University of Glasgow

INVESTIGATING THE EFFECTS OF ORAL MICROBIAL BIOFILMS ON ORAL EPITHELIAL CELLS

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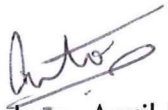
Declaration of Originality

I am aware of and understand the University's policy on plagiarism and I certify that this thesis is my own work, except where indicated by referencing.



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Abstract

Periodontal disease is associated with an inflammatory response to a pathogenic biofilm. The host response may cause gingival inflammation, which can progress to irreversible gingival recession, alveolar bone destruction and tooth loss. Enhanced understanding of the host-biofilm relationship may inform novel therapeutic approaches. A key molecule involved in inducing and mediating pro-inflammatory responses are the IL-17 cytokine family. An *in vitro* model system potentially provides a platform to investigate biofilm interaction with epithelial cells. The aim of this study was to develop *in vitro* mono-species and multi-species biofilms and investigate the survival of biofilms in cell culture conditions, and simultaneously assess the epithelial response to the bacterial biofilms and planktonic cells with respect to viablility, apoptosis and inflammatory mediators. This study also looked to determine whether IL-17A is expressed within and released from periodontal tissues and to investigate its role in the regulation of epithelial cell cytokine and chemokine production.

Mono- and multi-species biofilms of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* and *S. mitis* were developed, which were assessed for survival in cell culture conditions, recovery from biofilms and morphology. Gingival tissue from patients with chronic periodontitis or healthy controls were analysed for IL-17A gene expression by qPCR. Protein expression and cellular localization was determined by immunofluorescence. Single cell suspensions of gingival tissue were stimulated *in vitro* and IL-17A release assessed. Epithelial response after bacterial and IL-17A co-culture was assessed.

The individual bacteria survived preferentially in multi-species biofilm compared with mono-species biofilm in cell culture conditions. The viability, apoptosis and inflammatory mediator response depended on the type (pathogen or commensal) and form (planktonic or biofilm) of bacteria. Diseased gingival tissues expressed significantly higher levels of IL-17A mRNA than healthy samples. IL-17A localised to mast cells in the inflamed gingival tissue, and was released in cell culture supernatants following stimulation. Stimulation of epithelial cells with IL-17A resulted in the transcriptional regulation and release of numerous cytokines and chemokines.

The initial component of the entire investigation has provided a quantitative and qualitative assessment of both mono- and multi-species biofilms that can be used to investigate how oral biofilms interact with the host epithelium. The epithelial-biofilm co-culture model has demonstrated clear differences between **(i)** planktonic and biofilms, **(ii)** pathogens and commensals, and **(iii)** live and dead bacterial challenge. These observations and the utility of the model will provide a platform to investigate key questions relating to pathogen and host within the oral cavity and beyond. From this study, it appears that IL-17A plays an important role in the protective periodontal immune response to bacterial pathogens. The upregulation of acute inflammatory mediators (such as IL-8) will promote neutrophil recruitment and potentiate the removal of any invading microbial threat. Therefore it is important to understand the benefits of this cytokine, before systemic therapeutic agents are used to antagonise its actions. The hope for the future is to unravel the details of the mechanisms involved and thereby identify novel therapeutic targets for inflammatory and infectious disease.

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Abbreviations

Aa	*Aggregatibacter actinomycetemcomitans*
Ab	Antibody
ANOVA	Analysis of variance
AS	Artificial saliva with mucin
ATCC	American type culture collection
AI-2	Auto inducer-2
Bax	B-cell lymphoma 2 associated X protein
Bcl-2	B-cell lymphoma 2
BHI	Brain heart infusion
BHI+B	Brain heart infusion + 5% Blood
BOP	Bleeding on probing
BPE	Bovine pituitary extract
C5a	Complement 5 a
Ca	Calcium
CAL	Clinical attachment level
CAM	Camptothecin
CD	Cluster of differentiation
CDM	Chemically defined media
CFU	Colony forming unit
cm	Centimetre
CS	Thermanox® coverslip
cT	Threshold cycle
CT	Connective tissue
CLSM	Confocal laser scanning microscopy
CXCL1	Chemokine (C-X-C motif) ligand 1
CSF2	Colony stimulating factor 2
CSF3	Colony stimulating factor 3
CCL5	Chemokine (C-C motif) ligand 5
CCL3L3	Chemokine (C-C motif) ligand 3-like 3
CX3CR1	Chemokine (C-X3-C motif) receptor 1
CCL4	Chemokine (C-C motif) ligand 4
CXCL5	Chemokine (C-X-C motif) ligand 5
CXCL10	Chemokine (C-X-C motif) ligand 10

CXCL11	Chemokine (C-X-C motif) ligand 11
cDNA	Complementary Deoxyribo nucleic acid
CDFF	Constant-depth film fermenter
DNA	Deoxyribo nucleic acid
DMEM	Dulbecco's modified Eagle's media
DC	Dendritic cells
EGF	Epidermal growth factor
EF	Enrichment factor
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence in-situ hybridization
Fn	*Fusobacterium nucleatum*
g	Gram
G-CSF	Granulocyte - colony stimulating factor
GM-CSF	Granulocyte macrophage - colony stimulating factor
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
GEC	Gingival epithelial cells
h	Hour
HgA	Hemagglutinin adhesion
HBD	Human beta defensin
H_2	Hydrogen
HMDS	Hexamethyldisilazane
HA	Hydroxyapatite discs
IL	Interleukin
ITGB2	Integrin beta 2
ICAM-1	Intercellular adhesion molecule - 1
IFN	Interferon
ICAD	Inhibitor of caspase-activated DNase
kHz	Kilo hertz
kgp	Lysine-specific gingipains
LPS	Lipopolysaccharide
min	Minutes
mm	Millimetre
Mu	Mucosal
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase

MAMP	Microbe-associated molecular pattern
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
µg	Microgram
µL	Microlitre
mg	Milligram
NK	Natural killer cells
NOD	Nucleotide-binding oligomerization domain-containing protein
OPG	Osteoprotegerin
OKF6-TERT2	Oral mucosal immortalised keratinocyte cell line
OsO_4	Osmium tetroxide
PBS	Phosphate buffered saline
PBI	Papilla bleeding index
PI	Plaque index
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear
PRR	Pattern recognition receptor
PTFE	Polytetrafluoroethylene
Pg	*Porphyromonas gingivalis*
PCR	Polymerase chain reaction
PD	Probing depth
qPCR	Real time PCR
RgpA	Arginine-specific gingipains
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucelic acid
rpm	Revolutions per minute
RANTES	Regulated and normal T cell expressed and secreted
Sm	*Streptococcus mitis*
SAB	Schaedler's anaerobe broth
SD	Standard deviation
S100A8	S100 calcium binding protein A8
SEM	Standard error of mean
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- alpha
TEM	Transmission electron microscopy
TGF	Tumour growth factor

Th	T helper cells
Treg	T regulatory cells
TSB	Tryptic soy broth
TSB+B	Tryptic soy broth + 5% blood
THM	Tryptic soy broth + hemin + menadione
TLDA	Taqman low density array
TMB	3 3’ 5 5’ – tetra-methylbenzidine
w/v	Weight/volume
XTT	2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER 1:

INTRODUCTION

1.1 Periodontal disease

1.1.1 Clinical classification

Periodontal diseases include a long list of conditions involving the supporting structures of the tooth. Of these diseases, the most prevalent are dental-plaque induced gingivitis and chronic periodontitis. Gingivitis is defined as gingival inflammation without any loss of attachment and may be purely plaque related or may be exacerbated by local or systemic factors. Gingivitis may be associated with a slight increase in clinical probing depth as a result of erythema and swelling of the gingivae. This condition is entirely reversible with removal of the aetiological agent (dental plaque) with no permanent loss of periodontal attachment (Tatakis and Kumar, 2005). Periodontitis is defined as "an infectious disease resulting in inflammation within the supporting tissues of the teeth, progressive attachment loss and bone loss" (Flemmig, 1999). Periodontitis, classified as either chronic or aggressive, may present either as clinical probing depths greater than the depth of the physiological sulcus or as gingival recession or a combination of both (Novak et al., 2008). Chronic periodontitis, the most common, may be further described as mild moderate or severe (Lindhe et al., 2008). Aggressive periodontitis is a severe and widespread form, which is characterised by rapid attachment loss and bone destruction. Both chronic and aggressive periodontitis can occur in a localised form affecting a small number of teeth or a generalised form affecting the majority of the teeth.

The primary aetiological factor in periodontal disease is dental plaque (Socransky et al., 1998). It is a multi-factorial disease, which affects a significant proportion of the general population (60%) to some degree or another. Severe disease is, however, affects approximately 8% of the dentate adult population in Britain (Morris et al., 2001). There are a number of suggested reasons for an increase in susceptibility to periodontitis. The differences between patients have been suggested to be due to the amount of plaque, the microbial composition of the plaque or the host response to the presence of dental plaque. In fact, it is well recognised that the bacteria in dental plaque are responsible for the initiation of periodontal disease. This is supported by studies that confirm the association between the accumulation of dental plaque and periodontal disease and the absence of plaque in health (Löe et al., 1986). There is also clear evidence that removal of plaque will result in clinical

improvement and that when plaque control is poor the disease will recur (Becker et al., 1984, Axelsson et al., 2004).

The bacterial accumulation on teeth induces an inflammatory response in associated gingival tissues. The vast majority of patients who experience an accumulation of plaque will develop non-destructive periodontal inflammation (gingivitis) (Löe et al., 1978). Removal and disaggregation of the plaque leads to resolution of the classical signs of this inflammation (Löe et al., 1965, Theilade et al., 1966). Existing evidence indicates that gingivitis precedes the onset of periodontitis; however, not all gingivitis cases develop into periodontitis. This is because for the development of periodontitis, accumulation of plaque bacteria alone is not sufficient but a susceptible host as well (Listgarten et al., 1985, Lindhe et al., 1973, Löe et al., 1986, Page, 1999). Periodontitis is a disease that involves complex interactions of the biofilm with the host immune response and subsequent changes in bone and connective tissue homeostasis (Offenbacher et al., 2007, Tatakis and Kumar, 2005, Taubman et al., 2007).

This chapter will detail the microbiological and host factors associated with periodontal disease.

1.2 Microbiology of periodontal disease

Dental plaque has been defined as the diverse community of microorganisms, found on the tooth surface as a biofilm embedded in an extracellular matrix of polymers of host and microbial origin (Marsh, 2004). Essentially this is the basic definition of a biofilm, i.e. matrix enclosed microbial populations adherent to a surface and/or to one another (Ramage et al., 2010).

1.2.1 Biofilms

Historically microbiologists have focused upon free-living (planktonic) cells in pure-culture, resulting in the common perception that microorganisms are unicellular life forms. However, literature has emerged within the past 3 decades demonstrating a link between the surface attached growth state to microbial pathogenesis and human infection (Costerton et al., 1981a, Costerton et al., 1981b). Extensive research efforts since then have now revealed that a wide range of bacteria and fungi alternate between planktonic

and surface attached (sessile) multicellular communities, or biofilms, which is illustrated in Figure 1.1 (Ramage et al., 2010). Within their natural ecosystems most microbes have been shown to exist as attached communities of cells within an organized biofilm and not as planktonic organisms. Biofilms can form in the natural environment as well as inside the human host, and can be considered as complex cities of microbes that cooperatively interact in an altruistic manner (Coghlan, 1996). The advantages to an organism of forming a biofilm include protection from the environment, resistance of physical and chemical removal of cells, metabolic cooperation and a community based regulation of gene expression (Stoodley et al., 2002, Donlan and Costerton, 2002). In recent years there has been an increased appreciation of the role that microbial biofilms play in human medicine, particularly because microbes growing within biofilms exhibit unique phenotypic characteristics compared to their planktonic counterpart cells, including increased resistance to antimicrobial agents and protection from host defences (Donlan and Costerton, 2002, Ramage et al., 2010). Therefore, they pose a major problem to clinicians as they are difficult to treat, none more so than those associated with the oral cavity, specifically periodontitis.

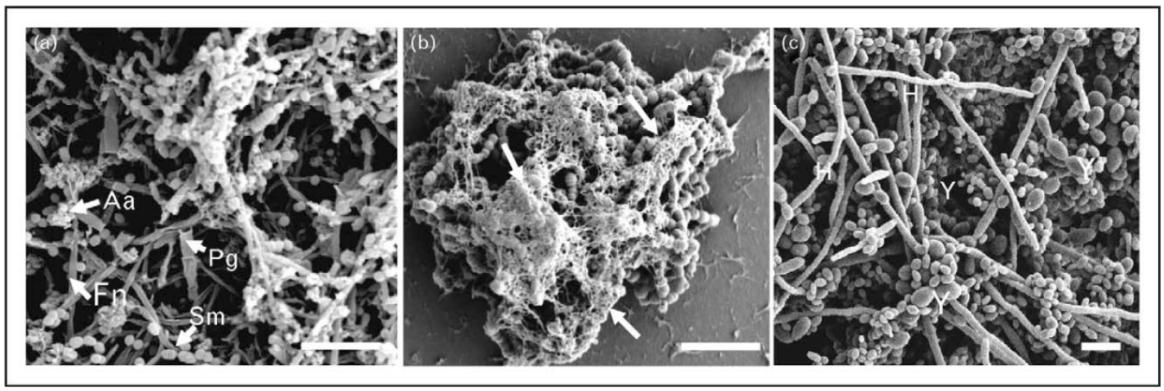


Figure 1.1: Scanning electron micrographs of clinically important biofilms formed in vitro. (a) Biofilm representative of subgingival periodontal plaque. The biofilm consists of *Streptococcus mitis* (Sm), *Fusobacterium nucleatum* (Fn), *Aggregatibacter actinomycetemcomitams* (Aa) and *Porphyromonas gingivalis* (Pg). **(b)** *Streptococcus mutans* biofilm. Note the polymeric matrix material (arrow) surrounding the coccoid cells because it was grown in media supplemented by 1% glucose. **(c)** *Candida albicans* biofilm. Note the combination of yeasts (Y) and hyphae (H) that form a lattice network to support the dense structure. The scale bar represents 10 μm. Taken from (Ramage et al., 2010).

1.2.2 Plaque biofilms

The microflora of the oral cavity is diverse and more than 700 bacterial species have been detected (Aas et al., 2005, Paster et al., 2006, Faveri et al., 2008). Nonetheless, in the maintenance of oral health and in the aetiology of oral diseases in humans, a relatively small number of bacterial species are thought to play an important role (Socransky et al., 2002, Kumar et al., 2005). The bacterial composition often changes from a sparse Gram-positive bacteria biofilm, usually found in healthy individuals, to more Gram-negative anaerobic rod bacterial biofilm, usually observed in diseased individuals. The biofilm communities are complex and dynamic structures that accumulate through sequential and ordered colonization of multiple oral bacteria (Kolenbrander et al., 2002). Phenotypical differences are observed in the oral bacteria growing in the biofilms and planktonic bacteria like increased tolerance to antibiotics, pH and oxygen (Bradshaw et al., 1998, Welin-Neilands and Svensater, 2007, Sedlacek and Walker, 2007). Bacterial species in dental biofilm communities interact with other bacteria in the biofilm either cooperatively or competitively through physiological and metabolic routes. Planktonic bacterial cells attach directly to the oral tissue surfaces or bind indirectly to other bacterial cells that have already been colonized (Kolenbrander et al., 2002). The communication

within the dental biofilms can occur through metabolic communication, genetic exchange and importantly, quorum-sensing (Chalmers et al., 2008, Sedgley et al., 2008). The defined and sequentially ordered steps in the formation of oral biofilms are outlined in Figure 1.2.

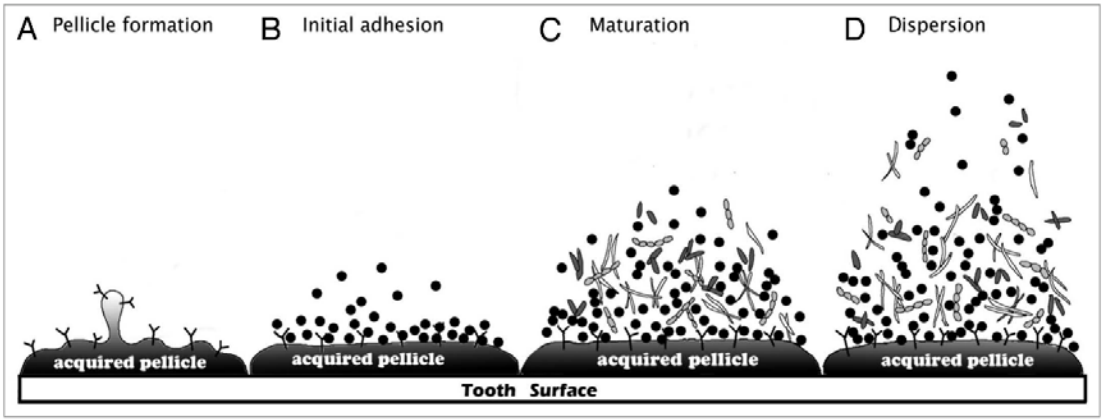


Figure 1.2: Oral biofilm formation. This diagram represents each step of oral biofilm formation. **(A) Pellicle formation**. The pellicle is a thin film derived from salivary glycoproteins attached to a clean tooth surface. **(B) Initial adhesion**. Pioneer bacteria in saliva recognize the binding proteins in acquired pellicle and attach to them. This adhesion is reversible. **(C) Maturation**. Different bacterial species coaggregate and mature biofilm forms. **(D) Dispersion**. Bacteria disperse from the biofilm surface and spread to colonize a new site. Taken from Huang and colleagues (Huang et al., 2011).

1.2.2.1 Adhesion to tooth surfaces

Bacterial adhesion to and subsequent colonization of the surfaces of teeth and tissues are the first steps towards the formation of dental biofilms. Oral bacteria such as streptococci can colonize the tooth surface by binding to the complex proteinaceous pellicle (Rogers et al., 2001, Kolenbrander et al., 2002). Many oral streptococci have that ability to bind to proteins such as alpha-amylase, proline-rich proteins, and proline-rich glycoproteins, and are recognized as early colonizers. This capability may present an advantage on the streptococci in establishing early dental plaque (Kolenbrander et al., 2002).

1.2.2.2 Co-aggregation among oral bacteria

Planktonic bacteria that cannot directly colonize the tooth surface may bind via receptors to the cell surfaces of early colonizers that adhere to the tooth surface. Co-aggregation is a specific cell-to-cell reaction that occurs between

distinct bacterial cells and is one of the most important mechanisms underlying oral bacterial colonization and dental biofilm formation (Kolenbrander et al., 2002). Early colonizers bind via adhesins to the complementary pellicle receptors on the tooth surface followed by secondary colonizers binding to early colonizers that are previously bound to the teeth. The process of bridging between a co-aggregation of cells consisting of more than 3 bacterial species is very important, because it connects a few species that are not co-aggregation partners. *F. nucleatum* can co-aggregate with many oral bacteria, including streptococci and obligate anaerobes like *P. gingivalis*. Therefore, *F. nucleatum* is a key component of dental biofilms and serves as a coordinator that bridges the late and early colonizers making it an "intermediate colonizer" (Kolenbrander et al., 2002). This is illustrated clearly through the schematic diagram in Figure 1.3.

1.2.2.3 Metabolic communication among oral bacteria

Oral bacteria use nutrients which are available from saliva, gingival crevicular fluid, food containing sugars, food debris, and metabolic products of other bacteria (Hojo et al., 2009). Metabolic communication among oral bacteria may occur through the excretion of a metabolite by one organism that can be used as a nutrient by a different organism. This can also occur through the breakdown of a substrate by the extracellular enzymatic activity of one organism that creates biologically available substrates for different organisms (Kolenbrander et al., 2002). For example, the hydrolysis of host glycoproteins by *S. oralis* and the subsequent utilization of released monosaccharides are important in the survival of this and other oral species (Byers et al., 1999).

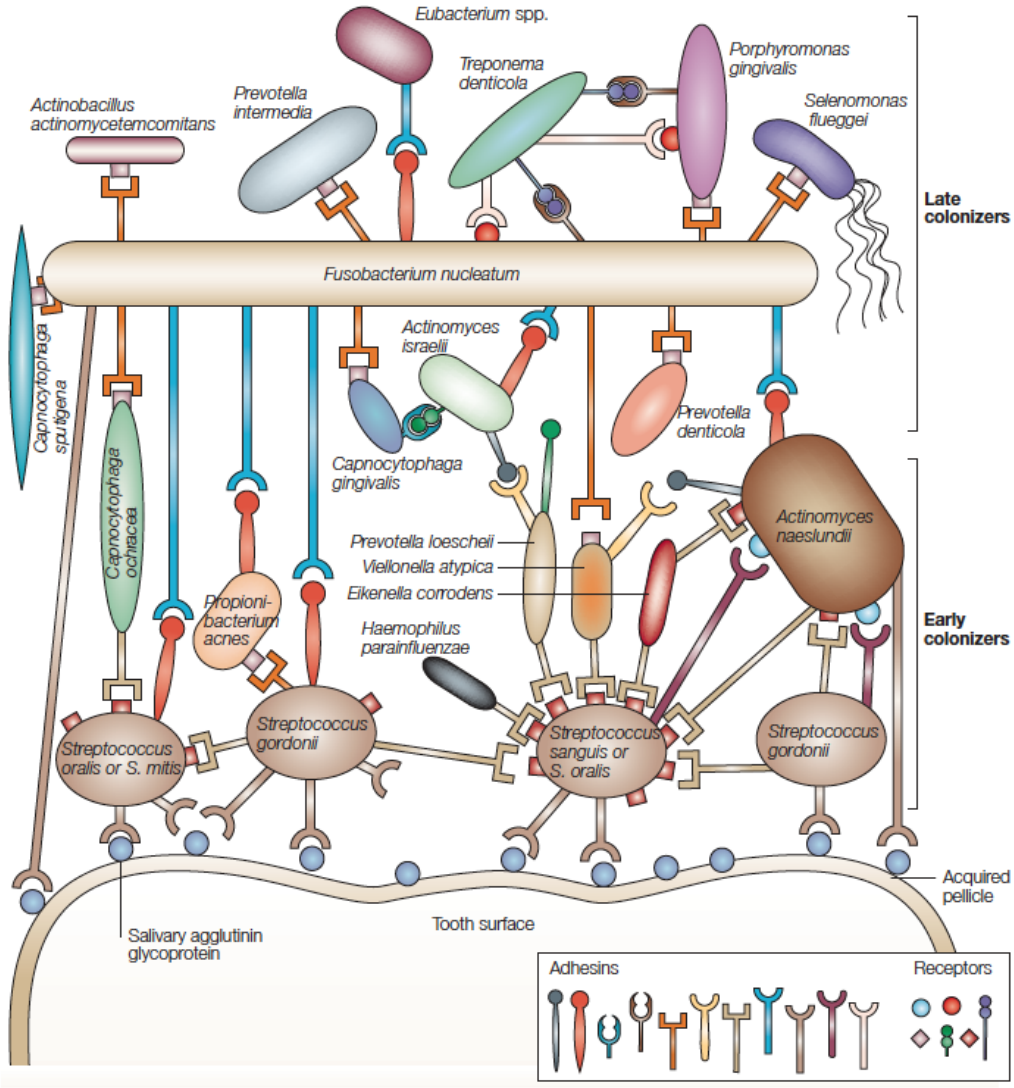


Figure 1.3: Bacterial colonization of a tooth surface. This shows the colonisation of bacteria through various adhesion molecules. The diagram shows the acquired pellicle on the tooth surface. Early colonisers mainly consisting of various streptococcal species bind the acquired pellicle. *Actinomyces* and *Veillonella* species associate closely with the streptococcus species and start forming the primary layer of the biofilm. Intermediate colonisers such as *F. nucleatum* adhere to members of the primary complex. Lastly, pathogenic bacteria such *A. actinomycetemcomitans* and *P. gingivalis* bind to the intermediate colonisers, thus forming a multi- species biofilm (Bakaletz, 2004).

1.2.2.4 Quorum-sensing

Quorum-sensing is a process of chemical communication among bacteria. It is defined as gene regulation in response to cell density, which influences various functions like virulence, acid tolerance and biofilm formation {Li, 2001 #40}. Quorum-sensing is considered an important bacterial function because bacteria within biofilms reach a high cell density (Hojo et al., 2009). Auto-inducer 2 (AI-2) is one of the most well-known signalling molecules associated with quorum-sensing for cross kingdom communication (Bassler et al., 1994). The synthesis of AI-2 is catalyzed by LuxS, an enzyme encoded by the *luxS* gene, which is conserved in the genome of a wide range of Gram-positive and Gram-negative bacteria. Many studies suggest that oral bacteria have a quorum-sensing system that depends on LuxS/AI-2 (Hojo et al., 2009). For example, LuxS-deficient *P. gingivalis* mutant was found to produce low levels of protease (Burgess et al., 2002) and an inability to form a mixed-species biofilm with *S. gordonii* (Lamont et al., 2002).

1.2.3 Dental plaque composition

A variety of Gram-positive (e.g., *Streptococcus* spp, *Actinomyces viscosus*) and Gram-negative species (e.g., *Campylobacter gracilis, Fusobacterium nucleatum*, *Prevotella intermedia*) have been implicated in gingivitis (Theilade et al., 1966, Macuch and Tanner, 2000, Kremer et al., 2000). The distinct role of the dental plaque in periodontitis has been established by various investigators in the field over many years. Historical studies indicate that periodontal diseases occur in response to the quantity of plaque (non-specific plaque hypothesis), whereas others implicate specific microbial species in the aetiology of the disease (specific plaque hypothesis) (Loesche, 1976, Loesche and Grossman, 2001). This concept is illustrated in Figure 1.4, where the acquisition of a 'foreign' bacteria can alter the balance of the microbial consortia, resulting a change from health to disease (Marsh, 2006).

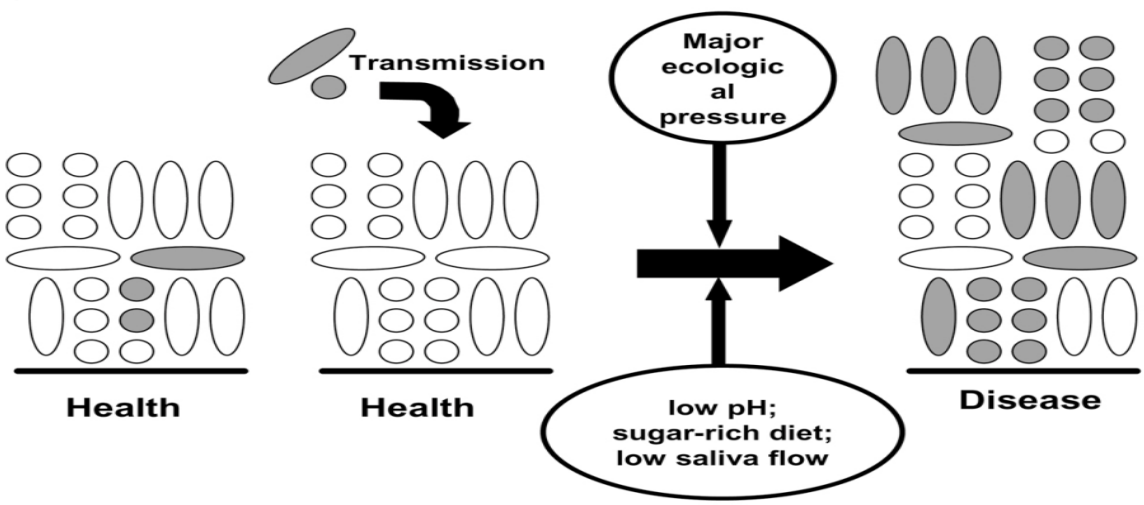


Figure 1.4: Schematic representation of the relationship between the microbial composition of dental plaque in health and disease. Potential pathogens (grey) may be present in low numbers in plaque, or transmitted in low numbers to plaque; both situations may be compatible with health. A major ecological pressure will be necessary for such pathogens to outcompete other members of the resident microflora (white) and achieve the levels (numerical dominance) needed for disease to occur. Possible ecological pressures for caries include a sugar-rich diet, conditions of low pH, or low saliva flow. Disease could be prevented not only by targeting the pathogen directly (*e.g.* with antimicrobial or anti-adhesion agents) but also indirectly by interfering with the ecological pressure responsible for the selection of the pathogen (Marsh, 2006).

Seminal studies of sub-gingival plaque by Socransky demonstrate that certain species frequently occur in complexes (Socransky and Haffajee, 2005). For example, the red complex of species, which includes *P. gingivalis, Tannerella forsythia* and *Treponema denticola,* is strongly associated with the clinical signs of periodontitis. *A. actinomycetemcomitans*is also strongly associated with certain forms of periodontal disease, specifically aggressive periodontitis (Berthold et al., 1986, Slots and Ting, 1999, Cortelli et al., 2005). A further group are classified as the amber species and these are less strongly associated with periodontal destruction. These include *Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, Fusobacterium nuc. vincentii, Fusobacterium nuc. nucleatum, Fusobacterium nuc. polymorphum and Fusobacterium periodonticum. Eiknella nodatum, Campylobacter gracilis, Streptococcus constellatus and Capnocytophaga rectus* (Socransky et al., 1998, Socransky and Haffajee, 2002, Zambon, 1985). Figure 1.5 illustrates the different complexes described by Socransky and colleagues (review (Socransky and Haffajee, 2005)). All of these species are commonly present at sites of periodontal destruction at the time of initial diagnosis and their elimination or

persistence is associated with prognosis after treatment. Each bacterium has specific virulence factors, which confer biological feasibility on their proposal as putative periodontal pathogens. The following section will focus on some of these key organisms in more detail, particularly those used in subsequent studies.

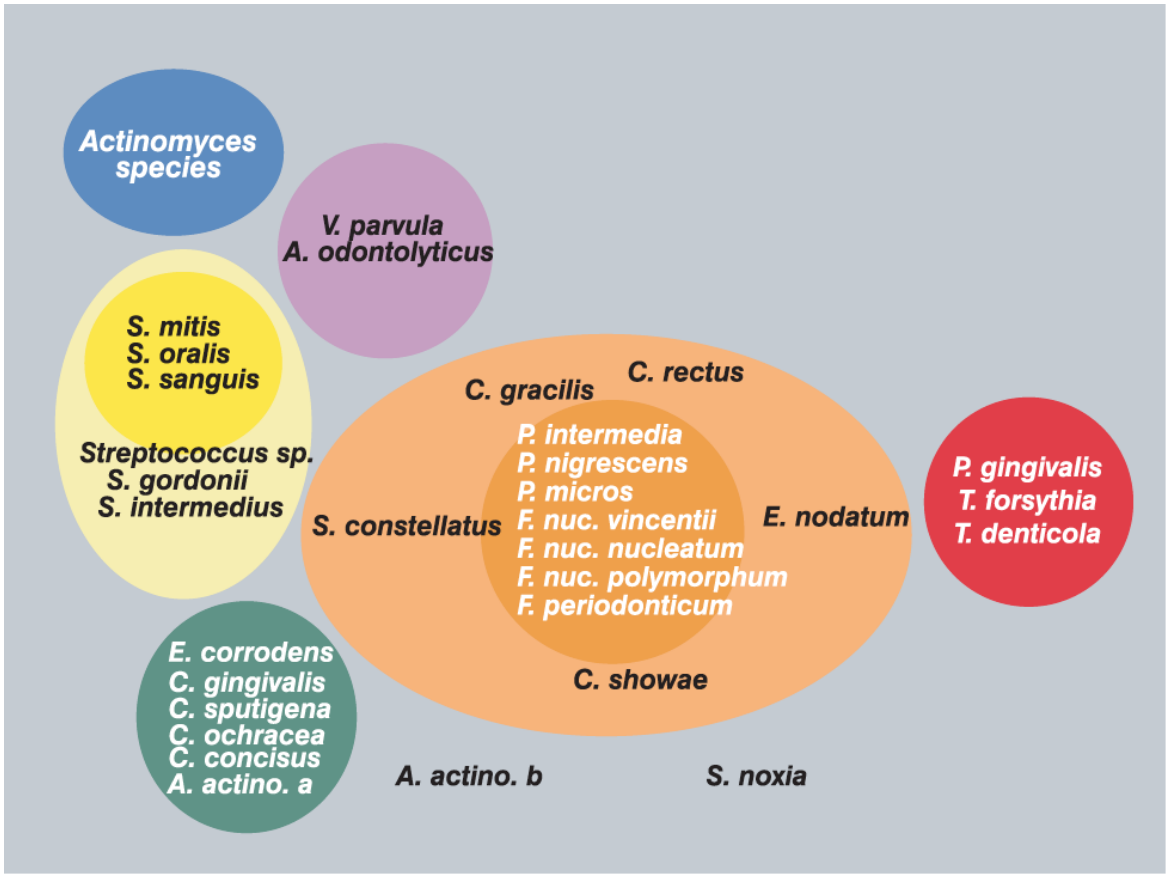


Figure 1.5: Diagrammatic representation of the relationships of species within microbial complexes and between the microbial complexes (Socransky and Haffajee, 2005).

1.2.3.1 *Porphyromonas gingivalis*

Porphyromonas gingivalis is a Gram-negative, anaerobic, non-motile, asaccharolytic coccobacilli associated with periodontal disease. This bacteria initially grows as white to cream coloured raised colonies on agar surfaces which starts to darken from the edge to the centre to a deep red to black colour with time (4-8 days) (Holt et al., 1999). *P. gingivalis* appears to colonise the inflamed gingival sulcus. In healthy subjects this is a 0.5 mm deep crevice between the tooth and the gum, the portal of entry and recess for periodontal biofilms. The subgingival sites of the oral cavity are the main areas colonized by *P. gingivalis* but has also been recovered from saliva, tongue, tonsils and supragingival plaque samples (Socransky and Haffajee, 1992). *P. gingivalis* makes up less than 5% of the cultivable subgingival flora of individuals with gingivitis but increases with advanced periodontal disease (Forng et al., 2000). For *P. gingivalis* to be associated with the disease, they have to accumulate in the subgingival dental plaque and start to proliferate in high numbers, as *P. gingivalis* can only be found in low numbers in a healthy mouth (Haffajee et al., 1998, Papapanou et al., 1997).

The microbial aetiology of periodontitis is inconclusive, but only a few specific bacteria, including *P. gingivalis* have been implicated as major pathogens of chronic periodontitis (Socransky et al., 1998), and has recently been assigned as a 'keystone pathogen' of periodontal disease, capable of causing a shift in microbial diversity even thought present in low numbers in the final microbial community (Hajishengallis et al., 2012). *P. gingivalis* produces a variety of virulence factors, including bioactive metabolic products, fimbriae and an array of proteolytic enzymes (Lamont and Jenkinson, 1998). Among the proteolytic enzymes, cysteine proteases (gingipains) have been implicated in at least 85% of the total proteolytic activity exerted by various strains of *P. gingivalis* and are considered major contributors to the pathogenic potential of *P. gingivalis* (Potempa et al., 1997). Gingipains, categorized into arginine-specific gingipains [Arg-gingipain-A (RgpA) and Arg-gingipain-B (RgpB0] and the lysine-specific gingipain [Lys-gingipain(Kgp)], are encoded by three different genes, referred to as *rgpA, rgpB* and *kgp*, which are conserved among laboratory and clinical strains of *P. gingivalis* (Curtis et al., 1999). *P. gingivalis* participate in the disease process sequentially by **(i)** adherence and colonization, **(ii)** nutrient acquisition,

(iii) neutralization of host defences, **(iv)** manipulation of inflammatory response and **(v)** tissue destruction, invasion and dissemination to tissue sites (Guo et al., 2010), which are now discussed.

(i) Adherence and colonization

Initial colonization of *P. gingivalis* is facilitated by its ability to adhere to a wide variety of biotic host surfaces, including epithelial cells, extracellular matrix (ECM) and other bacteria. Gingipains are involved directly or indirectly in this process and are therefore crucial in the initial stages of periodontal infection by *P. gingivalis* (Guo et al., 2010). Dental plaque is formed by the specific coaggregation of different species of oral bacteria (Kolenbrander et al., 2006, Rickard et al., 2003) and this leads to formation and maturation of a biofilm that facilitates the adherence and colonization by periodontal pathogens. *P. gingivalis*, which is a late colonizer of the bacterial biofilm on the tooth surface of sub-gingival sites, promotes co-aggregation by a variety of surface components, including lipopolysaccharides, carbohydrates, gingipain complexes and fimbriae, with the latter two being the best characterized (Guo et al., 2010). Discrete hemagglutinin-adhesin domains of RgpA, Kgp and hemagglutuinin A (HagA) have shown evidence in a direct role in coaggregation (Abe et al., 2004, Kamaguchi et al., 2003, Yamada et al., 2005). Therefore, *P. gingivalis* triple-knockout mutants devoid of gingipains (*rgpA rgpB kgp*$^-$), or devoid of proteins bearing hemagglutinin-adhesin domains (*hagA rgpA kgp*$^-$), exhibit no coaggregative activity (Abe et al., 2004).

The initial step of *P. gingivalis* attachment to the oral cavity is fimbriae-mediated [Long fimbriae (FimA) and short fimbriae (Mfa)] which are involved in cell invasion and induction of inflammatory responses (Amano, 2010). The maturation of the long fimbriae requires RgP activity (Nakayama et al., 1996). FimA, which is secreted onto the *P. gingivalis* surface in a precursor form requires Rgp to process it into the filamentous structure of the mature fimbriae (Shoji et al., 2004). The precursor form of FimA on the cell surface of Rgp devoid gingipain-null mutant can be converted into biologically active polymeric fimbriae by exogenous gingipains (Kato et al., 2007b).

Gingipains are potent non-fimbrial adhesions that actively bind several extracellular proteins (Pathirana et al., 2010) and also mediate tight adherence to epithelial cells and gingival fibroblasts (Chen and Duncan, 2004, Chen et al., 2001a), most of the binding provided by Kgp (Pathirana et al., 2007, Pathirana et al., 2008). Gingipains are also involved in *P. gingivalis* adherence through generation of cryptic ligands, by the degradation of extracellular matrix proteins by Rgps, which exposes the peptides with C-terminal arginine, for which FimA has specific affinity (Kato et al., 2007a, Kontani et al., 1997).

To summarise, gingipains are essential for *P. gingivalis* adherence to different sites within the oral cavity; either directly by acting as non-fimbrial adhesins or indirectly by arranging the assembly of fimbriae. The next step in the disease process for the pathogen is to acquire nutrients for growth and neutralize host defenses.

(ii) Nutrient acquisition

P. gingivalis, is a fastidious asaccharolytic bacteria which acquires energy by fermentation of both carbon and amino acid residues. The iron and porphyrin essential for the growth of *P. gingivalis* is provided by heme. Gingipains are responsible to a large extent to satisfy the nutritional needs of *P. gingivalis* by exploiting different sources of heme and iron in the human host (Guo et al., 2010).

Red blood cells are the largest reserves of heme and iron in the human hosts. *P. gingivalis* initially clumps the erythrocytes together and slowly lyses them to release the haemoglobin and the hemagglutinin activity is related to the hemagglutinin-adhesin domains of RgpA, Kgp and HgA (Shi et al., 1999). The slow hemolysis by *P. gingivalis* is not fully understood, although proteolytic activity has been implicated in this process (Chu et al., 1991). The study showed that the hemolysin was concentrated on the outer membrane of the bacteria with significant haemolytic activity concentrated on the outer membrane vesicles. The expression of haemolytic activity was increased by $Ca2^{+}$ and $Mg2^{+}$ ions while the activity was inhibited by proteinases K, trypsin, the proteinase inhibitors Na-P-tosyl-L-lysine chloromethyl ketone and benzamidine, the metabolic inhibitor M-chlorophenyl hydrazone, and iodoacetate. The effect of

heme limitation on increased haemolytic activity was also studied. Purified gingipains do not seem to have a direct involvement in hemolysis but a Kgp-null mutants show about half of the hemolytic activity of the wild-type strains or complemented mutants suggests a role for Kgp in hemolysis (Lewis et al., 1999).

Transferrin and lactoferrin act as stores of extracellular iron within mucosal surfaces. Hemoglobin leaking out from erythrocytes is taken up by haptoglobin, while the heme released from the erythrocytes are taken up by hemopexin and albumin. Because of these scavenging proteins, there is low concentration of free iron and heme in the physiological human fluids. Pathogenic bacteria uses siderophores, which are scavenger molecules, to get iron out from carriers of iron in the host (Raymond et al., 2003). As *P. gingivalis* lacks the siderophore action it uses gingipains to degrade haptoglobin, transferrin and hemopexin to grow on minimal media with serum human serum as the source of heme or iron (Sroka et al., 2001). The study showed that the lysine-specific gingipains Kgp can cleave haemoglobin, transferrin, haptoglobin and hemopexin. Degradation of hemopexin and transferrin in the human serum was also detected. However, the degradation of haemoglobin in serum by Kgp was not detected. The arginine-specific gingipains (RgpA and RgpB) also degraded hemopexin and transferrin in serum but only at high concentrations of the enzymes (Sroka et al., 2001).

P. gingivalis depends on peptides for its source of carbon and nitrogen for its growth, as it does not utilize free amino acids (Milner et al., 1996). Milner and colleagues developed a chemically defined media that used bovine serum albumin as the source of carbon and nitrogen, and alpha-ketoglutarate to prevent the blackening of the cells due to the deposition of metal sulphides. *P. gingivalis* uses its extensive proteolytic system, which is composed of endopeptidases (gingipains), oligopeptidase and di- and tripeptidylpeptidases, in a cascade-like manner, to fulfil this nutritional need (Potempa et al., 2000).

(iii) Neutralization of host defenses

After successful colonization of the oral mucosa by *P. gingivalis,* it has to confront the host defense system. The innate immune system presents the first obstacle, comprising of antimicrobial peptides and peptides, the complement system, neutrophils and tissue-resident macrophages. As *P. gingivalis* is

embedded deep in the biofilm, they resist the acute inflammatory reaction induced by neutrophil influx and complement activation. Hence, the second line of defense is activated, composed of antibodies, activated macrophages and T-cells. Even the concerted effects of both the systems (synchronized by the cytokine network) does not eliminate *P. gingivalis*, which makes the innate immune system cause extensive collateral damage to the surrounding gingival and periodontal tissues, with the pathologic changes associated with periodontitis (Guo et al., 2010).

The first line of mucosal defense against the invading bacteria is cationic antimicrobial peptides. Antimicrobial peptides, which are secreted from cells of the gingival tissues, include human beta-defensins, the cathelicidin-type peptide LL-37 and neutrophil-derived alpha-defensins, which maintain homoestasis in spite of constant exposure from a variety of invading bacteria (Diamond et al., 2008). This review states that antimicrobial peptides are secreted in the gingival epithelium in response to a small subset of bacteria and TLR ligands, via different pathways. The environments into which these peptides are secreted in the saliva and gingival crevicular fluid differ greatly and can affect their respective activities in host defense. Significantly increased levels of alpha-defensins and LL-37 were reported in the gingival crevicular fluid from chronic periodontitis patients in comparison with healthy controls (Puklo et al., 2008, Turkoglu et al., 2009). The Turkoglu study included 59 subjects who had their GCF levels of LL-37 and IL-18 measured using ELISA. Probing depth (PD), clinical attachment level (CAL), plaque index (PI), bleeding on probing, and papilla bleeding index (PBI) were also assessed for the chronic periodontitis and gingivitis patients and healthy controls. Elevated levels of LL-37 in GCF of chronic periodontitis patients were reported in comparison to other groups. There was also a positive relationship between the levels of LL-37 and PD, CAL, PI and PBI at the sites sampled. Puklo and colleagues (2008) collected GCF samples from 14 aggressive periodontitis patients, 17 chronic periodontitis patients and 9 healthy subjects. Samples were analysed for periodontopathogen load by real time PCR. The levels of methyloperoxidase and alpha-defensins (HNP 1-3) were determined by ELISA, and the level of cathelicidin (hCAP18/LL-37) assayed by Western blot. There was no correlation between myeloperoxidase concentration and the levels of LL-37 and HNP 1-3 in patient samples compared

to control. Aggressive periodontitis and chronic periodontitis patients showed elevated amounts of HNP 1-3 and those with chronic periodontitis had elevated levels of mature LL-37. The increased levels of both the peptides in chronic periodontitis correlated with the *P. gingivalis* load.

Chronic periodontitis does not resolve by itself unless there is therapeutic intervention due to the resistance of the bacterial biofilm to killing by the antimicrobial peptides (Guo et al., 2010). The sensitivity of *P. gingivalis* killing by LL-37 and human beta-defensins are strain dependent: ATCC 33277 is more susceptible than W50, W83 and ATCC 49417 (Ji et al., 2007, Joly et al., 2004, Ouhara et al., 2005). In the Ji study, 20 strains of 13 oral bacteria were tested for their susceptibility to LL-37 and human beta-defensin 3 by liquid dilution assay to determine the minimum inhibitory concentration (MIC). It reported that the MICs varied greatly depending on the strains and species. The study by Joly and colleagues (2004) tested the effectiveness of HBD-2 and HBD-3 against an array of oral microbes, including *P. gingivalis* by radial dilution assays on at least three strains of each species. The variability in MIC was strain specific rather than species specific. Ouhara and colleagues (2005) tested the antimicrobial activity of synthetic antimicrobial peptides human beta-defensin 1 (hBD-1), hBD-2, hBD-3 and LL-37 (CAP18) against oral bacteria, including six strains of *P. gingivalis*. Beta-defensin resistance was induced in *P. gingivalis* by pre-treating with 1 ng/mL of defensins, heat stress, oxidative stress and peroxide stress (Shelburne et al., 2005a), which in turn enhances the expression of *rgpA* and *rgpB* genes (Shelburne et al., 2005b). A study by Carlisle and co-workers (2009) used cell culture supernatants which fully or partially degraded human alpha-defensins and human beta-defensins after 30 minutes (Carlisle et al., 2009). Another study by Gutner and colleagues showed that *P. gingivalis* was capable of degrading LL-37 by utilizing its RgpA and RgpB (Gutner et al., 2009).

P. gingivalis has the ability to survive in the inflamed tissue environment from the attacks of phagocytic cells, neutrophils and macrophages. The interplay between phagocytic oxidative burst of the host and the oxidative stress response of *P. gingivalis* was investigated; here the oxidative burst by the neutrophils enhanced the survival of *P. gingivalis* (Mydel et al., 2006). *P. gingivalis* can also survive phagocytosis by macrophages by inducing cross-talk between CXCR4 and

TLR2 (Hajishengallis et al., 2008).The gingipains' role in evading the neutrophil and macrophage phagocytosis is either directly by degrading the receptors and cytokines essential for phagocyte functions or indirectly by C5a, which is used by *P. gingivalis* to weaken TLR2-dependent immunity (Wang et al., 2010).

(iv) Manipulation of the inflammatory response

Inflammation is a complex interaction between the cytokines and their receptors expressed by the host tissues and immune cells, in response to a foreign agent. The immune response is disrupted by the invading bacteria by targeting the cytokine mediators (Guo et al., 2010). *P. gingivalis* uses gingipains to disrupt the cytokines and cytokine receptor networks of the host, including interleukin-1 beta (IL-1 beta) (Sharp et al., 1998), interleukin-6 (IL-6) and its receptors (Banbula et al., 1999, Oleksy et al., 2002), interleukin-8 (IL-8) (Mikolajczyk-Pawlinska et al., 1998), interleukin 12 (IL-12) (Yun et al., 2001, Yun et al., 2002), interleukin-4 (IL-4) (Yun et al., 2003), interferon-gamma (Yun et al., 1999), tumor necrosis factor-alpha (Calkins et al., 1998), CD4/CD8 (Kitamura et al., 2002), CD14 (Sugawara et al., 2000) and intercellular adhesion molecule-1 (CD54) (Tada et al., 2003). *P. gingivalis* is able to manipulate the cytokine network through many factors in order to evade the host defence.

(v) Tissue destruction

Morphological changes associated with advanced periodontal disease include alveolar bone resorption and periodontal ligament destruction, which lead to attachment loss and periodontal pocket formation. Gingipains play an important role, directly and indirectly, in the remodelling of the pathologic tissue associated with the development and progression of periodontal disease by excessive expression of MMPs over their inhibitors (tissue inhibitors of metalloproteinases [TIMPs]), release of proteases from necrotic cells and activated neutrophils, inactivation of endogenous protease inhibitors and uncontrolled conversion of zymogenic MMPs into their proteolytically active forms (Guo et al., 2010). The broad consensus is that *P. gingivalis* cells, cell extracts, spent media or LPS can stimulate the secretion of MMPs at a higher level than TIMPs in dendritic cells (Jotwani et al., 2010), human periodontal ligament cells (Sato et al., 2009), human gingival fibroblasts (Zhou and Windsor, 2006) and an engineered human oral mucosa (Andrian et al., 2007). The latent

MMPs can be directly activated by gingipains (DeCarlo et al., 1997, Grayson et al., 2003). Inflammatory neutrophils are the main source of serine proteases and metalloproteases, which are released into the environment and usually neutralized by endogenous serine proteinase inhibitors. It has been shown that serine proteinase inhibitors are proteolytically inactivated by *P. gingivalis*-derived proteases (Nelson et al., 1999, Potempa et al., 1998, Into et al., 2006, Kantyka et al., 2009). If this occurs, the host proteases could attack various targets in the periodontium. This shows that *P. gingivalis* can be directly or indirectly involved in causing damage to periodontal tissues.

From this it can be concluded that the virulence factors of *P. gingivalis* are essential at every step of the infection: from attachment and colonisation, to nutrient acquisition, to evasion of host defences and to tissue invasion.

1.2.3.2 *Fusobacterium nucleatum*

Fusobacterium nucleatum are Gram-negative, anaerobic, non-motile, non-spore forming, spindle-shaped bacilli associated with periodontal disease. Colony morphology is not a consistent parameter of the Fusobacteria and is not sufficient for species identification (Tuner et al., 1992). It is one of the first Gram-negative species to become established in dental plaque and is considered as an intermediate colonizer, which helps in bridging the attachment of non-pathogens (commensals) and true pathogens (Kolenbrander, 2000, Kolenbrander et al., 2002). It is one of the most common species in both supragingival and subgingival plaque in both healthy individuals and patients with periodontal disease (Kononen et al., 1994, Moore and Moore, 1994, Ximenez-Fyvie et al., 2000). It is also one of the most common oral species isolated from extra-oral infections, including blood, brain, chest, lung, liver, joint, abdominal, obstetrical and gynaecological infections and abscesses (Signat et al., 2011). The ability of *F. nucleatum* to co-aggregate with many plaque bacteria suggests that it acts as a microbial bridge.

(i) Adhesion and co-aggregation

F. nucleatum is involved in both adhesion and co-aggregation reactions and seems to play a key role in the multi-species co-aggregation network found in the periodontal pocket (Bolstad et al., 1996). It is proposed that fusobacteria

act as a bridge between early and late colonisers. The early colonizers adhere to the tooth pellicle and co-aggregate with other early colonizers and also with *F. nucleatum*. Late colonizers such as *P. gingivalis, A. actinomycetemcomitans* and *T. denticola* co-aggregate almost exclusively with *F. nucleatum*, which seems to play an important role by bridging these co-aggregations with early colonizers (Kolenbrander et al., 1989, Kolenbrander et al., 1993, Kolenbrander and London, 1993). The adhesins in *F. nucleatum* are outer membrane proteins (OMPs) which are essential for their attachment to specific substrates as a first step of colonization. OMP is a receptor polypeptide involved in fusobacterial corncob coaggregations (Kaufman and DiRienzo, 1989). Attachment of early colonizers streptococci to fusobacteria is mediated through fimbriae on the surface of the cocci (Handley et al., 1985, Hasty et al., 1992) by at least two types of corncob receptors, one which binds to lipoteichoic acid and which that does not (DiRienzo and Rosan, 1984, Kaufman and DiRienzo, 1988, Kaufman and DiRienzo, 1989). *F. nucleatum* coaggregates with the late colonizer *P. gingivalis* through an OMP from *F. nucleatum* and a galactose-containing carbohydrate on *P. gingivalis* (Kinder and Holt, 1989, Kinder and Holt, 1993, Kolenbrander, 1988).

(ii) Immune response induction

F. nucleatum cell wall extracts have been shown to induce significant changes in the expression of genes associated with immune and defence responses which were mainly chemokines and cytokines, innate immune or inflammatory markers, antimicrobials, or protease inhibitors. The genes of defence responses include chemokines IL-8 and CXCL1, 3, 5 and 10, which attract neutrophils, monocytes, macrophages, lymphocytes and CSF2 and -3 that stimulate neutrophil development. *F. nucleatum* also up-regulated the genes encoding antimicrobial peptides and proteins. Multiple protease inhibitors were also up-regulated in response to *F. nucleatum* cell wall extracts (Signat et al., 2011). These inhibitors can target the proteases released by neutrophils (Magert et al., 2005) and bacteria (*P. gingivalis, T. denticola* and *T. forsythia*) (Curtis et al., 2001, Fenno et al., 2001, van der Reijden et al., 2006) and thereby control likely tissue damage. *F. nucleatum* can induce an inflammatory response by up-regulating pro-inflammatory cytokines and metalloproteases (MMP-9 and MMP-13) which has several functions in the regulation of inflammation. *F. nucleatum* can also secrete serine proteases which can damage the periodontal tissues. To

conclude, *F. nucleatum* has been shown to possess protective and aggressive qualities which asks the question if the bacteria is a commensal or a pathogen.

