are differences in both the epithelium and the underlying connective tissue which affects
the permeability of these regions and trans-mucosal adsorption (Landay and Schroeder 1979).

In general, the oral microbiome adapts to the mucosa epithelium and the connective tissues that act as a barrier against microbial toxic substances such as proteases and also serve as a physical barrier to these microbes (Rouabhia, Ross et al. 2002). It has been recognized that some cells play a role in the development of protective immunity against intracellular pathogens (Weinberg, Krisanaprakornkit et al. 1998; Mencacci, Bacci et al. 2000) by producing antimicrobial proteins, including definsins (Boyaka, Lillard et al. 1999). Accordingly, it can be concluded that the role of the oral mucosa is both immunological and biochemical rather than totally a physical barrier.

Specific and non-specific mucosal immunity contributes to the maintenance of oral health by limiting infection through continuous host monitoring of this microbiota (Smith and Taubman 1992). The innate host defence system prevents bacterial intrusion into host tissues although some bacteria have evolved mechanisms to evade this response by monitoring their surroundings and consequently modifying the host as desired (Kagnoff and Eckmann 1997; Darveau 2000; Huang, Kim et al. 2001). An adaptation exists between the colonizing organisms and the niches offered by the oral cavity for their attachment creating a homeostasis between the microbes present both as free microorganisms and dental plaque and the host (Janeway 1992).
4.5 Aims and objectives

Since synergistic and antagonistic interactions are essential to the formation, development and shape of oral biofilms, this chapter focuses on the nature of bacterial interactions between individual oral isolates. It was hypothesized that synergistic interactions between species predominate antagonistic ones especially interactions that facilitate a robust coexistence (Periasamy and Kolenbrander 2009). A modified cross streak method was adopted as a high throughput technique to investigate interactions between bacterial species isolated from the human oral cavity including species isolated from saliva and different oral sites (hard and soft tissues). This method is used for screening of antagonism (Lertcanawanichakul and Sawangnop 2011) but in the current chapter it was adapted for assessment of both growth antagonism and promotion by using a high cell density and enforced proximity of two tested species that facilitate the essential environments needed for synergistic (cross feeding, coordinate to better utilize nutrients) and antagonistic interactions (release of antimicrobials or compete for nutritional resources). Secondly, since the ability of oral bacteria to co-aggregate may be significant factor influencing biofilm formation by affecting the localization of species throughout a biofilm (Elias and Banin 2012), it was postulated that the frequency of coaggregation would be higher between combinations of species resulted in promotional growth interactions than ones resulted in antagonistic interactions.
4.6 Materials and methods

4.6.1 Isolation of oral bacteria
Samples of different oral sites and fresh saliva were obtained from a healthy subject who had received no recent antibiotic therapy or invasive dental treatment for at least one year before collecting the samples as mentioned in Sections 2.5.1 and 2.5.2. Isolation and enumeration of functional bacterial groups of saliva and oral samples were done as explained in Section 2.5.3.

4.6.2 Identification of oral bacterial isolates
In order to identify the isolates, bacterial DNA was extracted by boiling suspension of the samples for 5 min then centrifuging for 10 min at 11270 xg (Section 2.10.1.). DNA from the supernatant was amplified using primers directed to the V2-V3 region of eubacterial 16S rDNA namely 8FPL1 (5'-GAG TTT GAT CCT GGC TCA G-3') and 806R (5'-GGA CTA CCA GGG TAT CTA AT-3') (McBain, Bartolo et al. 2003) purchased from (Eurofins Genomics GmbH, Germany) (Section 2.10.2.). The quality and quantity of DNA obtained from extraction and PCR process was assessed by agarose gel electrophoresis (Section 2.10.3.). PCR products were purified using Qiaquick PCR purification kits (Qiagen, West Sussex, UK) (Section 2.10.4.). Sequence analysis was carried out on the purified products and identification took place using European Molecular Biology Laboratories’ (EMBL) prokaryote online database.

4.6.3 Cross-streak method
Bacteria isolated from oral cavity were screened for promotional or inhibitory interactions by a modified cross streak method (Krausse, Piening et al. 2005, Giudice, Brilli et al. 2007, Mangano, Michaud et al. 2009) using a 15x15 (225 tests),
32x32 (1024 tests) array of tests for different human oral sites isolated species and human fresh saliva isolated species, respectively. In addition, cross-activity between bacteria retrieved from different oral sites and saliva was investigated by testing promotional and inhibitory interactions of bacteria from oral sites against those isolated from saliva 15x32 (480 tests) and vice-versa (i.e. each strain was tested against one another). Test strains were grown overnight in Wilkins-Chalgren (10 ml) broth culture either aerobically or anaerobically. On the surface of a Wilkins-Chalgren agar plate 20 µl of each tested bacterial suspension (tester strain) was pipetted in a vertical line about 3cm in length. After drying, 20 µl of a second suspension containing another tested strain (target strain) was pippeted horizontally across the first streak and plates were incubated both aerobically and aerobically for each pair-wise combination at 37ºC for 24 hr. This was repeated for every possible pair-wise interaction resulting in 1729 cross-streaks of isolated strains.

Interactions were analysed visually and distinguished into three different interactivity clusters, termed: positive (growth promotion of one or both strains), negative (growth inhibition of one or both strains) or neutral (no reaction). Each plate was photographed using a digital camera (Cannon, Surrey, UK).

4.6.4 Coaggregation assay

4.6.4.1 Preparation of inocula for the coaggregation assays

Each strain was grown until stationary phase was reached, after which a loopful was transferred into Wilkins-Chalgren broth (10 ml) and incubated either aerobically or anaerobically as desired. After incubation cultures were centrifuged for 20 min at 24000 xg. The deposit was washed three times in coaggregation buffer consisting \([\text{CaCl}_2 \ (1 \times 10^{-4} \ M), \ \text{MgCl}_2 \ (1 \times 10^{-4} \ M), \ \text{NaN}_3 \ (0.02\%) \ \text{and NaCl} \ (0.15 \ M)}\)
(dissolved in 0.001 M Tris adjusted to pH 8.0]) (Cisar, Kolenbrander et al. 1979). The microbial suspensions were adjusted to give an OD600 nm of 1.0.

4.6.4.2 Visual, semi-quantitative assay

The methods followed for the coaggregation assay were according to (Cisar, Kolenbrander et al. 1979, Kolenbrander, Andersen et al. 1985). Autoaggregation was measured by the observation of the degree of aggregation of the bacterial inocula over a period of 60 min. visually. Inter-species coaggregation was evaluated by pairing the bacteria shown in Table (1) as following. Equal volumes (500 µl) of each of two microbial suspensions were mixed in sterile test tubes and mixed for 10 s by pipetting. After mixing, the suspensions were kept at room temperature for 15 min. and scored for coaggregation. The scoring system of Kolenbrander 1995 was used to evaluate the degree of coaggregation in the mixed suspensions by naked eye visualization. All assays were conducted in triplicate. The scoring system was as follows (0) clear suspension with no evidence of aggregation or coaggregation; (+1) finally dispersed detected aggregates; (+2) coaggregates formed immediately but suspended in turbid background; (+3) coaggregates settled rapidly and supernatant remained slightly cloudy; (+4) rapidly large coaggregates formed and settling leaving a water-clear supernatant.
4.7 Results

4.7.1 Identification of human oral cavity isolates

From the closest relatives (based on results of EMBL searches) a total of one hundred and three bacterial isolates incubated under aerobic and anaerobic conditions were identified. For saliva, nineteen strains were grown under anaerobic conditions and thirteen under aerobic conditions and for oral sites isolates six were grown anaerobically and nine were grown aerobically. These were selected for pairwise interactions. Among the 47 bacteria isolated 34% were streptococci (16 isolates), 19.14% were enterococci (9 isolates), 12.8% were lactobacilli, uncultured bacterial clones and staphylococci (6 isolates each) respectively, 4.26% were Acenitobacter (2 isolates), 2.1% were Paenibacillus motobuensis and 2.1% were Micrococcus luteus (1 isolate each). Figure 4.2 shows the strains isolated in genus-level from saliva and different oral sites.

![Figure 4.2 Isolated bacterial strains from human saliva and different oral cavity sites according to genus-level.](chart.png)
Table 4.1 BLAST search results from saliva and different oral sites isolates grown both aerobically and anaerobically subjected to 16SRNA gene sequencing

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a based on EMBL database searches. b max identity describes percentage similarity between the query and subject sequences over the length of the coverage area. QC: Query Coverage.
*Isolates selected for cross streak study.
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\(^a\) based on EMBL database searches. \(^b\) max identity describes percentage similarity between the query and subject sequences over the length of the coverage area. QC: Query Coverage. *Isolates selected for cross streak study. NS=No significant matching data.
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*a* based on EMBL database searches. *Max identity describes percentage similarity between the query and subject sequences over the length of the coverage area, QC: Query Coverage.

*Isolates selected for cross streak study. NS=No significant matching data.
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<td>KF777409.1</td>
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<td>87%</td>
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<td>89%</td>
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<td>93%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>Gmn12*</td>
<td>Gum</td>
<td><em>Streptococcus</em> sp. ChDC B364</td>
<td>KF733688.1</td>
<td>99%</td>
<td>0.0</td>
<td>99%</td>
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</tbody>
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\(^a\) based on EMBL database searches. \(^b\) max identity describes percentage similarity between the query and subject sequences over the length of the coverage area. QC: Query Coverage.

*Isolates selected for cross streak study. NS=No significant matching data.
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<td>Teeth</td>
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<td>0.0</td>
<td>100%</td>
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<td>Gmn12*</td>
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<td>*Streptococcus vestibularis F0396</td>
<td>HM596286.1</td>
<td>96%</td>
<td>0.0</td>
<td>99%</td>
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<td>Cheeks</td>
<td>*Streptococcus sp. JCM 5703</td>
<td>AB690250.1</td>
<td>100%</td>
<td>0.0</td>
<td>99%</td>
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<td>JX678678.1</td>
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<td>99%</td>
</tr>
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</table>

a based on EMBL database searches. b max identity describes percentage similarity between the query and subject sequences over the length of the coverage area, QC: Query Coverage.

*Isolates selected for cross streak study.

Letters in ID column refer to the stock code as following: For saliva: NB=total anaerobes, LB=lactobacilli, SB=streptococci, GB=Gram-negative anaerobes, AB=total aerobes. For different oral sites: Tga, Tgn=isolates from tongue (aerobic and anaerobic), Cka, Ckn=isolates from cheeks inner mucosa (aerobic and anaerobic), Upa, Upn=isolates from upper palate (aerobic and anaerobic), Tha, Thn=isolates from teeth surface (aerobic and anaerobic) and Gma, Gmn=isolates from gum (aerobic and anaerobic).

NS=No significant matching data.

**4.7.2 Growth curves construction for all selected isolates to be used in cross-streak assay and coaggregation assay**

Growth curves were constructed for all isolates (n=47), Figures (4.3-4.6) as described earlier in Section 2.9. All were used to assess the intra- and inter-species coaggregation interactions using a visual, semi-quantitative assay. The time at which the microorganism entered the stationary phase was determined for each isolated strain because microorganism coaggregation abilities are optimum at that phase of growth (Rickard, Leach et al. 2000). Stationary phases generally begun approximately 6 hours post-inoculation (Figures 4.3-4.6).
Figure 4.3 Bacterial planktonic growth rate of strains isolated from saliva by Wilkins Chalgren agar with Gram negative supplement under aerobic incubation (a, b and c) and anaerobic incubation (d). Error bars show standard deviation of optical density of bacteria (n=3).
Figure 4.4 Bacterial planktonic growth rate of strains isolated from saliva by using (a) TYCS media (n=5), (b) Rogosa agar media (n=4) under both aerobic and anaerobic incubation, (c) Wilkins Chalgren agar media under aerobic incubation (n=4) and (d) Wilkins Chalgren agar media under anaerobic incubation (n=6). Error bars show standard deviation of optical density of bacteria (n=3).
Figure 4.5 Bacterial planktonic growth rate of strains isolated from (a) tongue (n=4), (b) teeth (n=3), (c) upper palate (n=2) and (d) cheeks inner mucosa (n=2) under both aerobic and anaerobic incubation. Error bars show standard deviation of optical density of bacteria (n=3).
Figure 4.6 Bacterial planktonic growth rates of strains isolated from gum (n=4) under both aerobic and anaerobic incubation. Error bars show standard deviation of optical density of bacteria (n=3).

4.7.3 Cross-streak assay results of human oral isolates under aerobic and anaerobic conditions

47 species isolated from human fresh saliva (15 strains) and different oral sites (32 strains) were selected for the cross-streak study. Of 1729 cross-streaks done and incubated under aerobic conditions, 281 (16.25%) resulted in either growth promotion or inhibition whereas the remaining 1448 (83.75%) resulted in neither positive nor negative interaction and no mixed interactions were observed as shown in Table 4.2. In addition the incidence of promotional effects (15.21%) was much higher than antagonizing one (1.04%). When the same streak assays were incubated anaerobically, 280 (16.19%) resulted in either growth promotion or inhibition whereas the remaining 1449 (83.81%) resulted in neither positive nor negative interaction as shown in Table 4.3 and no mixed interactions observed. In
addition the incidence of promotional effects (15.33%) was much higher than the antagonizing ones (0.87%).

Table 4.2 Overview of the interactions occurring between all isolates cultured under aerobic conditions

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<th></th>
<th>Promotional</th>
<th></th>
<th>Antagonistic</th>
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<th>Neutral</th>
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<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
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<td>17.33</td>
<td>10</td>
<td>4.44</td>
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<td>Fresh saliva isolates</td>
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<tr>
<td>Different oral sites isolates with Fresh saliva isolates</td>
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<td>35.21</td>
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<td>1.25</td>
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</tbody>
</table>

The frequency of interactions of any type under aerobic and anaerobic conditions appeared to be different in those species incubated aerobically compared to anaerobically incubated ones, but these differences were not considered statistically significant (p= 0.96). Although the promotional interactions for different oral isolates frequency increased up to 50% when the same streaked isolates were incubated anaerobically. In general, the frequency of promotional interactions was higher than the antagonizing interactions both under aerobic or anaerobic conditions for all pair-wise combinations. Examples of synergistic, antagonistic and neutral interactions are shown in Figure 4.7.

