Microbial biofilm composition influences the host immune response

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Abstract

Periodontal disease (PD) is a multifactorial disease of the oral cavity affecting the majority of the population. Although not a direct cause of mortality, PD is a health concern because it affects the majority of the population and has a negative impact on oral health, ability to chew, appearance, quality of life, dental care costs and can lead to tooth loss. Dental plaque is a microbial biofilm, which is necessary but not sufficient for the development of periodontitis. The interactions between the biofilm and the host cells, both local tissue and immune cells, can lead to tissue destruction and ultimately tooth loss. Clinical management of periodontitis involves mechanical removal of plaque from the tooth surface. Treatment is time consuming, in some patients only partially successful and recurrence is common. Therefore, understanding how the host interacts with microbial biofilms in both health and PD will help improve treatments and identify novel targets for therapeutic and preventative strategies.

The hypothesis of this thesis is that the bacterial composition of oral biofilms may modulate host cell responses which contribute to the pathogenicity of PD. The overarching aim of this research was to develop an *in vitro* co-culture model system to study how biofilm composition can influence the host immune response.

The studies document the development of health-associated, intermediate and disease-associated biofilms with host tissue and immune cells, and the use of these models to test antimicrobial and anti-inflammatory compounds as potential treatments for PD.

The biofilms developed were assessed for survival in cell culture conditions and batch reproducibility by PCR and morphology visualised using SEM. The health-associated biofilm included *Streptococcus mitis, S. intermedius* and *S. oralis* (3-species); the intermediate biofilm additionally included *Veillonella dispar, Actinomyces naeslundii, Fusobacterium nucleatum* and *F. nucleatum* spp. *Vincentii* (7-species); and the disease-associated biofilm included further addition of *Porphyromonas gingivalis, Prevotella intermedia,* and *Aggregatibacter actinomycetemcomitans* (10-species). These biofilms were co-cultured with an oral epithelial cell line and primary gingival epithelial cells, as

well as neutrophils and a myeloid cell line. Host cell viability was assessed by AlamarBlue®/LDH and changes in mRNA and protein expression of chemokines and cytokines were assessed by quantitative PCR and ELISA/Luminex®, respectively. Cellular responses were further evaluated by microscopy and flow cytometry.

Generally, co-culture of health associated biofilms with host cells resulted in minimal impact on cell viability and generally low inflammatory gene expression and protein release, with some genes including CXCL5 and CCL1 being downregulated compared to the cells only control. Intermediate biofilms caused some cell death and a marked upregulation of inflammatory genes and protein release, including a 302.7 fold increase of epithelial cell IL-8 gene expression compared to the cells only control ($p<0.001$). These intermediate biofilms elicited significant upregulation of CD40 ($p<0.001$) and CD69 ($p<0.01$) expression on the monocyte cell line compared with untreated controls. Co-culture of the 10 species disease associated biofilms with host cells resulted in significant host cell death of both epithelial cells ($p<0.001$) and monocytes ($p<0.05$). The 10 species biofilm caused significantly increased pro-inflammatory gene expression, but only low levels of protein could be detected in the supernatants. Similar trends in upregulation of inflammatory gene expression but low levels of protein release was observed in co-culture with differentiated pro-monocytes, whereas upregulation of inflammatory gene expression and protein release in neutrophil co-cultures was observed.

The effect of antimicrobial and anti-inflammatory compounds, resveratrol and chlorhexidine, was evaluated using this model system. Prior treatment of epithelial cells with resveratrol and biofilm with chlorhexidine significantly reduced IL-8 release from epithelial cells in co-culture with biofilms for 4 ($p<0.001$) and 24 hours ($p<0.001$).

In conclusion, this research has developed and validated 3 complex multi-species biofilms to study host: biofilm interactions in vitro. Furthermore, using these models in co-culture with multiple host cell types, clear differences in the host response to different biofilms were observed. The variations in inflammatory response of host cells and oral biofilms observed in this study help further understanding of the complex host: biofilm interactions within the oral cavity

which contribute to PD. This model demonstrated its potential as a platform to test novel actives, highlighting its use a tool to study how actives can influence host: biofilm interactions within the oral cavity. Future use of this model will aid in greater understanding of host: biofilm interactions. Such findings are applicable to oral health and beyond, and may help to identify novel therapeutic targets for the treatment of PD and other biofilm associated diseases.

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List of publications based on thesis

Millhouse, E, Jose A, Sherry, L, Lappin, D, Patel N, Middleton A, Pratten J, Culshaw, S and Ramage G. 2014. Development of an *in vitro* periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules. BMC Oral Health 14, 80.

Related publications

Sherry L, **Millhouse E**, Lappin DF, Murray C, Culshaw S, Nile CJ and Ramage G. 2013. Investigating the biological properties of carbohydrate derived fulvic acid (CHD-FA) as a potential novel therapy for the management of oral biofilm infections. BMC Oral Health, 13, 47.

Shahzad M, **Millhouse E**, Culshaw S, Edwards CA, Ramage G and Combet E. 2015. Selected dietary (poly)phenols inhibit periodontal pathogen growth and biofilm formation. Food Funct. 6(3):719.

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Author's Declaration

I declare that I have carried out the work described in this thesis unless otherwise acknowledged or cited, under the supervision of Professor Gordon Ramage and Dr Shauna Culshaw. I further declare that this thesis has not, in whole or in part, been submitted for any other degree.

Emma Millhouse

Definitions/Abbreviations

AA	Aggregatibacter actinomycetemcomitans
AI-2	Auto inducer 2
AMP	Antimicrobial peptide
AN	Actinomyces naeslundii
ANOVA	Analysis of variance
APC	Antigen presenting cell
AS	Artificial saliva
BHI	Brain heart infusion broth
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CBA	Colombia blood agar
cDNA	Complementary deoxyribonucleic acid
CDFF	Constant depth film fermenter
CFU	Colony forming units
CHX	Chlorhexidine
CSF	Colony stimulating factor
CLSM	Confocal laser scanning microscopy
CSFE	Carboxyfluorescein succinimidyl ester
Ct	Cycle threshold
CV	Crystal violet
CVD	Cardiovascular disease
ddH_2O	Double distilled water
d-KSFM	Defined keratinocyte serum free medium
DMEM	Dulbecco's modified eagle's media
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FAA	Fastidious anaerobic agar
FACS	Fluorescent activated cell sorting
FCS	Foetal calf serum
FN	Fusobacterium nucleatum

FNV	Fusobacterium nucleatum ssp. vincentii
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCF	Gingival crevicular fluid
GF	Germ free
HA	Hydroxyapatite
hBD	Human ß defensin
HGEC	Human gingival epithelial cell
HMDS	Hexamethyldisilazane
IFNγ	Interferon γ
IgG/M/A	Immunoglobulin G/M/A
IL	Interleukin
KSFM	Keratinocyte serum free medium
LAD	Leukocyte adhesion deficiency
LDH	Lactate dehydrogenase
LL37	Cathelicidin
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κ-light-chain-enhancer of activated b cells
NLRP3	Nucleotide-binding oligomerization domain-like receptor 3
OD	Optical density
OKF6-TERT2	Oral keratinocyte cell line
OPG	Osteoprotegrerin
OTU	Operational taxonomic unit
PBS	Phosphate buffered saline
PD	Periodontal disease
PFA	Paraformaldehyde
PI	Prevotella intermedia
PG	Porphyromonas gingivalis
PGE_2	Prostaglandin E_2
PMA	Phorbol 12-myristate 13-acetate

PMN	Polymorphonuclear leukocyte
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor κ-B ligand
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute-1640 media
RSV	Resveratrol
SD	Standard deviation
SEM	Scanning electron microscopy
SI	Streptococcus intermedius
SO	Streptococcus oralis
SM	Streptococcus mitis
SPF	Specific pathogen free
Th1/2/17	T helper cell 1/2/17
TGF	Transforming growth factor
THP-1	Human monocytic cell line
TLR	Toll like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America
VD	Veillonella dispar
Vitamin D_3	1α,25-Dihydroxyvitamin D_3
WT	Wild-type

1 Introduction

1.1 Periodontal Disease

Periodontal diseases (PDs) are multifactorial diseases of the oral cavity. Microorganisms, which grow as a biofilm (plaque) on the surface of teeth, and microbial products initiate an inflammatory response lead to local tissue destruction and ultimately tooth loss. However, plaque is necessary but not sufficient to cause PD and many other local and systemic factors can contribute to the dysregulated inflammatory immune response seen in PD. This introduction shall provide a brief overview of the microbiological aspects of PD, the host immune response in PD, the models used to study PD, and finally the novel therapeutics used in an attempt to treat the disease.

1.1.1 Clinical classification of PD

PD includes highly prevalent conditions encompassing a range of inflammatory diseases involving the gingivae and supporting structures of the teeth (Williams, 1990). The two most common forms are plaque-induced gingivitis and periodontitis (Figure 1.1). Gingivitis is predominantly caused by the accumulation of bacterial biofilms on the teeth adjacent to the gingivae, but the host response to the biofilm can also be modified by a variety of factors including pregnancy, malnutrition and local and systemic diseases such as diabetes and leukaemia (Pihlstrom et al., 2005). This can cause increased bleeding upon probing at the site of infection due to erythema and swelling of the surrounding tissues; however, gingivitis does not affect the supporting structures of the tooth and is a reversible condition if dental plaque is removed (Theilade et al., 1966, Mariotti, 1999).

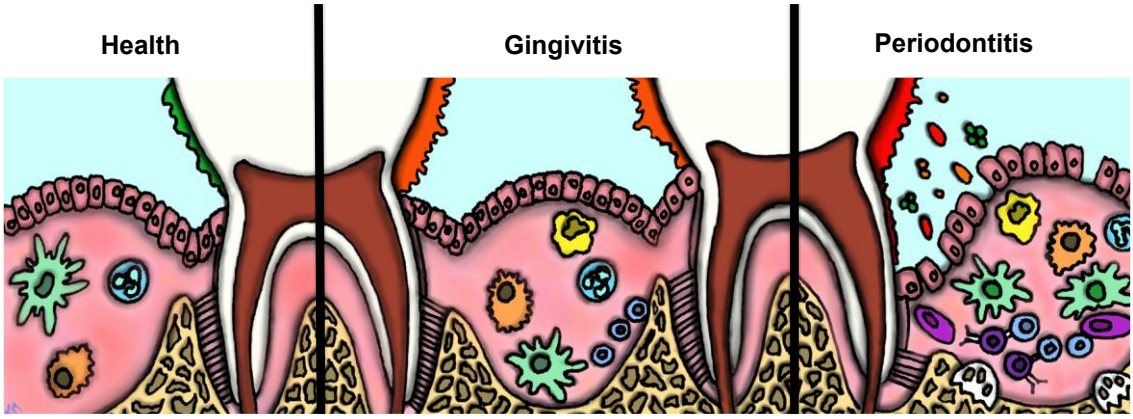


Figure 1.1: Progression from periodontal health to gingivitis or periodontitis In **periodontal health** biofilms accumulate on the tooth at the gum line with no inflammation and are in a state of homeostasis with the host. In **gingivitis** a build up of bacteria within the biofilm causes localised inflammation, with an increase of immune cells at the site and swelling of the gum forming a small gingival crevice. In **periodontitis** bacteria form a dysbiotic sub-gingival biofilm on the tooth which causes further inflammation, destruction of the alveolar bone and tooth loss. Image by Emma Millhouse.

Unlike gingivitis, periodontitis causes the destruction of tooth supporting structures, including the gingivae, periodontal ligament and the alveolar bone, ultimately resulting in tooth loss. Periodontitis also increases patients' risk of systemic diseases, including rheumatoid arthritis (RA), cardiovascular disease (CVD) and cancer (Casanova et al., 2014, Dietrich et al., 2013, Kaur et al., 2013). Periodontitis can be further divided into two subclasses; chronic and aggressive, and either form can be found localised to fewer than 30% of the teeth or generalised affecting the majority of the teeth in the oral cavity (Armitage, 1999). Chronic periodontitis is the most common form of the disease and ranges from mild to severe, presenting with deposits of plaque and calculus, inflammation at the site and a slow rate of disease progression (Armitage and Cullinan, 2010). In contrast, aggressive periodontitis is characterised by rapid loss of attachment and bone destruction and is typically observed in patients younger than those who present with chronic periodontitis (Armitage, 1999). Furthermore, noticeable differences in plaque accumulation have been observed between chronic and aggressive periodontitis, with generally relatively minimal

amounts of plaque and calculus present in aggressive periodontitis and some differences in the microbiology, namely a greater chance of harbouring *Aggregatibacter actinomycetemcomitans* in aggressive periodontitis than chronic periodontitis (Armitage, 2010, Douglass et al., 1990, Liljenberg and Lindhe, 1980, Fine et al., 2007).

PD is common throughout the UK. A survey in 2009 found only 17% of adults in the UK had healthy periodontal tissues, with 45% of adults having PD, and 9% of adults having severe periodontitis at one site or more (White et al., 2012). It is well established that dental plaque plays an essential role in the initiation of PD (Socransky, 1977). However, there are a variety of risk factors associated with susceptibility to PD and rate of disease progression including smoking, lifestyle, genetics and local and systemic diseases (Michalowicz et al., 1991, Ismail et al., 1983, Bawadi et al., 2011). The key role of individual susceptibility was described by Loe et al (1986) who observed three distinct patterns of loss of attachment; none, moderate and rapid in a population of Sri Lankan labourers exposed to the same environmental factors. Interestingly, there was no significant difference in oral hygiene status and gingivitis prevalence between these groups throughout the duration of the study, although there were some associations between these factors and those in the rapid attachment loss group (Loe et al., 1986).

For most patients' plaque accumulation will develop into non-destructive gingivitis which may precede periodontitis. However, not all gingivitis shall develop into periodontitis, with gingivitis only having an approximate 30% predictive value for progression to periodontitis (Loe, 1983). This is due to the understanding that plaque is necessary, but not sufficient, to cause periodontitis, and that shifts in plaque composition and host immune response result in dysbiosis of the oral cavity and ultimately disease (Hajishengallis, 2015). Therefore understanding both the microbiology of dental plaque and the host response to this is essential to understanding PD.

1.1.2 Microbiology of PD

1.1.2.1 Biofilms

Dental plaque is a microbial biofilm that forms on the surface of teeth. Biofilms are defined as 'a structured consortium of microbial cells surrounded by a self produced polymer matrix' (Costerton, 1995). Mono-species and multi-species biofilms exist and are prevalent in both nature, within the human host and on man-made structures with over 95% of bacteria estimated to exist as biofilms (Saini et al., 2011). Historically, it was believed that bacteria predominantly existed within a planktonic state, however, contemporary studies have more recently shown bacteria can also exist in adherent biofilm states that have a defined role in the pathogenesis of diseases, such as cystic fibrosis and candidiasis (Harriott and Noverr, 2011, Bjarnsholt, 2013, Mulcahy et al., 2014). Biofilms confer advantages to bacteria over planktonic states, including protection from the environment, resistance to chemical and physical removal of bacteria, and mutualistic co-operation between species present within the community (Sedlacek and Walker, 2007, Schwering et al., 2013, Falsetta et al., 2014). These characteristics play an important role in clinically relevant biofilms, with such communities being phenotypically different to their planktonic counterparts, allowing resistance to antimicrobials and evading host immunity (Donlan and Costerton, 2002, Thurlow et al., 2011). As a result these biofilms are clinically difficult to treat, and many studies now focus on understanding microbial biofilms in disease in an effort to find better ways to manage and eradicate them.

1.1.2.2 Dental Plaque

It is estimated that over 700 different bacterial species can reside within the human oral cavity, of which more than 400 reside within the periodontal pocket, with individuals reported to have approximately 100 different bacterial species present in dental plaque (Paster et al., 2006). These microbial biofilms have been shown to form by an ordered and sequential colonisation of bacterial species which, depending on the composition, can be linked to oral health or PD (Figure 1.2) (Kolenbrander et al., 2002, Kolenbrander et al., 2010a). Many of the

species present are difficult to culture and have only been found using 16S gene cloning and next-generation sequencing (Aas et al., 2005, Keijser et al., 2008). Distinct microbial profiles have been observed using these methods, illustrating differences in the microbial composition of different sites within the oral cavity, as well distinctions between health and disease (Huang et al., 2011b). In periodontitis, the microbial composition generally changes from a Gram-positive biofilm to a more complex Gram-negative anaerobic biofilm. Pioneering DNA hybridisation studies investigating the role of bacteria in periodontitis implicated a small number of bacterial species that are present in low amount that play an important role in the aetiology of the disease, with species such as *Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia* being strongly associated with chronic periodontitis (Socransky et al., 1998).

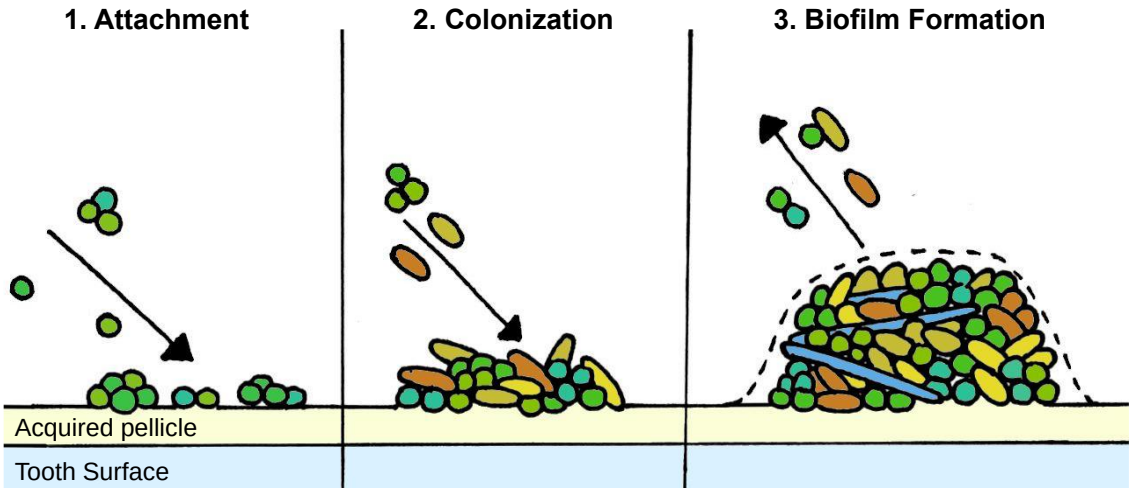


Figure 1.2: Oral biofilm formation

This diagram represents the steps of oral biofilm formation. **(1) Attachment:** Early colonising bacteria recognise salivary glycoproteins found within the acquired pellicle of the tooth surface and attach to them. **(2) Colonisation:** Early colonisers grow, co-aggregate with other oral species within the biofilm and facilitate the incorporation of late colonisers to the biofilm. **(3) Biofilm development**: As the biofilm matures, interactions between bacterial species occurs including quorum sensing and DNA transfer as well as competitive interactions such as bacteriocin production. Mature biofilms also provide a barrier function which protects bacteria from antibiotics and environmental changes. Bacteria can also disperse from the biofilm surface and spread to colonise a new site. Image adapted from (Hojo et al., 2009).

1.1.2.3 Adhesion to tooth surfaces

Biofilm formation begins with early colonising bacteria such as *Streptococcus* species, which constitute up to 60-90% of bacteria that re-colonise the tooth surface in the first 4 hours after professional cleaning (Nyvad and Kilian, 1987). These bacteria bind to the salivary pellicle found on the surface of enamel which is made up of a variety of components including enzymes, such as alpha-amylase, proline rich proteins, phosphatase rich proteins such as statherin, mucins and agglutinins (Kolenbrander et al., 2002).

1.1.2.4 Co-aggregation of plaque bacteria

Not all bacteria can bind directly to the salivary pellicle present on the surface of the tooth. For planktonic bacteria present in the oral cavity other bacterial species that have adhered to the tooth surface can become a binding site. This process is known as co-aggregation and is defined as "specific cell-cell interactions between genetically distinct cells" and plays an essential role in oral biofilm formation (Kolenbrander et al., 2002). As a result many oral bacterial strains have been studied *in vitro* to test their ability to co-aggregate with other oral bacterial species and the mechanisms of adhesion by which this occurs (Maeda et al., 2013, Sato and Nakazawa, 2014, Guggenheim et al., 2001b). Furthermore, co-aggregation of bacteria is species specific, with some species unable to adhere directly to others in biofilms without the presence of an intermediate bacterial species (Figure 1.3) (Ledder et al., 2008, Biyikoglu et al., 2012, Ammann et al., 2013a). The oral bacterium *Fusobacterium nucleatum* has been shown to co-aggregate with many bacterial species and therefore plays an essential role in 'bridging the gap' between early and late colonisers of oral biofilms (Bradshaw et al., 1998, Biyikoglu et al., 2012).

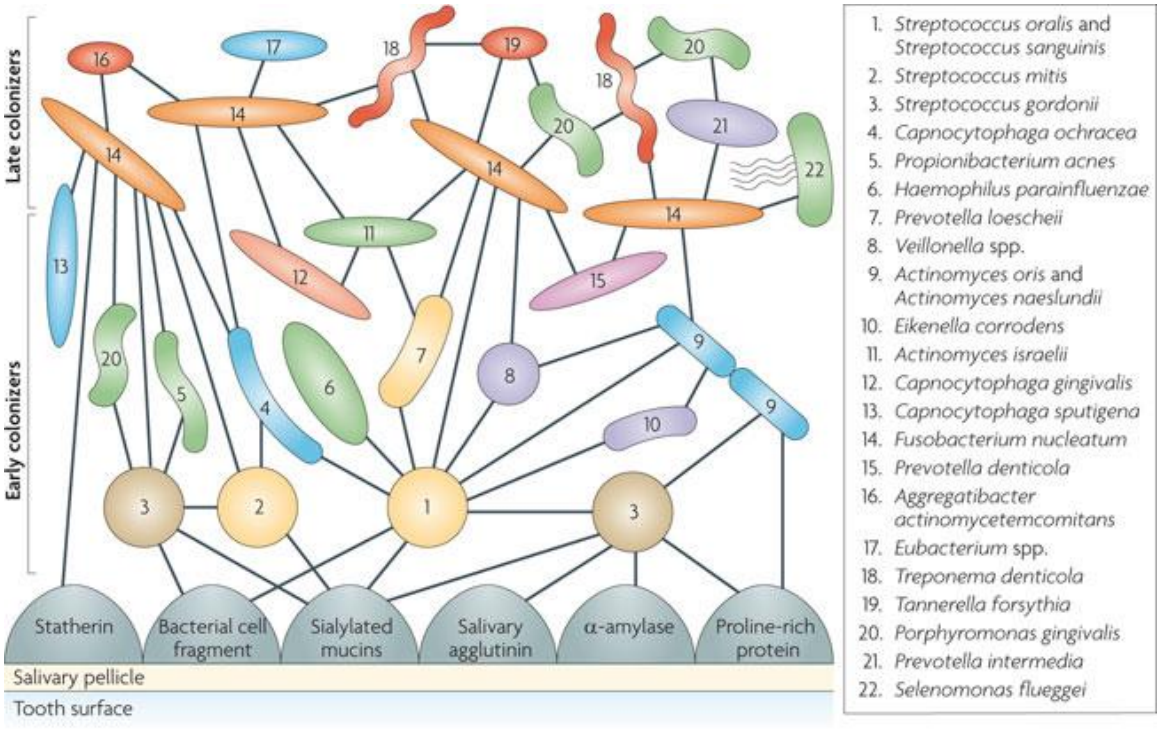


Figure 1.3: Bacterial colonisation of a tooth surface

This image shows the species specific colonisation of oral bacteria on the tooth surface. Early colonisers (predominantly *Streptococcus* species) are able to bind to the proteins found within the salivary pellicle. These species allow the incorporation of other species such as *Veillonella* species to the biofilm, which are unable to directly bind to the salivary pellicle themselves. Intermediate colonisers such as *F. nucleatum* show the ability to adhere with a large number of early colonisers of the biofilms while also facilitating the adhesion of late colonisers, typically associated with disease, such as *Porphyromonas gingivalis* to the biofilm (Kolenbrander et al., 2010a).

1.1.2.5 Microbial communication between plaque bacteria

Oral microbial biofilms are a dynamic, complex community. Their reliance on nutrients available from saliva, gingival crevicular (GCF) fluid and food debris ensures interspecies communication is essential and plays an important part of biofilm development (Kolenbrander, 2011). Mutualistic relationships are found within biofilms where metabolites produced by one species are used as a nutrient for another and often results in co-localisation of species that are

metabolically compatible. An example of this is the metabolic synergy between *P. gingivalis* and *T. denticola* which when grown together produce a significantly larger biomass than when grown as mono-species biofilms or with other bacterial species (Cogoni et al., 2012). *P. gingivalis* produces isobutyric acid which enhances *T. denticola* growth, whereas *T. denticola* produces succinic acid which enhances *P. gingivalis* growth (Grenier, 1992). Bacteria can also compete for nutrients, which in turn can shape the composition of oral biofilms. Studies have shown that some oral bacteria antagonise disease-associated bacteria within the oral cavity, such as inhibition of colonisation by the cariogenic bacteria *S. mutans* through the production of hydrogen peroxide by *Streptococcus sanguinis* and *Streptococcus gordonii* (Kreth et al., 2008, van Essche et al., 2013).

In microbial biofilms, bacterial distance and density also play an important role in communication. Quorum sensing is defined as the regulation of genes in response to cell density, influencing functions including virulence, antibiotic susceptibility and biofilm formation (Ahmed et al., 2007, Merritt et al., 2003, Novak et al., 2010). It is believed to play an important role in communication between Gram-positive and Gram-negative bacteria due to the highly conserved signalling molecules used. The most well known signalling molecule involved in quorum sensing is auto-inducer 2 (AI-2), which is encoded by the *luxS* gene and is conserved among many bacterial species, including *Streptococcus oralis, S. gordonii, A. actinomycetemcomitans* and *P. gingivalis* (Huang et al., 2011a). In oral biofilms, AI-2 plays an important role in promoting biofilm formation and studies have shown mutations in the *luxS* gene of *S. oralis* species results in failed biofilm formation with *Actinomyces naeslundii* (Rickard et al., 2006). Other studies have shown *luxS* deficient *P. gingivalis* was unable to form multi-species biofilms with other oral bacteria, produced low levels of protease and haemagglutinin and caused a reduced inflammatory response in culture with periodontal fibroblasts (Scheres et al., 2014, Burgess et al., 2002, McNab et al., 2003).

Biofilms also allow bacteria to exchange DNA due to the proximity of cells and the ability of DNA to be trapped in the extracellular matrix. A variety of oral bacteria have been reported to have transposons which allow for DNA transfer

and horizontal gene transfer and this has been reported between streptococcal species including *S. gordonii, S. oralis* and *S. sanguinis* in oral biofilms (Roberts et al., 2001, Warburton et al., 2007). This has been proposed to be beneficial to the communities within biofilms due to the ability of DNA release to stabilise the structural integrity of the biofilm and pass on beneficial traits such as antibiotic resistance (Kolenbrander et al., 2010a).

1.1.2.6 Dental plaque composition

Biofilms are dynamic environments and can form in a variety of locations in the oral cavity such as the tooth and root surfaces (both supra- and sub-gingival), dental implants, dentures, the tongue and other mucosal sites (Dewhirst et al., 2010, Aas et al., 2005). Studies investigating oral biofilms have observed distinct microbial biofilm profiles present at different hard and soft tissue sites within the oral cavity, with significant differences in the microbial composition of supra- and sub-gingival biofilms compared with the tongue, hard palate and saliva (Mager et al., 2003). This study also concluded that while many species can colonise multiple surfaces, composition overall at each site is different, with *Actinomyces* species colonising teeth in far greater numbers than at other sites. Furthermore, at the same sites distinct microbial profiles were observed when comparing health and disease states with increased levels of *Streptococcus* and *Veillonella* species in health and increased levels of *P. gingivalis* in disease (Jorth et al., 2014, Colombo et al., 2009). The role of dental plaque in PD has been extensively studied and hypothesis devised for the aetiology of disease. The 'non-specific hypothesis' states that if biofilms are allowed to grow uncontrollably this will inevitably lead to disease. Alternatively, the 'specific plaque hypothesis' states that there are single or limited numbers of specific pathogens within biofilms and specific forms of PDs will have specific bacterial aetiologies. For example, *P. gingivalis* is the most studied bacteria in PD due to its strong associations with periodontitis. *P. gingivalis* is present in 79% of patients with periodontitis patients compared with 25% of patients with good oral health and is proposed to be a keystone bacterial pathogen in periodontitis (Loesche, 1976, Loesche, 1979, Griffen et al., 1998). The 'ecological plaque' hypothesis states that there is a qualitatively distinct bacterial composition between healthy and diseased sites and a pathogenic shift is a result of

disrupted equilibrium. This disrupted equilibrium can occur due to lifestyle changes, including diet and smoking, and result in a change in the microbes that are found in the plaque. This change in environment can allow colonisation by virulent bacteria. Several key studies by Socransky's team characterized the composition of plaque from which bacteria were grouped into 'complexes' based on associations between bacterial species and prevalence in health and disease, observing differing microbial compositions at supragingival and subgingival sites (Figure 1.4-1.5) (Socransky et al., 1998, Haffajee et al., 2008).

1.1.2.7 Supragingival biofilms

As aforementioned, gingivitis precedes periodontitis, but not all gingivitis progresses to become periodontitis. Biofilm accumulation begins above the gum line as early as 4 hours after professional cleaning with significantly increased levels of streptococci such as *S. mitis* and *S. oralis*, and other early colonisers such as *Neisseria mucosa* and *Veillonella parvula* observed at this time (Teles et al., 2012). The most common species present in supragingival plaque were those of the orange, yellow and blue complexes, as described by Socransky (Figure 1.4) (Haffajee et al., 2008). Increased levels of *Actinomyces* species in supra-gingival biofilms were observed over time and attributed to the replacement of yellow complex *Streptococcus* species as the biofilm matures (Ritz, 1967). Furthermore, bacteria of the 'red complex', which is typically associated with PD and subgingival plaque, were strongly associated with increased levels of gingival redness, bleeding on probing, and could be found present in supragingival plaque from sites where sub-gingival samples were negative for the same species (Ximenez-Fyvie et al., 2000b).

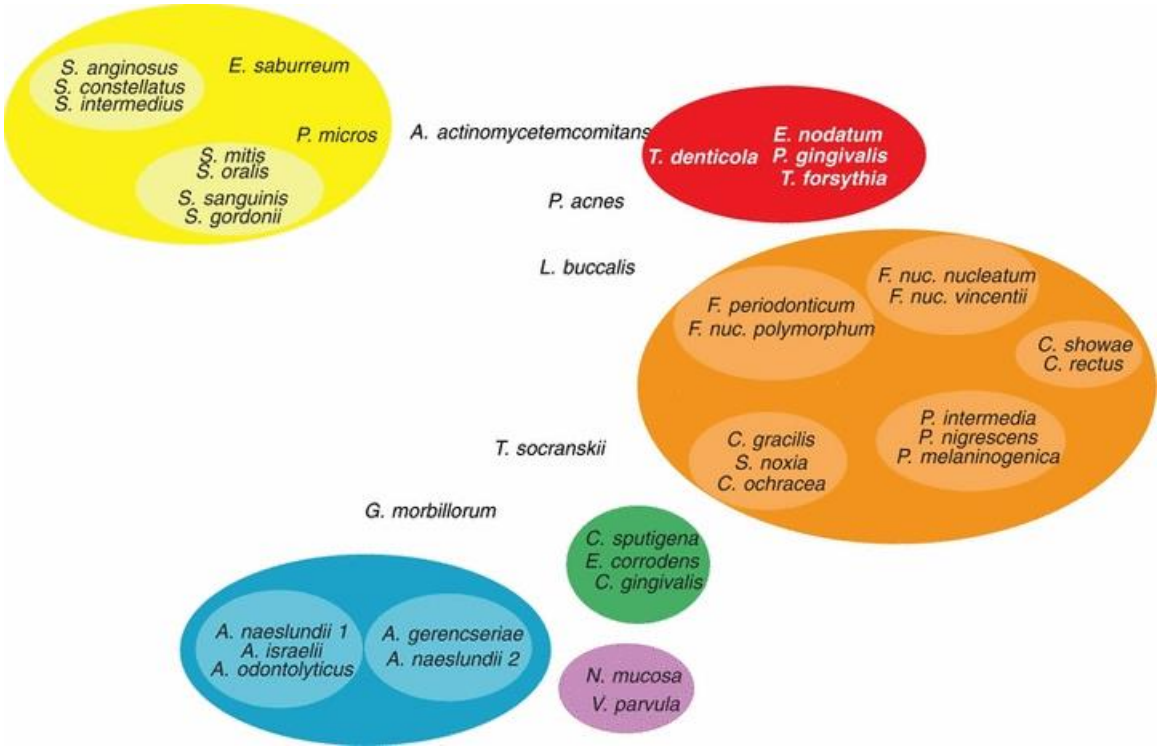


Figure 1.4: Supra-gingival biofilm complexes

Representation of the relationships of species in supragingival plaque (Haffajee et al., 2008).