1.2.3.3 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans are Gram-negative, microaerophilic and facultative anaerobic, non-sporulating, non-motile coccobacilli (Zambon, 1985, Pulverer and Ko, 1970). The colony morphology shows transparent rough surfaced and opaque smooth surfaced types. There is also a transparent smooth surfaced type, which is intermediate between the other two types. The colonial variation is associated with fimbriation (Inouye et al., 1990, Rosan et al., 1988, Scannapieco et al., 1987, Slots, 1982).

A. actinomycetemcomitans possess numerous virulence factors that can enhance its survival and may be involved in the pathogenesis of periodontitis (Fives-Taylor et al., 1999). These include the ability to attach to extracellular matrix proteins (Mintz and Fives-Taylor, 1999) and epithelial cells (Meyer and Fives-Taylor, 1994); antibiotic resistance (Roe et al., 1995); a bacteriocin (Hammond et al., 1987, Stevens et al., 1987); bone resorption by either endotoxin (Saglie et al., 1990) or surface-associated material (Meghji et al., 1995); a chemotactic inhibitor (Ashkenazi et al., 1992a); a collagenase (Robertson et al., 1982); a cytotoxin (Shenker et al., 1982a); Fc-binding proteins (Mintz and Fives-Taylor, 1994b); a leulotoxin (Baehni et al., 1979); immunosuppressive factors (Chen et al., 1991, Kinane et al., 1989, Kurita-Ochiai and Ochiai, 1996, Shenker et al., 1982b, Shenker et al., 1990); and the ability to invade epithelial cells (Meyer et al., 1991) and tissues (Christersson et al., 1987).

(i) Adhesion

A. actinomycetemcomitans increase their numbers in the dental plaque by aggregation, intra-generic and inter-generic coaggregation. Vesicles and extracellular amorphous material are likely to mediate the aggregation (Meyer and Fives-Taylor, 1993, Meyer and Fives-Taylor, 1994). While the intra-generic coaggregation by *A. actinomycetemcomitans* has not been demonstrated (Kolenbrander et al., 1990), inter-generic coaggregation has been demonstrated with *F. nucleatum* (Kolenbrander, 1989).

The adhesion of *A. actinomycetemcomitans* to the gingival crevice epithelium is an important step in the colonization and subsequent tissue destruction associated with periodontal disease. The adhesion of the bacteria to the tooth surface has also been investigated using an *in vitro* tooth model which showed that bacteria with fimbriae adhere 3- to 4-fold better than non-fimbriated strains (Rosan et al., 1988). *A. actinomyecetmcomitans* fimbriae (Holt et al., 1980, Scannapieco et al., 1983) are peritrichous (Scannapieco et al., 1987) and often occur in bundles (Rosan et al., 1988). The adhesion of *A. actinomycetemcomitans* has been shown to be strong with the epithelial cells with most of the strains (Meyer and Fives-Taylor, 1994). Cell surface ultramicroscopic structures that mediate adherence include fimbriae (Meyer and Fives-Taylor, 1994, Rosan et al., 1988), extracellular vesicles (Meyer and Fives-Taylor, 1993) and extracellular amorphous material (Meyer and Fives-Taylor, 1994). The fimbriae are implicated in the adherence of *A. actinomycetemcomitans* but some strains expressing the smooth colonial phenotype which have little or no fimbriae can still adhere to the epithelial cells (Meyer and Fives-Taylor, 1994, Mintz and Fives-Taylor, 1994a), indicating adhesion molecules or mechanisms unrelated to fimbriae. Poorly adherent *A. actinomycetemcomitans* which were suspended in extracellular amorphous material became coated with the material and exhibited increased adherence (Fives-Taylor et al., 1995). Vesicles, which are a major feature of the non-fimbriated *A. actinomycetemcomitans* surface, are associated with a non-fimbrial *A. actinomycetemcomitans* adherence mechanism (Meyer and Fives-Taylor, 1993). To conclude, the adhesion of *A. actinomycetemcomitans* to epithelial cells is multi-factorial with several adhesins and mechanisms playing a role.

(ii) Invasion of epithelial cells

Many pathogenic bacteria have the capability of penetrating and surviving within eukaryotic cells (Finlay and Falkow, 1997, Meyer et al., 1997). *A. actinomycetemcomitans* has been shown to penetrate the gingival epithelium (Christersson et al., 1987, Saglie et al., 1986). Based on *in vitro* models using cultured cell lines it has been shown that *A. actinomycetemcomitans* invasion of epithelial cells is a highly dynamic complex involving the attachment of bacteria to the host cell with initiation of some form of signalling, binding to a receptor,

entry into a vacuole, escape from the vacuole, rapid multiplication, intracellular spread, exit from the cell and cell-to-cell spread (Meyer et al., 1996). Many clinical and laboratory isolates have shown invading capabilities in a variety of cell lines but invasion efficiencies vary with individual strains and cell lines used (Blix et al., 1992, Meyer et al., 1991, Sreenivasan et al., 1993). The invasion is initiated by *A. actinomycetemcomitans* coming into contact with microvilli of the epithelial cells. The contact induces the epithelial cells to produce ruffled apertures and the bacteria seems to pass through these apertures into the interior of the host cell (Meyer et al., 1996). The invasion of phagocytes by *A. actinomycetemcomitans* involves the rearrangement of the host cytoskeletal network which is inhibited by cytochalsin-D demonstrating a role of actin in the invasion process (Brissette and Fives-Taylor, 1999). There are a few *A. actinomycetemcomitans* isolates which enter the cells via receptor-mediated endocytosis exhibit actin-independent entry, suggesting multiple mechanisms for the *A. actinomycetemcomitans* entry into the epithelial cells (Brissette and Fives-Taylor, 1999). Host-derived membrane bound vacuole takes up *A. actinomycetemcomitans* which the bacteria lyses to enter the cytoplasm (Sreenivasan et al., 1993). The bacteria now transits through the cell to adjacent cells via bacteria-induced protrusions. If there are no adjacent or neighbouring cells, bacteria are just released from the host cell (Meyer et al., 1996). Microtubules have been strongly implicated in the intra- and inter-cellular spread of *A. actinomycetemcomitans* and its release into the extra-cellular environment (Meyer et al., 1996). All these observations show a dynamic process by which *A. actinomycetemcomtans* invades the epithelial cells to enable it to spread to the gingival and connective tissue and initiate the tissue destruction associated with periodontal disease.

(iii) Immunosuppressive factors

A. actinomycetemcomitans has been shown to possess many factors to suppress the host defense mechanisms which play a major role in controlling the concentrations of bacteria in the dental plaque. The bacteria produces a protein which inhibits DNA, RNA and protein synthesis in mitogen-activated human T cells (Shenker et al., 1982a). The protein has also been shown to inhibit IgG and IgM synthesis by human lymphocytes (Shenker et al., 1982b). In addition to this, leukotoxin also impairs the ability of lymphocytes to respond to mitogens by

inhibiting DNA, RNA, protein, IgG and IgM synthesis (Rabie et al., 1988). *A. actinomycetemcomitans* secretes a low-molecular-weight compound which inhibits polymorphonuclear leukocyte chemotaxis which is the host's first line of defense against invading bacteria (Van Dyke et al., 1982). *A. actinomycetemcomitans* has also been shown to be capable of inhibiting polymorphonuclear leukocytes from producing certain compounds which aid in the the fusing of PMN leukocytes with lysosomes. This protein in *A. actinomycetemcomitans* inhibits the production of hydrogen peroxide by PMN leukocytes and some strains are naturally resistant to high concentrations of hydrogen peroxide (Ashkenazi et al., 1992b, Miyasaki et al., 1984). *A. actinomycetemcomitans* are also resistant to defensins that are found in neutrophils (Miyasaki et al., 1990).

(iv) Tissue destruction

A. actinomycetemcomitans has been shown to stimulate bone resorption by several different mechanisms: lipopolysaccharide (LPS) (Kiley and Holt, 1980), proteolysis-sensitive factor in microvesicles (Nowotny et al., 1982) and surface-associated material (Meghji et al., 1995). *A. acinomycetemcomitans* LPS (endotoxins) has been well characterized in tissue destruction (Kiley and Holt, 1980). Low concentrations of *A. actinomycetemcomitans* LPS stimulate macrophages to produce IL-1 α, IL-1 ß and TNF-α, which are cytokines involved in tissue inflammation and bone resorption (Saglie et al., 1990).

(v) Leukotoxin

Leukotoxin is one of the most studied virulence factors of *A. actinomycetemcomitans* which is produced by 56% of the strains isolated from aggressive periodontitis patients (Zambon et al., 1983). The leukotoxin binds to neutrophils, monocytes and a subset of lymphocytes to form pores in the membranes of these target cells (Iwase et al., 1990, Taichman et al., 1987) which reduces the ability of these cells to sustain osmotic homeostasis, resulting in cell death (Karakelian et al., 1998).

1.2.3.4 *Streptococcus mitis*

S. mitis are Gram-positive, facultative anaerobic, non-sporulating, non-motile, chains of cocci (Bensing et al., 2001). *S. mitis* is a primary coloniser which is

predominantly associated with the early stages of plaque formation and is a member of Socransky's yellow complex (Socransky and Haffajee, 2005). *S. mitis and other* streptococcal *species* can bind to the acquired pellicle through a number of adherence strategies such as proline-rich proteins (PRP-1) and lectin-like bacterial proteins. PRP-1 is found within the saliva and different regions of PRP-1's can bind to early colonisers such as streptococcal species. Lectin-like protein is a bacterial protein which can interact with pellicle-associated glycoproteins causing adhesion of primary colonisers to the pellicle (Marsh, 2006). This pioneer bacterium allows other bacteria to bind receptors such as adhesion molecules, which are present on its surface, leading to biofilm formation (Lindhe, 2008, Sardin et al., 2004).

(i) Commensal properties

S. mitis have generally been considered a somewhat benign oral streptococcus and a member of the oral commensal flora. The interaction of *S. mitis* with the host innate immune system is not known exactly. It has shown some strong immunomodulatory effects on human cells. *S. mitis* can induce the expression of human ß-defensin 2 (hBD2) after incubation with gingival epithelial cells (Eberhard et al., 2009), which is a host antimicrobial peptide which can kill oral pathogens. *S. mitis* is also very tolerant to the action of antimicrobial peptides (Nishimura et al., 2004, Ouhara et al., 2005). *S. mitis* also modulates the expression of IL-8, which is a pro-inflammatory chemokine responsible for the chemotaxis of neutrophils to the site of infection (Eberhard et al., 2009). *S. mitis* does not promote IL-8 expression on its own but with co-incubation of *S. mitis* with other oral pathogens *F. nucleatum* or *A. actinomycetemcomitans* can dampen the production of IL-8 induced in response to the pathogens (Zhang et al., 2008, Sliepen et al., 2009). From these observations, it can be concluded that *S. mitis* can supplement host immunity and can survive in the complex oral environment from the competition of oral pathogens, as a beneficial commensal.

Overall, these four oral bacteria that have been described all possess unique properties that allow them to persist within the oral cavity to different extents. However, it is clear from this literature that the immune system plays an important role in protecting the host from these bacteria. This will now be discussed.

1.3 Immunology of periodontal disease

Microbial plaque is considered necessary, but not sufficient for the progression of periodontal disease. Thus, the host response plays a pivotal role in disease pathogenesis (Graves, 2008, Preshaw, 2008). Many studies, both *in vivo* and *in vitro*, have confirmed the periodontopathic virulence of many key oral pathogens (Holt and Ebersole, 2005). Nevertheless, it has been clearly demonstrated that the presence of dental plaque does not necessarily lead to advanced periodontal disease. Moreover, key periodontal pathogens can be found at sites of relative periodontal health (Wolff et al., 1993, Van der Velden et al., 2006). Therefore, it can be concluded that in addition to the necessary role of the dental biofilm in initiating the inflammatory response, other host factors must also be involved (Kinane et al., 2006). These factors may determine whether the inflammatory response will be an effective protective/preventive one, or whether an inappropriate/inefficient inflammatory response leads to host induced tissue destruction.

The pathogenesis of periodontal disease is categorised into four stages, based on histopathological examination of the development of periodontal inflammation as a result of plaque accumulation. These stages are called the **(i)** initial, **(ii)** the early, **(iii)** the established, and **(iv)** the advanced lesion (Page and Schroeder, 1976). A brief description of each stage in lesion progression follows.

(i) Initial lesion

Without normal oral hygiene measures, within 2 - 4 days of plaque accumulation, the early inflammatory response is observed histologically. It is characterised by vasodilatation, loss of perivascular collagen, and active migration of neutrophils and monocytes into the periodontal tissues and junctional epithelium mediated by intercellular adhesion molecules (ICAM) and endothelial leucocyte adhesion molecules (ELAM) are observed. The exudation of serum proteins from the dilated capillaries leads to an increase in GCF fluid flow.

(ii) Early lesion

The early lesion presents after 7 - 14 days plaque accumulation. This is clinically detectable as gingivitis, with more pronounced vascular changes and an increase

in extra-vascular neutrophils. Histologically the inflammatory infiltrate consists of numerous lymphocytes (predominantly T lymphocytes), immediately below the proliferating basal cells of the junctional epithelium. Destruction of the gingival connective tissue occurs, through apoptosis of fibroblasts and a reduction in the collagen fibre network of the marginal gingivae, via host and pathogen derived matrix metalloproteinases (Page and Schroeder, 1976, Takahashi et al., 1995).

(iii) Established lesion

This is similar to the established with a shift in the cell population in the inflammatory infiltrate. Here, a large numbers of plasma cells are the main histological feature in older patients, whereas in younger patients the infiltrate continues to be dominated by lymphocytes (Fransson et al., 1996). Clinically, inflammation becomes more pronounced with an increase in swelling, and the development of false pocketing. T and B lymphocytes and antibodies and complement are present in the inflamed marginal gingivae and gingival sulcus.

(iv) Advanced lesion

At this stage the inflammatory lesion extends into the periodontal ligament and alveolar bone. There is destruction of connective tissue attachment to the tooth. The junctional epithelium migrates down the root surface to form a true periodontal pocket. Destruction of the periodontal ligament and the surrounding alveolar bone is mediated through matrix metalloproteinases and through enhanced osteolytic activity. Direct tissue damage can occur through direct cytotoxicity of bacterial products such as proteinases, collagenases, epitheliotoxin, cytolethal distending toxin, hemolysin, hydrogen sulphide and ammonia (Haffajee and Socransky, 1994). Moreover, dysregulation of host derived factors such as proteinases and proteinase inhibitors, matrix metalloproteinases (MMPs) and TIMPs, pro-inflammatory cytokines such as IL-1α, IL1β, TNFα and others, prostaglandins and the products of polymorphonuclear leukocytes lead to damage to the connective tissue attachment (Kornman, 2008).

1.3.1 Innate immune response to periodontal biofilms

The innate host response primarily involves the recognition of microbial components such as LPS, bacterial DNA, and peptidoglycan by the host cells and subsequent production of inflammatory mediators. The Toll-like receptors (TLRs), which are expressed by leukocytes and resident cells in the periodontal tissues, can activate the innate immune response by the binding to various bacterial components (Garlet, 2010, Mahanonda and Pichyangkul, 2007). The developing biofilm consists of initially Gram-positive cocci in health, changing to increased numbers of motile Gram-negative anaerobes in gingivitis and periodontitis (Moore and Moore, 1994, Socransky and Haffajee, 1997). Gram-negative bacteria possess cell wall endotoxin (lipopolysacharide [LPS]), a potent stimulator of TLR4. LPS is released from Gram-negative bacteria through cell lysis and becomes linked to the extracellular acute phase protein LPS binding protein before binding to CD14. This leads to transfer of LPS to the extracellular domain of the TLR4 receptor and subsequent TLR4 signalling (Akira, 2006). Gram-negative bacteria also activate TLR2 through their cell membrane proteins, TLR5 through flagella, TLR9 through recognition of bacterial CpG DNA and nucleotide-binding oligomerization domain-containing protein 1 and 2 (NOD 1,NOD 2) through peptidoglycan derivatives (Akira, 2006, Mogensen, 2009).

Periodontal pathogens have been reported to stimulate TLRs *in vitro*. For example, *P. gingivalis* LPS and fimbriae is a potent TLR2 agonists (Hirschfeld et al., 2001, Asai et al., 2001, Erridge et al., 2004). *A actinomycetemcomitans* and *F. nucleatum* LPS, and whole *P gingivalis* will stimulate (Kikkert et al., 2007, Nussbaum et al., 2009, Darveau et al., 2004, Mochizuki et al., 1999, Yoshimura et al., 2002). Moreover, many bacteria can initiate an immune response via TLR9, which also detects viable bacterial DNA (Bauer et al., 2001). It is therefore clear that the myriad of bacteria that are present in both health and increasing severity of periodontal disease will present a challenge to the innate immune response in the periodontal tissues. Following TLR activation, an intracellular signalling cascade occurs which can lead to the activation of transcription factors and subsequent inflammatory cytokine expression, leukocyte migration and tissue destruction (Nakamura et al., 2008, Ukai et al., 2008, Gelani et al., 2009, Lima et al., 2010). The nucleotide-binding oligomerization domain (NOD) and the inflammosome system have also been

suggested as possible accessory molecules in the induction of innate immune response against periodontal pathogens (Uehara and Takada, 2007, Okugawa et al., 2010, Bostanci et al., 2009).

The junctional epithelium is the front line between the host and the oral microflora. It is well equipped to recognise invading pathogens, with studies showing the presence of mRNA encoding TLR2, 3, 4, 5, 6 and 9 in gingival epithelial cells (Kusumoto et al., 2004). Tissue dendritic cells and Langerhans cells are also present within the gingival epithelium and the underlying connective tissue. These antigen-presenting cells express a wide range of TLRs including TLR1, 2, 3, 4, 5, 6, 8 and 10 (Mogensen, 2009, Mahanonda and Pichyangkul, 2007). These cells will monitor invasion of the bacteria or bacterial products initiating the adaptive immune response.

The alveolar bone is the supporting structure into which the periodontal ligament inserts and is the tissue that is ultimately destroyed by the inflammatory lesion of periodontal disease. Osteoblasts and osteoclasts involved in bone turnover also express TLR1, 4, 5, 6 and 9 (Asai et al., 2001) and TLR1, 2, 3, 4, 5, 6, 7, 8 and 9 (Itoh et al., 2003), respectively. It is therefore possible that TLR signalling within the bone can generate an inflammatory response to invading pathogens, leading to pathological resorption of bone through excessive or prolonged production of osteolytic host molecules, including: IL-1, Tumour Necrosis Factor-α (TNFα) and Prostaglandin E2 (PGE_2), which stimulate osteoblast inhibition and osteoclast activation and maturation through the receptor activator of nuclear factor kappa-B ligand/osteoprotegering (RANKL/OPG).

Many biological events in periodontal disease are strictly regulated by cell-cell interactions, which may be categorized into two forms: cognate (adhesive) interaction, achieved by mutual recognition between membrane bound cell-surface molecules, and cytokine-mediated interactions (Okada and Murakami, 1998). Adhesion molecules include ICAM-1 (Intercellular Adhesion Molecule-1, CD54) and ITGB2 (Integrin Beta 2, CD18), which stabilize cell-cell interactions and facilitate leukocyte migration across the endothelial barrier (Yang et al., 2005, Kotovuori et al., 1999).

1.3.2 Pro-inflammatory cytokines

Cytokines are a large and diverse family of soluble mediators including interleukins, colony stimulating factors, growth factors, and cytotoxic factors. Cytokines play an important role in numerous biological activities including proliferation, development, differentiation, homeostasis, regeneration, repair and inflammation (Okada and Murakami, 1998). Cytokines networks are an important aspect of periodontal inflammation, and subject to several excellent reviews, and are illustrated in their complexity in Figure 1.6 (Preshaw and Taylor, 2011, Kinane et al., 2011). Pro-inflammatory cytokines IL-1α and IL-1ß belong to the IL-1 family of cytokines, which has diverse activities and roles in immunity, inflammation, tissue breakdown, and tissue homeostasis (Dinarello, 1988, Gowen et al., 1986, Havemose-Poulsen and Holmstrup, 1997, Mizel, 1989, Mizel et al., 1981, Nguyen et al., 1991, Schmidt et al., 1982, Stashenko et al., 1987b, Tatakis, 1993, Stashenko et al., 1987a). TNF-α is a pro-inflammatory cytokine that is secreted mainly by monocytes and macrophages. It induces the secretion of collagenase by fibroblasts, resorption of cartilage and bone, and has been implicated in the destruction of periodontal tissue in periodontitis (Alexander and Damoulis, 1994, Chaudhary et al., 1992, Elias et al., 1987, Meikle et al., 1989). IL-1α, IL-1ß and TNF-α stimulates bone resorption and inhibit bone formation (Stashenko et al., 1987a, Nguyen et al., 1991, Tatakis, 1993). IL-6 is a pleiotrophic cytokine influencing immune responses and inflammatory reactions (Hirano T, 1991). IL-6 is of particular importance in the human B-cell responses, hence it has been speculated that the expansion of B-cells/plasma cells seen in periodontitis lesions may result from an increased production of IL-6 at diseased sites (Fujihashi et al., 1993). It has also been suggested that IL-6 plays an important role in the local regulation of bone turnover (Ishimi et al., 1990, Lowik et al., 1989, Kurihara et al., 1990).

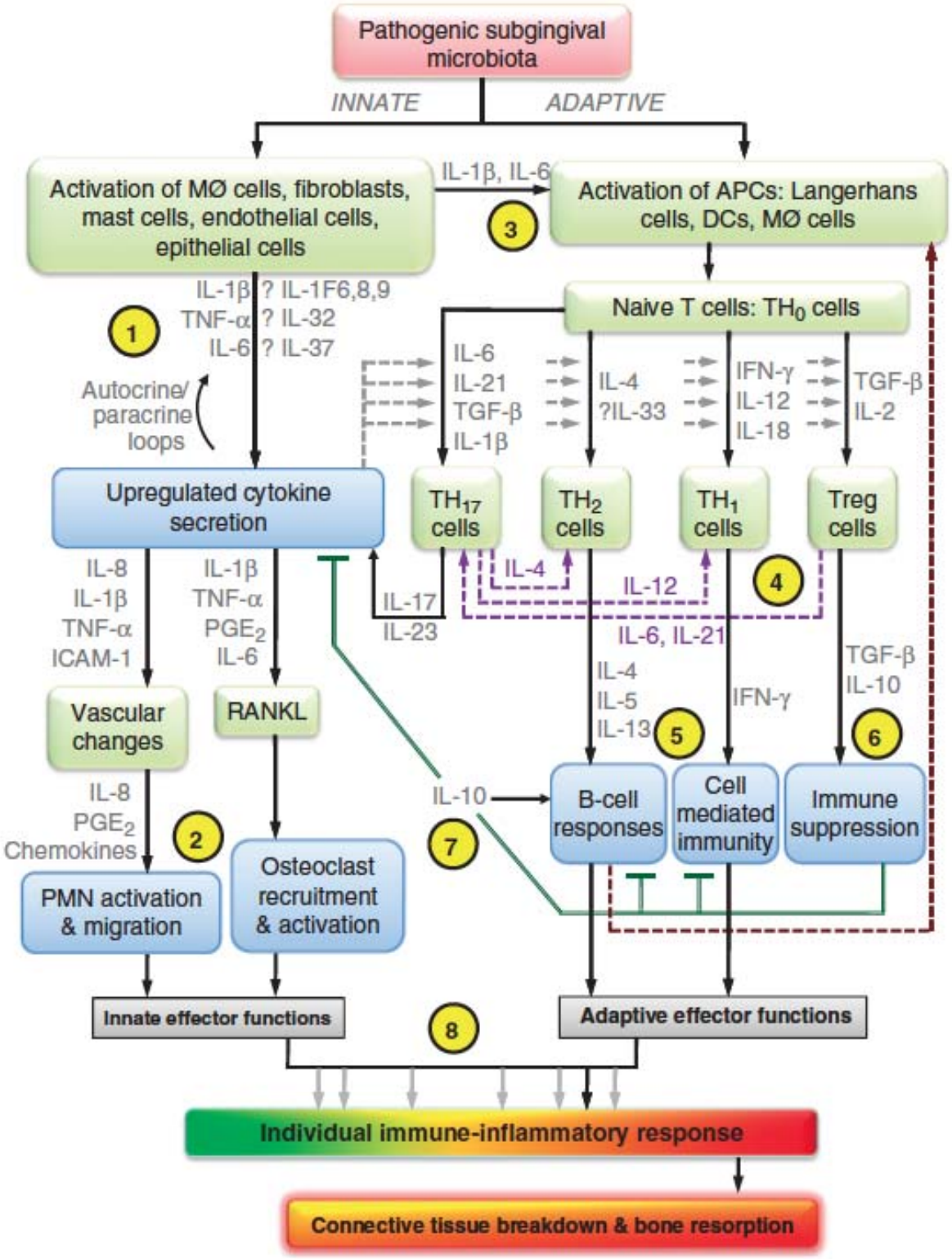


Figure 1.6: Cytokine networks in periodontal diseases. Schematic to illustrate the multiple interactions between cytokines and cellular functions in periodontal diseases. Taken from Kinane and colleagues (Kinane et al., 2011).

1.3.2.1 Interleukin-1 α and ß (IL-1 α/ ß)

IL-1 is a polypeptide, which has diverse activities and roles in immunity, inflammation, tissue breakdown, and tissue homeostasis (Dinarello, 1988, Gowen et al., 1986, Havemose-Poulsen and Holmstrup, 1997, Mizel, 1989, Mizel et al., 1981, Nguyen et al., 1991, Schmidt et al., 1982, Stashenko et al., 1987b, Tatakis, 1993, Stashenko et al., 1987a). IL-1 is synthesized by various cell types, including fibroblasts, lymphocytes, skin cells, macrophages, monocytes, vascular cells and bone cells, following its activation. IL-1α and IL-1ß belong to the IL-1 family of cytokines which have similar biological functions and bind to the same receptors found on many cell types (Okada and Murakami, 1998). Local excessive production of IL-1 by periodontal ligament cells stimulates gingival and periodontal ligament fibroblasts to induce the production of other mediators, prostaglandin E_2 (PGE2) and matrix degrading enzymes that can cause connective tissue destruction and attachment loss. IL-1 has also been implicated in the pathogenesis of periodontitis, which involves bone destruction (Okada and Murakami, 1998). IL-1 α and IL-1 ß have been shown to stimulate bone resorption and inhibit bone formation (Stashenko et al., 1987b, Nguyen et al., 1991, Tatakis, 1993) and also accentuates the bone resorptive actions of TNF-α (Bertolini et al., 1986, van der Pluijm et al., 1991). IL-1 ß has been shown to have significantly more potent in mediating bone resorption compared with IL-1 α and TNF-α (Alexander and Damoulis, 1994). IL-1 can also stimulate elevated production of matrix metalloproteinases (MMPs) (Havemose-Poulsen and Holmstrup, 1997), procollagenase (Meikle et al., 1989, Lark et al., 1990) and plasminogen activator (Mochan et al., 1988) but no significant change in the synthesis of TIMP (Meikle et al., 1989).

1.3.2.2 Interleukin-6 (IL-6)

IL-6, which belongs to the IL-6 family of cytokines, is a pleiotropic cytokine with a wide range of biological functions including influencing immune response, inflammatory reactions, acute phase response, oncogenesis and hematopoiesis (Kishimoto, 1989, Le and Vilcek, 1989, Sehgal, 1990, Heinrich et al., 1990, Hirano and Kishimoto, 1992, Van Snick, 1990). Stimulated monocytes, macrophages, T- and B- cells, fibroblasts, keratinocytes and endothelial cells have been shown to produce IL-6 after stimulation (Okada and Murakami, 1998). In the periodontium, IL-6 is expressed by osteoblasts (Feyen et al., 1989),

gingival fibroblasts (Takashiba et al., 2003) and periodontal ligament cells (Jonsson et al., 2008). Increased levels of IL-6 have been detected in the gingival crevicular fluid (Lin et al., 2005). IL-6 has been implicated in the local regulation of bone turnover (Lowik et al., 1989, Ishimi et al., 1990, Kurihara et al., 1990). The cytokine has also been shown to stimulate bone resorption in mouse calvarial bone (Palmqvist et al., 2002, Ahlen et al., 2002). Simultaneous treatment of mouse osteoblastic cells and bone marrow cells with soluble IL-6 and IL-6 receptors induced osteoclast formation, in an *in vitro* study (Tamura et al., 1993). These observations imply a role for IL-6 in the pathogenesis of periodontal tissue destruction in periodontal disease.

1.3.2.3 Tumor Necrosis Factor alpha (TNF-α)

TNF-α is a pro-inflammatory cytokine released by activated monocytes and macrophages (Okada and Murakami, 1998). TNF-α functions include inducing the up-regulation of adhesion molecules and chemokines which are involved in the cell migration to inflamed and infected sites (Peschon et al., 1998, Dinarello, 2000, Wajant et al., 2003, Kindle et al., 2006). The cytokine can also induce the secretion of collagenase by fibroblasts, resorption of cartilage and bone, and destruction of periodontal tissue (Elias et al., 1987, Meikle et al., 1989, Chaudhary et al., 1992). Both gingival crevicular fluid (GCF) and diseased periodontal tissues have shown high levels of TNF-α and showed positive correlation to MMP and RANKL expression (Graves and Cochran, 2003, Garlet et al., 2004). Animal studies also demonstrated that TNF-α plays a key role in inflammation and periodontal tissue destruction including bone resorption and loss of connective tissue attachment (Graves et al., 2008, Graves and Cochran, 2003). TNF-α also up-regulates the production of other pro-inflammatory cytokines, such as IL-1 ß and IL-6, which are also associated with inflammatory cell migration and bone resorption (Okada et al., 1997, Dinarello, 2000, Wajant et al., 2003, Kwan Tat et al., 2004, Musacchio et al., 2009, Graves, 2008, Fonseca et al., 2009).

1.3.2.4 Interleukin 17 (IL-17) family

IL-17A, which was initially identified in T cells (Liang et al., 2010), has been shown to be released from other cellular sources including γδ T cells, NK cells, neutrophils, eosinophils, LTi CD4 cells (Kimizuka et al., 2012) and mast cells

(Hueber et al., 2010). IL-17 A has the potential to induce production of cytokines (Fossiez et al., 1996, Numasaki et al., 2004a, Numasaki et al., 2004b), chemokines (Yang et al., 2008, Kawaguchi et al., 2003), matrix metalloproteinases (Yang et al., 2008, Park et al., 2005, Yagi et al., 2007) and antimicrobial peptides (Kao et al., 2004). IL-17 has been shown to participate in periodontal disease but the role which it plays, host protective or destructive, is not clear (Kramer and Gaffen, 2007). In sterile inflammatory situations, IL-17 is destructive but the protective role of IL-17 is in line with the protective role of Th17 cells in infectious diseases (Cua et al., 2003). Conversely, IL-17 has also been shown to take part in the destructive phase of periodontal disease (Oda et al., 2003). Figure 1.7 illustrates the central role it plays in periodontal disease and its interaction with other key cytokines.

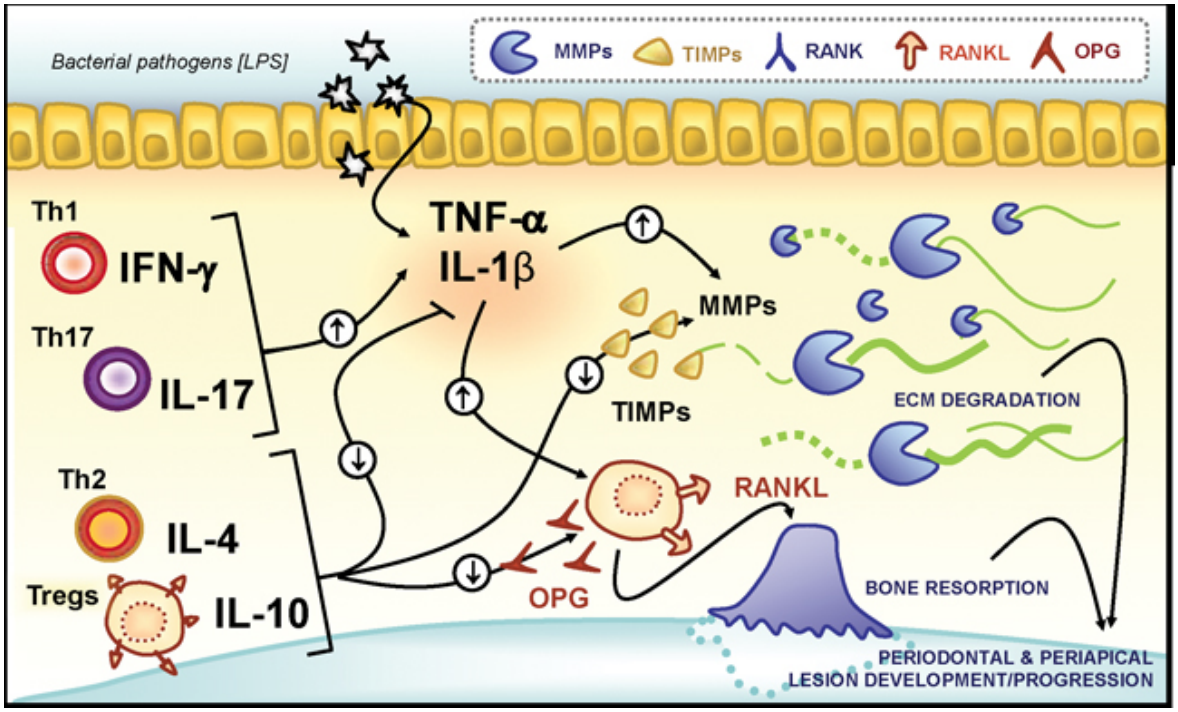


Figure 1.7: Cytokine regulation of matrix degradation and bone resorption in periodontal and periapical environments. The presence of microbial pathogens in periodontal and periapical environments trigger an initial production of proinflammatory cytokines, such as TNF-α and IL1ß, which stimulate expression and activation of MMPs that degrade extracellular connective tissue matrix. Cytokines such as TNF-α can stimulate osteoclastogenesis independently while other cytokines stimulate RANKL expression that leads to formation of osteoclasts and osteoclast activity. The combined innate and adaptive immune responses are likely to lead to the high levels of inflammation and bone resorption. These proinflammatory cytokines are thought to generate an amplification loop that contributes to periodontal and periapical lesion progression. Conversely, cytokines produced by Th2 cells and Tregs, such as IL-4 and IL-10 have the opposite effect, in part, through stimulating production of TIMPs and OPG as well as restrain inflammatory cytokine production. Taken from Graves and colleagues (Graves et al., 2011).

1.3.3 Chemokines

Development of the periodontal diseases seems to be related to the progression of the inflammatory cell infiltrate into the deeper periodontal tissues (Graves et al., 1998). The chemokines, which are found in the gingival tissue and GCF have been implicated in the immunopathogenesis of periodontal diseases (Silva et al., 2007).

1.3.3.1 Interleukin-8 (IL-8)

IL-8/CXCL8 is a chemoattractant of polymorphonuclear leukocytes (PMNs) which has been detected in healthy periodontal tissues and low subclinical inflammation closely associated with PMNs (Payne et al., 1993, Mathur et al., 1996). The levels of IL-8 increase rapidly in GCF preceding the clinical signs of disease (Zhang et al., 2002). In early periodontal lesions, PMNs are the first cell types found in high numbers (Garlet et al., 2005). Periodontitis patients show a drastic increase in the levels of IL-8 in both periodontal tissue and GCF which has been correlated with disease severity (Tsai et al., 1995). IL-8 production has been shown in gingival fibroblasts, gingival epithelial cells, endothelial cells, and in *in vitro* studies (Takashiba et al., 1992, Takigawa et al., 1994, Huang et al., 1998, Yumoto et al., 1999). Figure 1.8 illustrates the central role of the chemokine IL-8 in periodontal disease.

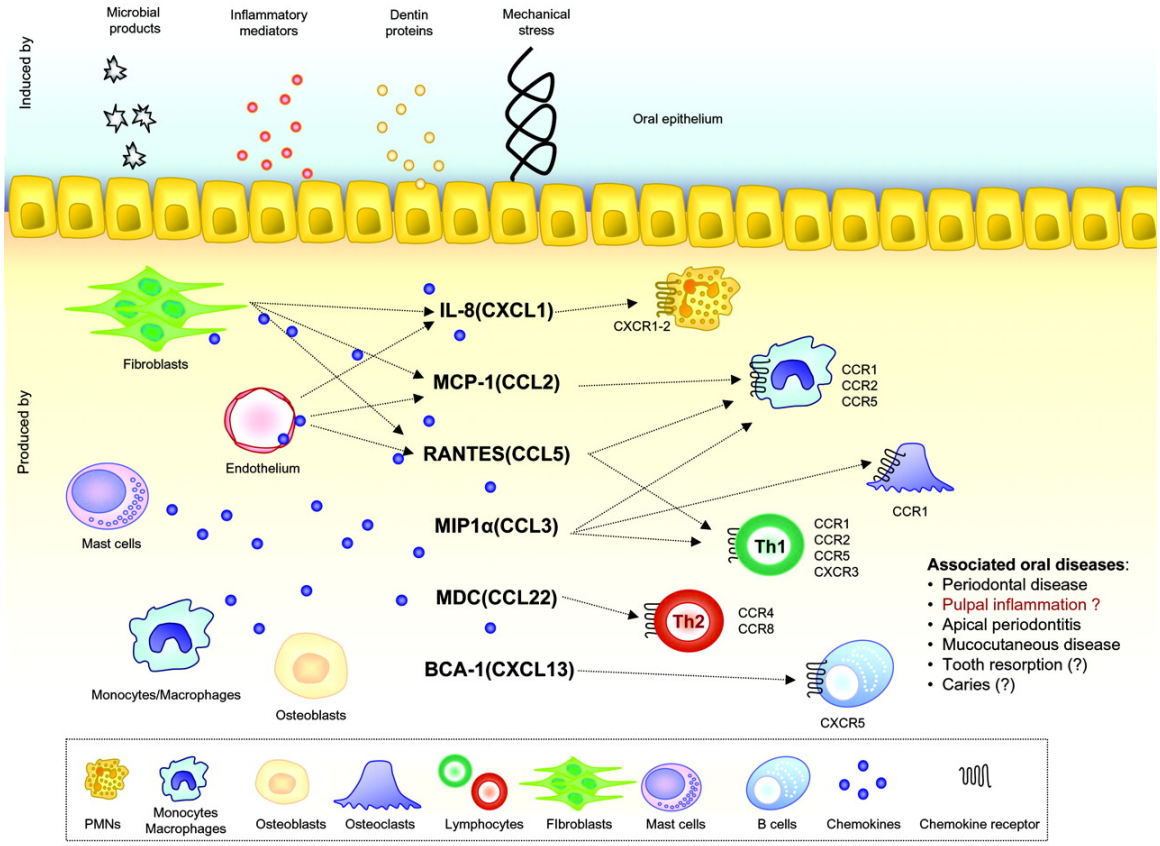


Figure 1.8: Chemokines and chemokine receptors in oral tissues: potential involvement in the induction and maintenance of inflammatory reactions. A schematic representation of chemokine and chemokine receptor networks in oral tissues. Chemokine expression can be triggered by microbial components, inflammatory mediators, host factors such as dentin proteins, or even by mechanical stress. Both resident (such as osteoblasts, fibroblasts, mast, epithelial, and endothelial cells) and inflammatory cells (polymorphonuclear leukocytes [PMNs], lymphocytes, monocytes/ macrophages) can be sources of chemokines in the oral environment. The chemokines produced in oral tissues selectively attract different cell types to the tissues, such as PMNs, lymphocyte subsets, monocytes/macrophages, and osteoclasts, and, consequently, can determine the course of inflammatory reactions and the clinical outcome of potentially associated diseases (Silva et al., 2007).

1.3.4 Antimicrobial peptides

It has been shown that gingival epithelial cells serve as physical barriers to microbial challenge and also play an active role in sensing and initiating host response, by expression of various pattern recognition receptors and other innate host defense molecules such as antimicrobial host defense peptides (Dale, 2002). Antimicrobial peptides are very important functional components of the innate defense system (Boman, 1995, Zasloff, 2002), which have strong antimicrobial activity against both Gram-positive and Gram-negative bacteria by disrupting the integrity of their cell membranes (Boman, 1995, Harder et al., 1997). They also contribute to the homeostasis of the microbe-host interactions through immunoregulation of innate and adaptive host response (Boman, 1995, Peschel and Sahl, 2006). Defense peptides consist of mainly defensins, cathelicidin LL-37 and histatins (Dale, 2002, Marshall, 2004).

1.3.5 Human defensins

Human defensins are classified into α and ß subforms based on the cysteine spacing and connecting patterns of three sulphide bonds (Ganz and Lehrer, 1995). Human ß-defensins are produced by the epithelial cells. Human ß-defensin 1 is constitutively expressed in various epithelial cells, suggesting a surveillance-like role in the absence of infection and a protective role in the presence of infection (Mathews et al., 1999, Zhao et al., 1996). Human ß-defensin 2 is an inflammation-induced defensin and can be detected in saliva and gingival (Mathews et al., 1999) which are active against Gram-negative bacteria but not so much against Gram-positive bacteria (Harder et al., 1997). Human ß-defensin 2 expression has been shown in inflamed tissues (Lehmann et al., 2002, Mathews et al., 1999). Exposure of human keratinocytes to inflammatory cytokines and microbes have shown induction of human ß-defensin 2, in *in vitro* studies, suggesting a more specialized role in the innate epithelial defence compared with human ß-defensin 1 (Krisanaprakornkit et al., 2000, Mathews et al., 1999).

1.4 Periodontal disease models

To facilitate our understanding of the complexity of periodontal disease, various *in vitro* and *in vivo* models have been used. The following section describes some of the key models and how these have contributed to the literature.

1.4.1 Human *in vivo* study models

The pathogenesis of human periodontal disease is a dynamic and complex process. Interpreting and translating data from studies, which have investigated the pathogenesis of periodontal disease in laboratory or animal models can be tricky. The optimal model to study this would obviously be in humans, even though this would be an expensive and highly regulated process. Experimental gingivitis model in man was first developed by Löe *et al* as early as 1965 (Löe et al., 1965). This was a seminal paper in understanding the causal aspects of gingivitis. This study significantly impacted on periodontal research because of the simple and instructive demonstration of the causal role of plaque bacteria in gingivitis. The experimental gingivitis model described by Löe *et al*. (1965) inspired many researchers in the field to modify and use this within their area of interest (Johnson et al., 1997, Biesbrock and Yeh, 2000, Offenbacher et al., 2010, Grant et al., 2010, Smith et al., 1978, Offenbacher et al., 2009). These studies investigated various aspects in the pathogenesis of gingivitis and other periodontal diseases. Cell mediated immune responses to plaque antigens was also investigated in an experimental gingivitis model in man (Smith et al., 1978). Offenbacher and co-workers performed investigated changes in gingival crevicular fluid inflammatory mediator levels and gingival transcriptome during the induction and resolution of experimental gingivitis in humans (Offenbacher et al., 2010, Offenbacher et al., 2009).

Of notable interest was the recent seminal study by Offenbacher and colleagues (Offenbacher et al., 2009). An experimental gingivitis study was performed in humans to understand changes in patterns of whole-transcriptome gene expression that occur during the induction and resolution of experimental gingivitis in humans. Gingival biopsy samples were collected from 14 subjects during a 28-day plaque-induced experimental gingivitis model. This was followed by treatment and resolution at days 28 through to day 35. Biopsy samples were collected at different sites within each subject at baseline (day 0), at the peak

of gingivitis (day 28), and at resolution (day 35) and processed using whole-transcriptome gene-expression arrays (Offenbacher et al., 2009). Interestingly, a high proportion of the genes demonstrating greatest differences between health and disease were genes encoding chemokines, cytokines, adhesion molecules, antimicrobial peptides.

Another practical use of these models is for the assessment of chemical agents, such as chlorhexidine, for their potential to inhibit plaque formation and prevent gingivitis (Löe and Schiott, 1970), which over time has been shown through systematic review to be an effective anti-plaque compound (Van Strydonck et al., 2012).

1.4.2 Animal *in vivo* study models

Prospective studies to investigate the mechanisms underlying the progression of periodontitis cannot always be answered by *in vivo* studies of humans due to important ethical considerations. Instead, several rodent models have been used to study host-bacterial interactions that have assisted in elucidating the mechanisms of periodontitis (Graves et al., 2008).

1.4.2.1 Calvarial model

A calvarial, or scalp, model was developed by Boyce and colleagues to study the effect of cytokines on osteoclastogenesis (Boyce et al., 1989). Other workers adapted this model to study host-bacterial interactions *in vivo* by inoculating *P. gingivalis* into the connective tissue overlying the calvarial bone to study the inflammatory infiltrate and fibroblast death stimulated by this organism (Graves et al., 2001, Leone et al., 2006). This model introduces the bacteria into the connective tissue but has no interactions with the epithelial cells. Therefore, no information about the immune response in the epithelial cells is obtained.

1.4.2.2 Oral gavage model

Most periodontal pathogens are not present in the oral microflora of mice and laboratory mouse strains, so alternative models introduced human strains of bacteria by oral gavage and the consequent impact on the periodontium has been investigated (Chang et al., 1988, Klausen et al., 1991). A mouse periodontitis model was developed, in which *P. gingivalis* oral gavage induced

pro-inflammatory cytokines, gamma interferon and interleukin-6, which contributed to alveolar bone loss (Baker et al., 1999, Lalla et al., 1998). Other studies have looked at similar immune responses involved in bone loss using other periodontal pathogens, including *A. actinomycetemcomitans* (Garlet et al., 2006) and *Tannerella forsythia* (Sharma et al., 2005). For enhanced reproducibility of the model, prior antibiotic treatment to reduce the oral flora and repeated inoculations of the organisms are needed, which leads to complexity of the model (Graves et al., 2008).

1.4.2.3 Airpouch model

The airpouch model was originally developed in mice and rats by the Willoughby group to study the function of synovial membrane by producing an airpouch subcutaneously followed by the analysis of both the exudate fluid and the epithelial lining of the airpouch to assess the acute immune response (Edwards et al., 1981). This model was adapted for periodontitis by Pouliot and co-workers to study the acute inflammatory response to *P. gingivalis* (Pouliot et al., 2000). By injecting *P. gingivalis* into the airpouch, Pouliot and colleagues demonstrated that *P. gingivalis* is a strong pro-inflammatory stimulus. Another observation was that PGE_2, which is an important marker in the pathogenesis of periodontitis, was found in neutrophils in addition to monocyte/macrophage in the inflammatory lesion (Pouliot et al., 2000). The main drawback of this model is that chronic inflammation experiments could not be performed because maintenance of the airpouch is a limiting factor, so inflammatory response could be assessed only in the acute context.

1.4.2.4 Tissue cage and Chamber model

The chamber model is a modification of the airpouch model to study the chronic aspect of inflammation. The chamber is a coiled stainless steel wire that is surgically implanted subcutaneously into the back of the mouse and the wound allowed to heal for 10 days. The interior of the chamber becomes epitheliasised and it also allows the injection of bacteria into the lumen of the chamber. Fluid can be aspirated from the chamber or the entire chamber can be excised for histological studies (Graves et al., 2008). This model was adapted for use in periodontal studies by Genco and colleagues mainly for the assessment of the virulence of bacteria (Genco et al., 1991). The chamber model was used to

demonstrate the development of acquired immune response to *P. gingivalis* and strain differences within the bacteria by injecting the bacteria into the chamber (Genco et al., 1991). It has also been utilised to examine the role of complement in the clearance of *P. gingivalis* (Schenkein, 1989), immunisation studies (Genco et al., 1992), and the activation of the kinin system which is involved in the breach of the vascular barrier and the subsequent dissemination of *P. gingivalis* (Hu et al., 2006). The main advantage of the chamber models is the ability to perform long-term experiments that can be adapted to chronic inflammation and pathogenesis experiments. The limitation of all animal models is that there is no single model that represents all aspects of the human periodontal disease (Graves et al., 2008).

1.4.3 *In vitro* study models

1.4.3.1 Multi-species biofilm models

Various plaque biofilm models have been used to study plaque formation, structure and antimicrobial susceptibility (Table 1.1). Guggenheim has used a defined multi-species model of supragingival and subgingival plaque to study structure, antimicrobial susceptibility and host-pathogen interaction (Guggenheim et al., 2001a, Guggenheim et al., 2009, Guggenheim and Meier, 2011, Guggenheim et al., 2001b). Figure 1.9 illustrates the Guggenheim multi-species biofilm structure as visualized by CLSM and TEM, showing the various bacteria in the model stained by multiplex fluorescent *in situ* hybridisation. Moreover, constant depth film fermenter (CDFF) models have been used to study the structure and spatial distribution of plaque bacteria (Netuschil et al., 1998).

These defined multi-species biofilm models have been used to mimic gingival plaque (Guggenheim et al., 2009, Guggenheim et al., 2004, Periasamy and Kolenbrander, 2009b, Peyyala et al., 2011b), while others have used undefined microcosm of bacteria from either pooled saliva or pooled plaque samples (Hope et al., 2012, Pratten, 2007). Table 1.1 summarises some of the key *in vitro* biofilm models reported in the literature that have been used to study supra-gingival and sub-gingival plaque structures. In some instances biofilms have been produced using undefined bacteria from pooled saliva or plaque samples. Although structurally the composition of the biofilm retains the complexities of the original sample (Pratten et al., 2003), it is difficult to delineate from these

the exact mechanisms used in host-pathogen interaction model when compared with the defined multi-species biofilm models.

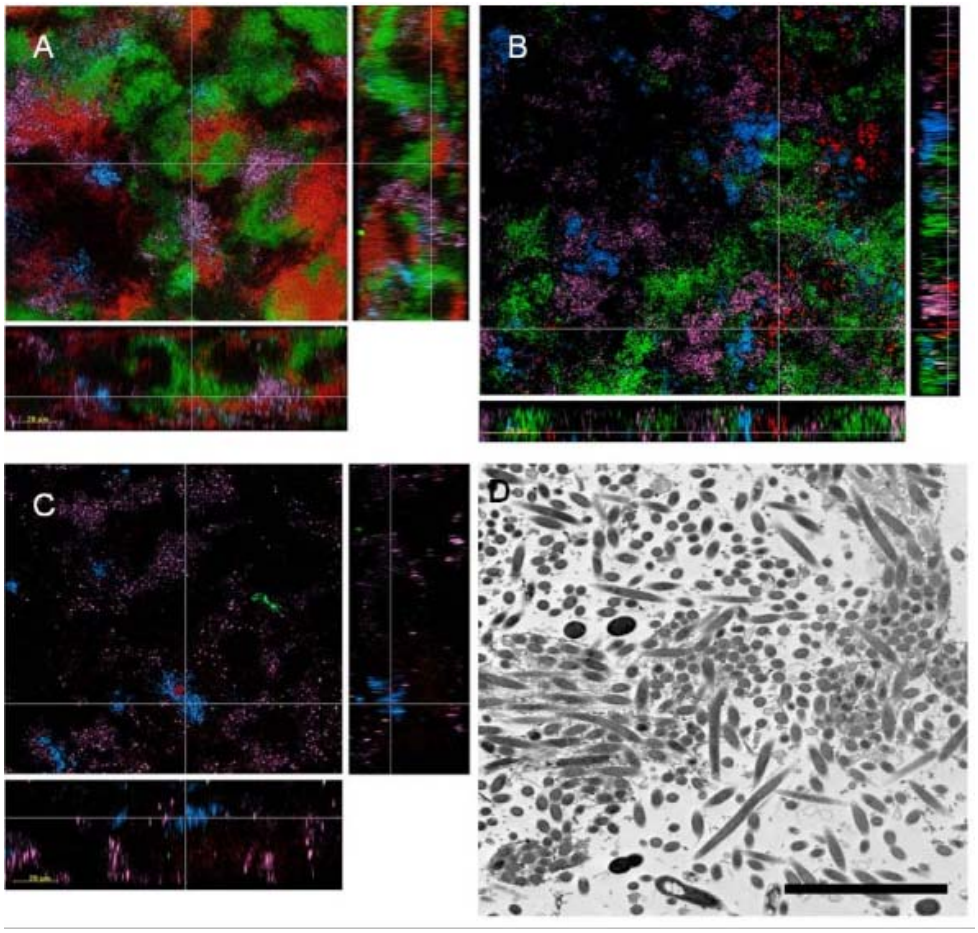


Figure 1.10: Example of model multi-species biofilm structure visualized by CLSM and TEM. CLSM images of a 9-species biofilm stained by multiplex FISH for **(A)** *V. dispar* (purple), *C. rectus* (blue), *F. nucleatum* (red), and *P. intermedia* (green), **(B)** *V. dispar* (purple), *A. naeslundii* (red), *S. intermedius* (green), and *S. oralis* (blue), and **(C)** *V. dispar* (purple), *T. forsythia* (green), *P. gingivalis* (red), and *C. rectus* (blue). Images are 1-*μ*m-thick transverse (large images), sagittal (right) and coronal (bottom) slices at the positions indicated by the fine lines. The length of the bars indicates 20 *μ*m. **(D)** TEM image of a multi-species biofilm demonstrating the predominance of varius cocci or very short rods (*S. oralis, S. intermedius, V. dispar, P. intermedia*) and of the fusiform *F. nucleatum* cells. Bar = 5 *μ*m (Guggenheim et al., 2009).