Table 4.3 Overview of the interactions occurring between all isolates cultured under anaerobic conditions

<table>
<thead>
<tr>
<th></th>
<th>Promotional</th>
<th></th>
<th>Antagonistic</th>
<th></th>
<th>Neutral</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Different oral sites isolates</td>
<td>68</td>
<td>30.22</td>
<td>11</td>
<td>4.89</td>
<td>146</td>
<td>64.89</td>
</tr>
<tr>
<td>Fresh saliva isolates</td>
<td>48</td>
<td>4.69</td>
<td>1</td>
<td>0.10</td>
<td>975</td>
<td>95.21</td>
</tr>
<tr>
<td>Different oral sites isolates with Fresh saliva isolates</td>
<td>149</td>
<td>31.04</td>
<td>2</td>
<td>0.42</td>
<td>329</td>
<td>68.54</td>
</tr>
</tbody>
</table>
The following tables show some of the cross-streak assays of the selected isolates (fresh saliva and different oral sites) against themselves and against each other under aerobic and anaerobic conditions.
Table 4.4 Overview of positive and negative interactions between bacteria isolated from different oral sites under \textit{aerobic} incubation:

<table>
<thead>
<tr>
<th>Species</th>
<th>E. ludwigii</th>
<th>S. salivarius</th>
<th>E. species Uncultured Streptococcus sp. clone SC004B32</th>
<th>S. mutans</th>
<th>S. species E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species Staph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
<th>S. species</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. ludwigii}</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. salivarius}</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M.Sw_oHS_10/11_8_2(1)</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{E. species} KK1</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Uncultured \textit{Streptococcus} sp. clone SC004B32</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. mutans} p11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. species}</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{E. gergoviae} TyB1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Ps. putida} NCB0308-456</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{E. mori} CB2B2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>\textit{E. species} Cd20b</td>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{Staph. species} O-10</td>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{E. species} CF-S19</td>
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<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. species} SR5</td>
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<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{M. luteus} OS-139</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>\textit{S. species} JCM 5703</td>
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<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(+{)} means synergistic interaction; (-{)} means antagonistic interaction and (0{)} means no interaction of any type.
**Table 4.5** Overview of positive and negative interactions between bacteria isolated from different oral sites under anaerobic incubation:

<table>
<thead>
<tr>
<th>species</th>
<th>E. ludwigii</th>
<th>S. salivarius</th>
<th>E. species</th>
<th>Uncultured Streptococcus sp. clone SC004B32</th>
<th>S. mutans</th>
<th>S. species</th>
<th>E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species</th>
<th>Steph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
<th>S. species</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. ludwigii</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. salivarius</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
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</tr>
<tr>
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<td>0</td>
<td>+</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>+</td>
<td>0</td>
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</tr>
<tr>
<td>Uncultured Streptococcus sp. clone SC004B32</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. mutans p11</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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<td>+</td>
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</tr>
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<td>E. gergoviae TyB1</td>
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<td>+</td>
<td>0</td>
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<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>Ps. putida NCB0308-456</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>+</td>
<td>0</td>
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<td>0</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. mori CB2B2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
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<td>0</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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</tr>
<tr>
<td>Staph. species O-10</td>
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<td>0</td>
<td>-</td>
<td>+</td>
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<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>E. species CF-S19</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>M. luteus OS-139</td>
<td>0</td>
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<td>0</td>
<td>+</td>
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<td>0</td>
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<td>0</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>0</td>
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</tr>
</tbody>
</table>

(+) means synergistic interaction; (-) means antagonistic interaction and (0) means no interaction of any type.
Table 4.6 Overview of the interactions occurring between isolates from fresh saliva selected for cross-streak analysis with isolates from different oral sites under aerobic incubation:

<table>
<thead>
<tr>
<th>Species</th>
<th>Acinet. baumannii</th>
<th>Staph. epidermidis</th>
<th>Strep. salivarius</th>
<th>Staph. aureus</th>
<th>Uncultured bacterium clone Ncd1405g02c1</th>
<th>Strep. agalactiae</th>
<th>Uncultured bacterium clone Ncd871h11c1</th>
<th>P. aeruginosa</th>
<th>Uncultured bacterium clone Sp2-lb12-3H</th>
<th>Strepto. anginosus</th>
<th>Entero. faecalis</th>
<th>Sp2-lib12-3H</th>
<th>L. fermentum</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterobacter ludwigii</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp. ChDC B364</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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</tr>
<tr>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
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</tr>
<tr>
<td><em>Streptococcus</em> sp. ChDC B364</td>
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<td>+</td>
<td>0</td>
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<td>+</td>
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</tr>
<tr>
<td><em>Ps. putida</em> NCB0308-456</td>
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</tr>
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<td>+</td>
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</tr>
<tr>
<td><em>Enterobacter</em> sp. Cd20b</td>
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<td>0</td>
<td>+</td>
</tr>
<tr>
<td><em>Staphylococcus</em> sp. O-10</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td><em>Enterobacter</em> sp. CF-S19</td>
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</tr>
<tr>
<td><em>Streptococcus</em> sp. SR5</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. luteus</em> OS-139</td>
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<td>+</td>
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<td>+</td>
<td>+</td>
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<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus</em> sp. JCM5703</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
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<td>+</td>
<td>+</td>
<td>0</td>
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</tr>
</tbody>
</table>

(+) means synergistic interaction; (-) means antagonistic interaction and (0) means no interaction of any type.
Table 4.7 Overview of the interactions occurring between isolates from fresh saliva selected for cross-streak analysis with isolates from different oral sites under anaerobic incubation:

<table>
<thead>
<tr>
<th>species</th>
<th>Acinet. lwoffii</th>
<th>Staph. epidermidis</th>
<th>Strept. salivarius</th>
<th>Staph. aureus</th>
<th>Uncultured bacterium Ncd1405g02c1</th>
<th>Streptococcus sp.</th>
<th>Entero. faecalis</th>
<th>Uncultured bacterium ncd871h11c1</th>
<th>P. motobuensis</th>
<th>Uncultured bacterium P2b12-3H</th>
<th>Strept. anginosus</th>
<th>Enter. faecium</th>
<th>Staph. aureus</th>
<th>L. fermentum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacter ludwigii</td>
<td>+</td>
<td>+</td>
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<td>0</td>
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<td>+</td>
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<td>0</td>
<td>0</td>
<td>+</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
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<td>+</td>
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<td>0</td>
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<td>-</td>
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<td>Uncultured Streptococcus sp. clone SC004B32</td>
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<td>Streptococcus sp. ChDC B364</td>
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<td>Enterobacter gergoviae TyB1</td>
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<td>Ps. putida NCB0308-456</td>
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<tr>
<td>Enterobacter mori CB2B2</td>
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<td>M. luteus OS-139</td>
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<td>+</td>
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<td>0</td>
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<td>Streptococcus sp. JCM5703</td>
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</tr>
</tbody>
</table>

(+) means synergistic interaction; (-) means antagonistic interaction and (0) means no interaction of any type.
Figure 4.7 Images showing different types of antagonistic, synergistic and neutral cross assay interactions. (a) Growth inhibition of *Paenibacillus motobuensis* (GBb1) by *Streptococcus* sp. JCM 5703 (CKn12), (b) No interaction occurring between *Streptococcus salivarius* (Tga22) and *Staphylococcus aureus* Z1588 (NBc2) and (c) Promotion of *Staphylococcus epidermidis* P8 (GBc9) by *Enterobacter* sp. CF-S19 (Upa32).

Figure 4.8 Cross-streak data comparing the percentage of pairings for each oral isolate that resulted in an antagonistic or synergistic interaction. Open bars, under aerobic incubation; closed bars, under anaerobic incubation.
4.7.4 Visual coaggregation assays

4.7.4.1 Coaggregation ability of oral sites isolates

In the present study visual coaggregation assay scores for the chosen isolates from different sites of the oral cavity namely, isolates from tongue (4), gum (4), upper palate (2), inner mucosa of cheeks (2) and teeth (3) were examined and it was observed that isolates from both the gum and tongue were the most productive co-aggregators. The most coaggregation ability was observed in *Pseudomonas putida* NCB0308-456 (Gma32), which coaggregates with all oral cavity isolates, and mostly scoring +4, +3.

The following pairings are the most considerable (scoring +3, +4): *Pseudomonas putida* NCB0308-456 (Gma32) with all the isolates from the gum namely, *Streptococcus mutans* P11 (Gmn12), *Streptococcus* sp. ChDC B364 (Gmn12*) and *Enterobacter gergoviae* TyB1 (Gma22) (scoring +4). *Streptococcus salivarius* M_Sw_oHS_10/11_8_2 (1) (Tga22) pairings with Uncultured *Streptococcus* sp. clone SC004B32 (Tgn32*) and *Enterobacter sp.* KK1 (Upa22) respectively; *Pseudomonas putida* NCB0308-456 (Gma32), and *Enterobacter mori* CB2B2 (Tha12) (scoring +3) followed by *Enterobacter sp.* KK1 (Upa22) pairings with Uncultured *Streptococcus* sp. clone SC004B32 (Tgn32), *Streptococcus mutans* P11 (Gmn12), *Streptococcus* sp. ChDC B364 (Tgn32) and *Pseudomonas putida* NCB0308-456 (Gma32) (scoring +3) as shown in Figure 4.11.

In addition, *Staphylococcus* sp. O-10 (Thn21) pairings with *Streptococcus mutans* P11 (Gmn12), *Enterobacter gergoviae* TyB1 (Gma22), *Streptococcus* sp. ChDC B364 (Tga22) and *Pseudomonas putida* NCB0308-456 (Gmn12) all scored +3.Finally, *Streptococcus* sp. JCM 5703 (Ckn12) pairings with all gum isolates all scored +3. (Table 4.7).
Figure 4.9 Coaggregation tubes contain suspensions of (left to right) between: *Streptococcus* sp. ChDC B364 (Tga22) and (1) *Enterobacter mori* CB2B2 (Tha12) Scoring +1, (2) *Enterobacter ludwignii* (Tga12) Scoring 0, (3) *Pseudomonas putida* NCB0308-456 (Gma32) Scoring +4, (4) *Staphylococcus* sp. O-10 (Thn21) Scoring +3 and (5) *Streptococcus* sp. ChDC B364 (Tga22) Scoring +2 15 min after mixing.

4.7.4.2 Coaggregation ability of human fresh saliva isolates

Tables 4.9a, b, and c show the results of coaggregation of pairs isolated from saliva which presented that these isolates behaved totally different from oral sites isolates as all of them had no ability to coaggregate with pairings of the isolates from saliva except one strain *Staphylococcus aureus* (GBb10) which coaggregated with all isolates (score +2) except uncultured bacilli bacterium clone MS064A-F08 (NBa2), *Streptococcus* sp. ChDC B363 (NBa1) and *Lactobacillus fermentum* KDLLL2-1 (GBc1). Additionally it has an autoaggregation score (+4).

4.7.4.3 Coaggregation of mixtures of oral sites and saliva isolates

No strong coaggregation ability was observed for the salivary isolates that were paired with isolates form other oral sites.
Table 4.8 Visual coaggregation assay scores of pairs of isolated bacterial species \((n=15)\) from different oral sites:

<table>
<thead>
<tr>
<th>species</th>
<th>E. ludwigii</th>
<th>S. salivarius</th>
<th>E. species</th>
<th>Uncultured Streptococcus sp. clone SC004B32</th>
<th>S. mutans</th>
<th>S. species</th>
<th>E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species</th>
<th>Staph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
<th>S. species</th>
</tr>
</thead>
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<td>S. salivarius</td>
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<td>+3</td>
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<td></td>
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</tr>
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</tr>
<tr>
<td>Uncultured Streptococcus sp.</td>
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<td>+4</td>
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</tr>
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<td>S. mutans</td>
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<td>+3</td>
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<tr>
<td>S. species</td>
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<td>+3</td>
<td>+2</td>
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<td></td>
</tr>
<tr>
<td>E. gergoviae</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Ps. putida</td>
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<td>+3</td>
<td>+3</td>
<td>+1</td>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.9a Visual assay coaggregation scores of pairs of isolated bacterial species (n=11) from saliva:

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<tr>
<th>species</th>
<th>S. species</th>
<th>Acenito. species</th>
<th>E. species</th>
<th>Uncultured bacterium clone nbw1070h04c1</th>
<th>S. anginosus</th>
<th>L. fermentum A011</th>
<th>L. fermentum SM38</th>
<th>L. fermentum FQ022</th>
<th>L. oris F0423</th>
<th>Strepto. sp.</th>
<th>Uncultured bacterium clone070058_178</th>
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</tr>
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<td>0</td>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.9b Visual coaggregation assay scores of pairs of isolated bacterial species (n=11) from saliva:

<table>
<thead>
<tr>
<th>species</th>
<th>Strepto. salivarius</th>
<th>L. sp. oral clone CX036</th>
<th>Strepto. salivarius TYE</th>
<th>Strepto. salivarius SAM3</th>
<th>Staph. aureus Z1588</th>
<th>Uncultured bacilli bacterium clone MS064A-F08</th>
<th>Strepto. sp.</th>
<th>Pa. motobuensis</th>
<th>Uncultured bacterium clone sp2-lib12-3H</th>
<th>S. anginosus</th>
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<td>+2</td>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.9c Visual coaggregation assay scores of pairs of isolated bacterial species (n=10) from saliva:

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<thead>
<tr>
<th>species</th>
<th>E. faecium</th>
<th>Staph. aureus</th>
<th>L. fermentum</th>
<th>Ac. lwofii</th>
<th>Staph. epidermidis</th>
<th>S. salivarius</th>
<th>Staph. aureus</th>
<th>Uncultured bacterium clone ncd 1405g02c1</th>
<th>S. sp.</th>
<th>E. faecalis</th>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.10a Visual coaggregation assay scores of pairs of (15) oral sites isolated strains and (11) saliva isolated strains:

<table>
<thead>
<tr>
<th>species</th>
<th>E. ludwigii</th>
<th>S. salivarius</th>
<th>E. species</th>
<th>Uncultured Streptococcus sp. clone SCC04B2</th>
<th>S. mutans</th>
<th>S. species</th>
<th>E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species</th>
<th>Staph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
<th>S. species</th>
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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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.10b Visual coaggregation assay scores of pairs of (15) oral sites isolated strains and (11) saliva isolated strains:

<table>
<thead>
<tr>
<th>species</th>
<th>E. ludwigi</th>
<th>S. salivarius</th>
<th>E. species</th>
<th>Uncultured Streptococcus sp. clone SC04B32</th>
<th>S. mutans</th>
<th>S. species</th>
<th>E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species</th>
<th>Staph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
Table 4.10c Visual coaggregation assay scores of pairs of (15) oral sites isolated strains and (10) saliva isolated strains:

<table>
<thead>
<tr>
<th>species</th>
<th>E. ludwigii</th>
<th>S. salivarius</th>
<th>E. species</th>
<th>Uncultured Streptococcus sp. clone SC04832</th>
<th>S. mutans</th>
<th>S. species</th>
<th>E. gergoviae</th>
<th>Ps. putida</th>
<th>E. mori</th>
<th>E. species</th>
<th>Staph. species</th>
<th>E. species</th>
<th>S. species</th>
<th>M. luteus</th>
<th>S. species</th>
</tr>
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<tbody>
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<tr>
<td>Strepto. salivarius SAM3</td>
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<tr>
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<tr>
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</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). Coaggregation scores shaded dark to light purple from (+4) to (+1).
isolates except coaggregation scored between *Streptococcus salivarius* M_Sw_oHS_1 0/11 _8_2 (1) (Tga22) isolated from the surface of the tongue with 28 out of 32 saliva isolates mostly scoring (+2) apart from pairing with *Staphylococcus aureus* (GBc3) which scoring (+4). Furthermore *Staphylococcus* sp. O-10 (Thn21) isolated from the surface of the teeth coaggregated with 23 out of 32 saliva isolates though the coaggregation score was ranging between (+1 and +2) as shown in Tables 4.10a,b,c. In conclusion, 787 coaggregation mixtures were tested were pairings from all oral cavity sites, 18.30% of these showed coaggregation ability (n=144). Percentage of coaggregation interactions examined ranged from 6.95% to 52.29%. Overall, 120 pairs of oral sites isolates tested and showed the strongest degree of coaggregation reactions (n=63, 52.5% coaggregation). Some degree of coaggregation (n=13, 6.95% coaggregation) were demonstrated between saliva isolates; pairings tested were 187. Mixtures of saliva and oral sites isolates (480 pairings) demonstrated some positive coaggregation reactions (n=68, 14.17% coaggregation) as shown in Figure 4.10.
Figure 4.10 Percentage of coaggregation reactions among isolated strains from human oral cavity.
4.8 Discussion

The human body comprises numerous indigenous microorganisms at different anatomical sites (Dethlefsen, McFall-Ngai et al. 2007). The oral microbial community is a complex, multispecies microbial community in which complex synergistic and antagonistic interactions occur while establishing themselves in the environment, both under euobiotic or dysbiotic conditions. In the synergistic interactions one resident stimulates the growth of one or more residents; such as cross-feeding (Belenguer, Duncan et al. 2006, Vartoukian, Palmer et al. 2010), competition for nutrients is another type of interaction taking place in addition to antagonistic interactions such as one resident releasing an antagonistic compound that inhibits the growth of another in the same community for example, bacteriocins production (Cintas, Casaus et al. 2001, Zhang, Chu et al. 2011). Moreover, many other interactions regarding virulence and interference with growth-dependent signalling mechanisms (Apolonio, Carvalho et al. 2007, Kuramitsu, He et al. 2007) take place. Additionally, oral biofilm development involves coaggregation and coadhesion of oral bacteria (Kolenbrander, Palmer et al. 2006), intra and inter-species interactions along with host interactions (Kreth, Merritt et al. 2009).