1.1.2.8 Sub-gingival biofilms

Sub-gingival biofilms are found attached to the root surface, below the gum line in the periodontal pocket, surrounded by GCF. At this site species diversity is higher than that of supra-gingival biofilms, with an increase of Gram-negative, anaerobic bacterial species (Paster et al., 2006). Subgingival plaque is most likely formed by the spread of supragingival plaque down into the gingival sulcus, with studies showing re-colonisation of pristine pockets occurring with the same species involved in supra-gingival biofilm formation (Quirynen et al., 2005). These early colonisers are typically aerobic or facultative anaerobic bacteria. However, as the biofilm matures an oxygen gradient forms, which allows obligate anaerobes to be incorporated. As a result, over time biofilm composition shifts from a Gram-positive aerobic population to a Gram-negative anaerobic one. As with supragingival biofilm, Socransky *et al* (1998) described complexes of bacterial associations and observed evidence to link the 'red complex' bacteria (*P. gingivalis*, *Prevotella intermedia* and *T. denticola*) with

symptoms of disease, particularly pocket depth and bleeding on probing (Figure 1.5).

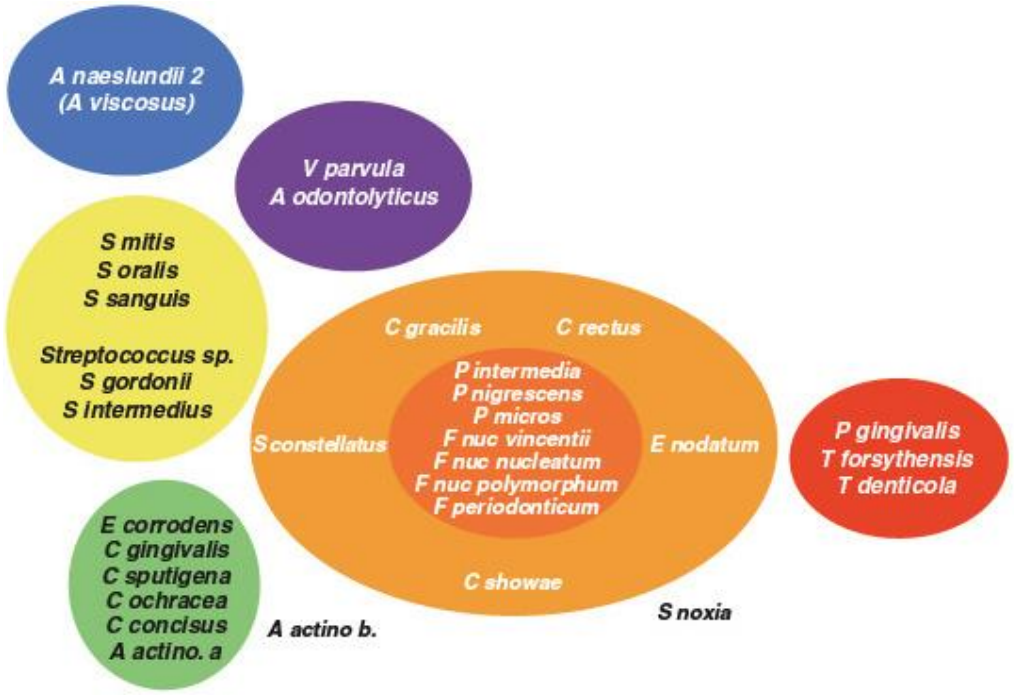


Figure 1.5: Sub-gingival biofilm complexes

Representation of the relationships of species in sub-gingival plaque (Socransky et al., 1998)

1.1.2.9 Microbial Dysbiosis

In the oral cavity the transition of health to disease is associated with a shift from the facultative bacteria in homeostasis with the host to a dysbiotic biofilm containing an increased number of species, in particular anaerobic bacteria. Dysbiosis is defined as a shift or imbalance of the relative abundance or influence of species within a microbiome that is associated with disease such as inflammatory bowel disease or periodontitis. This shift, combined with a dysregulated immune response, either due to immune subversion or host immunoregulatory defects, results in biofilm overgrowth and uncontrolled inflammation at the site. *P. gingivalis* has been extensively studied and shown to use a variety of virulence factors to modulate both the host immune response and the oral biofilm dynamics such as gingipains which can degrade inflammatory cytokines such as IL-8 produced by host cells (Palm et al., 2015, Tada et al., 2003). *P. gingivalis* is typically present in low numbers in microbial

plaque; however, a study by Hajishengallis *et al* (2011) observed that inclusion of small numbers of *P. gingivalis* in a biofilm altered the amount and composition of commensal bacteria present in plaque, and increased alveolar bone loss in specific pathogen-free mice (Hajishengallis et al., 2011). Furthermore, this study showed *P. gingivalis* alone was unable to cause bone loss when inoculated in germ-free mice. However, transmission of commensal bacteria to these germ-free mice resulted in bone loss. This suggests that *P. gingivalis* promotes periodontitis by altering commensal bacterial biofilms, resulting in inflammation and bone loss. Hence, *P. gingivalis* has been termed a 'keystone pathogen' involved in the microbial shift from periodontal health to disease (Darveau et al., 2012, Duran-Pinedo et al., 2014). Furthermore, *P. gingivalis* may also alter the commensal bacterial biofilm to its advantage, with studies reporting *P. gingivalis* directly causes *S. mitis* death and DNA fragmentation in a multi-species biofilm (Duran-Pinedo et al., 2014). While *P. gingivalis* has been the most extensively studied of these species, which in part was due to its relative ease of culture, both *T. forsythia, T. denticola* of the 'red complex' and other periodontal bacteria also cause a shift in the composition of oral plaque resulting in microbial dysbiosis.

1.1.3 Immunology of PD

Microbial plaque is considered necessary, but not sufficient, for the progression of PD, and that dental plaque accumulation does not necessarily progress to periodontitis. Many studies both *in vitro* and *in vivo* have demonstrated the role of pathogenic 'red complex' oral bacteria and PD. However, these species have also been found in periodontally healthy patients, albeit at lower frequencies than patients with PD (Yang et al., 2004, Ximenez-Fyvie et al., 2000a). It can therefore be assumed that while these species may initiate host inflammatory responses, other mediators must be involved which determine the progression of PD. These mediators may play a key role in determining the host's outcome, providing either a protective and preventative response or an ineffective inflammatory one, which results in host induced tissue destruction. The development of gingivitis and periodontitis can be divided into four stages based on histological examination of gingival tissues, which will be described below (Page and Schroeder, 1976).

1.1.3.1 Initial lesion

The initial inflammatory lesion develops within 2 - 4 days of plaque accumulation, at which time the early inflammatory response can be observed histologically. This stage is characterised by oedema, vasodilation, increased gingival fluid flow, loss of perivascular collagen and migration of polymorphonucelar leukocytes, such as neutrophils and monocytes, into the periodontal tissues and junctional epithelium in experimental gingivitis (Payne et al., 1975). At this stage the gingival crevice has a lower redox potential than other sites in the oral cavity, encouraging the growth of *Actinomyces* ssp. and other carbon dioxide requiring species (Moore et al., 1982). The lesion that develops is an acute inflammatory response by the gingival tissues to bacterial enzymes and metabolic products produced by the capnophilic species, which in turn increases the permeability of the junctional epithelium allowing increased GCF fluid flow containing PMNs, complement and antimicrobial compounds (Ohlrich et al., 2009).

1.1.3.2 Early lesion/ stable lesion

Approximately 4 - 7 days after the initial plaque accumulation, the developing lesion is clinically detectable as gingivitis. At this stage the lesion shows further histological changes including more pronounced vascular changes, increased GCF flow and widening of junctional epithelium (Page and Schroeder, 1976). The inflammatory infiltrate also begins to shift from one containing mainly neutrophils and monocytes to more macrophages and lymphocytes as well as some plasma cells at the periphery of the lesion. The extent of leukocyte infiltration is only marginally greater than that seen in the early lesion, with leukocytes comprising approximately 15% of the cell population. However, collagen degradation is more extensive, affecting 60-70% of the matrix (Page and Schroeder, 1976). There are clinical signs of gingival inflammation and bleeding on gentle probing around 12-21 days, at which point lymphocytes will make up approximately 70% of the cell infiltrate and PMNs consisting of approximately 10% (Seymour et al., 1983). The persistence of the plaque biofilm prevents the immune system from resolving the inflammation and fully eradicating the microbial challenge, which left untreated, develops into chronic gingivitis. Mechanical cleaning to remove the plaque at this stage allows for gingival tissue

repair and no permanent damage to the tissue. This early lesion may remain stable or progress to periodontitis.

1.1.3.3 Established lesion

In some cases, either due to host specific susceptibility or environmental factors, the lesion becomes dominated by an adaptive T cell/B cell/plasma cell immune response (Seymour et al., 1979b). Unlike the stable gingival lesions where the immune response is in balance with the disease, in the progressive lesion pro-inflammatory activity is dominant. Many of the Gram-negative bacteria associated with the progressive lesion such as *P. gingivalis* have been cited as polyclonal B cell activators and the growth of these could be responsible for the increased Th2 cells response observed, with Th2 cells also being involved in the destruction of extracellular microorganisms in both human and animal studies (Tew et al., 1989, Han et al., 2009). This stage is associated with high levels of inflammatory mediators including IL-1, IL-6, TNFα, PGE_2, further connective tissue breakdown, migration of the junctional epithelium in an apical direction and periodontal pocked formation (Honda et al., 2006).

1.1.3.4 Advanced lesion

The advanced lesion has similar cellular composition as the established lesion as it is dominated by lymphocytes (Thorbert-Mros et al., 2014). The lesion extends down the periodontal ligament and alveolar bone and shows clinical loss of attachment at the site. This connective tissue destruction is believed to be due to the effects of the immune response, notably fibroblasts and macrophages, and is mediated through matrix metalloproteinases (MMPs) and enhanced osteolytic activity, although direct damage can also occur via the cytotoxic effects of bacteria products such as proteinases and collagenases (Ohlrich et al., 2009, Haffajee and Socransky, 1994). This dysregulation of host derived factors results in damage to the connective tissue attachment.

It has been shown that PD has both states of activation and states of remission, characterised by active and inactive lesions and much of the loss of attachment is believed to be due to the repeated episodes of inflammation at lesion sites. The result of the progressive lesion is the major destruction of hard and soft

tissues by the host, due to inflammatory mediators as well as the activation of osteoclasts to initiate bone resorption.

1.1.3.5 Innate immune response to periodontal biofilms

Innate immunity is a consistent feature of both gingivitis and periodontitis and may be considered as comprised of mechanical, chemical and cellular elements. The mechanical element is the physical barrier function of epithelial cells, the chemical element comprises of soluble and cell associated proteins including patter-recognition receptors, cytokines and chemokines and the cellular element is made up of the cells of the gingival epithelium and underlying connective tissue which comprises of multiple cell types such as epithelial cells, tissue macrophages, neutrophils, dendritic cells, NK cells, γδ T cells and Langerhans cells. Their main role in the oral cavity is the defence against pathogens and maintenance of tissue integrity and homeostasis.

Recognition of microbial components such as bacterial lipopolysaccharide (LPS), DNA and peptidoglycan occurs primarily through toll-like receptors (TLRs) expressed on these cell types. Activation of TLRs results in an intracellular signalling cascade which can lead to activation of innate immune responses, activation and modulation of adaptive immune responses and production of antimicrobial mediators including antimicrobial peptides (AMPs), defensins, histatins and cathelicidins (Akira and Takeda, 2004). Gingival epithelial cells of the junctional epithelium are the front line between the host and oral bacteria. As such, these cells are well equipped to recognise pathogens and express TLR2, 3, 4, 5, 6 and 9 (Kusumoto et al., 2004). Langerhans cells and resident dendritic cells are also present in the gingival epithelium and express TLR1-8 and 10 (Agrawal et al., 2003). Furthermore TLR activation of these cells has been shown to play a critical role in downstream adaptive responses including the activation and differentiation of T cells (Iwasaki and Medzhitov, 2004). Unsurprisingly, in the oral cavity TLR expression has been shown to be increased in the gingivae of subjects with PD compared with healthy subjects, and is associated with the influx of both innate and adaptive immune cells into the site (Muthukuru et al., 2005).

Most Gram-negative bacteria that are associated with PD elicit a strong TLR2 and 4 response via TLR binding to bacterial peptidoglycans and LPS, respectively (Tietze et al., 2006). However, studies have shown *P. gingivalis* stimulates TLR1, TLR2, TLR4, TLR7 and CD14 *in vitro* (Scheres et al., 2011, Sun et al., 2010). A study by Maekawa et al (2014) also showed the ability of *P. gingivalis* to modulate the host response by degrading the protective TLR2-MyD88 response and activates the TLR2-Mal-P13K pathway which blocks phagocytosis and promotes inflammation (Maekawa et al., 2014b). Studies investigating the TLR expression in gingival tissue from chronic periodontitis patients observed increased levels of TLR2 and TLR9 expression compared with healthy individuals (Wara-aswapati et al., 2013). TLR9 recognises bacterial CpG DNA and stimulates inflammatory cytokine responses through NF-κB signalling. Studies showing differential cytokine responses to bacterial DNA, observed low inflammatory responses to commensal species such as *S. sanguinis* DNA and increased responses to pathogenic bacteria such as *P. gingivalis* (Sahingur et al., 2012). Evidently, the innate immune system in periodontal tissues is under constant challenge by oral bacteria in both health and PD.

1.1.3.6 Adaptive immune response to periodontal biofilm

In the oral cavity both T and B cells are present in health and in PD and are believed to play a critical role in disease pathogenesis. In general, antigen-presenting cells (APC), such as DCs and macrophages, capture and present antigen to T and B cells at which point activated CD4+ T cells produce subset specific cytokines that will define the adaptive immune response. Th1 and Th2 cells are associated with cellular and humoral responses, respectively, while T regulatory cells (Treg) have more suppressive functions (Murphy and Reiner, 2002). B cells are activated by Th2 cells and differentiate into plasma cells, which are able to produce high affinity antibodies against specific bacterial antigens. The inflammation due to the activation of the adaptive immune response is a key driver of connective tissue destruction and alveolar bone loss in periodontitis, with studies using T and/or B cell deficient mice observing decreased alveolar bone loss following oral infection with bacteria such as *P. gingivalis* (Baker et al., 1994, Baker et al., 2002).

Lindhe et al (1980) assessed the cellular composition of gingival tissue from patients with advanced periodontitis and reported plasma cells occupied 31% and lymphocytes occupied 5-10% of the lesion (Lindhe et al., 1980). Many studies have shown that in health and gingivitis, T cells dominate the tissue where as B cell numbers increase in periodontitis lesions under the influence of T cells, with active sites containing increased levels of lymphocytes compared with healthy or stable sites in the same patient (Amunulla et al., 2008, Seymour et al., 1979a, Berglundh and Donati, 2005). Furthermore, the development of gingivitis appears to be dominated by Th1 cells, while in periodontitis there is a shift towards the Th2 phenotype (Berglundh and Donati, 2005). PD mouse models have shown that IFNγ deficient mice are more resistant to alveolar bone loss following oral infection with *A. actinomycetemcomitans*, although a higher total bacterial load and the subsequent death of mice was observed (Garlet et al., 2008). This data suggests that the Th1 derived IFNγ is involved in protection from bacterial infection but also plays a role in tissue destruction and alveolar bone loss. Furthermore, other studies have investigated the role of bacteria in the Th1/Th2 balance, observing the 'red complex' bacteria *T. forsythia* causes a Th2 bias through TLR2 signalling on APCs which induced alveolar bone loss in mice (Myneni et al., 2011). More recently, focus has shifted towards Th17 cells and their role in PD due to studies reporting increased numbers of Th17 cells in gingival tissues of periodontitis patients compared with healthy controls (Adibrad et al., 2012, Cardoso et al., 2009). These cells are typically associated with inflammation, and in the gingival tissue of periodontitis patients have been found at sites of most inflammation, with increased numbers observed in at apical region compared with the coronal region and in active lesions compared with inactive lesions (Ohyama et al., 2009, Allam et al., 2011).

It is evident that B cells and plasma cells are the main cell type present in periodontitis lesions (Berglundh and Donati, 2005). Early studies investigating the role of B cells in PD measured significantly increased levels of IgG and IgM plasma cells in periodontitis patients compared with healthy controls (Mackler et al., 1977, Seymour and Greenspan, 1979). B cells have been shown to produce specific antibodies to periodontal bacteria but have also been shown to present antigen to T cells in periodontitis lesions (Orima et al., 1999). Studies have shown B cells stimulated with oral bacteria such as *P. gingivalis* and *P.*

intermedia up regulated CD83 and CD86 and in mixed leukocyte cultures B cells stimulated T cells to differentiate into Th1 cells and produce high levels of IFNγ (Mahanonda et al., 2002). This finding was further confirmed in a study by Gemmell *et al* (2002) using gingival tissues from periodontitis patients where it was reported that B cells were the main antigen presenting cells in active lesions (Gemmell et al., 2002). B cells also contribute to the destruction of alveolar bone where the deletion of IgD has been shown to reduce alveolar bone loss in mice following oral infection with *P. gingivalis* (Baker et al., 2009, Oliver-Bell et al., 2015).

1.1.3.7 Alveolar bone loss

The alveolar bone is the supporting structure of the tooth and is destroyed by the inflammatory lesion in periodontitis resulting in tooth loss. Bone resorption is a well-regulated process throughout the body, and in health bone formation and bone resorption occur continuously. However, in disease, this balance shifts and increased bone resorption occurs though osteoblast inhibition and increased osteoclast activation. Inflammatory mediators reported to stimulate osteoclast activation include IL-1ß, IL-6, TNFα, IL-17 and PGE_2 and the TNF family cytokine RANKL (Schett, 2011). RANKL is expressed by osteoblasts and other cell types including fibroblasts, T cells and B cells and induces the differentiation of osteoclasts from monocytes/macrophages as well as osteoclast activation (Bartold et al., 2010). RANKL is regulated in response to pro-inflammatory cytokines such as IL-1ß and TNFα; however, effects of RANKL can be blocked by its decoy receptor osteoprotegerin (OPG). In periodontitis patients, elevated levels of RANKL and low levels of OPG have been reported in the GCF fluid and periodontal tissues of periodontitis patients compared with healthy controls (Wara-aswapati et al., 2007, Baltacioglu et al., 2014). Studies have shown that the 90% of B cells and 50% of T cells within a periodontal lesion are RANKL positive and B cells produce RANKL in response to periodontal pathogens such as *A. actinomycetemcomitans* (Han et al., 2009, Kawai et al., 2006). *In vitro* co-cultures of gingival fibroblasts or human periodontal ligament and *P. gingivalis* also observed up regulation of RANKL and suppression of OPG highlighting the impact periodontal pathogens on alveolar bone loss (Belibasakis et al., 2007).

1.1.3.8 Soluble immune mediators in PD

Microbial biofilms activate and initiate the host inflammatory and immune response. In periodontitis the loss of periodontal tissue is widely accepted to be as a result of the host immune response to microbial biofilms. As a result of activation, chemical mediators are released by host cells, which in PD are generally believed to perpetuate inflammation at the site and contribute to local tissue destruction and alveolar bone loss. Some of the key mediators involved in this process are described below.

Complement

The complement system is a fundamental component of innate immunity and therefore plays an important role in maintaining oral health (Krauss et al., 2010). It is triggered through a variety of pathways, namely classical, alternative and lectin, and involves sequential activation of serum proteins, the result of which is the recruitment and activation of inflammatory cells, phagocytosis, microbial opsonisation and direct lysis of pathogens (Sarma and Ward, 2011). Complement is present in high concentrations in GCF, comprising of up to 70% of the serum (Schenkein and Genco, 1977a). Activated complement fragments are also present at higher concentrations in the GCF and gingival connective tissue of individuals with PD and are absent or in lower concentrations in healthy subjects (Schenkein and Genco, 1977b, Attstrom et al., 1975, Courts et al., 1977). This correlates with human experimental gingivitis models, where increases in complement were associated with clinical inflammation, plaque association and bleeding on probing (Patters et al., 1989). This suggests that excessive activation of complement is associated with periodontal inflammation. Recent *in vivo* studies using mice deficient in complement C5aR or use of a C5aR antagonist in WT mice observed resistance to PD with reduction of inflammatory cytokines including TNF, IL-1ß, IL-6 and IL-17A and reduced alveolar bone loss compared to controls (Abe et al., 2012, Liang et al., 2011). The ability of *P. gingivalis* to modulate the complement pathway has also been shown, with C3a and C5a receptor deficient mice resisting alveolar bone loss following *P. gingivalis* infection compared to WT controls (Hajishengallis et al., 2011).

Cytokines and chemokines

Cytokines are a large family of soluble mediators that bind to specific receptors on target cells to initiate cell signalling and subsequently phenotypic changes by regulating gene expression. Cytokines play a multitude of roles in the host, regulating homeostasis, cell proliferation, activation, differentiation and repair. Chemokines are a subset of cytokines that induce chemotaxis and are characterised by the spacing of the first two cysteine residues found in the protein into four families; C, CC, CXC, CX_3C where X is an amino acid. Both cytokines and chemokines play a critical role in inflammation, orchestrating both innate and adaptive responses in the host.

Within the oral cavity, innate immunity is predominantly activated through recognition of bacterial components via TLRs. Studies have suggested that commensal bacteria in the oral cavity stimulate a low level cytokine response in innate immune cells such as neutrophils, tissue macrophages and dendritic cells and host tissues including epithelial cells which may prime the host and maintain tissue integrity (Hasegawa et al., 2007). Furthermore, changes in microbial composition of plaque has also been reported to amplify the immune response leading to increased cytokine and chemokine production which may lead to chronic inflammation and destruction at the site (Handfield et al., 2005). The adaptive immune responses are dependant on the combinations of cytokines produced by dendritic cells and other APCs, which determines the differentiation of T cell subsets, each of which has a unique cytokine profile, and subsequently influences B cell development and function.

Many studies have investigated the role of cytokines and chemokines in PD, focusing on the response to specific bacteria, the role of single proteins in disease and more recently cytokine profiles associated with host cell responses to bacteria (Hasegawa et al., 2007, Taylor, 2010). Oral bacteria have been reported to cause secretion of a range of pro-inflammatory cytokines by host cells including IL-1α, IL-1ß, IL-6, IL-8, IL-10, IL-12, TNFα and IFNγ, which are involved in both innate and adaptive pathways (Preshaw and Taylor, 2011). The complexity of the interactions between cytokines and the innate and adaptive

immune responses in PD is described in Figure 1.6 and some key cytokine and chemokines studied in PD discussed further.

IL-1α and IL-1ß belong to the IL-1 super family of cytokines and play many roles in immunity, inflammation, connective tissue turnover and homeostasis. Produced by various cells including lymphocytes, fibroblasts, epithelial cells, macrophages and monocytes, studies have shown increased levels of IL-1ß present in GCF of periodontitis patients compared with healthy controls (Toyman et al., 2015). Increased levels of IL-1 have been associated with the increased production of tissue degrading enzymes such as prostaglandin E_2 and MMPs which can cause tissue destruction and attachment loss (Murayama et al., 2011). Both IL-1α and IL-1ß have also been shown to stimulate bone resorption and inhibit bone formation (Kayal, 2013).

The cytokine IL-6 plays a critical role in regulation and differentiation of B cell responses through T cell differentiation and also promote bone resorption (Baker et al., 1999). Studies have shown increased expression of IL-6 by gingival mononuclear cells in response to oral bacteria compared with mononuclear cells extracted from peripheral blood (Gemmell and Seymour, 1993). In the oral cavity, IL-6 is expressed by gingival fibroblasts and osteoblasts and increased levels have been reported in the GCF of periodontitis patients compared with healthy controls (Reinhardt et al., 1993). Furthermore, T cells in periodontitis patients also produce more IL-6 compared with T cells from healthy controls (Takeichi et al., 2000).

TNFα is a pro-inflammatory cytokine produced by monocytes and macrophages, which is able to cause vascular changes by up regulation of ICAM-1, VCAM-1 and E-selectin to recruit immune cells. This cytokine also promotes bone resorption, inhibits bone formation and increases collagenase secretion by fibroblasts resulting in destruction of periodontal tissue (Graves and Cochran, 2003). Studies have shown increased levels of TNFα present in both GCF and gingival tissues in periodontitis patients (Takeichi et al., 2000, Stashenko et al., 1991). TNFα also upregulates other pro-inflammatory cytokines such as IL-1ß and IL-6 which are associated with bone resorption. TNFα receptor deficient mice have reduced *P.*

gingivalis induced osteoclastogenesis compared with wild type controls (Graves et al., 2001, Papadopoulos et al., 2013).

IL-8 (CXCL8) is a pro-inflammatory chemokine that has been extensively used to measure inflammation in PD both *in vitro* and *in vivo* (Fukui et al., 2013, Dommisch et al., 2015). Produced by host tissues including gingival fibroblasts, gingival epithelial cells and innate immune cells, IL-8 levels are significantly increased in GCF and gingival tissues correlating with the severity of PD (Ertugrul et al., 2013, Teles et al., 2009, Noh et al., 2013). IL-8 expression is typically associated with migration of phagocytic cells to the site of infection however IL-8 has also been shown to have a direct effect on osteoclast differentiation and activity (Bendre et al., 2003). It should also be noted that IL-8 release occurs constitutively in the gingivae of healthy individuals and in germ-free mice, albeit at a lower level than in PD, which is believed to allow for chemotaxis of leukocytes into the gingivae during homeostasis (Darveau, 2009, Tonetti et al., 1994, Dixon et al., 2004).

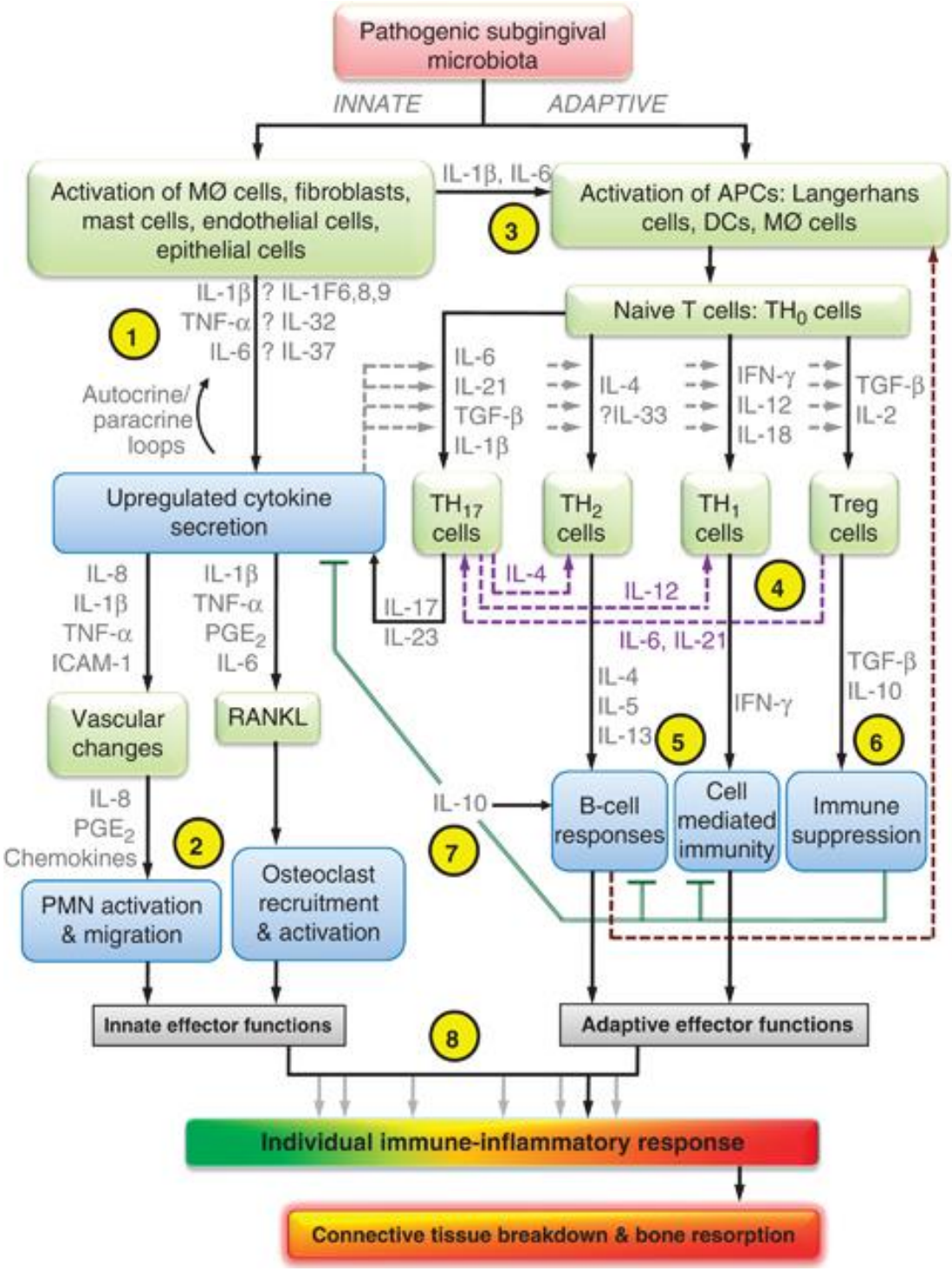


Figure 1.6: Cytokine networks in PD.

Diagram to represent the multiple interactions between cytokines and innate and adaptive immunity in PD (Kinane et al., 2011).

Antimicrobial peptides

Antimicrobial peptides (AMPs) are an essential component of the innate immune system. These mediators have strong antimicrobial activity against both Gram-positive and Gram-negative bacteria through the disruption of cell membrane integrity. Over 45 AMPs have been identified in the oral cavity, found both in saliva and in GCF (Gorr and Abdolhosseini, 2011). Studies have shown that AMPs are differentially regulated in PD, with 13 AMPs up regulated and 11 AMPs down regulated either at gene level or in saliva or GCF, although it has been suggested that down regulation of AMPs in saliva and GCF may be due to proteolytic degradation by bacterial proteases (Gorr, 2012). AMPs have a variety of biological activities that are believed to play a role in the innate defence against oral bacteria including direct antibacterial activity, inactivation of bacterial proteases and binding bacterial toxins. Some of the most commonly investigated AMPs include cathelicidin (LL-37) and defensins.

LL-37 is a multifunctional peptide expressed in saliva by epithelial cells and neutrophils. LL-37 has potent antibacterial activity and is able to kill both Gram-negative and Gram-positive bacteria including *S. gordonii*, *P. gingivalis* and *A. actinomycetemcomitans* in minimum inhibitory concentration (MIC) studies (Ji et al., 2007). However, its primary function is believed to be an immune cell activator due to the ability of LL-37 to act as a chemoattractant for neutrophils, monocytes and T cells and modulate the host pro-inflammatory response. LL-37 has been shown to directly bind heat killed *P. gingivalis, P. gingivalis* LPS and fimbriae subsequently inhibiting pro-inflammatory responses by human gingival fibroblasts, reducing IL-6, IL-8 and CXCL10 gene expression and protein release *in vitro* (Inomata et al., 2010). In the oral cavity, studies have also reported the presence of LL-37 in total GCF and shown its levels are increased in periodontitis patients compared with healthy controls (Puklo et al., 2008). Furthermore, in individual patients LL-37 levels are increased in GCF at inflamed sites compared with healthy sites and a lack of LL-37 production has been directly implicated in periodontitis development (Putsep et al., 2002, Dommisch et al., 2009).