1.4.3.2 Host-pathogen interaction models

Supra-gingival and sub-gingival *in vitro* biofilm models have been used to study antimicrobial agents against the biofilms, chemical and biological components of the biofilms and host-pathogen interaction. Guggenheim's group have investigated host-pathogen interaction using multi-species biofilm-epithelial cell co-culture model looking at apoptosis and inflammatory mediator response of the epithelium to bacterial biofilms (Belibasakis et al., 2011b, Belibasakis et al., 2011a). In these studies, primary gingival epithelial cells were challenged with the bacterial biofilms to study host-pathogen interactions. The Ebersole group used immortalized epithelial cell line OKF4 to co-culture with bacterial biofilms and planktonic cells to investigate host-pathogen interactions by investigating the inflammatory mediator response of the epithelial cells to the bacteria (Peyyala et al., 2012, Peyyala et al., 2011a).

Organotypic 3-dimensional tissue models have also been used to study host-pathogen interaction and test antimicrobials (Dongari-Bagtzoglou and Kashleva, 2006a, Gursoy et al., 2012, Oksanen and Hormia, 2002). Even though these tissue models are more representative of the oral and gingival mucosa *in vivo*, they are time-consuming and labour-intensive to develop. The Dongari-Bagtzoglou 3-D model takes about 2-3 weeks to develop with a sub-mucosal component to and the multi-layered epithelium (Dongari-Bagtzoglou and Kashleva, 2006a, Gursoy et al., 2012, Oksanen and Hormia, 2002). It uses immortalized OKF6-TERT2 oral epithelial cells, which has been used in the current study. This model has been used to study the Candidal and Candida-Streptococcal interaction with the tissue model (Dongari-Bagtzoglou and Kashleva, 2006b, Diaz et al., 2012), but has the capacity to be used in the context of periodontal disease. Other investigators have investigated the effect of *F. nucleatum* biofilms in the 3-D models (Gursoy et al., 2012). Primary oral epithelial cells have also been used to develop the 3-D tissue models (Moharamzadeh et al., 2009).

Commercially available organotypic tissue models have also been described, including Mattek® and SkinEthic®, which have human epithelial and gingival tissue models with a submucosal component. These models have been used for toxicological studies and host-pathogen interaction studies (Yadev et al., 2011,

Kimball et al., 2006). These studies show the potential of using the organotypic 3-dimensional tissue models for the study of host-pathogen interaction in periodontal disease. There is a range of models which can be used to investigate host-pathogen interaction in periodontal disease *in vitro* including monolayer of primary gingival epithelial cells, monolayer of immortalized oral epithelial cell lines, primary organotypic 3-D tissue models and organotypic 3-D tissue models using immortalized oral epithelial cells. Selection of the models for host-pathogen interaction would depend on the reproducibility of the model, time involved in its development, labour-intensity, cost factor and the application it is intended for.

Biofilm model	Media	Substrate	Innoculum	Application	Reference
Marsh's	Porcine gastric mucin, 2.5 g/L; KC1 2.5 g/L; proteose peptone 2 g/L; yeast extract 1 g/L; trypticase peptone 1 g/L ; cysteine hydrochloride 0.1 g/L; haemin, 0.001 g/L.	Polytetrafluoroethylene (PTFE)	**Multispecies biofilm:** *Streptococcus mutans* R9 *Streptococcus oralis* EF186 *Streptococcus sanguis* 209 *Lactobacillus casei* AC413 *Actinomyces naeslundii* WVU627 *Neisseria subfava* A1 078 *Veillonella dispar* ATCC 17745 *Porpbyromonas gingivalis* W50 *Prevotella nigrescens* T588 *Fusobacterium nucleaturn* ATCC 10953	Assay chemical and biological components of the biofilm. For example, pH	(Kinniment et al., 1996a) (Kinniment et al., 1996b)
Zurich	60% Human saliva, 10% Human serum, 30% modified fluid universal medium	Pellicle-coated HA disc	**Multispecies biofilm:** *C. rectus* OMZ 697 *F. nucleatum* subsp. *vincentii* KP-F2 (OMZ 596) *P. gingivalis* ATCC 33277T (OMZ 925) *P. intermedia* ATCC 25611T (OMZ 278) *T. forsythia* OMZ 1047 *T. lecithinolyticum* ATCC 700332T (OMZ 684), *V. dispar* ATCC 17748T (OMZ 493) *A. naeslundii* OMZ 745 *S. intermedius* ATCC 27335 (OMZ 512) *S. oralis* SK 248 (OMZ 607)	Epithelial cell apoptosis Inflammatory mediator response Antimicrobial testing	(Guggenheim et al., 2009) (Belibasakis et al., 2011b) (Belibasakis et al., 2011a) (Stathopoulou et al., 2010) (Guggenheim and Meier, 2011)

Kentucky	Brain heart infusion medium supplemented with 5 g hemin/mL and 1 g menadione/mL	Rigid gas-permeable hard contact lenses	**Multispecies biofilm:** *S. gordonii*, *S. oralis*, and *S. sanguinis* or *S. gordonii*, *A. naeslundii*, and *F. nucleatum* or *S. gordonii*, *F. nucleatum* and *P. gingivalis* **Monospecies biofilm:** *S. gordonii* *S. oralis* *S. sanguinis* *A. naeslundii* *F. nucleatum* *P. gingivalis*	Inflammatory mediator response	(Peyyala et al., 2011b) (Peyyala et al., 2012)
CDFF	Artificial saliva with mucin	HA discs	Undefined oral bacterial inoculum from pooled saliva	Antimicrobial testing	(Hope and Wilson, 2003) (O'Neill et al., 2002)
Florida	Trypticase soy broth	Pellicle-coated HA disc	Undefined subgingival bacterial inoculum	Antibiotic resistance studies	(Walker and Sedlacek, 2007) (Sedlacek and Walker, 2007)
Eastman	Artificial saliva with mucin	Bovine enamel discs	Undefined oral bacterial inoculum from pooled saliva	Antimicrobial testing	(Pratten, 2007) (Pratten et al., 2003) (Pratten and Wilson, 1999) (Pratten et al., 1998a) (Pratten et al., 1998b)

Table 1.1 Summary of *in vitro* supragingival and subgingival multi-species biofilm models. The table summarises some of the models which have been developed of supragingival and subgingival periodontal plaque biofilms. The models use a variety of growth media, substrates and inoculum for the biofilm development.

1.5 Summary and aims of study

From this brief review of the literature it can be seen that both microbiological and immunological factors are pivotally important for the development and progression/or otherwise of periodontal disease. Plaque, or microbial biofilm, resides at the gingival margin and on the root surface, setting up an interface with host cells and tissues. The composition of the microbial biofilm determines the extent of host cell interactions, whilst the highly adaptable pathogens within the biofilm aim to subvert the protective effects of host immune factors. Nevertheless, innate immune factors continue to protect the gingivae through cell signalling from the first line of defense, the epithelium. Both *in vivo* and *in vitro* models have been developed to investigate these complex interactions, which have provided greater knowledge into host-pathogen interactions.

To gain further insight into this, the aim of this study was to develop an *in vitro* multi-species biofilm model relevant to periodontal disease, which can be used to investigate host-pathogen interactions. The specific aims were as follows:

1) Develop a multi-species biofilm of periodontal biofilms
2) Create a host (epithelial) - pathogen (biofilm) model
3) Investigate how *in vitro* periodontal biofilms impact cellular responses
4) Investigate how cytokines modulate immune response in periodontal disease

CHAPTER 2:

DEVELOPMENT AND VALIDATION OF A PERIODONTAL BIOFILM MODEL

2.1 INTRODUCTION

Microbiologically the oral cavity is an important milieu where complex interplay exists between host immune factors and diverse microbial populations. This environment is abundant with heterogeneous planktonic bacterial suspensions in saliva or as adherent biofilm communities contained within distinct microenvironments (Kolenbrander, 2000). Molecular analysis of oral microbial communities by cloning and sequencing the bacterial 16S rRNA genes present has indicated that the oral cavity provides a habitat for approximately 700 species of bacteria, of which between 100 and 200 different species are present in the healthy mouth of any individual (Kolenbrander et al., 2010).

The gingival crevice represents an important site within the oral cavity where bacterial biofilm adheres to an enamel surface and bathes within gingival crevicular fluid. Here the biofilm (or plaque) is a diverse population of inter-connected bacteria that coexist within an oxygen-limited environment, that are anchored to the enamel surface by pioneer species, such as oral streptococci (Rogers et al., 2001, Kolenbrander et al., 2002). Understanding their growth requirements, interactions and composition will help provide clues as to how the biofilm is formed and regulated, and how this structure interacts with its environment. However, with hundreds of bacteria associated with both supra- and sub-gingival plaque it is difficult to delineate how each of these bacteria interacts specifically with its environment. Therefore, oral microbiologists have developed a variety of methods to grow and study periodontal biofilms.

Many groups have undertaken work to develop complex periodontal biofilm models using a variety of approaches (Belibasakis et al., 2011a, Guggenheim et al., 2009, Kinniment et al., 1996a, Kolenbrander et al., 2010, Stathopoulou et al., 2010, Hope and Wilson, 2006). Systems reliant on flow and perfusion, static models, media composition and selection of appropriate substrates are all considered paramount, and that is before consideration of the microbial composition and the research question being asked (Sissons, 1997).

Philip Marsh's group first reported the development of a 9-species oral biofilm using the CDFF. Spatially differentiated steady-state biofilm communities composed of obligate and facultative anaerobes were described (Kinniment et

al., 1996a), and these could be employed to evaluate orally relevant antimicrobial compounds (Kinniment et al., 1996b). More recently the model has been utilised to produce undefined biofilms created from inoculation from pooled saliva (Hope et al., 2002). This study illustrated differential viability of bacteria throughout different layers of the biofilms using vital dyes, which has implications for host-pathogen interactions. Moreover, the choice of growth media has implications for the final biofilm structural composition. For example, artificial saliva and a model glycoprotein have both been used in model biofilm systems (Kinniment et al., 1996a, Hope et al., 2012), whereas Guggenheim and colleagues (2009) reported the use a more orally relevant nutrient source, i.e. human saliva and human serum (Guggenheim et al., 2009). This is in stark contrast to other studies that have relied on conventional bacteriological media, such as brain heart infusion (Peyyala et al., 2011b), or a selection of different media such as chemically defined media (Standar et al., 2010). Clearly, these factors play a critical role in the resultant biofilms that are formed.

Other groups have used alternative methods to grow biofilms, including flow cell models. It has been recently reported that flow cells preferentially enable two- and three-species biofilms to demonstrate mutualistic behaviour compared to a static peg-based model (Periasamy and Kolenbrander, 2009a). These same systems have been used to demonstrate the structural and spatial role of *P. gingivalis* throughout biofilm formation (Periasamy and Kolenbrander, 2009b). However, these excellent models are technically complex and labour intensive, so others have opted for the more traditional static model. For example, a rigid gas permeable lens has recently been described for the development of a multi-species biofilm model (Peyyala et al., 2011b). Biofilms of early and late colonising species were produced and evaluated by qPCR and fluorescence microscopy. Here it was shown that substantial qualitative and quantitative differences were observed, with spatial heterogeneity between the biofilms evident. Notably, Guggenheim and colleagues described a sub-gingival biofilm model used for host-pathogen interaction studies that was grown on hydroxyapatite (HA) discs using nine species of periodontal bacteria (Guggenheim et al., 2009). These biofilms were shown to be thick (50um) with evidence of defined architecture, including microcolonies, with a considerable variety of cell morphotypes.

Collectively, these models show a plethora of different ways of developing periodontal biofilms, which has its advantages and disadvantages depending on its downstream applications. Therefore, the purpose of this piece of work was to design and develop a simple, reproducible and defined *in vitro* multi-species biofilm that could be used to investigate host cell interactions with sub-gingival biofilm.

2.2 AIMS

Gingival biofilms are heterogeneous with complex three-dimensional architecture, which are therefore difficult to reproduce accurately within the laboratory. Moreover, several key species of bacteria are implicated in periodontal disease progression, either through direct pathogenic mechanisms or indirectly through host mediated pathogenesis. In order to understand host-pathogen interactions more carefully the initial aim of this study was to:

1. Create a simplified and reproducible gingival biofilm model
2. Qualitatively and quantitatively compare and contrast mono- and multi-species biofilms

2.3 MATERIALS & METHODS

2.3.1 Growth conditions and standardisation of periodontal bacteria

Porphyromonas gingivalis ATCC 33277, *Fusobacterium nucleatum* ATCC 10953, *Aggregatibacter actinomycetemcomitans* ATCC 43718 and *Streptococcus mitis* ATCC 12261 were used in the course of these studies. The bacteria were stored in the -80^{o}C freezer in cryovials (Fisher Scientific, Loughborough, UK). From beads *P. gingivalis* and *F. nucleatum* bacteria were revived at 37^{o}C on fastidious anaerobic agar (Oxoid, Cambridge, UK) with 5% defibrinated horse blood (E&O laboratories, Bonnybridge, UK) in an anaerobic chamber (85% N_2, 10% CO_2 and 5% H_2, [Don Whitley Scientific Limited, Shipley, UK]) for 3 days and 2 days, respectively. From beads *A. actinomycetemcomitans* and *S. mitis* bacteria were revived at 37^{o}C on blood agar (Oxoid, Cambridge, UK) in microaerophilic conditions (5% CO_2, [Binder GmbH, Tuttlingen, Germany]) for 1 day. These bacterial strains were used for experimental procedures after the second passage. These strains were maintained on the respective agar plates as described above.

For experimental procedures, *P. gingivalis* and *F. nucleatum* were grown at 37^{o}C in Schaedler anaerobe broth (Oxoid, Cambridge, UK) for 2 days and 1 day, respectively. *A. actinomycetemcomitans* and *S. mitis* were grown at 37^{o}C in tryptic soy broth (Sigma, Poole, UK) supplemented with 0.8% w/v glucose (BDH, Poole, UK) and 0.6% w/v yeast extract (Oxoid, Cambridge, UK) for 1 day in 5% CO_2.

All four bacterial species were centrifuged individually at 3000 rpm for 5 min and the pellet washed 3 times with phosphate buffered saline (PBS; pH 7.4, Oxoid, Cambridge, UK). *P. gingivalis, F. nucleatum* and *A. actinomycetemcomitans* were standardized at an OD_{550} of 0.2 in a colourimeter (Model 45, Fisher Scientific, Loughborough, UK) to obtain approximately 1×10^8 cfu/mL. *S. mitis* was standardized at an OD_{550} of 0.5 in a colourimeter (Model 45, Fisher Scientific, Loughborough, UK) to obtain approximately 1×10^8 cfu/mL. This was confirmed by standard plate counting method using the Miles and Misra method (Miles et al., 1938).

2.3.2 Artificial saliva

Artificial saliva (AS) was prepared as previously described (Pratten et al., 1998b), containing the following constituents: porcine stomach mucins Type III (0.25% w/v), sodium chloride (0.35 w/v), potassium chloride (0.02 w/v), calcium chloride dihydrate (0.02 w/v), yeast extract (0.2 w/v), lab lemco powder (0.1 w/v), proteose peptone (0.5 w/v) in ddH_2O (Sigma, Poole, UK). Urea was then added to independently to a final concentration of 0.05% (v/v).

2.3.3 Development of bacterial biofilms

2.3.3.1 Mono-species biofilm

P. gingivalis *and F. nucleatum* were standardized to 1 $\times$ 10^7 cfu/mL in AS, Schaedler anaerobe broth, chemically defined medium (Milner et al., 1996), tryptic soy broth (Sigma, Poole, UK), tryptic soy broth + 5% defibrinated horse blood, brain heart infusion broth and brain heart infusion broth + 5% defibrinated horse blood. The standardized bacteria (500 µL) were transferred to 24 well plates (Corning, NY, USA) containing customised hydroxyapatite (HA) discs (13 mm diameter, 1.5 mm thick [Plasma Biotal Ltd, Tideswell, Derbyshire, UK]) and Thermanox™ coverslips (13 mm diameter [Thermo Scientific, NY, USA]). *P. gingivalis* was incubated at 37°C in an anaerobic environment for 48 and 96 h. *F. nucleatum* was incubated at 37°C in an anaerobic environment for 48 h. *A. actinomycetemcomitans* and *S. mitis* were standardized to 1 $\times$ 10^7 cfu/mL in AS, Schaedler anaerobe broth and chemically defined medium; and 500 µL transferred to 24 well plates (Corning, NY, USA) containing customised hydroxyapatite (HA) discs (13 mm diameter, 1.5 mm thick) and Thermanox™ coverslips (13 mm diameter). *A. actinomycetemcomitans* was incubated at 37°C in 5% CO_2 for 48 and 96 h. *S. mitis* was incubated at 37°C in 5% CO_2 for 48 h. For all bacterial species the media was replaced daily.

After incubation the HA discs were washed 3 $\times$ with sterile PBS to remove the non-adherent cells, and biofilms disaggregated by mild sonication in a sonic bath at 35 kHz (FB11021, Fisherbrand, Loughborough, UK) for 5 min. The sonicate was then used for viable cell counting using the Miles and Misra plate technique (Miles et al., 1938). This was performed in triplicate on at least two separate occasions for each organism.

2.3.3.2 Multi-species biofilm

S. mitis biofilms were formed for 24 h, as described above. The media was removed, and standardised *F. nucleatum* in AS added to the 24 h *S. mitis* biofilm. These were incubated at 37°C in an anaerobic environment for a further 24 h. The media was then removed and standardised *P. gingivalis* and *A. actinomycetemcomitans* in AS added to the *S. mitis*/*F. nucleatum* biofilm. This was incubated at 37°C in an anaerobic environment for a further 4 days, each day replacing the AS, to finally produce a four species biofilm (Figure 2.1). Total viable cell counts were performed on bacterial species-specific plates. This was performed in triplicate on at least two separate occasions.

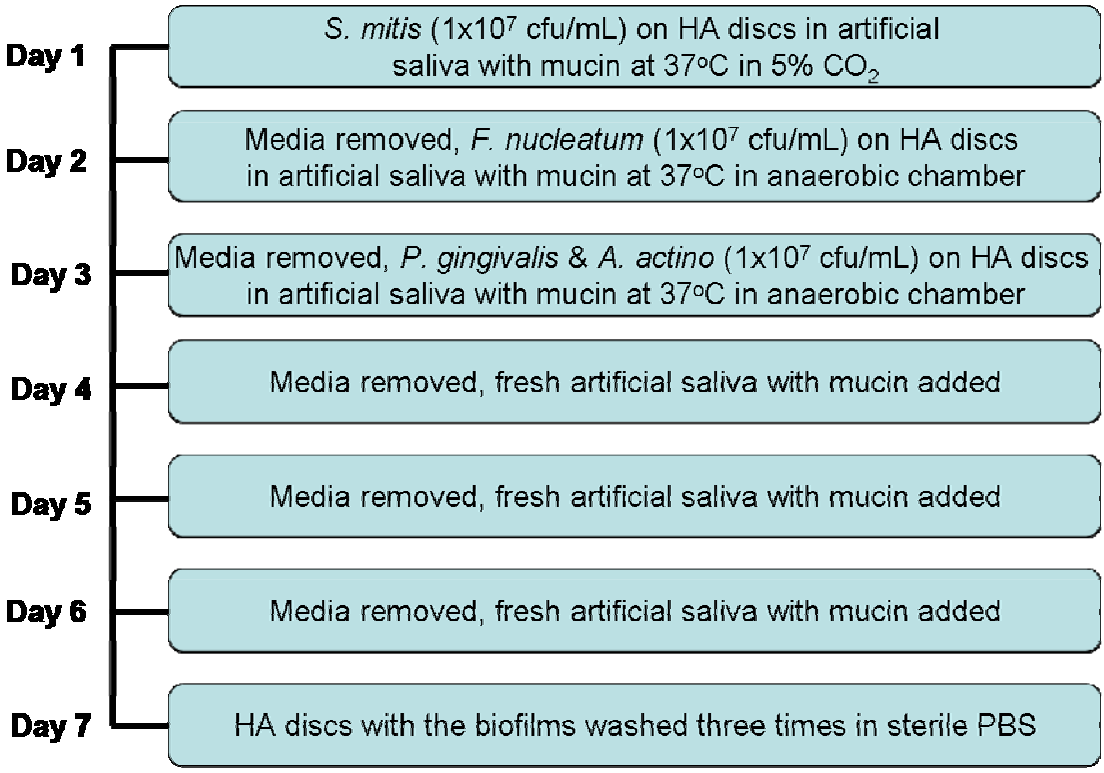


Figure 2.1: Development of mixed species biofilm. Diagrammatic representation of the sequence of events for developing the four-species biofilm consisting of *S. mitis*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* over a period of 7 days.

2.3.3.3 Bacterial species-specific agar plates

(i) *A. actinomycetemcomitans* **agar**: This agar contains 4% w/v of tryptic soy agar (Oxoid, Cambridge, UK) and 0.1% w/v of yeast extract (Fluka analytical, Sigma, Poole, UK) in ddH_2O. After autoclaving and cooling, 10 mL of 75 µg/mL bacitracin (Sigma, Poole, UK), 10 mL of 5 µg/mL vancomycin hydrochloride from *Streptomyces orientalis* (Sigma, Poole, UK) and 100 mL of filtered horse serum (E&O laboratories, Bonnybridge, UK) was added to the agar.

(ii) Mitis salivarius agar (*S. mitis***)**: The agar contains 9% w/v of mitis salivarius agar powder (MSA) (Fluka analytical, Sigma, Poole, UK) in ddH_2O. After autoclaving and cooling to 50°C, 1 mL of potassium tellurite solution (Fluka analytical, Sigma, Poole, UK) was added to the cooled agar.

(iii) *P. gingivalis* **agar:** These plates were commercially sourced and consisted of 42.5g of Columbia agar, 5 mL (0.1%) hemin, 1 mL (1%) vitamin K_1, 6.5 g of agar, 15 mg of nalidixic acid, 15.37 mg of colistin methanesulfonate, 10 mg of bacitracin, 5% sheep blood in 1 L of ddH_2O (Anaerobe systems, CA, USA)

(iv) Fastidious anaerobic agar for *F. nucleatum***:** The agar contains 4.6% w/v of fastidious anaerobic agar (E&O laboratories, Bonnybridge, UK) in ddH_2O. After autoclaving and cooling, 5% defibrinated horse blood (E&O laboratories, Bonnybridge, UK) was added to the cooled agar.

2.3.4 Impact of freezing on biofilm viability

To assess the effects of freezing the biofilm, as a means of creating reproducible biofilm stocks, media was removed from the mono- and multi-species biofilms, which were then sealed within 24 well plates and stored at -80°C. These were latterly revived by adding 500 µL of AS and incubating within respective environments at 37°C for 24 h. Total viable cell counts were then performed as described above to compare to the fresh biofilms. The experiment was performed in triplicate on least two separate occasions.

2.3.5 Survival of biofilms in cell culture media

Frozen biofilms were revived in AS overnight at 37°C in the respective environments at 37°C. The biofilms were maintained in defined keratinocyte

serum-free media (K-SFM, Invitrogen) for 4 and 24 h at 37°C in 5% CO_2. After incubation, the bacterial biofilms were washed three times with sterile PBS to remove the non-adherent biofilm, sonicated and viable counts obtained using standard plate counting method, as described above.

2.3.6 Scanning electron microscopy (SEM)

All biofilms on HA were examined by SEM, as previously described (Erlandsen et al., 2004). Briefly, the biofilms were fixed in 2% para-formaldehyde, 2% gluteraldehyde and 0.15M sodium cacodylate and 0.15% alcian blue (pH 7.4). The fixative was then replaced with 0.15M sodium cacodylate buffer and samples washed 3 × 5 min. A 1% (w/v) osmium tetroxide (OsO_4) was then added to an equal volume of 0.15M sodium cacodylate buffer for 1 h. Samples were rinsed 3 × with ddH_2O for 10 min. 0.5% (v/v) aqueous uranyl acetate was then added to the wells and incubated in the dark for 30 min at room temperature. Samples were then dehydrated in an ascending ethanol series (30%, 50%, 70%, 90%, absolute alcohol and dried absolute alcohol) then fixed in hexamethyldisilazane (HMDS, TAAB, Berks, UK) for 2 × 5 min in separate containers. These were then placed in a dessicator overnight. The fixed and dried denture base samples were sputter-coated with gold/paladium and viewed under a JEOL JSM-6400 scanning electron microscope.

2.3.7 Statistical analysis

The statistical analyses on the difference in viable bacteria recovered from the biofilms were performed with GraphPad Prism (San Diego, CA, USA) using two-tailed unpaired student t test. This method gives an unpaired two sample student t test with a confidence interval for the difference between the means. The unpaired t method tests the null hypothesis that the population means related to two independent, random samples from an approximately normal distribution are equal (Altman, 1991; Armitage and Berry, 1991). The significance level was set at $p < 0.05$ in a two-sided test.

2.4 RESULTS

2.4.1 Quantitative and structural analysis of mono-species biofilms grown in different media. Periodontal bacteria exist in the oral cavity as heterogeneous and structurally complex biofilms (Kolenbrander, 2000, Rosan and Lamont, 2000). However, in order to gain a clearer understanding of how different bacteria influence biofilm composition and architecture then analysis of biofilms formed by single species was first performed. Taking this reductionist approach mono-species biofilm development of *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis* was investigated, as described in section 2.3.3.

The quantitative recovery of bacteria from monospecies biofilms grown on Thermanox™ coverslips in the different media is presented in Table 2.1. *P. gingivalis* biofilms grew marginally better in AS (6.44 X 10^4 CFU/mm^2) compared to THM (5.41 X 10^4 CFU/mm^2). No viable *P. gingivalis* was recovered when grown in SAB. *F. nucleatum* biofilms grew preferentially in BHI+B (1.61 X 10^7 CFU/mm^2), followed by TSB (5.16 X 10^6 CFU/mm^2), AS (1 X 10^6 CFU/mm^2), SAB (3.25 X 10^5 CFU/mm^2) and TSB+B (1.93 X 10^5 CFU/mm^2). No growth was observed in the *F. nucleatum* biofilms grown in THM, CDM and BHI. *A. actinomycetemcomitans* biofilms grew preferentially in SAB (4.56 X 10^5 CFU/mm^2), followed by SAB (2.65 X 10^5 CFU/mm^2) and AS (1.62 x 10^5 CFU/mm^2). Finally, *S. mitis* biofilms grew preferentially in AS (7.90 X 10^5 CFU/mm^2), compared with those grown in SAB (2.25 x 10^2 CFU/mm^2) and THM (5.72 x 10^3 CFU/mm^2).

Table 2.1: Quantitative analysis of mono-species biofilms grown in different media on Thermanox™ coverslips for 48 h

	CFU/mm^2 (SD)							
	P. gingivalis		*F. nucleatum*		*A. actinomycetemcomitans*		*S. mitis*	
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
AS	6.44 X 10^4	1.15 X 10^4	1 X 10^6	4.57 X 10^5	1.62 X 10^5	1.94 X 10^3	7.90 X 10^5	3.87 X 10^4
SAB	NG		3.25 X 10^5	2.46 X 10^5	4.56 X 10^5	2.22 X 10^4	2.25 X 10^2	5.04
THM	5.41 X 10^4	1.98 X 10^4	NG		2.65 X 10^5	1.27 X 10^4	5.72 X 10^3	2.22 X 10^2
CDM	NG		NG		ND		ND	
TSB	NG		5.16 X 10^6	1.49 X 10^5	ND		ND	
TSB+B	NG		1.93 X 10^5	1.31 X 10^4	ND		ND	
BHI	NG		NG		ND		ND	
BHI+B	NG		1.61 X 10^7	6.22 X 10^5	ND		ND	

AS = Artificial saliva; SAB = Schaedlers anaerobic broth; THM = Tryptic soy broth supplemented with haemin and menadione; CDM = chemically defined media; TSB = Tryptic soy broth; TSB+B = TSB plus blood; BHI = Brain heart infusion broth; BHI+B = BHI plus blood; NG = No growth; ND = Not determined. (n=3)

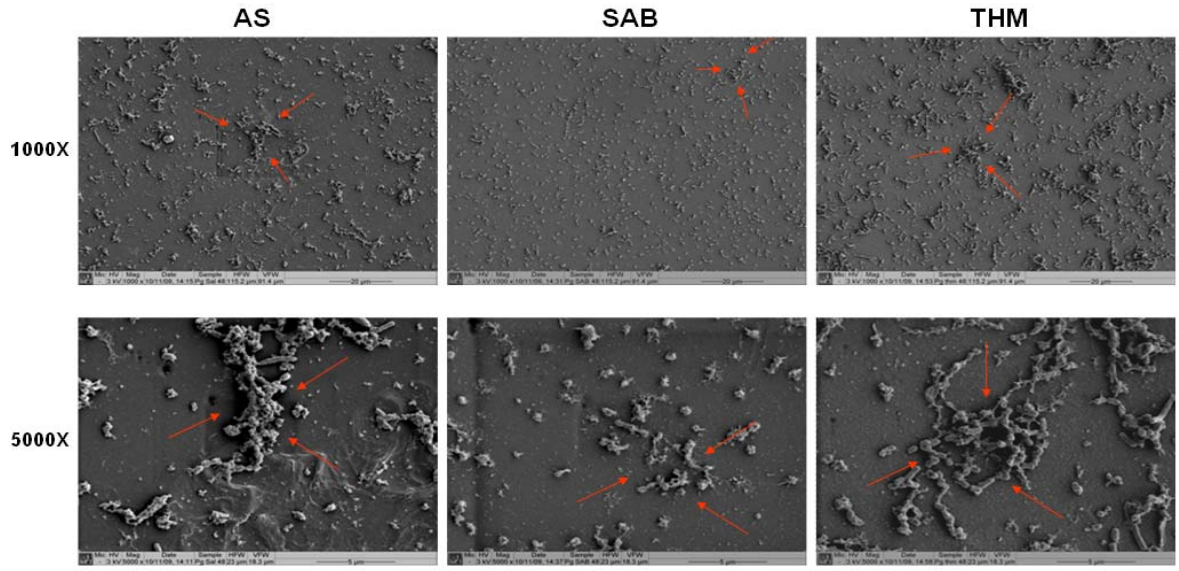
The structure of these monospecies biofilms was evaluated using SEM. For *P. gingivalis,* diffuse clusters of bacterial cells were observed at low magnification (1000x) when it was grown in AS and THM. In SAB the biofilms were very sparse with no evidence of 3-D architecture. At higher magnification (5000x), microcolonies of short rod-shaped *P. gingivalis* with extracellular matrix were observed when it was grown in AS and THM (Figure 2.2A). However, in SAB whilst occasional adherent cells were observed, there were no microcolonies with 3-D architecture.

F. nucleatum biofilms grown in AS and SAB were densely populated (1000x), particularly compared to the sparse monolayers of those grown in THM (Figure 2.2B). At higher magnification (5000x) the biofilms grown in AS and SAB were structurally complex, with those in AS showing the presence of matrix material.

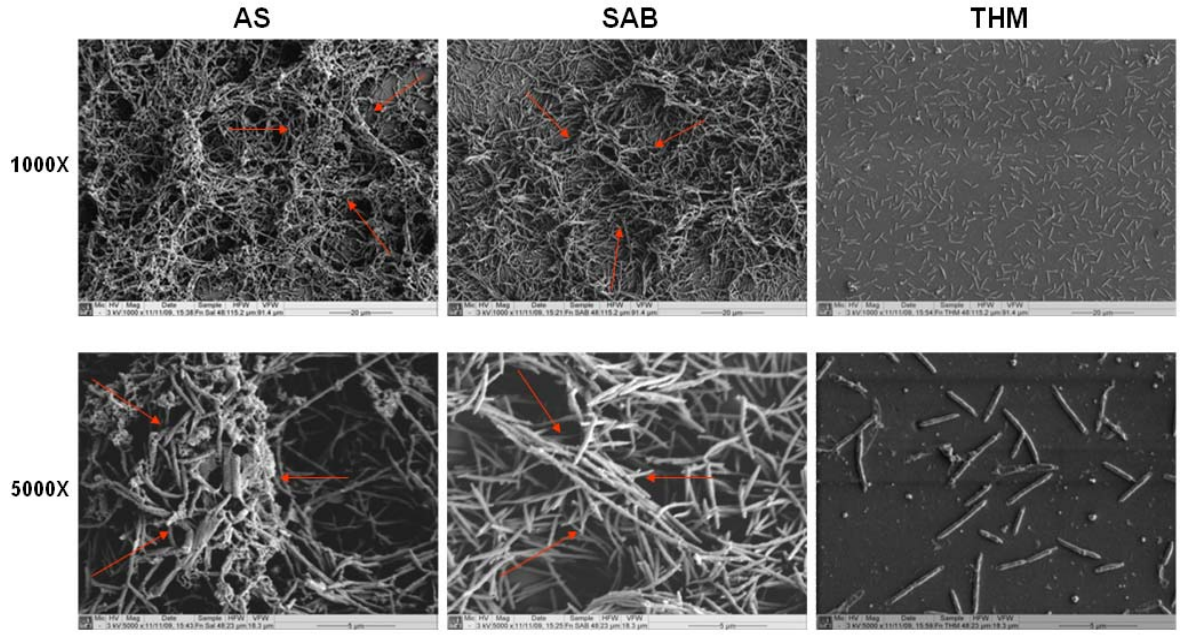
A. actinomycetemcomitans biofilms grew sparsely with minimal architectural complexity when it was viewed under low magnification in AS, SAB and THM (Figure 2.2C). However, under higher magnification (5000x) these biofilms showed evidence of microcolonies with matrix production within all the media tested.

S. mitis biofilms displayed contrasting biofilm architecture when grown in the different media. At low magnification (1000x) the biofilm grown in SAB was densely packed compared to those in AS and THM (Figure 2.2D). At higher magnification (5000x) the SAB grown biofilms were shown to be chains of cocci densely packed, whereas those in AS and THM showed microcolony architecture with pairs of cooci and matrix material.

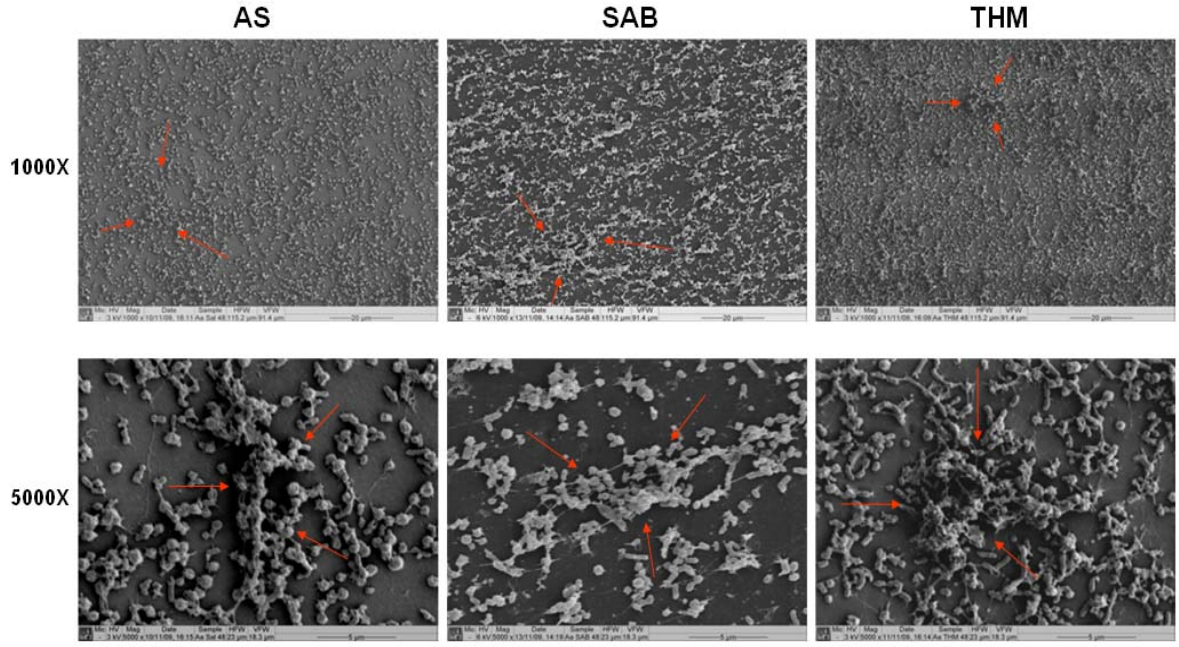
(A) *P. gingivalis* **biofilm**



(B) *F. nucleatum* **biofilm**



(C) *A. actinomycetemcomitans*



(D) *S. mitis* **biofilm**

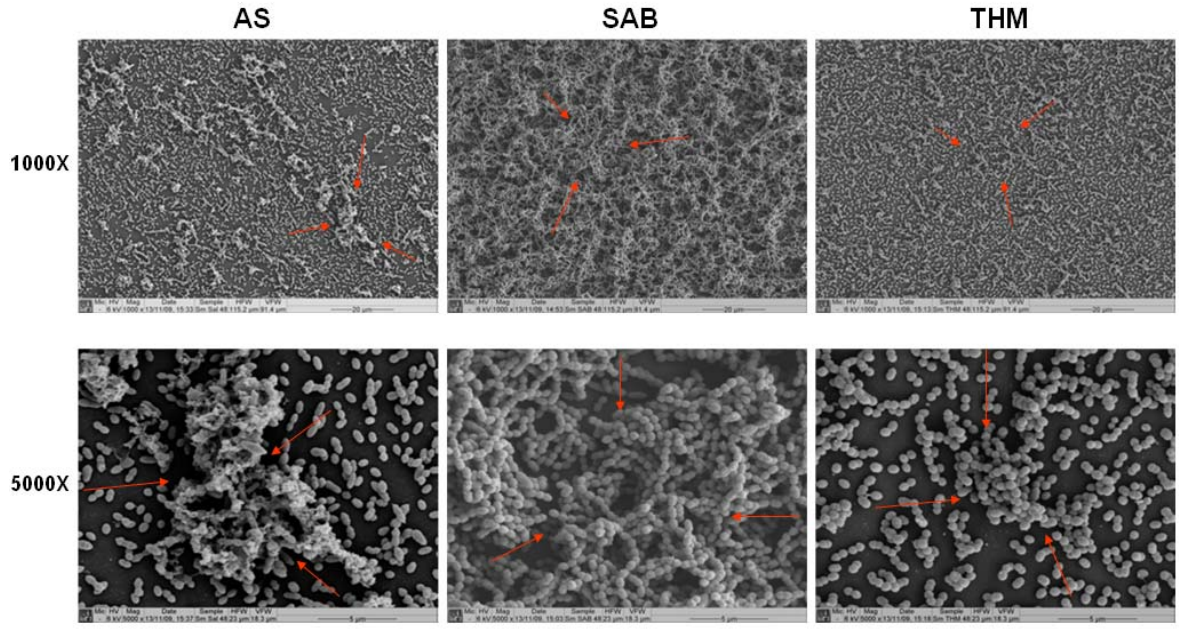


Figure 2.2: Scanning electron micrographs of 48 h biofilms grown in artificial saliva. (A) *P. gingivalis* and (B) *F. nucleatum* were grown as monospecies biofilms in AS, SAB and THM on Thermanox™ coverslips for 48 h in the anaerobic chamber at 37°C. (C) *A. actinomycetemcomitans* and (D) *S. mitis* were grown as monospecies biofilms in AS, SAB and THM on Thermanox™ coverslips for 48 h in 5% CO_2 at 37°C. The coverslips were dip-washed three times in sterile PBS before fixing for SEM. Biofilms were viewed under JEOL JSM-6400 scanning electron microscope using 1000x and 5000x magnifications. The red arrows denote microcolonies and extrapolymeric matrix.

2.4.2 Quantitative and structural analysis of *P. gingivalis* and *A. actinomycetemcomitans* biofilms grown in artificial saliva for 96 h. *F. nucleatum* and *S. mitis* yielded dense mono-species biofilms with 3-D structure when grown in AS for 48 h compared to *P. gingivalis* and *A. actinomycetemcomitans*, which were sparse monolayers. Therefore, to investigate if the poor biofilm phenotypes was a factor of time *P. gingivalis* and *A. actinomycetemcomitans* were grown in AS for 48 and 96 h at 37°C in the anaerobic chamber and 5% CO_2, respectively.

Significantly more bacteria were recovered from the 96 h *P. gingivalis* biofilm (5.38×10^5 CFU/mm^2, $p<0.0001$) compared with the 48 h biofilm (6.44×10^4 CFU/mm^2) (Figure 2.3). SEM analysis also revealed increased numbers of bacterial cells present as microcolonies of greater complexity after 96 h compared with the 48 h *P. gingivalis* biofilms (Figure 2.4A). Similarly, *A. actinomycetemcomitans* biofilms yielded significantly more viable bacterial cells after 96 h biofilm growth (2.61×10^5 CFU/mm^2, $p<0.0001$) compared with the 48 h biofilm (1.62×10^5 CFU/mm^2). SEM analysis showed greater coverage of the coverslip clearly covered in extra polymeric material.

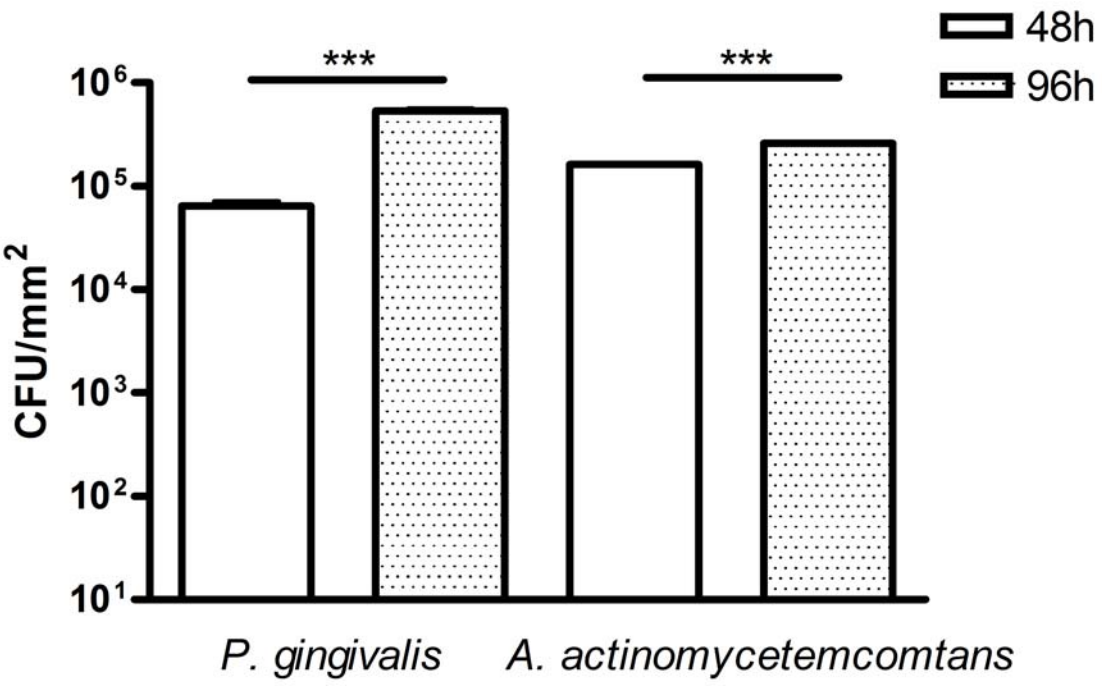
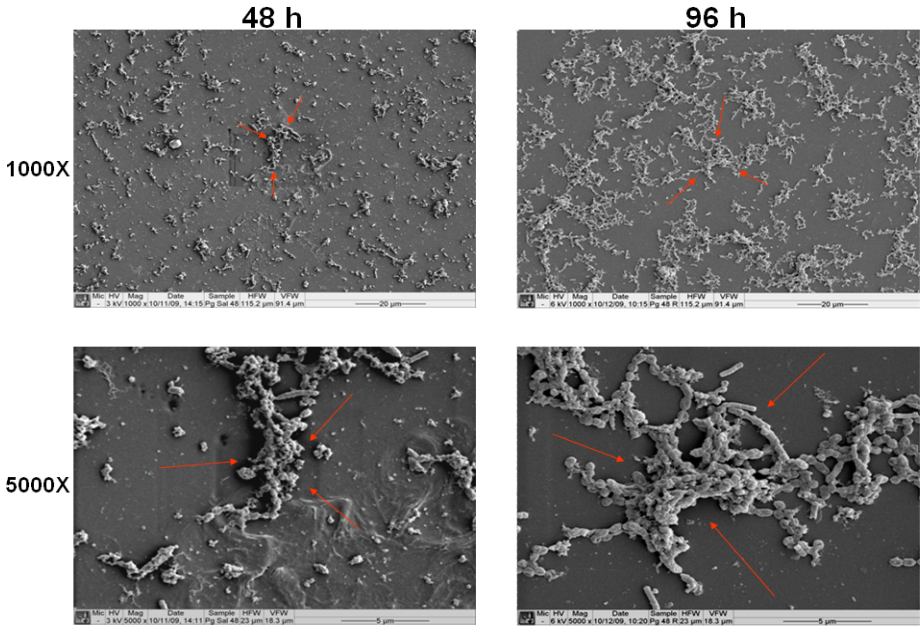


Figure 2.3: Viable cell recovery of *P. gingivalis* **and** *A. actinomycetemcomitans***.** *P. gingivalis* and *A. actinomycetemcomitans* were grown as monospecies biofilms in AS on Thermanox™ coverslips for 48 and 96 h in the anaerobic chamber and 5% CO_2 respectively at 37°C. The coverslips were dip-washed three times in sterile PBS before sonicating in sterile PBS for 5 min in an ultrasonic bath. Viable counts of the bacteria were obtained by performing Miles and Misra plate counting method on the bacterial agar plates. Data shown are viable bacteria recovered from the monospecies biofilms in CFU/mm^2. Five technical replicates were performed for three independent biofilms. (***p<0.0001).

(A) *P. gingivalis* **biofilm**



(B) *A. actinomycetemcomitans* **biofilm**

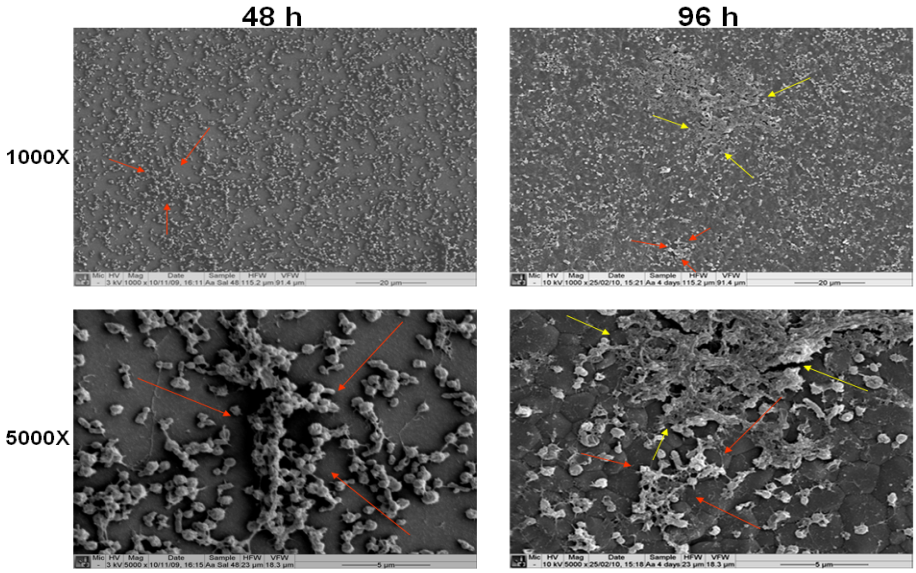


Figure 2.4: Scanning electron micrographs of 48 and 96 h *P. gingivalis* **and** *A. actinomycetemcomitans* **biofilms grown in artificial saliva on Thermanox™ coverslips.** (A) *P. gingivalis* and (B) *A. actinomycetemcomitans* were grown as monospecies biofilms in AS on Thermanox™ coverslips for 48 and 96 h in the anaerobic chamber and 5% CO_2 respectively at 37°C. The coverslips were dip-washed three times in sterile PBS before fixing for scanning electron microscopy. The bacterial biofilms were viewed under JEOL JSM-6400 scanning electron microscope using 1000X and 5000X magnifications. In the panels, the biofilms are shown between the red arrows and extracellular matrix within yellow arrows.

2.4.3 Quantitative analysis of monospecies biofilms grown on different substrates. The main biomineral component of the human hard tissues, tooth and bone, is hydroxyapatite (HA), which is represented by the formula $Ca_{10}(PO_4)_6(OH)_2$ (LeGeros, 2002, Santos et al., 2004). Therefore, HA surfaces were selected to investigate mono-species bacterial biofilm formation of *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*, grown in AS and compared to biofilms grown on Thermanox™ coverslips.

Significantly less viable bacteria were recovered from the *P. gingivalis* biofilm grown on HA (2.60×10^5 CFU/mm^2, $p<0.0001$) compared with those grown on CS (5.40×10^5 CFU/mm^2). Similarly, significantly less viable *F. nucleatum* and *A. actinomycetemcomitans* were recovered from those biofilms on HA compared with CS. For *F. nucleatum* biofilms 3.09×10^5 CFU/mm^2 were recovered from CS compared with 3.41×10^4 CFU/mm^2 from HA biofilms ($p<0.0001$), and for *A. actinomycetemcomitans* 4.22×10^4 CFU/mm^2 were recovered from CS compared with 8.03×10^2 CFU/mm^2 from HA ($p<0.0001$). *S. mitis* biofilms also showed a reduction in the viable recoverable bacteria on the HA compared with CS, but this was not significant (Figure 2.5).

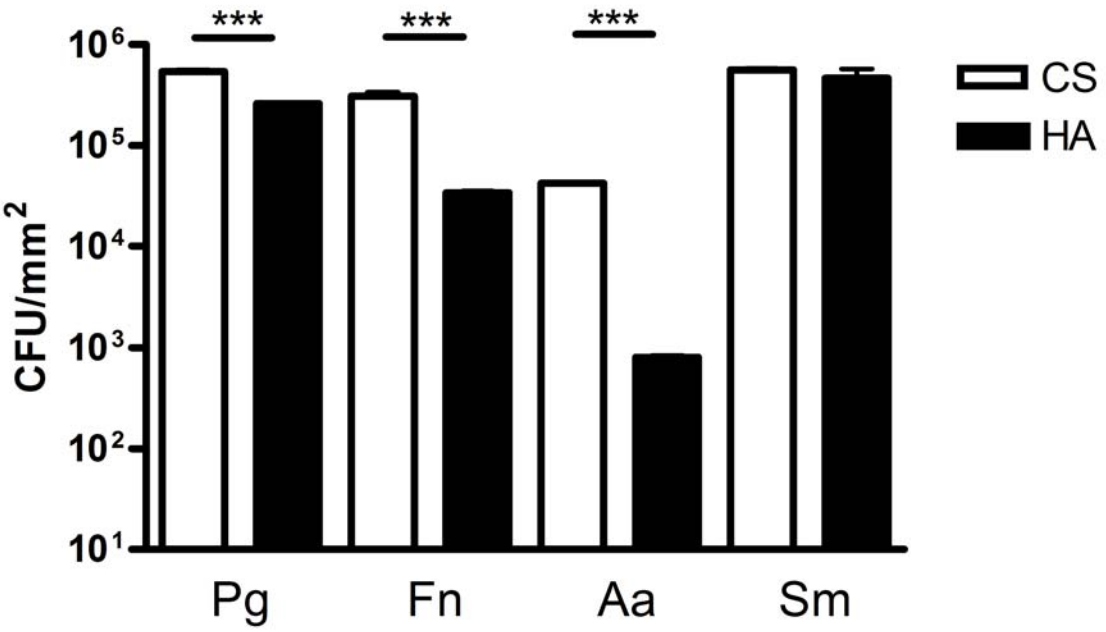


Figure 2.5: Viable cell recovery of optimised mono-species biofilms grown on Thermanox™ coverslips and hydroxyapatite discs. *P. gingivalis* and *A. actinomycetemcomitans* were grown as mono-species biofilms in AS on CS and HA substrates for 96 h in the anaerobic chamber and 5% CO_2, respectively, at 37^{o}C. *F. nucleatum* and *S. mitis* were grown as mono-species biofilms in AS on CS and HA substrates for 48 h in the anaerobic chamber and 5% CO_2, respectively, at 37^{o}C. Thereafter, biofilms were processed for total viable cell counting using the Miles and Misra plate counting method on the species-specific bacterial agar plates. Data shown are viable bacteria recovered from the monospecies biofilms in CFU/mm^2. Five technical replicates were performed for three independent biofilms. Statistical analysis was performed by two-tailed unpaired t test (***p<0.0001).

2.4.4 Quantitative analysis of multi-species biofilms grown by two different methods. Periodontal bacteria exist in the oral cavity as complex multi-species biofilms opposed to simple mono-species biofilms (Kolenbrander, 2000, Rosan and Lamont, 2000). Attachment and growth of these different bacterial species occur in a sequential manner to form dental plaque (Kolenbrander and London, 1993). Therefore, it was an aim of this study to create a defined multi-species biofilm. Biofilms were grown using two defined methods: (A) *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis* were mixed together at defined concentrations and grown collectively for 96 h (4 days) in AS, and (B) early colonising *S. mitis* grown in artificial for 24 h, followed by intermediate coloniser *F. nucleatum* for 24 h, followed by late colonisers *P. gingivalis* and *A. actinomycetemcomitans* for 96 h, as illustrated in Figure 2.1. Quantitative analysis was performed as described previously.