To investigate the interactions between isolates derived directly from the oral microbiota the determination of the frequency of synergistic and inhibitory interactions between bacterial isolates from the same oral cavity was investigated. Subsequently, the adhesion between these isolates was determined by observing their coaggregation ability. Data from this study demonstrated that the frequency of both promotional interactions and antagonistic interactions was greater between isolates from oral sites than salivary isolates under aerobic incubation (21.77% vs 5.57%) and under anaerobic incubation (35.11% vs 4.79%). It was noted that the
percentage of saliva interactions were higher under aerobic incubation and for oral sites isolates this increased under anaerobic conditions by up to one third. Furthermore it was shown that the incidence of promotional interactions was significantly higher than the incidence of antagonistic interactions for both saliva isolates and different oral sites isolates under aerobic (17.3 vs 5.37) and anaerobic (30.22 vs 4.69) environments. The frequency of promotional interactions remains high even when saliva isolates were streaked against different oral sites isolates and vice versa under both aerobic and anaerobic incubation.

There is a predominance of promotional interactions because many oral cavity organisms co-operate for better biofilm establishment as reported by many researchers (Ghigo 2001, Palmer, Kazmerzak et al. 2001, Filoche, Anderson et al. 2004).

Coaggregation is a specific mechanism of bacterial cell-to-cell adhesion and it is the most important step in biofilm establishment which is mediated by specific adhesin-receptor interactions (Rickard, McBain et al. 2004). Bacteria isolated from biofilms have previously been shown to have a higher rate of coaggregation in comparison to planktonic cells (Rickard, Gilbert et al. 2003).

Coaggregation efficiency was evaluated by a semi-qualitative method similar to several previous studies (Kolenbrander and Andersen 1989, Bradshaw, Marsh et al. 1998, Okuda, Kokubu et al. 2012). In this study coaggregation was assessed by a visual score system. The coaggregation ability of bacterial strains isolated from oral microbiota was examined using pair wise combinations.

The majority of isolates from different oral sites have the ability to coaggregate with each other and with some fresh saliva isolates. *Pseudomonas putida* NCB0308-456 (Gma32) being the strongest coaggregator which coaggregates with all isolates
from different oral cavity sites and mostly scoring +4, +3. This can be related to the fact that some strains of *Pseudomonas putida* have a broad metabolic versatility (Wu, Monchy et al. 2011) and are often highly antibiotic resistant strains (Molina, Udaondo et al. 2014). Additionally, Gjermansen, Morten et al. 2006 proved that *Pseudomonas putida* contains a large quantity of proteins that contain the domains GGDEF and EAL which induces the synthesis of biofilm matrix material and biofilm development (Gjermansen, Ragas et al. 2006). The remaining strong pair-wise interactions were between *Streptococcus* species or Enterobacter which is reflected in the fact that streptococci are primary dental plaque biofilm colonizers (Egland, Dû et al. 2001, Takahashi, Ruhl et al. 2002).

Bacterial isolates from human saliva generally did not coaggregate except for *Staphylococcus aureus* which coaggregated with 10 other saliva isolates scoring +2 as shown in Tables 4.9b and c. Moreover, it has an autoaggregation ability of +4. This was expected since a bacterium that cannot coaggregate will be more likely to be removed by salivary flow in the oral cavity. It is logical that saliva will contain microorganisms that lack coaggregation ability. When the isolates from different oral sites were paired with salivary isolates (tested against each other) there was some degree of coaggregation as shown in Tables 4.10 a, b and c. The most notable was that *Streptococcus salivarius* (Tga22) coaggregates with 88% of saliva isolates soring mostly +2 which is in agreement with published data describing streptococci in the oral cavity as predominant initial colonizers (Nyvad and Kilian 1990; Dige, Nilsson et al. 2007; Ding, Palmer et al. 2010) followed by *Staphylococcus* species (Thn21) which coaggregates with 72% of saliva isolates.

In conclusion, this analysis revealed that there was a predominance of promotional interactions between oral isolates from the same mouth. This is likely to
be because oral cavity organisms co-operate together during biofilm development, in common with many other ecosystems. Additionally, the majority of different oral sites isolates have the ability to coaggregate with each other and with some fresh saliva isolates however salivary isolates generally failed to coaggregate with each other or with different oral sites isolates.
CHAPTER 5

Assessing interactions between periodontal pathogens, human oral cells and probiotics
5.0 Abstract

In this chapter, the potential of the candidate dental probiotics *Lactobacillus rhamnosus* LGG, *Lactobacillus reuteri* and *Streptococcus salivarius* K-12 to protect primary human oral keratinocytes and a non-tumour-derived immortalized human oral epithelial cell line from the periodontal pathogens *Fusobacterium nucleatum, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* was assessed. Antagonism between the candidate probiotics and the pathogens was tested in binary culture using well-diffusion and spot on the lawn assays. The ability of live probiotics, lysates and cell filtrates to protect monolayers of the two oral epithelial cells from the cytopathic effects of *F. nucleatum, P. gingivalis* and *A. actinomycetemcomitans* was assessed by challenging confluent cell cultures in the presence or absence of probiotics or their extracts. All probiotics inhibited the growth of pathogens when applied simultaneously, and significantly decreased toxicity (p<0.05, in most mixtures). However there were differences in percentage of viability of treated cells according either to the probiotic pathogen mixture or to the type of treated cell line. The rank order of cytopathic effect for the pathogens was *F. nucleatum > P. gingivalis > A. actinomycetemcomitans* in both cell lines. In general LGG was had the greatest protective effect for both cell lines regardless of probiotic or pathogen used followed by *L. reuteri* and *S. salivarius* is the least. This study showed that tested probiotics and their extracts interact with both epithelial oral cell lines and protect them from the cytopathic effect of periodontal pathogens in addition to their extracts and that *LGG* conferred the best protection.
5.1 Introduction

5.1.1 Probiotics / replacement therapy

5.1.1.1 Replacement therapy

Bacterial replacement therapy or probiosis is an approach proposed replacement of potential pathogenic microorganisms with less virulent genetically modified organisms in the treatment of some diseases (Kailasapathy and Chin 2000). There are some important requirements for this type of therapy to be applied such as the used microorganism must not cause disease, must persistently colonize and should possess a high degree of genetic stability. Probiotics have been applied successfully in intestinal tract and in the vagina to reduce the incidence of some disorders by modulation of bacterial populations (Reid et al, 2003).

5.1.1.2 Probiotics

Recent approach in the management of oral microbiota is the use of probiotics (Haukioja 2010, Bizzini, Pizzo et al. 2012, Di Pierro, Adami et al. 2013, van Essche, Loozen et al. 2013, Pradeep, Kuttappa et al. 2014, Ishikawa, Mayer et al. 2015, Simón-Soro and Mira 2015). Probiotics have been defined according to the World Health Organisation (WHO) as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Joint 2002). These microorganisms belong to the natural human flora and can survive the acidity throughout transportation to the intestines and have been reviewed to achieve many targets in the digestive system such as prevention of cellular adhesion and invasion of pathogens (colonization resistance) and modification of intestinal environment and immune system modulation (Floch, Walker et al. 2011). The clinical potential of probiotics against several diseases has been evaluated by many studies, such as diarrhoea (Hempel, Newberry et al. 2012, Applegate, Walker et al. 2013), necrotizing
entero-colitis inflammatory bowel disease, viral infection and some allergic conditions namely, atopic eczema and rhinitis (Allen, Jordan et al. 2014).

Recently the use of probiotics to selectively remove oral pathogens from the oral microbiota has been evaluated for the prevention of oral diseases, including caries (Saha, Tomaro-Duchesneau et al. 2012, Twetman and Keller 2012, Cagetti, Mastroberardino et al. 2013, Schwendicke, Dörfer et al. 2014, Simón-Soro and Mira 2015).

Several strains used as probiotics including *Lactobacillus rhamnosus* GG, *L. casei*, *L. reuteri*, *L. plantarum*, *L. brevis* CD2, *Bifidobacterium animalis*, *Bifidobacterium lactis*, *Bifidobacterium longum* (Kimoto, Kurisaki et al. 1999, Zhou, Shu et al. 2000) and *Saccharomyces cerevisiae* (Sanders and Huis 1999) have been proposed to reduce incidence of caries, changes in both *mutans Streptococci* and *Lactobacilli* counts in plaque and plaque pH control. One of the uncertain characteristics of the use of the probiotics in oral cavity is whether the probiotics species will colonize the oral cavity and the length of microbial shift induced (Rao, Lingamneni et al. 2011). It was published that for the gastrointestinal tract short time colonization only is achieved (Ravn, Dige et al. 2012).

**5.1.2 Mechanisms of action of probiotics**

Various direct and indirect mechanisms of action have been attributed their purported beneficial effects in humans including, modulation of the mucosal immune system (Ulisse, Gionchetti et al. 2001; Lammers, Helwig et al. 2002), production of antimicrobials (Oh, Kim et al. 2006), enhanced barrier function (Madsen, Doyle et al. 1999), improvement of digestion and food absorption and moreover alteration of the gut microflora (Fioramonti, Theodorou et al. 2003; Hemaishwarya, Raja et al. 2013).
5.1.3 Probiotics and oral cavity

For a microorganism to be an effective oral probiotic it may be important to possess the ability to adhere to oral surfaces and then colonize these surfaces. Various studies have indicated that administered lactobacilli probiotics do not necessarily persist in dental plaque or saliva of individuals after long time consumption (Meurman, Antila et al. 1994; Busscher, Mulder et al. 1999; Krasse, Carlsson et al. 2005). Many studies have however demonstrated that Lactobacilli strains with probiotic properties such as Lactobacillus rhamnosus and Lactobacillus salivarius can be isolated from the oral cavity (Colloca, Ahumada et al. 2000; Teanpaisan and Dahlén 2006; Mayanagi, Kimura et al. 2009; Kaci, Goudercourt et al. 2014; Pradeep, Kuttappa et al. 2014). Lactobacillus species recovered from healthy human saliva include L. rhamnosus, L. salivarius, L. fermentum, L. casei, L. acidophilus and L. plantarum (Teanpaisan and Dahlén 2006). These organisms could play an important role in the microbiological balance in the oral cavity by many mechanisms such as competition for adhesion sites, inhibition of growth of pathogens and influence of local and systemic immune responses (Haukioja 2010). To study the attachment, adhesion and colonization of oral probiotics many model systems have been used, such as saliva-coated hydroxyapatite or hydroxyapatite coated with proteins, buffers or other substances according to the experiment requirement in addition to adhesion to oral epithelial cells. In this chapter the protection of oral epithelial cells from toxicity of some oral pathogens by different probiotic candidates was investigated.

5.1.4 Probiotics and periodontal disease

Recently probiotics has been suggested to have the potential to benefit oral health (Meurman 2005, Çaglar, Kavaloglu Cildir et al. 2006, Meurman and

Periodontitis caused by bacterial infection, predominantly by Gram negative anaerobic bacteria, which involve accumulation of plaque beyond gingival sulcus and host-immune response. When periodontal pathogens interact with periodontal tissues, may invade epithelial cells (Stamatova and Meurman 2009), endothelial cells (Dorn, Dunn et al. 1999) and fibroblasts (Amornchat, Rassameemasmaung et al. 2003). As a result of the lipopolysaccharides secreted by periodontal pathogens such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, the resulting chronic inflammation may affect the integrity of the tissue around the tooth with the recruitment of leucocytes, macrophages and neutrophils (Bhansali, Yeltiwar et al. 2013, Moosani, Sigal et al. 2014). Further investigations about the mechanism of action of purported oral probiotic species are needed.

It has been reported that probiotic species help in controlling and treatment of periodontal disorders caused by *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia* and *Porphyromonas gingivalis*.

Krasse *et al.* (2005) reported that *Lactobacillus reuteri* was effective in controlling gingivitis and plaque in patients with severe gingivitis after two weeks of probiotic intake (Krasse, Carlsson et al. 2005). Another study demonstrated a significant decrease in plaque index after oral administration of *Lactobacillus salivarius WB21* (Shimauchi, Mayanagi et al. 2008). Although it was reported that probiotics possess an antimicrobial against periodontal pathogens *in vitro* (Ishikawa, Aiba et al. 2003, Köll-Klais, Mändar et al. 2005).
5.2 Aims of this chapter

The aims for this chapter were to evaluate a panel of candidate oral probiotics and their extracts isolated from oral cavity, for their ability to protect confluent primary human oral keratinocytes (HOKs) and non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) from the cytopathic effects of the pathogens in the presence or absence of probiotics or their extracts.
5.3 Material and methods

5.3.1 Bacterial characterization

Table 5.1 Strains used in this study

<table>
<thead>
<tr>
<th>Oral pathogens</th>
<th>Bacterial strains</th>
<th>Gram stain</th>
<th>Oxidase reaction</th>
<th>Catalase reaction</th>
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<td></td>
<td><strong>Aggregatibacter actinomycetemcomitans</strong> ATCC 33384</td>
<td>G&lt;sup&gt;−&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><strong>Fusobacterium nucleatum ATCC 10953</strong></td>
<td>G&lt;sup&gt;−&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td><strong>Porphyromonas gingivalis ATCC 33277</strong></td>
<td>G&lt;sup&gt;−&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>_</td>
<td>v</td>
</tr>
<tr>
<td>Probiotic bacteria</td>
<td><strong>Lactobacillus rhamnosus GG ATCC 53103</strong></td>
<td>G&lt;sup&gt;+&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td><strong>Lactobacillus reuteri ATCC 55730</strong></td>
<td>G&lt;sup&gt;+&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td></td>
<td><strong>Streptococcus salivarius K-12</strong></td>
<td>G&lt;sup&gt;+&lt;/sup&gt;v&lt;sup&gt;e&lt;/sup&gt;</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

G<sup>−</sup>v<sup>e</sup> = Gram negative, G<sup>+</sup>v<sup>e</sup> = Gram positive, v = variable

Following the laboratory identification strains were identified by 16s RNA sequencing (Section 2.10.2). After sequence analysis was carried out on the purified PCR products and strains identified using European Molecular Biology Laboratories’ (EMBL) prokaryote online database (Section 2.10.5.).

Growth curves for each pathogenic and probiotic strain respectively were constructed in order to determine the time at which each strain entered the stationary phase after inoculation into broth culture used according to each strain. This is important because in logarithmic or stationary phase some probiotic bacteria may produce antimicrobial agents (bacteriocins) and in some cases the production is reportedly maximal at these phases of growth (Stoffels, Nes et al. 1992; Pilet, Doussset et al. 1995; Ogunbanwo, Sanni et al. 2003). Growth curves were
constructed according to the method mentioned in Section 2.7. The graphs were constructed using the mean values obtained from 9 replicas for each sample as shown in Figure 5.1.

5.3.2 Well-diffusion assay

The ability of *L. rhamnosus* GG ATCC 53103, *L. reuteri* ATCC 55730 and *S. salivarius* K-12 to inhibit the growth of periodontal pathogens *A. actinomycetemcomitans* ATCC 33384, *F. nucleatum* ATCC 10953 and *P. gingivalis* ATCC 33277 was evaluated using a well-diffusion assay. The test organisms were grown to stationary phase (c 10⁸ CFU/ml). The experiment carried out either by using bacterial cell culture, cell lysate or cell-free supernatant. Pathogenic bacteria cultures were diluted 1:100 in Wilkins Chalgren agar and 20 ml agar plates poured and left to set. Once set, cup cuts were done on agar (8 mm) wells and filled with 100 µl of the probiotic cell culture, lysate or cell-free supernatant of each tested probiotic organism. The plates incubated at 37°C anaerobically for 48h and the zones of inhibition calculated by measuring the diameter of the zone of inhibition using a ruler.