Defensins are a family of AMPs divided into α and ß subgroups based on their cysteine spacing and connecting sulphide bonds. Human α-defensins (human

neutrophil peptides) are primarily expressed in neutrophils, while human ß-defensins are predominantly expressed in epithelial cells (Gorr, 2012). Defensin expression in human gingival epithelial cells from periodontitis patients has been shown *in vitro* to be differentially regulated by disease-associated bacteria (which increased hBD-1 and hBD-3) and health-associated bacteria (which increased hBD-2 and hBD-3) (Vankeerberghen et al., 2005). Recent studies investigating the expression of hBDs in gingival tissue reported increased levels of hBDs in periodontitis patients compared with health controls (Liu et al., 2014). Human neutrophil peptides 1-3 have also been shown to be significantly increased in the GCF of both chronic and aggressive periodontitis patients compared with healthy controls (Puklo et al., 2008). Similarly to LL-37, defensins are antibacterial and are able to kill phagocytosed bacteria in neutrophils where defensins are stored in secretory granules. Defensins are also able to act as a chemoattractant and activator of antigen presenting cells such as dendritic cells and have been shown to produce hBD1-3 in response to oral bacteria such as *F. nucleatum* and *P. gingivalis* (Yin et al., 2010). Studies have also reported the ability of defensins to inhibit LPS stimulated host responses, observing inhibition of IL-1ß, IL-8 protein release and ICAM-1 surface expression on human gingival fibroblasts which has been stimulated with *P. intermedia* LPS (Lee et al., 2010).

1.1.3.9 Subversion of host immune responses by oral bacteria

The transition from health to disease in the oral cavity is due to both a shift from a commensal microbial biofilm to a pathogenic one and the subsequent dysregulated host immune response to the biofilm. The dysregulation of the host immune system can be due to immunoregulatory defects such as leukocyte adhesion deficiency (LAD) but in 'healthy' individuals pathogenesis may be caused by subversion of the host immune responses by oral bacteria present within the biofilms. Most studies investigating immune subversion by bacteria have focused on the pathogen *P. gingivalis*, however, other species can also modulate the host immune response. Furthermore, it is believed that the bacteria associated with PD require inflammation as a source of nutrients such as collagen peptides and haem-containing compounds from the breakdown of

tissue therefore require subversion mechanisms to allow inflammation at the site while evading killing by the immune system (Hajishengallis, 2014).

It is known that microbial pathogens are able to modulate or inhibit TLR-mediated immune responses by either down regulating TLR expression or blocking TLR signalling pathways (Teng, 2006). *P. gingivalis* has been shown *in vitro* to block neutrophil phagocytosis and promote inflammation through crosstalk between complement receptor C5aR and TLR2 (Maekawa et al., 2014b). This process not only protects *P. gingivalis* from neutrophil phagocytosis but also bystander bacteria such as *F. nucleatum,* which cannot modulate neutrophil responses alone. Belibasakis et al (2013) observed *P. gingivalis* presence in a multi-species biofilm reduced nucleotide-binding oligomerisation domain-like receptor 3 (NLRP3), an inflammasome involved in innate immune responses of PD, when co-cultured with human gingival fibroblasts (Belibasakis et al., 2013a). Furthermore, the exclusion of *P. gingivalis* from this biofilm partly restored NLRP3 expression, with suggestions that the down regulation of NLRP3 by *P. gingivalis* modified host inflammatory responses to promote biofilm survival.

Bacteria including *P. gingivalis, P. intermedia* and *T. denticola* have been shown to possess mechanisms that allow the inhibition and subversion of host complement activation independent of normal complement activating pathways. *P. gingivalis* uses gingipains to degrade complement molecules such as C3 which supresses the formation of the membrane attack complex or the binding of opsonins on the surface of the pathogen (Wingrove et al., 1992). Furthermore, *P. gingivalis* has been shown to be able to negatively regulate complement activation by binding the complement inhibitor C4b-binding protein using the arginine-specific gingipain RgpB resulting in inhibition of the complement cascade (Potempa et al., 2008). *P. intermedia* supresses complement activation by binding the complement inhibitor serine protease Factor I (Malm et al., 2012). *T. denticola* appears to take a different approach to hijacking the complement system. Unlike *P. gingivalis* and *P. intermedia* which supress the complement pathway, *T. denticola* uses the protease dentilisin to generate active fragments of iC3b that is readily opsonized by (Yamazaki et al., 2006). While this may appear counterproductive iC3b mediated phagocytosis has been

suggested to be a weak killing mechanism and may facilitate safe intracellular passage into cells, a method that is used by pathogens such as *Mycobacterium tuberculosis* (Krauss et al., 2010, Ernst, 1998).

Many oral bacteria also possess proteases that are able to degrade host chemokines to subsequently alter the local environment and modulate the host inflammatory response. Of these the most studied are the gingipains produced by *P. gingivalis*, which have been shown to degrade IL-6 and IL-8 produced by gingival epithelial cells *in vitro* (Stathopoulou et al., 2009, Palm et al., 2014). *T. denticola* has also been shown to be able to degrade IL-8 protein produced by gingival epithelial cells through the use of the protease dentilisin (Jo et al., 2014). The ability of bacteria to degrade chemokines may have a downstream effect on both reducing the immune response to bacteria and promoting chronic inflammation.

Oral bacteria are also thought to modulate the adaptive immune responses in PD. *P. gingivalis* has been shown to promote differentiation of Th17 cells at the expense of Th1 cells by suppression of Th1 promoting cytokines such as CXCL10 (Jauregui et al., 2013, Moutsopoulos et al., 2012). Other studies have proposed the ability of *P. gingivalis* to subsequently modulate the host antibody response, with an IgG1 (Th2) response produced by *P. gingivalis* immunised mice, which shifted to an IgG2a (Th1) response when mice were first infected with *F. nucleatum* (Gemmell et al., 2004).

1.1.4 PD models

To enhance our understanding of PD, a variety of *in vitro* and *in vivo* models have been used. Some of the key models, their contribution to the field and advantages and disadvantages are described below.

1.1.4.1 Human *in vivo* models

Due to the complexity of PD the optimal model for use in studies would obviously be in humans. However, this is a highly expensive and regulated process with many ethical considerations. The first experimental gingivitis model in man was developed by Löe et al (1965) and demonstrated the casual role of bacteria in the induction of gingivitis. Dental students refrained from brushing for 21 days demonstrating that plaque accumulation over time directly correlated with increased gingival scores (Loe et al., 1965). Restoring oral hygiene and removing the plaque eliminated the inflammation. This simple model subsequently allowed various research groups to research many aspects of PD including histology of gingival lesions, cellular inflammatory responses and markers in both gingival tissue and GCF and also test the potential of antimicrobial and anti-inflammatory compounds in reducing the clinical markers of PD (Eberhard et al., 2013, Dommisch et al., 2015, Leishman et al., 2013, Heasman et al., 1993).

As scientific technology advances this model has been utilised to study differences in the host gene expression and the biofilm development in experimental gingivitis beyond culture based methods and cellular histology. Offenbacher et al (2009) measured the changes in patterns of whole-transcriptome gene expression during the induction and resolution of experimental gingivitis, observing the greatest differences of gene expression between health and disease to be found in genes encoding for inflammatory chemokines and cytokines (IL-1α, IL-1ß, IL-8 RANTES, CSF-3), stress mediators (superoxide dismutase 2) and reduction in antimicrobial peptides (beta 4 defensin) (Offenbacher et al., 2009). The composition and development of dental plaque in experimental gingivitis patients has also been examined using 454-pyrosequencing over the course of two weeks, reporting significant increases in bacterial diversity, relative abundance and gingivitis associated bacteria, such

as *F. nucleatum*, over the time course and compared to healthy controls (Kistler et al., 2013). The use of the experimental gingivitis model has also be used to study supra- and sub- gingival plaque formation in smokers vs. non-smokers, finding lower levels of gingival inflammation and low plaque scores in smokers compared with non-smokers and observing lower species diversity of subgingival plaque of periodontitis in smokers compared with non-smokers, highlighting the role of environmental factors in PD (Branco et al., 2015, Camelo-Castillo et al., 2015). The ability to study species diversity as plaque transitions from health to disease has also opened the field to allow for studies to find biomarkers to predict PD risk and progression. Huang *et al* (2014) using sequencing to profile the microbial changes of plaque from health to PD in an experimental gingivitis model and found eight microbial taxa which they propose can distinguish health from disease with 94% accuracy in this model (Huang et al., 2014). Another study sequenced the sub-gingival plaque of chronic periodontitis patients and found 80 OTU biomarkers of disease and 17 biomarkers of health which they propose can diagnose periodontitis having shown an 88% success rate in their work (Szafranski et al., 2015).

It should be noted there are limitations when interpreting results from experimental gingivitis models with relation to periodontitis. Experimental gingivitis models are a short-term method of assessing an acute inflammatory response initiated over a short period of time (1-2 weeks) in response to an increased level of bacteria in oral plaque that does not cause local destruction. Chronic periodontitis is typically a slow process involving a dysregulated immune response to the oral biofilm, which results in alveolar bone loss. A study comparing the composition of experimental gingivitis and chronic periodontitis plaque in humans also observed significant differences between the composition of these groups (Kistler et al., 2013).

1.1.4.2 Animal *in vivo* models

Animal models offer scope for investigations of mechanism and interventions in disease processes, the initial investigations of which would be unethical in humans. Various species have been used to investigate the pathogenesis of PD and test novel therapeutics. PD can occur naturally or be experimentally

induced in animals although care must be taken when considering results and their relationship to human PDs. Some of the most frequently used animal models are described below.

Non-human primates such as rhesus monkeys and baboons are advantageous to use in PD models due to their anatomical, immunological and microbiological similarities with humans. Primates have oral structures and teeth that are similar to those in humans and are also susceptible to naturally occurring PD and have naturally occurring plaque containing bacterial species similar to those found in human plaque. Studies using non-human primates typically accelerate periodontitis by the use of ligatures to accelerate plaque accumulation. They have also been used to study periodontal healing, biomaterials and implant surgery (Branch-Mays et al., 2008, Smith et al., 1993). Periodontitis in primates arguably offers the closest resemblance to human disease; however due to the expense of the maintenance, ethical considerations and high susceptibility to infections such as tuberculosis of these animals they are not the most practical model and are only rarely used (Weinberg and Bral, 1999).

Dogs have provided an alternative model to studying naturally occurring PD (Albuquerque et al., 2012). The anatomy, aetiology and physiological mechanisms of PD in canines are also well studied due to the historical use of dogs in periodontal research and in clinical research in veterinary medicine (Sorensen et al., 1980, Kortegaard et al., 2008). All domestic dogs have an increased natural susceptibility for PD with age and have been used to investigate both gingivitis and periodontitis *in vivo* (Struillou et al., 2010). Studies have also shown that the subgingival plaque in dogs is similar to that in humans, with *P. gingivalis, F. nucleatum* and *T. forsythia* present naturally in this species (Di Bello et al., 2014). There are however some limitations to the use of dogs as a model for PD, like non-human primates the costs of maintenance are high. Additionally, PD in dogs encompasses the complete width of the marginal gingivae in comparison to the tissue lateral to the gingival pocket in human PD, which may be considered a further limitation of the model (Haney et al., 1995).

Mice and rats are commonly used to study PD due to the availability of experimental reagents and genetically modified animals, as well as their relatively low maintenance costs, small size, and prompt availability. Due to extensive research using both mice and rats the genetics and immune system of these species is very well understood and as a result several rodent models have been used to investigate the mechanisms involved in host and bacterial interactions of PD. The calvarial (scalp) model was developed by Boyce et al (1989) to investigate the effect of cytokines on osteoclastogenesis, showing repeated infusions of IL-1α caused increased calvarial bone resorption (Boyce et al., 1989). This model was subsequently adapted for PD to study the effect of oral bacteria on bone resorption and investigate the host-bacterial interactions (Kesavalu et al., 2002, Leone et al., 2006, Dunmyer et al., 2012). In particular the calvarial model was used to study the role of *P. gingivalis* in osteoclastogenesis of wild type and TNF receptor knockout mice observing that TNF activity plays a critical role in the bone resorption induced by *P. gingivalis* by modulating fibroblast apoptosis, neutrophil recruitment and osteoclast formation (Graves et al., 2001). The calvarial model requires stimulus to be injected directly into the connective tissue overlying the calvarial bone, which stimulates inflammatory pathways at the site; however, as a result this model has no interaction with epithelial cells and therefore any information regarding their role in downstream osteoclastogenesis is lost.

Most of the periodontal pathogens that are present in the human oral microflora are not present in laboratory rodent strains, therefore the oral gavage models were introduced (Chang et al., 1988, Klausen et al., 1991). Mice or rats are treated with antibiotics prior to repeated oral administration of bacteria in a solution, which allows investigation of the impact of specific bacterial species on the periodontium. As a result mouse periodontitis models have been developed in which oral gavage of periodontal bacteria including *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* induces reproducible alveolar bone loss (Baker et al., 2000b, Garlet et al., 2006, Sharma et al., 2005). The oral gavage model has also been utilised to investigate the role of different aspects of the host response on PD. Studies have shown increased resistance to alveolar bone loss in mice deficient in MHC class II responsive CD4 T cells compared with normal mice after infection of *P. gingivalis* by oral gavage, additionally blocking

complement receptor 3 has also been shown to increase *P. gingivalis* clearance and reduce alveolar bone loss using this model (Baker et al., 1999, Hajishengallis et al., 2007). While the oral gavage model is useful for investigating host: pathogen interactions there are limitations. Notably, infections usually use one or two specific periodontal bacteria such as *P. gingivalis* or *P. intermedia* of the many species that are present in the human oral microflora at concentrations far higher than those found within the oral cavity and typically require multiple exposures to invoke a response. Additionally, antibiotic exposure prior to infection in an attempt to supress the murine oral flora does not necessarily result in the complete removal of oral bacteria in mice, therefore infection with *P. gingivalis* may modify the microflora present to become destructive and favour pathogen survival and development of PD. To combat this many groups use specific pathogen free mice, that is mice which are guaranteed to be free of particular pathogens, or germ free mice, which have no bacteria present at all, to study the host responses to a single bacterial species.

The airpouch model was originally developed by the Willoughby group to study the function of the synovial membrane by producing a subcutaneous airpouch from which the exudate fluid and epithelial lining can be examined (Edwards et al., 1981). This model was extensively used in RA studies before it was adapted for periodontitis to investigate the acute inflammatory responses to *P. gingivalis,* observing that *in situ P. gingivalis* is a strong pro-inflammatory stimulus to innate immune cells causing high levels of leukocyte infiltration into the airpouch cavity following infection (Pouliot et al., 2000). This study reported that PGE_2, which is an important marker for pathogenesis in PD, is produced not only from monocytes and macrophages, as originally believed, but also neutrophils in the inflammatory lesion. The main limiting factor of this model is the short duration of the airpouch only allows for investigation of the acute inflammatory response. Subsequently, the airpouch model was modified by Genco et al (1991) into the chamber model to allow chronic inflammation to be studied (Genco et al., 1991). The chamber model uses a coiled stainless-steel wire implanted subcutaneously which is allowed to heal and epithelialize for 10 days before subsequent injection of bacteria into the chamber following which fluid can be aspirated from the chamber and analysis of immune cell infiltrate, soluble mediators and bacteria can be performed. This model has been used to

demonstrate acquired immune response to *P. gingivalis* following injection into the chamber as well as bacterial colonisation, growth, virulence of periodontal bacterial strains as well as local tissue damage by the host immune system in response to them (Genco et al., 1991, Wang et al., 2014, Polak et al., 2013).

Many animal models ranging from mice to non-human primates induce PD by placing ligatures around the teeth. This process results in the accumulation of dental plaque, ulceration of the sulcular epithelium, facilitates the invasion of periodontal bacteria into the connective tissue, alveolar bone loss and loss of periodontal attachment. This model allows for the study of the role of both bacteria and the host response in PD as well as the effects of systemic disease on PD progression. Application of topical antiseptics or antibiotics has been shown to reduce loss of attachment and bone resorption and in this model Gram-negative bacteria have also been shown to enhance osteoclastogenesis (Samejima et al., 1990, Luan et al., 2008, Kenworthy and Baverel, 1981). Studies investigating the role of the inflammatory response have reported reduced gingival inflammation, osteoclast formation and alveolar bone loss when prostaglandin inhibitors or doxycycline are applied and increased bone destruction when excess cytokines such as IL-1 or TNF are introduced (Bezerra et al., 2002, Koide et al., 1995, Bezerra et al., 2000).

Each animal model for PD has advantages and disadvantages (Table 1.1). Non-human primates are most similar to the human condition and dogs have naturally occurring periodontitis with a similar microflora; however, the cost of maintenance, ethical considerations and limited availability of reagents and genetic modifications of these animals can limit their use in studies of PD. Mice and rats are less expensive to maintain and much is known about their genetics which allows manipulation and subsequent genetically modified strains; however these species have anatomical differences and do not have the same microflora found in the human disease. There is no single animal model that represents all aspects of human PD but *in vivo* models are useful for investigating the immune response to oral bacteria and how downstream cellular interactions can influence the oral cavity (Graves et al., 2012).

Table 1.1: Animal models in PD studies

Animal Model	Advantages	Disadvantages	References
Non-human primates	Similar oral cavity to humans Naturally occurring PD Similar bacterial species present	Expensive to maintain Ethical considerations High susceptibility to infections	(Maekawa et al., 2014a) (Ebersole et al., 2000) (Roberts et al., 2004)
Dogs	Naturally occurring PD	Expensive to maintain Dentition differs to humans	(Ji et al., 2015) (Albuquerque et al., 2014) (Shimizu et al., 2009)
Rodents	Low maintenance costs Defined models of PD Ease of handling Gene knock out technology available Large number of reagents available	Naturally resistant to periodontitis Different microflora from humans Large numbers required for studies	(Abe and Hajishengallis, 2013) (Barros et al., 2011) (Hajishengallis et al., 2011) (Polak et al., 2009)

A variety of animal models are used in PD studies. This table summarises some of the advantages and disadvantages of using each model and studies of PD in each species.

1.1.4.3 *In vitro* biofilm models

1.1.4.4 Multi-species biofilm models

Many studies have shown the diversity of supra- and sub-gingival biofilms and the associations of certain species with health and disease from human dental plaque. To further understand the role of plaque and specific bacteria in PD, various oral biofilm models have been developed to study biofilm formation, biofilm structure and antimicrobial susceptibility. There are a wide range of options and variables to consider when developing a biofilm, such as inoculum, culture media and conditions, substrate and bacteria species (Ammann et al., 2012).

Biofilm models of supra- and sub-gingival plaque have been created from both defined species or from undefined bacteria from pooled saliva or plaque samples (Table 1.2). Biofilm models from undefined samples typically contain more bacterial species than defined samples due to the species diversity present in the oral cavity. These samples typically contain the composition and complexity of the original samples; however, it is difficult to reproduce these models and delineate the exact mechanisms used compared with the defined biofilm models in studies (Li et al., 2014a, Hope et al., 2012, Maske et al., 2014, Pratten et al., 2000b).

As a result many research groups have developed defined biofilm models of supra- and sub-gingival plaque to study biological components of biofilms such as pH, antimicrobial susceptibility, formation, structure, antibiotic resistance and species-specific interactions within biofilms (Table 1.2). A ten species biofilm was developed by Kinniment et al (1996), grown in a constant depth film fermenter (CDFF) in complex medium to study biofilm growth kinetics, spatial distribution, reproducibility and antimicrobial susceptibility (Kinniment et al., 1996b, Kinniment et al., 1996a). This model was then adapted by Shu et al (2003) who replaced *S. sanguinis* with *S. salivarius* and *A. viscosus* with *A. naeslundii* to create a biofilm of nonureolytic bacteria to test the role of urease enzymes on the stability of the biofilm observing a requirement for these enzymes to ensure bacteria diversity following carbohydrate challenge (Shu et al., 2003). A five species caries biofilm model consisting of *S. oralis*, *A.*

naeslundii, S. mitis, S. downei and *S. sanguinis* was used to study biofilm formation in the presence of bovine milk osteopontin demonstrating reduction in overall biomass following treatment (Schlafer et al., 2012). Guggenheim and colleagues developed both supragingival and subgingival plaque models grown on pellicle coated hydroxyapatite (HA) disks with pooled human saliva for the study of biofilm structure and distribution, antimicrobial susceptibility and host-pathogen interactions (Guggenheim et al., 2001a, Guggenheim et al., 2009, Guggenheim et al., 2001b, Hofer et al., 2011). More recently this model has been advanced to include new species including *T. denticola* to study growth kinetics and spatial composition studied (Ammann et al., 2012). These biofilms can also include fungi; the six species supra-gingival plaque model developed by Thurnheer et al (2003) containing *A. naeslundii, F. nucleatum, S. oralis, S. sobrinus, V. dispar* and *Candida albicans* was used to investigate the diffusion of macromolecules throughout the biofilm (Thurnheer et al., 2003).

1.1.4.5 Host- pathogen interaction models

Supragingival and subgingival biofilms have been used to study host-pathogen interactions in PDs. The subgingival model developed by Guggenheim *et. al.* (2009) was co-cultured with primary human gingival epithelial cells to measure apoptosis and the inflammatory mediator response to oral biofilms, demonstrating that the biofilm caused increased cell death and pro-inflammatory mediator release over time (Guggenheim et al., 2009). The group then continued to investigate host-pathogen interactions using human gingival fibroblasts to compare the immune response to both supragingival and subgingival biofilms, observing increases in PGE_2, IL-6 and RANKL expression in co-cultures with sub-gingival biofilms compared with supra-gingival (Belibasakis et al., 2011b, Belibasakis and Guggenheim, 2011). A notable study was performed by transcriptional profiling of gingival fibroblasts with sub-gingival biofilms and sub-gingival biofilms without the 'red complex' bacteria. Here, genes encoding pro-inflammatory responses including cytokines, chemokines, toll-like receptors and heat shock protein were up regulated in response to both biofilms compared to the cells only control, although no differences in gene expression between the biofilm co-cultures was observed (Belibasakis et al., 2014).

The Ebersole group used biofilms in co-culture with the immortalised epithelial cell line OKF4 to understand host-pathogen interactions, investigating the inflammatory mediator response of epithelial cells to bacteria. Their studies showed that mono-species biofilms cause increased pro-inflammatory cytokine release compared with planktonic bacteria, with 'health-associated' bacteria causing lower cytokine release than 'disease-associated' bacteria (Peyyala et al., 2011, Peyyala et al., 2012). Further work by this group investigating the pro-inflammatory protein release by OKF4 epithelial cells co-cultured with multi-species biofilm models with differing bacterial compositions, reported differential protein release to each of the biofilms. IL-6, IL-8, Gro-1α and fracktalkine protein release by OKF4 cells was increased in *S. gordonii*/*A. naeslundii*/*F. nucleatum* biofilm co-cultures compared with *S. gordonii*/ *S. oralis* *S. sanguinis* biofilms and *S. gordonii*/*F. nucleatum*/*P. gingivalis* biofilms suggesting oral biofilm composition may play a role in the epithelial cell inflammatory response (Peyyala et al., 2013).

Organotypic 3-dimensional tissue models are potentially more representative of the oral and gingival mucosa *in vivo* than the 2D monolayer models typically used. However, they are labour intensive, expensive and time-consuming methods. When comparing the responses of 3D and 2D gingivae models, mRNA expression of cytokeratin proteins in monolayers of gingival epithelial cells was shown to be significantly lower than in a 3D organotypic model which has similar levels of cytokeratin expression as gingival tissue samples (Bao et al., 2014a). Additional studies testing dental resins on both monolayers and 3D cultures of gingival epithelial cells observed significant increases in cell death and loss of cell function in monolayers following treatment compared with the 3D model (Tomakidi et al., 2000). A 3-dimensional organotypic model of the oral mucosa was developed by Dongari-Bagtzoglou et al (2006) using the human oral keratinocyte cell line OKF6-TERT2 to create a multi-layered tissue (Dongari-Bagtzoglou and Kashleva, 2006). This model was used to study *Candida* biofilm formation and interaction with oral streptococci and the 3-dimensional tissue model (Diaz et al., 2012, Banerjee et al., 2013). Other 3-dimensional tissue models using primary gingival keratinocytes and fibroblasts have been used to study cell invasion and epithelial proliferation in culture with *F. nucleatum* biofilms, observing the ability of *F. nucleatum* to invade the superficial layers of

a 3D gingival tissues and cause increased MMP release (Dabija-Wolter et al., 2012, Pollanen et al., 2012). A study by Dickinson et al (2011) investigated the ability of different periodontal bacteria to invade oral tissue by culturing *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans* or *S. gordonii* with primary gingival epithelial cells cultured as a 3-dimensional tissue. The study reported differential invasion into the tissue, with *P. gingivalis* invading epithelial cells intracellularly, *F. nucleatum* and *A. actinomycetemcomitans* entering the tissue but not invading cells and *S. gordonii* remaining on the surface of the tissue (Dickinson et al., 2011).

One of the main limitations of *in vitro* 3-dimensional tissue models is that they are time consuming, with most models taking 2-3 weeks to form a suitable tissue (Dongari-Bagtzoglou and Kashleva, 2006). Commercially available organotypic 3-dimensional tissue models have been developed including MatTek®'s EpiGingival 3D tissue model which uses human derived epithelial cells grown on culture inserts. This model has been used to study antimicrobial activity and the host immune response to both oral multi-species oral biofilms and viruses (Hai et al., 2006, Belibasakis et al., 2013b, Yang et al., 2011). Notably, differences in the tissue IL-8 protein release was reported in studies co-culturing the 3D gingival tissue with the 10 species Zurich model with or without the 'red complex' bacteria, suggesting 'red complex' species may differentially regulate IL-8 in gingival epithelia and show the potential of using the organotypic 3-dimensional tissue models for the study of host-pathogen interactions in PD (Belibasakis et al., 2013b).

These examples show there are a large range of models that can be used to study host-pathogen interactions in PD and careful consideration must be taken to use an appropriate model for the scientific questions being asked in each study.

Table 1.2: Summary of *in vitro* multi-species biofilm models.

Biofilm model	Media	Substrate	Inoculum (Multi-species biofilm)	Application	Reference
Marsh	Porcine mucin Potassium chloride Proteose peptone Yeast extract Typticase peptone Cysteine hydrochloride Haemin	Polytetrafluoroethylene	*Streptococcus mutans* *Streptococcus oralis* *Streptococcus sanguinis* *Lactobacillus casei* *Actinomyces viscosus* *Neisseria subflava* *Veillonella dispar* *Porphyromonas gingivalis* *Prevotella nigrescens* *Fusobacterium nucleatum*	Growth kinetics Spatial distribution Antimicrobial susceptibility	(Kinniment et al., 1996b) (Kinniment et al., 1996b)
Zurich	Human saliva Human serum	Pellicle coated hydroxyapatite disk	*Streptococcus oralis* *Streptococcus intermedius* *Actinomyces naeslundii* *Veillonella dispar* *Prevotella intermedia* *Porphyromonas gingivalis* *Fusobacterium nucleatum* spp. vincentii *Campylobacter recuts* *Tannerella forsythia* *Treponema lecithinolyticum* *Tremponema denticola*	Growth kinetics Spatial distribution Antimicrobial susceptibility Epithelial cell viability Inflammatory mediator response	(Guggenheim et al., 2001a) (Guggenheim et al., 2009) (Guggenheim et al., 2001b) (Belibasakis et al., 2014) (Belibasakis and Guggenheim, 2011) (Ammann et al., 2012)

Kentucky	Brain heart infusion broth Hemin Menadione	Rigid gas permeable hard contact lenses	*Streptococcus gordonii* *Streptococcus oralis* *Streptococcus sanguinis* or *Streptococcus gordonii* *Actinomyces naeslundii* *Fusobacterium nucleatum* or *Streptococcus gordonii* *Fusobacterium nucleatum* *Porphyromonas gingivalis*	Epithelial cell inflammatory protein release	(Peyyala et al., 2011) (Peyyala et al., 2012) (Peyyala et al., 2013)
Eastman	Sodium chloride Potassium chloride Calcium chloride dihydrate Proteose peptone Lab Lemco powder Yeast extract Porcine stomach mucin	Bovine enamel disks	Undefined oral bacteria for pooled saliva	Antimicrobial testing Biofilm structure Biofilm composition	(Pratten et al., 1998) (Pratten and Wilson, 1999) (Pratten et al., 2000b) (Pratten et al., 2000a) (Pratten et al., 2003) (Dalwai et al., 2006)

1.1.5 Periodontal chemotherapeutics

The main aim of treatment of PD, in particular periodontitis, is to halt the progression of the disease and prevent further alveolar bone loss. Ideally, lost periodontal tissue is regenerated, but in many cases this remains an unachievable goal. Currently, the mainstay of treatment for PD involves the removal of both supra- and sub-gingival plaque by mechanical debridement by dentists combined with oral hygiene instructions for patients. Many studies have shown clinical improvement when plaque is removed and regression when plaque reoccurs (Tonetti et al., 2015, Becker et al., 1984, Checchi et al., 2002).

However, treatment is not always completely effective for all patients, some may find only limited improvement following treatment even with impeccable standards of oral hygiene, while others may regress over time after successful periodontal therapy. There are many factors that may influence the outcome of periodontal therapy. Smoking is a significant risk factor for periodontitis and subsequently many patients who do not respond well to PD treatment are smokers (Van der Velden et al., 2003). Additionally, systemic diseases such as diabetes mellitus can impact the response to treatment (Costa et al., 2013, Izuora et al., 2015). Poor patient compliance with oral hygiene regimes, and failure to return for maintenance treatments increases the likelihood of disease regression (Lee et al., 2015). Due to the fact that mechanical debridement alone may not effectively control infection, combined with limitations in the standards of plaque control that can be achieved by all patients, there have been many efforts to supplement mechanical therapy with other forms of chemotherapeutics in an attempt to maintain gingival health.

The most common forms of chemotherapeutics used for the management of PD are antimicrobial agents designed to reduce plaque and microbial re-colonisation through bacteriostatic and bactericidal properties. Loe *et. al.* (1970) reported inhibition of plaque formation and gingival inflammation in test subjects when using a 0.2% chlorhexidine (CHX) solution and from the many subsequent studies conducted CHX has been shown to be an effective adjunctive for the treatment of gingivitis and in the short term reduction of plaque in periodontitis (Loe and Schiott, 1970, Calderini et al., 2013, Manthena et al., 2014). Other topical

antimicrobial agents including hydrogen peroxide, povidone iodine, as well as essential oils such as tee tree oil, have been shown to be effective at reducing overall plaque accumulation and improve gingivitis and short term periodontitis treatment outcomes when used as an adjunct to mechanical debridement (Hossainian et al., 2011, Sahrmann et al., 2012, Feng et al., 2011). More recently local drug delivery devices such as CHX chips have been used at disease sites that are difficult to treat due to pocket depth, or lack of access for mechanical debridement (John et al., 2015). However, outcomes of studies using these are conflicting, with some finding improvement and others finding no difference in gingival index, probing pocket depth and clinical attachment level scores of chronic periodontitis patients when used in conjunction with mechanical debridement (Cosyn and Wyn, 2006).

Systemic antibiotics are used in treatment of some cases of periodontitis, in particular aggressive periodontitis, or in cases where patients have not responded to treatment as expected (Lang et al., 2015). Many studies have shown that systemically administered antimicrobials improve clinical scores in conjunction with mechanical debridement (Mombelli et al., 2015, Soares et al., 2014, Casarin et al., 2012). However, due to increased levels of antibiotic resistant bacteria found in the oral cavity combined with potential side effects for patients, the use of antimicrobials in the treatment of PD has become a controversial topic and novel methods of biofilm control remain desirable (Xie et al., 2014, Kuriyama et al., 2007).

Novel antimicrobials for use in periodontal therapy have become an increasingly popular area of research. Polyphenols are a family of compounds naturally derived from fruits, vegetables, herbs and spices and include phenolic acids, proanthocyanidines and flavonoids (Palaska et al., 2013). Cranberry polyphenols have been shown to inhibit biofilm formation of *F. nucleatum* and *P. gingivalis* and attenuate *P. gingivalis* gingipains actives (Yamanaka et al., 2007). Polyphenols from other blackcurrant and lingonberry juices have also been shown to inhibit co-aggregation of *S. mutans* with *F. nucleatum* and *A. naeslundii* (Riihinen et al., 2011). In addition to their antibacterial properties, polyphenols also possess potent anti-inflammatory effects with studies showing inhibition of *P. gingivalis* adhesion and reduction of inflammatory IL-1ß, IL-6, IL-

8 and TNFα protein release by oral carcinoma cells following polyphenol treatment compared with untreated controls (Lohr et al., 2011). The combination of antimicrobial and anti-inflammatory properties of polyphenols makes them a potentially useful target for periodontal therapy research (Shahzad et al., 2015).

It is well established that much of the local tissue destruction observed is a result of the inflammatory immune response of host in response to the bacterial challenge. Therefore, there are an increasing number of studies focusing on the potential of new chemotherapeutics that target the host response as an alternative to antimicrobials.