Mixed species biofilms formed by two different methods yielded statistically more recoverable viable bacteria when they were grown on HA compared with CS (Figure 2.6). The mixed species biofilm grown by the basic combination of all 4 bacteria (A) yielded more total recoverable bacteria when it was grown on HA (3.71×10^5 CFU/mm^2, $p<0.001$) compared with CS (2.87×10^5 CFU/mm^2). Multi-species biofilms formed by the sequential method (B) contained statistically greater quantities of bacteria on HA (3.44×10^6 CFU/mm^2, $p<0.001$) compared with CS (2.28×10^6 CFU/mm^2). Of both methods statistically greater quantities of bacteria were recovered from the mixed species grown on HA using the sequential method (3.44×10^6 CFU/mm^2, $p<0.0001$) compared with the combined method (3.71×10^5 CFU/mm^2).

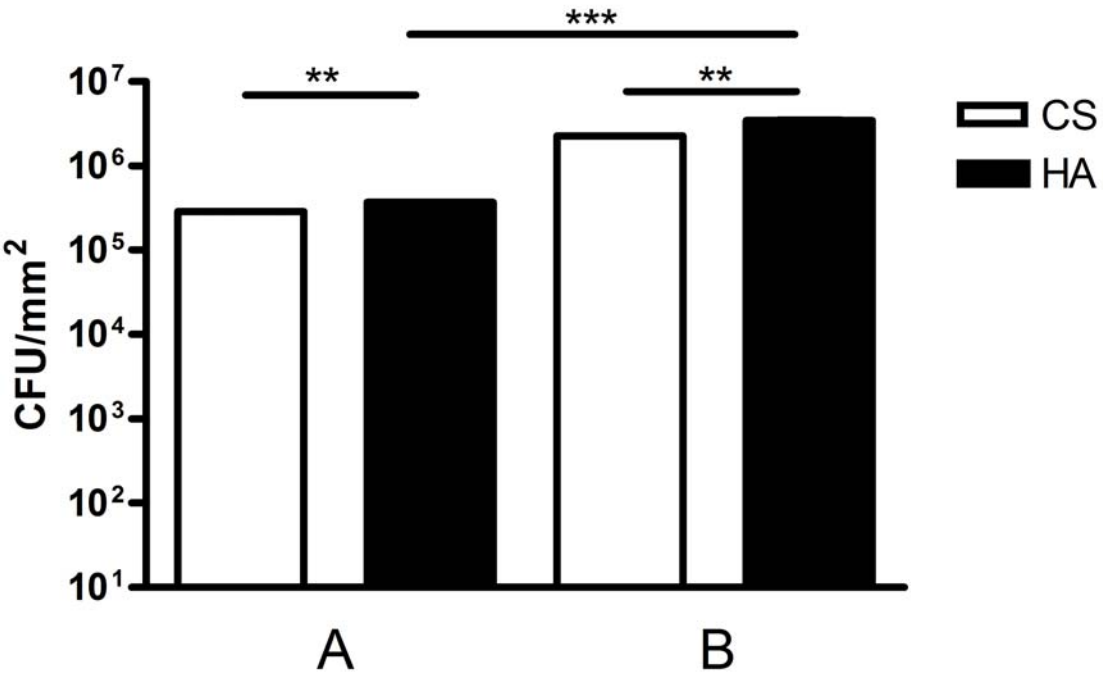


Figure 2.6: Viable cell recovery of multi-species biofilms grown by two different methods on Thermanox™ coverslips and hydroxyapatite discs. (A) *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis* were grown together on CS and HA substrates in AS for 96 h in the anaerobic chamber at 37°C. (B) *S. mitis* was grown in AS for 24 h in 5% CO_2, followed by *F. nucleatum* for 24 h in the anaerobic chamber, followed by *P. gingivalis* and *A. actinomycetemcomitans* for 96 h in the anaerobic chamber at 37°C. Thereafter, biofilms were processed for total viable cell counting using the Miles and Misra plate counting method on the species-specific bacterial agar plates. Data shown are viable bacteria recovered from the multi-species biofilms in CFU/mm^2. Five technical replicates were done for three independent biofilms. Statistical analysis was performed by two-tailed unpaired t test (**p<0.01, ***p<0.001).

2.4.5 Quantitative and structural analysis of fresh and frozen mono-species and multi-species biofilms grown on hydroxyapatite discs. Mono-species and multi-species bacterial biofilm growth is lengthy and labour intensive, therefore to improve efficiency of the model system the effects of freezing the biofilm as a means of creating biofilm stocks was explored, as described in section 2.3.4.

Significantly more viable bacteria were recovered from the fresh *P. gingivalis* biofilms (1.97×10^5 CFU/mm^2, $p<0.001$) compared with the frozen biofilms (7.67×10^4 CFU/mm^2) (Figure 2.7). The micrographs appeared to show similar bacterial quantities for the fresh and frozen *P. gingivalis* biofilms, but the *P. gingivalis* revived from the frozen biofilms cellular structure was more defined and absent of extrapolymeric matrix (Figure 2.8A). In contrast, significantly greater viable bacteria recovered from frozen *F. nucleatum* biofilms (7.22×10^6 CFU/mm^2, $p<0.01$) than the fresh biofilms (7.97×10^5 CFU/mm^2). The structure of the fresh *F. nucleatum* biofilm was comparable to its frozen form, as was the cellular abundance (Figure 2.8B). Viable bacteria recovered from *A. actinomycetemcomitans* and *S. mitis* biofilms did not show significant differences when its fresh and frozen forms were compared. Fresh *A. actinomycetemcomitans* biofilms yielded 1.99×10^5 CFU/mm^2 compared to 1.76×10^5 CFU/mL. The micrographs revealed comparable bacterial structure and abundance for the fresh and frozen (Figure 2.8C). Viable bacteria recovered from fresh *S. mitis* biofilms yielded 6.90×10^5 CFU/mm^2 compared to 7.14×10^5 CFU/mm^2). Visually bacterial numbers were comparable, both showing similar cocci structure in its fresh and frozen forms (Figure 2.8D).

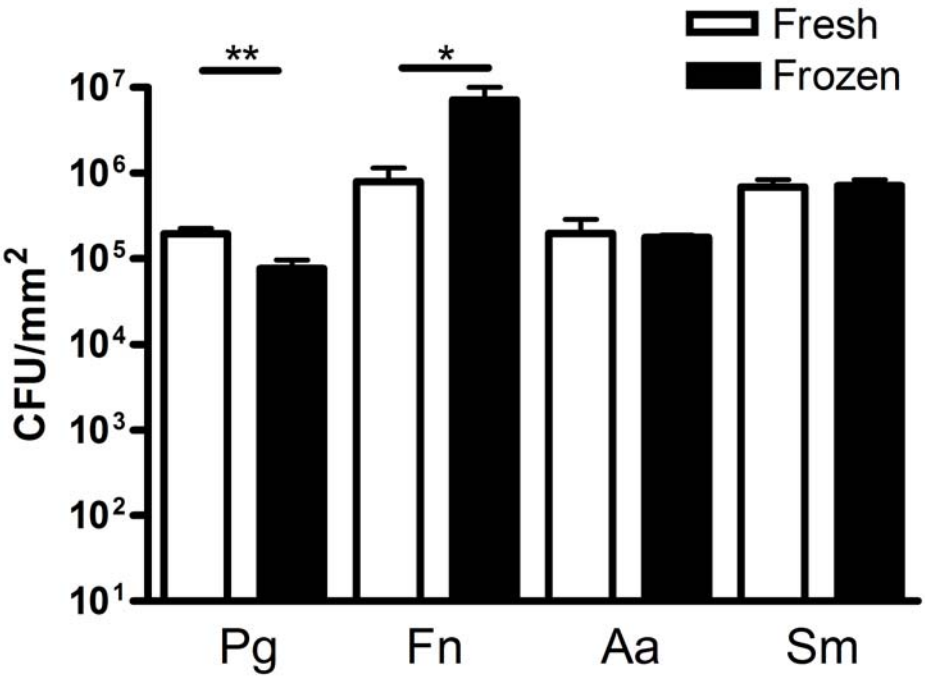
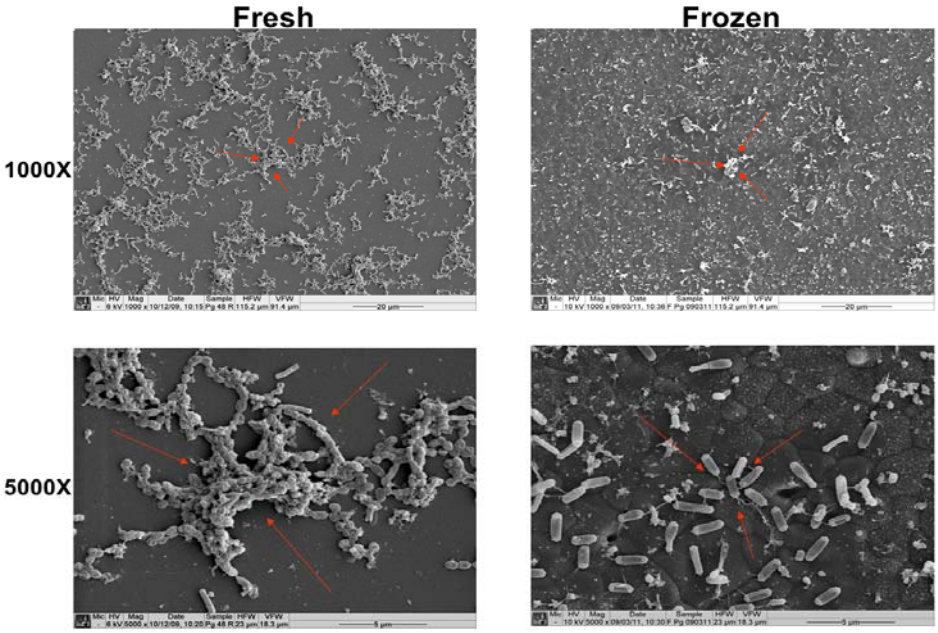
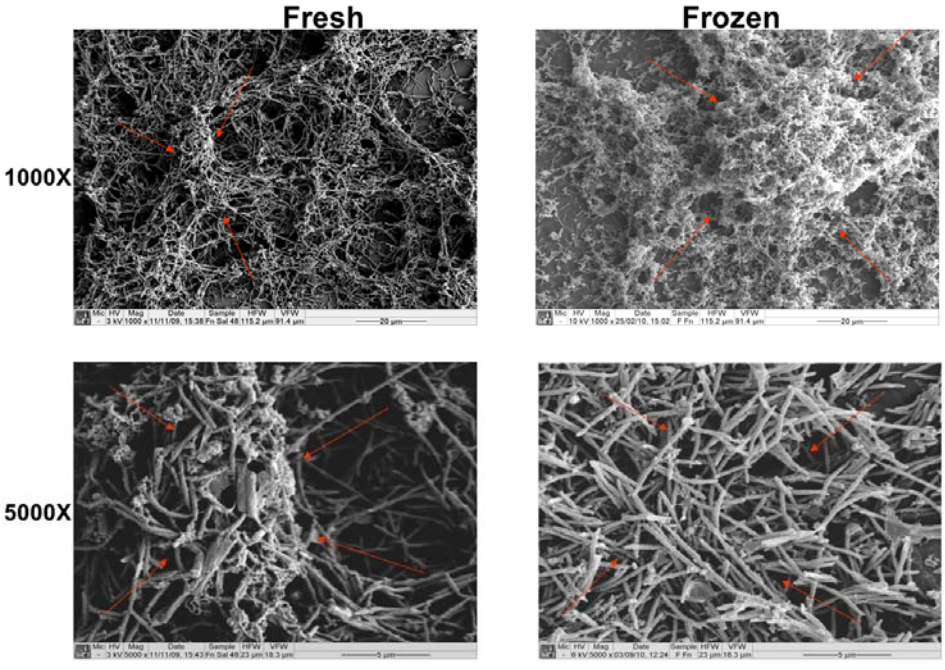


Figure 2.7: Viable cell recovery of fresh and frozen optimised mono-species biofilms grown on hydroxyapatite discs. *P. gingivalis* and *A. actinomycetemcomitans* were grown as mono-species biofilms in AS on HA discs for 96 h in the anaerobic chamber and 5% CO_2, respectively, at 37°C. *F. nucleatum* and *S. mitis* were grown as mono-species biofilms in AS on HA for 48 h in the anaerobic chamber and 5% CO_2 respectively at 37°C. The AS was removed; plates sealed with parafilm and kept in the -80°C freezer till used. The frozen biofilms were revived by thawing the biofilms and incubating with fresh AS within respective environments at 37°C for 24 h. Thereafter, biofilms were processed for total viable cell counting using the Miles and Misra plate counting method on the species-specific bacterial agar plates. Data shown are viable bacteria recovered from the mono-species biofilms in CFU/mm^2. Three replicates were performed for two independent experiments. Statistical analysis was performed by two-tailed unpaired t test (*p<0.01, **p<0.001).

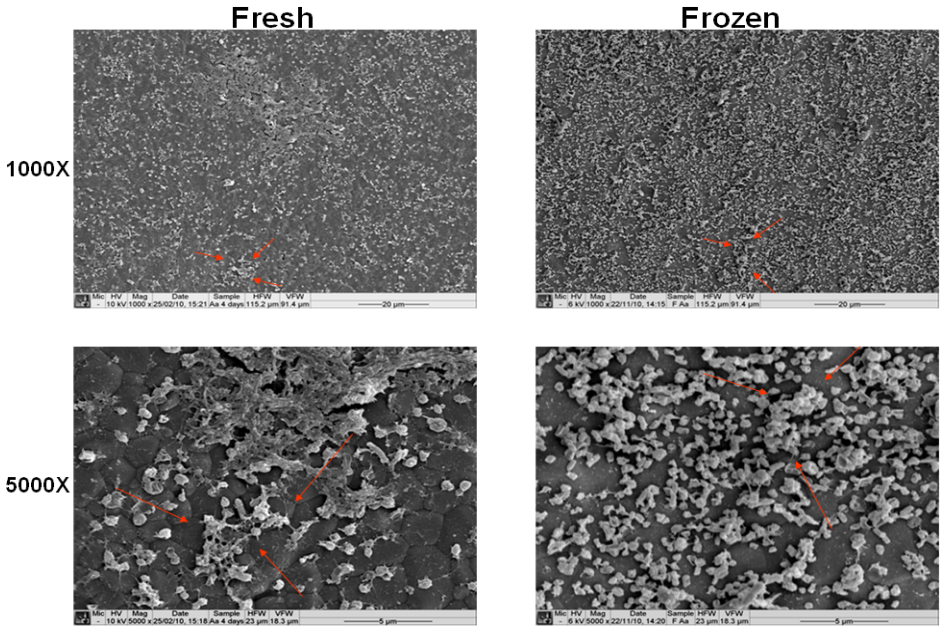
(A) *P. gingivalis* **biofilm**



(B) *F. nucleatum* **biofilm**



(C) *A. actinomycetemcomitans* **biofilm**



(D) *S. mitis* **biofilm**

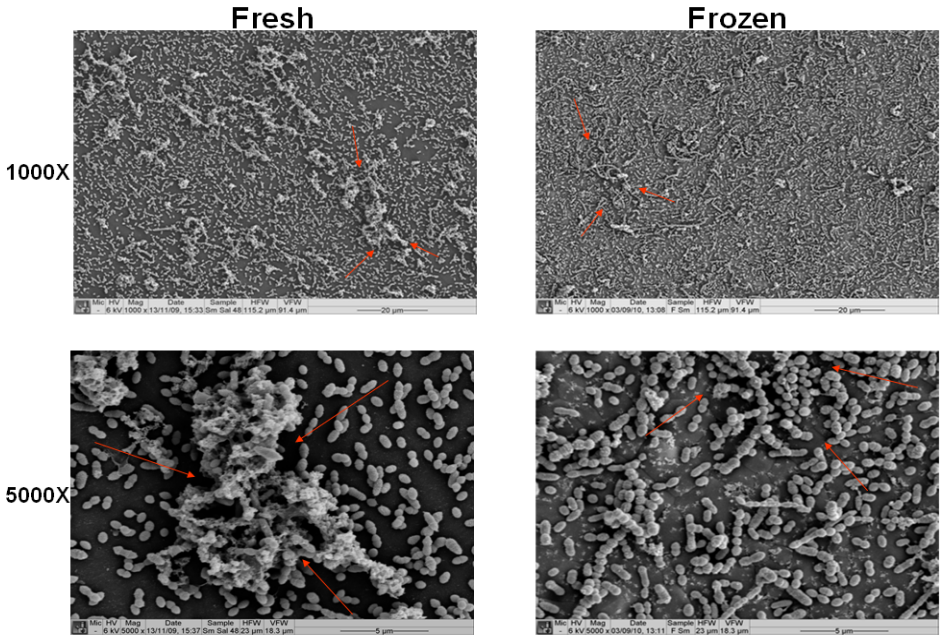


Figure 2.8: Scanning electron micrographs of fresh and frozen mono-species biofilms grown on hydroxyapatite discs. (A) *P. gingivalis* and (C) *A. actinomycetemcomitans* were grown as monospecies biofilms in AS on HA for 96 h in the anaerobic chamber and 5% CO_2, respectively, at 37°C. (B) *F. nucleatum* and (D) *S. mitis* were grown as mono-species biofilms in AS on HA for 48 h in the anaerobic chamber and 5% CO_2, respectively, at 37°C. The AS was removed; plates sealed with parafilm and kept in the -80°C freezer till used. The frozen biofilms were revived by thawing the biofilms and incubating with fresh AS within respective at 37°C for 24 h. The biofilms were dip-washed three times in sterile PBS before fixing for scanning electron microscopy. The bacterial biofilms were viewed under JEOL JSM-6400 scanning electron microscope using 1000X and 5000X magnifications. The bacterial biofilms are shown between the red arrows on the panels.

Significantly more viable *P. gingivalis* were recovered from the fresh multi-species biofilms (1.10 x 10^6 CFU/mm^2, p<0.01) compared with the frozen biofilms (5.73 x 10^5 CFU/mm^2) (Figure 2.9), whereas marginally more viable *F. nucleatum* were recovered from the frozen multi-species biofilms (4.57 x 10^6 CFU/mm^2) compared with the fresh biofilms (3.54 x 10^6 CFU/mm^2). Comparable quantities of viable *A. actinomycetemcomitans* were recovered from fresh multi-species biofilms (1.91 x 10^6 CFU/mm^2) compared with the frozen biofilms (1.75 x 10^6 CFU/mm^2). Finally, significantly more *S. mitis* was recovered from the fresh multi-species biofilms (1.29 x 10^6 CFU/mm^2, p<0.0001) compared with the frozen biofilms (6.13 x 10^5 CFU/mm^2).

Structural analysis by SEM of the fresh and frozen multispecies biofilms revealed showed similar bacterial distribution, which contained 3-D architecture in both types of biofilm. At higher magnification (5000x), the four constituent bacteria were shown to be present as indicated by arrows on the micrographs for both the fresh and frozen biofilms (Figure 2.10A and B).

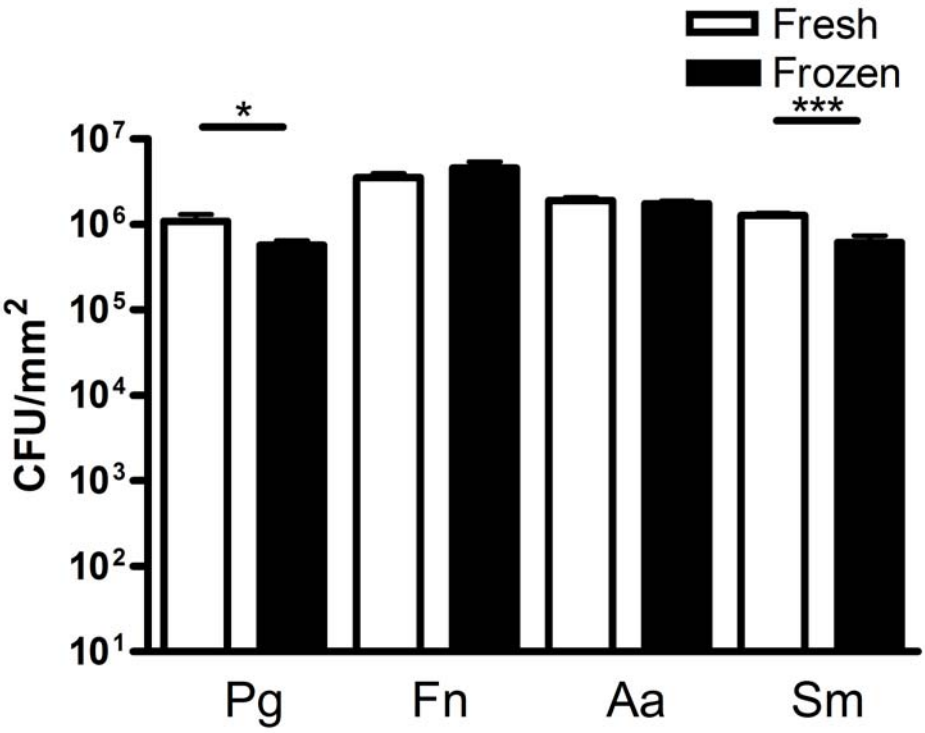
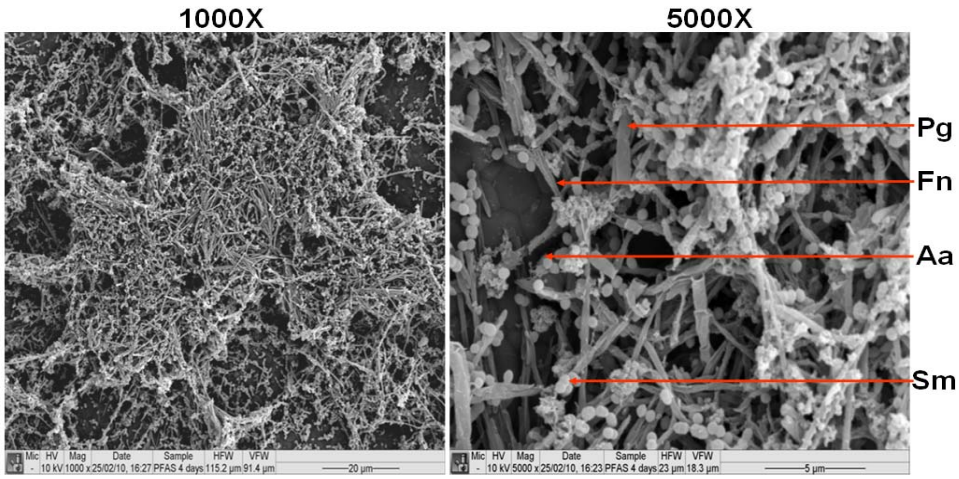


Figure 2.9: Viable cell recovery of fresh and frozen multi-species biofilms grown on hydroxyapatite discs. *S. mitis* was grown in AS for 24 h in 5% CO_2, followed by *F. nucleatum* for 24 h in the anaerobic chamber, followed by *P. gingivalis* and *A. actinomycetemcomitans* for 96 h in the anaerobic chamber at 37^{o}C. The AS was removed; plates sealed with parafilm and kept in the -80^{o}C freezer till used. The frozen biofilms were revived by thawing the biofilms and incubating with fresh AS within respective at 37^{o}C for 24 h. Thereafter, biofilms were processed for total viable cell counting using the Miles and Misra plate counting method on the species-specific bacterial agar plates. Data shown are viable bacteria recovered from the multi-species biofilms in CFU/mm^2. Three replicates were done for two independent experiments. Statistical analysis was performed by two-tailed unpaired t test (*p<0.01, ***p<0.0001).

(A) Fresh multi-species biofilm



(B) Frozen multi-species biofilm

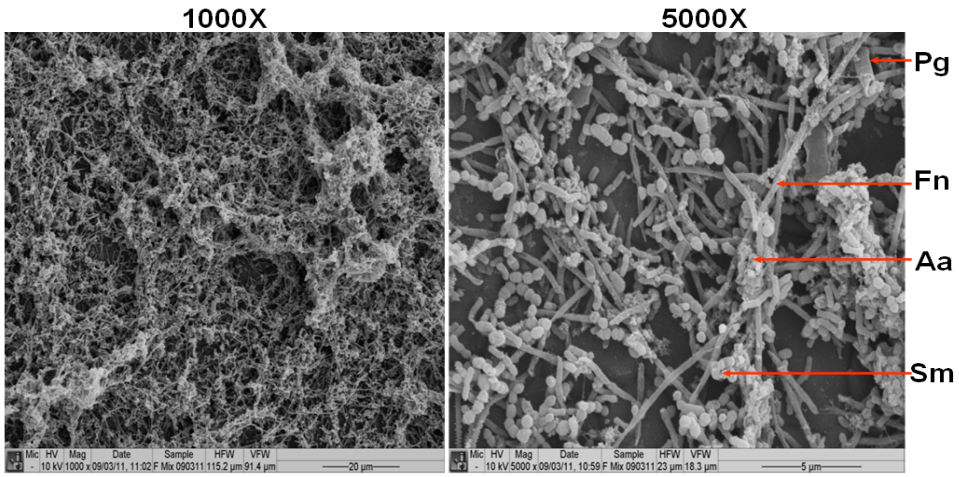


Figure 2.10: Scanning electron micrographs of (A) fresh and (B) frozen multi-species biofilms grown on hydroxyapatite discs. *S. mitis* was grown in artificial for 24 h in 5% CO_2, followed by *F. nucleatum* for 24 h in the anaerobic chamber, followed by *P. gingivalis* and *A. actinomycetemcomitans* for 96 h in the anaerobic chamber at 37°C. The AS was removed; plates sealed with parafilm and kept in the -80°C freezer till used. The frozen biofilms were revived by thawing the biofilms and incubating with fresh AS within respective at 37°C for 24 h. The biofilms were dip-washed three times in sterile PBS before fixing for scanning electron microscopy. The bacterial biofilms were viewed under JEOL JSM-6400 scanning electron microscope using 1000x and 5000x magnifications.

2.4.6 Survival of mono-species and multi-species biofilms in cell culture conditions. Periodontal bacteria survive under a range of harsh microaerophilic and hypoxic conditions within the oral cavity. The ultimate aim of the study was to investigate host-pathogen interactions between these biofilms and epithelial cells. Therefore, investigating the survival of the periodontal bacterial biofilms under cell culture conditions was a critical component of these studies. To investigate the survival of periodontal bacterial biofilms under cell culture conditions, mono-species and multi-species biofilms were suspended in cell culture media as described in section 2.3.5.

Significantly less viable bacteria were recovered from the *P. gingivalis* mono-species biofilms grown in cell culture media after both 4 h (3.62×10^3 CFU/mm^2, $p<0.001$) and 24 h (7.74×10^2 CFU/mm^2, $p<0.001$) compared with those control biofilms not exposed to cell culture media (7.67×10^4 CFU/mm^2). *F. nucleatum* biofilms showed a significant reduction in viable bacterial numbers within cell culture media after 4 h (5.63×10^5 CFU/mm^2, $p<0.01$) and 24 h (2.99×10^4 CFU/mm^2, $p<0.01$) compared with bacteria not maintained in cell culture conditions (7.23×10^6 CFU/mm^2). For *A. actinomycetemcomitans* biofilms, a significant reduction in viable bacteria was observed after 4 h (3.17×10^4 CFU/m^2, $p<0.0001$) in cell culture media compared with the bacteria not maintained in cell culture conditions (1.75×10^5 CFU/mm^2), but not at 24 h. Finally, significantly more viable *S. mitis* were recovered after 24 h (3.08×10^7 CFU/mm^2, $p<0.01$) in cell culture media compared with the bacteria not maintained in cell culture conditions (7.14×10^5 CFU/mm^2). A marginal reduction in viable *S. mitis* was observed after 4 h (4.72×10^5 CFU/mm^2) in cell culture media compared with the bacteria not maintained in cell culture conditions (Figure 2.11).

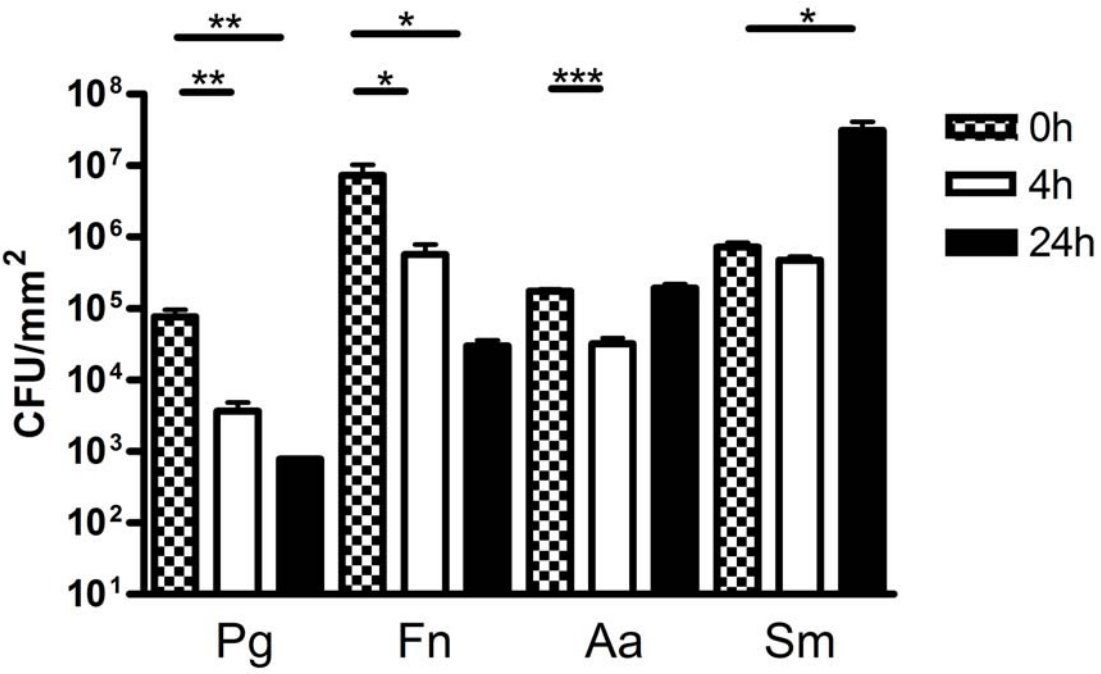


Figure 2.11: Survival of monospecies biofilms in cell culture conditions. The mono-species biofilms were grown in AS on HA discs. The biofilms were dip-washed three times in sterile PBS before adding into 24-well cell culture plates containing 1 mL of defined K-SFM and incubated at 37^{o}C in 5% CO_2 for 4 and 24 h. The biofilms were dip-washed three times in sterile PBS before sonicating in sterile PBS for 5 min in an ultrasonic bath. Total viable counts of the bacteria were obtained by performing Miles and Misra plate counting method on the bacterial agar plates. Data shown are viable bacteria recovered from the mono-species biofilms in CFU/mm^2. Three replicates were done for two independent experiments. Statistical analysis was performed by two-tailed unpaired t test (*p<0.01, **p<0.001, p<0.0001).

Viable *P. gingivalis* recovered from multi-species biofilms was shown to be significantly greater after 24 h (9.89 x 10^5 CFU/mm^2, p<0.01) in cell culture conditions compared with the biofilms not maintained in cell culture conditions (5.73 x 10^5 CFU/mm^2). *P. gingivalis* recovered from the multi-species biofilms not maintained in cell culture conditions and which were maintained in these conditions for 4 h (5.70 x 10^5 CFU/mm^2) were comparable. There was significantly less viable *F. nucleatum* recovered from multi-species biofilms after 4 h (7.39 x 10^5 CFU/mm^2, p<0.0001) and 24 h (1.72 x 10^6 CFU/mm^2, p<0.001) in cell culture conditions compared with those recovered from the biofilms not maintained in cell culture media (4.57 x 10^6 CFU/mm^2). Similarly, viable *A. actinomycetemcomitans* recovered from multi-species maintained in cell culture media for 4 h (7.03 x 10^5 CFU/mm^2, p<0.0001) and 24 h (7.79 x 10^5 CFU/mm^2, p<0.001) were significantly less compared with those recovered from the biofilms not maintained in cell culture (1.75 x 10^6 CFU/mm^2). *S. mitis* recovered from multi-species biofilms was significantly less after 4 h (8.61 x 10^4 CFU/mm^2, p<0.001) in cell culture media compared with those which were not maintained (6.13 x 10^5 CFU/mm^2). More viable *S. mitis* was recovered from multi-species biofilms maintained in cell culture media for 24 h (1.34 x 10^6 CFU/mm^2) compared with those that were not maintained (Figure 2.12).

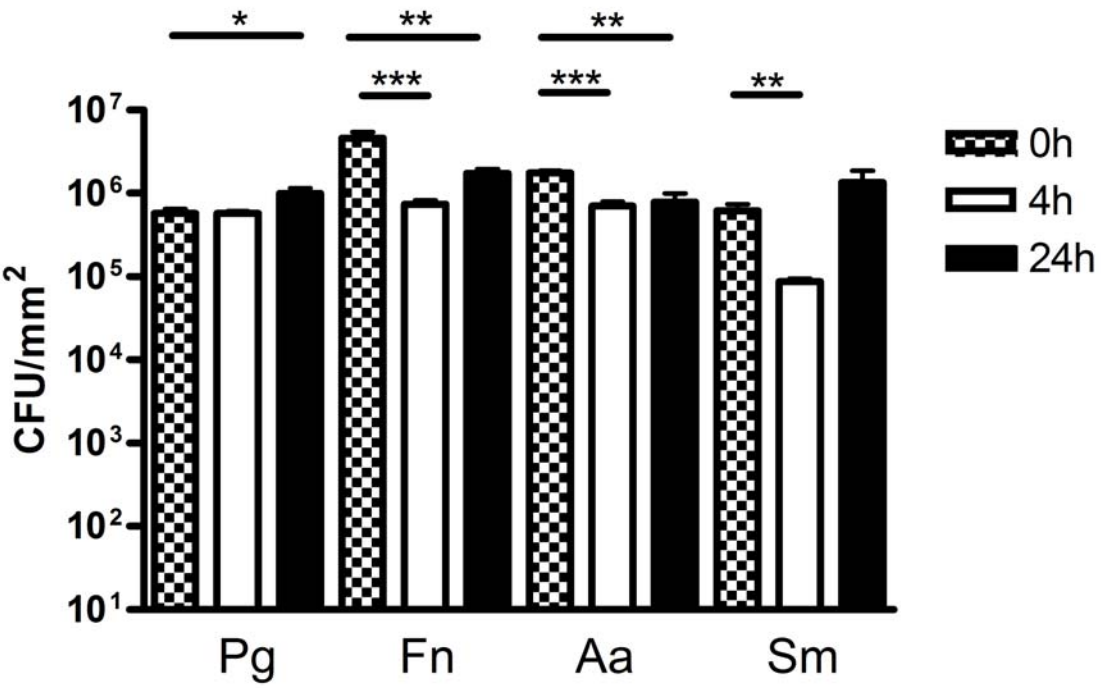


Figure 2.12: Survival of multispecies biofilms in cell culture conditions. The multi-species biofilms were grown in AS on HA. The biofilms were dip-washed three times in sterile PBS before adding into 24-well cell culture plates containing 1 mL of defined K-SFM and incubated at 37^{o}C in 5% CO_2 for 4 and 24 h. The biofilms were dip-washed three times in sterile PBS before sonicating in sterile PBS for 5 mins in an ultrasonic bath. Total viable counts of the bacteria were obtained by performing Miles and Misra plate counting method on species-specific bacterial agar plates. Data shown are viable bacteria recovered from the multi-species biofilms in CFU/mm^2. Three replicates were done for two independent experiments. Statistical analysis was performed by two-tailed unpaired t test (*p<0.01, **p<0.001, p<0.0001).

2.5 DISCUSSION

The data described herein reports a simple and reproducible *in vitro* model capable of comparing and contrasting the composition and structural arrangement of mono- and multi-species periodontal biofilms. This model has been shown to grow optimally on HA discs grown in AS over a defined time period of 2-4 days and 7 days, respectively. Biofilms of a multi-species nature were shown to be the most complex, which were densely packed with structural heterogeneity. Conversely, those mono-species biofilms of *P. gingivalis* and *A. actinomycetemcomitans* displayed sparse monolayers with occasional micro-colonies observed. The ability of these mono-species biofilms to survive in cell culture conditions was poor compared to the multi-species biofilm. Moreover, the multi-species biofilms were conducive to freezing. Some of the caveats of the development of this model will now be discussed.

Initially, in order to evaluate the bacterial composition of the biofilms, total viable cell counts were performed using specialised media, as described elsewhere (Hunt et al., 1986). For mono-species biofilms the resultant colony counts was minimally variable in AS under optimised conditions, ranging from ~10^5 to 10^6 CFU/mm^2 for all four species. However, this could not be verified through SEM analysis. For example, densely packed biofilms of *F. nucleatum* were in stark contrast to those of *P. gingivalis*. This difference could be explained by the dense nature of the *F. nucleatum* biofilms, which would be difficult to make into a homogenous suspension following sonication. Therefore, clumps of dislodged cells may have represented only one colony, therefore impacting and underestimating the cell counts, as has been reported elsewhere (Coco et al., 2008, Yadev et al., 2011). This may be an inherent problem with this methodology for biofilm enumeration (Childers et al., 2011). In fact, several other studies have relied on molecular techniques to quantify their biofilm numbers. qPCR was shown to be an effective method for enumerating biofilm composition in a three-species lens model system (Peyyala et al., 2011b). The data from this study showed substantial differences in cell numbers when mono-species and multi-species biofilm were compared, often 2 or 3 log differences, and which were consistently 1-2 log above what was reported herein. However, the numbers are similar to those reported from a 9-species for both *F. nucleatum* and *P. gingivalis*, of approximately 10^7 CFU/mL (Kinniment et al.,

1996a). Nevertheless, consideration of nuclease activity should be given, as this has been shown to alter qPCR sensitivity (Nadkarni et al., 2009).

The multi-species biofilms in this study were also quantitatively superior (1 log) to the mono-species biofilms. In this case the SEM analysis clearly showed structural differences. The biofilms showed evidence of 3-D architecture, spatial heterogeneity and an increased biomass. However, SEM may not have been the optimal method for this analysis due to the inherent problems with SEM and post-processing, i.e. dehydration of sample resulting in altered morphology and ultrastructure. Confocal laser scanning microscopy (CLSM) may have been useful in this context for both qualitative and quantitative analysis, but was not available at the time of this work. It was interesting that the cells within this biofilm multi-species environment were able to flourish compared to those grown as mono-species biofilms. Biofilms of this structural complexity have been reported by others, both *in vitro* and *in vivo* (Walker and Sedlacek, 2007, Zijnge et al., 2012). Various studies have consistently reported that once the cells within the biofilm are heterogeneous then the resultant biofilms form a robust biofilm with stable proportions of each organism present (Guggenheim et al., 2009, Periasamy and Kolenbrander, 2009b, Peyyala et al., 2011b). However, the use of colony counts is limited in these studies.

The use of molecular tools combined with fluorescence microscopy has become more frequently utilised by researchers as an alternative (Childers et al., 2011). Recently, Sanchez and coworkers (2011) reported the use of CLSM and RFLP to study biofilm development of a 6-species sub-gingival model on HA (Sanchez et al., 2011). Here it was used to show the developmental characteristics, structural composition and relative abundance of each bacterial species as the biofilms reached a steady state. Similarly, Guggenheim and coworkers (2009) demonstrated the structural composition of the nine species by using CLSM and transmission electron microscopy (TEM). Using fluorescence *in situ* hybridisation (FISH) it was shown that the biofilms after 64.5 h were approximately 40-60um, and TEM demonstrated a morphological abundance of short cocci and rods. Peyyala and colleagues (2011) also examined the three species biofilms under CSLM, where they found substantial differences in the quantities and distribution of the species in the multi-species biofilm. As observed from the studies

mentioned above multi-species biofilm development is a very complex and time-consuming process. This study has tried to create a simple multi-species periodontal biofilm, which could be used in industrial setting to screen active compounds in a quick and reproducible manner. Here an organism characterised for its pioneer status was used (*S. mitis*). Furthermore, *F. nucleatum* was used, which is one of the first Gram-negative species to become established in dental plaque and is considered as an intermediate colonizer, helping bridge attachment of non-pathogens (commensals) and true pathogens (Kolenbrander, 2000, Kolenbrander et al., 2002). These two pivotal organisms provided the structural stability of the biofilm in the early phases of growth, allowing the successful introduction of *A. actinomycetemcomitans* and *P. gingivalis*, which judging from SEM analysis were unable to form structurally secure biofilms. The sequential attachment of the periodontal bacteria on the tooth surface to form the periodontal plaque biofilm has been reported by various researchers (Socransky et al., 1998). In this study HA was used, representative of enamel. This has been shown to be an acceptable substrate for studies of this nature (Guggenheim et al., 2009). However, alternative substrates, such as contact lenses have been used (Peyyala et al., 2011b). Collectively each of the organisms selected in this study represent bacteria that play a critical function in periodontal biofilm formation, and which also represent organisms across the pathogenic spectrum. This was an essential requirement for downstream host-pathogen analysis.

Overall, the data from this study and those performed by other groups demonstrate that although biofilm models are diverse in terms of the substrate, nutrients and the specific oral bacteria used, the multi-species biofilms that are formed are largely similar, i.e. being spatially heterogeneous and abundant in different species. However, given that the end-point of this study was to investigate host-pathogen interactions, it was important to evaluate how the biofilm responded to an environment optimised for cell culture. Here it was shown that the mono-species biofilms, particularly *P. gingivalis* and *F. nucleatum* (obligate anaerobes), did not tolerate the 5% CO_2 conditions well compared to *S. mitis*. Interestingly, within the multi-species biofilm there was a minimal fluctuation of the cells over 24 h incubation. Given that the gingival crevice has differential microenvironments that enables different bacteria to

survive and flourish, it is likely that an element of mutualism enables the four species to maintain a homeostatic balance within the biofilm, as has been shown elsewhere (Periasamy and Kolenbrander, 2009b). Several analogous studies have recently reported biofilm models to investigate host-pathogen interactions (Belibasakis et al., 2011b, Belibasakis et al., 2011a). From these studies it is not apparent that any consideration has been given to how the multi-species biofilms from these studies respond to cell culture environments. Moreover, these biofilms are able to survive freezing, as shown from our investigations. The structure and composition is maintained when revived. This was critical in developing robust and reproducible biofilms for downstream testing in a host-pathogen co-culture system.

In summary, this initial component of the entire investigation has provided a quantitative and qualitative assessment of both mono- and multi-species biofilms that can be used to investigate how oral biofilms interact with the host epithelium.

CHAPTER 3:

ASSESSING IMPACT OF PERIODONTAL BIOFILMS ON EPITHELIAL CELLS

3.1 INTRODUCTION

The previous chapter presented a novel biofilm model for use in host-pathogen interaction studies. Various oral bacteria associated with periodontal disease have been shown to possess virulence factors that aid intracellular invasion, intracellular persistence and host cell apoptosis (programmed cell death), of which *P. gingivalis* is a paradigm (Curtis et al., 2005, Curtis et al., 2011, Curtis et al., 2001, Kinane et al., 2008). These are factors that have the potential to significantly affect mammalian cell viability and function.

Apoptosis is prevalent in the gingivae at sites of chronic bacteria-induced inflammation (Koulouri et al., 1999, Tonetti et al., 1998), particularly in the superficial cells of the junctional epithelium (Tonetti et al., 1998). The caspases (cysteine-dependent aspartate-specific proteases) are of central importance to apoptotic signalling network which are activated in most cases of apoptotic cell death (Bratton et al., 2000). So far, 14 different members of the caspase family have been identified which are divided into two subfamilies, initiator and executor caspases. The initiator caspases are activated by forming heteromeric complexes with accessory molecules. This is followed by the cleavage and subsequent activation of downstream caspases, such as caspases 3 and 6 (Nakata, 2000). Cleavage of a select group of substrates by downstream caspases (Stroh and Schulze-Osthoff, 1998) is responsible for the dismantling of essential cell components, which results in the morphological and biochemical changes that characterize apoptotic cell death.

A frequently activated death protease is caspase-3 which is also required for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis (Porter and Janicke, 1999). Caspase-3 is also required for apoptotic chromatin condensation and DNA fragmentation in all cell types (Woo et al., 1998, Janicke et al., 1998, Oberhammer et al., 1993). Thus caspase-3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, but it may also function before or at the stage when commitment to loss of cell viability is made (Porter and Janicke, 1999). The degradation of nuclear DNA into nucleosomal units is one of the best-characterized biochemical features of apoptotic cell death (Wyllie, 1980, Earnshaw, 1995). When cells are

induced to undergo apoptosis, caspases, in particular caspase-3, cleave ICAD to dissociate the CAD:ICAD complex, allowing CAD to cleave chromosomal DNA (Nakata, 2000). Oral bacteria, including *P. gingivalis*, have been shown to use this strategy to survive within the gingivae.

P. gingivalis has been shown to induce apoptosis in a time and dose dependent manner with significant apoptosis occurring between 12 and 24 h hours of challenge via a gingipain-dependent (arginine or lysine) mechanism (Stathopoulou et al., 2009). This was demonstrated was through caspase-3 activity. It was shown that live bacteria strongly up-regulate intrinsic apoptotic pathway, such as caspase-3, -8, -9, and that DNA fragmentation was present. It was also shown that *P. gingivalis* blocks camptothecin-mediated apoptosis of epithelial cells, up-regulates anti-apoptotic molecule expression Bcl-2 expression, and down-regulates pro-apoptotic molecule Bax expression (Nakhjiri et al., 2001). These studies collectively indicate that *P. gingivalis* has multiple mechanisms to interfere with apoptosis and cellular functions, unlike other bacteria of the oral cavity that are considered commensals, such as oral streptococci. However, its distant relatives, *S. pneumoniae*, have been shown to induce apoptosis (Marriott and Dockrell, 2006).

Modulation of cellular functions by periodontal bacteria has also been reported elsewhere. For example, apoptosis (characterized by Annexin-V staining) was shown to increase in the gingival epithelial cells (GEC) multi-layers following challenge with *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*. In contrast, *P. gingivalis* induced a transient increase in early apoptotic markers, which returned to control levels after 24 h (Dickinson et al., 2011). It was also shown that *P. gingivalis* was able to up-regulate the anti-apoptotic pathways in GECs in a time-dependent manner (Mao et al., 2007). Therefore, apoptotic events depend not only on the species present but also on the duration/frequency of interaction. Moreover, it was shown that *P. gingivalis* blocks apoptotic pathways in GECs through manipulation of the JAK/Stat pathway (Mao et al., 2007). This controls the intrinsic mitochondrial cell death pathways. As *P. gingivalis* is predominantly located intracellularly, this is thought to represent a strategy of the bacteria to prolong the life of its eukaryotic host cell (Mao et al., 2007). Clearly, *P. gingivalis* is adept at living

within differential microenvironments of the gingival crevice and resisting immune defence mechanisms.

3.2 AIM

In order to investigate the impact that a multi-species biofilm model has upon oral epithelial cells when compared to mono-species biofilms, the direct cellular effects must first be investigated. For this component of the study *P. gingivalis* and *S. mitis* were selected for use, each representing a pathogenic and commensal mono-species biofilm. Each of these was compared to a multi-species biofilm, as described in Chapter 2.

Therefore, the aim of these experiments was to investigate the responses of oral epithelial cells to bacterial biofilms with respect to **(i)** viability and **(ii)** apoptosis.

3.3 MATERIALS AND METHODS

3.3.1 Oral keratinocyte cell line growth and maintenance

OKF6-TERT2 cells (kind gift of the Rheinwald laboratory, Brigham and Woman's Hospital, Boston) are an immortalized human oral keratinocyte cell line. These cells have been immortalized by forced expression of telomerase, and resemble primary oral keratinocytes in studies of cytotoxicity or inducible cytokine and beta-defensin expression (Dongari-Bagtzoglou and Kashleva, 2003). All cell culture media and supplements were purchased from Invitrogen (Paisley, Scotland) unless otherwise indicated.

OKF6-TERT2 cells were cultured in keratinocyte serum-free medium (KSFM) [37010-022 Invitrogen, Paisley, UK] supplemented with 100 IU penicillin, and 100 µg/mL streptomycin, 25 µg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF) and 0.3 mM $CaCl_2$ (0.4 mM total Ca^{2+}). BPE and EGF were filter-sterilised (0.2 µm). Cells were seeded at 5 x 10^5 cells/mL in a 75 cm^2 cell culture flask (Corning, NY, USA). Cells were passaged at approximately 90% confluence, with 0.05% Trypsin EDTA. Following detachment of the cells the trypsin was neutralized with 15 mL of Dulbecco's modified Eagle's media (Sigma, Poole, UK) supplemented with 10% foetal calf serum (Sigma, Poole, UK). Cells were washed in Hanks balanced salt solution (Sigma, Poole, UK). The cells (20 µL) were mixed with Trypan blue (10 µL) (Sigma, Poole, UK) to obtain a dilution factor of 1.5. The cells were then transferred to a haemocytometer and viewed under the microscope to count the cells and study cell morphology. Dead cells were observed as blue because of the loss of cell wall integrity, which allows the Trypan blue to enter the cells. Live healthy cells were round and transparent, which were counted using the haemocytometer grids. The cells were re-seeded at around 5 x 10^4 cells/ mL (1 x 10^6 cells / flask). For assays, cells were cultured with defined-KSFM to remove batch variability found in BPE. In defined-KSFM, BPE is replaced with defined growth-promoting additives including insulin and fibroblast growth factor (proprietary solution). Also, BPE has only 4-week stability in medium (may deteriorate before this), compared to 3 months stability of defined-KSFM.

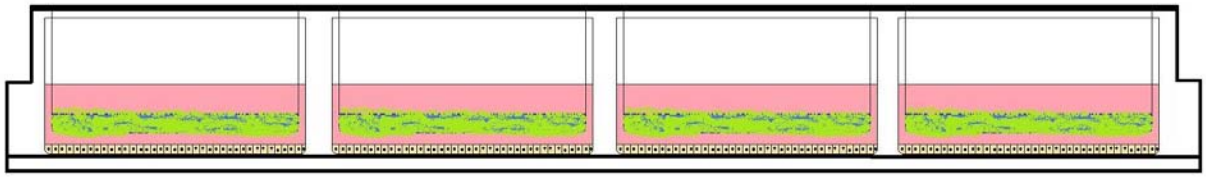
Frozen stocks of OKF6-TERT2 epithelial cells were also prepared. The epithelial cells (2 x 10^6 cells/mL) were resuspended in DMEM containing 20% foetal calf

serum. Equal volumes of the resuspended epithelial cells and 20% DMSO were transferred to a cryo tube to obtain a final volume of 1 mL. The vials were kept in an insulated box in the -80^{o}C freezer overnight, for slow cooling, and then transferred into liquid nitrogen for prolonged storage. The cells were revived from frozen by thawing the cells rapidly in a 37^{o}C water bath and transferring the cells into a cell culture flask containing warm K-SFM. The cells were passaged after it reached 90% confluence. The OKF6-TERT2 cells were harvested after the third or fourth passage for use in the experiments.

3.3.2 Development of an epithelial biofilm co-culture model

The OKF6-TERT2 cells were harvested, seeded at a density of 1 x 10^5 cells per well in 24 well cell culture plates (Corning, NY, USA) with Thermanox™ coverslips in a total volume of 1 mL of defined K-SFM. When the cells reached 80-90% confluence, the cells were washed once with defined K-SFM to remove the non-adherent cells. The epithelial cells were then challenged with previously frozen inverted biofilms (as described in chapter 2). These were adhered using Vaseline® to hanging cell culture inserts (Millipore, MA, USA) and inserted into 24 well plates with the epithelial cells at the bottom of the well, as illustrated in Figure 3.1. The biofilms on the HA discs were separated from the epithelial cells on the bottom of the well by 0.5 mm gap, which modelled the gingival crevicular fluid filled space. After 4 and 24 h, cell culture supernatants were harvested and stored at -80^{o}C for subsequent protein evaluation.

(A)



(B)

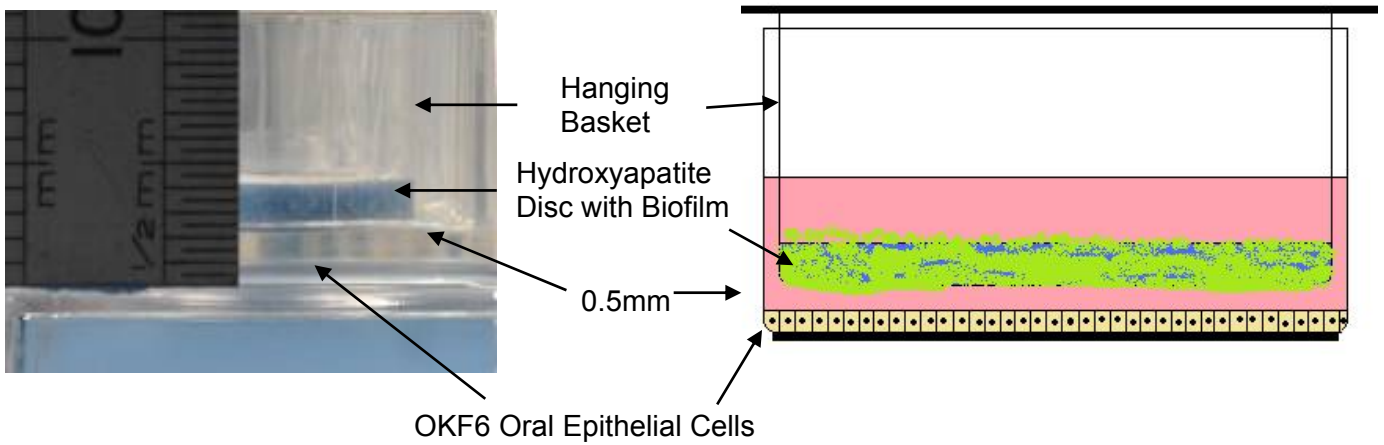


Figure 3.1: Epithelial biofilm co-culture model system. (A) Transverse view of hanging basket design. Hanging inserts (Millipore) with the HA discs were introduced into each well of a 24 well plate (Corning). **(B)** Close-up transverse view of the hanging insert, HA disc and 0.5 mm space between the HA disc and epithelial cells. An inverted HA disc was secured to the hanging insert using sterile Vaseline®.