5.3.3 Spot-on-the-lawn Assay

Plates of Wilkins Chalgren agar were spotted with 20 µl of an overnight broth culture of *L. rhamnosus* GG ATCC 53103, *L. reuteri* ATCC 55730 and *S. salivarius* K-12. Sterile Wilkins Chalgren broth used as a negative control; plates were then incubated at 37°C anaerobically for 48 h. An overnight culture of *A. actinomycetemcomitans* ATCC 33384, *F. nucleatum* ATCC 10953 or *P. gingivalis* ATCC 33277 was diluted 1:100 in 10ml (0.8%) Wilkins Chalgren agar; mixed well and poured over the plates spotted with the probiotics; incubated anaerobically at
37°C anaerobically for 24 h. Zones of inhibition size around the spots measured by using a ruler.

5.3.4 The effect of probiotic species on the viability of two oral cell lines

Two different human oral cell lines were used namely, Human oral keratinocytes (HOKs, Sciencell Research Laboratories, USA) and Non-tumour-derived, immortalized human oral epithelial cells (GMSM-K) (Gilchrist, Moyer et al. 2000) to assess the effect of probiotics on their viability. Cells were maintained as mentioned in section 2.15 and 2.16. The species selected were *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12. Cells were exposed to $10^8$ CFU/ml of each probiotic cell suspension for 24 h to investigate the effects of probiotics on HOKs and GMSM-Ks viability using trypan blue exclusion assay (section 2.18). Uninfected cells included as a control. Additionally the effects of probiotics lysate and supernatant culture filtrates on the viability of cells were assessed.

5.3.5 The effects of periodontal pathogens on the viability of two oral cell lines

Cells were exposed to $10^6$ CFU/ml of bacterial suspension of *A. actinomycetemcomitans*, *F. nucleatum* or *P. gingivalis* for 24 h to determine their effects on HOKs and GMSM-Ks viability. This was monitored using trypan blue exclusion assay (Section 2.18). Uninfected cells included as a control.
5.4. Results

5.4.1 Growth curves

Growth curves were constructed and used to determine the beginning of stationary phase of probiotics as shown in Figure 5.1. These demonstrate that the probiotic species entered the stationary phases at approximately 11-13 h post inoculation; whereas pathogenic strains began the log phases for *A. actinomycetemcomitans* and *P. gingivalis* at approximately 4h post inoculation and for *F. nucleatum* at 8h post inoculation.
Figure 5.1 Bacterial planktonic growth rates of periodontal pathogens and probiotic strains. Error bars show standard deviation of optical density of bacteria (n=3).

5.4.2 Well-diffusion Assay

The antimicrobial capability of probiotic strains, strains lysate and supernatant culture filtrate were evaluated using well-diffusion assay (Section 5.3.2). Zones of inhibition were visible when all pathogens were treated with cultures or filtrates of probiotics respectively incubated anaerobically as presented in Tables 5.2-5.4. For bacterial suspension of probiotics the greatest effect was observed with *S. salivarius* K-12 against the growth of *A. actinomycetemcomitans* (ZOI = 41 ± 2) and the least was the effect of *L. rhamnosus* GG against *F. nucleatum* (ZOI = 11 ± 2) as shown in Table 5.2. The same pattern observed with probiotic lysates and supernatant culture filtrates regarding the greatest and the least antagonistic effect of probiotic and affected pathogen as shown in Tables 5.3 and 5.4.
Table 5.2 Zones of inhibition (ZOI) of tested pathogenic strains by bacterial suspensions of probiotics by well-diffusion assay (n=3):

<table>
<thead>
<tr>
<th>Organism</th>
<th>L. rhamnosus ZOI (mm)</th>
<th>L. reuteri ZOI (mm)</th>
<th>S. salivarius K-12 ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>11 ± 2</td>
<td>15 ± 1</td>
<td>20 ± 0</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>19 ± 1</td>
<td>15 ± 2</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>33± 2</td>
<td>24 ± 0</td>
<td>41 ± 2</td>
</tr>
</tbody>
</table>

Table 5.3 Zones of inhibition (ZOI) of tested pathogenic strains by lysate derived from probiotics by well-diffusion assay (n=3):

<table>
<thead>
<tr>
<th>Organism</th>
<th>L. rhamnosus ZOI (mm)</th>
<th>L. reuteri ZOI (mm)</th>
<th>S. salivarius K-12 ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>10 ± 0</td>
<td>13 ± 1</td>
<td>18 ± 0</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>17 ± 1</td>
<td>14 ± 2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>27 ± 2</td>
<td>20 ± 1</td>
<td>32 ± 1</td>
</tr>
</tbody>
</table>

Table 5.4 Zones of inhibition (ZOI) of tested pathogenic strains by SCF derived from probiotics by well-diffusion assay (n=3):

<table>
<thead>
<tr>
<th>Organism</th>
<th>L. rhamnosus ZOI (mm)</th>
<th>L. reuteri ZOI (mm)</th>
<th>S. salivarius K-12 ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>10 ± 1</td>
<td>15 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>09 ± 1</td>
<td>12 ± 0</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>19 ± 1</td>
<td>16 ± 1</td>
<td>28 ± 2</td>
</tr>
</tbody>
</table>
Figure 5.2 Well-diffusion assays.

Representative image of one experiment demonstrating: (a) inhibition of *P. gingivalis* growth by *L. rhamnosus* GG and (b) inhibition of *A. actinomycetemcomitans* by *S. salivarius* K-12. (LGG= *L. rhamnosus* GG, LGG lys= *L. rhamnosus* GG lysate, S. s K12= *S. salivarius* K-12).

5.4.3 Spot-on-the lawn Assay

Data of spot-on-the lawn inhibition assay showed that there was no inhibition effect proven by any of probiotic supernatant filtrate while bacterial suspensions showed large zones of inhibition for all probiotics against all pathogens respectively as presented in Table 5.5. Probiotic lysates showed some degree of inhibition but were very small and not all probiotic lysates inhibit the targeted strains as shown in Table 5.6.
Table 5.5 Zones of inhibition (ZOI) of tested pathogenic strains by bacterial suspensions of probiotics by spot-on-the lawn assay (n=3):

<table>
<thead>
<tr>
<th>Organism</th>
<th>L. rhamnosus ZOI (mm)</th>
<th>L. reuteri ZOI (mm)</th>
<th>S. salivarius K-12 ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>17± 1</td>
<td>13± 1</td>
<td>17± 2</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>11.5</td>
<td>12± 3</td>
<td>14± 1</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>15± 2</td>
<td>11± 0</td>
<td>14± 1</td>
</tr>
</tbody>
</table>

Table 5.6 Zones of inhibition (ZOI) of tested pathogenic strains by lysate derived from probiotics by spot-on-the lawn assay (n=3):

<table>
<thead>
<tr>
<th>Organism</th>
<th>L. rhamnosus ZOI (mm)</th>
<th>L. reuteri ZOI (mm)</th>
<th>S. salivarius K-12 ZOI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. nucleatum</td>
<td>2± 1</td>
<td>1± 0</td>
<td>4± 1</td>
</tr>
<tr>
<td>P. gingivalis</td>
<td>2± 1</td>
<td>0</td>
<td>3± 1</td>
</tr>
<tr>
<td>A. actinomycetemcomitans</td>
<td>1± 1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 5.3 Spot-on-the lawn assays.

Representative image of one experiment demonstrating: (a) inhibition of *P. gingivalis* growth by LGG, L. r. and S. k12; (b) inhibition of *F. nucleatum* by LGG, L. r. and S. k12 (LGG= *L. rhamnosus* GG, L. r.= *L. reuteri*, S. s K12= *S. salivarius* K-12).
5.4.4 The effect of probiotic species on the viability of HOKs and GMSM-Ks

The viability of human oral keratinocytes (HOKs) and non-tumour-derived, immortalized human oral epithelial cells (GMSM-Ks) in presence of three different probiotics was assessed. The selected species were *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12; at a concentration of $10^8$ CFU/ml. As a result the exposure of HOKs to *L. rhamnosus* GG and *L. reuteri* did not cause significant cell death with 92% ±0.45 ($p=0.07$, n=3) and 93% ±0.9 ($p=0.05$, n=3) respectively compared to non-treated cells following 24h incubation. However, the viability of monolayers treated with *S. salivarius* K-12 reduced significantly to 82% ±0.24 ($p=0.024$, n=3) compared to 97% ±0.2 in control monolayer as shown in Figure 5.4.

**Figure 5.4** The effect of different probiotic strains on human oral keratinocytes viability (HOKs).

LGG and LR did not cause HOKs death with 92% ±0.45 ($p=0.07$, n=3) and 93% ±0.9 ($p=0.08$, n=3) respectively of cells remaining viable following 24h incubation. However SK12 reduced HOKs monolayer viability significantly to 82% ±0.24 ($p=0.024$, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *$p<0.05$. 
The same assessment applied on GMSM-Ks and the results were the same as there was no significant decrease in the viability of monolayers treated with both *L. rhamnosus* GG, *L. reuteri* with 93% ±0.63 and 91% ±0.9 (p=0.16, n=3) respectively of cells remaining viable following 24h incubation. However *S. salivarius* K-12 reduced GMSM-Ks monolayer viability significantly to 69% ±0.71 (p=0.006, n=3) compared to 97% ±0.19 in control monolayer as presented in Figure 5.5. Although there was a significant killing effect of both monolayers treated with *S. salivarius* K-12 but used alongside the lactobacilli species as they widely used in oral products to investigate there effect in protecting both monolayers from toxic effect of oral periodontal pathogens.

**Figure 5.5** The effect of different probiotic strains on Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks).

LGG and LR did not caused GMSM-Ks death with 93% ±0.63 and 91% ±0.9 (p=0.16, n=3) respectively of cells remaining viable following 24h incubation. However SK12 reduced GMSM-Ks monolayer viability significantly to 69% ±0.71 (p=0.006, n=3) compared to 97% ±0.19 in control monolayer. Results are expressed as mean ±SEM, p<0.05.
5.4.5 The effects of oral pathogens on the viability of HOKs and GMSM-Ks

When control (uninfected) HOKs were incubated for 24h, approximately 97% ±0.2 of the cells remained viable; whereas, the percentage of HOKs that remained viable following 24h infection with $10^6$ CFU/ml of three selected different periodontal pathogens respectively was significantly lower. *F. nucleatum* decreased the viability of treated cells to 34% ±0.75 ($p=0.00027$, n=3), *P. gingivalis* decreased the viability of treated cells to 40% ±0.78 ($p=0.00041$, n=3) and *A. actinomycetemcomitans* decreased the viability to 51% ±0.97 ($p=0.0091$, n=3) as presented in Figure 5.6.

![Figure 5.6](image_url)

**Figure 5.6** The effect of different periodontal pathogens on human oral keratinocytes (HOKs) viability.

*A. actinomycetemcomitans* decreased the viability of HOKs to 51% ±0.97 ($p=0.0091$, n=3), *P. gingivalis* decreased the viability of treated cells to 40% ±0.78 ($p=0.00041$, n=3) and *F. nucleatum* decreased the viability of treated cells to 34%±0.75 ($p=0.00027$, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p<0.05.*
Control (uninfected) GMSM-Ks were incubated for 24h and approximately 97% ±0.19 of the cells remained viable; whereas, the percentage of GMSM-Ks that remained viable following 24h infection with $10^6$ CFU/ ml of three selected different periodontal pathogens respectively was significantly low as shown in Figure 5.7. *F. nucleatum* decreased the viability of treated cells to 13% ±0.1 ($p=0.00021$, n=3), *P. gingivalis* decreased the viability of treated cells to 21% ±0.2 ($p=0.0005$, n=3) and *A. actinomycetemcomitans* decreased the viability to 52% ±0.33 ($p=0.00034$, n=3).

**Figure 5.7** The effect of different periodontal pathogens on human oral keratinocytes (GMSM-Ks) viability.

*A. actinomycetemcomitans* decreased the viability of GMSM-Ks to 52% ±0.33 ($p=0.00034$, n=3), *P. gingivalis* decreased the viability of treated cells to 21% ±0.2 ($p=0.0005$, n=3) and *F. nucleatum* decreased the viability of treated cells to 13% ±0.1 ($p=0.00021$, n=3) compared to 97% ±0.19 in control monolayer. Results are expressed as mean ±SEM, *$p<0.05$*.
5.4.6 Specific probiotic species protect HOKs from the toxic effect of tested periodontal pathogens

The ability of \textit{L. rhamnosus} GG, \textit{L. reuteri} and \textit{S. salivarius} K-12 to protect HOKs from the effects of \textit{F. nucleatum}, \textit{P. gingivalis} and \textit{A. actinomycetemcomitans} was investigated. HOKs were exposed simultaneously to a combination of $10^6$ CFU/ml of pathogen strains respectively and $10^8$ CFU/ml of each probiotic. After 24h incubation of treated monolayers with each of mixtures of pathogens and probiotics respectively it has been demonstrated that there was a significant higher percentage of viability with some tested combinations and some insignificant effects.

\textit{A. actinomycetemcomitans} combined with \textit{L. rhamnosus} GG, \textit{L. reuteri} or \textit{S. salivarius} K-12 increased the viability of the cells to (90% ±0.84, \textit{p}=0.014; 65% ±0.27, \textit{p}=0.12 and 53% ±0.22, \textit{p}=0.45 respectively, \textit{n}=3) as shown in Figure 5.8; in comparison to monolayers infected with \textit{A. actinomycetemcomitans} presented previously in Figure 5.5. Treatment of monolayers with a combination of \textit{P. gingivalis} with \textit{L. rhamnosus} GG, \textit{L. reuteri} or \textit{S. salivarius} K-12 changes the viability of cells in comparison to treated cells with \textit{P. gingivalis} alone (40% ±0.78, \textit{p}= 0.00041, \textit{n}=3) as illustrated in Figure 5.9. HOKs treated with a combination of \textit{F. nucleatum} with \textit{L. rhamnosus} GG, \textit{L. reuteri} or \textit{S. salivarius} K-12 increased the viability to (69% ±0.83, \textit{p}= 0.0005; 74% ±0.65, \textit{p}= 0.005; 81% ±0.66, \textit{p}= 0.01 respectively, \textit{n}=3) compared with the viability after treatment with pathogen alone (34% ±0.75, \textit{p}= 0.00027) as shown in Figure 5.10.
**Figure 5.8** The effect of *A. actinomycetemcomitans* on human oral keratinocytes (HOKs) viability.

*A. actinomycetemcomitans* decreased the viability of HOKs to 51% ±0.97 (*p*= 0.0091, n=3), *A. actinomycetemcomitans* combined with *L. rhamnosus GG, L. reuteri* or *S. salivarius K-12* increased the viability of the cells to (90% ±0.84, *p*=0.014; 67% ±0.31, *p*=0.12 and 55% ±0.71, *p*=0.45 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
Figure 5.9 The effect of *P. gingivalis* on human oral keratinocytes (HOKs) viability.

*P. gingivalis* decreased the viability of HOKs to 40% ± 0.78, (*p* = 0.00041, n=3). *P. gingivalis* combined with *L. rhamnosus GG, L. reuteri* or *S. salivarius* K-12 increased the viability of the cells to (75% ± 0.46, *p*=0.001; 77% ± 0.08, *p*=0.0025 and 81% ± 0.01, *p*=0.06 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
Figure 5.10 The effect of *F. nucleatum* on human oral keratinocytes (HOKs) viability.