Pro- and anti-inflammatory cytokines play an important role in the pathogenesis of PD and therefore may be a potential target for therapy by either neutralising cytokines, blocking cytokine receptors or activating immunosuppressive pathways. The use of anti-cytokine therapy has been studied extensively as a treatment of RA, with the use of the anti-TNFα antibody Infliximab (Remicade) reducing inflammation of experimental arthritis. Both Infliximab and the genetically engineered TNFαRII Etanercept (Enbrel) have both been used in successful trials for treatment of Crohn's disease, psoriatic arthritis, RA, psoriasis and Ankylosing spondylitis (Waykole et al., 2009, Wooley et al., 1993). The IL-1R agonist Anakinra (Kineret) has also been used to reduce inflammation and cartilage destruction in RA (Mertens and Singh, 2009). Studies investigating potential of the IL-1 and TNFα antagonists as PD chemotherapeutics observed reduction of alveolar bone loss, reduced loss of tissue attachment and inhibition of inflammatory immune cells into the gingivae of non-human primates with experimental periodontitis following local treatment (Delima et al., 2001, Assuma et al., 1998). Use of recombinant human IL-11 in the twice-weekly treatment of ligature-induced periodontitis in dogs also found a significant reduction in clinical attachment and radiographic bone loss, suggesting that cytokine therapies may be a potential chemotherapeutic for the treatment of PD (Martuscelli et al., 2000).

Modulation of cell signalling pathways has also been proposed as a target for PD therapy. Predominantly activated by cytokines the mitogen-activated protein

kinase (MAPK) pathway is involved in host inflammation and is made up of three groups; extracellular signal-regulated kinases (ERK1/2), c-jun N-terminal kinases (JNKs) and p38. The p38 MAPK signalling pathway is involved in LPS induced inflammatory cytokine production and a variety of inhibitors have been produced which inhibit this pathway (Gulati et al., 2014). Studies have shown periodontal bone loss is significantly reduced in rats with treatment of a p38α MAPK inhibitor twice per week compared with a sham control when each group was exposed to Gram-negative bacterial LPS by oral gavage and gingival injections three times per week (Kirkwood et al., 2007). As signalling pathways are shared by a variety of cytokines, they are believed to be more potent than current cytokine strategies; however, more studies are required to determine if there are any risks associated.

Statins are a class of drugs that reduce cholesterol levels, particularly low-density lipoprotein cholesterol, and are usually prescribed to prevent (cardiovascular disease CVD). However, these drugs also have potential anti-inflammatory effects by blocking intermediate metabolites of the mevalonate pathway (Lazzerini et al., 2007). Recently, studies have suggested the use of statins to be beneficial as a chemotherapeutic of periodontitis. Statins have been shown to have an anti-inflammatory effect on periodontal ligament cell exposed to LPS with reductions of IL-1β, IL-6 and TNFα following treatment (Estanislau et al., 2015). Levels of pro-inflammatory cytokines IL-1β, IL-6 and TNFα have also been reported to be reduced in GCF of PD patients following systemic administration of statins (Suresh et al., 2013, Fentoglu et al., 2012). Statins can inhibit LPS induced osteoclastogenesis and subsequently reduce alveolar bone loss in experimental periodontitis models (Jin et al., 2014). Another study by Kim *et. al.* (2011) reported increased osteoblast differentiation of mouse periodontal ligament cell following statin treatment and suggested a role for statins in regenerating periodontal hard tissue (Kim et al., 2011). MMPs are considered critical in periodontium destruction, and secretion of MMP-1, MMP-2, MMP-8 and MMP-9 has been shown to be significantly decreased in macrophages following statin treatment (Luan et al., 2003). Taken together these studies indicate potential for statins as a chemotherapeutic for PD.

These studies show the potential for both antimicrobial and anti-inflammatory compounds to act as supplements to mechanical debridement to increase the success of periodontal therapy. However, at this point in time it is believed there is not enough direct evidence to recommend a specific protocol for the use of adjunctive compounds for periodontal therapy (Lang et al., 2015). Therefore, further studies are required to understand the potential benefits these compounds can have on treatment of PDs.

1.1.6 Summary and aims

The literature reviewed demonstrates that in the oral cavity both microbiological and immunological factors play an important role in the development and progression, or lack thereof, in PD. The microbial biofilm on the surface of the tooth develops at the gingival margin, in close proximity with host tissues and immune cells. Host: biofilm interactions are key and the composition of the biofilm is related to the host response, with keystone pathogens such as *P. gingivalis* able to subvert the immune response to maintain disease status. This immune response begins with the first line of defence, the epithelium and incorporates all the innate and adaptive immune cells. A variety of *in vitro* and *in vivo* models have been developed to investigate oral microbial biofilms and the interactions between oral biofilms and the host, advancing our understanding of the complex interactions which occur and gaining insight into how to treat them. *In vitro* models provide a defined and reproducible system to study the interactions between host cells and oral biofilms and the use of multiple models with varying compositions can allow the study of the host response to oral biofilms in both oral health and disease.

To further the current understanding of these interactions, the aim of this study was to develop three different reproducible, well characterised multi-species biofilm models which contain bacteria that are 'health-associated', 'intermediate' and 'disease-associated' to investigate host: pathogen interactions. The specific aims were as follows:

1. Develop three multi-species biofilm models of differing microbial compositions

2. Investigate how composition of *in vitro* biofilms impacts cellular responses in co-culture with:

    a. Epithelial cells

    b. Neutrophils

    c. Monocytes/macrophages

3. Investigate the potential for the co-culture models use in testing novel anti-inflammatory and antimicrobial actives

2 Materials and Methods

2.1 Bacterial based methods

2.1.1 Bacterial culture and standardization

A selection of laboratory strains of commensal and pathogenic bacteria associated with oral biofilm disease were used in this study, including *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* ATCC 27335, *Streptococcus oralis* ATCC 35037 and *Aggregatibacter actinomycetemcomitans* OSM 1123, which were grown and maintained at 37°C on Colombia blood agar (CBA) [Oxoid, Hampshire, UK] in 5% CO_2. *Fusobacterium nucleatum* ATCC 10593, *Fusobacterium nucleatum* ssp. *vincentii* ATCC 49256, *Actinomyces naeslundii* ATCC 19039, *Veillonella dispar* ATCC 27335, *Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas gingivalis* W83 and *Porphyromonas gingivalis* W50 were maintained at 37°C on fastidious anaerobic agar (FAA) [Lab M, Lancashire, UK] under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2 [Don Whitley Scientific Limited, Shipley, UK]). All isolates were stored in Microbank® vials (Pro-Lab Diagnostics, Cheshire, UK) at -80°C until required.

P. gingivalis strains, *F. nucleatum, F. nucleatum* ssp. *vincentii* were propagated in 10 mL Schaedler's anaerobic broth [Oxoid, Hampshire, UK], *V. dispar, A. naeslundii* and *P. intermedia* were grown in 10 mL of brain heart infusion broth (BHI) [Sigma-Aldrich, Dorset, UK]. *S. mitis, S. intermedius, S. oralis* and *A. actinomycetemcomitans* were grown in 10 mL tryptic soy broth (TSB) [Sigma-Aldrich, Dorset, UK] supplemented with 0.6% yeast extract [Formedium, Hunstanton, UK] and 0.8% glucose [Sigma-Aldrich, Dorset, UK]. Cultures were washed by centrifugation (3000 rpm) and resuspended in 10 mL phosphatase buffered saline (PBS) [Sigma-Aldrich, Dorset, UK]. All cultures were then standardised and adjusted to a final working concentration of 1 x 10^8 cells/mL for further studies.

2.1.2 Artificial Saliva

All biofilm cultures were grown using artificial saliva (AS) as previously described (Pratten et al., 1998). This was comprised of porcine stomach mucins (0.25% w/v) [Sigma-Aldrich, Dorset, UK], sodium chloride (0.35 w/v) [VWR, Leuven, Belgium] potassium chloride (0.02 w/v) [VWR, Leuven, Belgium], calcium chloride dihydrate (0.02 w/v) [VWR, Leuven, Belgium], yeast extract (0.2 w/v) [Formedium, Hunstanton, UK], lab lemco powder (0.1 w/v) [Oxoid, Hampshire, UK] and proteose peptone (0.5 w/v) [Sigma-Aldrich, Dorset, UK] in ddH_2O [Thermo Scientific, DE, USA]. Urea [Sigma-Aldrich, Dorset, UK] was diluted in ddH_2O (40% w/v) and added to a final concentration of 0.05% (v/v) in AS.

2.1.3 Single species *P. gingivalis* biofilms culture

Single species biofilms of *P. gingivalis* were prepared in 24 well plates [Corning, NY, USA] containing Thermanox™ coverslips (13mm diameter, [Fisher Scientific, Loughborough, UK]). Standardised suspensions of 1 x 10^7 CFU/mL, at 0.2 OD_{550}, were prepared in 500 µL of AS for each *P. gingivalis* strain. Biofilms were grown for 4 days at 37°C in the anaerobic chamber with supernatants removed and fresh AS added each day.

2.1.4 Multi-species biofilms culture (4 species)

A simple 4 species multi-species periodontal biofilm model consisting of *S. mitis, F. nucleatum, A. actinomycetemcomitans* and *P. gingivalis* was previously developed (Jose, 2013). Biofilms were prepared in 24 well plates [Corning, NY, USA] containing Thermanox™ coverslips (13mm diameter, [Fisher Scientific, Loughborough, UK]). For the addition of each bacterial species to the biofilm a standardised suspension of 1 x 10^7 CFU/mL was prepared in 500 µL of AS. *S. mitis* biofilms were grown for 24 hours at 37°C, 5% CO_2, supernatant was then removed and standardised *F. nucleatum* in AS was added and the biofilms incubated anaerobically for a further 24 hours. The supernatant was removed and finally the standardised *P. gingivalis* and *A. actinomycetemcomitans* in AS added to the biofilm and incubated at 37°C in the anaerobic chamber for a further 4 days. Each day supernatants were removed and fresh AS added.

2.1.5 Generation of multi-species biofilms (3, 7 &10 species)

Three *in vitro* biofilm models were developed to represent and recapitulate the stages from health to disease in periodontitis. Biofilms were prepared in 24 well plates [Corning, NY, USA] containing Thermanox™ coverslips (13mm diameter, [Fisher Scientific, Loughborough, UK]). For the addition of each bacterial species to the biofilm a standardized suspension of 1 x 10^7 CFU/mL was prepared in 500 µL of AS.

A three species biofilm model containing *S. mitis, S. intermedius* and *S. oralis* was developed to model 'health-associated' biofilms in the oral cavity. All species were added together and incubated at 37°C in 5% CO_2 for 4 days with spent supernatants being removed and replaced with fresh AS daily.

A seven species biofilm model containing *S. mitis, S. intermedius* and *S. oralis*, as well as *F. nucleatum, F. nucleatum* ssp. *vincentii, A. naeslundii and V. dispar* was developed to model an 'intermediate' biofilms, transitioning from health to a diseased state. Briefly, *S. mitis, S. intermedius* and *S. oralis* were grown for 24 hours and incubated at 37°C in 5% CO_2. Next, supernatant was removed and standardized *F. nucleatum, F. nucleatum* ssp. *vincentii*, *A. naeslundii* and *V. dispar* were added to the biofilms and incubated at 37°C in the anaerobic cabinet for 4 days, with spent supernatants being removed and replaced with fresh AS daily.

A ten species biofilm model was formed as described in the 7 species, but with the addition of *P. gingivalis, P. intermedia and A. actinomycetemcomitans* which was standardized and added on the third day. Biofilms were incubated at 37°C in the anaerobic cabinet for 4 days, with spent supernatants being removed and replaced with fresh AS daily.

Biofilms were used directly after culture or AS removed and stored at -80°C until required. Frozen biofilms were revived by the addition of 500 µL of AS, incubating for 24 hours in the anaerobic cabinet before experimental use.

2.1.6 Growth kinetics of *P. gingivalis*

The growth kinetics of *P. gingivalis* was assessed. Each strain was standardised to 1 x 10^4 CFU/mL in Schaedler's anaerobic broth with 200 µL added to each well of a 96 well round bottomed plate. The plate was incubated at 37°C in the anaerobic chamber and the absorbance was measured at 650 nm every 3 hours following shaking at 100 rpm for 30 seconds prior to reading. Each strain was tested with 8 replicate cultures and repeated on three independent occasions. Negative controls containing Schaedler's anaerobic broth only were included for background correction.

2.1.7 Bacterial quantification: Miles and Misra

Live *P. gingivalis* bacteria were quantified using the Miles and Misra method (Miles et al., 1938). Briefly, biofilms were removed from Thermanox™ coverslips in a sonic bath at 35 kHz for 10 minutes in 1 mL of PBS. The inoculum was serially ten-fold diluted from neat supernatant to 10^{-8} in PBS. For each dilution, 20 µL was dropped-plated in triplicate on FAA plates and left to dry on the bench for 30 minutes before being cultured at 37°C in the anaerobic cabinet for 48 hours. Following incubation, colonies were then counted at each dilution where the number of colonies ranged between 30 – 300 and the CFU calculated as follows: CFU = mean no. of colonies in dilution x 50 x dilution factor. The experiment was performed on three samples per group and repeated on three separate occasions.

2.1.8 Biomass quantification (crystal violet)

To quantify biomass, biofilms were washed with PBS and allowed to air dry for 1 hour at 37°C. 100 µL of 0.05 (w/v) of crystal violet (CV) solution was added to each biofilm and incubated at room temperature for 30 minutes to allow for the uptake of the dye. Following incubation the CV solution was removed and biofilms were washed using ddH_2O to remove any excess dye. Next, 100 µL of 100% ethanol was added to samples to de-stain each biofilm and mixed thoroughly to ensure complete removal. Contents of each well were then transferred to a fresh 96 well flat-bottom micro titre plate for measurement. The biomass was quantified spectrophotometrically by reading absorbance at

570 nm in a microtitre plate reader [FLUOStar Omega, BMG Labtech, VA, USA]. All absorbance values were blank corrected based upon the negative control where no biofilms were formed.

2.1.9 Impact of freezing on biofilm viability

To assess the effects of freezing on the biofilm, as a means of creating biofilm stocks, AS was removed from the biofilms, which were then sealed within 24 well plates and stored at -80ºC. They were latterly revived by adding 500 µL of AS and incubating in at 37ºC in the anaerobic chamber for 24 hours. Total cell counts were then performed by qPCR as described in section 2.1.14. The experiment was performed in triplicate on three separate occasions.

2.1.10 Survival of biofilms in cell culture media

Frozen biofilms were revived in AS overnight at 37ºC in the anaerobic cabinet. The biofilms were maintained in defined-KSFM (d-KSFM) [Invitrogen, Paisley, UK] for 4 and 24 hours at 37ºC in 5% CO_2. After incubation biofilms were washed with sterile PBS to remove non-adherent bacteria and total cell counts performed as described previously section 2.1.14.

2.1.11 Exogenous IL-8 degradation assay

Single strain *P. gingivalis* biofilms were cultured as described section 2.1.3 in a 24 well plate. At maturation AS was removed and replaced with 500 µL of Schaedler's anaerobic broth containing 300 pg/mL exogenous IL-8 [PeproTech, London, UK] then incubated at 37ºC in the anaerobic chamber for 0, 1, 4 and 24 hours. A bacteria-free control containing 300 pg/mL of exogenous IL-8 without biofilm was used to measure natural degradation of IL-8 over time. At each time point supernatants were removed from the culture and stored at -20ºC until required, at which time the remaining IL-8 in each sample was measured by ELISA (section 2.3.8) Each strain was tested in triplicate on three independent occasions.

2.1.12 Live, dead, filtered and unfiltered conditions for *P. gingivalis* co-culture

A variety of conditions were measured in the co-culture of *P. gingivalis* strains with OKF6-TERT2 oral epithelial cells. For co-culture of epithelial cells with live planktonic *P. gingivalis*, strains were cultured in broth and standardized as described in section 2.1.1. Standardised bacteria were resuspended in d-KSFM with growth-promoting additives of which 500 µL was added to co-culture. For co-culture with methanol fixed (dead) planktonic *P. gingivalis*, bacteria were cultured and standardized as described for live planktonic *P. gingivalis* co-culture, then resuspended in 100% methanol for 30 minutes on a shaking platform 200 rpm at 37°C in the anaerobic cabinet. Next, bacteria were washed in triplicate using PBS to remove excess methanol and resuspended in d-KSFM for use in co-culture. For co-culture with biofilms supernatants, biofilms were cultured for 24 hours in d-KSFM at 37°C in the anaerobic cabinet. Following incubation, supernatants were removed and passed through a 0.22 µm Ministart® hydrophobic filter [Sartorius, Surrey, UK] before use in co-culture. Finally, for co-culture with unfiltered *P. gingivalis* biofilm supernatants biofilms were cultured for 24 hours in d-KSFM at 37°C in the anaerobic cabinet. Following incubation, supernatants were removed and 500 µL used for co-cultures.

2.1.13 Extraction of DNA from bacteria

DNA extraction of bacteria was performed using the MasterPure® Gram Positive DNA purification kit [Epicentre®, Cambridge, UK]. Biofilm samples were sonicated in 1 mL of PBS for 10 minutes and the supernatant transferred to a 1.5 mL microfuge tube and centrifuged at 13000 rpm for 10 minutes to pellet the sample. For planktonic samples 1 mL of supernatant was transferred to a 1.5 mL microfuge tube and centrifuged at 13000 rpm for 10 minutes to pellet the sample. The supernatants were then discarded and 150 µL of TE buffer (10 mM Tris-HCl, 1mM EDTA) [Epicentre®, Cambridge, UK] was added to each sample to resuspend the pellet and 1 µL of Ready-lyse™ lysozyme solution [Epicentre®, Cambridge, UK] added to break down the bacterial cells. Samples were incubated at 37°C for 2.5 hours. Following this a solution containing 1 µL proteinase K (50 µg/mL) and 150 µL of Gram positive cell lysis solution from the MasterPure® Gram Positive DNA purification kit was added to each sample and

incubated at 65ºC for 15 minutes. Samples were cooled to 37ºC and stored on ice for 5 minutes before DNA precipitation. Next, to each lysed sample 175 µL of MPC protein precipitation reagent [Epicentre®, Cambridge, UK] was added and vortexed for 10 seconds. Samples were then centrifuged at 13000 rpm for 10 minutes at 4ºC with the resultant supernatant transferred to a clean microfuge tube and 1 µL of RNase A (5 µg/mL) added and incubated for a further 30 minutes at 37ºC. 500 µL of isopropanol was then added to each sample which was mixed by inverting each tube 40 times before centrifugation at 13000 rpm for 10 minutes at 4ºC. Finally, the DNA pellet was washed with 70% ethanol and resuspended in 35 µL of TE buffer.

2.1.14 Quantification of bacteria (PCR)

To quantify bacteria by PCR, one microliter of extracted DNA, as outlined in the previous section, was added to a master mix containing 12.5 µL SYBR® GreenER™ [Life technologies, Paisley, UK]. 9.5 µL of UV-treated RNase-free water [Life technologies, Paisley, UK]. and 1 µL of 10 µM forward/reverse primers for each bacteria (Table 2.1). For each experiment three independent replicates were analysed in triplicate using the MxProP Quantitative PCR machine and MxProP 3000 software [Stratagene, Amsterdam, Netherlands]. The thermal profile was 95ºC for 10 minutes to allow for DNA polymerase activation and 40 amplification cycles of 95ºC denaturation for 30 seconds, 55ºC annealing for 60 seconds and 72ºC extension for 60 seconds. A dissociation curve was performed following the final amplification cycle for confirmation of only one product. This cycle consisted of 95ºC for 60 seconds, 55ºC for 30 seconds and 92ºC for 30 seconds. A non-template control replacing DNA with sterile water was included in each experiment to rule out the presence of contamination. The cycle threshold (Ct) was set automatically and Ct values for all samples of interest.

Table 2.1: Primer sequences used for bacterial PCR

Target	Primer Sequence (5'-3')	Reference
Streptococcus species (*S. mitis, S. intermedius* and *S. oralis)*	F-GATACATAGCCGACCTGAG R- CCATTGCCGAAGATTCC	(Periasamy et al., 2009)
F. nucleatum and *F. nucleatum* ssp. *vincentii*	F-GGATTTATTGGGCGTAAAGC R- GGCATTCCTACAAATATCTACGAA	(Sanchez et al., 2014)
A. naeslundii	F-GGCTGCGATACCGTGAGG R- TCTGCGATTACTAGCGACTCC	(Periasamy et al., 2009)
V. dispar	F-CCGTGATGGGATGGAAACTGC R- CCTTCGCCACTGGTGTTCTTC	(Periasamy and Kolenbrander, 2009)
P. gingivalis	F-GCGCTCAACGTTCAGCC R-CACGAATTCGCCTGC	(Boutaga et al., 2003)
P. intermedia	F-CGGTCTGTTAAGCGTGTTGTG R- CACCATGAATTCCGCATACG	(Loozen et al., 2011)
A. actinomycetemcomitans	F-GAACCTTACCTACTCTTGACATCCGAA R- TGCAGCACCTGTCTCAAAGC	(van der Reijden et al., 2010)

2.1.15 Generating standard curves for bacteria

Quantification of each bacterial species was carried out by standardising each bacterial species to 1 x 10^8 CFU/mL. DNA was extracted from bacteria as described in section 2.1.13 using ten-fold serial dilutions of bacteria ranging from 1 x 10^3 to 1 x 10^8 CFU/mL, and qPCR performed as described in the previous section. Standard curves were constructed by plotting the Ct values against the known diluted quantities of DNA. These were calculated by log transforming the known concentrations of DNA then using non-linear regression analysis (Sigmoidal-dose response – variable slope) to interpolate the Ct values from these known concentrations as numbers of bacteria as a standard curve for each bacterial species. The R^2 value was used to determine the fit of the line calculated as follows; $R^2 = 1- SS_{residuals} / SS_{total.}$ R^2 values ranging between 0.95-1.0 were considered to show a good fit of the line and validate the use of PCR to determine bacterial quantities. Each dilution of DNA was assessed in triplicate for each primer set.

2.1.16 Live dead PCR

To quantify the number of live and dead bacteria in a biofilm a live/dead qPCR method was used as described in (Alvarez et al., 2013). This method uses propidium monoazide which is able to penetrate dead cells due to loss of cell membrane structure. Once inside the cell propidium monoazide binds to double stranded DNA (ds-DNA), which when exposed to bright light results in a permanent modification of the ds-DNA. This modification makes it unable to be used as a qPCR template and allows the discrimination by live and dead cells by qPCR.

Briefly, biofilms grown on Thermanox™ coverslips were sonicated at 35 kHz in a sonic bath for 10 minutes and the sonicate transferred to a RNase-free microfuge tube. 5 µL of 10 mM of propidium monoazide [Sigma-Aldrich, Dorset, UK] was added to each sample with additional samples containing no propidium monoazide included as a control. All samples were then incubated in the dark at room temperature for 10 minutes to allow for uptake of propidium monoazide into the cells. Samples were then placed on ice on a rocking platform and exposed to a 650W halogen light positioned 20 cm away for 5 minutes. Following exposure, DNA could then be extracted from samples as described in section 2.2.13 or stored at -20ºC until required. Following DNA extraction, biofilm composition could then be quantified by qPCR as described in section 2.1.14. Samples containing propidium monoazide only amplify live cells and control samples without propidium monoazide amplify all cells. Thus, the number of dead cells can also be quantified by subtracting the number of live cells by the number of total cells in each sample group. Each experiment was conducted in triplicate and repeated on three separate occasions.

2.1.17 Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on mature biofilms. Following maturation, biofilms were carefully washed using PBS before fixation in 2% paraformaldehyde, 2% gluteraldehyde, 0.15 M sodium cacodylate and 0.15% (w/v) alcian blue, pH 7.4 overnight. The fixative was discarded and replaced with 0.15 M sodium cacodylate buffer and stored at 4ºC until processing. Samples were then prepared for SEM as previously described (Erlandsen et al.,

2004). Briefly, samples were washed three times for 5 minutes with 0.15 M cacodylate to ensure all gluteraldehyde had been removed. Following this, samples were then treated with 1% osmium tetroxide solution containing 0.15 M sodium cacodylate (1:1) and incubated in the fume hood for 1 hour. After incubation samples were then washed three times for 10 minutes in distilled water before treatment with 0.5% uranyl acetate and incubated for a further hour in the dark. Uranyl acetate was then removed from the samples and washed with water before a series of dehydration steps were carried out where 2 x 5 minute washes of 30, 50, 70 and 90% alcohol followed by 4 x 10 minute washes of absolute and dried absolute alcohol were used. Hexamethyldisilazane (HMDS) was used to dry the samples by soaking for 5 minutes before transferring to a fresh plate containing HMDS. All samples were then placed in a desiccator overnight to allow evaporation of any residual drying agent. The specimens were then mounted and sputter-coated with gold in an argon filled chamber and viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop software [Adobe, San Jose, CA, USA].

2.2 Cell based methods

2.2.1 Growth and maintenance of OKF6-TERT2

OKF6-TERT2 cells [Rheinwald Laboratory, Brigham and Woman's Hospital, Boston, USA] are an immortalised human oral keratinocyte cell line developed through the forced expression of telomerase (Dickson et al., 2000, Dongari-Bagtzoglou and Kashleva, 2006). These cells were maintained in keratinocyte serum-free medium (KSFM) [37010-022 Invitrogen, Paisley, UK] supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF) and 0.4 mM $CaCl_2$. Cells were seeded at 5 x 10^4 cells/mL in a 75cm^2 cell culture flask [Corning, NY, USA] and passaged at 90% confluence. To do this the adherent monolayer of cells was detached using 0.05% trypsin EDTA which was then neutralised with 15 mL of Dulbecco's modified Eagle's media (DMEM) [Sigma-Aldrich, Dorset, UK]. Following this cells were washed in Hanks balanced salt solution [Sigma-Aldrich, Dorset, UK] containing 10% foetal calf serum (FCS) [Sigma-Aldrich, Dorset, UK] to inactivate the trypsin and resuspend in 5 mL of KSFM before counting using a haemocytometer with 20 µL of cells mixed with 10 µL of trypan blue [Sigma-Aldrich, Dorset, UK] to give a dilution factor of 1.5. Cells were viewed under a light microscope and round, healthy clear cells counted using the appropriate grid. Trypan blue stains dead cells blue through the loss of cell wall integrity and these cells were therefore omitted during the counting process. Cells were re-seeded at 1 x 10^5 cells/flask in KSFM.

Frozen stocks of OKF6-TERT2 oral epithelial cells were prepared for long term storage in liquid nitrogen. Cell suspensions were standardised to 2 x 10^6 cells/mL in DMEM supplemented with 20% FCS. Equal volumes of 20% dimethyl sulfoxide (DMSO) and the OKF6-TERT2 cells were transferred to a cryotube and stored at minus 80^{o}C in an insulated box overnight to ensure the cells were cooled slowly before final storage in liquid nitrogen. To retrieve cells from frozen stocks, cryovials were thawed at 37^{o}C before transferring into a cell culture flask containing KSFM. For experiments, OKF6-TERT2 cells were seeded at 1x 10^5 cells/mL in d-KSFM where BPE is replaced with defined keratinocyte-SFM Growth supplement.

2.2.2 Primary cell culture

Primary human oral keratinocytes [Tebu-bio, Peterborough, UK] cultured from the oral mucosa of 58 year old male were used as a comparator with the OKF6-TERT2 cell line. Briefly, cells were seeded at 2.5 x 10^5 cells/flask in KSFM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 25 µg/mL bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF) and 0.4 mM $CaCl_2$ and maintained until 80% confluence. To passage cells, KSFM was removed and 5 mL of Acutase® [Sigma-Aldrich, Dorset, UK] added to disaggregate the cells from the surface before washing with DMEM to remove the Acutase®. Cells were resuspended in 5 mL of KSFM before counting using a haemocytometer with 20 µL of cells mixed with 10 µL of trypan blue [Sigma-Aldrich, Dorset, UK] to give a dilution factor of 1.5. Cells were viewed under a light microscope and viable cells counted using the appropriate grid. Cells were re-seeded at 1 x 10^5 cells/flask in KSFM.

Frozen stocks of primary oral keratinocytes were prepared as described for OKF6-TERT2 cells in the previous section. For experiments, primary oral keratinocytes were seeded at 1x 10^5 cells/mL in d-KSFM where BPE is replaced with growth-promoting additives and not used beyond passage 4 as per manufacturer's instructions.

2.2.3 Co-culture of epithelial cells and biofilms

OKF6-TERT2 cells were seeded at 1 x 10^5 cells/mL in 24 well plates in d-KSFM and incubated overnight at 37°C 5% CO_2. Following incubation cells were washed with PBS and 500 µL of fresh d-KSFM added to each well. Biofilms were attached to the underside of a hanging cell culture insert [Millipore, Massachusetts, USA] using Vaseline®, then placed adjacent to the OKF6-TERT2 cell monolayer. Figure 2.1 shows a schematic of the co-culture system. Epithelial cells were incubated with the biofilm for 4 and 24 hours at 37°C in 5% CO_2. Following stimulation, supernatants and cell lysates were retained to assess changes in gene and protein expression of a variety of pro-inflammatory mediators.

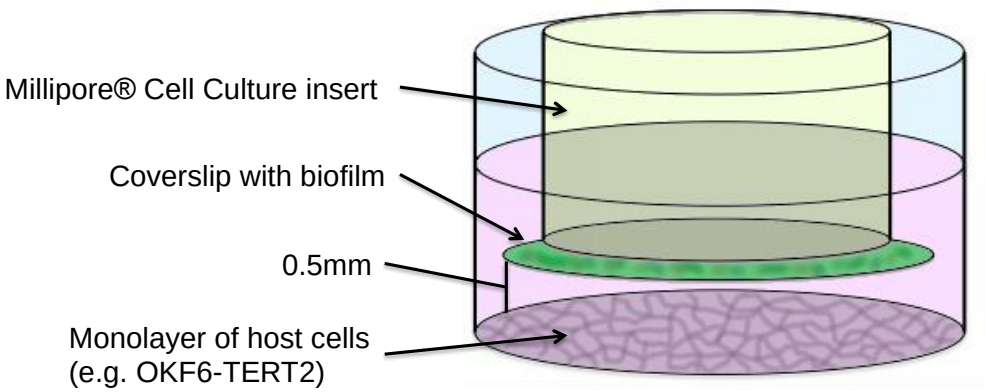


Figure 2.1: Host cell: biofilm co-culture model system
Hanging baskets with Thermanox™ coverslips containing multi-species biofilms were introduced into each well of a 24 well plate. Inverted coverslips were secured to the hanging basket using sterile Vaseline® with a 0.5 mm space between the biofilm and cell monolayer.

2.2.4 Inverted biofilm co-culture

To investigate any gravitational effect of biofilm co-culture with epithelial cells an inverted biofilm co-culture experiment was performed. Briefly, the co-culture model described in section 2.2.3 was inverted to allow biofilms to grow on the 24 well plate surface and OKF6-TERT2 cells to be hanging over. This was achieved by seeding OKF6-TERT2 oral epithelial cells at 1 x 10^5 cells/mL, using 500 µL on Thermanox™ coverslips and incubated overnight in KSFM at 37°C 5% CO_2. Following incubation, cells were washed in PBS and attached to the underside of a hanging cell culture insert [Millipore, Massachusetts, USA] using Vaseline®. This would be inserted into 24 well plates containing multi-species biofilms on the plate surface. Cells were incubated in d-KSFM for 4 and 24 hours in 37°C 5% CO_2 before removal of supernatants for further analysis. Figure 2.2 shows a schematic of the inverted co-culture system.

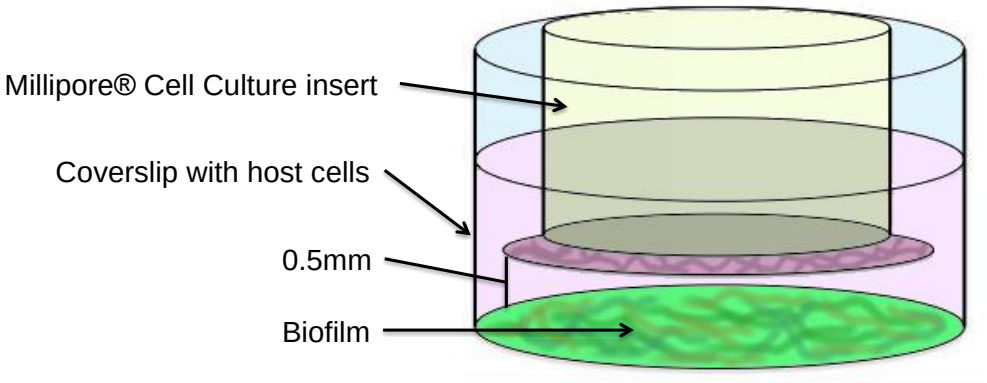


Figure 2.2: Inverted co-culture model system

Biofilms were grown on the surface of 24 well plates. Hanging baskets with Thermanox™ coverslips containing host cells were introduced into each well of a 24 well plate. Inverted coverslips were secured to the hanging basket using sterile Vaseline® with a 0.5 mm space between the biofilm and cell monolayer.