3.3.3 Assessment of epithelial cell viability

The viability of epithelial cells challenged with bacterial biofilms for 4 and 24 h were analysed using AlamarBlue® dye (Invitrogen, CA, USA). The epithelial cells were washed with fresh K-SFM and replaced with fresh K-SFM containing 10% AlamarBlue® for 4 h. AlamarBlue® is a sensitive oxidation-reduction indicator that fluoresces and changes colour upon reduction by living cells. The reduction of Alamar blue is believed to be mediated by mitochondrial enzymes (O'Brien et al., 2000). Viable cells elicit a colour change from blue to pink. The percentage reduction of the absorbance values was calculated using the AlamarBlue® colorimetric calculator, as per manufacturer's instructions, which indicates the viability of the epithelial cells.

3.3.4 Histone-associated cell death detection apoptosis ELISA

The OKF6-TERT2 epithelial cells challenged with bacterial biofilms for 4 and 24 h were analyzed for cell death by determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) by using the Cell Death Detection ELISA^{PLUS} (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. The assay is based on a quantitative sandwich-enzyme-immunoassay-principle using monoclonal antibodies, directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates.

To obtain epithelial cells with induced apoptosis, the OKF6-TERT2 epithelial cells were incubated at 37^{o}C for 24 h with a titration of camptothecin (CAM, Sigma, Poole, UK) in serially declining concentrations from 4 µg/mL to 2 ng/mL. After incubation, the cells were lysed by adding 1 mL of Lysis buffer (provided with the kit) for 30 min at room temperature. The lysed cells were stored at -80^{o}C for subsequent use.

The biofilm challenged epithelial cells were also lysed as mentioned above and the cell lysates stored. Before use in the assay the cell lysates were centrifuged at 200 x *g* for 10 min. The lysates (20 µL) of the CAM and biofilm treated cells were transferred into the streptavidin coated microtitre plate with 80 µL of the Immunoreagent. The plate was incubated under gentle shaking (300 rpm) for 2 h at room temperature. After thorough rinsing using Incubation buffer, ABTS

solution was added to the wells and incubated by shaking until sufficient colour was developed. The stop solution was added to the ABTS solution. The developed colour was measured at 405 nm against ABTS solution + ABTS stop solution as a blank (reference wavelength 490 nm). The values from the double absorbance measurements of the samples were averaged and the background value subtracted from each of these averages. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values was calculated using the formula:

$$\text{Enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without CAM treatment)}}$$

$$\text{mU} = \text{absorbance } (10^{-3})$$

3.3.5 Statistical analysis

The statistical analyses on the difference in viable bacteria recovered from the biofilms were performed with GraphPad Prism (San Diego, CA, USA) using two-tailed unpaired student t test. This method gives an unpaired two sample student t test with a confidence interval for the difference between the means. The unpaired t method tests the null hypothesis that the population means related to two independent, random samples from an approximately normal distribution are equal (Altman, 1991; Armitage and Berry, 1991). The significance level was set at $p < 0.05$ in a two-sided test.

RESULTS

3.4.1 Metabolic changes in epithelial cells after challenge with bacterial biofilms. To investigate changes in cellular metabolism that may reflect changes in cell viability, OKF6-TERT2 epithelial cells were challenged with live or methanol-fixed (dead) mixed or single species biofilms (mixed: *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm and *S. mitis* biofilm for 4 and 24 h. The viability of the epithelial cells was assessed with fresh media containing 10% AlamarBlue® and the percentage reduction of the absorbance values was calculated using the AlamarBlue® colorimetric calculator which indicates the viability of the epithelial cells. Data shown are percent survival of the epithelial cells relative to OKF6-TERT2 cells cultured with cell culture medium (medium control). Statistical analysis was performed using an unpaired t-test.

Epithelial cells challenged with live mixed or single species biofilms maintained their viability for 4 h, However, a reduction in viability was observed after 24 h. Assuming 100% viability after culture in medium alone, epithelial cells challenged with live mixed species biofilm demonstrated 93% survival after 4 h, compared with 57% after 24 h ($p<0.001$) (Figure 3.2). Interestingly, the viability of the epithelial cells after 4 h of challenge with live *P. gingivalis* and *S. mitis* was maintained but the viability dropped to 19% ($p<0.0001$) and 60% ($p<0.001$) respectively after 24 h. The viability of the epithelial cells reduced to about 54% after 4 h when challenged with dead mixed species, *P. gingivalis* and *S. mitis* biofilm. The epithelial cell viability reduced further after 24 h challenge when challenged with dead mixed species biofilm (47%). However, the epithelial cell viability reduced to 87% ($p < 0.001$) and 71% after 24 h challenge with dead *P. gingivalis* and *S. mitis* biofilms, respectively. Overall, the epithelial cell viability reduced after 24 h challenge with live biofilms compared with 4 h. Live *P. gingivalis* biofilm caused more epithelial cell death than the other biofilms after 24 h.

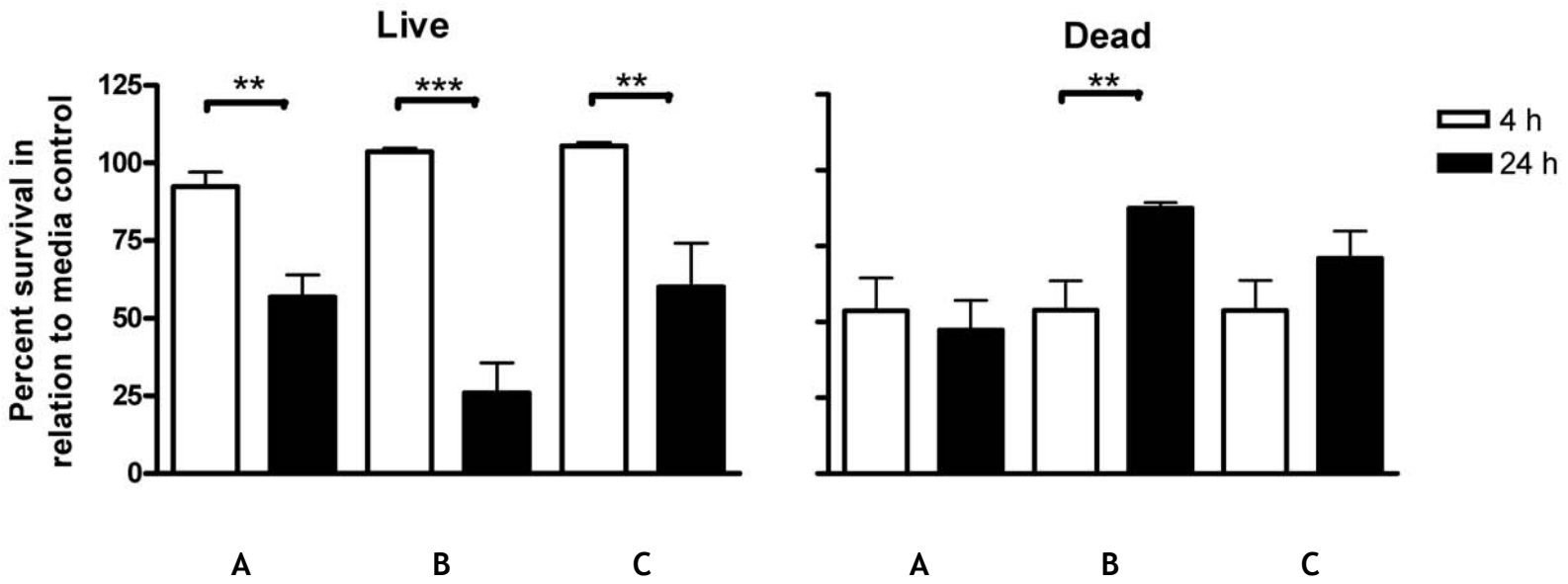


Figure 3.2: Survival of epithelial cells after challenge with live or dead bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** multi-species, **(B)** *P. gingivalis* and **(C)** *S. mitis* biofilm for 4 and 24 h. The media was removed from the epithelial and washed with fresh media before replacing with fresh media containing 10% AlamarBlue™. Cell viability was calculated as percentage of the difference between the reductions of Alamar Blue in treated cells versus controls. Results are presented as percentage of controls, means ± SEM of triplicate measurements of two independent experiments and analysed using an unpaired t-test (**p<0.001; ***p<0.0001).

3.4.2 Apoptosis of epithelial cells after bacterial biofilm challenge assessed by DNA fragmentation. The plaque bacterial biofilms may express numerous virulence factors which promote host cell apoptosis (Kinane et al., 2008). To characterize the biofilm-associated apoptosis of epithelial cells under various conditions, OKF6-TERT2 cells were challenged with live or methanol-fixed (dead) mixed- or mono-species biofilms (mixed: *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm and *S. mitis* biofilm for 4 and 24 h. The apoptosis of the epithelial cells were determined by the detection of DNA fragmentation by ELISA. Statistical analysis was performed using an unpaired t-test.

Unchallenged OKF6-TERT2 cells were used as negative controls and the positive control was OKF6-TERT2 cells challenged with 4 µg/mL camptothecin for 24 h. Data represents the enrichment factor (EF) of the specific mono- and oligo-nucleosomes in the cytoplasm of the epithelial cells.

Increased DNA fragmentation was observed in the epithelial cells after 24 h challenge with bacterial biofilms compared with 4 h (Figure 3.3). Time-dependent increase in DNA fragmentation was observed in epithelial cells challenged with live mixed species (4 h, 1.9 EF; 24 h, 3.6 EF), *P. gingivalis* (4 h, 1.6 EF; 24 h, 8.6 EF [$p<0.01$]) and *S. mitis* biofilm (4 h, 1.6 EF; 24 h, 6.8 EF). Dead *P. gingivalis* biofilm (11.2 EF) induced greater DNA fragmentation in the epithelial cells compared with live biofilms (8.6 EF) after 24 h challenge. DNA fragmentation was observed more in the epithelial cells stimulated with live or dead mixed-species and mono-species biofilms compared with the unstimulated medium control after 24 h. Significantly increased DNA fragmentation was observed in the epithelial cells after it was challenged with live and dead *P. gingivalis* (Live; $p<0.01$, Dead; $p<0.05$) and *S. mitis* biofilm (Live; $p<0.05$, Dead; $p<0.05$) compared with unstimulated media control. Overall, some degree of cell death of the epithelial cells due to DNA fragmentation was observed after challenge with bacterial biofilms.

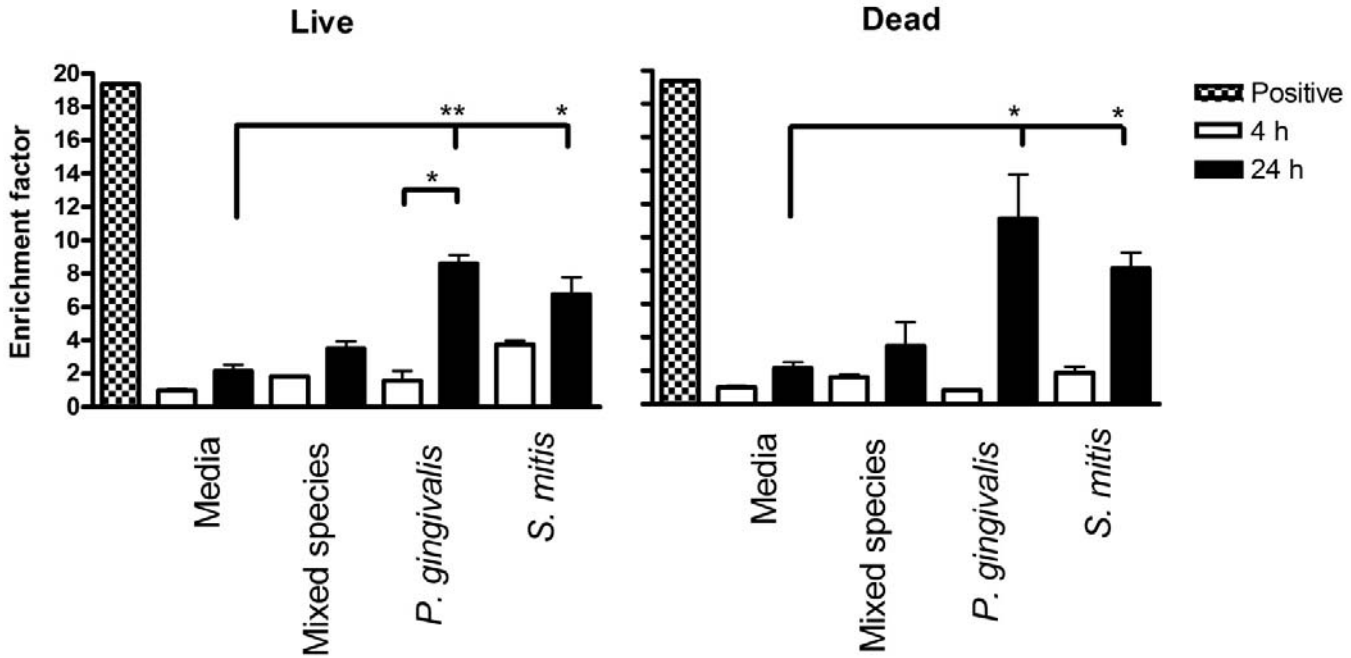


Figure 3.3: Histone-associated apoptosis of epithelial cells after challenge with bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with mixed species, *P. gingivalis* and *S. mitis* biofilm for 4 and 24h. Medium control was unchallenged OKF6-TERT2 cells. Positive control was OKF6-TERT2 cells challenged with camptothecin 4 µg/mL. Histone concentration in the epithelial cells was assessed by ELISA. Data represents the enrichment factor of mono- and oligo-nucleosomes released into the cytoplasm of the epithelial cells. Values are means ± SEM of two independent wells of two independent experiments that were analysed using an unpaired t-test (*$p<0.05$; **$p<0.001$).

3.5 DISCUSSION

A pathogenic (*P. gingivalis*) and commensal (*S. mitis*) mono-species biofilm were compared with a multi-species biofilm, both dead and live, with respect to epithelial cell viability and apoptosis. It was demonstrated that the OKF6-TERT2 epithelial cells challenged with both live multi-species biofilms or *P. gingivalis* and *S. mitis* mono-species biofilms for 24 h resulted in reduced cell viability when compared both media control and 4 h stimulated cells. Dead biofilms on the other hand were shown to variably reduce cell viability at both 4 and 24 h. When apoptosis was evaluated, both live and dead biofilms showed a progressive increase from 4 to 24 h, with *P. gingivalis* showing the greatest levels, followed by *S. mitis* and then multi-species biofilms. Collectively, these data show that biofilms have a negative impact on cellular function, which will now be discussed (Zhang et al., 2008).

In this study, all biofilms were shown to affect cellular viability, and have variable effects upon apoptosis. This is in agreement with other studies, which have shown *P. gingivalis* planktonic cells to negatively impact cellular viability of GECs (Johansson et al., 1996, Sandros et al., 1993). Johansson *et al* (1996) showed that *P. gingivalis* ATCC 33277 were cytotoxic to the target cell lines used after 24 h of incubation. Guggenheim and colleagues also demonstrated that their 9-species biofilm model affected apoptosis and cell morphology (Guggenheim et al., 2009), which is supported from observations within mono-species infection cell culture models (Stathopoulou et al., 2009). In contrast, however, other studies that have used multi-species periodontal biofilm models have failed to take this into account for their downstream analysis (Peyyala et al., 2012). This has direct implications for the interpretation of cellular effects, particularly cytokine analysis. The study discussed herein also tried to investigate the epithelial cell morphology after incubation with the bacterial biofilms, using Rapi-Diff II stain pack. Due to technical difficulties in the capture of the images, the data has not been presented here.

For cell viability assessment Alamar blue is often used as an assay to determine cytotoxicity of the epithelial cells exposed to different stimuli. For example, it has been used in studies of oral epithelial cells (TR146) and in a reconstituted human

epithelial (RHE) organotypic model to evaluate the effects of dentally relevant compounds (Moharamzadeh et al., 2008, Boyle et al., 2010). Alamar blue is a sensitive oxidation-reduction indicator that fluoresces and changes colour upon reduction by living cells. The reduction of Alamar blue is believed to be mediated by mitochondrial enzymes (O'Brien et al., 2000). There are several other commonly used cytotoxicity screening assay that are currently being used, including ATP measurement (Untch et al., 1994), MTT (Mosmann, 1983), neutral red (Babich and Borenfreund, 1991), XTT (Ramage et al., 2012), membrane integrity/LDH release (Korzeniewski and Callewaert, 1983), macromolecular synthesis (Grojean et al., 2000) and glutathione depletion (Baker et al., 1990). A study compared the Alamar blue assay to the most common *in vitro* toxicity assay, MTT, by evaluating the cytotoxicity of 117 drugs on a human hepatoma cell line HepG2 (Hamid et al., 2004). It was shown that MTT assay was less sensitive compared with the Alamar blue assay in detecting an effect for certain drugs, as defined by the improved Z-factor, a statistical parameter of assay quality.

In this study, it was shown that live and dead mixed species, *P. gingivalis* and *S. mitis* biofilms all induced cell death in epithelial cells after 24 h of stimulation, as evidenced by DNA fragmentation (Figure 3.3). These results agree partly with previous reports on epithelial cells (Stathopoulou et al., 2009). They showed that live *P. gingivalis* induced more apoptosis in epithelial cells after 24 h of challenge, while the dead *P. gingivalis* did not, using the same assay system. Two other studies contradicted these results, stating that *P. gingivalis* did not induce apoptosis in epithelial cells even after 24 h (Nakhjiri et al., 2001, Dickinson et al., 2011). It was shown that even though apoptosis in GEC multilayers was increased following challenge with *A. actinomycetemcomitans, F. nucleatum* or *S. gordonii,* only *P. gingivalis* induced a transient increase in early apoptotic markers, which returned to control levels after 24 h. Nakhjiri and co-workers (2001) also showed that *P. gingivalis* inhibited apoptosis in epithelial cells by up-regulation of the anti-apoptotic molecule Bcl-2. Both these studies used planktonic *P. gingivalis* instead of biofilms for the challenge of the epithelial cells. This may explain the differences in apoptosis observed in the study described herein, as the different cell phenotypes, i.e. biofilm and planktonic behave differently (Lo et al., 2009).

The reason why the dead biofilms induced more apoptosis in the epithelial cells compared with the live after 24 h is not clear. One hypothesis is that the there was still some residual methanol, which was used to fix the biofilms, retained within the biofilms which could have reacted with the epithelial cells. Another hypothesis is that the dying cells may be releasing some undefined secreted protein or molecule, which may induce apoptosis in the epithelial cells (Kravchenko et al., 2011). In this study, only DNA fragmentation was used to ascertain the apoptosis in the epithelial cells. Evaluation of apoptotic factors, such as Bax (Bcl-2 associated X protein), Caspase 3 and anti-apoptotic factors (Bcl-2) may provide a more thorough understanding of the overall role of apoptosis in these cellular interactions.

Apoptosis of host cells by *P. gingivalis* has been attributed to gingipains (Urnowey et al., 2006, Sheets et al., 2005, Sheets et al., 2006, Chen et al., 2001b, Kobayashi-Sakamoto et al., 2006). Gingipains are cysteine proteases produced by *P. gingivalis* that are either secreted or membrane bound and are arginine or lysine specific (Kadowaki et al., 2000). For future studies, gingipain deficient *P. gingivalis* mutants could be used, or wild-type *P. gingivalis* pre-treated with specific gingipain inhibitors. Another useful study would be to use filtered cell-free supernatants to stimulate the epithelial cells to evaluate if gingipains on their own can induce apoptosis.

From this study, it was concluded that bacterial biofilms have the ability to induce cytotoxicity and apoptosis in epithelial cells. This induction is dependent on the type of the biofilm (pathogen or commensal) and the form (live or dead) of the biofilm.

CHAPTER 4:

THE INFLAMMATORY RESPONSE OF ORAL EPITHELIUM TO PERIODONTAL BIOFILMS

4.1 INTRODUCTION

Periodontal disease is induced by bacterial biofilms that accumulate at the gingival margin. A series of aberrant inflammatory responses are initiated in periodontal tissues by a small subset of gram-negative periodontal bacteria, including *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* (Feng and Weinberg, 2006). These microbes possess numerous potent virulence factors aimed at neutralizing local host defences and destroying periodontal tissues, as evidenced from studies in the previous chapter. However, periodontal diseases progresses in only a limited number of individuals harbouring the pathogens, suggesting a complex multifactorial aetiology relating to an imbalance between host and pathogen (Kinane et al., 2007). Therefore, the interactions between the microbes and the immune system have been a major topic of investigation with regard to aetiology of periodontal disease.

There are numerous microbiological factors affecting the host-oral bacteria relationship. For the purposes of these investigations, these have been categorized as:

1. Bacterial species and whether they are pathogens or commensals?
2. Whether the bacteria are present in planktonic or biofilm state?
3. Whether the bacteria are dead or alive?

Oral bacteria can be broadly classified as commensals or pathogens (Feng and Weinberg, 2006). Previous studies have concluded that different dental plaque bacteria, both in planktonic and biofilm states, induce different host response profiles depending on the their pathogenic or commensal status (Hasegawa et al., 2007, Krisanaprakornkit et al., 2000, Sliepen et al., 2009, Stathopoulou et al., 2010, Zhang et al., 2008). For example, it has been shown that *F. nucleatum* preferentially induced IL-6 and IL-8 production from epithelial cells compared to *S. gordonii* (Hasegawa et al., 2007). This was also shown in a study of primary human gingival epithelial cells (Stathopoulou et al., 2010). Moreover, in this same study cellular challenge with *P. gingivalis* was shown to produce high levels of IL-1ß, and challenge with *A. actinomycetemcomitans* induced high levels of IL-8.

Host-pathogen interaction studies previously used putative periodontal pathogens in planktonic state to stimulate human primary cells or cell lines (Mans et al., 2006). However, the periodontal bacteria in dental plaque exist as a complex microbial biofilm (Kolenbrander, 2000, Rosan and Lamont, 2000); therefore *in vitro* planktonic bacteria are far disconnected from the *in vivo* situation. A number of investigators have more recently used mono-species and multi-species periodontal bacterial biofilms to study host-pathogen interactions and demonstrated differences in the host response to biofilms and planktonic bacteria (Oscarsson et al., 2008, Daep et al., 2008, Ramsey and Whiteley, 2009, Guggenheim et al., 2009, Peyyala et al., 2011b). The periodontal pathogen *A. actinomycetemcomitans* strain D7S, in both planktonic and in biofilm form were shown to release free-soluble surface material independent of outer membrane vesicles. This material enhanced production of pro-inflammatory cytokines (IL-1ß, TNFα, IL-6, IL-8, MIP-1 ß), as evidenced by using a cytokine antibody array. There was enhanced pro-inflammatory cytokine production with the biofilms as compared with the planktonic form (Oscarsson et al., 2008).

Until recently, heat-killed bacteria were the method of choice to study host-pathogen interactions *in vitro*. Bacteria considered putative periodontal pathogens elicit a strong inflammatory response. When these bacteria are killed cytokines that are released early (IL-1ß) and later (IL-6 and IL-8) are reversed; i.e., IL-1ß levels were reduced and IL-6 and IL-8 levels enhanced (Eskan et al., 2008, Hasegawa et al., 2007, Stathopoulou et al., 2010, Zambon, 1985). The differential regulation of the host response to live and dead bacteria should be considered when conducting host-pathogen relationship studies *in vitro*.

Microbial plaque is considered necessary, but not sufficient, for the progression of periodontal disease. Thus, the host response plays a pivotal role in disease pathogenesis. Many biological events in periodontal disease are strictly regulated by cell-cell interactions, which may be categorized into two forms: cognate (adhesive) interaction, achieved by mutual recognition between membrane bound cell-surface molecules, and cytokine-mediated interactions (Okada and Murakami, 1998). Adhesion molecules include ICAM-1 (Intercellular Adhesion Molecule-1, CD54) and

ITGB2 (Integrin Beta 2, CD18), which stabilize cell-cell interactions and facilitate leukocyte migration across the endothelial barrier (Yang et al., 2005, Kotovuori et al., 1999). Cytokines are a large and diverse family of soluble mediators including interleukins, colony stimulating factors, growth factors, and cytotoxic factors. Cytokines play an important role in numerous biological activities including proliferation, development, differentiation, homeostasis, regeneration, repair and inflammation (Okada and Murakami, 1998). Epithelial cells are not only physical barriers but also a source of potent cytokines and other soluble mediators including antimicrobial peptides. Antimicrobial peptides, such as human beta-defensins and S100A8 are produced by epithelial cells and induced by microbial products, inflammatory cytokines and epidermal growth factor (McCormick and Weinberg, Hiroshima et al.).

Offenbacher and colleagues (2009) performed an experimental gingivitis study in humans to understand changes in patterns of whole-transcriptome gene expression that occur during the induction and resolution of experimental gingivitis in humans using bioinformatics tools (Offenbacher et al., 2009). Gingival biopsy samples were collected from 14 subjects during a 28-day stent-induced experimental gingivitis model, followed by treatment, and resolution at days 28 through 35 were analysed using gene-expression arrays. Biopsy samples were collected at different sites within each subject at baseline (day 0), at the peak of gingivitis (day 28), and at resolution (day 35) and processed using whole-transcriptome gene-expression arrays (Offenbacher et al., 2009). Interestingly, a high proportion of the genes demonstrating greatest differences between health and disease were genes encoding chemokines, cytokines, adhesion molecules, antimicrobial peptides, and a number of these have been selected for an *in vitro* model of experimental gingivitis.

4.2 AIMS

The aim of this study was to investigate the response of oral epithelial cells to bacterial biofilms with respect to **(i)** biofilms versus planktonic microbes, **(ii)** live biofilms versus dead biofilms and, **(iii)** pathogenic biofilms versus commensal biofilms. The response of the epithelium was investigated by assessing cytokine and chemokine mRNA and protein expression at various times following exposure to biofilms.

4.3 MATERIALS AND METHODS

4.3.1 Gene expression studies

Analysis of inflammatory mediators was initially performed using gene expression analysis using two different methods. A list of genes of interest are shown in Table 4.1

4.3.1.1 RNA extraction and purification

Biofilms were formed as described in chapter 2 and epithelial cells challenged as described in chapter 3. RNA extraction and purification from these epithelial cells was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Buffer RLT (350 µL) was added to the monolayer epithelial cells in the 24 well cell culture plates (Corning, NY, USA) to dislodge and lyse the cell, mixed and transferred to RNA free microcentrifuge tubes. An equal volume of 70% ethanol was added to the lysed cells, mixed and transferred to the RNeasy spin column and centrifuged at 13,000 rpm for 15 sec. The RNA on the spin column membrane was washed with buffer RW1 by centrifugation at 13,000 rpm for 15 sec. DNase 1 solution (Qiagen, Hilden, Germany) was added directly to the membrane and incubated at room temperature for 10 min. The membrane was then washed with buffer RW1 by centrifugation at 13,000 rpm for 15 sec. The RNA on the membrane was precipitated by adding buffer RPE and centrifuged at 13,000 rpm for 15 sec and then again for 2 min. The RNA was then eluted into RNase free water and stored in -80^{o}C freezer.

Table 4.1: List of genes assessed in the study

Gene	Name	Class	Functions
1. IL-1 α	Interleukin-1 alpha	Cytokine	Initiates cytokine cascade & vascular adhesion molecule expression
2. IL-1 ß	Interleukin-1 beta	Cytokine	Initiates cytokine cascade & vascular adhesion molecule expression
3. IL-6	Interleukin-6	Cytokine	Pro-inflammatory pleiotrophic cytokine
4. TNF α	TNF-alpha	Cytokine	Regulation of immune cells
5. CSF2	GM-CSF	Cytokine	Stimulates stem cells to produce granulocytes & monocytes
6. CSF3	G-CSF	Cytokine	Initiates differentiation, proliferation and survival of PMNs & macrophages
7. IL-8	Interleukin-8	Chemokine	PMN chemoattractant
8. CXCL1	Gro alpha	Chemokine	PMN chemoattractant
9. CXCL3	Gro gamma	Chemokine	Suppresses hematopoietic progenitor cell proliferation
10. CXCL5	ENA78	Chemokine	Chemotaxis of neutrophils promoting angiogenesis
11. CCL5	RANTES	Chemokine	Promotion of inflammatory infiltrate & T-cell chemotaxis
12. CCL4	MIP-1 beta	Chemokine	Attracts $CD4^+$ T lymphocytes
13. CX3CR1	Chemokine receptor 1	Chemokine	Mediates leukocyte migration and adhesion
14. CCL3L3	Chemokine ligand 3 like 3	Chemokine	Immune response and chemotaxis
15. CXCL10	IP-10	Chemokine	Chemotactic effect on activated T, NK and TH-1 cells
16. CXCL11	I-TAC	Chemokine	Interferon inducible T-cell alpha chemoattractant
17. ICAM1	Intercellular adhesion molecule-1	Adhesion molecule	Stabilizing cell-cell interactions
18. ITGB2	Beta integrin 2	Adhesion molecule	Facilitate leukocyte migration across endothelial barrier
19. DEFB4	Beta-defensin 2	Antimicrobial peptide	Antimicrobial activity against Gram negative & monocyte chemoattractant
20. S100A8	S100 calcium binding protein A8	Antimicrobial peptide	Phagocytic granule protein
21. PROK2	Prokineticin 2	Angiogenic	Regulates angiogenesis
22. NFκB	Nuclear factor kappa-light-chain enhancer of activated B cells	Pathway	Regulates immune response to infection
23. TBP	TATA binding protein	Endogenous	Transcription factor that specifically bind to DNA sequence called TATA box
24. GAPDH	Glyceraldehyde 3-phosphate dehyrogenase	Endogenous	Catalyses the conversion of glyceraldehydes 3-phosphate

4.3.1.2 RNA quantification and quality check

A NanoDrop 1000 spectrophotometer (Thermo Scientific, DE, USA) was used to assess RNA concentration and quality. The pedestal was wiped clean and 1.5 µL of RNA samples in solution added to the pedestal to quantify the RNA. An A_{260} reading of 1.0 is equivalent to 40 ng/µL of RNA and the OD at 260 nm is used to determine the RNA concentration in a solution. The quality of the RNA was assessed by the ratio of the absorbance at 260 nm and 280 nm. Pure RNA has an A_{260}/A_{280} of 2.1 and a value of 1.8 and 2.1 indicates that the RNA is pure.

4.3.1.3 Reverse transcription

The purified RNA was reverse transcribed into complementary DNA (cDNA) using high capacity RNA to cDNA kits (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. To reverse transcribe the RNA, 500 ng of RNA was added to each reverse transcription reaction. A no-RT control was also included for each RNA sample, where reverse transcriptase enzyme was not included in the reaction. This was to ascertain that there was no genomic DNA in any of the cDNA samples.

4.3.1.4 Real time PCR analysis of cDNA samples using SYBR® Green

SYBR® Green and the primers were purchased from Invitrogen (Paisley, Scotland) unless otherwise indicated. For each condition, 1 µL of cDNA sample was PCR-amplified using 200 nM of the IL-8 and GAPDH primers (0.5 µL forward and reverse primers at 10 µM), as illustrated in Table 4.2. Each 25 µL real-time PCR reaction contained the following: 1 µL of 500 ng/µL cDNA, 0.5 µL forward and reverse primer (10 nM), 12.5 µL SYBR® Green and 10.5 µL of molecular biology grade dH_2O. Reactions were performed in triplicate in 0.2 mL optical tube strips (Agilent Technologies, South Queensferry, West Lothian, UK) using the Mx3000P QPCR machine (Stratgene, Amsterdam, Netherlands). A standard dissociation curve protocol was included after the 40 amplification cycles to confirm that only one product was made. The threshold cycle (C_t) was automatically determined and verified manually. Amplification plots were observed at the logarithmic scale and threshold selected to be above any baseline fluorescence, in the region where

amplification was exponential and where the majority of replicates gave similar C_t values, *i.e.* where triplicate amplification plots were parallel. IL-8 was normalised using GAPDH as a housekeeping gene. No-RT controls were included to check for DNA contamination and no template controls, with water, were run to rule out other contamination problems. The no-RT and no-template controls should ideally show no amplification and therefore no C_t values.

Table 4.2: PCR primer sequences of IL-8 and GAPDH

Target	Primer sequence	Binding site	Product size (Base pairs)
IL-8 F	CAGAGACAGCAGAGCACACAA	21-41	170
IL-8 R	TTAGCACTCCTTGGCAAAAC	171-190	
GAPDH F	CAAGGCTGAGAACGGGAAG	282-300	133
GAPDH R	GGTGGTGAAGACGCCAGT	397-414	

4.3.1.5 Real time PCR analysis of cDNA samples using Taqman® Low Density Array (TLDA)

For gene expression analysis Custom-designed ABI microfluidic Taqman® Low Density Array ([TLDA] Applied Biosystems, Foster City, CA, USA) was used in a reverse transcriptase polymerase chain reaction (RT-PCR) process using the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). This allowed 384 real time PCR reactions to be carried out simultaneously in order to determine the transcription of several genes. The TLDA used in this study was designed to contain gene expression assays to measure mRNA levels of NFκB, PROK2, CSF3, ITGB2, IL-8, IL-1α, CCL5, IL-1β, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, GM-CSF, GROα, IL-6, ICAM1, DEFB4 and CXCL5 (Offenbacher *et al*, 2009). Two control genes, TBP and GAPDH were utilized to span the relative abundance/Ct range of the genes on the card. A total of 100 uL reaction mixture with 20 µL cDNA template, 30 µl of RNAse free water and 50 µL of TaqMan universal master mix (Applied Biosystems, Foster City, CA, USA) was added to each port of TLDA after gentle pipetting to mix. Thermal cycler conditions were as follows: 10 min at 94.5°C and 30 s at 97°C and 1 min at 59.7°C for 40 cycles and thermal cycling and fluorescence detection was performed on Applied Bio-Systems

ABI Prism 7900HT Sequence Detection System with ABI Prism 7900HT SDS Software 2.1.

4.3.1.6 Gene expression analysis

Gene expression was quantified using the comparative threshold (C_t) method as suggested by the manufacturer. This technique utilises the formula $2^{-\Delta\Delta Ct}$ in order to calculate the relative number of gene transcripts (Livak and Schmittgen, 2001). The C_t value represents the PCR cycle at which the amplified gene target reaches a defined threshold. 40 PCR cycles were performed; accordingly the C_t values can range from 0 to 40. C_t values for each immunological molecule were calculated. C_t values were calculated for the bacterial stimulated samples and the unstimulated medium controls. The C_t of the endogenous control genes was also calculated. GAPDH was calculated to be the most reliable endogenous control. The ΔC_t value was calculated [$\Delta C_t = C_t$ (target gene) − C_t (GAPDH)]. This was performed for each gene in the bacterial stimulated samples and the unstimulated medium control samples. The relative expression of each gene was then calculated with the formula $\Delta\Delta C_T = \Delta C_t$ (positive sample) − ΔC_t (control sample). This was the expressed in terms of fold change relative to the control sample with the formula [fold change = $2^{-\Delta\Delta CT}$] (Livak and Schmittgen, 2001). For down regulated genes (value less than 1) the negative inverse of the value was taken. This allowed down regulation to be expressed as a negative value.

4.3.2 Protein expression studies

4.3.2.1 Protein analysis by human cytokine protein array

A human cytokine protein array kit, Proteome Profiler panel A (ARY005, R&D Systems, Abingdon, UK), was used to measure cytokine production from OKF6-TERT epithelial cells in response to medium control, mixed biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm and *S. mitis* biofilm for 24 h. The kit contains nitrocellulose membranes pre-blotted with duplicates of 36 capture antibodies to human cytokine proteins (Figure 4.1). Figure 4.1A illustrates the position of each protein on the nitrocellulose membrane, and Figure 4.1B illustrates an example of the array following processing. Cell supernatants can be mixed with a cocktail of biotinylated detection antibodies and

incubated with the membrane. Any cytokine/detection antibody complex will bind to the membrane via the capture antibody. Proteins are detected using streptavidin-horseradish peroxidase and chemiluminescent detection reagents. The level of light produced at each spot is proportional to the amount of bound cytokine. The densitometry analysis was performed on X-ray film using the Quantity One® 1-D Analysis Software (Gel Doc™, Bio-Rad, Hertfordshire, UK) by calculating the adjusted volume of the spots from the pixel density after subtracting from the background.

(A)

+ve	C5a	CD40	G-CSF	GM-CSF	GROα	I-309	sICAM-1	IFNγ	+ve
	IL-1α	IL-1β	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
	IL-10	IL-12p70	IL-13	IL-15	IL-17	IL-17E	IL-23	IL-27	
	I-32α	IP-10	I-TAC	MCP-1	MIF	MIP-1α	MIP-1β	Serpin E1	
+ve	RANTES	SDF-1	TNF-α	sTREM-1	-ve				

(B)

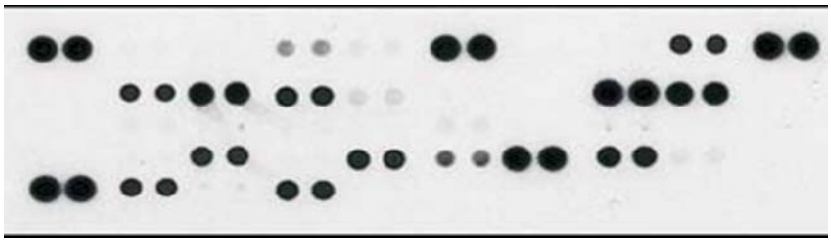


Figure 4.1: Human cytokine protein array. (A) Schematic of the membrane layout. **(B)** Example of a processed membrane.

4.3.2.2 Protein analysis by Luminex® multiplex beads

Supernatants harvested from OKF6 epithelial cells at 4 and 24h after bacterial biofilms and planktonic cells challenge were tested for the presence of IL-1ß, TNF-α, IL-6, IL-8, GM-CSF and G-CSF using Luminex® multiplex beads (Invitrogen, UK) according to the manufacturer's instructions. Briefly, 25 µL of 1 x beads (2.5×10^6 beads/mL/cytokine) with defined spectral properties were covalently conjugated to analyte specific monoclonal antibodies and then pipetted into each well of a 96 well filter bottom microplate. The conjugated beads were exposed to 50 µl of cell culture supernatant sample or standard solution containing a known concentration of each cytokine [IL-1 ß (7330 pg/mL), TNF-α (7410 pg/mL), IL-6 (5350 pg/mL), IL-8 (10010 pg/mL), GM-CSF (9820 pg/mL), G-CSF (28670 pg/mL)] and incubated at room temperature on an orbital shaker (500-600 rpm) for two hours in the dark. After three washes and filtration by vacuum manifold to remove unbound protein,

biotinylated detection antibodies specific for a different epitope on the analyte was added to the reaction and incubated for 1 hour at room temperature on an orbital shaker (500 – 600 rpm). Following this incubation step, two sets of washes and vacuum filtration were performed to remove unbound antibody. To the reaction mixture, 100 µL of Streptavidin – R Phycoerythrin (Streptavidin-RPE) was added, which binds with high affinity to the biotinylated antibodies. A further three sets of washes and vacuum filtrations were then performed to remove any unbound material. The beads were then resuspended in 100 µl of working wash solution. The reaction mixtures were analysed using Luminex® 100 hardware (Luminex, USA) and the concentrations of the analytes were determined using the software compared with a standard curve for each assessment. The instrument monitors the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. The standard curves were obtained using a five parameter algorithm. Samples from two separate experiments were tested.

4.3.2.3 Protein analysis by Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants harvested from OKF6 epithelial cells at 4 and 24h, after challenge with bacterial biofilms and planktonic cells, were analyzed for the presence of CXCL1 (Gro-α), CXCL10 (IP-10) and CCL5 (RANTES) using ELISA (Peprotech, London, UK), according to the manufacturer's instructions. Fresh solution of antigen-specific antibody (capture antibody) was prepared in PBS (1.0 µg/mL) and 100 µL aliquots added to each well of Nunc Maxisorp® Flat Bottom Microtiter® Plates (Fisher, Loughborough, UK). The plates were sealed and incubated at room temperature overnight. After washing, blocking agent (1% bovine serum albumin [BSA]) was added to the wells and incubated for at room temperature to block non-specific proteins. The plates were washed again and standards, samples and controls added to the wells. The plates were incubated at room temperature. After washing, biotinylated antigen-specific antibody (detection antibody) was added to each well and incubated at room temperature. After washing and drying, avidin-peroxidase conjugate was added to each well and incubated at room temperature for 30 minutes. After washing and drying, 3,3',5,5'-tetra-methylbenzidine (TMB, R&D systems, Abingdon, UK) was added to each well and the developing optical density read at 5 minute intervals using a plate reader (FLUOstar Omega. BMG Labtech, VA,

USA) set at 405 nm with a 650nm wavelength correction. A standard curve was generated using a four-parameter algorithm to calculate the concentration of the cytokines in the samples. The standard curve was obtained by plotting the mean absorbance for each standard (x-axis) against the target protein concentration (y-axis) using Omega Analysis Software. The R^2 for the standard curve, which predicts the outcome of one value from the other, must be greater than 0.98.

4.3.3 Statistical analysis

The statistical analyses on the difference in gene expression and the protein release from the epithelial cells after the different treatments were performed with GraphPad Prism using one-way analysis of variance (ANOVA) with Bonferroni correction. One-way ANOVA is a method used to compare means of two or more samples with numerical data. The ANOVA tests the null hypothesis that samples in two or more groups are drawn from populations with the same mean values (Howell, 2002). Bonferroni correction is a method used to counteract the problem of multiple comparisons. The problem of multiplicity occurs when there is an increase in the number of hypotheses in a test, there is also a likelihood of witnessing a rare event, and a chance to reject the null hypotheses when it's true. Bonferroni correction is the most naive way to address this issue (Dunnett, 1955). Differences were considered significant if $p<0.5$.

4.4 RESULTS

4.4.1 IL-8 mRNA expression in epithelial cells stimulated with *P. gingivalis* biofilms or planktonic cells. IL-8 release is a key function of epithelial cells, promoting neutrophil recruitment and activation (Tonetti et al., 1994). Therefore, this chemokine was investigated initially as a likely candidate for demonstrating epithelial cell regulation. To investigate the variability of IL-8 production of epithelial cells co-cultured with bacterial biofilms or planktonic cells, OKF6-TERT2 epithelial cells were challenged with both live or dead (heat-killed) *P. gingivalis* biofilms and planktonic cells for 4 and 24 h. IL-8 mRNA expression was assessed by SYBR® Green real time reverse-transcriptase PCR. Gene expression was normalised to GAPDH endogenous control. Data presented as fold change in gene expression relative to OKF6-TERT2 cells cultured only with cell culture medium ('medium control'). Statistical analyses were performed by one-way ANOVA with Bonferoni correction using GraphPad Prism (San Diego, CA, USA). Differences were considered significant if $p<0.5$.

Live *P. gingivalis* biofilm (4 h: ~61-fold vs medium control; 24 h: ~32-fold vs medium control) induced greater levels of IL-8 expression compared with live planktonic cells (4 h: ~2-fold vs medium control; 24 h: ~13-fold vs medium control). Dead biofilms and planktonic cells induced minimal changes in the IL-8 expression (maximal ~10-fold increase vs medium control) (Figure 4.2). To conclude, *P. gingivalis* biofilms induce more IL-8 expression than the planktonic cells.

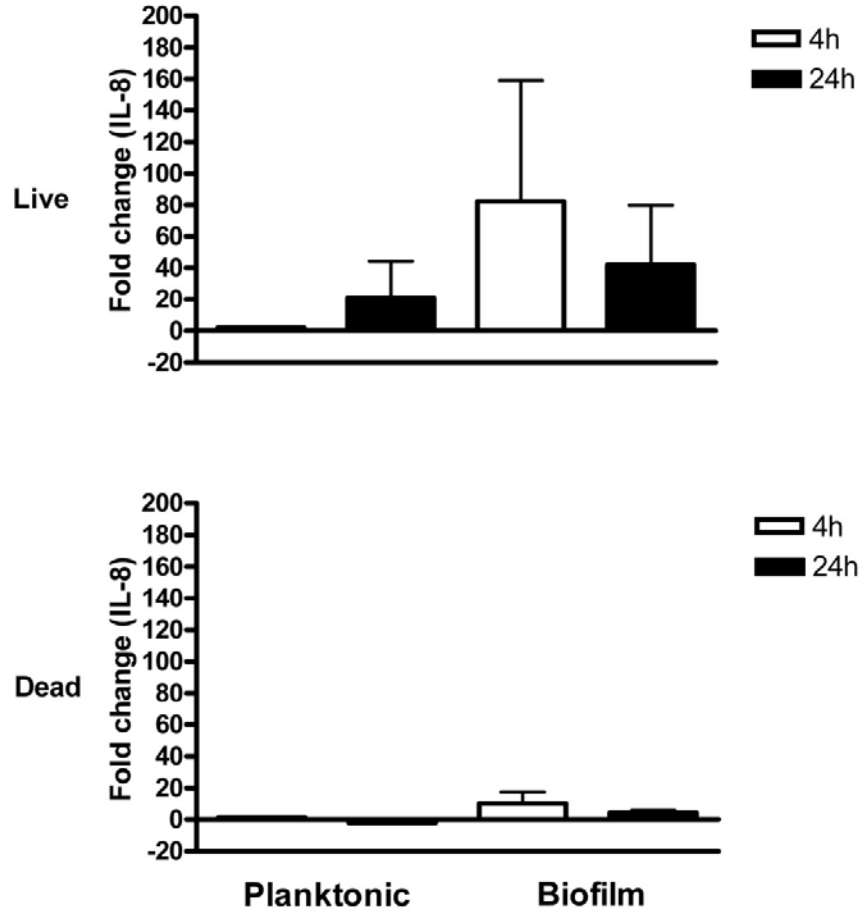


Figure 4.2: IL-8 mRNA expression in OKF6-TERT2 epithelial cells challenged with live or dead (heat-killed) ***P. gingivalis*** **biofilms or planktonic cells**. OKF6-TERT2 epithelial cells were challenged with *P. gingivalis* biofilm and planktonic cells for 4 and 24 h. mRNA expression was assessed by SYBR® Green real time PCR for IL-8. Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to medium control.

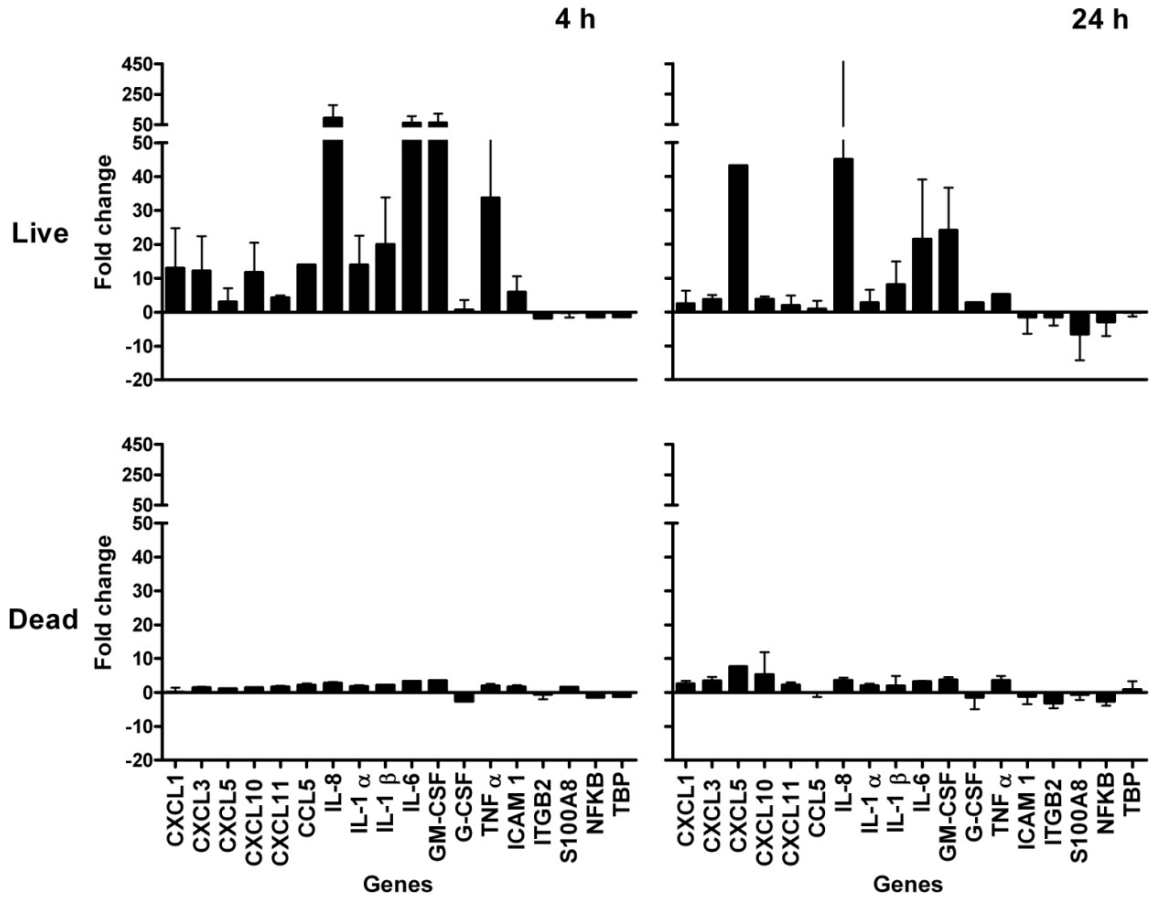
4.4.2 Expression of a panel of gingivitis-related genes in epithelial cells stimulated with *P. gingivalis* biofilms or planktonic cells. Marked changes in IL-8 expression were observed following stimulation with the bacterial biofilms. To investigate the effect of *P. gingivalis* biofilms or planktonic cells on a broader spectrum of the epithelial cell inflammatory armamentarium, expression of a panel of gingivitis related genes was next investigated (gene functions described in Table 4.1. The studies in the preceding section (4.4.1) were extended to include analysis of a larger panel of genes (NFκB, PROK2, CSF3, ITGB2, IL-1α, CCL5, IL-1β, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, CSF2, GROα, IL-6, ICAM1, DEFB4, CXCL5 and TBP). Gene expression was assessed by TaqMan® low-density array (TLDA). Gene expression was normalised to GAPDH endogenous control and then data expressed as fold change relative to OKF6-TERT2 cells cultured only with cell culture medium. Bacterial biofilm co-culture with epithelial cells resulted in little change in gene expression of CXC3R1, CCL3L1, CCL4, ICAM1, ITGB2, S100A8, DEFB4, NFκB, and PROK2 (Figure 4.3 A & B).

Minimal changes in chemokine gene expression were observed with both dead *P. gingivalis* biofilm and planktonic cells after 4 and 24 h, compared with medium control. Some chemokine gene expression was observed with live *P. gingivalis* planktonic cells after 24 h only. Similar to the observed effects on IL-8, a greater increase of CXCL1, CXCL3, CXCL5, CXCL10, CXCL11, CCL5 and IL-8 mRNA expression was observed following co-culture with live *P. gingivalis* biofilm compared with planktonic cells after 4 and 24 h (Figure 4.3 A & B). CXCL1 expression was greater following stimulation with live biofilms (~13-fold vs medium control) compared with planktonic cells (~1-fold vs medium control) after 4 h. Overall, epithelial cell chemokine gene expression was greater following co-culture with the biofilms compared with the planktonic cells.

Similar to the chemokine expression, minimal changes were observed with live and dead *P. gingivalis* planktonic cells, and dead *P. gingivalis* after 4 and 24 h. The expression of IL-1α, IL-1β, IL-6, CSF2 and TNF-α were elevated following co-culture with the live *P. gingivalis* biofilms compared with planktonic cells after 4 and 24 h (Figure 4.3 A & B). Overall, inflammatory cytokine mRNA expression in epithelial

cells was increased more following stimulation with biofilms compared with planktonic cells.