*F. nucleatum* decreased the viability of HOKs to 34% ±0.75, (p= 0.00027, n=3). *F. nucleatum* combined with *L. rhamnosus GG*, *L. reuteri* or *S. salivarius K-12* increased the viability of the cells to (79% ±0.83, *p*=0.0005; 74% ±0.65, *p*=0.005 and 81% ±0.67, *p*=0.01 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
5.4.7 Specific probiotic species protect GMSM-Ks from the toxic effect of tested periodontal pathogens

The ability of *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12 to protect GMSM-Ks from the effects of *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* was investigated. GMSM-Ks were exposed simultaneously to a combination of $10^6$ CFU/ml of pathogen strains respectively and $10^8$ CFU/ml of each probiotic. After 24h incubation of treated monolayers with each of pathogens combined with each of probiotics respectively it has been demonstrated that there was a significant higher percentage of viability with some tested combinations and some insignificant effects.

*A. actinomycetemcomitans* combined with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 increased the viability of the cells to (76% ±0.74, $p=0.07$; 71% ±0.73, $p=0.09$ and 67% ±0.84, $p=0.08$ respectively, n=3) as shown in Figure 5.11; than monolayers infected with *A. actinomycetemcomitans* presented previously in Figure 5.7. Treatment of monolayers with a combination of *P. gingivalis* with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 changes the viability of cells to (64% ±0.05, $p=0.075$; 51% ±0.79, $p=0.077$ and 65% ±0.51, $p=0.16$ respectively, n=3) in comparison to treated cells with *P. gingivalis* alone (21% ±0.12, $p= 0.0005$, n=3) as illustrated in figure 5.12. GMSM-Ks treated with a combination of *F. nucleatum* with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 increased the viability to (65% ±0.58, $p= 0.0067$; 72% ±0.86, $p= 0.005$; 61% ±0.32, $p= 0.04$ respectively, n=3) compared with the viability after treatment with pathogen alone (13% ±0.1, $p= 0.0002$) as shown in Figure 5.13.
Figure 5.11 The effect of *A. actinomycetemcomitans* on Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) viability.

*A. actinomycetemcomitans* decreased the viability of GMSM-Ks to 50% ±0.89 (*p* = 0.0003, n=3). *A. actinomycetemcomitans* combined with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 increased the viability of the cells to (76% ±0.74, *p*=0.07; 71% ±0.73, *p*=0.09 and 67% ±0.84, *p*=0.08 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
Figure 5.12 The effect of *P. gingivalis* on Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) viability.

*P. gingivalis* decreased the viability of GMSM-Ks to 21% ±0.12 (*p* = 0.0005, n=3). *P. gingivalis* combined with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 increased the viability of the cells to (64% ±0.05, *p*=0.003; 51% ±0.79, *p*=0.041 and 65% ±0.51, *p*=0.017 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
**Figure 5.13** The effect of *F. nucleatum* on Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) viability.

*F. nucleatum* decreased the viability of GMSM-Ks to 13% ±0.1, (*p* = 0.0002, n=3). *F. nucleatum* combined with *L. rhamnosus GG*, *L. reuteri* or *S. salivarius K-12* increased the viability of the cells to (65% ±0.58, *p* = 0.0067; 72% ±0.86, *p* = 0.005; 61% ±0.32, *p* = 0.04 respectively, n=3) compared to 97% ±0.2 in control monolayer. Results are expressed as mean ±SEM, *p*<0.05.
5.4.8 The effect of probiotic species lysates and spent culture fluid on HOKs and GMSM-Ks monolayers

It was confirmed after screening for protection of probiotic candidate to both oral tested cell lines from infection of periodontal pathogens that there is a degree of protection by some of tested probiotics, though there were differences in the degree of protection according to pathogen probiotic combination investigated. Subsequently, the effects of bacterial lysates (Section 2.6) and spent culture fluid (Section 2.5) were investigated to test whether a live organism is required for the protection effect. At first bacterial lysates and spent culture fluid (extracted from $10^8$ CFU/ml of each tested probiotic respectively) impact on the cell lines were screened and it was found that for *L. rhamnosus GG* lysate and spent culture fluid on HOKS there was no impact significantly on the viability of monolayers ($p=0.096$ and $p=0.069$, n=3 respectively) as shown in Figure 5.14 (a) and for GMSM-Ks were ($p=0.007$ and $p=0.01$, n=3 respectively) as presented in Figure 5.14 (b) while *S. salivarius K*-12 lysate or spent culture fluid did significantly decrease the viability of both cell lines the same as the live bacteria did. All results showed in Figure 5.14 (a) and (b).

5.4.9 The effect of probiotic species lysates and spent culture fluid on HOKs and monolayers infected with periodontal pathogens

For both *P. gingivalis* and *F. nucleatum* all probiotics extracts reduced the toxicity of pathogens to the extent that the viability of HOKs was for *P. gingivalis* combined with *L. rhamnosus GG* lysate and spent culture fluid(67% ±0.80 $p=0.01$ and 60% ±0.81 $p=0.003$, n=3 respectively) and with *L. reuteri* lysate or spent culture
Figure 5.14 (a) Effect of different probiotic strains live bacteria and extracts (lysate and supernatant culture fluid) on human oral keratinocytes viability (HOKs). (b) Effect of different probiotic strains live bacteria and extracts (lysate and supernatant culture fluid) on Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) viability.
Fluid (72% ±0.91 \ p= 0.0087 and 60% ±0.81 \ p=0.003, \ n=3 \ respectively) and finally with \textit{S. salivarius} \textit{K-12} lysate and spent culture fluid the viability increased to (85% ±0.81 \ p= 0.0006 and 81% ±0.92 \ p=0.008, \ n=3 \ respectively) compared with 40%±0.78 in those infected solely with \textit{P. gingivalis} as shown in Figure 5.15.

Bacterial lysate and spent culture fluid of \textit{L. rhamnosus} \textit{GG} reduced the toxicity of \textit{F. nucleatum} to the extent that the viability of infected HOKs was (72% ±0.64 \ p= 0.01 and 70% ±0.11 \ p=0.005, \ n=3 \ respectively) compared with 34% ±0.75 \ p=0.00027 in those infected with \textit{F. nucleatum} alone (Figure 5.16). \textit{L. reuteri} lysate or spent culture fluid (74% ±0.65 \ p= 0.005 and 61% ±0.46 \ p=0.026, \ n=3 \ respectively) and finally with \textit{S. salivarius} \textit{K-12} lysate and spent culture fluid the viability increased to (82% ±0.08 \ p= 0.0029 and 73% ±0.49 \ p=0.0015, \ n=3 \ respectively). The effect of probiotic extracts on the toxicity of \textit{A. actinomycetemcomitans} on HOKs was different from the effects of \textit{L. rhamnosus GG, L. reuteri} and \textit{S. salivarius} \textit{K-12} that all were significantly protective while with \textit{A. actinomycetemcomitans; L. rhamnosus GG lysate and spent culture fluid were protective and increasing the viability of cells to (84% ±0.94 \ p= 0.007 and 76% ±0.86 \ p=0.047, \ n=3 \ respectively) compared with 51% ±0.97 in those infected with \textit{A. actinomycetemcomitans} alone. \textit{L. reuteri} lysate was protective and increased reduced the toxicity of pathogen (79% ±0.40 \ p= 0.029, \ n=3) however spent culture fluid increased the viability of the cells in comparison to ones treated with pathogen only but this increase was not significant (68% ±0.97 \ p= 0.12, \ n=3) and finally with \textit{S. salivarius} \textit{K-12} lysate and spent culture fluid the viability increased to (63% ±0.85 \ p= 0.22 and 75% 0.22 \ p=0.057, \ n=3 \ respectively). Data presented in Figure 5.17.
Figure 5.15 All probiotics lysates and spent culture fluids protect HOKs from *P. gingivalis*.

HOKs were treated with either live *L. rhamnosus* GG (P.g.+LGG) or a lysate (P.g.+LGG LYS) or a spent culture fluid (P.g.+LGG SCF) simultaneously with *P. gingivalis*; treated with either live *L. reuteri* (P.g.+LR) or a lysate (P.g.+LR LYS) or a spent culture fluid (P.g.+LR SCF) simultaneously with *P. gingivalis* or treated with *S. salivarius K*-12 either live (P.g.+SK-12) or a lysate (P.g.+SK-12 LYS) or a spent culture fluid (P.g.+SK-12 SCF) simultaneously with *P. gingivalis*.

In all cases, the percentage of HOKs remaining viable after 24h incubation with *P. gingivalis* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM,* p<0.05, NS= non-significant.
Figure 5.16 All probiotics lysates and spent culture fluids protect HOKs from *F. nucleatum*.

HOKs were treated with either live *L. rhamnosus GG* (F.n.+LGG) or a lysate (F.n.+LGG LYS) or a spent culture fluid (F.n.+LGG SCF) simultaneously with *F. nucleatum*; treated with either live *L. reuteri* (F.n.+LR) or a lysate (F.n.+LR LYS) or a spent culture fluid (F.n.+LR SCF) simultaneously with *F. nucleatum* or treated with *S. salivarius K-12* either live (F.n.+SK-12) or a lysate (F.n.+SK-12 LYS) or a spent culture fluid (F.n.+SK-12 SCF) simultaneously with *F. nucleatum*.

In all cases, the percentage of HOKs remaining viable after 24h incubation with *F. nucleatum* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM,* p<0.05, NS= non-significant
Figure 5.17 All probiotics lysates and spent culture fluids protect HOKs from *A. actinomycetemcomitans*.

HOKs were treated with either live *L. rhamnosus* GG (A.a.+LGG) or a lysate (A.a.+LGG LYS) or a spent culture fluid (A.a.+LGG SCF) simultaneously with *A. actinomycetemcomitans*; treated with either live *L. reuteri* (A.a.+LR) or a lysate (A.a.+LR LYS) or a spent culture fluid (A.a.+LR SCF) simultaneously with *A. actinomycetemcomitans* or treated with *S. salivarius* K-12 either live (A.a.+SK-12) or a lysate (A.a.+SK-12 LYS) or a spent culture fluid (A.a.+SK-12 SCF) simultaneously with *A. actinomycetemcomitans*.

In all cases, the percentage of HOKs remaining viable after 24h incubation with *A. actinomycetemcomitans* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM, *p*<0.05, NS= non-significant.
5.4.10 The effect of probiotic species lysates and spent culture fluid on GMSM-Ks and monolayers infected with periodontal pathogens

For both *P. gingivalis* and *F. nucleatum* all probiotics extracts reduced the toxicity of pathogens. The same effect was seen when HOKs treated with the same manner and again only some probiotics and their extracts did not protect cells from the toxicity of *A. actinomycetemcomitans*.

The viability of GMSM-Ks treated with *P. gingivalis* combined with *L. rhamnosus* GG lysate and spent culture fluid was (62% ±0.64 *p*= 0.00057 and 64% ±0.47 *p*=0.0028, *n*=3 respectively) and with *L. reuteri* lysate or spent culture fluid (57% ±0.91 *p*= 0.006 and 53% ±0.83 *p*=0.01, *n*=3 respectively) and finally with *S. salivarius* K-12 lysate and spent culture fluid the viability increased to (58% ±0.35 *p*= 0.007 and 53%± 0.95 *p*=0.01, *n*=3 respectively) compared with 21% ±0.12 in those infected solely with *P. gingivalis* as shown in Figure 5.18.

Bacterial lysate and spent culture fluid of *L. rhamnosus* GG reduced the toxicity of *F. nucleatum* to the extent that the viability of infected GMSM-Ks was (69% ±0.34 *p*= 0.0003 and 65% 0.01 *p*=0.003, *n*=3 respectively) compared with 13% ±0.16 *p*=0.0002 in those infected with *F. nucleatum* alone (Figure 5.19). *L. reuteri* lysate or spent culture fluid (67% ±0.26 *p*= 0.001 and 67% ±0.25 *p*=0.005, *n*=3 respectively) and finally with *S. salivarius* K-12 lysate and spent culture fluid the viability increased to (61% ±0.33 *p*= 0.002 and 63% ±0.22 *p*=0.002, *n*=3 respectively) as shown on Figure 5.19.

The effect of probiotic extracts on the toxicity of *A. actinomycetemcomitans* on GMSM-Ks was different from the effects of *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12 that all were significantly protective to cells treated with *P. gingivalis*.
and *F. nucleatum* while with *A. actinomycetemcomitans; L. rhamnosus* GG lysate and spent culture fluid were protective and increasing the viability of cells to (72% ±0.41 *p= 0.002 and 66% ±0.01 *p=0.018, n=3 respectively) compared with 52% ±0.33 in those infected with *A. actinomycetemcomitans* alone. *L. reuteri* lysate was protective and reduced the toxicity of pathogen (74% ±0.63 *p= 0.007, n=3) however spent culture fluid increased the viability of the cells in comparison to ones treated with pathogen only but this increase was not significant to protect cells from toxicity of pathogen alone (57% ±0.66 *p= 0.06, n=3) and finally with *S. salivarius K-12* lysate the viability increased to (73% ±0.23 *p= 0.0038, n=3) and spent culture fluid did not protect cells from toxicity of pathogen (52% ±0.53 *p= 0.36, n=3). Data presented in Figure 5.20.
Figure 5.18 All probiotics lysates and spent culture fluids protect GMSM-Ks from *P. gingivalis*.

GMSM-Ks were treated with either live *L. rhamnosus GG* (P.g.+LGG) or a lysate (P.g.+LGG LYS) or a spent culture fluid (P.g.+LGG SCF) simultaneously with *P. gingivalis*; treated with either live *L. reuteri* (P.g.+LR) or a lysate (P.g.+LR LYS) or a spent culture fluid (P.g.+LR SCF) simultaneously with *P. gingivalis* or treated with *S. salivarius K-12* either live (P.g.+SK-12) or a lysate (P.g.+SK-12 LYS) or a spent culture fluid (P.g.+SK-12 SCF) simultaneously with *P. gingivalis*.

In all cases, the percentage of GMSM-Ks remaining viable after 24h incubation with *P. gingivalis* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM, *p*<0.05, NS= non-significant.
Figure 5.19 All probiotics lysates and spent culture fluids protect GMSM-Ks from *F. nucleatum*.

GMSM-Ks were treated with either live *L. rhamnosus* GG (F.n.+LGG) or a lysate (F.n.+LGG LYS) or a spent culture fluid (F.n.+LGG SCF) simultaneously with *F. nucleatum*; treated with either live *L. reuteri* (F.n.+LR) or a lysate (F.n.+LR LYS) or a spent culture fluid (F.n.+LR SCF) simultaneously with *F. nucleatum* or treated with *S. salivarius* K-12 either live (F.n.+SK-12) or a lysate (F.n.+SK-12 LYS) or a spent culture fluid (F.n.+SK-12 SCF) simultaneously with *F. nucleatum*.

In all cases, the percentage of GMSM-Ks remaining viable after 24h incubation with *F. nucleatum* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM,* *p*<0.05, NS= non-significant.
Figure 5.20 All probiotics lysates and spent culture fluids protect GMSM-Ks from *A. actinomycetemcomitans*.

GMSM-Ks were treated with either live *L. rhamnosus GG* (A.a.+LGG) or a lysate (A.a.+LGG LYS) or a spent culture fluid (A.a.+LGG SCF) simultaneously with *A. actinomycetemcomitans*; treated with either live *L. reuteri* (A.a.+LR) or a lysate (A.a.+LR LYS) or a spent culture fluid (A.a.+LR SCF) simultaneously with *A. actinomycetemcomitans* or treated with *S. salivarius K*-12 either live (A.a.+SK-12) or a lysate (A.a.+SK-12 LYS) or a spent culture fluid (A.a.+SK-12 SCF) simultaneously with *A. actinomycetemcomitans*.