2.2.5 THP-1 cell culture

THP-1 cells [Invitrogen, Paisley, UK] are a leukemic pro-monocytic cell line acquired from a 1 year old male with acute monocytic leukaemia (Tsuchiya et al., 1980). These cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) [Sigma-Aldrich, Dorset, UK] supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin. Cells were cultured at 37ºC at 5% CO_2 until 80% confluence at which point cells were washed and resuspended in 5 mL of RPMI-1640 before counting using a haemocytometer with 20 µL of cells mixed with 10 µL of trypan blue [Sigma-Aldrich, Dorset, UK] to give a dilution factor of 1.5. Cells were viewed under a light microscope and live counted using the appropriate grid. Cells were re-seeded at 2 x 10^5 cells/flask in RPMI-1640. Frozen stocks were generated as described in section 2.2.1. For experimental use, cells were counted and seeded into wells at 5 x 10^5 cells/mL in 24 well plates.

2.2.6 THP-1 differentiation

THP-1 cells were differentiated into monocyte/macrophage like cells using two different methods; 1α,25-Dihydroxyvitamin D_3 (vitamin D_3) [Enzo Life Sciences, Farmingdale, NY, USA] and phorbol 12-myristate 13-acetate (PMA) [Sigma-Aldrich, Dorset, UK] as it has been shown these methods induce differential signalling pathways which may result in different differentiation states (Schwende et al., 1996). Briefly, for differentiation using vitamin D_3, cells were resuspended in RPMI-1640 containing 10% FBS only and seeded at 5 x 10^5 cells/mL containing 100 nM of vitamin D_3 and incubated for 3 days at 37°C 5% CO_2 to allow differentiation. Once differentiated, supernatants containing unadhered THP-1 cells were removed, washed with PBS and 500 µL fresh RPMI-1640 containing 10% FBS (v/v) added to the cells 30 minutes prior to experiments. For differentiation using PMA the method described by Daigneault and colleagues (2010) was followed (Daigneault et al., 2010). Cells were resuspended in RPMI-1640 containing 10% FBS only and seeded at 5 x 10^5 cells/mL and 200 nM of PMA added and cultured for 3 days at 37°C 5% CO_2 to allow differentiation. Differentiation was then enhanced by removing supernatant containing excess cells and washing with PBS before adding fresh RPMI-1640 containing 10% FBS (v/v) for a further 5 days. Prior to experimental use cells were washed again with PBS and 500 µL fresh RPMI-1640 containing 10% FBS (v/v) added to the cells. THP-1 differentiation was measured by flow cytometry as described in section 2.2.10.

2.2.7 THP-1 co-culture

THP-1 cells were co-cultured with multi-species biofilms as described in epithelial cell co-culture in section 2.2.3. Briefly, cells were seeded at 5 x 10^6 cells/mL, 500 µL of which was added to each well of a 24 well plate and differentiated if required as described in section 2.2.6. Cell were co-cultured with biofilms for 4 and 24 hours at 37°C 5% CO_2, after which time supernatants and cell lysates were removed and stored at -20°C for further analysis.

2.2.8 Co-culture with conditioned media

THP-1 cells were seeded at 5 x 10^6 cells/mL in a 500 µL a 24 well plate and differentiated using either PMA or vitamin D_3 as described in section 2.2.6. Following differentiation, cells were washed with PBS and 250 µL of supernatants from epithelial cells and biofilm co-culture experiments was added. The supernatants from the co-culture were centrifuged at 10,000 g for 10 minutes to pellet and remove any bacteria prior to culture with THP-1 cells. Additionally, as OKF6-TERT2 oral epithelial cells were co-cultured in d-KSFM a further 250 µL of RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS) [Sigma-Aldrich, Dorset, UK] supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin was added to the THP-1 cell culture to total 500 µL. THP-1 cells were cultured with 1:1 ratio of conditioned media from epithelial cell co-cultures: fresh RPMI for 4 and 24 hours at 37°C 5% CO_2 at which time supernatants and cell lysates were removed and stored at -20°C for further analysis.

2.2.9 THP-1 adhesion assay

To quantify the adherence of naïve THP-1 cells to the surface of a 24 well plate following stimulation, cells were stained with 2 µL /mL of the green fluorescent dye carboxyfluorescein succinimidyl ester (CFSE) [Sigma-Aldrich, Dorset, UK] and incubated at 37°C 5% CO_2 for 30 minutes. Next, cells centrifuged at 1000 g for 5 minutes and washed in PBS to remove any excess dye before seeding at 5 x 10^6 cells/mL in 24 well plates containing Thermanox™ coverslips. At this time THP-1 cells were incubated for 4 and 24 hours with either 3, 7 or 10 species biofilms using hanging baskets as described in section 2.2.3 or by adding 200 nM of PMA or 100 nM of vitamin D_3. Following incubation coverslips were removed and dip washed in PBS then fluorescence was measured on a plate reader [FLUOStar Omega, BMG Labtech, VA, USA] using 485nm excitation and 520nm emission. All absorbance values were blank corrected based upon the negative control where no cells were present. Additionally, adhesion of cells was visualised using a fluorescent microscope [Motic®, Model BA400, Hong Kong, China]. Briefly, Thermanox™ coverslips were inversely mounted on glass slides [BDH Laboratory Supplies, Poole, UK] using Vector mount [Vector Labs, Peterborough, UK] and visualised on the FITC filter channel at multiple magnifications.

2.2.10 Flow cytometric analysis

For flow cytometric analysis, stimulated THP-1 cells were removed from co-culture using a cell scraper and washed three times in sterile PBS by centrifugation at 400 g for 5 minutes to remove and bacteria present. Cells were then transferred to a 96-well round bottomed Costar® microtitre plate [Sigma-Aldrich, Dorset, UK] and washed with 250 µL of FACS buffer (PBS with 2% v/v FCS and 0.01% NaN_3). Following this, cells were centrifuged at 400 g for 5 minutes at 4°C, supernatants discarded and cells resuspended in 50 µL FcR blocking buffer [Affymetrix, Santa Clara, CA, USA] and incubated for 15 minutes at 4°C. All eBioscience® antibodies [Affymetrix, Santa Clara, CA, USA] for extracellular staining and concentrations per test are described in table 2.2. Antibodies (with the exception of the viability dye) were added to each sample and incubated for a further 20 minutes on ice. Next, 100 µL of PBS was added and samples were centrifuged at 400 g for 5 minutes at 4°C after which supernatants were discarded and samples were washed in PBS a further 3 times by centrifugation at 400 g for 5 minutes at 4°C. For samples which required viability dye at this stage 100 µL of viability dye at 1 µg/mL made up in PBS was added to samples and incubated for 20 minutes at 4°C before washing three times in PBS by centrifugation at 400 g for 5 minutes at 4°C. Untreated cells, single stains controls and isotype controls were used for calibration and to ensure specific binding, respectively. Samples were then resuspended in 200 mL PBS and 200mL FACS buffer in FACS tubes ready for analysis. Data were acquired on a MACSQuant® [Miltenyi Biotech, Surrey, UK] and analysed using FlowJo software [Treestar, USA].

Table 2.2 Antibodies used in flow cytometric analysis

Antibody	Concentration (µg/test)
Anti-Human CD69 PE	0.015
Anti-Human CD40 FITC	0.5
Anti-Human CD14 APC	0.25
Fixable Viability Dye eFlour® 450	n/a

2.2.11 Neutrophil culture

Neutrophils were provided by Ana Adrados Planell from the Institute of Infection, Immunity and Inflammation, University of Glasgow for use in co-culture experiments.

Neutrophils were counted using a haemocytometer and non-viable cells excluded on the basis of trypan blue staining and resuspended at 2 x 10^5 cells /500 µL or 5 x 10^5 cells /500 µL depending on the downstream analysis in complete RPMI containing 2% FCS on glass coverslips and incubated for 1 hour at 37°C 5% CO_2 prior to experimental use.

2.2.12 Neutrophil co-culture with biofilms

Neutrophils were co-cultured with multi-species biofilms as described in epithelial and THP-1 cell co-culture in section 2.2.3. Briefly, neutrophils were seeded at 5 x 10^5 cells/500 µL of which 500 µL was added to each well of a 24 well plate. Cell were co-cultured with biofilms for 4 and 16 hours at 37°C 5% CO_2 after which time supernatants and cell lysates were removed and stored at -20°C for further analysis.

2.2.13 Neutrophil extracellular traps (NETs) formation

To visualise NETS following co-culture with multi-species biofilms, neutrophils were seeded at 2 x 10^5 cells/500 µL. Next, 500 µL was added to each well of a 24 well plate containing glass coverslips and cells were co-cultured with biofilms for 4 and 16 hours at 37°C 5% CO_2. Following incubation, coverslips were removed and fixed with 500 µL of 2% paraformaldehyde (PFA) overnight at 4°C or 2-4 hours at room temperature. Coverslips were then washed with PBS for 5 minutes in triplicate and then incubated for 1 minute with 0.5% Triton X-100 to permeabilize the cells before three further washes in PBS for 1 minute each. Following washing, 100 µL of blocking buffer (PBS plus 5% horse serum) was added to each coverslip and incubated for 30 minutes at 37°C in a humid chamber. Next 100 µL of primary antibodies diluted in blocking buffer (Table 2.4) were added and incubated for 1 hour at 37°C before washing for 5 minutes in PBS in triplicate. Secondary antibodies (Table 2.3) diluted in blocking buffer

were then added at 100 µL per coverslip and incubated for 1 hour at 37ºC before washing a further 3 times in PBS for 5 minutes. DNA was then stained using 1mg/mL Hoechst 33342 [Life technologies, Paisley, UK] for 5 minutes and then washing in PBS. Finally, coverslips were mounted and dried at room temperature overnight before storage at 4ºC until microscopy. NETS were visualised using a Zeiss LSM 510 Meta confocal microscope using DAPI, Cy3 and Cy5 filters.

Table 2.3 Primary and Secondary antibodies for NET staining

Primary Antibody	Concentration	Secondary Antibody	Concentration
Neutrophil elastase antibody (M-18)(goat) [Santa Cruz Biotechnology, Heidelberg, Germany]	1:200	Alexa 647 donkey (anti-goat) [Life technologies, Paisley, UK]	1:300
Anti-histone H3 (citrulline R2 + R8 +R17) α-H3cit (rabbit) [Abcan®, Cambridge, UK]	1:1000	Cy3 mouse anti-rabbit (anti-rabbit) [Life technologies, Paisley, UK].	1:300

2.3 Molecular Methods

2.3.1 RNA extraction

Following stimulation of host cells with biofilms, RNA was extracted using the RNeasy Mini kit [Qiagen Ltd, Crawley, UK] in accordance with the manufacturer's instructions. Host cells were lysed with the addition of 350 µL of buffer RLT to each well. The resultant lysate was transferred to an RNase free microfuge tube and mixed with 350 µL of 70% ethanol. Each 700 µL sample was then transferred to an RNeasy spin column placed within a 2 mL collection tube and centrifuged at 13000 rpm for 15 seconds, with the flow-through discarded. DNA contamination of isolated total RNA was addressed using a DNase kit [Qiagen Ltd, Crawley, UK], as per manufacturer's instructions. For DNase digestions DNase I stock solution [Qiagen, Crawley, UK] was prepared by injection 500 µL of RNase free water into the DNase vial using a needle and syringe and gently mixed. Aliquots of the stock solution were made and stored a -20^{o}C. For use in RNA extractions, 350 µL of buffer RW1 was added to the spin column and centrifuged at 13000 rpm for 15 seconds and flow-through discarded. A solution containing 10 µL of DNase I stock and 70 µL buffer RDD was added directly onto the RNeasy column membrane and incubated at room temperature for 20 minutes. Next, buffer RW1 was added to the column and centrifuged to 13000 rpm for 15 seconds where again flow-though was discarded.

Following DNase digestion, 700 µL of buffer RW1 was added to the spin column, centrifuged at 13000 rpm for 15 seconds and flow-though discarded. Following this, two wash stages using 500 µL of buffer RPE and centrifugation at 13000 rpm for 15 seconds occurred before removal of the spin column into a fresh 2 mL collection tube for an additional centrifugation at 13000 rpm for 1 minute to dry the membrane. The column was then placed in a 1.5 mL microfuge tube and 30 µL of RNase free water added directly to the membrane before centrifugation at 13000 rpm for 1 minute to elute the RNA. Finally the resultant RNA was placed back upon the membrane and centrifuged again to ensure the maximum release of RNA from the column. RNA was then collected and quantified using a spectrophotometer or stored at -20^{o}C until required.

2.3.2 RNA Quantification

To assess the concentration and quality of the RNA extracted from host cells, a NanoDrop 1000 spectrophotometer [Thermo Scientific, DE, USA] was used. Before RNA was measured the pedestal was wiped clean and background corrected with 1.5 µL of RNase free water. Each RNA sample was then measured and the RNA concentration recorded as ng/µL. The purity of the RNA was also determined using the ratio of the absorbance at 260 and 280 nm, where RNA with a 260/280 ratio greater than 1.8 was deemed to be of high enough quality for gene expression studies. Samples were either used immediately for cDNA synthesis or stored at -20^{o}C until required.

2.3.3 cDNA synthesis

Complementary DNA (cDNA) was synthesised using the high-capacity cDNA reverse transcription kit [Life technologies, Paisley, UK]. For each samples a 2 x RT master mix was made using 2.0 µL 10 x RT buffer, 0.8 µL 25 x dNTP mix (100 mM), 2.0 µL of 10 x RT random primers, 1 µL of Multiscribe reverse transcriptase, 1 µL of RNase inhibitor and 3.2 µL of RNase-free water. 10 µL of 2 x RT master mix was added to 10 µL of RNA sample and centrifuged at 1000 rpm for 2 minutes to remove air bubbles. Samples were then loaded on to the "T professional basic gradient thermocycler" [Biometra, Gooettingen, Germany] using the following thermal cycling conditions: 10 minutes at 25^{o}C followed by 120 minutes at 37^{o}C and finally 5 minutes at 85^{o}C. Samples were then placed on ice to be used immediately for qPCR or stored at -20^{o}C until required.

2.3.4 qPCR of genes using SYBR® GreenER™

Gene expression was analysed using SYBR® Green [Invitrogen, Paisley, UK] based qPCR using *GAPDH* as a housekeeping gene (Barber et al., 2005). The primers used are shown in the table below (Table 2.4). Briefly, 1 µL of cDNA was added to a master mix containing 12.5 µL of SYBR® GreenER™, 10 µL of RNase-free water and 1 µL of forward/reverse primers. The thermal profile was as follows: 2 minutes at 50^{o}C, 10 minutes at 95^{o}C, followed by 40 cycles of 15 seconds at 95^{o}C and 60 seconds at 60^{o}C. For each primer set a dissociation curve was performed to confirm the presence of only one product. For this the thermal cycle was as

follows: 60 seconds at 95ºC, 30 seconds at 55ºC and 30 seconds at 95ºC. Three independent replicates for each parameter were analysed in duplicate using the MxProP Quantative PCR machine and MxProP 3000 software [Stratagene, Amsterdam, Netherlands].

Gene expression was normalised to the housekeeping gene *GAPDH* according to the $2^{-\Delta Ct}$ method, and then quantified using the $2^{-\Delta\Delta Ct}$ method to calculate the expression of gene transcripts relative to the media control (Livak and Schmittgen, 2001). Using *GAPDH* as an endogenous control, the ΔCt value was calculated (ΔCt = Ct (gene of interest) – Ct (GAPDH)). This was performed for each gene tested. The relative gene expression of each gene was the calculated using the formula ΔΔCt = ΔCt (test sample) – ΔCt (control samples). This was also able to be expressed as fold change relative to the control samples with the formula (fold change = $2^{-\Delta\Delta Ct}$).

Table 2.4 Primer sequences used in cell qPCR

Target	Primer Sequence (5'-3')	Reference
IL-8	F- CAGAGACAGCAGAGCACACAA R- TTAGCACTCCTTGGCAAAAC	(Ramage et al., 2012)
IL-1B	F- TCCCCAGCCCTTTTGTTGA R- TTAGAACCAAATGTGGCCGTG	(Locati et al., 2002)
TNFa	F- CCCCAGGGACCTCTCTCTAATC R- GGTTTGCTACAACATGGGCTACA	(Boeuf et al., 2005)
CXCL5	F- CCCTGGGTTCAGAGACCTCCA R- CCAGAAAATTTTGGACGGTGGAAACA	(Awang et al., 2014)
GAPDH	F- CAAGGCTGAGAACGGGAAG R- GGTGGTGAAGACGCCAGT	(McKimmie et al., 2008)

2.3.5 Real time PCR analysis using RT^2 Profiler Array

Following initial gene expression analysis using single primer set to measure gene expression. Analysis was carried out using real time PCR (qPCR) with a custom designed RT^2 Profiler PCR Array [Qiagen, Crawley, UK]. These arrays use SYBR® GreenER™ based real-time PCR but allow for the detection of multiple genes of interest simultaneously.

2.3.6 Reverse transcription by RT^2 First Strand kit

cDNA for use in the multiplex assays 'RT2' was generated using the RT2 first strand kit [Qiagen, Crawley, UK]. An initial mixture containing 2 µL of 5 x gDNA elimination buffer and 10 µL of RNA was incubated at 42^{o}C for 5 minutes before chilling on ice for 3 minutes. Following this a reverse transcription mix containing 4 µL of 5 x RT buffer, 1 µL of primer and external control mix, 2 µL of reverse transcriptase enzyme mix and 3 µL of RNase-free water was added to the 10 µL of gDNA elimination mixture and incubated for a further 15 minutes at 42^{o}C. The reaction was stopped by heating the samples to 95^{o}C for 5 minutes following which 91 µL of RNAse-free water was added to each reaction tube. cDNA was then either used immediately with the RT^2 Profiler or stored at -20^{o}C until required.

2.3.7 Gene expression analysis using the RT^2 Profiler

A master mix prepared with SYBR® GreenER™, cDNA and RNase-free water and 24 µL was added to each well of the custom RT^2 Profiler plate which already contained the forward and reverse primers of the genes of interest. In the case of our studies the genes of interest were *IL-1, IL-1β, IL-6, TNFα, CSF2, CSF3, IL-8, CXCL1, CXCL3, CXCL5, CCL1* and *GAPDH*. Thermal cycler conditions were as follows: 10 minutes at 95^{o}C followed by 40 cycles of 15 seconds at 95^{o}C and 60 seconds at 60^{o}C. Two replicates of each condition were used in the RT^2 profiler and the experiment was performed on two separate occasions.

2.3.8 ELISA

Supernatant from co-cultures were retained to assess the release of pro-inflammatory protein by ELISA. ELISA kits for TNFα [Invitrogen, Paisley, UK], CCL5 [Peprotech, London, UK], IL-8 [Invitrogen, Paisley, UK], IL-6 [Invitrogen, Paisley, UK] and IL-1β [Invitrogen, Paisley, UK] were used according to manufacturer's instructions. Capture antibody (1 µg/mL) was prepared in NA_2HCO_3 and 100 µL added to each well of a Nunc™ Maxisorp® flat bottomed microtitre plate [Fisher, Loughborough, UK]. Plates were sealed and incubated overnight at 4^{o}C. Contents were then discarded and washed with 300 µL of wash buffer of PBS containing 500 µL Tween 20 /L. Plates were then blocked with 300

µL of assay buffer containing 0.5% bovine serum albumin (BSA) for 1 hour at room temperature to block non-specific binding. After incubation, contents were discarded and 100 µL of each sample loaded in duplicate as well as standards of known concentrations ranging from 2000 – 31.25 pg/mL. At this time detection antibody was diluted to 0.04 µg/mL in assay buffer and added to each well containing sample or standard. Plates were then sealed and incubated for 2 hours at room temperature on a shaking platform at 700 rpm. Following incubation the contents of the plate were discarded and 100 µL of detection antibody diluted to 0.04 µg/mL in assay buffer added to each well before a further incubation of 2 hours at room temperature at 700 rpm. Next, the plate was washed and 50 µL of a 1/2500 dilution of streptavidin-HRP in assay buffer was added to each well for a further 30 minute incubation shaking at 700 rpm. Finally, the supernatants were discarded and 100 µL of 3,3',5,5'-tetra-methylbenzide (TMB) [R&D Systems, Abingdon, UK] was added to each well and incubated in the dark for 30 minutes before addition of 100 µL 1 mM HCL to stop the reaction. Absorbance was read using a plate reader [FLUOstar Omega BMG Labtech, VA, USA] at 405 nm with a 650 nm wavelength correction. A standard curve was constructed by plotting the mean absorbance for each standard against the appropriate protein concentration and the R-squared calculated using a computer program [Omega analysis software, VA, USA]. Results were calculated using a 4-parameter curve fit to determine the concentration of protein release in samples tested. All samples were tested in triplicate on three individual occasions.

2.3.9 Luminex

Supernatants harvested from cells after co-culture were tested for the presence of IL-1ß, TNFα, IL-8, IL-6 and CSF2 using Luminex® multiplex beads [Invitrogen, Paisley, UK] according to the manufacturer's instructions. 25 µL of 1x beads (2.5 x 10^6 beads/mL/cytokine) stock solution with defined spectral properties covalently conjugated to specific monoclonal antibodies, diluted in working wash solution was added to a 96 well filtered bottom plate provided in the kit and incubated for 30 seconds before washing by vacuum manifold. Next, 50 µL of cell culture supernatant and standards were added to the appropriate wells and incubated on an orbital shaker (500 rpm) for 2 hours at room temperature in the

dark. Known concentration of standards provided by the manufacturer were as follows; IL-1β (11000 pg/mL), TNFα (4000 pg/mL), IL-8 (5200 pg/mL), IL-6 (4700 pg/mL) and CSF2 (10000 pg/mL). After a further three washes using the vacuum manifold to remove unbound protein, 100 µL of biotinylated detection antibodies were added to each well and incubated for a further 1 hour at room temperature on an orbital shaker (500 rpm). After incubation two washes were performed using the vacuum manifold to remove any excess antibody and 100 µL of Streptavidin-R Phycoerythrin (Streptavidin-RPE) added incubated for 30 minutes on the orbital shaker (500 rpm) at room temperature. Finally, the plate was washed a final three times using the vacuum manifold before addition of 100 µL working wash solution to allow the reaction to be analysed using Luminex® 100 hardware [Luminex, USA]. The standard curves were obtained using a five-parameter algorithm and samples measured were from three independent experiments.

2.3.10 Caspase-3 ELISA

OKF6-TERT2 epithelial cells were challenged with bacterial biofilms for 4 and 24 hours and apoptosis measured by determining the presence of caspase-3 using the Caspase-3 (active) human ELISA kit [Invitrogen, Paisley, UK] according to the manufacturer's instructions. Epithelial cells were collected in PBS by using a cell scraper to remove them from the bottom of 24 well plates. Cells were then washed in PBS three times before being centrifuged for 10000 rpm to obtain a cell pellet. Supernatants were discarded and cells were lysed for 30 minutes at room temperature with 1 mL of cell extraction buffer [FNN0011, Invitrogen, Paisley, UK] supplemented with 1 mM phenylmethanesulfonyl fluoride [Sigma-Aldrich, Dorset, UK] and 500 µL protease inhibitor cocktail [Sigma-Aldrich, Dorset, UK] per 5 mL cell extraction buffer. Lysates were then centrifuged at 13000 rpm for 10 minutes at 4^{o}C and the clear lysates used immediately or stored at -80^{o}C until required.

Caspase-3 (active) Human ELISA kit is a solid phase sandwich ELISA. Cell lysates, diluted 1:10 in standard diluent buffer and diluted standards (ranging from 2.5-0.39 pg/mL) were added to the appropriate microtitre wells of the plate provided and incubated for 2 hours at room temperature. The plate provided

had been pre-coated with a monoclonal antibody specific for human caspase-3. Following incubation wells were washed using the wash buffer provided and complete drying ensured before the addition of caspase-3 detection antibody and further incubation for 1 hour at room temperature. After incubation wells were washed again with wash buffer and anti-Rabbit HRP added to the wells for 30 minutes at room temperature. Wells were then washed for a final time before the addition of stabilized chromogen added and colour change observed for 30 minutes at which point stop solution was added and plate read at 450nm. The plate reader was banked using a chromogen blank composed of stabilized chromogen and stop solution. A standard curve from which the unknown samples could be quantified was generated using a four-parameter algorithm to calculate the concentration of caspase-3 in the samples.

2.3.11 LDH assay

To measure cell viability after co-culture an LDH-cytotoxicity colorimetric assay kit II [Promega, Wisconsin, USA] was used according to manufacturer's instructions. Lactate dehydrogenase (LDH) is a stable enzyme in all cell types released into supernatant after damage to cell membrane. Briefly, cells supernatants from co-cultures were plated in a 96-well round bottomed Costar® microtitre plate [Sigma-Aldrich, Dorset, UK] with blank media controls and a LDH standards ranging from 1000 – 3.9 ng/mL. 100 µL of LDH reaction mix was added to each well and incubated for 30 minutes at room temperature. After incubation, the reaction was stopped by adding 10 µL of stop solution provided and absorbance read at 450 nm with a reference wavelength of 650 nm. Percentage cytotoxicity was measured as per manufacturer's instructions. The calculation is as follows: Cytotoxicity (%) = (Test sample – untreated control)/(Positive control – untreated sample)

2.3.12 AlamarBlue® viability assay

AlamarBlue® [Invitrogen, Paisley, UK] was used to measure both bacterial and cell viability following stimulation. Biofilms and cell supernatants were removed and then biofilms and cells were carefully washed with PBS before addition of AlamarBlue® at 1:10 dilution in appropriate media and incubated for 4 hours at 37^{o}C in 5% CO_2. Following incubation, the absorbance was read at 570 nm and

the reference wavelength at 600 nm. The percentage reduction in viability was calculated according to the manufacturer's instructions. To calculate the percentage viability the following calculation was used;

$[(\varepsilon_{0X})\lambda_2 A\lambda_1 - (\varepsilon_{0X})\lambda_1 A\lambda_2 / (\varepsilon_{RED})\lambda_1 A'\lambda_2 - (\varepsilon_{RED})\lambda_2 A'\lambda_1] \times 100$

λ_1 = 570 nm λ_2 = 600 mn

$(\varepsilon_{0X})\lambda_2$ = 117,216, $(\varepsilon_{0X})\lambda_1$ = 80,586, $(\varepsilon_{RED})\lambda_1$ = 155,677, $(\varepsilon_{RED})\lambda_2$ = 14,652

$A\lambda_1$ = OD reading for test well, $A\lambda_2$ = OD reading for test well

$A'\lambda_1$ = OD reading for negative control, $A'\lambda_2$ = OD reading for negative control

For each experiment this was performed in triplicate of three separate occasions.

2.3.13 Use of active compounds in periodontal co-culture

Chlorhexidine (CHX) [Sigma-Aldrich, Dorset, UK] and resveratrol (RSV) [Sigma-Aldrich, Dorset, UK] were used to test the potential of the co-culture model as a novel compound testing platform. CHX solution was prepared at 0.01, 0.05 and 0.2% (v/v) in d-KSFM for antimicrobial testing. Biofilms were treated for 30 minutes with each concentration before washing with PBS to remove any excess compound before co-culture with oral epithelial cells. RSV powder was solubilised in ddH_2O prior to preparation in d-KSFM at 0.01, 0.05 and 0.5% v/v for use in subsequent cell stimulation studies. Oral epithelial cells were treated for 30 minutes with each concentration prior to washing with PBS to remove and excess compound before co-culture with multi-species biofilms. Co-culture with OKF6-TERT2 oral epithelial cells was followed as described in section 2.2.3.

2.3.14 Statistical analysis

Graph production, data distribution and statistical analysis were performed using GraphPad Prism (version 4; La Jolla, CA, USA). Data was assessed to confirm it conformed to a normal distribution before and after data transformations using a D'Agostino and Pearson omnibus normality test. When the mean of two groups were compared Student t-tests was used if data was normally distributed. If the mean of more than two groups was compared a one-way analysis of variance (ANOVA) was used to investigate significant differences between independent groups of data that approximated to a Gaussian distribution. When the mean of two or more groups at multiple time points was compared a two-way ANOVA was used. A Bonferroni correction (2-way ANOVA) or Tukey's post test (1-way ANOVA) was applied to the p value to account for multiple comparisons of the data. The number of comparisons was determined by the number of groups which were compared each analysis. Non-parametric data was analysed using the Mann-Whitney U-test to assess differences between two independent sample groups. Student t-tests were used to measure the statistical differences between the ΔCt values of the two independent groups assessed in gene expression studies, although the data may be represented as percentage or fold change in the figures. Statistical significance was achieved at $p < 0.05$ for all analysis.

3 The role of *Porphyromonas gingivalis* strain variation in oral biofilms and influencing epithelial host responses *in vitro*

3.1 Introduction

The obligate anaerobe *Porphyromonas gingivalis* is a Gram-negative coccobacillus that is implicated in the pathogenesis of PD (Schmidt et al., 2014, Alpagot et al., 1996). Part of Socransky's 'red complex', *P. gingivalis* is found in healthy and diseased oral biofilms with prevalence ranging from 10-25% in health and 70-90% in disease (Griffen et al., 1998, Klein and Goncalves, 2003, Alpagot et al., 1996). An opportunistic pathogen, *P. gingivalis* is considered a 'keystone' species in PD with the ability to alter the quantity and composition of oral commensal microflora, which can disrupt the host-microbial balance and cause inflammation (Hajishengallis et al., 2011).

P. gingivalis has been classified into virulent and avirulent strains using animal models (Grenier and Mayrand, 1987, Laine and van Winkelhoff, 1998). Strain diversity has been shown to play a role in soft-tissue abscess formation (Neiders et al., 1989, Ebersole et al., 1995), periodontal bone loss (Baker et al., 2000a), serum antibody response (Katz et al., 1996), systemic immune response (Marchesan et al., 2012, Vernal et al., 2009), cell invasion (Eick et al., 2002) and death in a *Drosophila melanogaster* model (Igboin et al., 2011). Strain specific variation of *P. gingivalis* has also been associated with disease severity in humans (Griffen et al., 1999, Amano et al., 2000, Igboin et al., 2009).

Many of the known virulence factors of *P. gingivalis* are found to vary between strains, which may attribute to pathogenicity observed in animal models and humans (Imai et al., 2005, Yoshino et al., 2007). Molecular studies suggest that the genetic diversity of strains may also correlate with pathogenicity (Igboin et al., 2009, Amano et al., 2000, Asai et al., 2005). Capsular strains of *P. gingivalis* have been shown to be more virulent that their non-encapsulated counterparts (Brunner et al., 2010, Kunnen et al., 2012). Although the precise reason for this virulence is unknown, a variety of capsular components have been shown to differ between *P. gingivalis* strains, which may contribute to varying virulence. These include lipopolysaccharides (LPS) (Shapira et al., 1998), proteases including gingipains (Bodet et al., 2005, Grenier et al., 2003, Jayaprakash et al., 2014), peptidylkarginine deiminase (Moelants et al., 2014), haemagglutinins (Liu

et al., 2011), fimbriae (Nakagawa et al., 2002, Miura et al., 2005) and outer membrane proteins (Imai et al., 2005).

P. gingivalis is considered a 'keystone' species in PD, however, it has been shown that approximately 25% of healthy individuals can have this bacteria present in oral plaque without consequence (Griffen et al., 1998). Additionally, studies have observed that some genotypes of *P. gingivalis*, such as W83, are more associated with chronic periodontitis and periodontal abscesses (Yoshino et al., 2007). It is therefore important to study the role strain variation plays in biofilm formation and epithelial immune cell responses to understand the role strain variation plays in PD susceptibility and progression.

3.2 Aims

It is hypothesized that *P. gingivalis* strain variation may influence PD severity in humans. Therefore, the aim of this chapter was to examine the role of strain variation of *P. gingivalis* ATCC 33277, W83 and W50 in relation to virulence in single- and multi-species biofilm formation, and assess the ability of these to influence host epithelial cell responses. Additionally, the results would allow an appropriate strain to be chosen for further multi-species biofilm studies in this thesis.