(A) *P. gingivalis* **biofilm**



(B) *P. gingivalis* **planktonic**

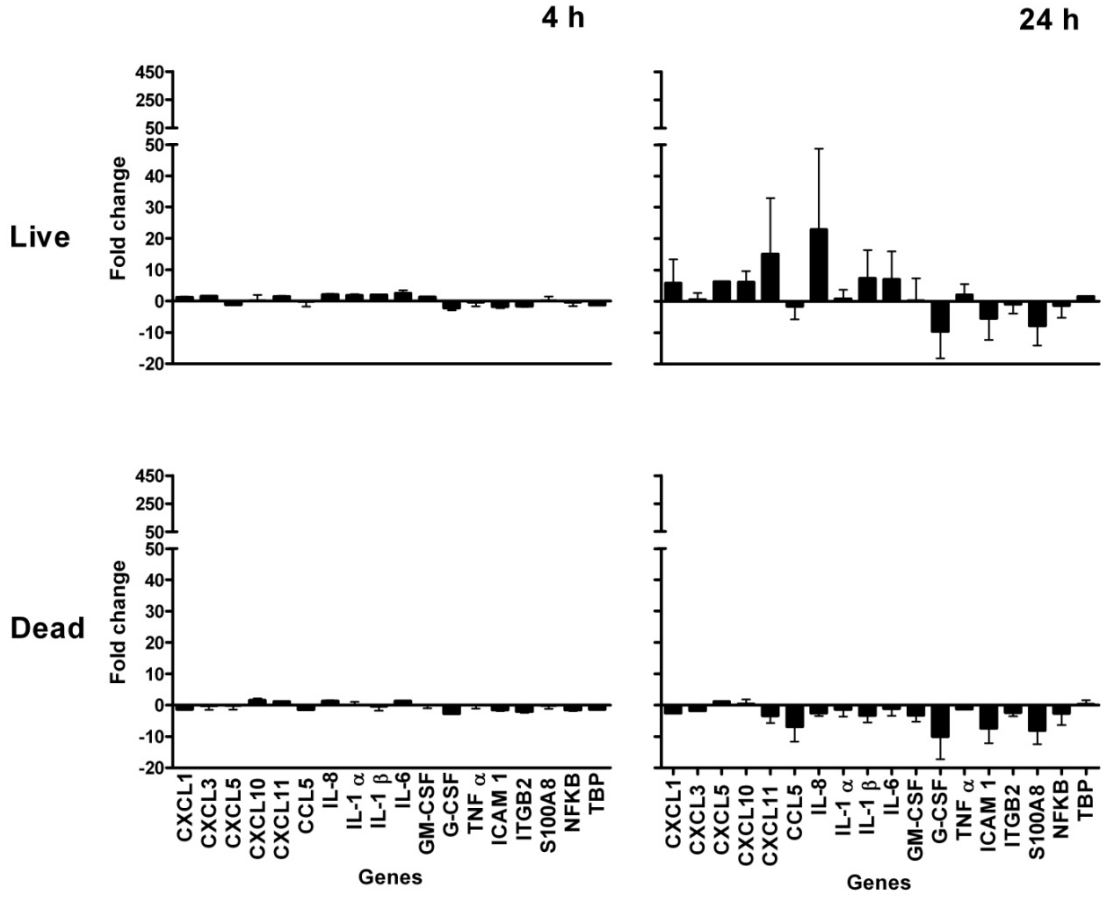


Figure 4.3: Expression of a panel of gingivitis-related genes in epithelial cells with *P. gingivalis* **biofilms or planktonic cells.** OKF6-TERT2 epithelial cells were challenged with (A) *P. gingivalis* biofilm and (B) *P. gingivalis* planktonic cells for 4 and 24 h. mRNA expression were assessed by TLDA for gingivitis-related genes (NFκB, PROK2, CSF3, ITGB2, IL-8, IL-1α, CCL5, IL-1β, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, GM-CSF, GROα, IL-6, ICAM1, DEFB4, CXCL5). Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to medium control.

4.4.3 IL-8 mRNA expression in epithelial cells stimulated with mixed and mono-species bacterial biofilms. The data presented above demonstrate that *P. gingivalis* biofilms induced greater levels of chemokine and cytokine mRNA expression in epithelial cells than planktonic cells. To investigate i) time-dependant, ii) live versus dead, and iii) pathogen versus commensal variability of IL-8 production by epithelial cells co-cultured with more bacterial biofilms, OKF6-TERT2 epithelial cells were challenged with live or dead (methanol-fixed) multi- or mon-species biofilms (multi: *P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm, and *S. mitis* biofilm for 1, 2, 4, 6, 8, 12, 18, 24 and 48 h. IL-8 mRNA expression was assessed by SYBR® Green real time RT-PCR. Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to OKF6-TERT2 cells cultured only with cell culture medium ('medium control').

(i) Time-dependent regulation of IL-8. Live and dead multi-species biofilms induced time dependent up-regulation of IL-8 mRNA. Elevated IL-8 mRNA expression was evident after 6 h (~200-fold) and there was a continued modest increase in IL-8 mRNA expression until 24 h (~1250-fold). IL-8 mRNA expression remained constant from 24 to 48 h (Figure 4.4A), suggesting the maximal response was reached by 24 h. There were similar changes in IL-8 mRNA expression upon stimulation with live *P. gingivalis* biofilm (Figure 4.4B). Live *S. mitis* biofilm induced somewhat erratic changes in IL-8 mRNA expression. The IL-8 mRNA expression was increased after 2 h (~150-fold), decreased after 4 h (~3-fold), increased again after 6 h (~125-fold) and decrease again after 8 h (~65-fold) (Figure 4.4C). These data demonstrate that IL-8 mRNA expression is regulated over time, and that this regulation varies depending on the nature of the biofilm to which the cells are exposed.

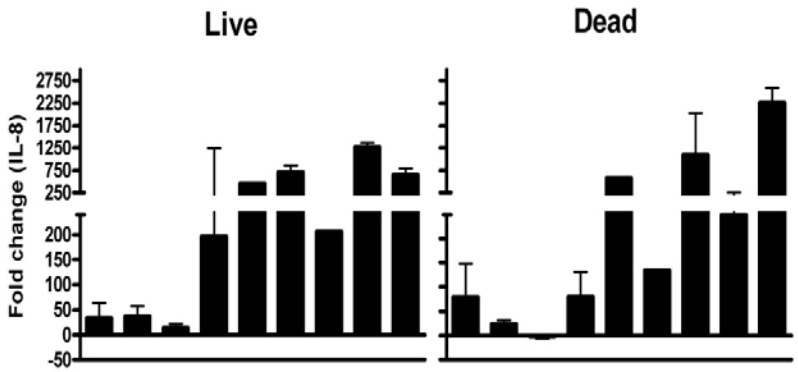
(ii) IL-8 mRNA expression upon co-culture with live versus dead biofilms. Live mixed biofilm induced similar IL-8 mRNA expression compared with dead mixed biofilm (Figure 4.4A). IL-8 mRNA expression was elevated following stimulation with live, but not dead *P. gingivalis* nor live *S. mitis* biofilm. Stimulation with dead *P. gingivalis* or *S. mitis* biofilms resulted in minimal changes in IL-8 mRNA expression compared with medium control (maximal ~19-fold for *P. gingivalis*) (Figure 4.4B &

C). To conclude, live bacterial biofilms induce more IL-8 expression than dead biofilms.

(iii) IL-8 mRNA expression following co-culture with pathogenic or commensal biofilms. The pathogenic *P. gingivalis* or multi-species biofilms elicited more IL-8 expression than the commensal biofilm (Figure 4.4). Only the live *P. gingivalis* biofilm caused increased IL-8 mRNA expression. This demonstrates that pathogens and commensals differentially regulate IL-8 expression in epithelial cells.

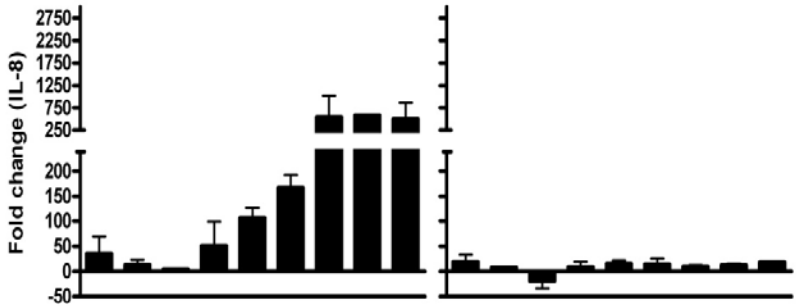
(A)

Mixed



(B)

P. gingivalis



(C)

S. mitis

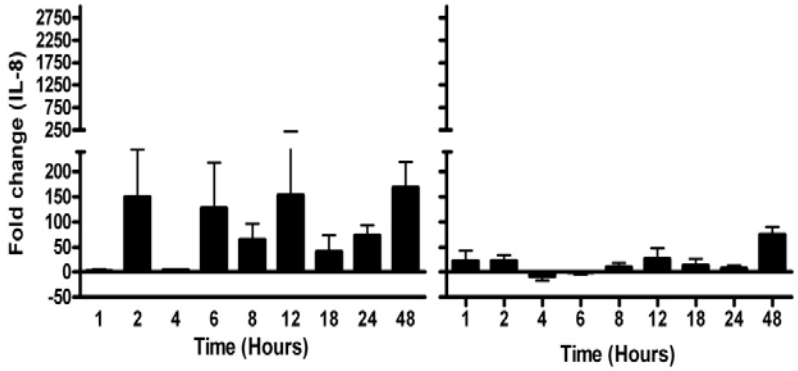


Figure 4.4: IL-8 mRNA expression in OKF6-TERT2 epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** mixed biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), **(B)** *P. gingivalis* biofilm and **(C)** *S. mitis* biofilm for 1, 2, 4, 6, 8, 12, 18, 24 and 48 h. mRNA expression was assessed by SYBR® Green real time PCR for IL-8. Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to medium control.

4.4.4 Expression of a panel of gingivitis-related genes in epithelial cells with multi- or mono-species biofilms. Marked changes in IL-8 expression were observed following stimulation with different biofilms. To investigate the biofilm effect on a broader spectrum of the epithelial cell inflammatory armamentarium, expression of a panel of gingivitis related genes was next investigated (gene panel selection described in Table 4.1). The studies in section 4.4.3 were extended to include analysis of a larger panel of genes (NFκB, PROK2, CSF3, ITGB2, IL-8, IL-1α, CCL5, IL-1ß, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, GM-CSF, GROα, IL-6, ICAM1, DEFB4, CXCL5 and TBP). Gene expression was assessed by TaqMan® low density array (TLDA). Gene expression was normalised to GAPDH endogenous control and then data expressed as fold change relative to OKF6-TERT2 cells cultured only with cell culture medium. Bacterial biofilm co-culture with epithelial cells resulted in little measurable gene expression of CXC3R1, CCL3L1, CCL4, ICAM1, ITGB2, S100A8, DEFB4, NFκB, and PROK2.

(i) Time-dependent regulation of genes. The kinetics of chemokine and cytokine gene regulation was investigated in epithelial cells co-cultured with bacterial biofilms. Similar to the effects on IL-8, there was time dependent upregulation of CXCL1 and CXCL3 following co-culture with live mixed species biofilm and *P. gingivalis* biofilm (Figure 4.5A & B; 4.6 A, B & D).

Inflammatory cytokines, IL-1α, IL-1ß, IL-6, GM-CSF and TNFα, were upregulated in a time-dependant manner following co-culture with live multi-species biofilm from 1 to 6 h (Figure 4.5A; 4.6 E, F, G, I & J). For instance, TNFα gene expression increased from 6.54 fold after 1 h, then 60.85 fold and 129.56 fold after 4 and 6 h, respectively (Figure 4.5A; 4.6J). Live *P. gingivalis* biofilms induced mRNA upregulation of all cytokines analysed from 1 to 4 h in a time-dependent manner (Figure 4.5B; 4.6 E-J). After 6 h, there were minimal changes in cytokine gene expression indicating an early transcriptional upregulation following co-culture with the *P. gingivalis* biofilm. Thus, a range of inflammatory chemokines and cytokines are regulated in a time-dependent manner in the epithelial cells co-cultured with bacterial biofilms.

ii) Gene expression upon stimulation with live versus dead biofilms. Similar to the observed effects on IL-8, a greater increase in CXCL1 and CXCL3 mRNA expression was observed following co-culture with live mixed species biofilm compared with dead biofilms after 4, 6 and 24 h (Figure 4.5A & B; 4.6A, B & D). There was elevated expression of CXCL1, CXCL3, CXCL5, CXCL10, CXCL11, CCL5 and IL-8 following co-culture with live compared with dead *P. gingivalis* biofilm after 4 and 6 h (Figure 4.5B; 4.6A-D). Live *S. mitis* biofilms induced more CXCL1, CXCL3, CXCL10, CXCL11, CCL5 and IL-8 gene expression after 6 and 24 h compared with dead biofilms (Figure 4.5C; 4.6A-D). Overall, epithelial chemokine gene expression was greater following co-culture with live biofilms compared with dead.

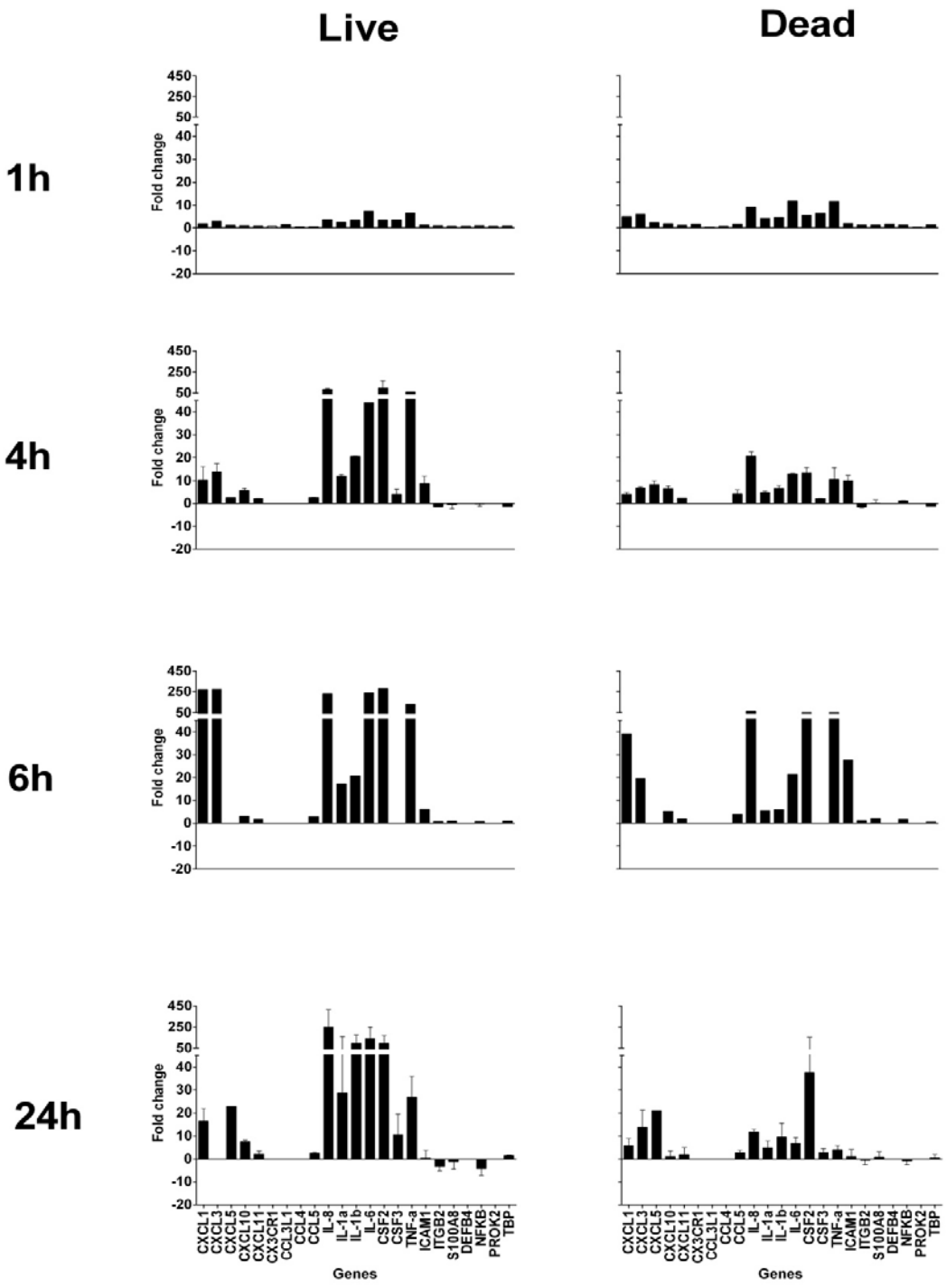
The expression of IL-1α, IL-1ß, IL-6, CSF2 and TNFα was elevated following co-culture with live mixed species and *P. gingivalis* biofilms compared with dead after 4, 6 and 24 h (Figure 4.5A & B; 4.6E, F, G, I & J). CSF3 expression was increased by co-culture with live mixed and *P. gingivalis* biofilm only after 4 and 24 h, as no detectable expression was observed after 6 h (Figure 4.6H). Live *S. mitis* biofilms induced a greater increase in expression of IL-1α, IL-1ß, IL-6, CSF2 and TNFα after 6 and 24 h compared with dead biofilm (Figure 4.5C; 4.6E, F, G, I & J). Overall, inflammatory cytokine mRNA expression was increased more with live biofilms compared with dead.

iii) Gene expression following co-culture with pathogenic or commensal biofilms. Chemokine and cytokine mRNA expression was investigated to evaluate differences in the epithelial cell response to pathogenic (multi-species and *P. gingivalis*) or commensal (*S. mitis*) biofilms.

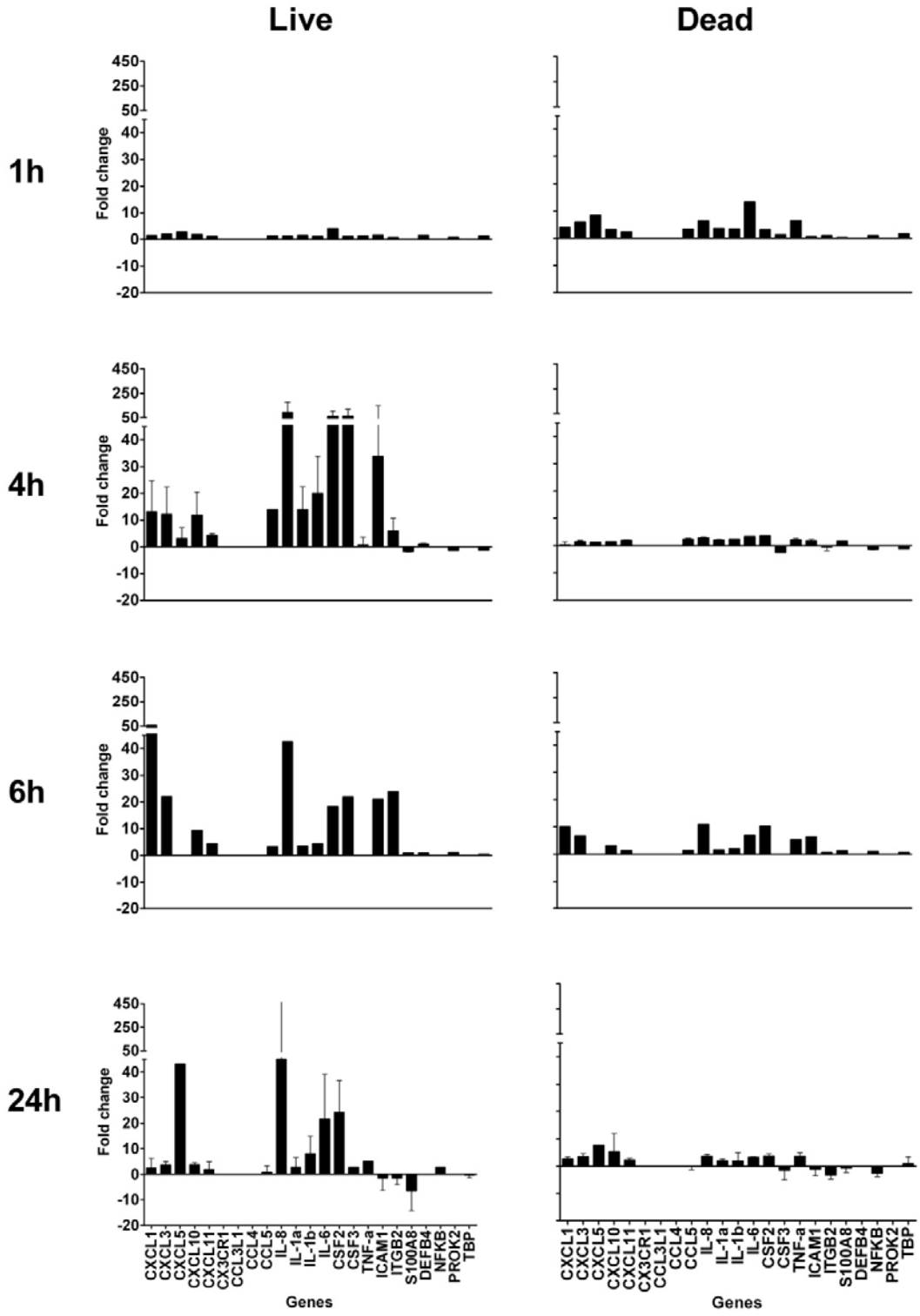
Co-culture with pathogenic biofilms induced more CXCL1, CXCL3, CXCL5, CXCL10, CXCL11, CCL5 and IL-8 expression than co-culture with commensal biofilm after 4 h (Figure 4.5A, B & C; 4.6A-D). Interestingly, pathogenic mono-species *P. gingivalis* biofilm induced generally greater chemokine expression than the mmulti-species biofilm; only CXCL3 expression was greater following stimulation with the mixed biofilm compared with *P. gingivalis* biofilm.

Increased cytokine mRNA expression (IL-1α, IL-1β, IL-6, CSF2 and TNFα) was observed following co-culture with the pathogenic biofilms compared with commensal biofilm after 4 h (Figure 4.5A, B & C; 4.6E, F, G, I & J). *P. gingivalis* induced greater IL-1α and IL-6 expression than the mixed biofilm. Overall, pathogenic biofilms show a greater propensity to increase mRNA expression of numerous chemokines and cytokines than commensal biofilms.

(A) Multi-species biofilm



(B) *P. gingivalis* **biofilm**



(C) *S. mitis* **biofilm**

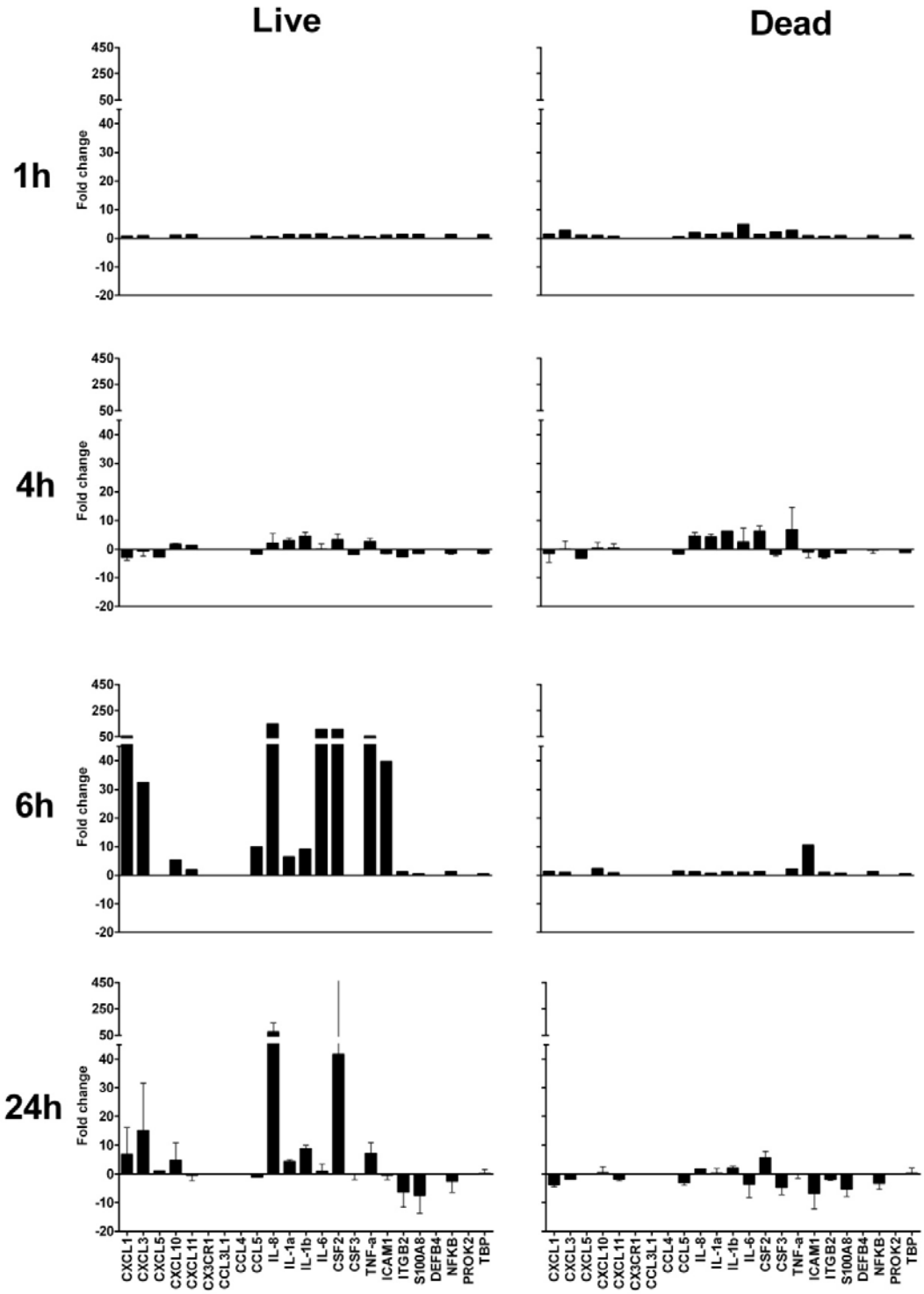
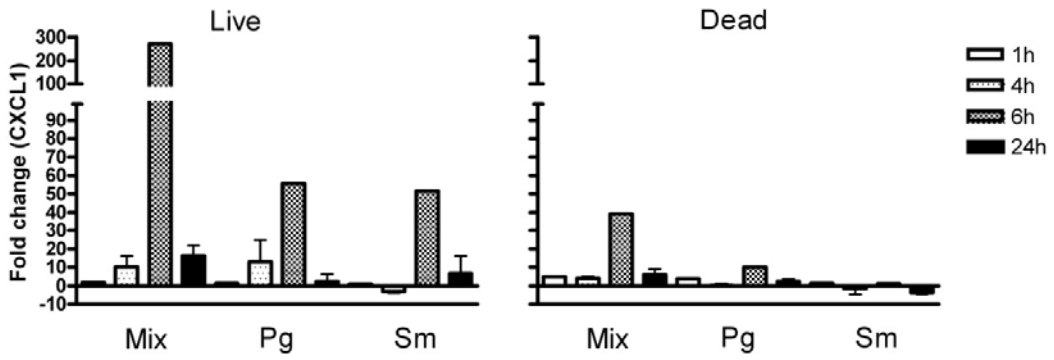
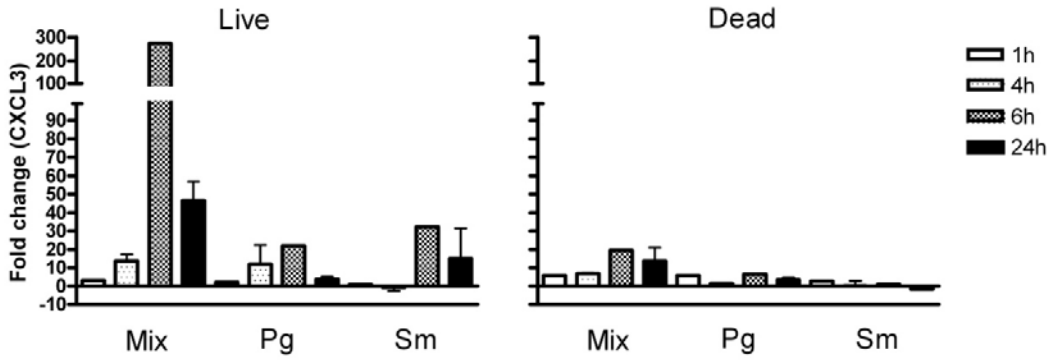


Figure 4.5: Expression of a panel of gingivitis-related genes in epithelial cells with multi- or mono-species biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), **(B)** *P. gingivalis* biofilm and **(C)** *S. mitis* biofilm for 1, 4, 6 and 24 h. mRNA expression was assessed by TLDA for gingivitis-related genes (NFκB, PROK2, CSF3, ITGB2, IL-8, IL-1α, CCL5, IL-1β, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, GM-CSF, GROα, IL-6, ICAM1, DEFB4, CXCL5). Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to medium control. These graphs show a global view of the data, which includes all the inflammatory cytokine genes expressed by the epithelial cells after co-culture with the different biofilm conditions at all the time points tested.

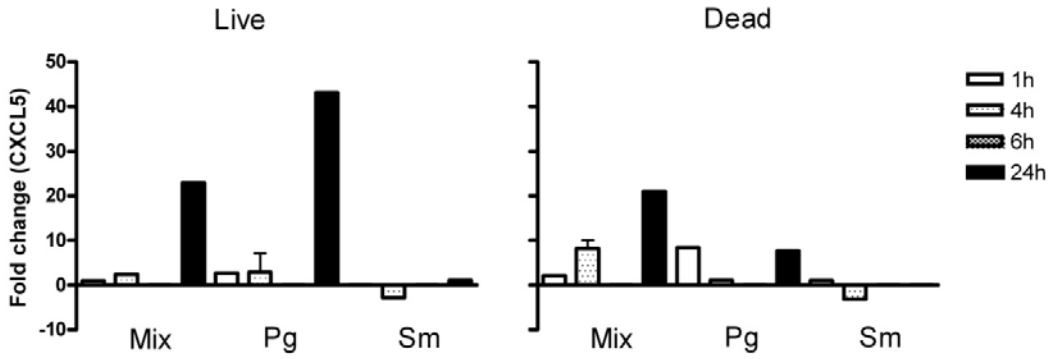
(A) CXCL1



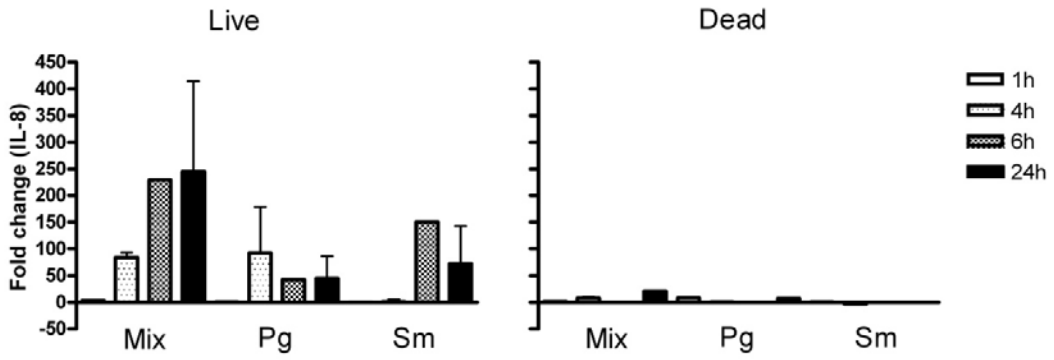
(B) CXCL3



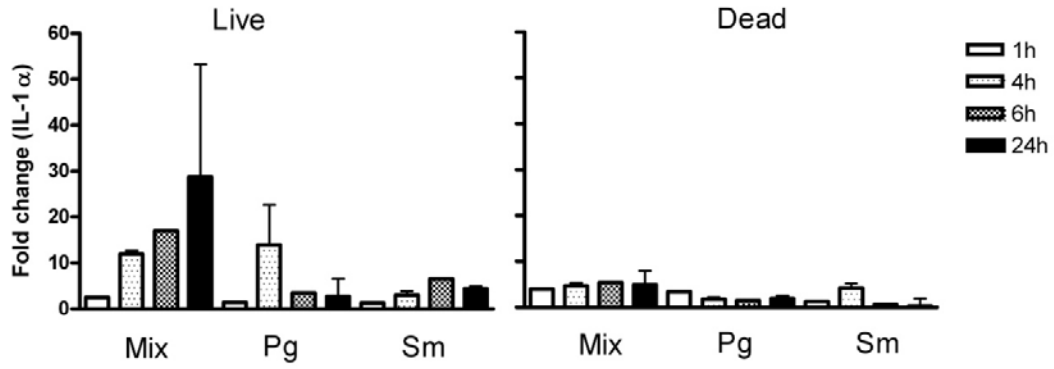
(C) CXCL5



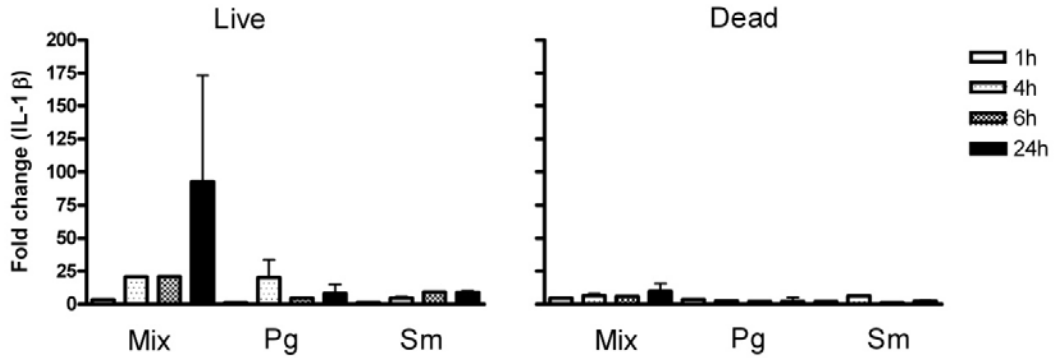
(D) IL-8



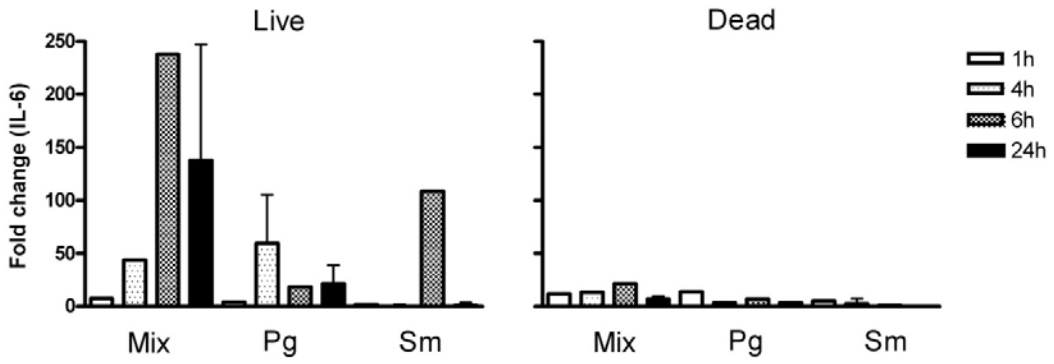
(E) IL-1α



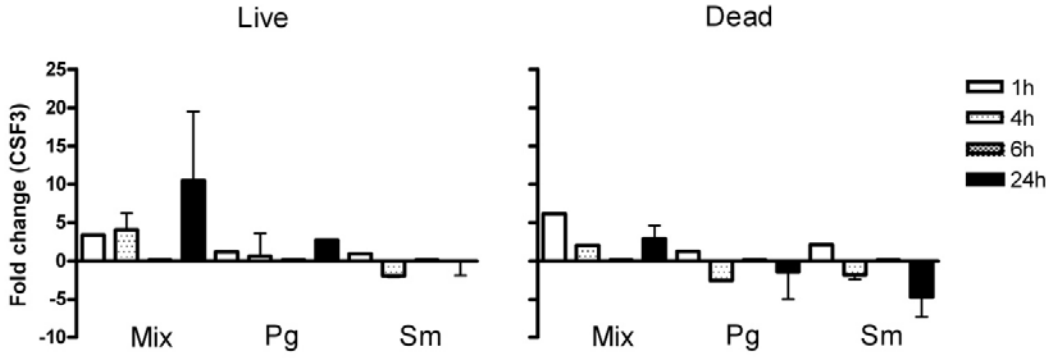
(F) IL-1β



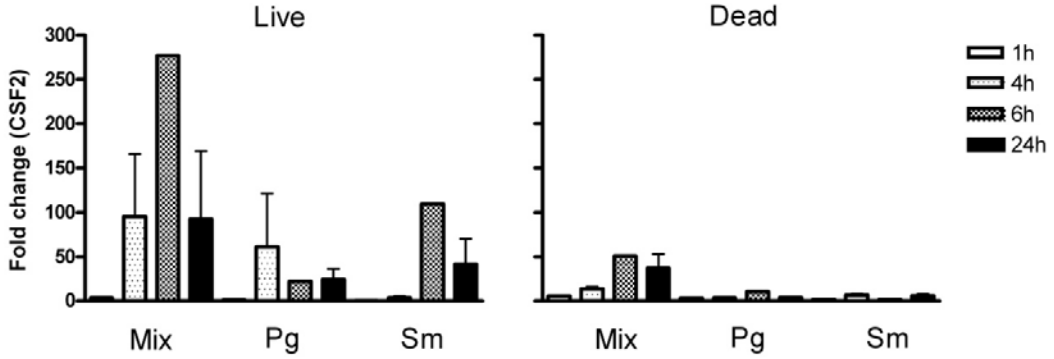
(G) IL-6



(H) CSF3



(I) CSF2



(J) TNFα

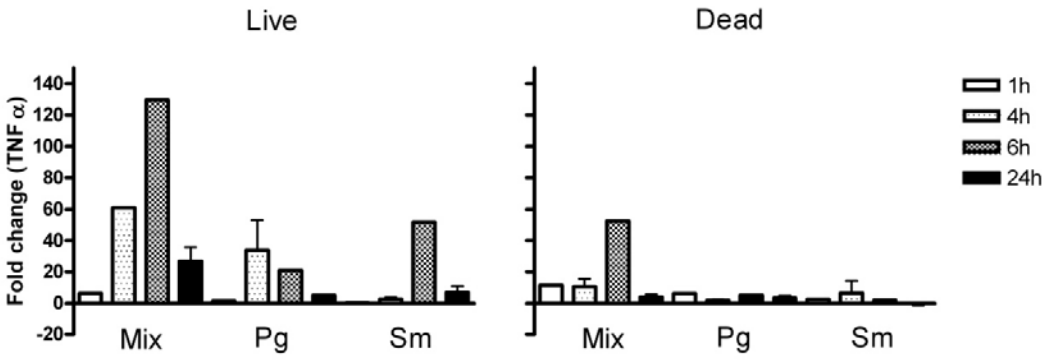


Figure 4.6: Kinetics of mRNA expression in OKF6-TERT2 epithelial cells challenged with live and dead (methanol-fixed) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm, *S. mitis* biofilm for 1, 4, 6 and 24h. mRNA expression were assessed by TLDA for gingivitis-related genes **(A)** CXCL1, **(B)** CXCL3, **(C)** CXCL5, **(D)** IL-8, **(E)** IL-1 α, **(F)** IL-1 ß, **(G)** IL-6, **(H)** G-CSF, **(I)** GM-CSF and **(J)** TNF-α. Gene expression was normalised to GAPDH endogenous control. Data shown are gene expression as fold change relative to unstimulated control cells. These graphs represent the same data as in Figure 4.6 but are presented as changes in individual genes.

The gene expression profiles of the epithelial cells challenged with the bacteria can be summarised from the heat map shown in Figure 4.7. Greater levels of gene expression are shown in red and lower levels of gene expression in green. In the right hand panel of the heat map, within the yellow box, the two left hand columns are the unstimulated cells. What is somewhat surprising is that there is some cytokine and chemokine gene regulation but not as much as might be expected compared with the unstimulated cells. This panel consists of epithelial cells stimulated with commensal bacteria (in either biofilm or planktonic state) or stimulated with pathogenic bacteria such as *P. gingivalis* in a planktonic state. However, the left hand panel of the heat map, within the blue box, shows striking changes in gene expression, i.e. a range of chemokines and cytokines are upregulated by the epithelial cells when these pathogens were presented to the cells as a biofilm.

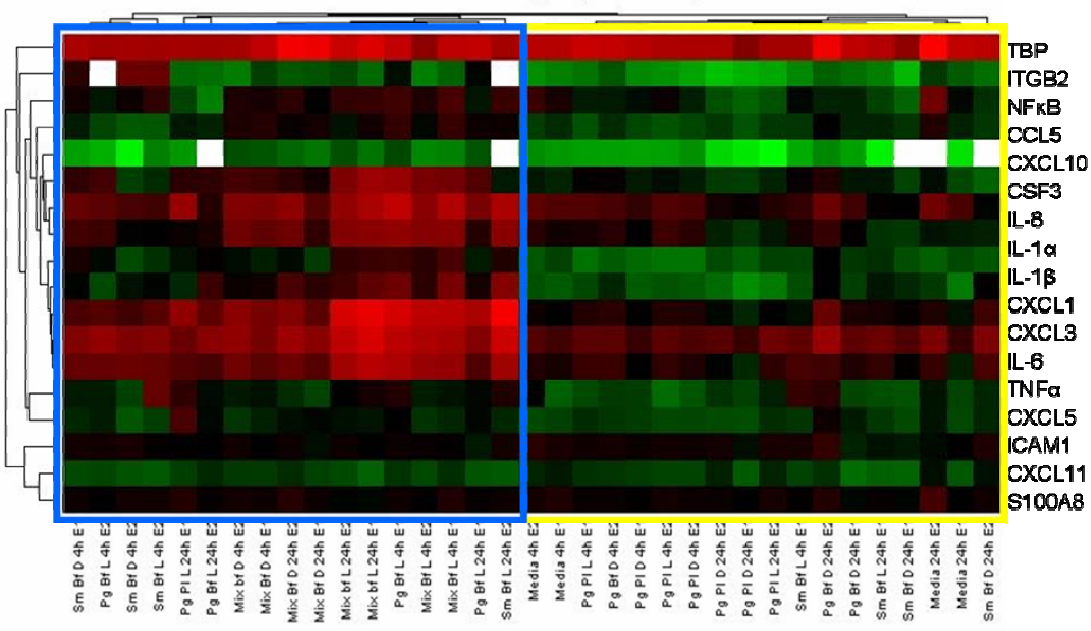


Figure 4.7: mRNA expression in OKF6-TERT2 epithelial cells challenged with live and dead (methanol-fixed) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with live and dead (methanol-fixed) multi-species biofilm (*P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm, *S. mitis* biofilm and *P. gingivalis* planktonic cells. mRNA expression was assessed by TLDA for gingivitis-related genes (NFκB, PROK2, CSF3, ITGB2, IL-8, IL-1α, CCL5, IL-1ß, CXCL3, CCL3L3, CX3CR1, S100A8, CCL4, CXCL10, CXCL11, TNFα, GM-CSF, GROα, IL-6, ICAM1, DEFB4, CXCL5). Gene expression was normalized to GAPDH endogenous control. The gene expression is presented as heat maps, which graphically display results of hierarchical clustering based on the ΔCT values using Pearson's Correlation, showing a global view of the average linkage of the various genes of duplicate measurements of two independent experiments. The x-axis represents the different bacterial conditions used to stimulate the epithelial cells. The y-axis represents the gingivitis-related genes that were expressed after stimulation with the different bacterial conditions. Each square represents the expression of one of the genes on the y-axis to the corresponding bacterial condition on the x-axis. The brighter the shade of red, the greater the up-regulation of the genes. The brighter the shade of green, the greater the down-regulation of the genes.

4.4.5 IL-8 release from epithelial cells stimulated with bacterial biofilms or planktonic cells. IL-8 mRNA expression was increased in epithelial cells co-cultured with *P. gingivalis* biofilms compared with planktonic cells (Section 4.4.1). To investigate whether this change in gene expression translated to protein expression and release, epithelial cells were co-cultured with bacterial biofilms and planktonic cells. OKF6-TERT2 epithelial cells were challenged with live and dead (heat-killed) *P. gingivalis* biofilms and planktonic cells for 4 and 24 h. IL-8 release from the epithelial cells was assessed by ELISA. Statistical analyses were performed by one-way ANOVA using GraphPad Prism program (San Diego, CA, USA) with the Bonferonni correction. Bonferonni correction was used because many different data sets were being compared simultaneously. Differences were considered significant if $p<0.05$.

Minimal changes in IL-8 release were observed after 4 h culture with dead *P. gingivalis* planktonic bacteria, or live *P. gingivalis* biofilm or planktonic cells co-cultured for 4 and 24 h. IL-8 protein release was significantly increased in cell culture supernatants of epithelial cells stimulated with dead *P. gingivalis* biofilms after 4 ($p<0.01$) and 24 h ($p<0.001$) compared with medium controls. Dead *P. gingivalis* biofilms induced significantly more IL-8 release after 4 (308 pg/mL, $p<0.001$) and 24 h (962 pg/mL, $p<0.001$) compared with planktonic cells at the same time points, 4 (43 pg/mL) and 24 h (63 pg/mL) (Figure 4.8). To conclude, *P. gingivalis* biofilms induce more IL-8 release than the planktonic cells.

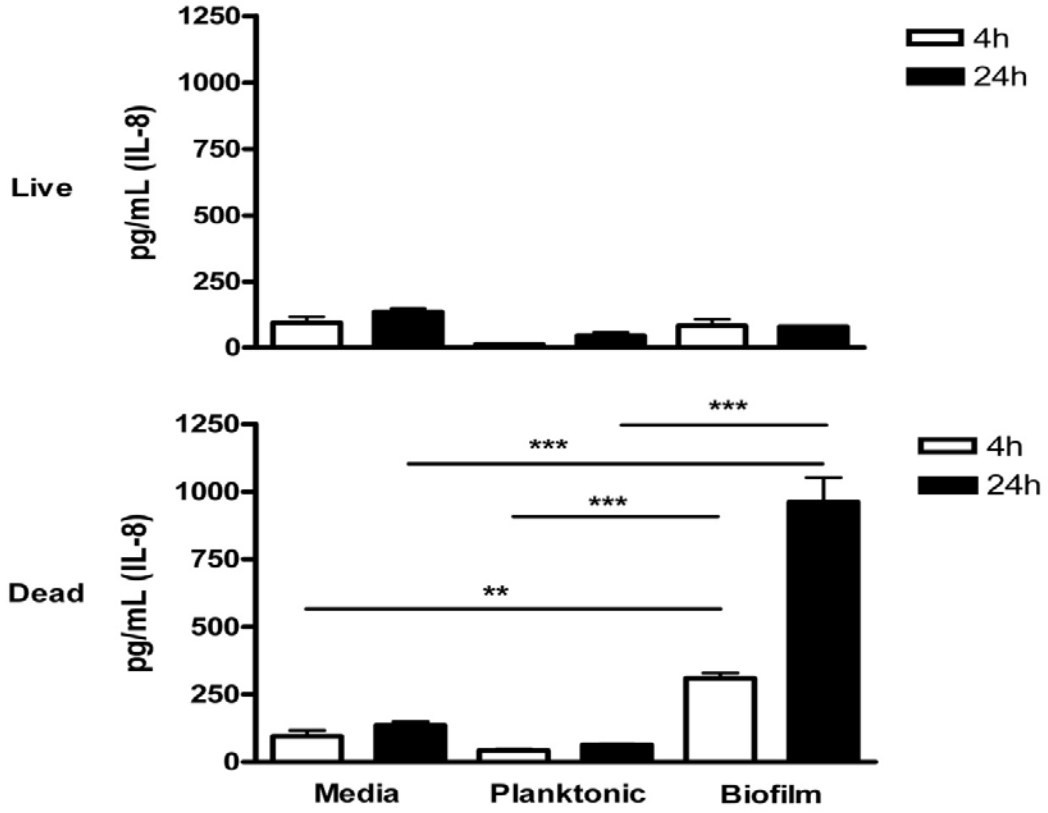


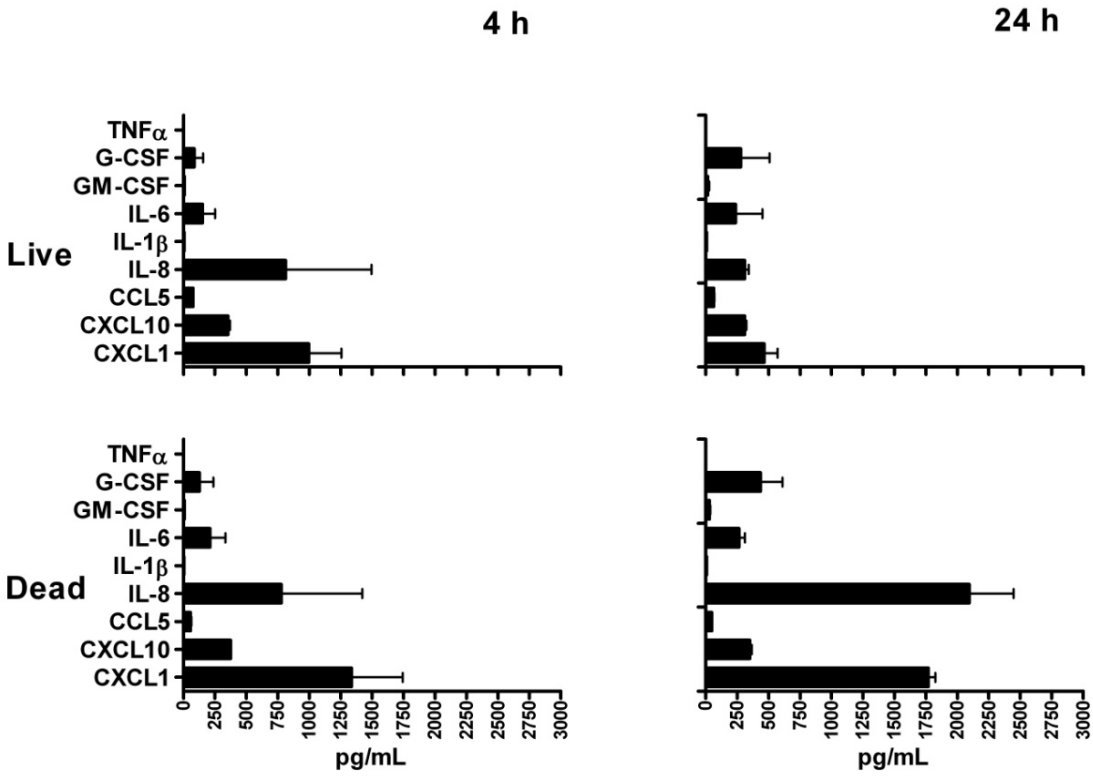
Figure 4.8: IL-8 release from OKF6-TERT2 epithelial cells challenged with live and dead (heat-killed) ***P. gingivalis*** **biofilms and planktonic cells.** OKF6-TERT2 epithelial cells were challenged with *P. gingivalis* biofilms or planktonic bacteria for 4 and 24 h. IL-8 release was assessed by ELISA. Data shown are protein concentrations in pg/mL. Values are means ± SEM of triplicate measurements of two independent experiments. Statistical comparisons are to the medium control and biofilms versus planktonic cells (**p<0.01, ***p<0.001).

4.4.6 Chemokine and cytokine release from epithelial cells co-cultured with bacterial biofilms or planktonic bacteria. The previous studies demonstrate changes in the IL-8 protein release were observed following stimulation with the bacterial biofilms. Biofilms induced expression of numerous cytokine and chemokine genes and therefore to investigate the biofilm and planktonic effect on a range of gingivitis-related proteins, these studies were extended to include analysis of protein products of a number of the upregulated genes, including: CXCL1, CXCL10, CCL5, IL-1β, IL-6, CSF2, CSF3 and TNF-α). Release of IL-1β, IL-6, IL-8, CSF2, CSF3 and TNF-α were assessed by Luminex®, while CXCL1, CXCL10 and CCL5 were assessed by ELISA. Data shown are protein concentrations in pg/mL.

Minimal changes in the release of CCL5 were observed following stimulation with *P. gingivalis* biofilms and planktonic bacteria after 4 and 24 h. CXCL10 release remained constant with *P. gingivalis* biofilms and planktonic cells after 4 and 24 h. Similar to the observed effects on IL-8, greater increase of CXCL1 release was observed following co-culture with dead *P. gingivalis* biofilm (4 h: 1334 pg/mL; 24 h: 1768 pg/mL) compared with planktonic cells after 4 (1029 pg/mL) and 24 h (904 pg/mL) (Figure 4.9A & B). Overall, the chemokine protein release was greater following co-culture with biofilms compared with the planktonic bacteria in the epithelial cells.

The inflammatory cytokine protein release from the epithelial cells was minimal with the planktonic cells after 4 and 24 h. Similarly, IL-1β, GM-CSF and TNFα protein release was minimal with the biofilms. Marginally higher protein release of IL-6 and G-CSF was observed from epithelial cells co-cultured with biofilms compared with planktonic cells (Figure 4.9A & B). To conclude, inflammatory cytokine protein release was marginally increased with the biofilms compared with the planktonic cells. To summarise, biofilms induce more protein release compared with planktonic cells in the epithelial cells. Interestingly, more protein release was observed with dead *P. gingivalis* compared with live.