In all cases, the percentage of GMSM-Ks remaining viable after 24h incubation with *A. actinomycetemcomitans* was significantly higher than in cells treated with pathogen alone. Results expressed as the mean ± SEM, *p*<0.05, NS= non-significant.
5.5 Discussion

Periodontitis is a common inflammatory disease caused by a complex polymicrobial infection causing destruction of bone and teeth-supporting tissue due to the chronic inflammatory reaction (Kadowaki, Takii et al. 2007; Palm, Khalaf et al. 2013). Some previous reports suggesting that the use of probiotic bacteria is a promising approach to treat chronic inflammatory diseases and, since conventional treatments alone may not be sufficient to control periodontitis (Armitage 2002), new strategies are required and probiotics could potentially fulfil this role (Bizzini, Pizzo et al. 2012).

To study the capability of selected probiotic strains (L. rhamnosus GG, L. reuteri and S. salivarius K-12) to be used as a proposed therapy to periodontal disorders, an initial investigation to their ability to inhibit the growth of some oral pathogens (A. actinomycetemcomitans, F. nucleatum and P. gingivalis) was investigated. It is well known that probiotics can inhibit the growth of some pathogenic bacteria as proven by many researchers (Dasari, Shouri et al. 2014; Gomes, Miyazak et al. 2015; Gómez, Ramiro et al. 2016; Amat, Subramanian et al. 2017). Some published data have shown that probiotic L. reuteri ATCC 55730 affected the growth of S. mutans and consequently reduced the incidence of cavities (Astekar, Sidhu et al. 2014; Cortés-Dorantes, Ruiz-Rodríguez et al. 2015; Nozari, Motamedifar et al. 2015). On further research Nissen et al. 2014 have been reported that Lactobacillus salivarius and L. gasseri have the ability to reduce the virulence of A. actinomycetemcomitans which is one of the putative oral pathogens (Nissen, Sgorbati et al. 2014).

Well-diffusion assays and spot-on-the lawn assays were performed for the selected probiotic strains against three different indicator periodontal pathogens
(Sections 5.3.2 and 5.3.3). All the tested probiotics and their extracts did inhibit the growth of pathogens (Tables 5.2-5.6) and (Figures 5.2 and 5.3).

Furthermore, this work investigated whether the selected probiotic candidates could potentially be used to protect oral host tissues (epithelial oral cells) from the cytopathic effect of oral pathogens using primary cell line (human oral keratinocytes) and immortalized cell line (non-tumour-derived immortalized human oral epithelial cells) as models. Generally mucosal epithelial cells have many functions including its role in the innate immune system and generation of molecules affecting growth, development and function of itself and other cells in addition to maintaining the balance between health and disease (Kagnoff and Eckmann 1997; Okada and Murakami 1998). Gingival epithelium surrounding the tooth and attached to the tooth surface and functions as a protective barrier against pathogens in dental plaque and has a role in innate host defence (Han, Shi et al. 2000; Joly, Maze et al. 2004; Ouhara, Komatsuzawa et al. 2005). A. actinomycetemcomitans, F. nucleatum and P. gingivalis were selected to be investigated because they have been considered as putative periodontal pathogens linked to several forms of periodontal diseases (Han, Shi et al. 2000). Therefore, the focus of our study was in the interactions between these strains and oral epithelial cells.

Initially, experiments determined the effect of periodontal pathogens on primary and immortalized cell viability. All pathogens reduced the viability of both monolayers when cells incubated with pathogens for 24 h. (Figures 5.6 and 5.7). Compared to pathogens, L. rhamnosus GG and L. reuteri, showed no significant effects on viability of both monolayers following 24h incubation (Figures 5.4 and 5.5). However, S. salivarius K-12 to some extent reduced the viability of the monolayers to 82% for
HOKs (Figure 5.4) and 69% for GMSM-Ks (Figure 5.5) in comparison to control viability (97%).

When both cell lines monolayers incubated with mixtures of pathogens and probiotics bacterial suspensions respectively, *P. gingivalis* and *F. nucleatum* with *L. rhamnosus* GG, *L. reuteri* or *S. salivarius* K-12 respectively yielded a protective effect noted as a significant increase in the number of viable cells, in comparison to the monolayers infected with pathogens solely (Figures 5.8-5.13) but for *A. actinomycetemcomitans*, HOKs monolayers were protected only by addition of *L. rhamnosus* GG and for GMSM-Ks none of the probiotics protects the monolayers from the toxicity of *A. actinomycetemcomitans*, though there was some increase in the number of viable cells in comparison to cells treated with pathogen solely but this increase was not significant. This means that the protective effect is not absolute but it is species dependent which is evidenced by fact that not all probiotic species tested were able to confer protection to both monolayers in the presence of different pathogens. *L. rhamnosus* GG was in most combinations the maximum protective species especially in protecting HOKs from cytopathic effect of *A. actinomycetemcomitans* as it increased the viability of the cells up to 40% while all other probiotics were unable to protect monolayers. There was an important characteristic regarding HOKs monolayers as it was observed that cell cultures beyond fourth passage were more susceptible to pathogens as they reach senescence and were impossible to get any reliable results at passage five and beyond that all the work was done in passages 3 and 4.

Since it was demonstrated that viable bacterial suspensions of different probiotic species were protective to both cell lines especially from the toxicity of *P. gingivalis* and *F. nucleatum* later it was investigated if probiotic lysates or the spent
culture fluids can also afford protection to the monolayers as this was observed in another study in our group using *L. rhamnosus* lysate and spent culture fluid to protect NHEK monolayers (Mohammedsaeed, McBain et al. 2014). All probiotic lysates and cell filtrates were similarly protect both monolayers and the increase in the viability of the cells was highly significant to all treated mixtures as shown in Figures (5.15, 5.16, 5.18 and 5.19). Furthermore, all probiotic lysates protected GMSM-Ks from *A. actinomycetemcomitans* (Figure 5.20) and only *S. salivarius K-12* lysate among other probiotic species did not confer protection to HOKs as data presented in Figure 5.17. In all experiments lysates derived from all probiotics respectively afforded higher protection against all pathogens respectively than cells filtrates which is in accordance with other reports (Prince, McBain et al. 2012; Mohammedsaeed, McBain et al. 2014). These data are in agreement with other reports as there are many studies proved the protecting effect of probiotic species to epithelial cells from cytopathic effects of pathogens, for example viable *Lactobacilli* probiotics provided a barrier which reduced the response of the host intestinal epithelium to *E. coli* O157:H7 and *E. coli* O127:H6 infections (Sherman, Johnson-Henry et al. 2005). Another study demonstrated that *Lactobacillus delbrueckii ssp. bulgaricus* B-30892 can inhibit cytopathic effects and adhesion of pathogenic *Clostridium difficile* to Caco-2 cells (Banerjee, Merkel et al. 2009). Moreover it was reported that some *Lactobacillus* and *Bifidobacterium* species were effective in displacing *Salmonella typhimurium* and *Escherichia coli* H10407 from a Caco-2 cell layer (Candela, Perna et al. 2008). Furthermore epithelial cell toxicity caused by *Streptococcus pyogenes* can be reduced by *Lactobacilli* (Maudsdotter, Jonsson et al. 2011). Additionally two previous studies in our group showed that different *Lactobacilli* species protect NHEK monolayers from the toxic effect of
*Staph. aureus* (Prince, McBain et al. 2012; Mohammedsaeed, McBain et al. 2014). However to our knowledge there are no published data that probiotics confer protection to human oral keratinocytes or immortalized gingival cells from putative periodontal pathogens toxicity.

In summary, the three tested probiotics, in addition to their extracts, interacted with both oral epithelial cells and protected them, especially from the cytopathic effect of *P. gingivalis* and *F. nucleatum* but only LGG protected monolayers from the toxicity of *A. actinomycetemcomitans*. *L. rhamnosus* GG conferred the greatest level of protection. The results obtained suggest that it is possible to incorporate the administration of probiotics in periodontitis prevention. Since host-pathogen interactions require further investigation with respect to probiosis, and there is a need for model with which to test outcomes and mechanisms of action which may include immunomodulation, an invertebrate system was adapted and applied in Chapter 6.
CHAPTER 6

Evaluation of anti-infective potential of probiotics in a *Galleria mellonella* model system
6.0 Abstract

Recently probiotics has been suggested to have a role in oral medicine and dentistry due to their immunomodulation ability. In this chapter the invertebrate *Galleria mellonella* model system was established as a model for testing periodontal pathogens. *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* and *Streptococcus salivarius* K-12 were tested for the ability to confer protection against the periodontal pathogens *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. Microorganisms were delivered to larvae by intra-haemocoelic injection, with the pathogen and probiotic being injected simultaneously into different pro-legs or as a mixture in the same pro-leg. Additionally, the prophylactic effects of prior probiotic pre-treatment were investigated. Data indicated that all pathogens were able to kill *G. mellonella* thus establishing the utility of the model whilst the probiotic strains were not lethal to the larvae at concentration up to $10^7$ cells/larvae. Moreover it was observed that prophylactic treatment with probiotics was able to re-establish protection levels similar to un-treated larvae. In conclusion, data presented in this chapter indicate that the larval model is suitable to study oral pathogens and probiosis in addition to that selected probiotic candidates showed an anti-infective potential in a *G. mellonella* model system.
6.1 Introduction

6.1.1 Invertebrate infection models
The use of alternative (surrogate or mini-host models) models to mammalian infection for studying pathogenic microorganisms has significantly increased (Swanson and Hammer 2000; Casadevall 2005; Chamilos, Samonis et al. 2011) due to a number of advantages including the fact that higher animals are not required (Ball, Goldberg et al. 1995). In addition to the realization that the microbial virulence mechanisms are common between different hosts (Rahme, Stevens et al. 1995), larvae are simple to work with and have a rapid life cycle in comparison to higher animals. Invertebrates have also been selected due to their resemblances of the innate immune systems to higher animals.

6.1.2 Similarities between vertebrates and invertebrates as infection models
The immune system consists of the acquired immune system and the innate immune system. The primary defence mechanism against any invading organisms is the innate immune system. Invertebrates lack the acquired immune system (specific immune system) however invertebrates and mammals share many common mechanical and genetic features of the innate immune system (Boehm 2006; Hirano, Das et al. 2011; Boehm, McCurley et al. 2012). For example, insects have the capability of increasing cellular and humoral responses to invading pathogens; but no antigen-triggered proliferative responses have yet been demonstrated in dissimilarity to vertebrates and it has been reported that direct and indirect immune recognition in insects occurs through receptors, such as Toll (Râmet, Manfruelli et al. 2002; Lemaitre and Hoffmann 2007).
Humoral and cellular elements are the components of the innate immune response. In humoral immunity invertebrates secrete antimicrobial peptides which are similar to vertebrates’ defensins (Kavanagh and Reeves 2004; Vogel, Altincicek et al. 2011). Additionally vertebrates and invertebrates are similar in terms of oxidative burst mechanisms and lysozyme induction (Kavanagh and Reeves 2004).

In the cellular immunity response, phagocytosis is similar in both vertebrates and invertebrates but is known as haemocytes in invertebrates the mechanism of engulfing and killing the invading microbe however, is the same (Strand 2008). Ecologically, bacteria and invertebrate interactions are common in most environments. In invertebrates physical barriers and antimicrobials protect them from microbial attack in a similar way to vertebrates (Tzou, De Gregorio et al. 2002). In this study, the greater wax moth *Galleria mellonella* injected with oral pathogens to evaluate the utility of probiotics to protect it from the toxicity of pathogens that was recognized in early stages by melanisation effect on larvae as shown in Figure 6.1.

### 6.1.3 *Galleria mellonella* as infection model

*Galleria mellonella*, the caterpillar of the greater wax moth (*Lepidoptera: pyralidae*) is widely used as a non-mammalian model system of infection which has significant logistical and ethical advantages over mammalian models. It has been used to study host-pathogen interactions by many researchers using different microorganisms including bacteria and fungi (Morton, Barnett et al. 1983; Miyata, Casey et al. 2003; Mylonakis, Moreno et al. 2005; Fedhila, Daou et al. 2006; Aperis, Fuchs et al. 2007; Seed and Dennis 2008). *Galleria* represents a potent, reliable and established model system for innate immunity studies. It is used at present as a host system to rebuild rapid mutual adaptations during host-parasite coevolution (Vilcinskas 2010).
Caterpillars can be incubated at the appropriate human pathogen incubation temperature (37°C) and precise inoculum doses can be administered by injection to the caterpillar body. Moreover, the host responses of the caterpillar can be assessed through both the cellular response mediated by phagocytic cells and the humoral immune response pathway mediated by antimicrobial peptides (AMPs) (Kavanagh and Reeves 2004). In addition this model can be used to assess antimicrobial efficacy as shown previously by many researchers (Mylonakis, Moreno et al. 2005; Aperis, Fuchs et al. 2007). Among the advantages of this model are that it is cost effective and the statistical power of the assay can be increased as large numbers of larvae can be infected with each tested strain.

Figure 6.1 Melanisation of *G. mellonella* is part of the infection process in which larvae colour changed from cream colour to grey or black as a sign of immune response. This photo shows larvae injected with *A. actinomycetemcomitans* (a) lysate and (b) Cell free extract directly after injection (less than 30 seconds).
6.2 Aims and objectives of this chapter

The caterpillar of the greater wax moth *Galleria mellonella* has been utilized to study host-pathogen interactions using various microorganisms for example, *Bacillus cereus* (Fedhila, Daou et al. 2006), *Pseudomonas aeruginosa* (Jander, Rahme et al. 2000; Miyata, Casey et al. 2003) and *Proteus mirabilis* (Morton, Barnett et al. 1983) in addition to many pathogenic fungi (Mylonakis, Moreno et al. 2005; Fuchs, O'Brien et al. 2010). Many reports established a significant correlation between the virulence of an organism in *G. mellonella* and mammalian models (Jander, Rahme et al. 2000; Mylonakis, Moreno et al. 2005). Additionally it has been confirmed that *G. mellonella* was used as a model to assess the efficacy of antimicrobial agents (Mylonakis, Moreno et al. 2005; Aperis, Fuchs et al. 2007).

The objective of this chapter was to utilize *G. mellonella* larvae to study virulence effects of different periodontal pathogens and to determine whether any probiotic protection effect against their pathogenicity can be evaluated. Based on the capacity of the probiotics *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* and *Streptococcus salivarius* K12 to protect confluent primary human oral keratinocytes (HOKs) and Non-tumour-derived immortalized human oral epithelial cells (GMSM-Ks) from the cytopathic effects of periodontal pathogens (*P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum*) in the presence or absence of probiotics or their extracts as shown in chapter five. In this chapter, we investigated *in vivo* the effects of these probiotics on the virulence of periodontal pathogens using the *Galleria mellonella* model system.
6.3 Materials and Methods

6.3.1 Infection model

*G. mellonella* (Live Foods Direct, Sheffield, United Kingdom) was used for all experiments. The larvae were the last instar stage, and were selected based on their weight (275-300 mg), the presence of a fresh cream colour and no grey markings. All larvae were used within three days from shipment.

6.3.2 Bacterial suspension preparation

A 10 ml volume of each of the species listed in Table 6.1 was prepared as mentioned in Section 2.6. Each larva received aliquots of 5 µl of this bacterial suspension injected directly to the hemocoel.