3.3 Results

3.3.1 Strain variation does not affect planktonic growth of P. gingivalis

The growth kinetics of *P. gingivalis* ATCC 33277, W83 and W50 were initially assessed to determine if strain variation played a role in growth rate during planktonic culture. Each strain was standardized to 1 x 10^4 bacteria/mL in Schaedler's anaerobic broth and automatically quantified at designated time points using a spectrophotometer (OD 650nm). Time points were selected based on the current literature citing planktonic growth of *P. gingivalis* taking approximately 48 hours to reach stationary growth phase (Christopher et al., 2010). No significant difference was observed between strains at each time point (Figure 3.1). From these data it was concluded that the log phase of growth for experimental use was between 18 and 39 hours, which was in agreement with previous findings (Grenier et al., 2001). However *P. gingivalis* does not typically reside planktonically *in vivo*, therefore the next stage was to determine if *P. gingivalis* strain variation played a role in biofilm formation.

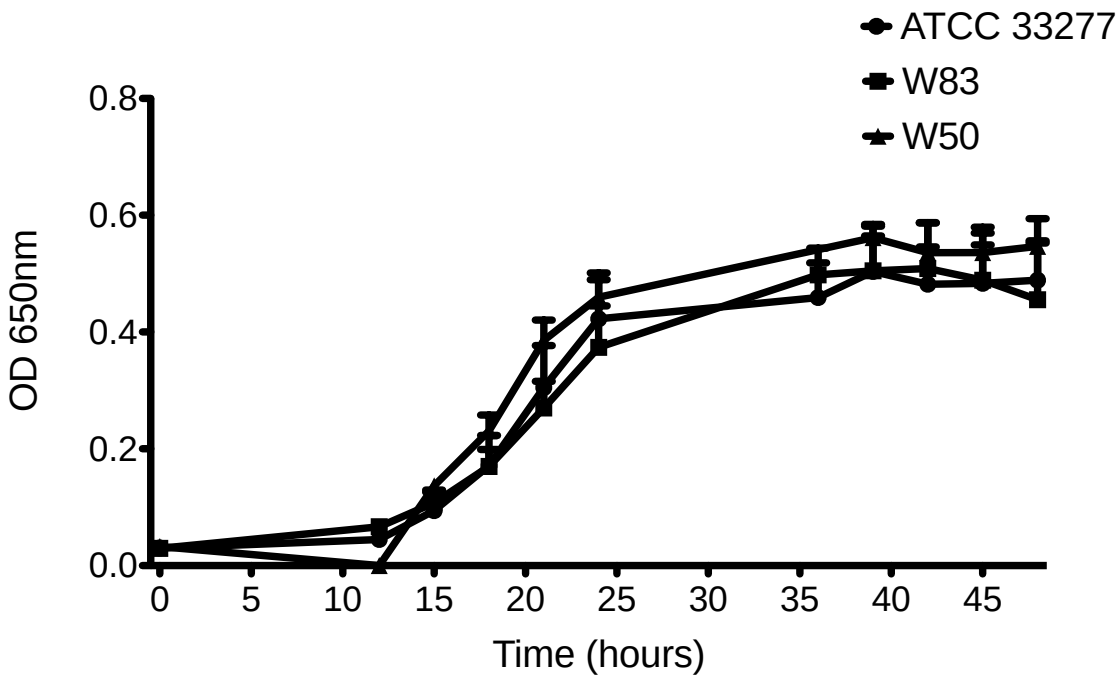


Figure 3.1: Planktonic growth of *P. gingivalis* strains over time

P. gingivalis ATCC 33277, W83 and W50 were standardized at $1x10^4$ bacteria/ mL and grown for 48 hours in Schaedler's anaerobe broth under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2). Absorbance was measured every three hours where possible using the FLUOstar plate reader at 650nm. Data shown are mean ±SD of three independent experiments each performed with eight cultures. Statistical analysis was performed using a one-way ANOVA to compare each strain at each time point.

3.3.2 Strain variation impacts biofilm formation of P. gingivalis in vitro

To determine if strain variation played a role in biofilm formation each strain was grown as described in section 2.1.3. Biofilm formation was assessed by a variety of methods, including evaluating the total biomass, enumeration of cell numbers by agar plate counts and qPCR, and visual inspection by SEM. Collectively, these data showed that significant differences were observed between strains when comparing biofilm formation.

When measuring the biomass of each *P. gingivalis* strain, i.e. the total cellular and ECM composition, the total biomass measured by crystal violet showed significant differences between strains (Figure 3.2). The biomass of *P. gingivalis* W83 single-species biofilms was 0.43 OD, which was significantly higher than 0.095 OD of ATCC 33277 ($p<0.05$) and 0.095 OD of W50 ($p<0.05$) biofilms.

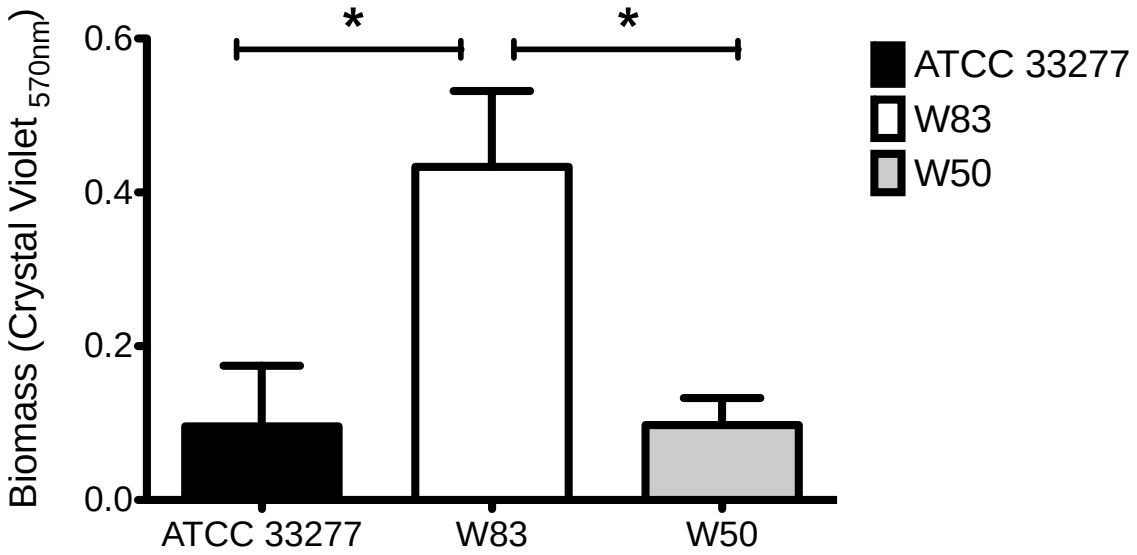


Figure 3.2: Biomass of mature single species *P. gingivalis* biofilms.
P. gingivalis ATCC 33277, W83 and W50 were standardized at $1x10^7$ bacteria/ mL and grown for four days in AS under anaerobic conditions. Growth was measured by crystal violet staining of the biofilms and measured using FLUOstar plate reader at 570nm. Data shown are mean ±SD of four independent experiments each with triplicate cultures normalized to the AS control. Statistical analysis was performed using a one-way ANOVA to compare each strain (* $p<0.05$).

As previously stated, biofilms are made up of both the bacteria and ECM which the bacteria produce. Although significant differences in the total biomass between each *P. gingivalis* strain was observed, this does not determine the total number of bacteria found in each biofilm. Therefore, to further elucidate if strain variation played a role in biofilm formation, quantification *P. gingivalis* in biofilms was then measured by plate counting and qPCR.

First, the Miles and Misra counting method (Miles et al., 1938) was used on sonicated biofilms to measure the number of live CFUs recoverable from each biofilm (Figure 3.3). Differences were shown between the number of recoverable bacteria in each biofilm, with significant differences between W83 ($P<0.01$) and both the ATCC 33277 and W50 strains, which contained 1.89 and 1.85 times less cells, respectively.

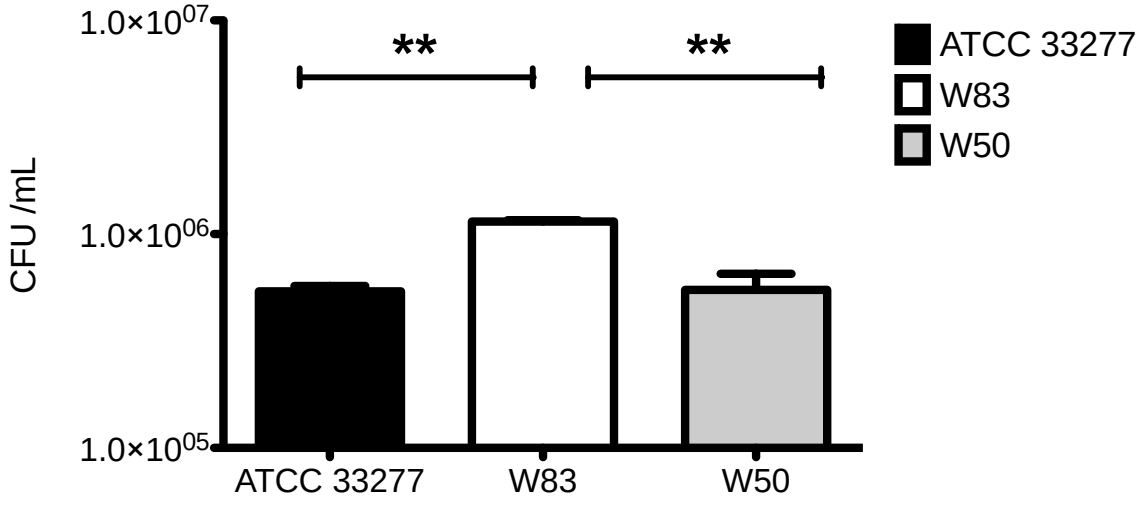


Figure 3.3: Viable cell recovery from single species *P. gingivalis* biofilms
P. gingivalis strain ATCC 33277, W83 and W50 were grown as single species biofilms for 4 days in the anaerobic chamber. Bacteria were sonicated in PBS for 10 minutes in a sonic bath and viable bacteria enumerated by using Miles and Misra plate counting method on FAA plates supplemented with 5% horse blood. Data shown are mean ±SD of nine independent biofilms enumerated in triplicate. Statistical analysis was performed using a one-way ANOVA to compare each strain (** $p<0.01$).

To supplement the quantification data using the Miles and Misra method, *P. gingivalis* was also quantified using qPCR with which the total number of bacteria present in each biofilm was measured. Combined, these two methods provided a live and total number of *P. gingivalis* in each biofilm to give a better indication as to whether strain variation influences biofilm formation.

Before biofilms could be quantified by qPCR methodologies, DNA from known concentrations of each *P. gingivalis* strain was used to prepare a standard curve that could be extrapolated to determine known bacterial counts (Figure 3.4)

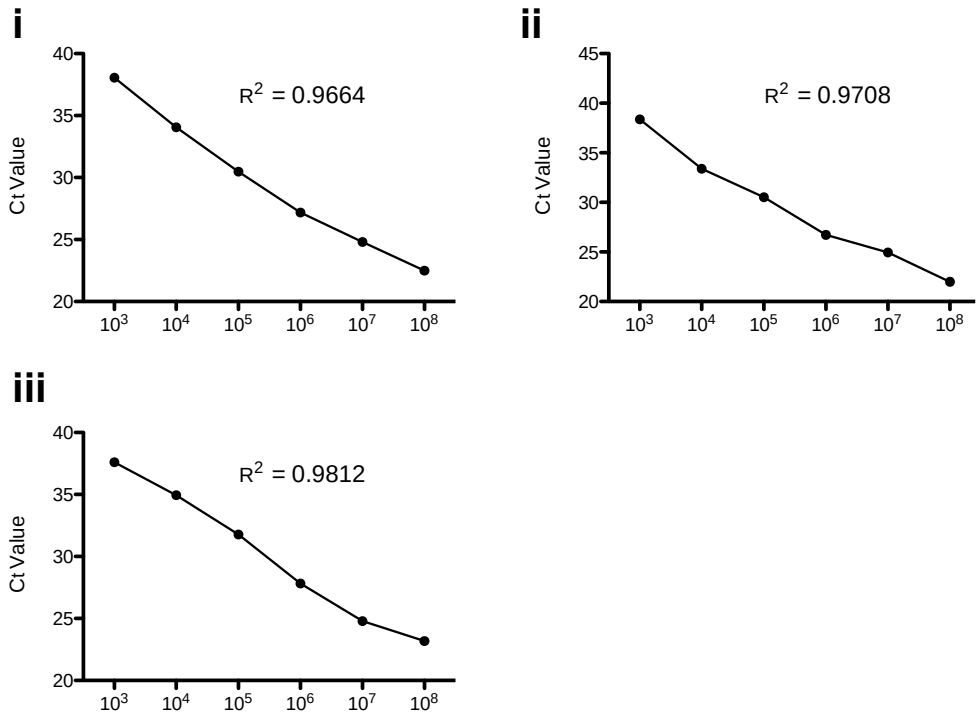


Figure 3.4: Standard curves of *P. gingivalis* strains

Ten-fold dilutions of DNA from known concentrations of *P. gingivalis* ATCC 33277 (i), W83 (ii) and W50 (iii) were used for qPCR and their equivalent Ct used for the preparation of a standard curve. Unknown bacterial counts could then be quantified by extrapolating from the standard curve. Each DNA dilution was assessed in triplicate.

This method allowed the quantification of the total number of bacteria present in each *P. gingivalis* biofilm to be enumerated (Figure 3.5). Significant differences in the total number of bacteria were present in each biofilm. *P. gingivalis* W83 single species biofilms contained 8.6 x 10^6 total bacteria, while both *P. gingivalis* ATCC 33277 and W50 single species biofilms contained significantly less bacteria, with a total bacteria count of 1.49 x 10^4 ($p<0.01$) and 6.23 x 10^3 ($p<0.01$) respectively.

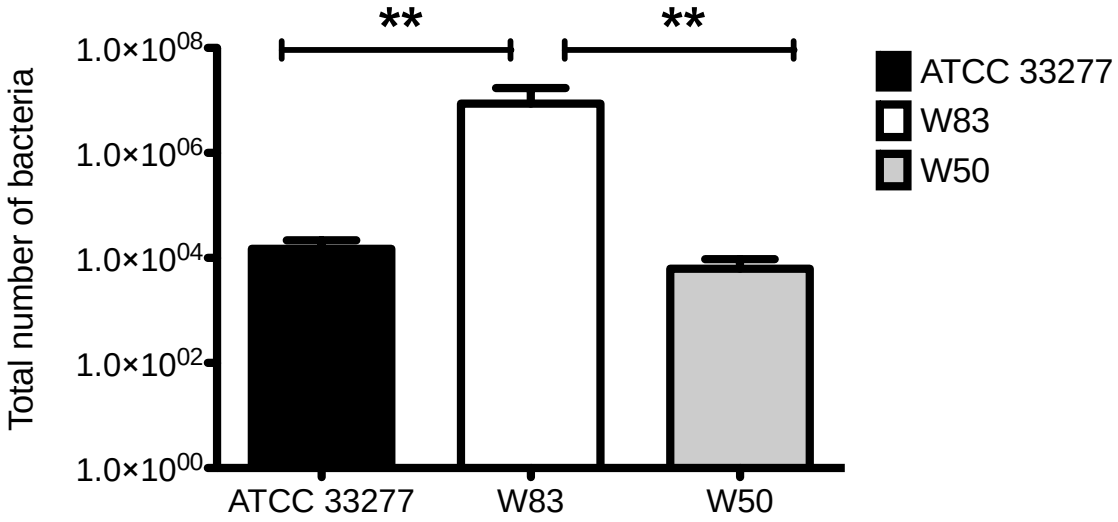


Figure 3.5: Total number of *P. gingivalis* in single species biofilms

P. gingivalis strain ATCC 33277, W83 and W50 were grown as single species biofilms. Bacteria were sonicated in PBS for 10 minutes and DNA was extracted using a Masterpure® Gram Positive DNA extraction kit. Each strain quantified using SYBR GreenER based qPCR. All samples were assayed in triplicate on the separate occasions. Data represents mean ±SD. Statistical analysis was performed using a one-way ANOVA to compare each strain (** $p<0.01$).

SEM analysis of each *P. gingivalis* strain as a single species biofilm was performed to evaluate and role of strain variation on biofilm architecture (Figure 3.6). At low magnification (1500x) the *P. gingivalis* W83 biofilm appears to be the most complex biofilm compared with ATCC 33277 and W50. At higher magnifications (5000x and 10000x) ECM can be observed on the biofilms of all three *P. gingivalis* strains; however, it is more prevalent in the biofilms of *P. gingivalis* W83 and in some part of the W50 biofilms.

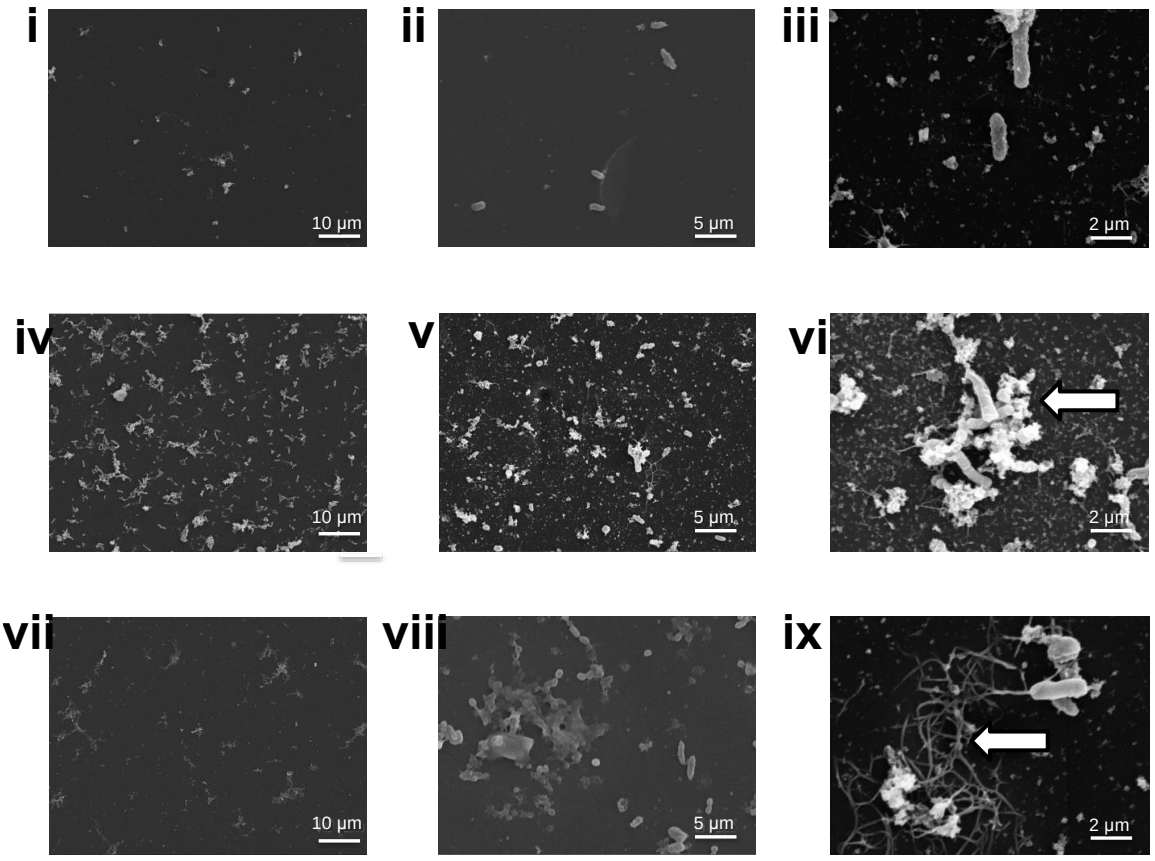


Figure 3.6: SEM of mature *P. gingivalis* single species biofilms

P. gingivalis biofilms were grown for 4 days then analysed by SEM at 1500x (i, iv, vii), 5000x (ii, v, viii) and 10000x (iii, vii, ix). Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope. Single species biofilms of *P. gingivalis* ATCC 33277 (i, ii, iii), W83 (iv, v, vi) and W50 (vii, viii, ix) were compared to assess biofilm formation and architecture. At highest magnification more ECM (highlighted by arrows) was visible in W83 (vi) and W50 (ix) biofilms compared with ATCC (iii).

3.3.3 Strain variation impacts exogenous IL-8 degradation in vitro

The ability of *P. gingivalis* to degrade inflammatory chemokines such as IL-8 is considered to be one of the main virulence factors of the species (Moelants et al., 2014). To assess whether strain variation plays a role in the ability of *P. gingivalis* to degrade IL-8, *P. gingivalis* biofilms were cultured with 300pg/mL exogenous IL-8 and degradation of the chemokine measured at 0, 1, 4, and 24 hours by ELISA (Figure 3.7). Exogenous IL-8 in bacteria free media was used as a control to measure any natural degradation of IL-8 over time. All three strains of *P. gingivalis* strains caused significant degradation of IL-8 *in vitro* compared with the bacteria free control at 1, 4 and 24 hours. *P. gingivalis* W50 reduced exogenous IL-8 by 42.33% ($p<0.01$), 47.03% ($p<0.001$) and 44.1 % ($p<0.01$) at 1, 4 and 24 hours, respectively. *P. gingivalis* ATCC 33277 significantly decreased exogenous IL-8 by 46.73% ($p<0.001$), 46.2% ($p<0.001$) and 89.87% ($p<0.001$) compared with the bacteria free control at 1, 4 and 24 hours, respectively. *P. gingivalis* W83 significantly decreased the levels of exogenous IL-8 in culture by 83.37% ($p<0.001$) and 97.23% ($p<0.001$) at 1 and 4 hours. At 24 hours the levels of IL-8 in the W83 culture were undetectable. IL-8 degradation in culture with *P. gingivalis* W83 was significantly higher than both ATCC 33277 and W50 at 1 ($p<0.01$), 4 ($p<0.001$) and 24 ($p<0.001$) hours.

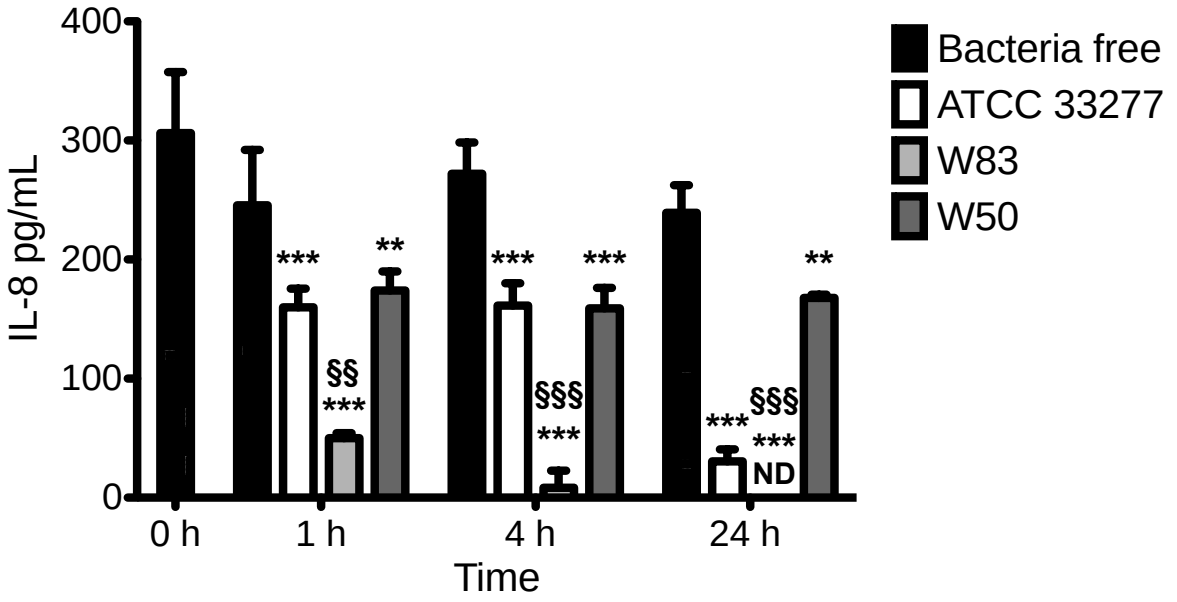


Figure 3.7: Exogenous IL-8 degradation by *P. gingivalis* biofilms over time
P. gingivalis ATCC33277, W83 and W50 biofilms were cultured with 300pg/mL of exogenous IL-8 and remaining IL-8 measured at 1, 4 and 24 hours. Exogenous IL-8 without bacteria present was used as a control. IL-8 was quantified at each time point by ELISA. All samples were assayed in triplicate on three independent occasions. Data represents mean ±SD (** $p<0.01$, *** $p<0.001$, §§ $p<0.01$, §§§ $p<0.001$). Statistical analysis was performed using a two-way ANOVA.

3.3.4 Strain variation does not influence epithelial cell IL-8 protein response in co-culture

As significant differences in IL-8 degradation were observed between *P. gingivalis* strains, it was then important to determine if strain variation played a role in the epithelial cell response to *P. gingivalis*. Using the hanging basket co-culture model as described in method 2.2.3, *P. gingivalis* biofilms were co-cultured with the orally relevant epithelial cell line OKF6-TERT2 for 4 and 24 hours and IL-8 protein release measured by ELISA (Figure 3.8 i). *P. gingivalis* was also co-cultured with oral epithelial cells and live planktonic bacteria (ii) as well as methanol fixed planktonic bacteria (iii). Filtered (iv) and unfiltered (v) supernatants from biofilm culture were also used. *S. mitis* was used as a

negative control for each of the experiments. At 24 hours, co-culture of *S. mitis* biofilms resulted in a 4.55 fold change in IL-8 protein release, which was significantly increased compared to a fold change of 0.17 for ATCC 33277 biofilms ($p<0.001$), 0.10 for W83 biofilms ($p<0.001$) and 0.25 for W50 biofilms ($p<0.001$). Fold change was relative to a cells only control containing cells and media only. At 4 hours and between each *P. gingivalis* strain at each time point there was no significant difference. Additionally, no significant differences were observed in the IL-8 levels measured between strains or the control after co-culture with live planktonic bacteria or filtered supernatants from bacterial biofilms at either time point. In co-cultures using unfiltered biofilm supernatants an increase in the IL-8 levels produced by epithelial cells was observed at 4 hours, with *P. gingivalis* W50 being significantly ($p<0.05$) higher than that of the *S. mitis* control. However, no significance was observed between *P. gingivalis* strains at either time point. Using methanol fixed bacteria in co-culture a significantly lower level of IL-8 was observed in *P. gingivalis* ATCC 33277 ($p<0.001$) ($p<0.01$), W83 ($p<0.001$)($p<0.001$) and W50 ($p<0.001$)($p<0.01$) at both 4 and 24 hours, compared with the *S. mitis* control. Additionally, significant differences were observed between *P. gingivalis* W83 ($p<0.01$) and W50 in methanol fixed co-cultures.

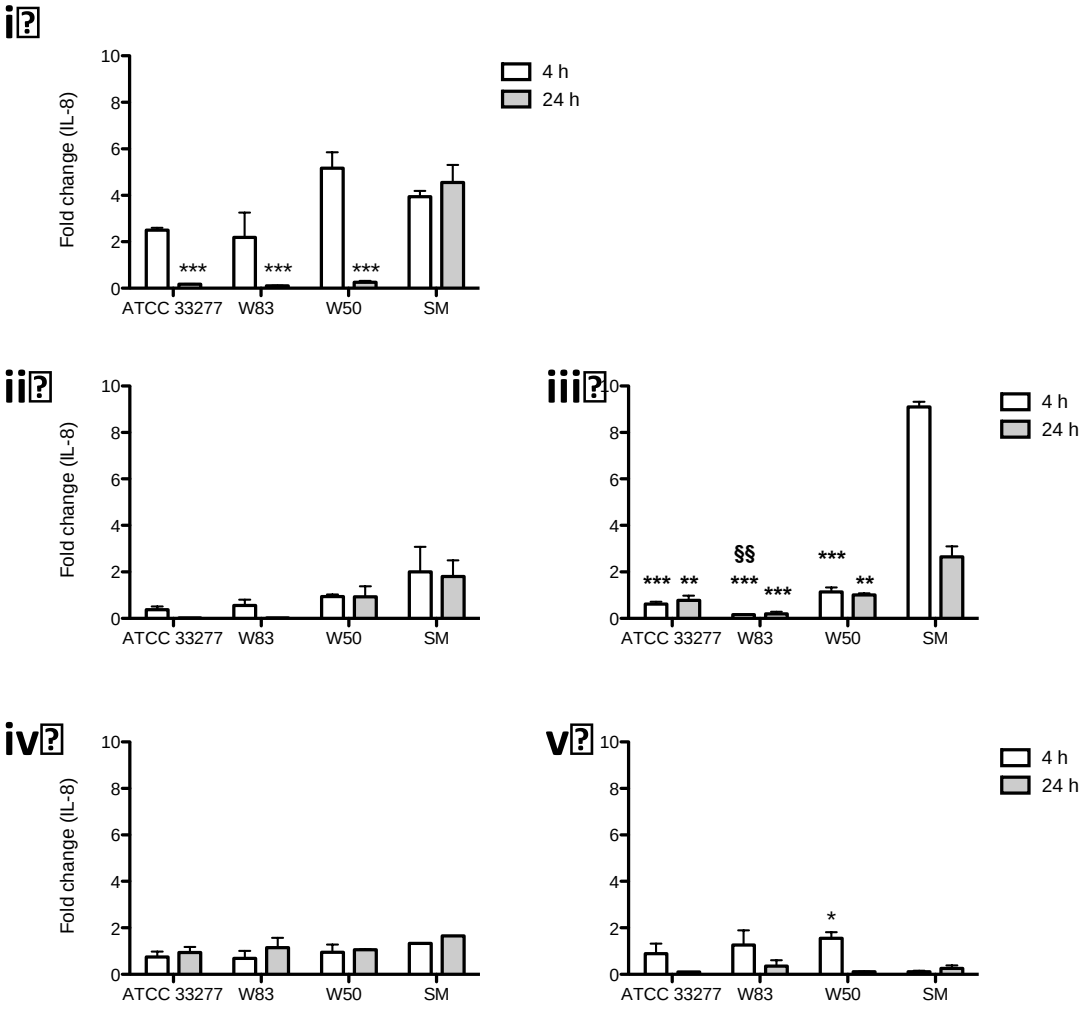


Figure 3.8: Epithelial cell IL-8 response to *P. gingivalis*

P. gingivalis ATCC 33277, W83 and W50 were co-cultured with oral epithelial cells (OKF6-TERT2) to measure IL-8 production at 4 and 24 hours. *P. gingivalis* was co-cultured as (i) biofilms, (ii) live planktonic cells, (iii) methanol fixed planktonic cells, (iv) filtered biofilm supernatants and (v) unfiltered biofilm supernatants. *S. mitis* was used as a control in each condition. IL-8 protein release was measured by ELISA. All samples were assayed in triplicate on three independent occasions. Data represents mean ±SD (* $p<0.05$ ** $p<0.01$, *** $p<0.001$ compared with *S. mitis* control) (§§ $p<0.01$ compared with *P. gingivalis* ATCC 33277 and W50). Statistical analysis was performed using a two-way ANOVA.

3.3.5 Strain variation does not influence multi-species biofilm formation or composition

While it is essential to understand strain variation in *P. gingivalis* species by investigating each as a single species biofilm, in the oral cavity *P. gingivalis* is found in biofilms with other oral bacterial species (Bao et al., 2014b, Socransky et al., 1998). To investigate if *P. gingivalis* strain variation plays a role in biofilm formation, a simple and reproducible multi-species biofilm model representative of sub-gingival plaque was developed containing *S. mitis, F. nucleatum, A. actinomycetemcomitans*, as well as each *P. gingivalis* strain (Section 2.1.4). During the growth of these biofilms biomass was measured by the crystal violet method described in section 2.1.8 (Figure 3.9), biofilm composition of mature biofilm quantified by qPCR (Figure 3.11), and architecture investigated by SEM (Figure 3.12).