(A) *P. gingivalis* **biofilm**



(B) *P. gingivalis* **planktonic**

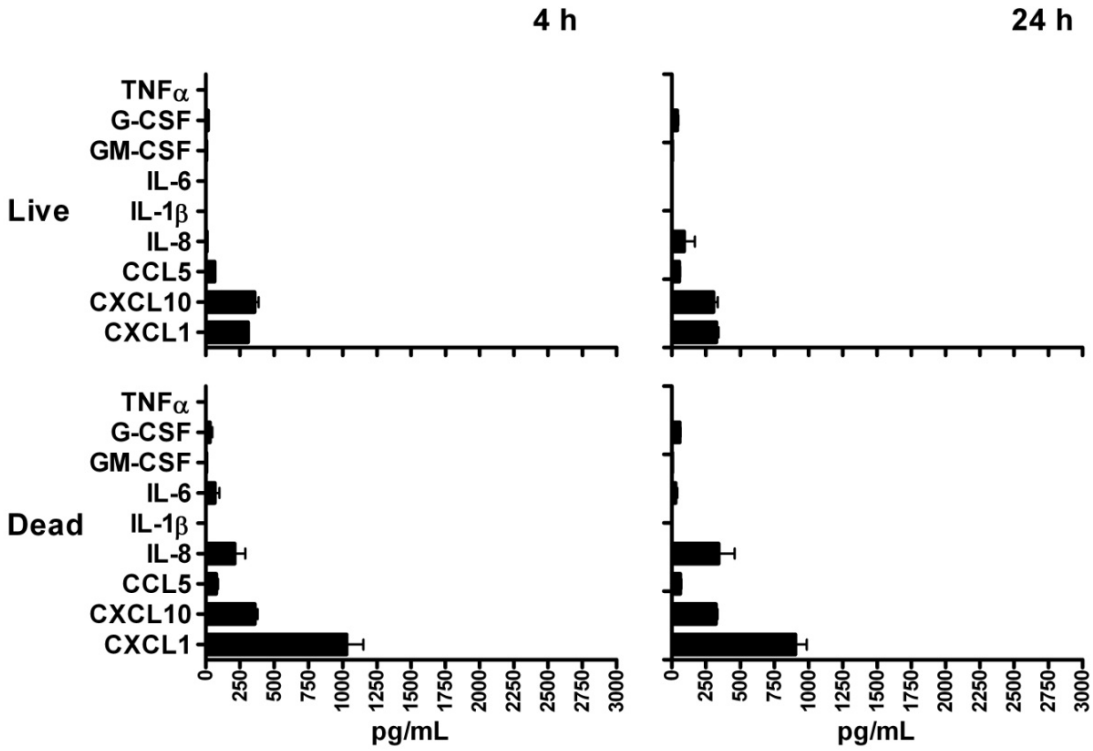


Figure 4.9: Protein release of a panel of gingivitis-related proteins in epithelial cells with bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** *P. gingivalis* biofilm & **(B)** *P. gingivalis* planktonic cells for 4 and 24 h. Protein release of IL-1β, IL-6, IL-8, GM-CSF, G-CSF and TNFα in the cell culture supernatants was assessed by Luminex® while CXCL1, CXCL10 and CCL5 were assessed by ELISA. Data shown are protein concentrations in pg/mL. Values are means ± SEM of two independent experiments.

4.4.7 Protein release of IL-8 in epithelial cells stimulated with bacterial biofilms. The characteristics and advantages of using IL-8 have been described previously. It was also noted that the biofilms induced greater protein release than the planktonic cells in the epithelial cells. To investigate the time-dependant, live versus dead and pathogen versus commensal variability of IL-8 protein release from the epithelial cells with more gingivitis-related bacterial biofilms, OKF6-TERT2 epithelial cells were challenged with live and dead (methanol-fixed) multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm, *S. mitis* biofilm for 1, 2, 4, 6, 8, 12, 18, 24, 48 and 72 h. Protein release of IL-8 was assessed by ELISA. Data shown are protein concentrations in pg/mL.

(i) Time-dependent release of IL-8. Live multi-species and *P. gingivalis* biofilms induced time-dependent IL-8 release from the epithelial cells. Time-dependent increase of IL-8 was observed with dead mixed species biofilm from 8 to 18 h, after which it remained stable. IL-8 concentration was 121, 311 and 732 pg/mL after 8, 12 and 18 h, respectively. Dead *P. gingivalis* and *S. mitis* induced somewhat erratic changes in IL-8 protein release (Figure 4.10A, B & C). These data suggest that IL-8 protein release is regulated over time but the regulation varies depending on the nature of the biofilm to which the cells are exposed.

(ii) IL-8 protein release upon co-culture with live versus dead biofilms. Live mixed biofilm induced similar IL-8 protein release with dead mixed biofilm (Figure 4.10A). IL-8 protein release was higher following stimulation with live (12 h: 314 pg/mL; 18 h: 204 pg/mL), but not dead *P. gingivalis* (12 h: 86 pg/mL; 18 h: 87 pg/mL) nor dead *S. mitis* biofilm (12 h: 95 pg/mL; 18 h: 43 pg/mL). Elevated IL-8 concentrations were observed with live *S. mitis* biofilm compared with dead Figure 4.10B & C). To conclude, live bacterial biofilms induce marginally more IL-8 protein release than dead biofilms.

(iii) IL-8 protein release following co-culture with pathogenic or commensal biofilms. The protein release of IL-8 was investigated to evaluate differences in the epithelial cell response to pathogenic (multi-species and *P. gingivalis*) or commensal (*S. mitis*) biofilms. The pathogenic dead mixed species biofilms elicited more IL-8 protein release than the dead pathogenic monospecies biofilm and commensal biofilm (Figure 4.10). This demonstrates that pathogens differentially regulate IL-8 protein release in epithelial cells, and this depends on the type of pathogenic biofilm.

IL-8 ELISA on the cell culture supernatants was useful in determining that the epithelial cells were releasing proteins upon co-culture with bacterial biofilms. This also showed that the protein release occurred in a time-dependant manner, and also showed the live versus dead and pathogen versus commensal variability.

(A) Mixed

(B) *P. gingivalis*

(C) *S. mitis*

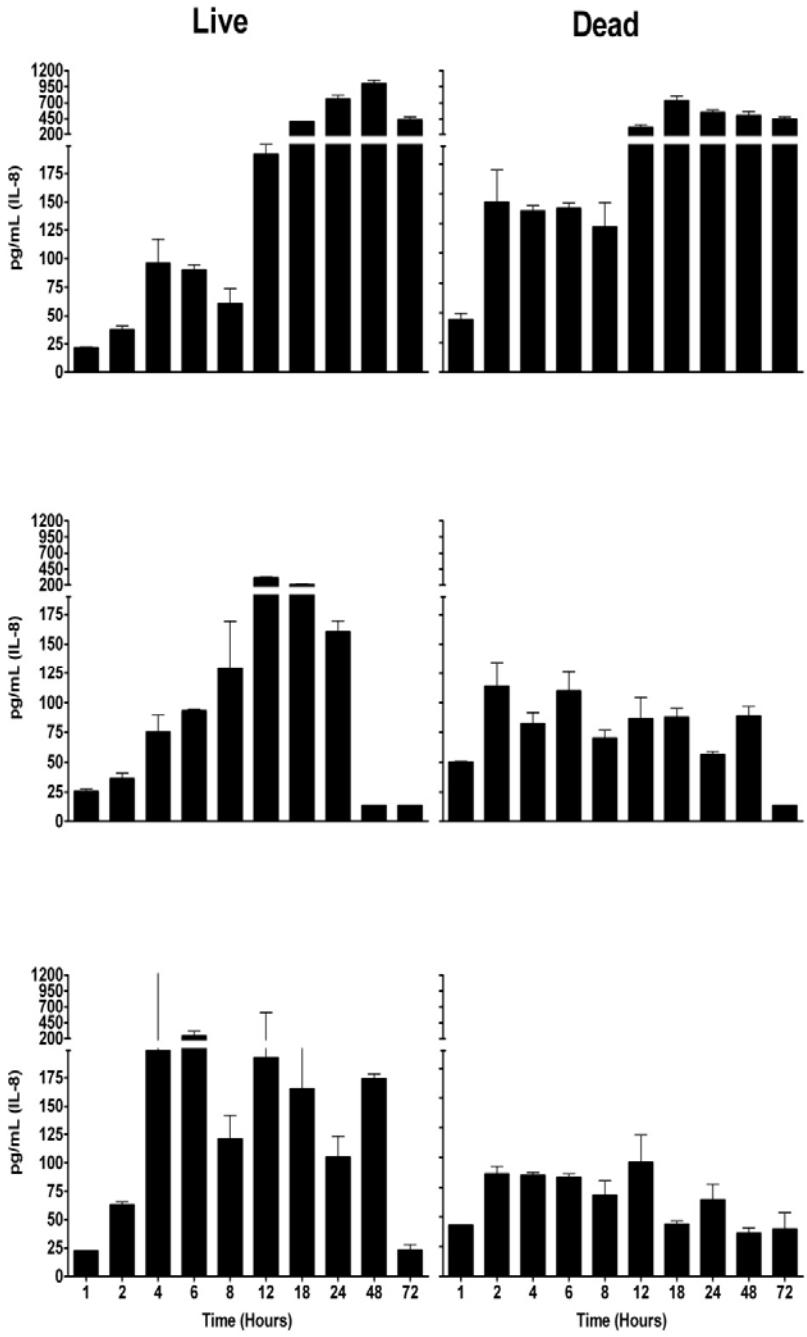


Figure 4.10: Protein release of IL-8 in OKF6-TERT2 epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), **(B)** *P. gingivalis* biofilm and **(C)** *S. mitis* biofilm for 1, 2, 4, 6, 8, 12, 18, 24, 48 and 72 h. Protein concentrations of IL-8 in the cell culture supernatants were measured by ELISA. Data shown are protein concentrations in pg/mL. Values are means ± SEM of duplicate measurements of two independent experiments.

4.4.8 Protein release in epithelial cells stimulated with bacterial biofilms on multiplex protein array. For a quick and easy evaluation of cytokines and chemokines in the cell culture supernatants of epithelial cells with bacterial biofilms, Proteome Profiler™ antibody arrays (RnD systems, Inc, UK) were used. OKF6-TERT2 epithelial cells were challenged with medium, multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*) (Mix), *P. gingivalis* biofilm (Pg) and *S. mitis* biofilm (Sm) for 24 h. The cell culture supernatants were used in the human cytokine array. The cell culture supernatants were mixed with a cocktail of biotinylated detection antibodies, and then incubated with the human cytokine array. The array was then incubated with streptavidin-HRP followed by chemiluminescent detection. Light was emitted at each spot in proportion to the amount of cytokine bound. Array images shown are from 5 min exposures to X-ray film (Figure 4.12). The densitometry analysis was performed on X-ray film using the Quantity One® 1-D Analysis Software (Gel Doc™, BioRad) by calculating the adjusted volume of the spots from the pixel density after subtracting from the background (Supplementary Figure). Data was also represented as heat map of the protein concentration as fold change relative to medium control (Table 4.3).

The chemokines, cytokines and acute phase proteins which were present in the cell culture supernatants of the epithelial cells co-cultured with the bacterial biofilms appeared as duplicate spots on the X-ray films. When densitometry analysis was performed on the X-ray films, the adjusted volume of the spots of IL-1α and IL-1ß were observed more with live *P. gingivalis* compared with dead (Figure 4.11). Similar IL-1 ra volume was observed with live and dead mixed species and *P. gingivalis* biofilms. Only live *S. mitis* biofilms induced increased IL-1 ra volume compared with dead (Figure 4.11). IL-6 protein appeared on the X-ray films only in the supernatants of epithelial cells challenged with dead *S. mitis* biofilm (Figure 4.11). Dead mixed species (3657 pixel intensity/mm^2), *P. gingivalis* (559 pixel intensity/mm^2) and *S. mitis* biofilms (3412 pixel intensity/mm^2) induced more IL-8 protein volume on the X-ray films compared with live mixed species (2039 pixel intensity/mm^2), *P. gingivalis* (0 pixel intensity/mm^2) and *S. mitis* (0 pixel intensity/mm^2) biofilm (Figure 4.11). Marginally more sICAM1 and G-CSF protein

volumes were observed with live *P. gingivalis* biofilms compared with the dead biofilms, while dead mixed species biofilms induced more volume of the same proteins compared with live (Figure 4.11). Increase GM-CSF volumes were observed on the X-ray films with the dead mixed species, *P. gingivalis* and *S. mitis* biofilms compared with live (Figure 4.11). Serpin E1 and MIF volumes were higher with medium control compared with the bacterial biofilms (Figure 4.11).

Data was also represented as heat map of the protein concentration as fold change relative to medium control (Table 4.3). The heat map summarises the chemokine and cytokine protein volumes in the cell culture supernatants from epithelial cells co-cultured with bacterial biofilms. More IL-8, G-CSF, GM-CSF and ICAM1 protein concentrations were observed with the dead mixed biofilm compared with the live. Dead *P. gingivalis* biofilms induced more IL-8 protein volume compared to the live biofilms. IL-1 α, IL-6, IL-8 G-CSF and GM-CSF protein volumes were increased more with dead *S. mitis* compared to live. To conclude, dead bacterial biofilms induce more cytokine and chemokine release from the epithelial cells compared with live.

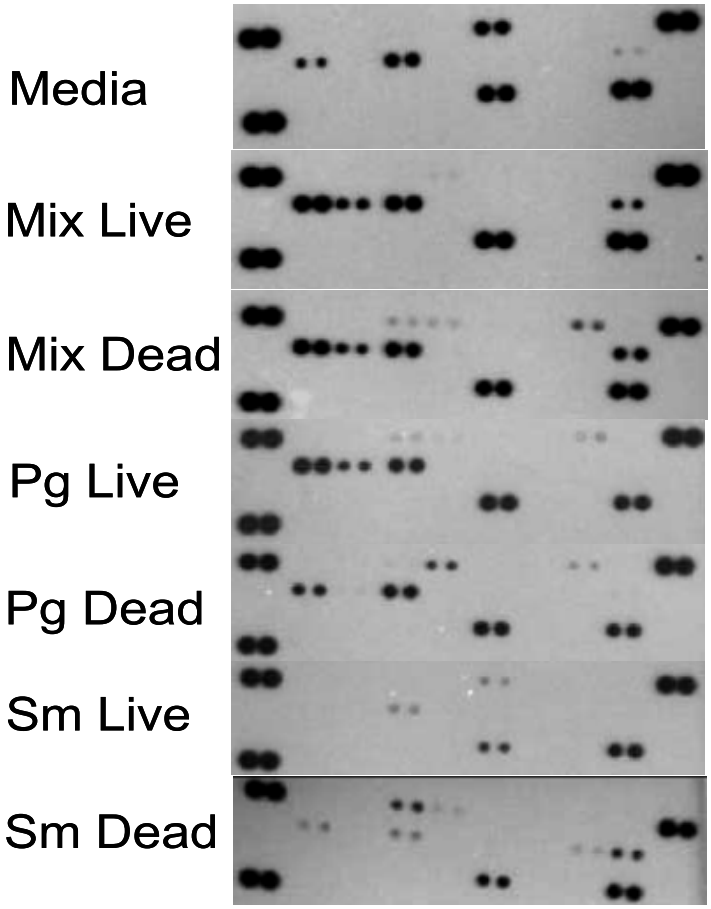


Figure 4.11: Human cytokine array of proteins from epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with media, multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*) (Mix), *P. gingivalis* biofilm (Pg) and *S. mitis* biofilm (Sm) for 24 h. The cell culture supernatants were mixed with a cocktail of biotinylated detection antibodies, and then incubated with the human cytokine array. The array was then incubated with streptavidin-HRP followed by chemiluminescent detection. Light was emitted at each spot in proportion to the amount of cytokine bound. Array images shown are from 5 min exposures to X-ray film.

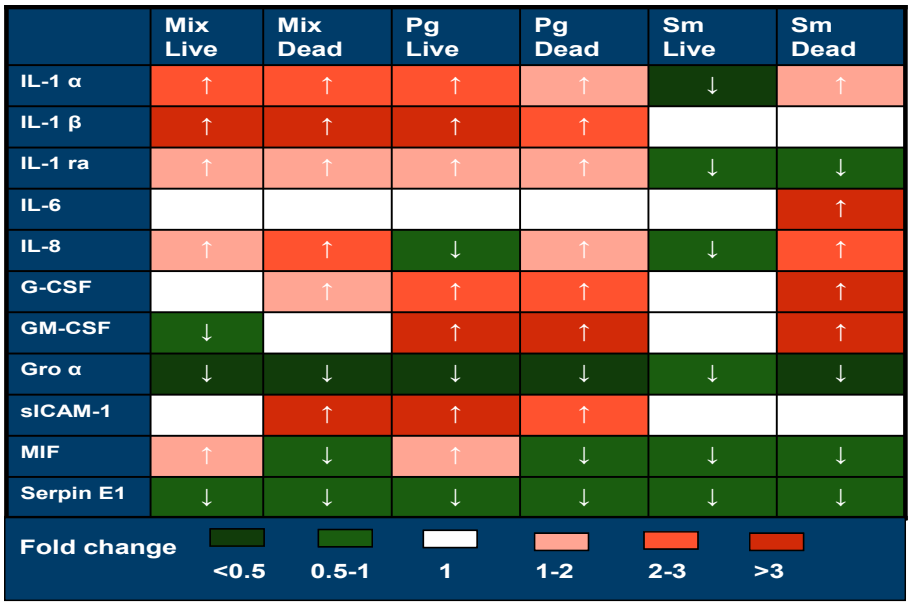


Table 4.3: Densitometry analysis of human cytokine array of proteins from epithelial cells challenged with live and dead (methanol-fixed) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with media, muti-species biofilm (*P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* and *S. mitis*) (Mix), *P. gingivalis* biofilm (Pg) and *S. mitis* biofilm (Sm) for 24 h. The cell culture supernatants were mixed with a cocktail of biotinylated detection antibodies, and then incubated with the human cytokine array. The array was then incubated with streptavidin-HRP followed by chemiluminescent detection. Light was emitted at each spot in proportion to the amount of cytokine bound. Arrays were exposed to the X-ray film for 5 minutes. The densitometry analysis was performed on X-ray film using the Quantity One® 1-D Analysis Software (Gel Doc™, BioRad) by calculating the pixel density after subtracting from the background. Data is represented as heat map of the protein concentration as fold change relative to unstimulated control cells represented as 1.

4.4.9 Protein release of a panel of gingivitis-related proteins in epithelial cells with bacterial biofilms. Marked changes in IL-8 protein release were observed following stimulation with the different biofilms. To investigate the time-dependant, live vs dead and pathogen vs commensal variability of protein release of the inflammatory cytokines (IL-1β, IL-6, G-CSF, GM-CSF, TNF-α) by the epithelial cells with bacterial biofilms, OKF6-TERT2 epithelial cells were co-cultured with live and methanol-fixed (dead) mixed species, *P. gingivalis* and *S. mitis* biofilms for 1, 4, 6, 8, 12, 18 and 24 h. Protein release into the cell culture supernatants was assessed by Luminex®. Bacterial biofilm co-culture with epithelial cells resulted in little measurable concentrations of IL-1β, GM-CSF and TNFα in the cell culture supernatants.

(i) Time-dependent protein release. Time-dependent regulation of inflammatory cytokines was investigated in epithelial cells co-cultured with the bacterial biofilms. Similar to IL-8 release, there was time-dependent increase of protein concentration of IL-6 in the epithelial cells co-cultured with live and dead mixed species biofilms (Figure 4.12A; 4.13A & B; Table 4.4A). Dead mixed species and *P. gingivalis* biofilms induced regulation of IL-6 and G-CSF in a time-dependent manner only after 8 h of stimulation suggesting a later translational regulation of the proteins (Figure 4.12A & B; 4.13B & C; Table 4.4A & B). These data demonstrate that the proteins are regulated over time, and that this regulation varies depending on the nature of the biofilm to which the cells are exposed.

(ii) Protein release upon stimulation with live versus dead biofilms. A greater increase in IL-8, IL-6 and G-CSF protein concentrations was observed following co-culture with dead mixed species biofilm compared with live biofilms (Figure 4.12A; 4.13A-C; Table 4.4A). For instance, after 24 h of stimulation with mixed species biofilm induced increased release of IL-8 with dead biofilms (6994 pg/mL) compared with live biofilms (602 pg/mL). There was higher IL-8 concentration with dead *P. gingivalis* and *S. mitis* biofilms compared with live only after 24 h (Figure 4.12B & C; 4.13A; Table 4.4B & C). The concentration of G-CSF was elevated in epithelial cells co-cultured with dead *P. gingivalis* and *S. mitis* biofilm only after 24 h (Figure 4.12B & C; 4.13C; Table 4.4B & C). Overall, the cytokine and chemokine

release was increased more with the dead biofilms compared with live in the epithelial cells.

(iii) Protein release following co-culture with pathogenic or commensal biofilms. The protein release of inflammatory cytokines was investigated to evaluate differences in the epithelial cell response to pathogenic (multi-species and *P. gingivalis*) or commensal (*S. mitis*) biofilms. Increased protein release of IL-8, IL-6 and G-CSF was observed following co-culture with the pathogenic biofilms compared with the commensal biofilms. Furthermore, pathogenic mixed species biofilm induced greater cytokine release than the mono-species *P. gingivalis* biofilm (Figure 4.12 & 4.13; Table 4.4). Overall, pathogenic biofilms show a greater tendency to increase protein release of the cytokines than commensal biofilms.

To summarize, the data demonstrated that the chemokine and cytokine protein regulation occurred in a time-dependent manner depending on the nature of the biofilm. Interestingly, the protein release was increased more with the dead biofilms compared with the live biofilms in the epithelial cells. The pathogenic biofilms also show a greater propensity to increase protein release than the commensal biofilms.

(A) Mixed species biofilm

Cytokine	IL-8		IL-1β		IL-6		GM-CSF		G-CSF	
BIOFILM / TIME	L	D	L	D	L	D	L	D	L	D
1 h	55.99	138.81	0.77	0.77	18.31	58.08	14.26	14.26	32.10	50.92
4 h	777.91	1896.46	19.11	16.48	275.25	609.16	15.44	41.23	99.15	361.53
6 h	407.57	568.32	4.74	10.67	113.21	471.12	14.26	36.85	108.48	320.42
8 h	459.92	1001.44	2.56	10.99	56.79	219.26	14.26	14.26	93.30	208.33
12 h	661.23	1854.72	2.18	21.56	57.17	498.09	14.26	21.39	129.39	446.57
18 h	992.20	5750.75	8.37	34.25	135.27	1087.33	14.26	118.42	158.90	1072.69
24 h	602.52	6994.63	3.73	24.23	194.74	972.22	4.75	87.03	54.68	1543.09

(B) *P. gingivalis* **biofilm**

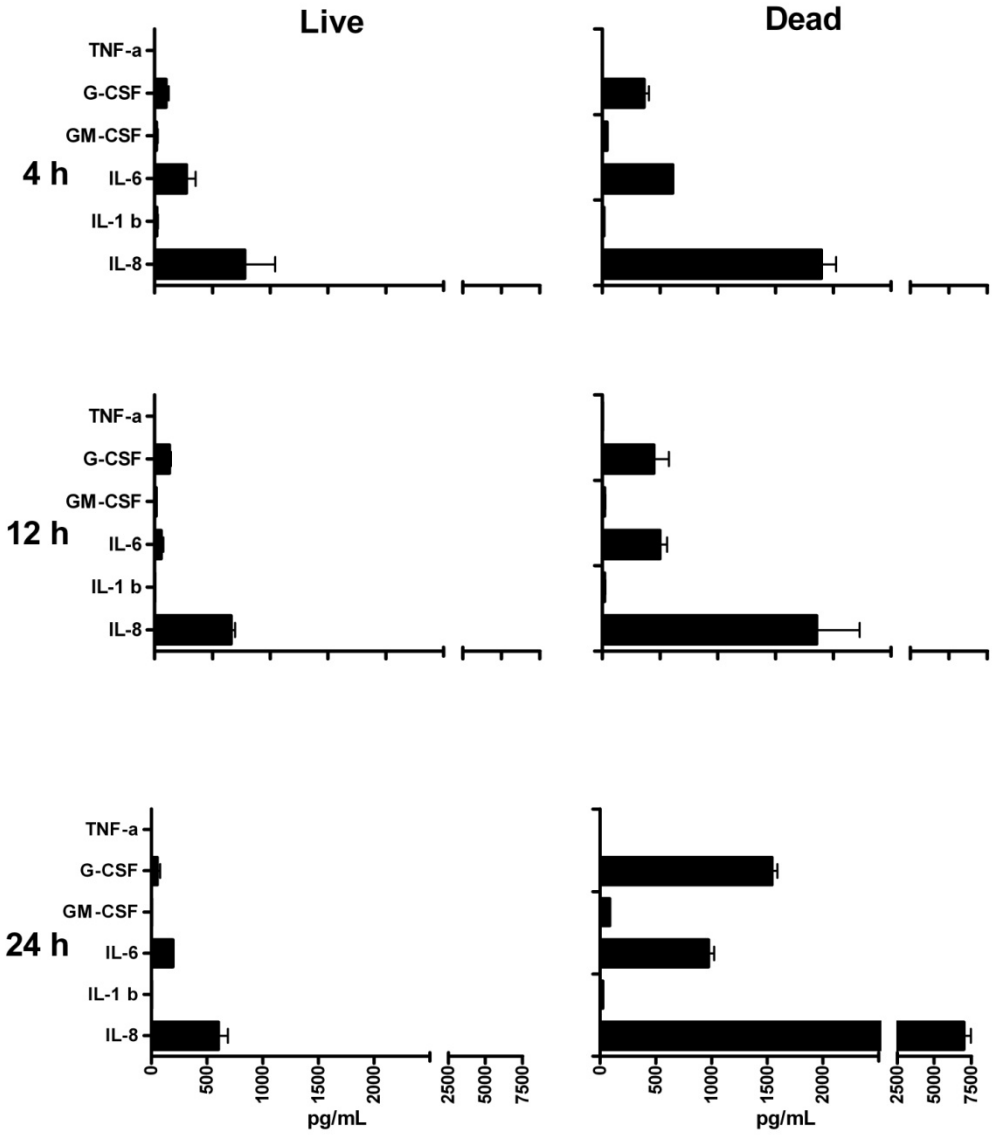
Cytokine	IL-8		IL-1β		IL-6		GM-CSF		G-CSF	
BIOFILM / TIME	L	D	L	D	L	D	L	D	L	D
1 h	42.49	72.67	0.77	0.77	12.16	29.48	14.26	14.26	32.10	32.10
4 h	812.62	777.58	5.06	4.56	152.53	211.03	7.14	7.14	85.48	127.39
6 h	517.10	305.15	11.27	7.92	62.28	181.89	14.26	14.26	197.31	114.68
8 h	1152.38	307.81	8.78	3.42	148.24	106.92	14.26	14.26	492.67	32.10
12 h	1013.65	276.99	11.63	3.81	87.57	108.23	21.39	14.26	218.71	83.67
18 h	600.84	361.10	13.50	6.52	48.80	146.37	14.26	14.26	274.41	124.76
24 h	310.96	2095.94	5.54	6.75	236.93	265.89	15.44	31.49	277.81	436.45

(C) *S. mitis* **biofilm**

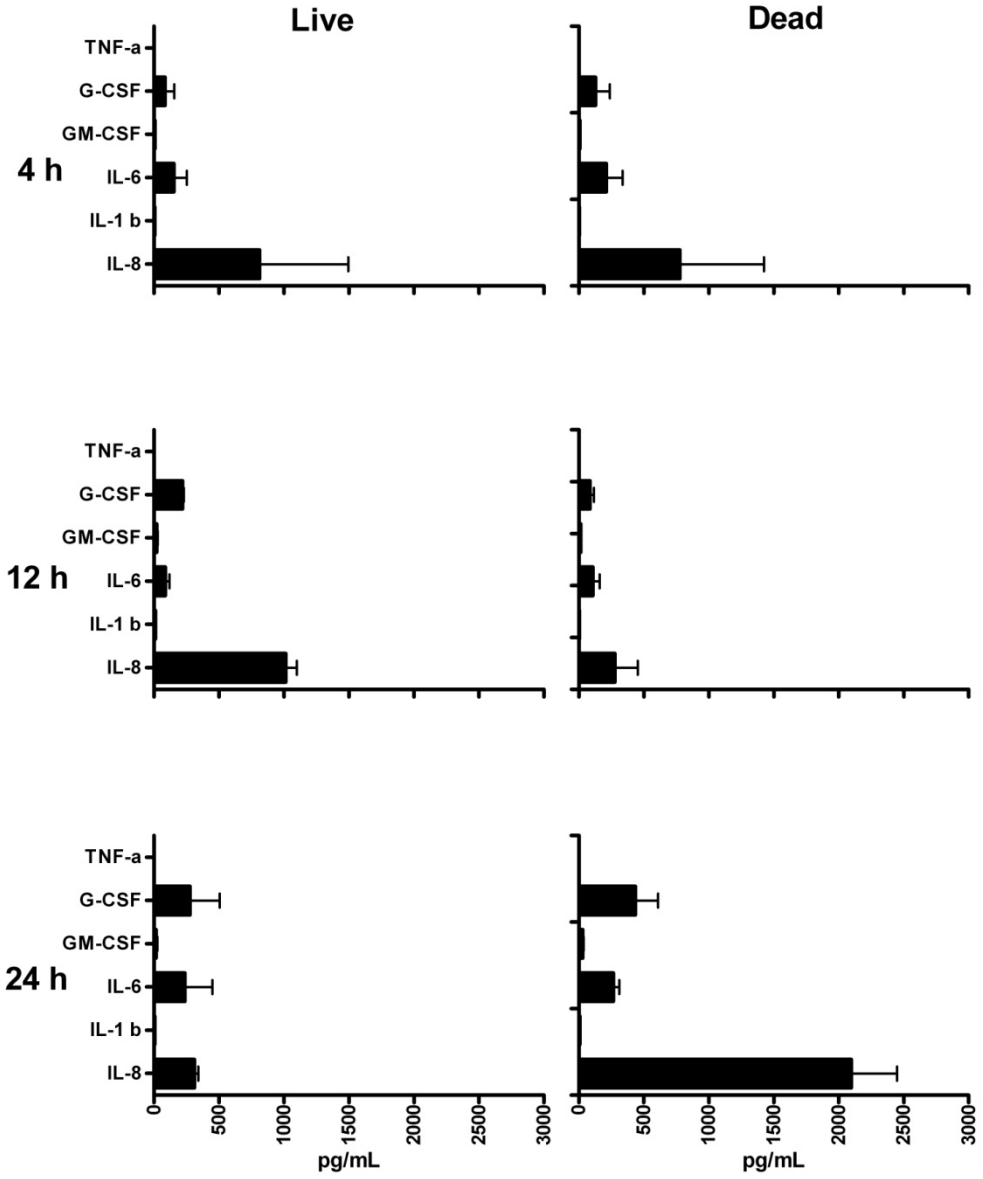
Cytokine	IL-8		IL-1β		IL-6		GM-CSF		G-CSF	
BIOFILM / TIME	L	D	L	D	L	D	L	D	L	D
1 h	61.45	82.22	0.77	0.77	19.91	39.36	14.26	14.26	13.27	32.10
4 h	224.16	372.94	0.36	0.36	67.84	63.19	4.75	4.75	15.75	49.94
6 h	993.30	254.63	18.87	11.50	586.22	229.76	53.76	14.26	225.38	94.63
8 h	849.03	306.31	11.93	3.81	337.12	125.93	43.98	14.26	158.90	114.68
12 h	727.50	486.49	16.38	8.32	473.32	165.20	53.76	14.26	249.39	119.50
18 h	551.71	230.79	21.89	5.22	574.70	143.47	59.44	14.26	222.36	76.59
24 h	577.38	1457.35	2.37	0.54	143.62	112.71	4.75	7.14	75.25	150.20

Table 4.4: Protein release in OKF6-TERT2 epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with **(A)** mixed biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), **(B)** *P. gingivalis* biofilm, **(C)** *S. mitis* biofilm for 1, 4, 6, 8, 12, 18 and 24 h. Protein concentrations in the cell culture supernatants were measured by Luminex® for inflammatory cytokine panel of genes (IL-8, IL-1 β, IL-6, G-CSF, GM-CSF and TNF-α). Data shown are protein concentrations in pg/mL. Values are means of duplicate measurements of two independent experiments.

(A) Mixed species biofilm



(B) *P. gingivalis* **biofilm**



(C) *S. mitis* **biofilm**

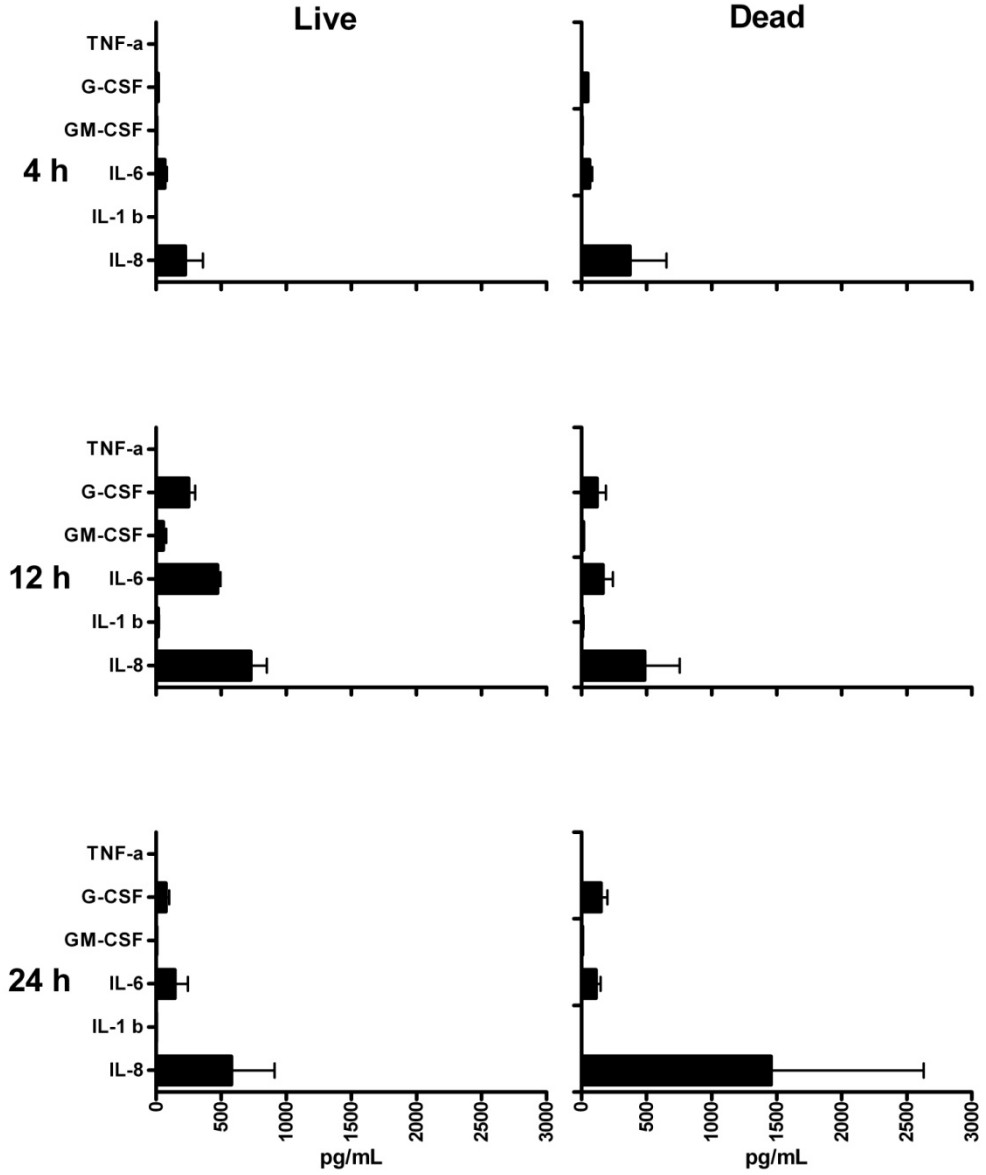
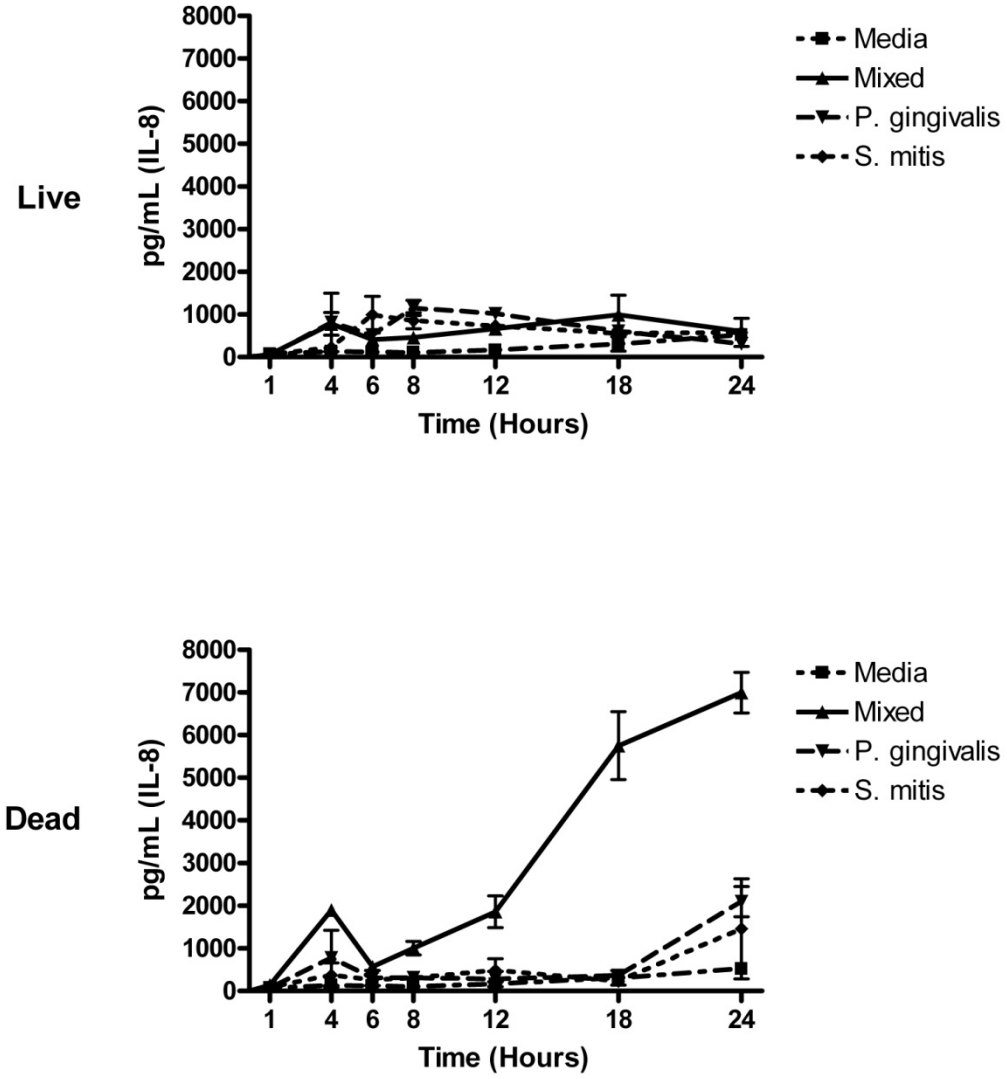
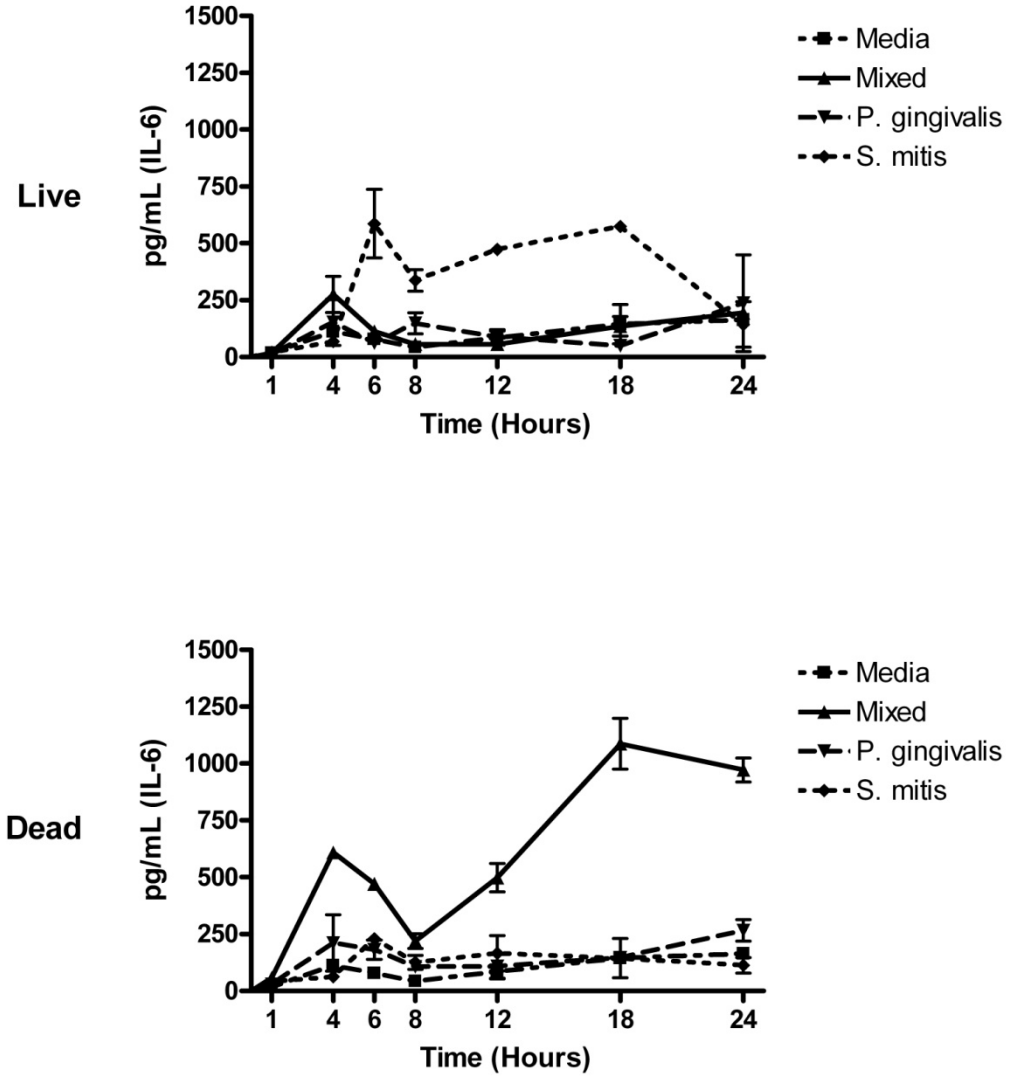


Figure 4.12: Protein release in OKF6-TERT2 epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with (A) mixed biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), (B) *P. gingivalis* biofilm, (C) *S. mitis* biofilm for 4, 12 and 24 h. Protein concentrations in the cell culture supernatants were measured by Luminex® for inflammatory cytokine panel of genes (IL-8, IL-1 ß, IL-6, G-CSF, GM-CSF and TNF-α). Data shown are protein concentrations in pg/mL. Values are means ± SEM of duplicate measurements of two independent experiments. These graphs show a global view of the data which includes all the inflammatory cytokine proteins released by the epithelial cells after co-culture with the different biofilm conditions at the time points tested.

(A) IL-8


Live
pg/mL (IL-8)
8000
7000
6000
5000
4000
3000
2000
1000
0
1
4
6
8
12
18
24
Time (Hours)
Media
Mixed
P. gingivalis
S. mitis
Dead
pg/mL (IL-8)
8000
7000
6000
5000
4000
3000
2000
1000
0
1
4
6
8
12
18
24
Time (Hours)
Media
Mixed
P. gingivalis
S. mitis

(B) IL-6


Live
pg/mL (IL-6)
1500
1250
1000
750
500
250
0
1
4
6
8
12
18
24
Time (Hours)
Media
Mixed
P. gingivalis
S. mitis
Dead
pg/mL (IL-6)
1500
1250
1000
750
500
250
0
1
4
6
8
12
18
24
Time (Hours)
Media
Mixed
P. gingivalis
S. mitis

(C) G-CSF

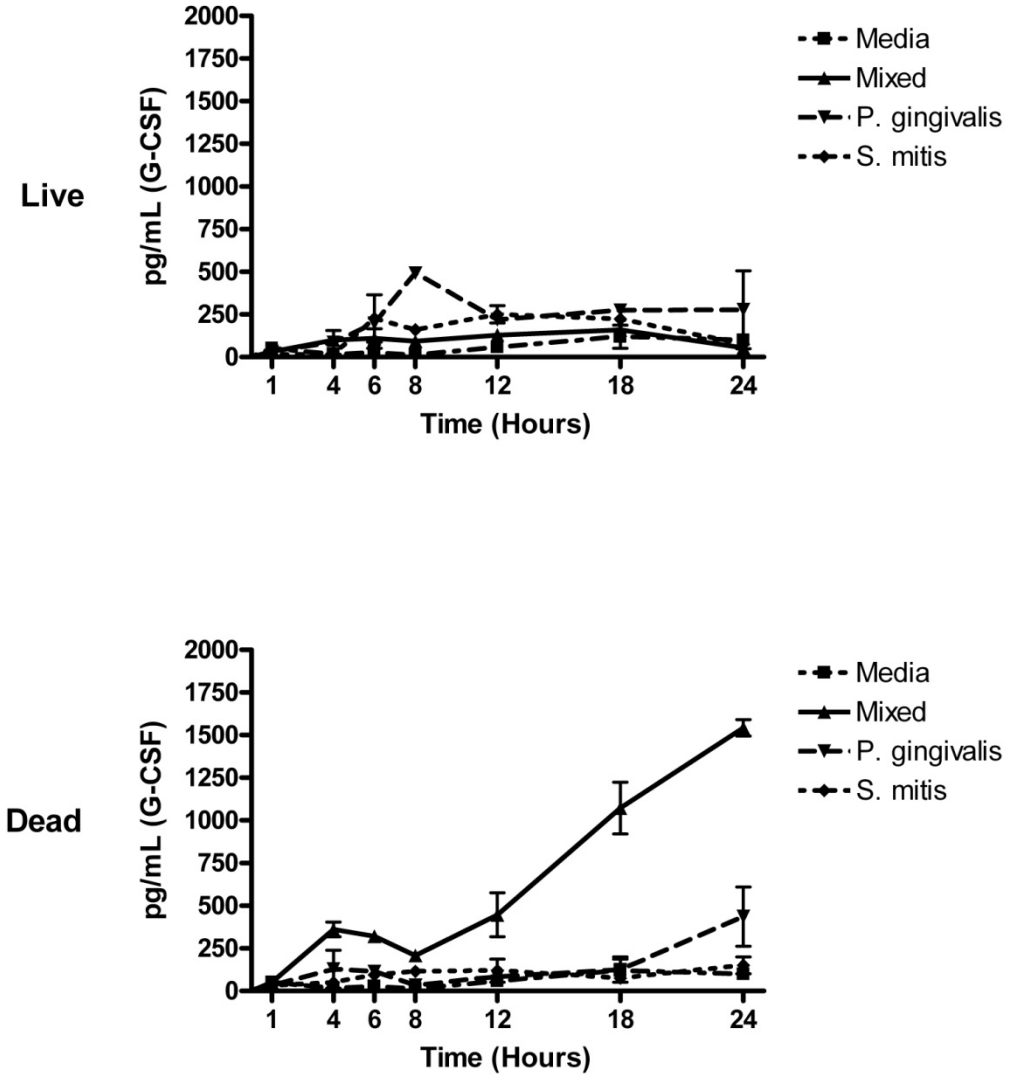


Figure 4.13: Protein release in OKF6-TERT2 epithelial cells challenged with live and methanol-fixed (dead) bacterial biofilms. OKF6-TERT2 epithelial cells were challenged with media, multi-species biofilm (*P. gingivalis, F. nucleatum, A. actinomycetemcomitans* and *S. mitis*), *P. gingivalis* biofilm and *S. mitis* biofilm for 1, 4, 6, 8, 12, 18 and 24h. Protein concentrations in the cell culture supernatants were measured by Luminex® for inflammatory cytokine panel of genes **(A)** IL-8, **(B)** IL-6 and **(C)** G-CSF. Values are means ± SEM of duplicate measurements of two independent experiments. These graphs represent the same data as in Figure 4.13, but based on individual proteins.

4.5 DISCUSSION

The current study investigated interactions between oral bacteria and the epithelium, aiming to further understand the aetiology and pathogenesis of periodontal disease. The epithelial-bacterial biofilm co-culture model created in earlier chapters 2 and 3 makes strides in part to successfully recapitulate the inflammatory changes in the host (epithelial cells) upon challenge with bacteria responsible for periodontal disease. Overall, the current study demonstrated: **(i)** the inflammatory response of the epithelium was different after stimulation with pathogenic or commensal bacterial biofilms, **(ii)** variability in the response of the epithelium when stimulated with bacteria in planktonic state compared with stimulation with biofilms, and **(iii)** stimulation of the epithelial cells with live or dead bacterial biofilms yielded differential inflammatory response from the epithelial cells.

In this study the chemokines, including CXCL1, CXCL3, CXCL5, CXCL10, CXCL11 and IL-8 were expressed more in the epithelial cells stimulated with pathogenic biofilms compared with commensal biofilms. These chemokines are responsible for the recruitment of immune cells responsible for innate (neutrophils) and adaptive (T cells) immune response to the site of inflammation. For example, IL-8 acts as a neutrophil chemoattractant aiding in the migration and extravasation of neutrophils into the sites of inflammation (Tonetti et al., 1994). Furthermore, greater levels of expression of the inflammatory cytokines including IL-1α, IL-1ß, IL-6, TNF-α, CSF2 (GM-CSF) and CSF3 (G-CSF) was observed when epithelial cells were stimulated with pathogenic biofilms compared with commensal biofilms. The increased activities of these chemokines and cytokines by the pathogenic mixed species and *P. gingivalis* biofilm compared with the commensal biofilms in this model agrees with what other investigators observed (Guggenheim et al., 2009, Peyyala et al., 2012, Peyyala et al., 2011a). Therefore, the current model may have a role to play in studies to understand the effects of host response to pathogens and commensals.

Pathogens (mixed species, *P. gingivalis*) were shown to induce greater inflammatory response in the epithelial cells compared with the commensals (*S. mitis*) at the mRNA and protein level, which was time dependant. In the current study lower levels of IL-8 production were observed from epithelial cells

stimulated by commensal bacteria compared with the pathogens, whereas *P. gingivalis* induced high levels of IL-8. Previous studies have also concluded that different dental plaque bacteria induce different host response profiles depending on their pathogenic or commensal status (Eberhard et al., 2009, Hasegawa et al., 2007, Krisanaprakornkit et al., 2000, Stathopoulou et al., 2010, Sliepen et al., 2009). In the study performed by Eberhard *et al* (2009), IL-8 mRNA expression was higher when the GECs were stimulated with *S. mutans* and naturally formed multi-species biofilms compared with *S. mitis* biofilm. Similar results were observed for this study where the pathogenic multi-species and mono-species biofilms induced greater gene expression in the epithelial cells compared with the commensal *S. mitis* biofilm. It was also shown that planktonic *S. mitis, S. salivarius* and *S. sanguinis* induced production of IL-8 from the epithelial cells and fibroblasts at a similar level to uninfected cells, whereas *A. actinomycetemcomitans* and *F. nucleatum* induction was significantly higher (Sliepen et al., 2009). Moreover, Hasegawa and co-workers (2007) showed that *S. gordonii*, which is a commensal in the oral cavity, induced significantly less gingival epithelial cell transcriptome compared with the pathogenic *P. gingivalis* and *A. actinomycetemcomitans* (Hasegawa *et al*, 2007). The study also showed IL-6 and IL-8 production was induced more by *F. nucleatum* compared with *S. gordonii*, a stereotypical commensal. In summary, these studies have demonstrated that inflammatory mediators produced by the host, depends on the pathogenic properties of the bacteria involved.

Biofilm formation is a key pathogenic characteristic of bacteria, particularly amongst those residing within the oral cavity (Ramage et al., 2010). It has been demonstrated from these studies that the putative periodontal pathogen *P. gingivalis* induces a greater IL-8 response in the epithelium at the mRNA and protein level in its biofilm state compared with planktonic bacteria. Previous studies have demonstrated differences in the host response to biofilms and planktonic bacteria (Daep et al., 2008, Oscarsson et al., 2008, Ramsey and Whiteley, 2009, Peyyala et al., 2011a). Oscarsson *et al* (2008) showed that there was enhanced IL-8 production from biofilms compared with planktonic *A. actinomycetemcomitans*. Using a biofilm rather than planktonic bacteria to study host-pathogen interaction is more representative with the *in vivo* situation because the periodontal bacteria in the dental plaque exist as complex microbial

consortia (Kolenbrander, 2000, Rosan and Lamont, 2000). Lo *et al* (2009) demonstrated that bacterial biofilms, which are dominant within the oral cavity, behave in a different manner to planktonic bacteria, as a comparative transcriptomic analysis of the *P. gingivalis* biofilm and planktonic cells revealed a difference in the genomic expression between the biofilms and planktonic bacteria (Lo et al., 2009). It was shown that several of the putative virulence determinants, sialidase (extracellular protease) and ADP-heptose-LPS heptosyltransferase (thiol protease), were up-regulated in biofilms compared to planktonic bacterial cells. Also, the transcriptomic profiling indicated a biofilm phenotype of slow growth rate and reduced metabolic activity. The altered gene expression profiles observed herein and elsewhere reflect the adaptive response of *P. gingivalis* to survive in a mature biofilm.