**Table 6.1 Organisms used in *G. mellonella* assay**

<table>
<thead>
<tr>
<th>Category</th>
<th>Strains used</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium nucleatum</em> ATCC 10953</td>
<td></td>
</tr>
<tr>
<td>Pathogenic species</td>
<td><em>Porphyromonas gingivalis</em> ATCC 33277</td>
</tr>
<tr>
<td></td>
<td><em>Aggregatibacter actinomycetemcomitans</em> ATCC 33384</td>
</tr>
<tr>
<td>Probiotic species</td>
<td><em>Lactobacillus rhamnosus</em> GG ATCC-53103</td>
</tr>
<tr>
<td></td>
<td><em>Lactobacillus reuteri</em> ATCC-55730</td>
</tr>
<tr>
<td></td>
<td><em>Streptococcus salivarius</em> K-12</td>
</tr>
</tbody>
</table>
6.3.3 Cell free culture preparation

A 10 ml culture of each of the pathogenic species listed in Table 6.1 was prepared as mentioned in Section 2.7. An aliquot (5 µl) was injected directly to the hemocoel of caterpillar. For confirmation that this spent culture fluid was free from any residual viable bacteria a 100 µl of the spent culture fluid was distributed on the surface of agar plate and incubated for 48 h at 37°C either anaerobically or at 10% CO₂ atmosphere according to the microorganism used to produce the spent fluid.

6.3.4 Preparation of bacterial lysates

Bacterial lysates were prepared as explained in Section 2.8. A 100 µl volume of the sonicated culture fluid was distributed on the surface of an agar plate and incubated for 48 h at 37°C either anaerobically or at 10% CO₂ atmosphere according to the microorganism used to produce the lysate to confirm the removal of any viable bacteria. A 5 µl of this lysate was injected directly to the hemocoel of larvae.

6.3.5 Galleria mellonella pathogenicity assay

Larvae of G. mellonella were stored at 4°C and used within three days and were kept for 30 min at room temperature prior to injection. Overnight cultures of each microorganism were pelleted (3220 xg, 15 min) and suspended in PBS, this was repeated twice. Cultures were adjusted to an OD₆₀₀nm of 0.1. To deliver microorganisms in G. mellonella a known quantity (0.14x10⁴-0.8x10⁵ CFU/ larva) of microorganism (5 µl) was delivered directly to the hemocoel through an injection in the last left pro-leg using a 26 gauge needle Hamilton syringe (Sigma, UK). PBS (5 µl) was injected to the control group in addition to the no treatment control group.
Prior to use the syringe was sterilized by autoclaving. The syringes were cleaned thoroughly with 70% alcohol and then rinsed with sterile distilled water twice between every sample. Multiple injections can be used such as in the case of injecting both pathogenic and probiotic species but the injection sites must not be the same in order to reduce trauma at the site of injection which may lead to an increase in larvae death. The right last pro-leg was used in this case. Infected insects were incubated in petri dish in groups of ten at 37°C in the dark for the duration of the experiment from 5-7 days.

6.3.6 Larval mortality recording

Larval mortality was checked on a daily basis over a period of a week. Larvae that had turned black and that were not moving in response to a gentle shaking of the dish or touching with a pipette tip were considered dead. Dead larvae were removed from the petri dish and the death was recorded. The experiment end point was designated by either the death of all the larvae in the tested groups or the conversion of larvae into pupa. Five petri dishes containing 10 caterpillars each were assigned to each experiment and control groups (50 caterpillars total for each sample). Dead *G. mellonella* were placed into sterile universals (30 ml) and homogenised in 10 ml of sterile PBS. This suspension was then serially diluted and spot plated onto Wilkins Chalgren agar to calculate bacterial load per individual larva. The experiments were terminated once 2 of the control individuals had died or pupated.
6.3.7 Data analysis

Values were considered significantly different if the $p$ value was less than 0.05. Data were plotted as survival curves using the Kaplan–Meier estimator in Excel. The survival probability at any particular time was calculated by the formula given below:

$$S_t = \frac{\text{Number of subjects living at the start} - \text{Number of subjects died}}{\text{Number of subjects living at the start}}$$
6.4 Results

6.4.1 Infection assays in the *Galleria mellonella* model

*G. mellonella* has been had not previously been studied in relation to periodontal pathogens infection. In this chapter we characterise putative periodontal pathogens pathogenesis in *G. mellonella* infection model.

6.4.2 Periodontal pathogens induces *Galleria mellonella* mortality

All three pathogens caused death of at least 50% of larvae. However *P. gingivalis* and *F. nucleatum* caused significantly (p< 0.05) higher mortality than *A. actinomycetemcomitans*. Data are presented in Table and Figure 6.2.

### Table 6.2 Numbers of *G. mellonella* larvae surviving various pathogenic and probiotic infections (n=10) over 5 days, $10^4$ - $10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis. All caterpillars survive probiotic infection.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>F. nucleatum</em> ATCC 10953</td>
<td>0.17x10$^4$</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td><em>P. gingivalis</em> ATCC 33277</td>
<td>0.14x10$^4$</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td><em>A. actinomycet.</em> ATTC 33384</td>
<td>0.24x10$^5$</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td><em>L. reuteri</em> ATCC 55730</td>
<td>0.56x10$^5$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>L. rhamnos. GG</em> ATCC 53103</td>
<td>0.70x10$^5$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>S. salivarius</em> K12</td>
<td>0.80x10$^5$</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NTC</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived. NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1
Figure 6.2 Kaplan-Meier survival curves for *A. actinomycetemcomitans*, *F. nucleatum* and *P. gingivalis*. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.

Additionally, bacterial lysates and broth culture filtrates of pathogens affect the viability of larvae (Tables 6.3a, b and c) however the mortality rate was less than with bacterial suspensions in all pathogen extracts as clearly shown in Figure 6.3a, b and c. No mortality was observed in both controls (non-treated control and PBS control). These results validate that *G. mellonella* is susceptible to infection with selected periodontal pathogens.

None of the probiotic candidates (*L. rhamnosus GG, L. reuteri* and *S. salivarius K-12*) that were injected into larvae induced any mortality to *G. mellonella* even after incubation for 168 h. These results demonstrate that *G. mellonella* is not susceptible to selected probiotics species and therefore can be used for protection assessment. Data presented in Table 6.2.
Table 6.3 Number of *G. mellonella* larvae surviving various pathogens and their filtrates infections (n=10) over 5 days, $10^{4}$ - $10^{6}$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

(a) Strains tested | bacterial dose (CFU/10 µl) | No. alive (/10) at time-points | MTTD (hrs)
|-------------------|-----------------------------|-----------------------------|--------------
| **Day 0 1 2 3 4 5** | **Hour 0 24 48 72 96 120** | **MTTD (hrs)** |
| *F. nucleatum* ATCC 10953 | 0.17x10$^{4}$ | 10 2 1 1 0 0 | 72 |
| *F. nucleatum* lysate | | 10 10 9 9 8 8 | * |
| *F. nucleatum* SCF | | 10 10 7 7 4 4 | 96 |
| PBS | | 10 10 10 10 10 10 | * |
| NTC | | 10 10 10 10 10 10 | * |

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1

(b) Strains tested | bacterial dose (CFU/10 µl) | No. alive (/10) at time-points | MTTD (hrs)
|-------------------|-----------------------------|-----------------------------|--------------
| **Day 0 1 2 3 4 5** | **Hour 0 24 48 72 96 120** | **MTTD (hrs)** |
| *P. gingivalis* ATCC 33277 | 0.14x10$^{4}$ | 10 2 1 1 0 0 | 72 |
| *P. gingivalis* lysate | | 10 10 9 9 8 8 | * |
| *P. gingivalis* SCF | | 10 10 10 10 10 10 | * |
| PBS | | 10 10 10 10 10 10 | * |
| NTC | | 10 10 10 10 10 10 | * |

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1

(c) Strains tested | bacterial dose (CFU/10 µl) | No. alive (/10) at time-points | MTTD (hrs)
|-------------------|-----------------------------|-----------------------------|--------------
| **Day 0 1 2 3 4 5** | **Hour 0 24 48 72 96 120** | **MTTD (hrs)** |
| *A. actinomycet.* ATTC 33384 | 0.24x10$^{5}$ | 10 5 4 3 2 2 | 48 |
| *A. actinomycet.* lysate | | 10 8 6 6 6 6 | * |
| *A. actinomycet.* SCF | | 10 8 7 7 7 7 | * |
| PBS | | 10 10 10 10 10 10 | * |
| NTC | | 10 10 10 10 10 10 | * |

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1
Figure 6.3 Kaplan-Meier plot of survival after infection with (a) *F. nucleatum*, (b) *P. gingivalis* and (c) *A. actinomycetemcomitans* living bacterial suspension, spent culture fluid and bacterial lysate respectively. Control group was included in the assay in which *G. mellonella* were injected with PBS. n= 10 larvae.
6.4.3 Effect of injecting mixture of pathogen and probiotic in the same pro-leg on G. mellonella mortality

When S. salivarius K-12 was injected with F. nucleatum and A. actinomycetemcomitans there was a higher larval viability 24 h post-injection than when G. mellonella was injected with the pathogens alone as shown in Tables and Figures 6.4 and 6.6. No effect was observed when the larvae were injected in a mixture with P. gingivalis (Table and Figure 6.5) however L. reuteri had greater effect when injected with P. gingivalis and less effect regarding the increase of larval viability when injected with F. nucleatum and A. actinomycetemcomitans. Unexpectedly, L. rhamnosus GG had some effect on G. mellonella viability when injected in a mixture with F. nucleatum and P. gingivalis and increased the mortality of larvae when injected in a mixture with A. actinomycetemcomitans than the effect of pathogen alone. Data are presented in Table and Figure 6.6.

Table 6.4 Numbers of G. mellonella larvae surviving Fusobacterium nucleatum and probiotic mixtures infections (n=10) over 5 days, 10^4 - 10^6 CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>0.17x10^4</td>
<td>10 2 1 1 0 0</td>
<td>72</td>
</tr>
<tr>
<td>F. nucleatum + L. reuteri</td>
<td>0.17x10^4 0.56x10^6</td>
<td>10 4 2 2 2 2</td>
<td>120</td>
</tr>
<tr>
<td>F. nucleatum + L. rhamnosus GG</td>
<td>0.17x10^4 0.70x10^6</td>
<td>10 2 2 2 2 2</td>
<td>120</td>
</tr>
<tr>
<td>F. nucleatum + S. salivarius K12</td>
<td>0.17x10^4 0.80x10^6</td>
<td>10 8 8 8 8 8</td>
<td>*</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived. NTC = non treated control. Bacterial cultures were adjusted to an OD600 of 0.1
Figure 6.4 Kaplan-Meier survival curves for *F. nucleatum* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.

Table 6.5 Numbers of *G. mellonella* larvae surviving *P. gingivalis* and probiotic mixtures infections (n=10) over 5 days, $10^4 - 10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>$0.14 \times 10^4$</td>
<td>10 3 1 0 0 0</td>
<td>72</td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. reuteri</em></td>
<td>$0.14 \times 10^4$, $0.56 \times 10^5$</td>
<td>10 6 6 6 6 6 6</td>
<td>*</td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. rhamnosus GG</em></td>
<td>$0.14 \times 10^4$, $0.70 \times 10^5$</td>
<td>10 4 3 3 3 3 120</td>
<td>120</td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>S. salivarius K12</em></td>
<td>$0.14 \times 10^4$, $0.80 \times 10^5$</td>
<td>10 2 0 0 0 0 48</td>
<td>48</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1.
Figure 6.5 Kaplan-Meier survival curves for *P. gingivalis* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS.

Table 6.6 Numbers of *G. mellonella* larvae surviving *A. actinomycetemcomitans* and probiotic mixtures infections (n=10) over 5 days, $10^4 - 10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>$0.24 \times 10^5$</td>
<td>10 3 0 0 0 0</td>
<td>48</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>L. reuteri</em></td>
<td>$0.24 \times 10^5$ $0.56 \times 10^5$</td>
<td>10 3 0 0 0 0</td>
<td>48</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>L. rhamnosus GG</em></td>
<td>$0.24 \times 10^5$ $0.70 \times 10^5$</td>
<td>10 0 0 0 0 0</td>
<td>24</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>S. salivarius K12</em></td>
<td>$0.24 \times 10^5$ $0.80 \times 10^5$</td>
<td>10 5 4 4 4 4</td>
<td>120</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived. NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1.
Figure 6.6 Kaplan-Meier survival curves for *A. actinomycetemcomitans* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS.

6.4.4 Effect of injecting of pathogen and probiotic simultaneously in different pro-legs on *G. mellonella* mortality

The effects of injecting pathogens and probiotics together in larvae were evaluated. This was in order to exclude any effects due to an inhibitory interaction between microorganisms. The effect on the viability of *G. mellonella* was different when pathogens and probiotic species were injected simultaneously but not in a mixture on different pro-legs (5 µl) each. The viability of *F. nucleatum* was increased when injected simultaneously but on different pro-legs with all probiotics and the most effective was when injected with *S. salivarius* K-12 as shown in Table and Figure 6.7.
Table 6.7 Numbers of *G. mellonella* larvae surviving *Fusobacterium nucleatum* and probiotic infections simultaneously in different pro-legs (n=10) over 5 days, 10^4 - 10^6 CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis. All probiotics conferred protection to caterpillars post-infection.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>0.17x10^4</td>
<td>10 2 1 1 0 0</td>
<td>72</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>L. reuteri</em></td>
<td>0.17x10^4 0.56x10^5</td>
<td>10 4 4 4 4 4</td>
<td>120</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>L. rhamnusus GG</em></td>
<td>0.17x10^4 0.70x10^5</td>
<td>10 3 3 3 3 3</td>
<td>120</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>S. salivarius K12</em></td>
<td>0.17x10^4 0.80x10^5</td>
<td>10 6 6 6 6 6</td>
<td>*</td>
</tr>
<tr>
<td>PBS</td>
<td>10 10 10 10 10 10</td>
<td>* NO MTTD values as &gt;50% survived.</td>
<td></td>
</tr>
<tr>
<td>NTC</td>
<td>10 10 10 10 10 10</td>
<td>* NO MTTD values as &gt;50% survived.</td>
<td></td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.

NTC = non treated control. Bacterial cultures were adjusted to an OD_{600} of 0.1

Figure 6.7 Kaplan-Meier survival curves for *F. nucleatum* and probiotics injected in different pro-legs. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.
The effect when *P. gingivalis* was injected with probiotics was different because only *L. reuteri* increased the viability of *G. mellonella* than the one injected with pathogen alone as shown in Table 6.8 while with other two probiotics the viability was less than the larvae injected with *P. gingivalis* alone as presented in Figure 6.8.

**Table 6.8** Numbers of *G. mellonella* larvae surviving *P. gingivalis* and probiotic infections simultaneously in different pro-legs (n=10) over 5 days, $10^4 - 10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>0.14x10^4</td>
<td>10 3 1 0 0 0</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. reuteri</em></td>
<td>0.14x10^4 0.56x10^5</td>
<td>10 3 3 3 3 3</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. rhamnosus GG</em></td>
<td>0.14x10^4 0.70x10^5</td>
<td>10 2 1 1 1 1</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>S. salivarius K12</em></td>
<td>0.14x10^4 0.80x10^5</td>
<td>10 2 0 0 0 0</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>10 10 10 10 10 10</td>
<td></td>
<td>*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTC</td>
<td>10 10 10 10 10 10</td>
<td></td>
<td>*</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD<sub>600</sub> of 0.1
Figure 6.8 Kaplan-Meier survival curves for *P. gingivalis* and probiotics injected in different pro-legs. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.