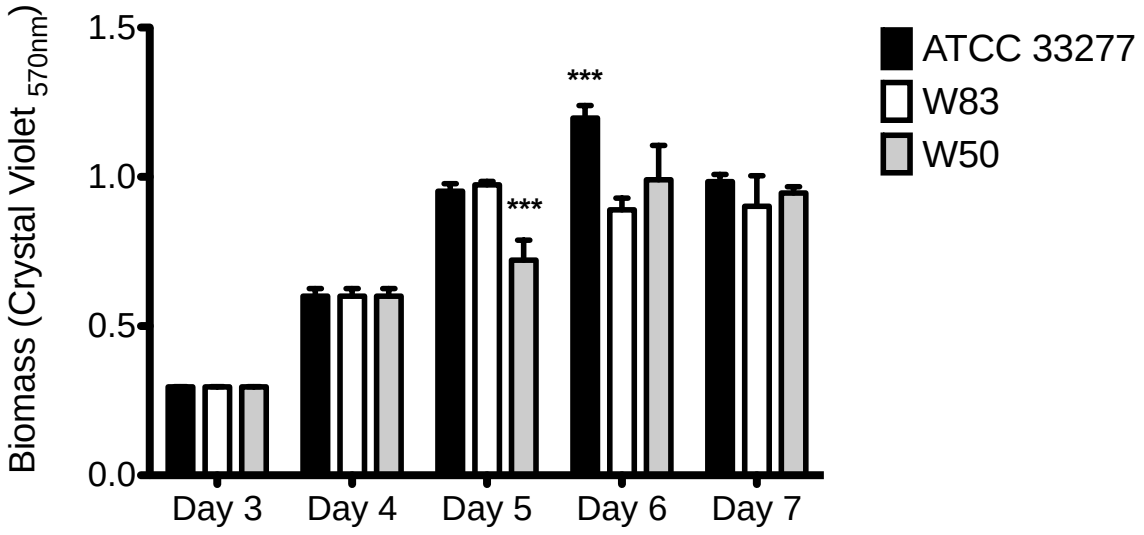


Figure 3.9: Biomass of multi-species biofilm containing *P. gingivalis* strains
Multi- species periodontal biofilms containing *P. gingivalis* ATCC 33277, W83 and W50 were grown on Thermanox™ coverslips. From day 3 *P. gingivalis* strain were added and biomass measured by crystal violet. All samples were assayed in triplicate on three independent occasions. Data represents mean ±SD (*** $p<0.001$). Statistical analysis was performed using a one-way ANOVA at each time point.

During the growth of multi-species biofilms significant differences were observed after the addition of *P. gingivalis* on day 3. On day 5, multi-species biofilms containing *P. gingivalis* W50 have a significantly lower biomass of 0.7 OD ($p<0.001$) compared to both ATCC 33277 and W83 with an OD of 0.9. On day 6 the biomass of multi-species biofilms containing *P. gingivalis* ATCC 33277 was significantly higher at a 1.19 OD ($p<0.001$) compared to those containing W83 and W50, which had an OD of 0.99 and 0.88, respectively. Mature biofilms did not show any significant difference in biomass in relation to varying *P. gingivalis* strains.

To further investigate if *P. gingivalis* strain variation played a role in mature biofilms composition biofilms were quantified further by qPCR. As previously, DNA from known concentrations of *S. mitis* (i), *F. nucleatum* (ii) and *A. actinomycetemcomitans* (iii) were used to prepare a standard curve that could be extrapolated to determine unknown bacterial counts (Figure 3.10).

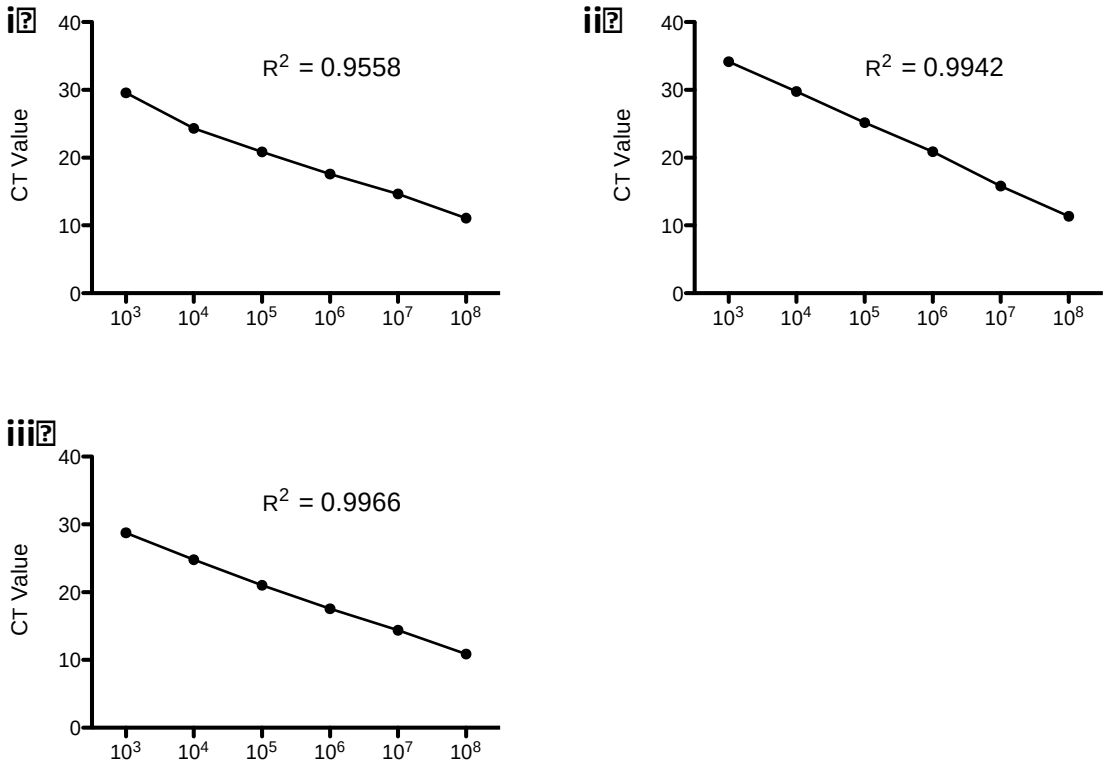


Figure 3.10: Standard curves of each species within multi-species biofilms
Ten-fold dilutions of DNA from known concentrations of *S. mitis* (i), *F. nucleatum* (ii) and *A. actinomycetemcomitans* (iii) were used for qPCR and their equivalent Ct used for the preparation of a standard curve. Unknown bacterial counts could then be quantified by extrapolating from the standard curve. Each DNA dilution was assessed in triplicate.

Biofilm were grown on Thermanox™ coverslips as previously described containing *P. gingivalis* ATCC 33277, W83 or W50. Mature biofilms were sonicated and DNA extracted using a Masterpure® Gram Positive DNA extraction kit and composition assessed using qPCR (Figure 3.11). Although no statistically significant differences were observed in the total number of *P. gingivalis* in each biofilm, biofilms containing *P. gingivalis* W83 contained 6x more *P. gingivalis* bacteria than those containing ATCC 33277 and W50. A significant difference in *F. nucleatum* was observed in biofilms containing *P. gingivalis* W83, with 3.99×10^7 total *F. nucleatum* compared to 2.2×10^7 ($p<0.05$) and 7.5×10^6 ($p<0.001$) total *F. nucleatum* in biofilms containing ATCC 33277 and W50, respectively.

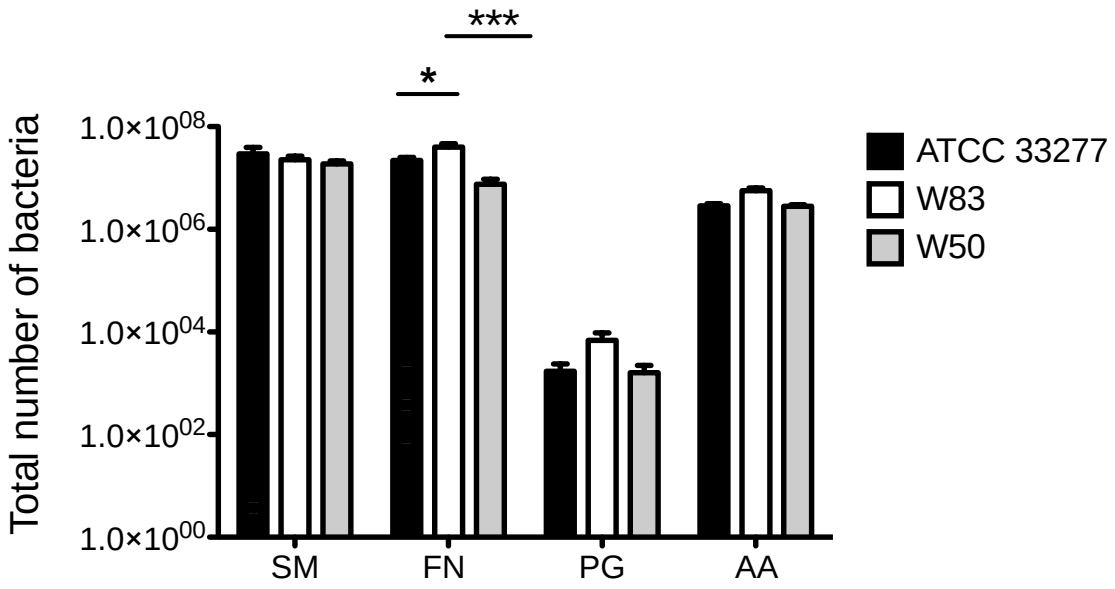


Figure 3.11: Total number of each species within multi-species biofilms
P. gingivalis strain ATCC 33277, W83 and W50 were grown in multi-species biofilms. Bacteria were sonicated and DNA was extracted using a Masterpure® Gram Positive DNA extraction kit. Each strain quantified using SYBR GreenER based qPCR. All samples were assayed in triplicate on the separate occasions. Data represents mean ±SD (* $p<0.05$ *** $p<0.001$). Statistical analysis was performed using a one-way ANOVA for each species within the biofilm.

The architecture of multi-species biofilms containing *P. gingivalis* ATCC 33277, W83 and W50 was analysed by SEM (Figure 3.12). At both low (1500x) and high (5000x) magnification no apparent *P. gingivalis* strain specific differences in overall composition of the biofilm were observed.

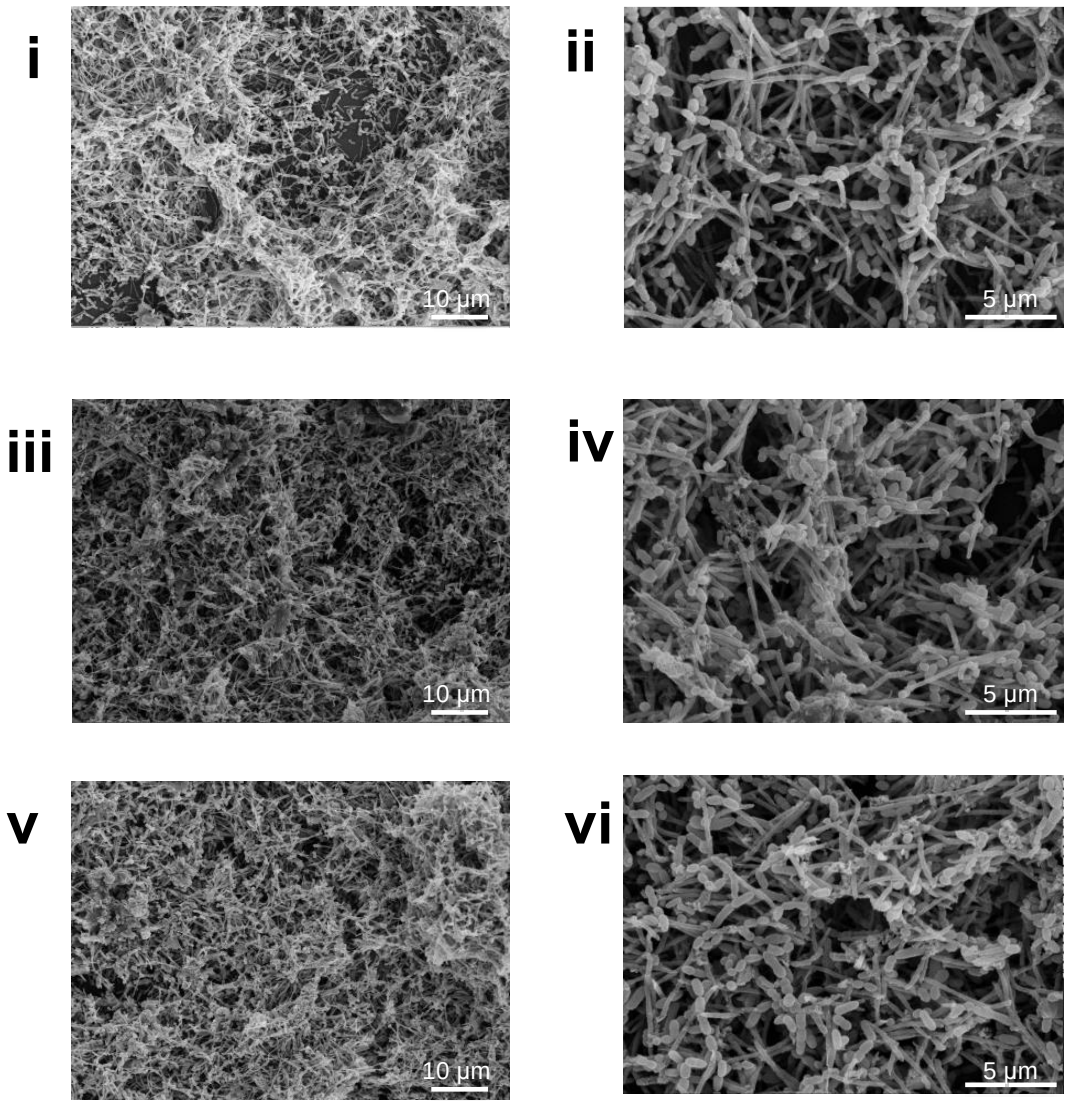


Figure 3.12: SEM of *P. gingivalis* strains within multi-species biofilms
Multi-species biofilms were analysed by SEM at 1500x (i, iii, v) and 5000x (ii, iv, vii). Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope. Multi-species biofilms containing *P. gingivalis* ATCC 33277 (i, ii), W83 (iii, iv) and W50 (vi vii) were compared to assess biofilm formation and architecture.

3.3.6 Strain variation influences epithelial cell IL-8 response in multi-species biofilms

To investigate if the *P. gingivalis* strain variation plays a role in the IL-8 response to multi-species biofilms by oral epithelial cells, multi-species biofilms containing *P. gingivalis* ATCC 33277, W83 and W50 were co-cultured with OKF6-TERT2 cells as previously described section 2.2.3 for 4 and 24 hours and IL-8 protein release measured by ELISA (Figure 3.13). A co-culture containing no biofilm was used as a control. At 4 hours no significant differences in IL-8 release by the epithelial cells in response to the biofilms was observed. At 24 hours in multi-species biofilms containing *P. gingivalis* W50 349 pg/mL IL-8 was observed, significantly higher than biofilms containing ATCC 33277 or W83, where 111.1 pg/mL ($p<0.001$) and 58.7 pg/mL ($p<0.001$) of IL-8 was observed, respectively.

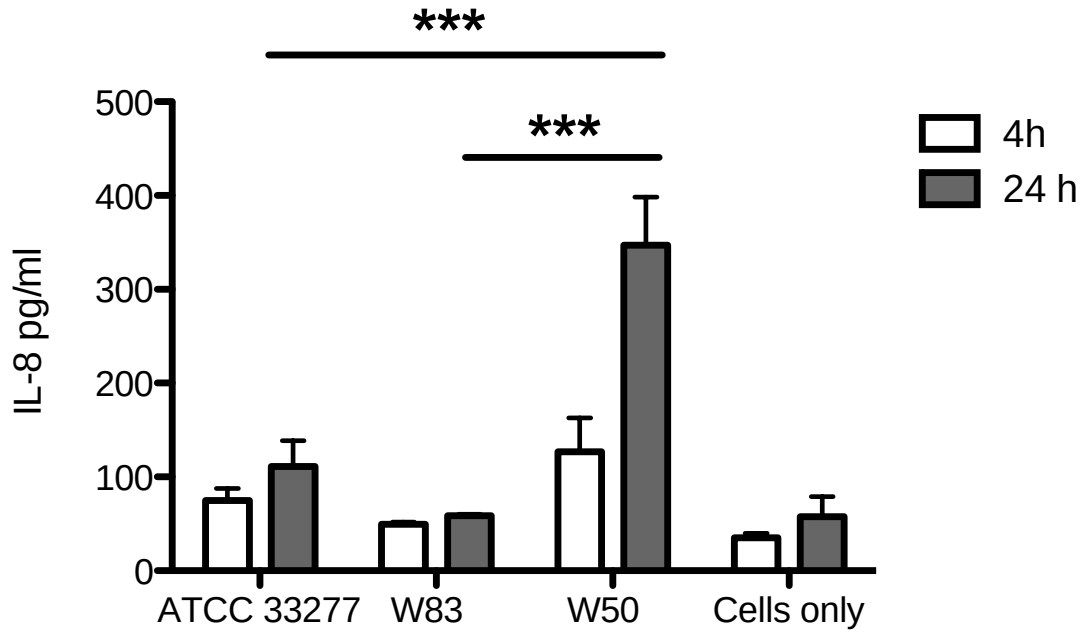


Figure 3.13: Epithelial cell IL-8 protein response to multi-species biofilms in co-culture

Multi- species periodontal biofilms containing *P. gingivalis* ATCC 33277, W83 and W50 were grown on Thermanox™ coverslips. Biofilms were co-cultured with oral epithelial cells as described previously for 4 and 24 h. IL-8 protein release was measured by ELISA. All samples were assayed in triplicate on the separate

occasions. Data represents mean ±SD (*** $p<0.001$). Statistical analysis was performed using a one-way ANOVA for each time point.

3.4 Discussion

P. gingivalis is a keystone pathogen which has been shown to influence the oral microflora, which in turn may contribute to disease (Hajishengallis et al., 2011). Furthermore *P. gingivalis* strains are classed as virulent or avirulent due to the association of certain strains with disease (Griffen et al., 1999). It is therefore important when investigating within the field of PD, both *in vitro* and *in vivo*, to consider the strain of *P. gingivalis* being used and its relevance to the study. For the future work outlined in this thesis creating a complex multi-species biofilm model it was imperative a pathogenic strain was chosen for use in the disease-associated biofilm, therefore three *P. gingivalis* strains were investigated. Strains ATCC 33277, W83 and W50 were selected based on their frequency of use for *in vitro* biofilm modelling (Bao et al., 2014b, Biyikoglu et al., 2012, Bercy and Lasserre, 2007). *P. gingivalis* W83 and W50 are considered virulent strains, while ATCC 33277 is considered avirulent (Chen et al., 2004). The data presented in this chapter show subtle differences between strains when both cultured as single-species and multi-species biofilms, which may be important considerations when modelling host-pathogen interactions.

In the single-species biofilm cultures an increase in the total number of W83 bacteria by CFU/mL and qPCR was reported when compared to both W50 and ATCC 33277. There was also a trend towards this with respect to overall biomass. Many previous studies report finding little or no biofilm formation when *P. gingivalis* is grown as a single species (Periasamy and Kolenbrander, 2009). A study by Biyikoğlu et. al. (2012) reported biofilm formation occurred only in less virulent strains such as ATCC 33277 when compared to W83 and W50, which are classed as more virulent strains in single species biofilm formation (Biyikoglu et al., 2012). A caveat to this is that in these studies bacteria are cultured for less that 20 hours compared to our own studies were bacteria are cultured for 4 days. Other studies have shown all three strains tested herein are able to grow

as single-species biofilms when cultured for more than 24 hours; however, strain variation was not compared (Aruni et al., 2012, Capestany et al., 2006, Lo et al., 2009). Additionally, Kuboniwa et al (2009) observed variations in biofilm formation based on fimbriae length and type of proteinase release when using mutant strains (Kuboniwa et al., 2009). This study showed that long fimbriae (FimA) promoted early biofilm formation while short fimbriae and lysine-specific proteases had a more suppressive function and strain specific variations within fimbriae and proteases may relate to the differential biofilm formation observed in these studies.

The ability of *P. gingivalis* single-species biofilms to degrade IL-8 was also observed, with W83 being most proficient compared to ATCC 33277 and W50. *P. gingivalis* produce arginine-specific-gingipains and lysine-specific-gingipains, known to degrade cytokines, the genes of which are highly conserved between strains (Stathopoulou et al., 2009). However, it has been reported that the amounts and forms of gingipains produced and secreted is strain dependant (Potempa et al., 1995). This may explain the strain dependant differences observed in this work, and correlates with studies by others where more virulent strains are more effective at cleaving cytokines such as IL-8 compared to their avirulent counterparts (Jayaprakash et al., 2014, Fletcher et al., 1998).

Significant degradation of IL-8 observed in co-culture of OKF6-TERT2 oral epithelial cells with *P. gingivalis* biofilms compared to *S. mitis* biofilms confirms our previous finding of the specific ability of *P. gingivalis* to degrade IL-8. Interestingly significant reductions in IL-8 protein release by OKF6-TERT2 cells were observed in co-culture with methanol fixed planktonic *P. gingivalis* compared to methanol fixed *S. mitis*. This may be due to a lack of response by the OKF6-TERT2 cells to the *P. gingivalis* as the methanol fixation prevents *P. gingivalis* from releasing virulence factors or shedding LPS which is known to stimulate pro-inflammatory cytokines from resident immune and tissue cells (Dias et al., 2008). Alternatively, the low IL-8 protein release observed when using single species *P. gingivalis* biofilms may be due to the fact that host cells do not respond highly to *P. gingivalis*. A study by Palm et al (2014) found no significant increase in CXCL8 protein release when human gingival fibroblasts

(HGF) were cultured with planktonic or heat killed *P. gingivalis* W50 and ATCC 33277 which may suggest OKF6-TERT2 epithelial cells may not respond to *P. gingivalis* in planktonic states (Palm et al., 2014), a finding correlating with the data produced by Peyyala et al who found a significantly lower IL-8 response by oral epithelial cells to planktonic cells when compared to single species bacterial biofilms (Peyyala et al., 2011). Live planktonic *P. gingivalis* and supernatants from biofilms and live bacteria elicit no significantly different response compared with their *S. mitis* controls. A study co-culturing *P. gingivalis* with human gingival epithelial cells observed IL-8 protein release of less than 50 pg/mL for both live and heat killed *P. gingivalis* at MOI 10:1 (Stathopoulou et al., 2010). This suggests that IL-8 degradation plays a minor role in host responses to *P. gingivalis* alone and correlates with our own findings of a low IL-8 response to *P. gingivalis* biofilms, planktonic cells, methanol fixed cells and supernatants. Alternatively, although gingipains are able to be secreted it is possible that there are not enough present in the supernatants of our experiments to cause an effect on the IL-8 response by OFK6-TERT2 cells.

As previously discussed, *P. gingivalis* is not found alone in the oral cavity and studies have shown many mutualistic relationships between it and other species of the oral microbiome (Periasamy and Kolenbrander, 2009). To investigate *P. gingivalis* strain heterogeneity in multi-species biofilms we used the simple four species biofilm model developed in our lab (Sherry et al., 2013). In a multi-species biofilm the strain of *P. gingivalis* did not affect the final biomass, however, there was significantly more *F. nucleatum* bacteria when biofilm composition was quantified by qPCR. *F. nucleatum* has been shown to facilitate *P. gingivalis* adhesion to multi-species biofilms due to its ability to co aggregate with both aerobic and anaerobic bacteria (Diaz et al., 2002, Bradshaw et al., 1998). Other studies investigating the role of *P. gingivalis* on biofilm formation observed no change in biofilm composition (with the exception of *Tannerella forsythia*) using a variety of strains, including both arginine-gingipain and lysine-gingipain mutants (Bao et al., 2014b).

When our multi-species biofilms containing the *P. gingivalis* strains were co-cultured with OKF6-TERT2 oral epithelial cells no significant differences where

found between strains at 4 hours, however, at 24 hours significantly more IL-8 was present in cultures using biofilms containing *P. gingivalis* W50 compared with W83 or ATCC 33277. As previously discussed, gingipains and other proteases produced by *P. gingivalis* play an important role in the virulence of the species and its ability to degrade pro-inflammatory cytokines. Additionally, strain specific variation of the quantity and forms of gingipains may further segregate *P. gingivalis* at a species specific level. Multi-species biofilms containing *P. gingivalis* ATCC 33277 have also been shown to significantly reduce IL-8 protein levels over time in co-culture with gingival epithelial cells, whereas biofilms without this species were unable to do so (Belibasakis et al., 2013b). Studies have observed that *P. gingivalis* W50 sheds significantly higher amounts of bacterial LPS and low levels of secreted gingipains compared to *P. gingivalis* ATCC 33277 (Jayaprakash et al., 2014), either one of which may contribute to the results observed in our study.

One of the main limitations of this study was the ability to grow *P. gingivalis* as single species biofilms. As can be seen in the SEM images of each of the strains grown as single species *P. gingivalis* forms scant biofilms with little coverage across the surface of the coverslip. Studies of *P. gingivalis* biofilm formation show that species differ in their ability to form biofilms, with one study showing *P. gingivalis* W83 and W50 unable to form single species biofilms, ATCC 33277 able to adhere to the surface to form a biofilm but not increase biomass over time, and *P. gingivalis* 381 able to form a biofilm and grow on the surface when cultured for 4-16 hours (Biyikoglu et al., 2012). Both W83 and W50 have been reported to form biofilms in a 40 day culture by Lo et. al. (2009) and Ang et al (2008) who analysed the transcriptome and proteome, respectively. These studies observed up regulation of C-terminal domain family surface adhesins and proteinases including arginine-specific proteases, hemagglutinin protein A and zinc carboxypeptidase as well as metabolic enzymes and transport related genes in *P. gingivalis* biofilms compared with planktonic cells (Lo et al., 2009) (Ang et al., 2008). Time may play a role in the ability of *P. gingivalis* to form a single species biofilm as the cultures described herein are for 96 hours as opposed to 4-16 hours where no biofilm formation was observed and 40 days where biofilm formation was. The time allowed for biofilms to develop may also play a role in

how epithelial cells respond to *P. gingivalis*, as little response was seen in co-culture in our work. Future studies investigating the role of *P. gingivalis* strain variation may benefit from leaving *P. gingivalis* single species biofilms to develop longer into more robust biofilms.

From this work *P. gingivalis* W83 was selected for further studies of complex multi-species biofilms. This was due to its potent ability to degrade IL-8 observed co-culture with both single and multi-species biofilms and confirmation by others of its virulent status and association with PD (Griffen et al., 1998).

CHAPTER FINDINGS

Biofilms of *P. gingivalis* W83 have an increased composition compared to W50 and ATCC 33277.

Biofilms of *P. gingivalis* W83 degrade exogenous IL-8 better than W50 and ATCC 33277.

4 Development and validation of three multi-species oral biofilm models representing the phases of plaque development

4.1 Introduction

The oral cavity is home to a large number of complex multi-species microbial communities that play an important role in both the health and disease of teeth and oral tissue. Over 700 bacterial species are associated with the oral cavity, identified using both culture based and genomic techniques, with between 100 and 200 species present in healthy mouth of any individual (Wade, 2011, Paster et al., 2006).

Oral bacterial communities form complex, highly organized, biofilms (plaque) on hard surfaces such as teeth and soft surfaces including the oral tissue (Zijnge et al., 2010). Biofilm structures are generated by microbial succession, a non-random process influenced by interactions between bacterial species, as well as between bacteria and the local physical and chemical environment (Kolenbrander et al., 2010b). The ability of microbial communities to alter the local environment can enable disease-associated succession of pathogenic bacteria into biofilms, which in turn can result in diseases such as periodontitis and caries (Colombo et al., 2009). Due to the large number of bacteria associated with both supra- and sub-gingival plaque, studying the roles of bacteria in oral biofilms is difficult. Therefore, multiple *in vitro* models have been devised to study oral biofilms and their interactions, both within the biofilm itself and within the local environment.

Many groups have developed multi-species biofilm models for investigating biofilm formation, architecture, and interactions with host cells using a variety of methods (Guggenheim et al., 2009, Peyyala et al., 2013, Belibasakis et al., 2011a, Shapiro et al., 2002, Blanc et al., 2014, Foster and Kolenbrander, 2004). These models use defined bacterial consortia to provide well-controlled conditions, using both static and flow systems. Within these systems there are variables such as inoculum, including bacterial culture media, human saliva and different bacterial densities, and substrates such as rigid gas permeable lenses or hydroxyapatite disks, each confer unique advantages depending on the research questions being investigated (Ammann et al., 2012). Static models contain limited nutrients and aeration, but enable rapid quantification of

biomass, species-specific roles in biofilm formation, and allow orally relevant antimicrobial compounds to be evaluated (Belibasakis and Thurnheer, 2014, Kinniment et al., 1996b, Ammann et al., 2013a). Flow cell models are more labour intensive and typically require specialized equipment; however, they allow studies of physical and chemical resistance of biofilms as well as preferential examination of bacterial species in systems where replenishing nutrients and shear forces play a role (Periasamy and Kolenbrander, 2009, Corbin et al., 2011, Saunders and Greenman, 2000). Collectively, these studies show the wide range of options and variables to consider when developing oral biofilms, each which have advantages and disadvantages depending on downstream applications.

Most studies focus on a single multi-species biofilm model when examining research questions, and currently little is known about differing biofilm characteristics when biofilms contain health-associated bacteria compared with disease-associated bacteria. While it is known that different bacterial species present in plaque are associated with either health or disease, few studies model the differences in biofilm development and composition when shifting between the two states (Teles et al., 2012). Previous studies modelling microbial population shifts have focused on the effect of antimicrobials on disease biofilm models, finding shifts in the biofilm composition following treatment (Ready et al., 2002, McBain et al., 2003). Dalwai *et. al* (2006) observed shifts in the microbial composition of both dual species and complex microcosm models when removed from aerobic conditions and exposed to a microaerophilic atmosphere more associated with gingivitis (Dalwai et al., 2006). Using confocal laser scanning microscopy this study also found the distribution of dead bacteria in the biofilm was dynamic, moving from being predominantly at the attachment site to more widely observed throughout the biofilm as conditions shifted from health to disease. More recently, Peyyala *et. al.* (2013) used three different multi-species biofilm models to show that biofilm composition can also influence epithelial cell protein response in co-culture (Peyyala et al., 2013). These findings emphasise the need to further understand the unique characteristics of multi-species biofilms due to microbial composition and how this may influence the host response in different states.

4.2 Aims

It is believed that the composition of oral biofilms may play a role in the modulation of the host immune response and subsequent disease progression in the oral cavity.

Therefore, the aim of this study was to create and validate three reproducible defined multi-species biofilm models *in vitro* to mimic health-associated, intermediate and disease-associated plaque, containing orally relevant bacteria which could be used in further downstream analyses.

4.3 Results

4.3.1 Quantification of bacteria for developing biofilm models

Based on the current literature the bacteria selected for the three multi-species biofilm models were are follows; *S. mitis, S. intermedius, S. oralis, F. nucleatum, F. nucleatum* ssp. *vincentii, A. naeslundii, V. dispar, P. gingivalis, P. intermedia* and *A. actinomycetemcomitans* (Guggenheim et al., 2009, Socransky et al., 1998). An initial step was taken to ensure optimum growth of these bacteria in the laboratory and determine the optical density to standardize them to 1 x 10^8 CFU/mL for use in the models, a concentration which other groups use when developing models of their own (Guggenheim et al., 2001a, Park et al., 2014).

S. mitis, *S. intermedius*, *S. oralis* and *A. actinomycetemcomitans* were grown in TSB in 5% CO_2 for 24 hours. *V. dispar*, *A. naeslundii* and *P. intermedia* were grown in BHI for 48 hours in the anaerobic cabinet and *F. nucleatum*, *F. nucleatum* ssp. *vincentii* and *P. gingivalis* were grown in Schaedlers anaerobic broth in the anaerobic cabinet for 48 hours. Following incubation all bacteria were standardized at a range of different OD's and counted using the Miles and Misra plate counting method. From this we observed that an 0.5 OD_{550} for SM, SI, SO and VD and an 0.2 OD_{550} for FN, FNV, AN, PI, PG and AA gave ~1 x 10^8 CFU /mL for each species (Figure 4.1).