Studying host-pathogen interaction using bacterial biofilms instead of planktonic bacteria is more representative to the *in vivo* situation. At initiation of this thesis work no published studies existed that specifically investigated the host-pathogen interaction using a multi-species biofilm model at an orally relevant interface. However, subsequently several studies have appeared in the literature (Guggenheim et al., 2009, Guggenheim et al., 2001b, Peyyala et al., 2011a, Peyyala et al., 2012). These groups independently reported the creation of multi-species biofilm systems specifically created to model the oral epithelial inflammatory response. Peyyala and colleagues (2012) reported the use of planktonic, mono- and multi-species (3) biofilms containing *Streptococcus sanguinis, Streptococcus oralis, Streptococcus gordonii, Actinomyces naeslundii, Fusobacterium nucleatum, and Porphyromonas gingivalis* on rigid gas-permeable contact lenses. Challenge of OKF4 epithelial cells for up to 24h from planktonic or biofilm forms of these species induced Gro-1α, IL1α, IL-6, IL-8, TGFα, Fractalkine, MIP-1α, and IP-10. Interestingly, in agreement with this study herein *P. gingivalis* biofilms significantly inhibited the production of inflammatory mediators. Moreover, oral streptococcal species in both biofilms and planktonic forms showed poor stimulation, as did our study. This study did not however appear to monitor cell survival or bacterial parameters within the biofilm, and it was limited to only 3-species within the biofilm. Guggenheim and colleagues also produced a model was based on the Zurich biofilm model (Guggenheim et al., 2004). This 9-species sub-gingival biofilm model was shown to invoke apoptosis

of cells, and trigger the release of pro-inflammatory mediators, whilst degrading cytokines through biofilm-specifically generated enzymes. Whilst this group undertook a detailed systematic approach to evaluating the effect of the biofilm on primary gingival epithelial cells, they did not provide a direct comparison to planktonic and mono-species biofilms, thus limiting the interpretation of the role of each bacteria within the biofilm.

The selection of bacterial species within the biofilm plays a key role in defining the immune response. Interestingly, the dead bacterial biofilms induced greater chemokine and cytokine release from the epithelial cells compared with the live biofilms. Conversely, live bacterial biofilms induced greater chemokine and cytokine mRNA expression in epithelial cells compared with dead biofilms. Therefore, transcription and translation of inflammatory mediators was not consistent. This study and other studies have shown that periodontal pathogen bacteria, especially *P. gingivalis*, induce transcriptional up-regulation of chemokine and cytokine expression in the epithelial cells, but often inflammatory mediators are modulated downstream (Guo et al., 2008). *P. gingivalis* has been shown to inhibit IL-8 accumulation at two levels: (i) IL-8 degradation by proteinases and (ii) IL-8 regulation by unidentified factors (Darveau et al., 1998, Mikolajczyk-Pawlinska et al., 1998, Huang et al., 2001, Zhang et al., 1999). *P. gingivalis* gingipains may play a vital role in the evasion of host defenses by disrupting cytokine signalling networks (Andrian et al., 2006). Gingipains cleave and degrade most pro-inflammatory cytokines, including IL-1 ß (Fletcher et al., 1997), IL-6 (Banbula et al., 1999), TNF-α (Calkins et al., 1998), and IL-8 (Mikolajczyk-Pawlinska et al., 1998, Zhang et al., 1999). Killing *P. gingivalis* by methanol-fixation would have essentially immobilized the gingipains rendering it inactive to degrade the cytokines and chemokines. Another question that arises is if the chemokine or cytokine transcription actually translates into protein production? Sandros *et al* (2000) investigated whether cytokine transcription was translated into protein production by performing immunohistochemistry (IHC) on epithelial cells with biotinylated α-human IL-1ß, IL-2, IL-6, IL-8 and TNF-α to visualise intracellular cytokines (Sandros et al., 2000). All the infected samples showed positive reactions in 80-90% of the cells, while no apparent differences were observed among the 4 tested cytokines, suggesting that any effect of inflammatory

mediators was result of direct effect against the mature protein. This suggests that the changes in cytokine and chemokine expression within the different parameters may have been the result of live periodontal pathogens, including *P. gingivalis* due to the degrading properties of gingipains. However, we cannot discount entirely the potential for technical problems.

In summary, the current study showed differential immune responses to the pathogenic and commensal biofilms in epithelial cells. Even though both the pathogens and commensals had effects on the chemokine and cytokine expression and release, the pathogens induced more expression and release by the epithelial cells. Epithelial cells stimulated with mono-species biofilms does not reflect the immune responses demonstrated by a multi-species biofilm but it is useful in characterizing each individual bacterial species, which serves as a useful comparison in order to delineate complex inflammatory responses induced by multispecies biofilms. The current study was performed on monolayer of immortalized human oral keratinocytes but in the oral cavity, the bacterial biofilms come into contact with gingival tissue, which consists of multilayer of epithelial cells and connective tissue. The present study was performed *in vitro* and the conclusions drawn from these results must be interpreted with caution. Further studies are required to understand the workings of the host with regards to the bacterial biofilms and vice versa. The current study has established an in vitro model system, which allows to clearly differentiate, *in vitro*, the different response to commensals versus pathogens. This is a critical step to understanding both periodontal and other diseases. The hope for the future is to unravel the details of the mechanisms involved and thereby identify novel therapeutic targets for inflammatory and infectious disease.

CHAPTER 5:

ROLE OF INTERLEUKIN-17A IN PERIODONTAL DISEASE

5.1 INTRODUCTION

The previous chapter investigated a panel of cytokines and chemokines that were shown to be associated with experimental gingivitis, in particular those mediators produced by epithelial cells, which are central to orchestrating the immune response. These molecules were released from epithelial cells as a means of alerting the immune system that periodontal biofilms were present. Recent data indicates that early cell migration to the gingivae in periodontitis is, surprisingly, strongly mediated by a cytokine associated more with the adaptive response, IL-17A (Yu *et al.*, 2007), which is key molecule to inducing and mediating numerous pro-inflammatory responses (Aggarwal and Gurney, 2002).

Interleukin-17A (IL-17A) was initially identified in T cells (Liang et al., 2010), although subsequent studies identified numerous other cellular sources of IL-17A, including γδ T cells, NK cells, neutrophils, eosinophils, LTi CD4 cells (Kimizuka et al., 2012) and mast cells (Hueber et al., 2010). IL-17A induces cytokine and growth factor production predominantly via signalling through the IL-17RA and IL-17RC heterodimer. IL-17RA is ubiquitously expressed, especially within hematopoietic tissues, and also by epithelial cells, endothelium, and fibroblasts (Gaffen, 2009). A protective role for IL-17 in bacterial and fungal infections emerged through studies in IL-17/IL-17RA deficient animals, which are susceptible to mucosal candidiasis (Takahashi et al., 2005), *Klebsiella pneumoniae* (Conti et al., 2009), *Legionella pneumonia* (Allam et al., 2011) and *Porphyromonas gingivalis* (Yu et al., 2007). In the latter model investigating IL-17R deficient animals, IL-17 conferred protection by promoting neutrophil migration to the gingival tissues. The absence of IL-17R signalling significantly compromised the antimicrobial effects of infiltrating neutrophils, rendering the animals more susceptible to periodontal disease.

IL-17A has been implicated in the pathogenesis of many chronic inflammatory diseases, such as Rheumatoid Arthritis, Psoriasis, Crohn's Disease and Multiple Sclerosis (Korn et al., 2009). Numerous studies of models of arthritis, a diseases process with notable parallels to periodontal disease, demonstrate IL-17 may contribute to initiation and perpetuation of chronic, destructive inflammation (Miossec et al., 2009). Neutralising IL-17A is therapeutically beneficial, and a neutralising anti-IL17A monoclonal antibody has demonstrated promise as a

treatment for Rheumatoid Arthritis in human trials (Genovese et al., 2010). Therefore, IL-17 plays a potentially double-edged role in periodontal disease (PD), with the ability to both protect against infection and perpetuate inflammation.

This conundrum is highlighted by findings in aged mice, which, in contrast to the findings in IL-17RA deficient animals, demonstrate increased periodontal bone loss, with associated substantial elevation of local IL-17A mRNA (Duarte et al., 2010). In human studies, IL-17A mRNA expression is elevated in the gingival crevicular fluid and tissues of individuals with chronic periodontitis, compared with healthy controls (Vernal et al., 2005, Beklen et al., 2009). Systemically elevated serum IL-17A has been documented in patients with aggressive periodontitis (Schenkein et al., 2010). Further highlighting the association of IL-17A with infection and inflammation, successful periodontal therapy results in a reduction in IL-17 expression (Cua and Tato, 2010). However, further studies have suggested that periodontitis may associate with reduced IL-17 in saliva (Zhao et al., 2011) and reduced concentration of IL-17 in the GCF of patients with GAP (Dutzan et al., 2011). Therefore, it may seem that in certain patients, at certain stages, IL-17 may mediate conflicting roles of both promoting protective neutrophil recruitment and perpetuating destructive inflammation. IL-17 is an attractive target for modulating bone destructive diseases including periodontitis. We sought to investigate alternative cellular sources of IL-17A, establish if it might be released from the tissue, and investigate potential effects of IL-17A on local epithelium.

5.2 AIMS

The aim of this study was to determine whether IL-17A is expressed within and released from periodontal tissues and to investigate its role in the regulation epithelial cell cytokine and chemokine production.

5.3 MATERIALS AND METHODS

5.3.1 Human periodontal tissues

Gingival tissues (n = 34) were obtained from patients with written consent, undergoing open flap debridement in the Unit of Periodontics at Glasgow Dental Hospital. Ethical review and approval was provided by the West of Scotland Research Ethics Committee. Patients undergoing open flap debridement had probing pocket depths of ≥ 6.0 mm, with clinical attachment loss of ≥ 4.0 mm, which persisted after non-surgical treatment. Patient age ranged from 38-64 years with a mean age of 47 years. Healthy control tissues were obtained from patients with clinically healthy periodontal tissues undergoing surgical crown-lengthening procedures.

5.3.2 mRNA and protein detection

Tissue samples were stored in RNA Later (Ambion) at -80°C prior to RNA extraction using RNeasy Fibrous Tissue Kit (Qiagen), as section 4.3.1, but with addition of a proteinase-K digest step. Cultured cells were processed as in Section 4.3.1. Reverse transcription, and Real time PCR was carried as in section 4.3.1, with primers as in table 4.2 and additionally for IL-17A: Forward primer – GGAATCTCCACCGCAATGAG, Reverse primer – ACACCAGTATCTTCTCCAGCC. Data were analyzed Proteins in cell culture supernatants were detected by either EILSA (IL-6, IL-8 – both eBiosource, Invitrogen) or Luminex™ (G-CSF and GM-CSF – both Invitrogen) according to the manufacturer's instructions and as described in section 4.3.2.

5.3.3 Fluorescent microscopy and immunohistochemistry

Five μm sections paraffin embedded periodontal tissues were deparaffinized and rehydrated. The 0.5% hydrogen peroxidase/methanol incubation and heat retrieval in 0.5 M citrate buffer (pH 6) was followed by incubation in 2.5% species/2.5% human serum with Avidin D (Vector Laboratories, Petersborough, U.K.). Staining for 1 h with mouse anti-mast cell tryptase [MCT] [0.43 mg/ml; Dako UK], was followed by 30 min incubation with biotinylated Abs (1:200; Vector Laboratories) with subsequent staining with streptavidin QDot605 (1:250; Invitrogen, Paisley, U.K.) for 45 min. Goat anti–IL-17 (5 mg/ml; R&D Systems, Abingdon, U.K.) was added overnight at 4^{o}C, then incubated with a biotinylated Ab for 30 min, and stained with Avidin FITC (1:500; Vector Laboratories) for 45

min. Slides were mounted with Vectashield containing DAPI (Vector Laboratories) and analyzed on a fluorescent imaging microscope (BX50; Olympus, Essex, U.K.). Images were captured using Apple Open laboratory software. For immunhistochemistry, prepared periodontal tissue sections were incubated with goat anti–IL-17 overnight, incubated with ImmPRESS reagent anti-goat Ig for 30 min (Vector Laboratories), and signal-developed using the peroxidase sub- strate nickel diaminobenzidine tetrahydrochloride (Vector Laboratories).

5.3.4 Cell culture

OKF6-TERT oral epithelial cells (kind gift of Rheinwald laboratory, Brigham and Woman's Hospital, Boston**)** were grown at 37°C, with 5% CO_2 in 75 cm^2 cell culture flask (Corning NY, USA) in KSFM (Invitrogen). The cells were replated and grown to confluence in defined KSFM (Invitrogen) then stimulated as indicated in the figure legends. Cells were used were between passage 13 and 15. Periodontal tissue was transferred immediately after collection to chilled RPMI (Invitrogen), then a single cell suspension prepared using Liberase™ (Invitrogen) according to the manufacturer's instructions. Cells were cultured at $2x10^6$/ml in conditions indicated in the figure legends.

5.3.5 Statistical analysis

Microsoft Excel and GraphPad Prism were used to process raw data and implement statistical analysis in conjunction with graphical design. Differences in mRNA abundance in healthy and periodontitis tissue biopsies as assessed by real-time PCR analysis were analysed by the Mann-Whitney U-test. Mann-Whitney U-test is a non-parametric statistical hypothesis test for assessing whether one of two samples of independent observations tends to have larger values than the other. This test can be used when the data is not normally distributed; if the variances for the two conditions are noticeably different or if the data are measurements on an ordinal scale (allowing for rank order) (Kruskal, 1957). Differences between the mean cytokine protein levels between stimulated and control samples were analysed by one-way ANOVA the Levene's test to test for the equality of variances in the samples and a *post hoc* Dunnett t-Test. Levene's test is used to test if different samples have equal variances. Statistical tests like ANOVA assume that variances are equal across groups or samples and Levene test can be used to verify that assumption (Levene, 1960).

Dunnett's test compares group means where all the groups are compared against one control group. The goal of this test is to identify groups whose means of the distributions are significantly different from the mean of the control group (Dunnett, 1964). Correlations between the cytokine levels were assessed using a bivariate Pearson correlation analysis. The bivariate Pearson correlation analysis is used to test the correlation between variables. Results are between -1 and 1. A result of -1 means that there is a perfect negative correlation between the two values, while a result of 1 means that there is a positive correlation between the two variables (Rodgers and Nicewander, 1988).

5.4 RESULTS

5.4.1 Expression of IL-17A in human periodontal tissues. IL-17A gene expression was significantly elevated in diseased compared with healthy human periodontal tissues (Figure 5.1). Protein expression was confirmed by immunohistochemistry (Figure 5.2). There was considerable variability in the number of IL-17 positive cells within the diseased tissue samples, ranging from between 2 and 4 positive cells per field of view to over 20 positive cells per field of view. The healthy tissue did not demonstrate any IL-17A positive cells (data not shown). In order to identify the cellular source of the IL-17A protein, samples were initially investigated for CD3 and variable numbers of CD3 positive cells were found infiltrating the diseased tissue. When assessed by immunoflouresence, the CD3 positive cells did not account for all the IL-17 present, therefore, as previous studies document high expression of IL-17 by mast cells in inflamed tissues (Hueber et al., 2010), therefore, IL-17 expression by mast cells was investigated. A composite overlay (Figure 5.3) demonstrates that the mast cells were predominantly IL-17A positive.

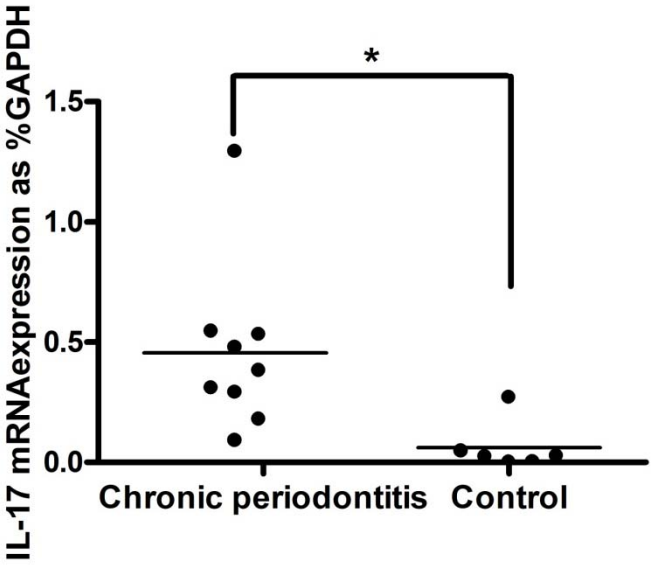


Figure 5.1: Expression of IL-17A in human periodontal tissue. IL-17 mRNA expression in gingival tissue obtained from patients with chronic periodontitis (n = 9) or healthy controls (n = 6). Each dot represents an individual patient sample. The difference in the IL-17A mRNA expression between the diseased and healthy tissue samples were assessed by Mann Whitney U test (* $p < 0.005$).

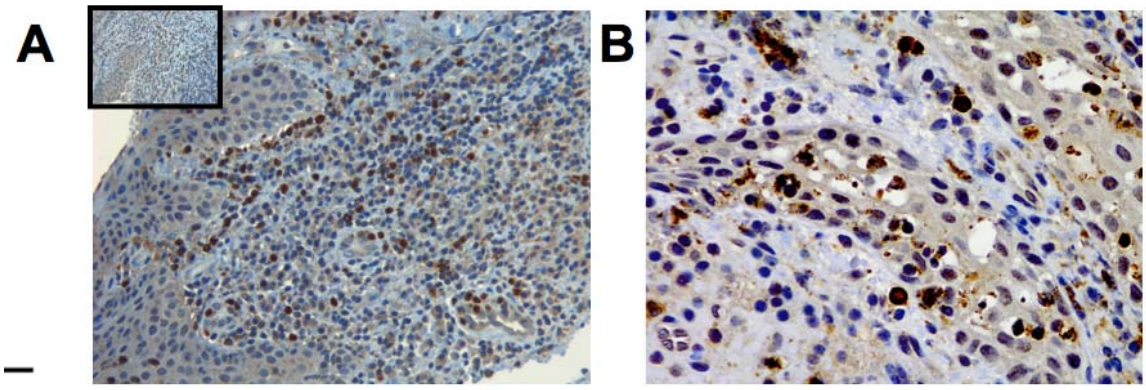


Figure 5.2: Expression of IL-17A in human periodontal tissue by immunohistochemistry. Sections of paraffin embedded periodontal tissue, from patients with chronic periodontitis, demonstrating IL-17A positive cells stained brown (inset box shows isotype control) at 100 X **(A)** and 400 X **(B)**.

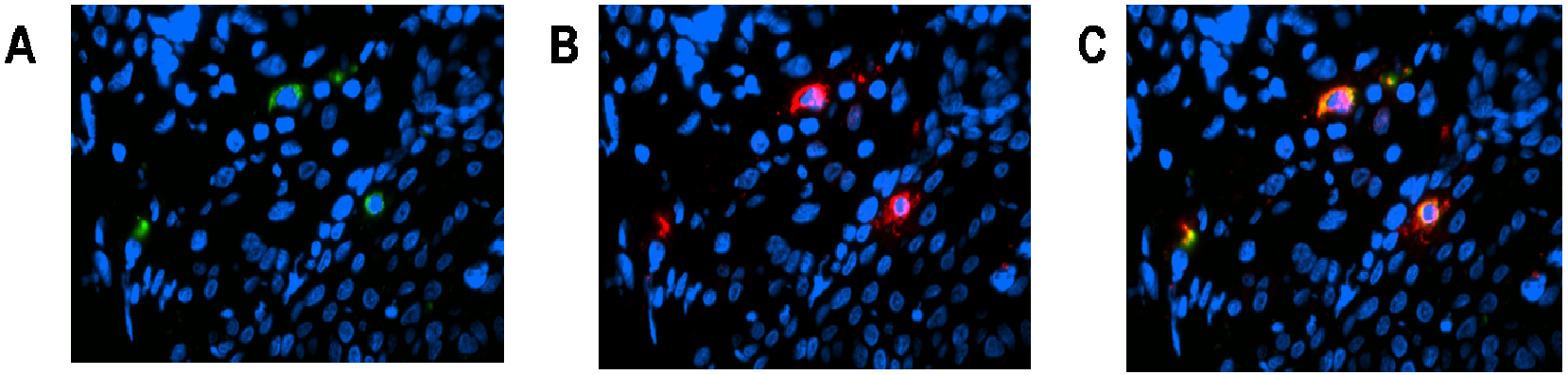


Figure 5.3: Expression of IL-17A in human periodontal tissue by Immunofluorescence. Immunofluorescence demonstrating IL-17A in green **(A)**, mast cells in red **(B)**, and composite overlay showing co-localization of IL-17A to mast cells **(C)**.

5.4.2 Release of IL-17A from periodontal tissue. Single cell suspensions obtained from diseased human periodontal tissues were stimulated with PMA/ionomycin for 48 h. Only following stimulation, IL-17A was detectable by ELISA in the cell culture supernatants of 9 out of 10 patients' samples. There was an apparent dichotomy: cultures from 50% of the patients releasing considerably more IL-17A than the other samples. There was no correlation between these patients' clinical condition (probing pocket depths at surgical site or age) and the concentration of IL-17A released. Interestingly, IL-17 release was not stimulated by LPS (either *P. gingivalis* or *E. coli*, data not shown). Next, the release of other inflammatory cytokines into the cell culture supernatants was investigated to establish if the IL-17 release reflected a different inflammatory profile overall. Considerable IFN was released only upon cell stimulation, (Figure 5.4B). TNFα was found in high concentrations in both the unstimulated and the stimulated cell culture samples (Figure 5.4C). IL-1ß release was highly variable amongst the unstimulated and stimulated samples (Figure 5.4D). IL-6 concentrations decreased upon stimulation compared with the unstimulated samples (Figure 6.4E). High concentrations of IL-10 were released from both the unstimulated and stimulated samples (Figure 5.4F). There was a possibility of a link between the baseline concentration of IL-6 and the final concentration of IL-17A, however this was not statistically significant. Due to the small number of patient samples no link or correlation between the different cytokines could be inferred with certainty. Nevertheless, it was noted in baseline samples that IL-1ß, IL-6 and IL-10 concentrations were all correlated (all > 0.730, $p < 0.025$). These apparent relationships were lost on stimulation, where after IL-6 correlated with IFNγ (0. 733, $p=0.024$). No correlation between IL-17A concentrations and the other cytokines could be found, therefore there was no apparent 'profile' or cassette of cytokines associated with particular patient samples.

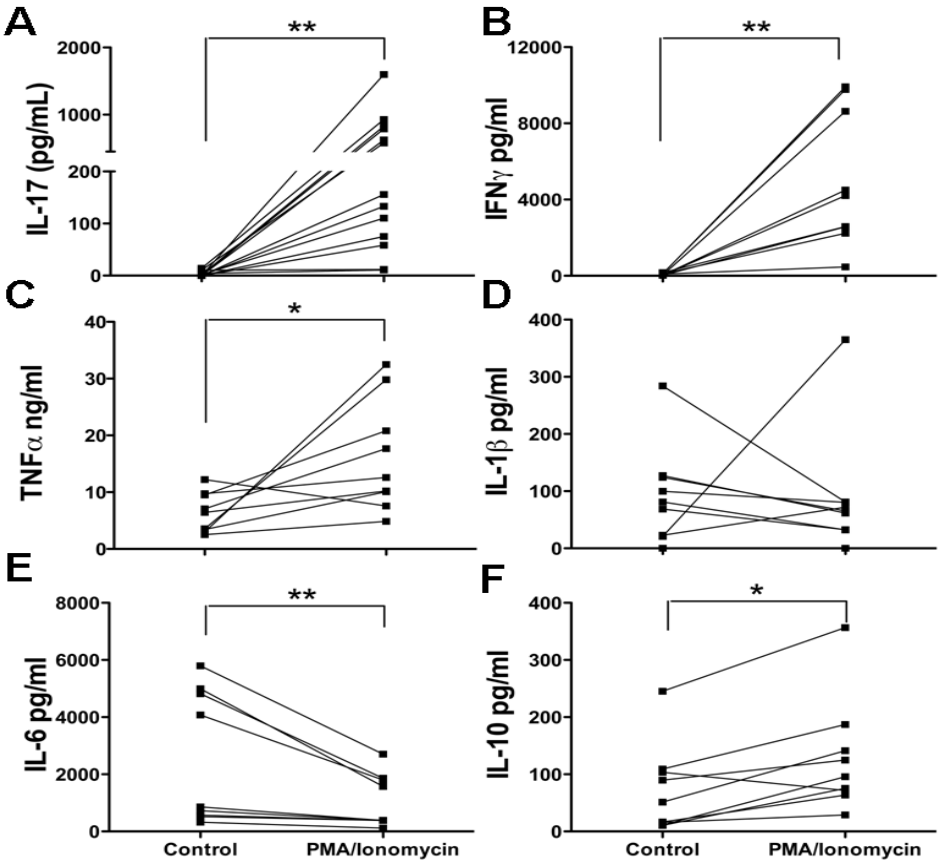


Figure 5.4: Cytokine release from tissues obtained from patients with chronic periodontitis. Diseased periodontal tissue samples (n=10) were enzymatically digested to single cell suspensions and then stimulated with PMA/Ionomycin for 48 hours. Cytokine release was assessed by Luminex™ of cell culture supernatants. Each point represents an individual patient sample. Data shown are cytokine concentrations of **(A)** IL-17A, **(B)** IFNγ, **(C)** TNFα, **(D)** IL-1ß, **(E)** IL-6 and **(F)** IL-10 in pg/mL in the stimulated and unstimulated samples. Differences between pre- and post-stimulation levels of cytokines secreted by periodontal tissue cells were assessed by paired sample Wilcoxan signed ranks test. (* $p < 0.05$; ** $p < 0.005$).

5.4.3 Effect of IL-17A on OKF6 oral epithelial cells. It was hypothesised that IL-17 released in the periodontium likely acts locally on epithelial cells and this was first investigated by confirming expression of the IL-17 Receptor (IL-17R) subunits on the oral epithelial cell line, OKF6, by PCR (data not shown). Culture with IL-17A for 4 and 24 h resulted a dose and time dependent upregulation of a number of chemokine and cytokine genes (Figure 5.5), including CXCL-1, -3 -5, IL-8, CCL5, IL-1α, IL-1ß, GMCSF and GCSF. Protein release was investigated by ELISA and Luminex™ of cell culture supernatants. OKF6 cells demonstrated a dose response to IL-17A, which resulted in a statistically significant release of IL-8, IL-6, G-CSF and GM-CSF after 4 h stimulation ($p = 0.037$, $p = 0.0040$, $p = 0.037$ and $p = 0.0012$, respectively) and after 24 h for all but the GM-CSF ($p = 0.0023$, $p = 0.0040$, $p = 0.035$ and $p = 0.060$ respectively) (Figure 5.6). IL-1α and TNFα were also analysed, however, no protein product was detected in the culture fluids (data not shown). Thus, IL-17A appeared to have potent effects on epithelial cells, inducing a dose and time dependent upregulation of numerous chemokines and cytokines by oral epithelial cells.

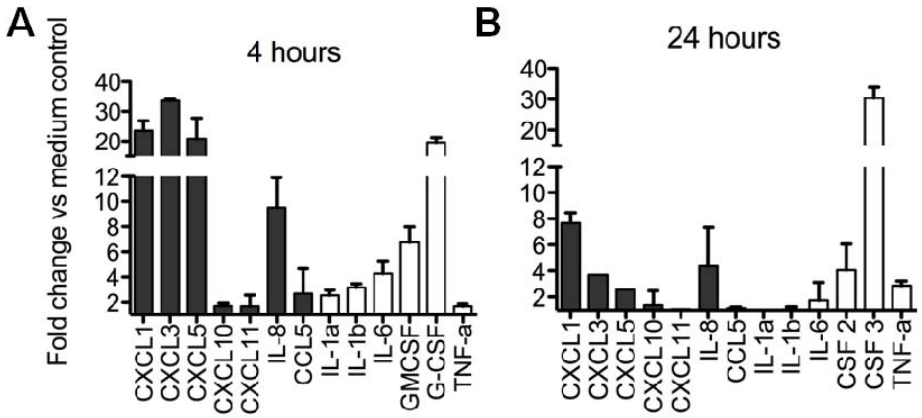


Figure 5 .5: Gene expression changes in epithelial cells stimulated with IL-17. OKF6 cells were stimulated with 100 ng/ml IL-17A for 4 h (A) and 24 h (B). Gene expression was analysed by TaqMan Low Density Array (TLDA) allowing multiple genes to be analysed simultaneously. Graphs demonstrate fold change in gene expression following IL-17A stimulation. Data are mean fold change +/- SEM of duplicate measurements from two independent experiments.

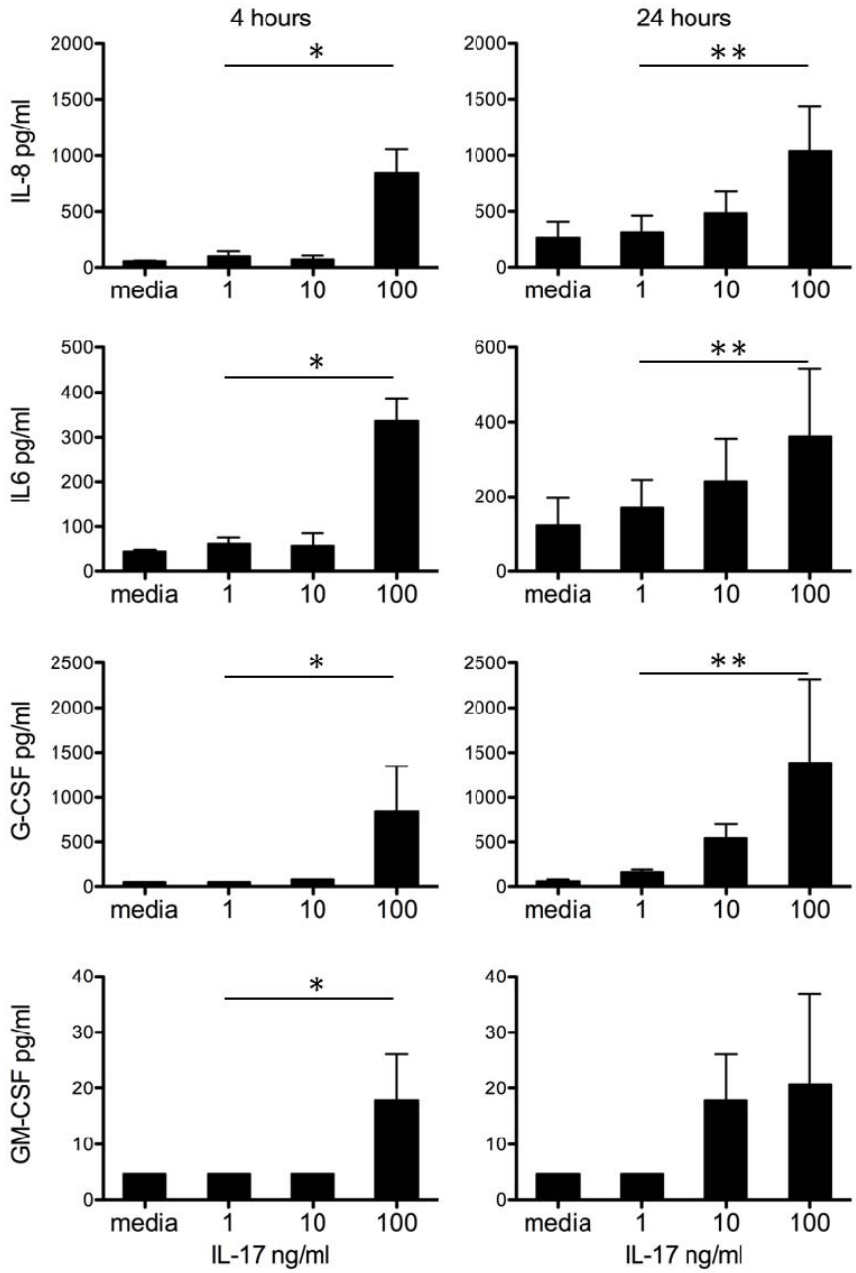


Figure 5.6: Cytokine and chemokine release from epithelial cells stimulated with IL-17A. OKF6 cells were stimulated with 1, 10 or 100 ng/ml IL-17A for 4 h or 24 h, after which the cell culture supernatants were analysed by ELISA/Luminex™. Data are mean +/- SEM of triplicate measurements of two or more independent experiments. The difference in dose response of the IL-17A with respect to IL-8, IL-6, G-CSF and GM-CSF release from the epithelial cells were assessed by Jonckheere-Terpstra Test (*$p < 0.05$, **$p < 0.005$).

5.5 DISCUSSION

IL-17A expression was significantly increased in diseased, chronically inflamed periodontal tissues, compared with healthy tissues, and a considerable proportion of this IL-17 localised to mast cells. Moreover, IL-17 could be released in response to calcium and protein kinase C agonists. IL-17A may locally activate a number of cells including oral epithelial cells resulting in increased chemokine and cytokine expression. This study is, to the best of our knowledge, the first to document the expression of IL-17 in mast cells in periodontal disease. Previous studies have documented elevated IL-17A mRNA expression in chronically inflamed periodontal tissues compared with gingivitis models and healthy controls (Vernal et al., 2005, Takahashi et al., 2011, Beklen et al., 2009), and elevated IL-17A protein in GCF (Vernal et al., 2005). These data confirm these findings and imply that the IL-17 detected in GCF is likely to be predominantly derived from local cells rather than the serum, although elevated serum IL-17A has been reported in patients with aggressive periodontitis (Schenkein et al., 2010).

Following periodontal treatment both local and systemic effects on IL-17 expression have been documented (Perregaux and Gabel, 1998, Cua and Tato, 2010), suggesting that the elevated IL-17 expression is driven either directly by the pathogenic plaque biofilm or secondarily to mediators induced by the biofilm. Cardoso *et al* elegantly demonstrate the presence of Th17 cells in the periodontitis lesion (Cardoso et al., 2009), and the current study suggests that these cells, and mast cells, are likely to both provide sources of IL-17 in these tissues. Recent data suggests that at different stages of disease, periods of disease intensity and even at different locations within lesions adjacent to the same tooth, there may be variable expression of IL-17 as the cellular composition of the lesion changes (Liang et al., 2006). The role of the mast cell in periodontal disease although intriguing, remains relatively poorly understood (Berglundh and Donati, 2005, Gemmell et al., 2004). Both mucosal (Mu) and connective tissue (CT) mast cells are consistently identified in both health and inflammatory disease in the oral cavity (Gemmell et al., 2004, Steinsvoll et al., 2004). The density of mast cells is increased in chronic periodontitis and, as would be expected the Mu mast cells appear to dominate the oral mucosa (Gemmell et al., 2004). Mast cells produce numerous cytokines (Galli and Tsai,

2010) implicated in periodontal pathogenesis, including IL-1 and TNFα, and the current study described their capacity to produce IL-17. It could be argued that in the context of bacterial infection, the protective roles of IL-17 at mucosal surfaces may dominate the pathogenic. Clearly, the timing of such cytokine release is likely to be critical to its ultimate role. Mast cells are positioned to rapidly release numerous mediators capable of promoting neutrophil recruitment, which is essential to early bacterial control, but likely contributes to later stages of chronic inflammation. Thus, perhaps mast cell derived cytokines and other mediators offer potential novel therapeutic targets.

Here it was also documented that IL-17 exerts a broad range of functions in oral epithelial cells, which extends previous studies investigating IL-17 augmentation of TLR mediated IL-1ß and TNFα release from gingival epithelial cells (Beklen et al., 2009). Given the previously reported synergism between both IL-17A and IL-17F and IL-22 (Liang et al., 2006, Kolls et al., 2008) this was investigated in the current study system; however, no additional effect above that of IL-17 was observed. The lack of release from the epithelial cells of IL-1α and TNFα is somewhat surprising, given their apparent gene regulation, however, this may reflect the requirement for processing prior to release (Perregaux and Gabel, 1998). Similarly, TNFα requires processing by TACE ADAM17 (Pradines-Figueres and Raetz, 1992).

From this data, it appears that IL-17A plays an important role in the protective periodontal immune response to bacterial pathogens. The upregulation of acute inflammatory mediators (such as IL-8) will promote neutrophil recruitment and potentiate the removal of any invading microbial threat. Therefore it is important to understand the benefits of this cytokine, before systemic therapeutic agents are used to antagonise its actions. The previous chapter discussed the potential roles that the innate inflammatory mediators play in the pathogenesis of periodontal disease. Some demonstrated protective roles and some destructive. IL-17 seems to play a role in the protective periodontal immune response by the release of IL-8 to counter the bacterial pathogens. It would be interesting to investigate the IL-17 expression using the epithelial cell-biofilm co-culture model used in the previous chapters to understand the effects of the bacterial biofilms in the IL-17 expression.

CHAPTER 6:

DISCUSSION

6.1 INTRODUCTION

6.1 Summary

Periodontal disease involves a complex network of interactions between bacterial (biofilm and planktonic) and cellular responses. This series of investigations has demonstrated this phenomenon through a reductionist approach, i.e. the successful development of an epithelial-bacterial biofilm co-culture model system, which recapitulates the inflammatory changes in host epithelial cells upon challenge with bacteria associated with periodontal disease. In summary, this model has demonstrated clear differences between **(i)** planktonic and bacterial biofilms, **(ii)** pathogens and commensals and **(iii)** live and dead bacterial challenge. These observations and the utility of the model will provide a platform to investigate key questions relating to pathogen and host within the oral cavity and beyond.

6.2 *In vivo* studies of periodontal disease

In vivo study models have been used extensively to investigate the pathogenesis of periodontal disease. These models include human and various animal models. The quest for understanding the pathogenesis of periodontal disease is rooted in an important human experimental gingivitis study almost half a century ago (Löe et al., 1965). This study significantly impacted on periodontal research because of the simple and instructive demonstration of the causal role of plaque bacteria in gingivitis. Similar studies latterly showed that cell-mediated immune response played an important role in the process (Smith et al., 1978). Recently, Offenbacher's group evaluated the inflammatory mediators in the GCF during the induction and resolution of experimental plaque-induced gingivitis in humans (Offenbacher et al., 2010, Offenbacher et al., 2009). Ultimately, these studies showed that the destructive inflammatory mediators produced in response to the bacterial plaque are reversed once the causative factor (bacterial plaque) is removed. Even though these studies were instrumental in understanding periodontal disease, prospective studies to investigate the mechanisms underlying the progression of periodontal disease cannot be answered by *in vivo* studies of humans alone due to important ethical considerations (Williams et al., 2012). This is especially true given the growing body of literature showing an association between periodontal disease and systemic health (Offenbacher et al., 2010, Offenbacher et al., 2009, Lockhart et al., 2012).

To overcome these ethical limitations animal models have been used extensively to study host-bacterial interactions and have assisted in elucidating the pathogenesis of periodontal disease (Graves et al., 2012). These studies have helped demonstrate the clinical progression of periodontitis as subclinical gingivitis, clinical gingivitis and periodontal breakdown. However, as in human models there are limitations, including species differences and diverse microflora, they only provide a 'snapshot' of the disease process, and of course the ethical implications of animal use (Gruber and Hartung, 2004). To illustrate this point, a murine Calvarian model was used to investigate host-bacterial interactions with *P. gingivalis* (Boyce et al., 1989, Graves et al., 2001, Leone et al., 2006). This model was not performed within an oral environment and also provides no information relating to the interactions with the epithelial tissues, therefore its validity must be questioned. Moreover, an oral gavage model using human periodontal pathogens to study the consequent impact on periodontium has been used extensively (Baker et al., 1999, Chang et al., 1988, Garlet et al., 2005, Klausen et al., 1991, Lalla et al., 1998, Sharma et al., 2005). These key pathogens are not indigenous mouse oral microflora, so prior antibiotic treatment is required to reduce the resident microflora, so the impact on the host and its translation to human oral disease is questionable. Similar arguments can be made about tissue cage, chamber models and airpouch models (Edwards et al., 1981, Pouliot et al., 2000, Genco et al., 1991, Genco et al., 1992). Ultimately, the main drawback of these animal models is that they do not accurately recapitulate human periodontal disease, creating more questions than answers. Moreover, *P. gingivalis* from Socransky's red complex is touted as the alpha-periodontal pathogen (Socransky et al., 1998), and is therefore subject to the majority of studies (Hajishengallis et al., 2012). Yet, increasing evidence from recent studies indicate that bacteria such as *Filifactor alocis* appear to play a significant role in periodontal disease (Schlafer et al., 2010). Moreover, the contribution of uncultivable bacteria and multispecies consortia should not be discounted (Peters et al., 2012). Modelling a large number of experimental parameters in animal models is therefore problematic, so *in vitro* studies provide an opportunity to refine and reduce these parameters.

6.3 *In vitro* modelling of periodontal disease

Modelling biofilms associated with periodontal disease has been subject to numerous studies over the years (Hope et al., 2012, Marsh, 2006, Periasamy and Kolenbrander, 2009b, Peyyala et al., 2011b, Pratten, 2007). Defined and undefined biofilm consortia have been evaluated to provide insight into how oral bacteria interact with one another (Lamont and Jenkinson, 1998). However, recent studies have evolved this concept to include mammalian cells in an attempt to understand how these interact with the host (Dickinson et al., 2011, Belibasakis et al., 2011b, Belibasakis et al., 2011a, Guggenheim and Meier, 2011, Peyyala et al., 2011a, Peyyala et al., 2012). These have a number of advantages over *in vivo* periodontal disease models, the most obvious being the ethical argument. These controlled and reproducible systems enable evaluation of periodontal biofilms in 'real-time', allowing for the analysis of both biofilm consortia and cellular responses and how they modulate one another. The control of the system is of critical importance particularly when dissecting immune pathways. Guggenheim's group pioneered the use of *in vitro* host-pathogen interactions using a multi-species biofilm-epithelial cell co-culture model to investigate the cellular response to oral biofilms (Belibasakis et al., 2011b, Belibasakis et al., 2011a, Guggenheim and Meier, 2011), an innovative approach that has been recently emulated (Peyyala et al., 2011a, Peyyala et al., 2012). These studies have independently shown differences in cytokine and chemokine response to the different bacterial biofilms and planktonic cells.

The aim of the work presented herein was to develop a simple *in vitro* periodontal biofilm model to study host-pathogen interactions with respect to pathogen versus commensal, and biofilms versus planktonic cells. Ultimately, the study was aimed at creating a model that could be used to evaluate the effects of various bioactive molecules associated with oral health. To this end a simple *in vitro* 4-species biofilm model was successfully produced. This minimalist approach was employed because models, which use pooled saliva or pooled plaque samples are associated with problems of reproducibility (Hope et al., 2012). The current model delivered mono-species and multi-species biofilms grown on hydroxyapatite in artificial saliva through the sequential addition of the bacteria. The advantage to this approach was reproducibility, growth on a biologically relevant substrate and in a biologically relevant media, and

development of a biofilm in a manner similar to plaque. However, the limited number of bacterial species involved can be construed as a flaw to the model. However, in this scenario this was used as an advantage because the limited parameters enabled careful dissection of their contribution to inflammatory mediators. Therefore, this model can be used for high throughput analysis of host-pathogen interactions without worrying about ethical approvals for using human biological samples.

Specifically, the model showed that the individual bacteria preferentially survive when grown as multi-species biofilms compared with mono-species biofilms in cell culture conditions, and was successfully used to investigate host-pathogen interactions. This is in agreement with previous multi-species biofilm models, demonstrating mutualistic behaviour (Periasamy and Kolenbrander, 2009b). The current study also showed differential immune responses of various cytokines and chemokines to the pathogens and commensals. Even though both the pathogens and commensals affected the expression and release of inflammatory mediators, the pathogens preferentially induced expression and release by the epithelial cells as illustrated by the defined array patterns. Moreover, there is a high level of confidence in the data generated as IL-8 gene expression was observed following *P. gingivalis* biofilm challenge, whereas the release of IL-8 did not correlate with the gene expression. This is explained by gingipain release, a key immunomodulatory component of *P. gingivalis* (Mikolajczyk-Pawlinska et al., 1998). Therefore, the hypothesis that this model system could differentiate clearly between commensal and pathogenic, and planktonic and biofilms, was satisfactorily demonstrated. This offers improvements on others studies that investigated protein release without gene expression (Peyyala, 2012 #360), or focussed predominantly only on pathogenic biofilms (Guggenheim et al., 2009).

In summary, a novel *in vitro* model has been developed which successfully recapitulates the inflammatory changes in the host (epithelial cells) upon challenge with bacteria responsible for health and disease in periodontal disease.

6.4 Pathogens, commensals and the immune system

There have been several studies pertaining to the gut or intestinal epithelium (Pott and Hornef, 2012, Brandtzaeg, 2011, Brandtzaeg, 2010). These studies discuss how immune homeostasis in the gut environment is modulated by adaptive and innate immune responses and coordinated by antigen-presenting cells. Immune homeostasis in the oral cavity could also be explained in a similar manner. The oral cavity also consists of a range of commensals and pathogens that are in a state of homeostasis with the epithelium. This may be due to the fact that the innate immune system is highly active in the healthy tissues (Darveau, 2010). An imbalance of this system can contribute to the destruction of the periodontal tissue (Page and Kornman, 1997). Also, the recognition that both the commensals and pathogens can initiate innate immune responses through TLRs, which recognise microbes (Beutler et al., 2003). The current study demonstrated clear differences in the epithelial cell responses after challenge with commensals and pathogens. This was in agreement with a study showing similar epithelial cell responses to pathogenic *P. gingivalis* and commensal *S. gordonii*. This group investigated the gingival epithelial responses to pathogens and commensals *in vitro* using a co-culture model (Dickinson et al., 2011, Hasegawa et al., 2007). The oral commensal *S. gordonii* and opportunistic commensal *F. nucleatum* were shown to induce the gingival epithelial cell transcriptome less significantly compared with the oral pathogens *P. gingivalis* and *A. actinomycetemcomitans*. The limitation of this study was that the bacteria were in suspension when cultured with the epithelial cells. The study by Jeff Ebersole's group also investigated the differential epithelial cell response to commensal and pathogen biofilms with respect to protein release of various cytokines and chemokines (Peyyala et al., 2012). The current study showed similar protein profile with IL-8 and IL-1α where there was lower production of these proteins with *P. gingivalis* and *Streptococcus* species stimulation. In summary, all these studies show that pathogens and commensals behave differently when they come in contact with the epithelial cells. The exact mechanisms have not been fully understood.

It is not known how exactly the commensal *S. mitis* interacts with the innate immune system. It has been shown that *S. mitis* is remarkably tolerant to human ß-defensin 2 (hBD-2) and other antimicrobial peptides (Nishimura et al., 2004,

Ouhara et al., 2005). In addition to this, *S. mitis* can also modulate the expression of the pro-inflammatory chemokine IL-8 by not promoting the IL-8 expression (Eberhard et al., 2009). Taken together, these data indicate that *S. mitis*, as a beneficial commensal, can supplement host immunity by maintaining tissue homeostasis. On the other hand, the pathogenic mixed species and *P. gingivalis* biofilms boasts of a huge array of virulence factors, which can induce the innate immune response. The putative periodontal pathogen *P. gingivalis* attaches to the epithelial cells using fimbriae and gingipains and invades the cells {Weinberg, 1997 #231; Guo, 2010 #60}. The lipopolysaccharide (LPS) with its lipid A component in *P. gingivalis* induces a strong response from the host immune systems as it binds the toll-like receptor complex which promotes the secretion of pro-inflammatory cytokines in the epithelial cells including many other cell types (Loppnow et al., 1990). *P. gingivalis* uses gingipains to disrupt the cytokines and cytokine receptor networks of the host, including IL-1 beta (Sharp et al., 1998), interleukin-6 (IL-6), interleukin-8 (IL-8) (Mikolajczyk-Pawlinska et al., 1998), interferon-gamma (Yun et al., 1999) and TNF-alpha (Calkins et al., 1998). This could explain why protein release of these cytokines and chemokines does not correlate with the gene expression. All these factors prove the pathogenic potential of *P. gingivalis*. The host immune system does not rely totally on epithelial cells to act as defense against pathogenic plaque. A mixture of cells and other inflammatory mediators play a role in maintaining homeostasis within the gingival crevicular space.

6.5 IL-17 A – Important protection against pathogens

A protective role for IL-17 in oral microbial infections emerged through studies in IL-17/IL-17R deficient animals, which are susceptible to mucosal candidiasis (Takahashi et al., 2005) and *P. gingivalis* (Yu et al., 2007). The study by Yu *et al* demonstrated increased periodontal disease susceptibility in IL-17R deficient animals and elucidates to a protective role of IL-17 against *P. gingivalis* induced periodontal disease by promoting the migration of neutrophils to the site of infection to combat the pathogenic threat. However, a recent study by Eskan *et al* using an ageing mouse model of periodontal disease demonstrated that elevated IL-17 levels are associated with excessive neutrophil infiltration through the inhibition of development endothelial locus 1 (Del1) and the promotion of lymphocyte function associated antigen 1 (LFA-1) dependant

recruitment of neutrophils (Eskan et al., 2012). Therefore, these contrasting studies suggest that although IL-17 is required to protect mucosal surfaces of the periodontium from potential pathogens an over excessive IL-17 response may promote the pathogenesis of periodontal disease. Indeed, clinical studies have shown elevated levels of IL-17 mRNA in gingival tissue of periodontal disease patients (Vernal et al., 2005, Takahashi et al., 2011, Beklen et al., 2009) and elevated IL-17A protein levels in GCF (Vernal et al., 2005), serum and saliva of patients with periodontal disease (Ozcaka et al., 2011, Schenkein et al., 2010). Furthermore, IL-17 levels in serum and saliva have been demonstrated to correlate with clinical parameter of periodontal disease and IL-17 levels have been shown to fall after periodontal therapy (Duarte et al., 2010, Schenkein et al., 2010).

The current study confirmed that elevated levels of IL-17 mRNA in gingival tissue are associated with periodontal disease. This also suggests that levels of IL-17 detected in GCF by other groups are likely to be primarily derived from local cells rather than serum. In this study we demonstrated that mast cells may be a key source of IL-17 in the periodontium and although oral epithelial cells themselves do not express IL-17 they can respond to exogenous IL-17 by upregulating the expression of a range of pro-inflammatory mediators including IL-8, IL-6, TNF-α, IL-1 α and the colony stimulating factors. The upregulated expression of these mediators by oral keratinocytes maybe a key mechanism by which IL-17 protects mucosal surfaces from pathogens. For example, increased expression of CXCL8 (IL-8) expression will promote neutrophil recruitment to the site of infection and potentiate the removal of any microbial threat. However, studies of models of arthritis, a diseases process with notable parallels to periodontal disease, demonstrate IL-17 may contribute to initiation and perpetuation of chronic, destructive inflammation (Miossec et al., 2009). Furthermore, mast cells have also been demonstrated to be one of the key IL-17 producing cells in the synovium of patients with arthritis (Hueber et al., 2010). Therefore, it is interesting to speculate that mast cells through the expression of IL-17 may play a key role in the regulation of the periodontal immune response and contribute to the pathogeneisis of periodontal disease.

6.6 Future studies

These studies have developed a simple co-culture model to investigate host-pathogen interactions in the context of oral disease. This provides the opportunity to evaluate how small molecules of anti-microbial or immuno-modulatory capacity interact with biofilms and host cells. These would prove useful for the development of novel oral healthcare products. From a basic scientific standpoint this system would be useful to evaluate the role of virulence factors, such as gingipains, through the use of defined mutants. It would be particularly interesting to supplement the biofilm with other oral pathogens and to evaluate the contribution these make through analogous studies described herein. Finally, and potentially the most interesting, would be to investigate the immunity in more detail. Given the interesting IL-17 data generated and the role of the mast cells, it would be useful to introduce new mammalian cells types either singly or as a 3-D cell culture model. This would provide the opportunity to evaluate the individual component that these cells types play in oral health whilst concomitantly evaluating their impact on plaque biofilms.

6.7 Conclusion

In summary, the current study has shown differential immune responses to the pathogenic and commensal biofilms in epithelial cells. Even though both the pathogens and commensals had effects on the chemokine and cytokine expression and release, the pathogens induced more expression and release by the epithelial cells. Epithelial cells stimulated with mono-species biofilms does not reflect the immune responses demonstrated by a multi-species biofilm but it is useful in characterizing each individual bacterial species. The current study was performed on monolayer of immortalized human oral keratinocytes, but in the oral cavity bacterial biofilms come into contact with gingival tissue, which consists of multilayer of epithelial cells and connective tissue. The present study was performed *in vitro* and the conclusions drawn from these results must be interpreted with caution. Further studies are required to understand the workings of the host with regards to the bacterial biofilms and vice versa. The current study has established an *in vitro* model system, which allows to clearly differentiate, *in vitro*, the different response to commensals versus pathogens. This is a critical step to understanding both periodontal and other diseases. This

study also investigated a novel cytokine IL-17, which has been shown to have a protective role against the influx of the periodontal pathogens. The hope for the future is to unravel the details of the mechanisms involved and thereby identify more novel therapeutic targets for inflammatory and infectious disease.

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