*L. reuteri* increased the viability of larvae when injected with *A. actinomycetemcomitans* on different pro-legs while both *L. rhamnosus* GG and *S. salivarius* K-12 effect on the number of viable larvae was less than when *A. actinomycetemcomitans* injected alone 24 h post-infection (Table and Figure 6.9).
Table 6.9 Numbers of *G. mellonella* larvae surviving *A. actinomycetemcomitans* and probiotic infections simultaneously in different pro-legs (n=10) over 5 days, 10^4 - 10^6 CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>0.24x10^5</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.56x10^5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td><strong>A. actinomycetemcomitans</strong> + <em>L. reuteri</em></td>
<td>0.24x10^5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.70x10^5</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td><strong>A. actinomycetemcomitans</strong> + <em>L. rhamnosus GGG</em>*</td>
<td>0.24x10^5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>+ <em>S. salivarius</em> K12</td>
<td>0.80x10^5</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>NTC</strong></td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.
NTC = non treated control. Bacterial cultures were adjusted to an OD600 of 0.1

Figure 6.9 Kaplan-Meier survival curves for *A. actinomycetemcomitans* and probiotics injected in different pro-legs. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.
6.4.5 Effect of injecting probiotic 24h pre-injection with pathogen in different pro-legs on *G. mellonella* mortality

There was a decrease in an activation rate of all treated larvae when they were challenged for 24 h with probiotics and this result applied to all probiotics and pathogens. None of the larvae were killed when *F. nucleatum* was injected to larvae that were pre injected with *L. rhamnosus* GG or *S. salivarius* K-12. Furthermore there was a highly significant decrease in mortality of larvae pre-treated with *L. reuteri* as presented in Table and Figure 6.10.

**Table 6.10** Numbers of *G. mellonella* larvae surviving *Fusobacterium nucleatum* and probiotic infections simultaneously in different pro-legs (n=10) over 5 days, $10^4$ - $10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis. All probiotics confer protection to caterpillars post-infection.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0 24 48 72 96 120</td>
<td></td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>$0.17 \times 10^4$</td>
<td>10 2 1 1 0 0</td>
<td>72</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>L. reuteri</em></td>
<td>$0.17 \times 10^4$ 0.56$ \times 10^6$</td>
<td>PC 10 10 10 9 8 8</td>
<td>*</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>L. rhamnosus</em> GG</td>
<td>$0.17 \times 10^4$ 0.70$ \times 10^6$</td>
<td>PC 10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td><em>F. nucleatum</em> + <em>S. salivarius</em> K12</td>
<td>$0.17 \times 10^4$ 0.80$ \times 10^6$</td>
<td>PC 10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td>10 10 10 10 10 10</td>
<td>*</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived. NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1.
**Figure 6.10** Kaplan-Meier survival curves for *F. nucleatum* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the insect die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.

Data shown in Table 6.11 show the effects of injecting *P. gingivalis* on larvae that were pre-treated for 24 h with probiotics and clearly revealed that there was total protection for larvae pre-treated with *L. reuteri* whereas the least protection was conferred by *L. rhamnosus* GG.
Table 6.11 Number of *G. mellonella* larvae surviving *P. gingivalis* and probiotic mixtures infections (n=10) over 5 days, $10^4$ - $10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
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<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>$0.14 \times 10^4$</td>
<td>10 2 1 1 0 0</td>
<td>72</td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. reuteri</em></td>
<td>$0.14 \times 10^4$, $0.56 \times 10^5$</td>
<td>PC 10 10 10 10 10 10 *</td>
<td></td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>L. rhamnosus GG</em></td>
<td>$0.14 \times 10^4$, $0.70 \times 10^5$</td>
<td>PC 10 2 1 1 1 1 120</td>
<td></td>
</tr>
<tr>
<td><em>P. gingivalis</em> + <em>S. salivarius</em> K12</td>
<td>$0.14 \times 10^4$, $0.80 \times 10^5$</td>
<td>PC 10 8 6 4 4 4 120</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>10 10 10 10 10 10 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTC</td>
<td>10 10 10 10 10 10 *</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived.

NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1

Figure 6.11 Kaplan-Meier survival curves for *P. gingivalis* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.
Table 6.12 Number of *G. mellonella* larvae surviving *A. actinomycetemcomitans* and probiotic mixtures infections (n=10) over 5 days, $10^4 - 10^6$ CFU doses. Median time to Death (MTTD) values were calculated by Kaplan-Meier survival analysis.

<table>
<thead>
<tr>
<th>Strains tested</th>
<th>Bacterial dose (CFU/10 µl)</th>
<th>No. alive (/10) at time-points</th>
<th>MTTD (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hour 0</td>
<td>24</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td>$0.24 \times 10^5$</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>L. reuteri</em></td>
<td>$0.24 \times 10^5$, $0.56 \times 10^5$</td>
<td>PC</td>
<td>10</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>L. rhamnosus GG</em></td>
<td>$0.24 \times 10^5$, $0.70 \times 10^5$</td>
<td>PC</td>
<td>10</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em> + <em>S. salivarius K12</em></td>
<td>$0.24 \times 10^5$, $0.80 \times 10^5$</td>
<td>PC</td>
<td>10</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NTC</td>
<td></td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Light grey = <100% alive. Dark grey = 0% alive. * NO MTTD values as >50% survived. NTC = non treated control. Bacterial cultures were adjusted to an OD$_{600}$ of 0.1

Figure 6.12 Kaplan-Meier survival curves for *A. actinomycetemcomitans* and probiotics. The curves start at 10 (all alive) and drop down the y-axis as the larvae die. The point at which the curve ends indicates the number of surviving caterpillars at the end of assessment. Control group was included in the assay in which caterpillars were injected with PBS. n= 10 larvae.
6.5 Discussion

In a previous investigation, Vilela et al. (2015) used *Galleria mellonella* to study the effect of *Lactobacillus acidophilus* on *Candida albicans* infection and in addition they established the lactobacilli were not pathogenic to the larvae (Vilela, Barbosa et al. 2015). The authors reported that the larvae were simple to manipulate, infect and can survive 37°C while other invertebrate models survive only at 25°C (Nathan 2014). Furthermore it has shown promise as an infection model in bacterial research pathogenesis (Ramarao, Nielsen-Leroux et al. 2012; Cook and McArthur 2013; Mukherjee, Raju et al. 2013). In comparison with other invertebrate infection model systems the *G. mellonella* host immune defence mechanisms are the closest to those encountered in humans (Nathan 2014; Köhler 2015).

The first objective of the current chapter was to establish the *G. mellonella* model for infection with periodontal pathogen and secondly, to confirm that the three tested probiotic strains *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12 are not pathogenic to the larvae. The final objective was to determine whether the virulence in *G. mellonella* after co-infection with probiotics matched the virulence of pathogens solely. (i.e. if the probiotic candidates confer protection against the lethal activity of periodontal pathogens).

Data showed that all tested oral pathogens were lethal to *G. mellonella* after intra-haemocoelic injection indicating that *G. mellonella* can probably be used to study periodontal pathogens virulence (at the mortality level). Both *F. nucleatum* and *P. gingivalis* caused 100% killing of all larvae tested (n=10) in each group after 3 days of incubation while 20% survived after 5 days of incubation post-infection with *A. actinomycetemcomitans*. Likewise, bacterial lysates and broth culture filtrates of
pathogens affected the viability of larvae (Tables 6.3a, b and c) however the mortality rates were less than with bacterial suspensions in all pathogen extracts as clearly shown in Figure 6.3a, b and c. No mortality was observed in both controls (non-treated control and PBS control). These results indicate that *G. mellonella* is susceptible to infection by the selected periodontal pathogens. Interestingly this data is in agreement with many previous studies describing that the *G. mellonella* model is suitable to be used in pathogenesis research (Champion, Cooper et al. 2009; Inglis, Gardner et al. 2009; Mukherjee, Raju et al. 2013), in addition to other microorganisms such as *Candida albicans* (Ribeiro, Barros et al. 2017).

Prior to the study of the effects of probiotics in the development of pathogenicity of tested pathogenic strains it was observed that the chosen probiotic strains did not cause death of the larvae in concentrations up to $10^7$ cells/larvae for 7 days post-infection. This demonstrated low pathogenicity in the *G. mellonella* model. In another study *G. mellonella* was used as a model to study probiotic bacteria and it was reported that it was not virulent to this host (Joyce and Gahan 2010; Vilela, Barbosa et al. 2015). By injecting the pathogen and probiotic in different sites in the larvae it was found that the mortality rate was variable. Initially, a mixture of probiotic and pathogen were injected from each strain tested. In general all probiotics tested conferred protection to infected larvae. Some probiotic strains provided better protection against pathogens than others but this phenomenon was variable according to the pathogen/probiotic combination used, for example *S. salivarius* K12 offered the best protection against *F. nucleatum* pathogenicity whilst larvae infected with *P. gingivalis* had no protection at all when injecting simultaneously with *S. salivarius* instead *L. reuteri* showed some degree of protection.
Additionally it was detected that mixing the pathogen and the probiotic together before injection into the larvae correlated to an increase in protection. This could be due to one of them inhibiting the other or altering its viability in the mixture which was noted as a decrease in the protection when compared to the results when they injected simultaneously in different pro-legs.

Moreover, it was observed that the mortality levels in *F. nucleatum* injected 24 hr. after LGG administration, *F. nucleatum* injected after *S. salivarius* K12 and *P. gingivalis* injected after *L. reuteri* administration were very similar to the results observed in the PBS control group. These observations suggest that prophylactic treatment with probiotics was able to re-establish protection levels similar to uninfected larvae. This may be due to immunomodulation in the *G. mellonella* model that may affect in the survival of larvae during infection. Likewise in another study it was demonstrated that co-infection of *G. mellonella* with *L. acidophilus* and *C. albicans* reduced the number of yeast cells in the larval haemolymph and increased the survival of caterpillars (Vilela, Barbosa et al. 2015).

In summary, the selected probiotic candidates showed varied probiotic activity against *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans*. These strains represent new potential probiotics that may possibly be used to control periodontal infections. Additionally it was clearly shown in this study that prior exposure to a probiotic dose activates *G. mellonella* immunity which allows the larvae to overcome lethal infection by pathogens.
CHAPTER 7

Conclusions
7.0 Overview

The general aim of this doctoral project was to explore the bacterial interactions in the oral cavity with allochthonous microorganisms and with cells of mucosal origin. As well as investigating the interaction between oral bacteria, the potential of candidate dental probiotic bacteria towards protection of human oral host tissues was assessed using oral epithelial cells and periodontal pathogens.

Thesis chapters cover the exposure of multi-species communities in which bacteria interact within a microbial biofilm, to antimicrobial formulations (Chapter 3), investigations into the positive and negative interactions between bacteria derived from the human oral microbiota (Chapter 4), potential protection of oral epithelial cells from the cytopathic effect of oral pathogens by probiotics (Chapter 5). Finally, the putative anti-infective potential of probiotics in the invertebrate Galleria mellonella model system was investigated (Chapter 6). As previously reported, data showed that bacteria were significantly more susceptible in planktonic culture than in colonies or when grown in biofilms, supporting the commonly observed phenomenon of biofilm recalcitrance as many studies demonstrated that standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infections as several pathogens associated with chronic infections, including Pseudomonas aeruginosa in cystic fibrosis pneumonia, Streptococcus pneumoniae in chronic otitis media, Staphylococcus aureus in chronic rhinosinusitis and enteropathogenic Escherichia coli in recurrent urinary tract infections, are linked to biofilm formation (Costerton, Stewart et al. 1999; Davies, Ellwood et al. 2003; Hall-Stoodley and Stoodley 2009). The reason behind biofilms resistance attributed to poor antimicrobials penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells (Stewart 2002). The rank order of
effectiveness of the test formulations held true in most cases for MIC, MBC and MBEC with respect to the least and most effective agent, regardless of test bacterium. However the rank order according to viability mapping was markedly different in relation to antimicrobials used and may represent a more realistic indicator of in situ effectiveness. The current data indicate that all of test formulations showed a variable degree of antibacterial activity both in planktonic and biofilm mode of growth and the susceptibility decreased in biofilms. Formulations containing the bisphenol microbicide triclosan or the bis-biguanide chlorhexidine were most effective. Coaggregation reportedly plays an important role in biofilm formation (Kolenbrander, 1988, Kolenbrander 2000). Likewise, coadhesion, which is an interaction between a planktonic cell and a surface-attached cell have the same role in bacterial colonization (Bos et al. 1994). It was hypothesized that synergistic interactions between species will be more common than those marked by antagonistic, within isolates from one person oral microbiota.

The incidence of positive interactions was higher than the incidence of negative interactions and the neutral interactions dominated. It could be concluded that within the highly populated oral microbial ecosystem cooperation plays a greater role in biofilm development and maintenance than competition with other species. Additionally, the frequency of both promotional interactions and antagonistic interactions were more common between bacteria isolated from oral biofilms. The incidence of coaggregation among oral site isolates was higher between pairings that had promotional interactions in cross assays. The majority of oral sites isolates had the ability to coaggregate with each other and with some fresh saliva isolates however, salivary isolates almost failed to coaggregate with each other or with different oral sites isolates which indicated that the adhesion of salivary bacteria to
oral surfaces is facilitated by other oral bacteria as it is lacking the coaggregation ability. Previous studies have suggested that adhesion of salivary *B. adolescentis* OLB6410 to tooth surfaces is probably mediated by other oral bacteria as it is lacking coaggregation ability (Nagaoka et al. 2008).

Probiotic bacteria are may be beneficial to health although further development is required (Kalliomäki, Salminen et al. 2001; McFarland 2006; Osborn and Sinn 2007). In this thesis, interactions between periodontal pathogens, human oral cells and probiotics (Chapter 5) were assessed. Data show that protection of human oral keratinocytes (HOKs) and immortalized human oral epithelial cells (GMSMKs) depends on the species of bacterium used as probiotic. *Lactobacillus rhamnosus* GG and *Lactobacillus reuteri* were well tolerated by both cell types *in vitro*. Interestingly, although it has been reported that *Streptococcus salivarius* K-12 has comparatively low pathogenic potential (Burton, Wescombe et al. 2006) it had a significant negative effect on viability of both cell types. All probiotics and their extracts, including *Streptococcus salivarius* K-12 conferred some protection to human oral keratinocytes and immortalized human oral epithelial cells from the cytopathic effect of *F. nucleatum* and *P. gingivalis*. For *A. actinomycetemcomitans*, only *Lactobacillus rhamnosus* GG and its extracts could protect human oral keratinocytes from its toxicity while for immortalized epithelial cells the lysates of the three probiotics are protective against the cytopathic effects of *A. actinomycetemcomitans*.

An *in vivo* model used to study the pathogenesis of periodontal bacteria listed above. The anti-infective potential of probiotics in a *Galleria mellonella* model system was evaluated. Data indicated that all pathogens were able to kill *G. mellonella* thus establishing the utility of the model whilst the probiotic strains were not lethal to the larvae at concentration up to $10^7$ cells/larvae. The selected probiotic candidates
showed an anti-infective potential in a *G. mellonella* model system. Moreover it was observed that prophylactic treatment with probiotics was able to re-establish protection levels similar to un-treated larvae.

### 7.1 Future work

In terms of the study in Chapter 3, regarding the effect of growing bacteria in the biofilm mode and the effect of that on exposure of multi-species communities to antimicrobial formulations, examining the response of abstracted oral bacterial communities of eight oral hygiene formulations, the study could be expanded to consider inter individual variation in biofilm response.

In Chapter 4, the identification of compounds involved in the antagonistic reactions such as bacteriocins would be an interesting addition to the work done so far. Additionally, the species that have been shown to coaggregate could be tested for coaggregation with more bacterial isolates from other sites of the body. Data presented in Chapters 5 and 6 show that *L. rhamnosus* GG, *L. reuteri* and *S. salivarius* K-12 confer protection against the periodontal pathogens *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* both *in vivo* and *in vitro*. However, further work is required to elucidate the mechanisms by which they protect the oral cells and may prevent bacterial pathogenesis. This could include the study of innate immune responses of HOKs, GMSMKs and *G. mellonella* to probiotics and pathogenic bacteria. Analysis of the expression of antimicrobial peptides (AMPs) by HOKs and GMSMKs in response to probiotic bacteria and pathogenic strains would also be of interest. The use of HOKs and GMSMs as an *in vitro* model of oral cavity tissues and *G. mellonella* as an *in vivo* model were of value but, following further preclinical testing, human volunteer studies are required to ascertain with certainty whether the protection observed *in vitro* can be translated to the clinic.
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