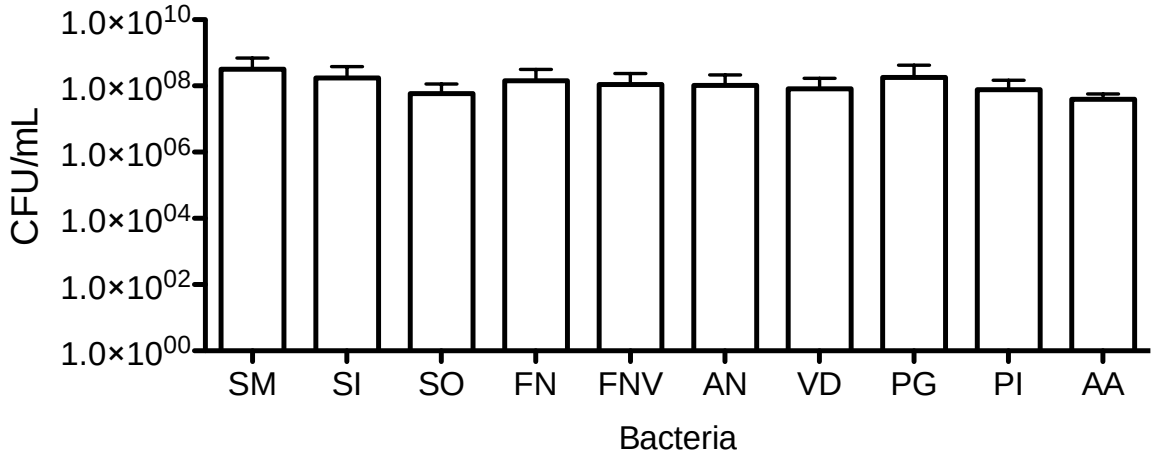


Figure 4.1: Enumeration of standardized bacteria

Bacterial species *S. mitis* (SM), *S. intermedius* (SI), *S. oralis* (SO), *F. nucleatum* (FN), *F. nucleatum ssp. vincentii* (FNV), *A. naeslundii* (AN), *V. dispar* (VD), *P. gingivalis* (PG), *P. intermedia* (PI) *and A. actinomycetemcomitans* (AA) were cultured in broth in either anaerobic conditions or 5% CO_2. Following culture, bacteria were standardized to $1x10^8$ CFU/mL at 0.5 OD_{550} for SM, SI, SO and VD, and 0.2 OD_{550} for FN, FNV, AN, PG, PI, and AA. Viable bacteria were enumerated using Miles and Misra plate counting method on appropriate agar plates. Data shown are mean ±SD of three independent cultures enumerated in triplicate.

In order to quantify species within multi-species biofilms a sensitive and specific method was required. One method available is quantitative PCR, which uses species specific primers to determine species present in both clinical plaque samples and the total composition of multi-species biofilm models (Ammann et al., 2013b, Fernandes et al., 2014). For each species used in the multi-species biofilms known concentrations of DNA (1×10^3-10^8 CFU /mL) were used to prepare a standard curve to determine unknown bacterial counts from both planktonic bacteria and biofilms (Figure 4.2). The R^2 value was used to determine the fit of the line. Each bacterial species R^2 values ranged between 0.95-0.99 suggesting that the extraction method used at each concentration gave reliable recovery rates for qPCR quantification.

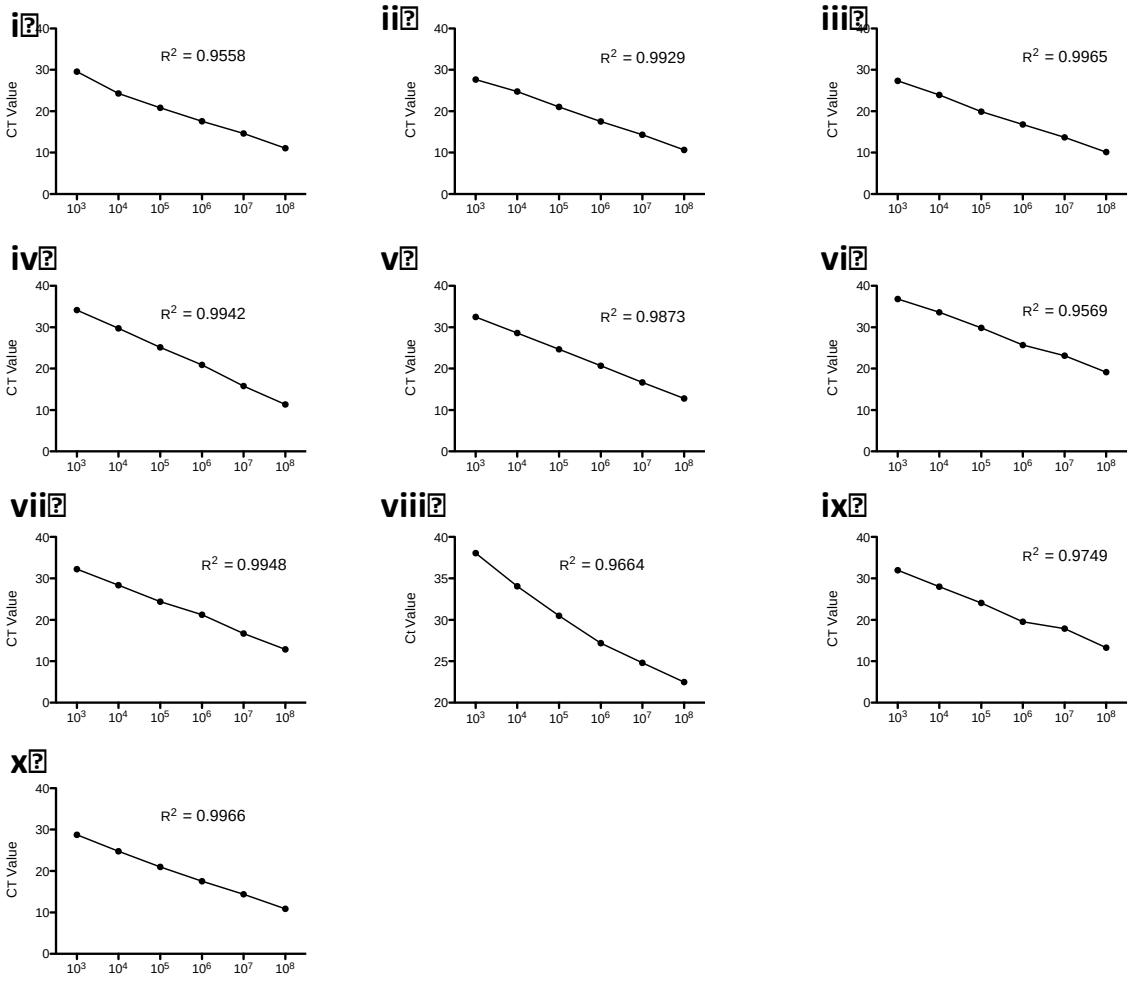


Figure 4.2: Generation of standard curves by qPCR

Planktonic bacterial species *S. mitis* (SM) [i], *S. intermedius* (SI) [ii], *S. oralis* (SO) [iii], *F. nucleatum* (FN) [iv], *F. nucleatum ssp. vincentii* (FNV) [v], *A. naeslundii* (AN) [vi], *V. dispar* (VD) [vii], *P. gingivalis* (PG) [viii], *P. intermedia* (PI) [ix] *and A. actinomycetemcomitans* (AA) [x] were cultured in broth and standardized to $1x10^8$ bacteria/mL. Ten-fold dilutions of DNA from known concentrations were extracted using the MasterPure™ gram positive DNA purification kit and quantified using SYBR® GreenER based qPCR. The equivalent Ct and each concentration were used for the preparation of a standard curve. Unknown bacterial counts could then be quantified by extrapolating from the appropriate curve. Using linear regression analysis R^2 was determined. Data shown are mean ±SD of three independent cultures enumerated in triplicate for each primer set.

Each species was then tested for its ability to grow in artificial saliva (AS), the medium used to grow the multi-species biofilm models. This was validated using both qPCR to measure total bacteria and Miles and Misra to measure viable bacteria and evaluate any differences (Figure 4.3). Each bacterial species was standardized to 1 x 10^8 CFU /mL and diluted to 1 x 10^7 CFU/mL in AS, where it was grown for 72 hours in either 5% CO_2 or anaerobically as appropriate for each species. The results show all bacteria were able to survive in AS, as shown by the viable CFU counts (Figure 4.3 i), and there was no statistical significant differences between enumeration by Miles and Misra plate counting method and the quantification of each species by qPCR when statistical analysis was performed using a two-tailed unpaired t-test. (Figure 4.3 ii).

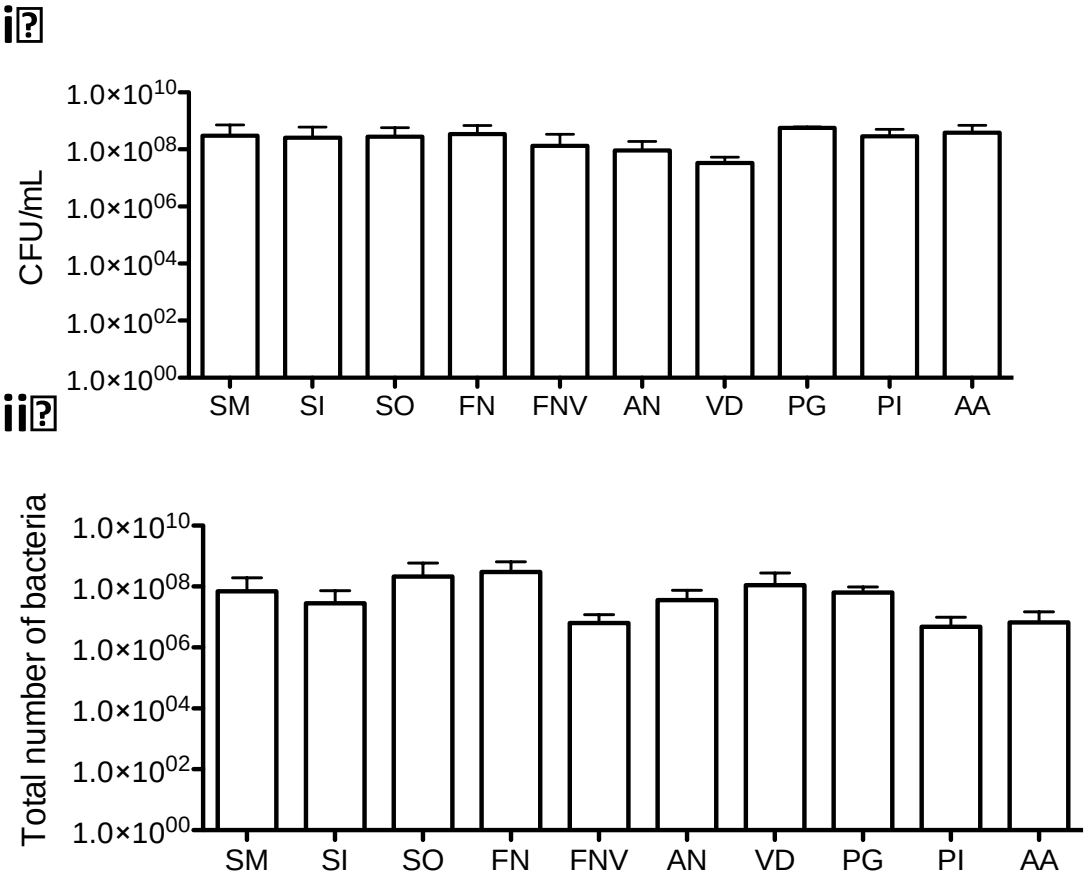


Figure 4.3: Enumeration of bacteria grown in AS by Miles and Misra and qPCR
Planktonic bacterial species *S. mitis* (SM), *S. intermedius* (SI), *S. oralis* (SO), *F. nucleatum* (FN), *F. nucleatum ssp. vincentii* (FNV), *A. naeslundii* (AN), *V. dispar* (VD), *P. gingivalis* (PG), *P. intermedia* (PI) *and A. actinomycetemcomitans* (AA) were cultured in broth and standardized and diluted to 1 x 10^7 bacteria/mL. Bacteria were then added to AS and cultured for 72 hours in either 5% CO_2 or anaerobic conditions. Viable bacteria were enumerated using Miles and Misra plate counting method on appropriate agar plates [i]. Total bacteria were quantified using SYBR® GreenER based qPCR [ii]. Data shown are mean ±SD of 3 cultures per bacteria performed in triplicate. Statistical analysis was performed using a two-tailed unpaired t-test.

4.3.2 Analysis of multi-species biofilm maturation and composition

Once each bacterial species was known to survive in AS, multi-species biofilms were developed. The three plaque phases selected were: a health-associated 3 species biofilm containing *S. mitis, S. intermedius* and *S. oralis;* an intermediate 7 species biofilm (biofilms progressing from health to disease) containing *S. mitis, S. intermedius, S. oralis, F. nucleatum, F. nucleatum* ssp. *vincentii, A. naeslundii* and *V. dispar*; and a 10 species disease-associated biofilm containing *S. mitis, S. intermedius, S. oralis, F. nucleatum, F. nucleatum* ssp. *vincentii, A. naeslundii, V. dispar, P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*. The architecture of each mature biofilm was then assessed by SEM, where noticeable physical differences were observed between each model (Figure 4.4). The 3 species biofilms produced a flattened but evenly distributed biofilm across the surface (Figure 4.4 i-iii), whereas the 7 species biofilms showed formation of heterogeneous micro-colonies on the surface, which were distributed unevenly across the surface (Figure 4.4 iv-vi). Finally, 10 species biofilms were the most complex in terms of biomass and composition, with a topographically heterogeneous 3D structure covering the entire surface (Figure 4.4 vii-ix).

To further investigate the development of multi-species biofilms over time, SEM analysis of the 10 species biofilm was assessed each day during culture (Figure 4.5). These photomicrographs show the initial broad covering of the surface by *Streptococcus* species, similar to the 3 species biofilm, after which time bacteria co-aggregate in various micro-colonies, which were observed in the mature 7 species biofilms. These micro-colonies then appear to expand and merge across the entire surface over time resulting in the complex 3D architecture associated with the 10 species biofilms.

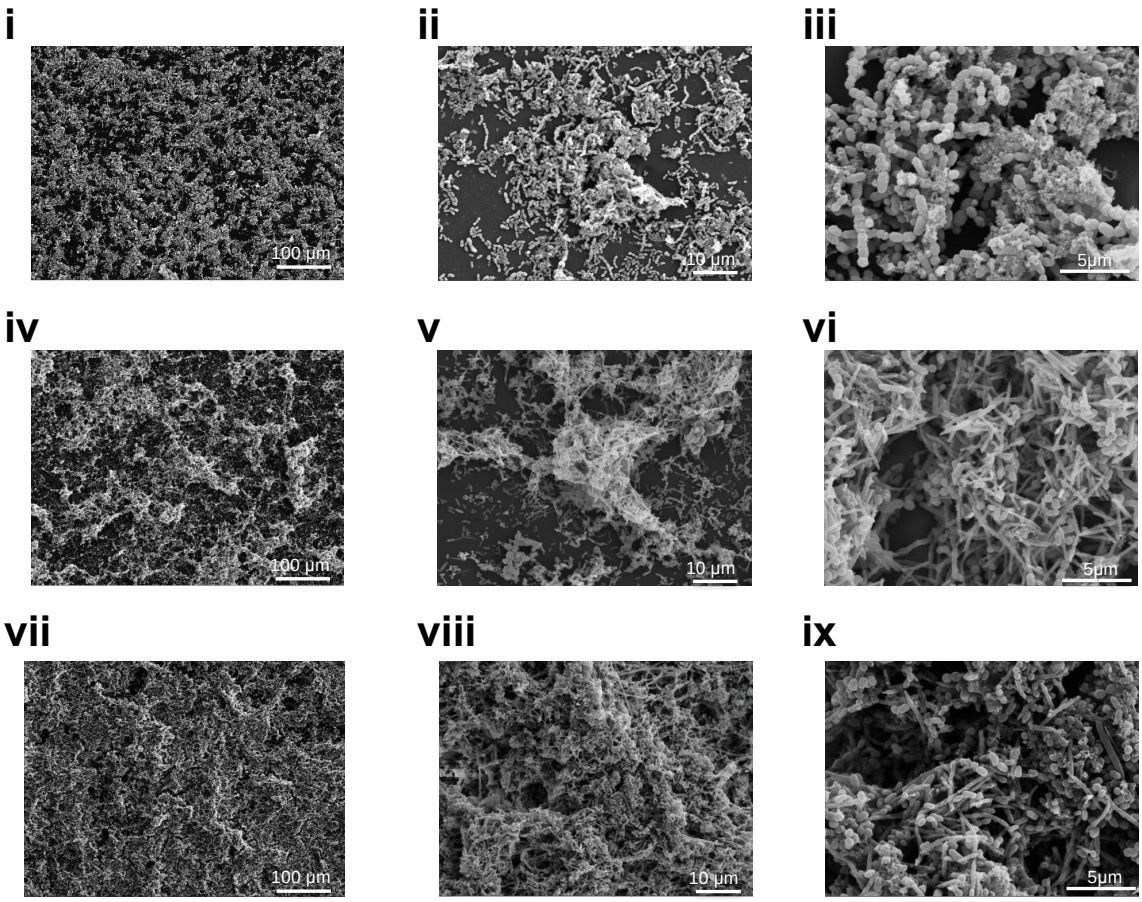


Figure 4.4: SEM analysis of 3, 7 and 10 species biofilms

Three [i-iii], 7 [iv-vi] and 10 [vii- ix] species biofilms grown on Thermanox® coverslips were analysed by SEM at 200x [i, iv, vii], 1500x [ii, v, viii] and 5000x [iii, vi ix]. These were processed and viewed on a JEOL JSM-6400 scanning electron microscope and images assembled using Photoshop software.

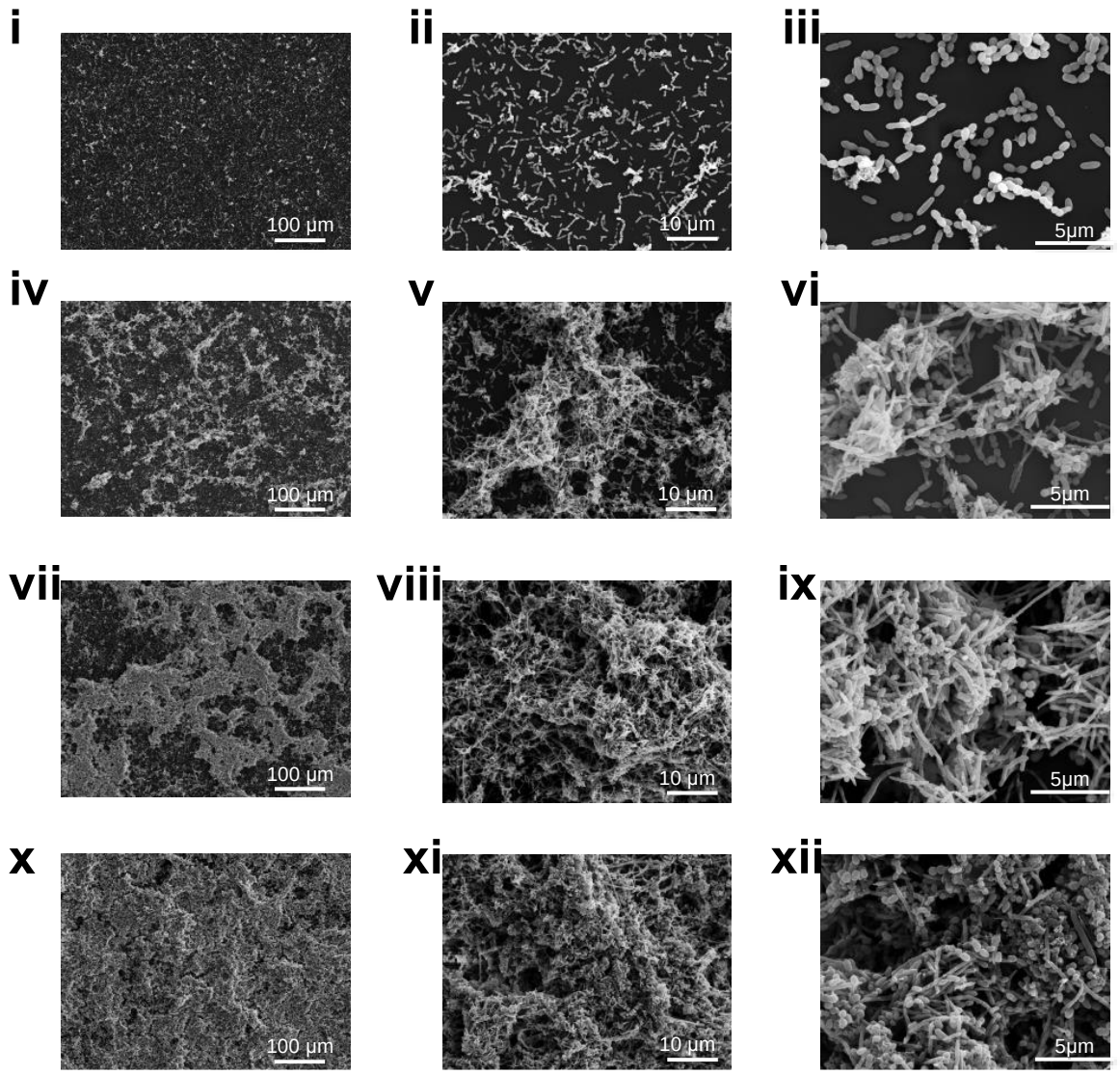


Figure 4.5: Maturation of multi-species biofilms

Ten species biofilms were grown on Thermanox® coverslips and biofilm formation was assessed by SEM during development at 24 [i-iii], 48 [iv-vi], 72 [vii-ix] and 168 [x-xii] hours at 200x [i, iv, vii, x], 1500x [ii, v, viii, xi] and 5000x [iii, vi, ix, xii]. Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope and images assembled using Photoshop software.

The composition of each mature biofilm was assessed using SYBR® GreenER based qPCR to ensure all bacterial species were present and also investigate the quantity of species within each biofilm (Figure 4.6). The 3 species biofilms contained 5.1 x 10^6 (100%) total *Streptococcus* species (Figure 4.6 i). The 7

species biofilms contained 1.6 x 10^7 (4.2%) total *Streptococcus* species; 1.4 x 10^7 (63.3%) total *F. nucleatum* species; 1.2 x 10^7 (32.34%) *A. naeslundii* and 2.1 x 10^6 (0.04%) *V. dispar* (Figure 4.6 ii). The mature 10 species contained 1.0 x 10^7 (11%) total *Streptococcus* species; 1.4 x 10^7 (17%) *F. nucleatum* species; 2.4 x 10^7 (59.9%) *A. naeslundii*; 3.2 x 10^6 (0.12%) *V. dispar*; 1.6 x 10^4 (3.72%) *P. gingivalis*; 2.9 x 10^6 (5.5%) *P. intermedia* and 1.7 x 10^6 (2.6%) *A. actinomycetemcomitans* (Figure 4.6 iii).

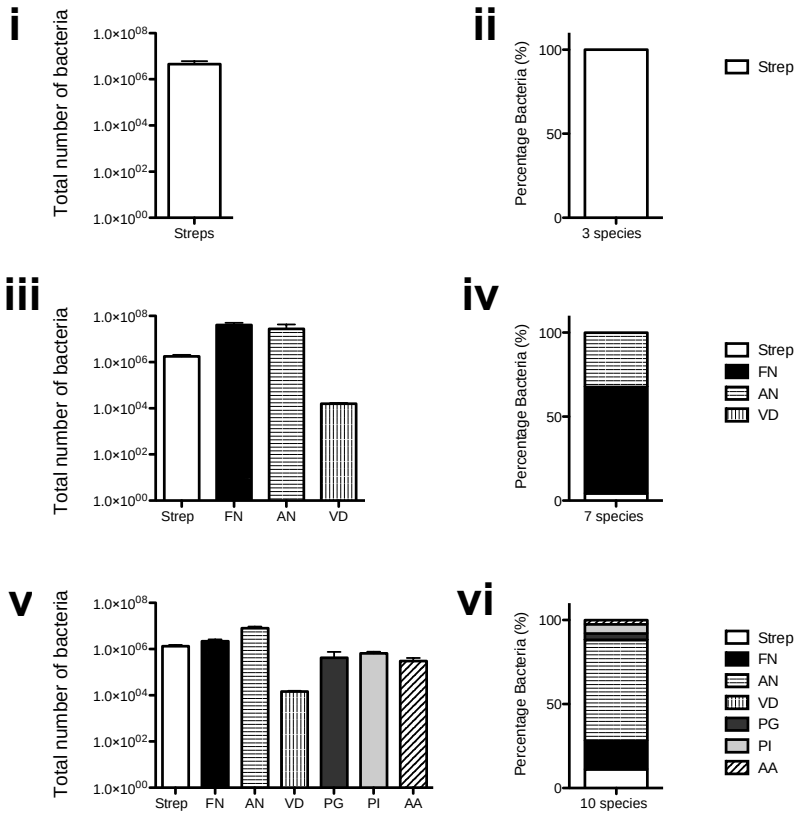


Figure 4.6: Quantification of Biofilms in AS

Three [i, ii], 7 [iii, iv] and 10 [v, vi] species biofilms were grown on Thermanox® coverslips and sonicated for 10 minutes to detach the biofilm from the coverslip. Total bacterial DNA was extracted from each biofilm using Masterpure® Gram positive DNA extraction kit. The total number of each species [i, iii, v] and composition of each biofilm [ii, iv, vi] was quantified using SYBR® GreenER based qPCR for the 3, 7 and 10 species biofilms from previously calculated standards of each bacterial species. Data shown are mean ±SD for total number of bacteria and mean only for biofilm composition of 3 independent experiments performed in triplicate.

To further understand the composition of the biofilms, a novel SYBR® GreenER based qPCR technique as described in section 2.1.14 was used to determine the proportion of live bacteria in the total number of each bacterial species present (Figure 4.7). In the mature 3 species biofilms 1.9 x 10^5 live *Streptococcus* bacteria were present, significantly lower than the 5.6 x 10^7 total number of *Streptococcus* species ($p<0.01$)(Figure 4.7 i). The mature 7 species biofilms showed no significant differences between the number of live bacteria and the total bacteria (Figure 4.7 ii). Finally, in the mature 10 species biofilms significant differences between the live and total number of bacteria present were observed in the *Streptococcus* species and *A. naeslundii*, with a log difference of 3.31 ($p<0.05$) and 3.13 ($p<0.01$) respectively (Figure 4.7 iii).

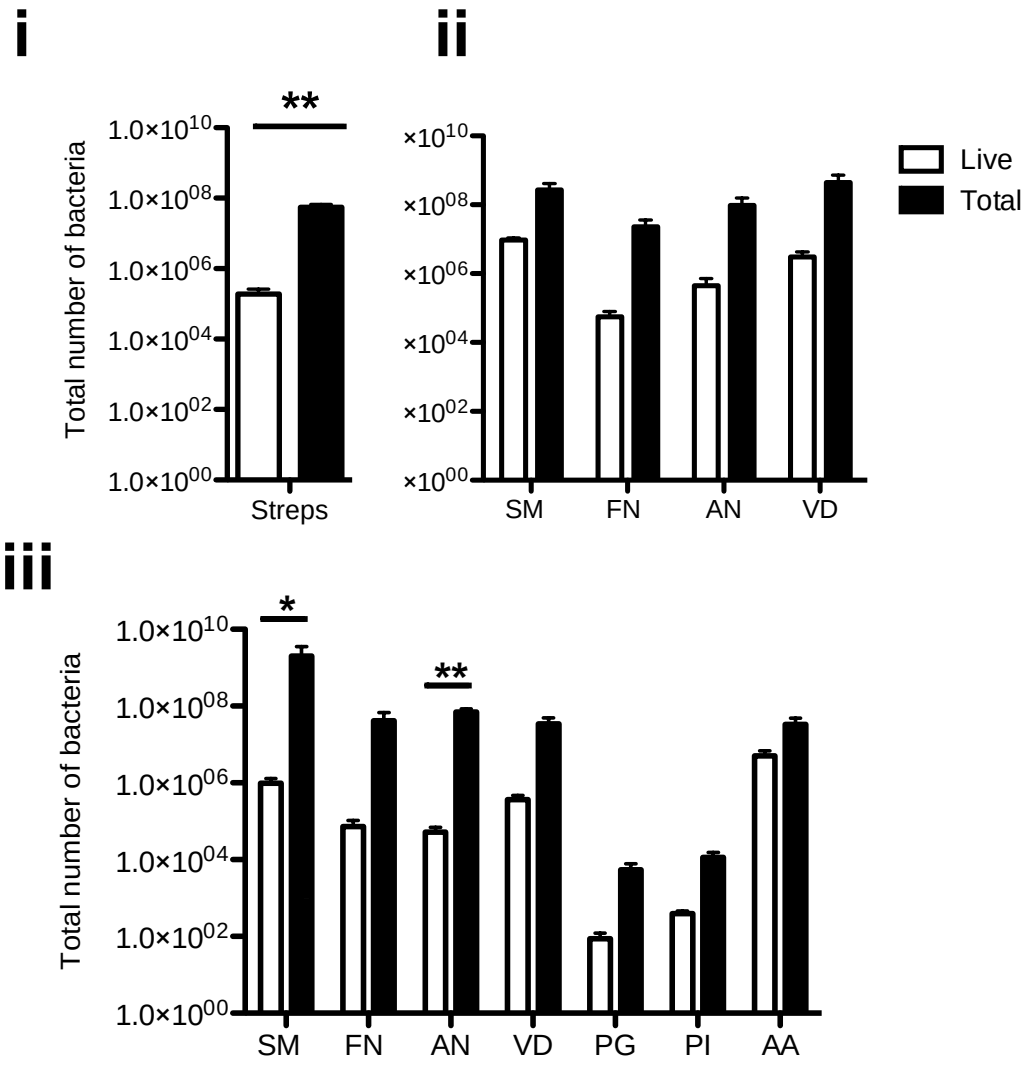


Figure 4.7: Quantification live and total bacteria in multi-species biofilms

Three [i], 7 [ii] and 10 [iii] species biofilms were sonicated to detach the biofilm from the coverslip and half the samples treated with 5 µL of 10 mM of propidium monoazide, incubated for 10 minutes in the dark before a 5 minute exposure to a 650W halogen light to determine the proportion of live bacteria. Control biofilms were not treated with propidium monoazide. Bacterial DNA from biofilms was then extracted using the Masterpure Gram-positive DNA extraction kit and each species quantified using SYBR® GreenER based qPCR. Data shown are mean ±SD of 3 independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed unpaired t test (*$p<0.05$, ** $p<0.01$).

4.3.3 Quantitative analysis of fresh and frozen multi-species biofilms

Due to the lengthy and labour intensive nature of growing multi-species biofilms the effects of freezing biofilms as a means of creating biofilm stocks was investigated to maximise efficiency and ensure quality control. The composition of fresh mature 3, 7 and 10 species biofilms were compared with biofilms which had been stored in -80^{o}C and recovered by culture at 37^{o}C in anaerobic conditions in AS for 24 hours prior to DNA extraction and bacterial quantification (Figure 4.8). The data show no significant differences between bacterial species in fresh and frozen 3, 7 and 10 species biofilms.

Three species biofilms were not significantly different between fresh and frozen, with total *Streptococcus* species quantified at 4.5 x 10^6 total bacteria in fresh biofilms and 1.3 x 10^7 bacteria in frozen biofilms (Figure 4.8 i). Additionally, 7 species biofilms showed no significant differences between fresh and frozen, with total *Streptococcus* species quantified at 1.7 x 10^6 and 2.0 x 10^7; *F. nucleatum* at 4.0 x 10^6 and 3.9 x 10^7; *A. naeslundii* at 2.7 x 10^7 and 8.3 x 10^7 and *V. dispar* at 1.5 x 10^4 and 3.5 x 10^4 for fresh and frozen biofilm, respectively (Figure 4.8 ii). Finally,10 species biofilms also showed no significant differences between fresh and frozen with total *Streptococcus* species quantified at 1.3 x 10^6 and 1.9 x 10^6; *F. nucleatum* at 2.1 x 10^6 and 7.9 x 10^6; *A. naeslundii* at 8.0 x 10^6 and 1.6 x 10^7; *V. dispar* at 1.4 x 10^4 and 1.8 x 10^3; *P. gingivalis* at 4.1 x 10^5 and 9.7 x 10^3; *P. intermedia* at 6.5 x 10^5 and 1.7 x 10^6 and *A. actinomycetemcomitans* at 3.0 x 10^5 and 4.9 x 10^4 for fresh and frozen biofilms, respectively (Figure 4. 8 iii).

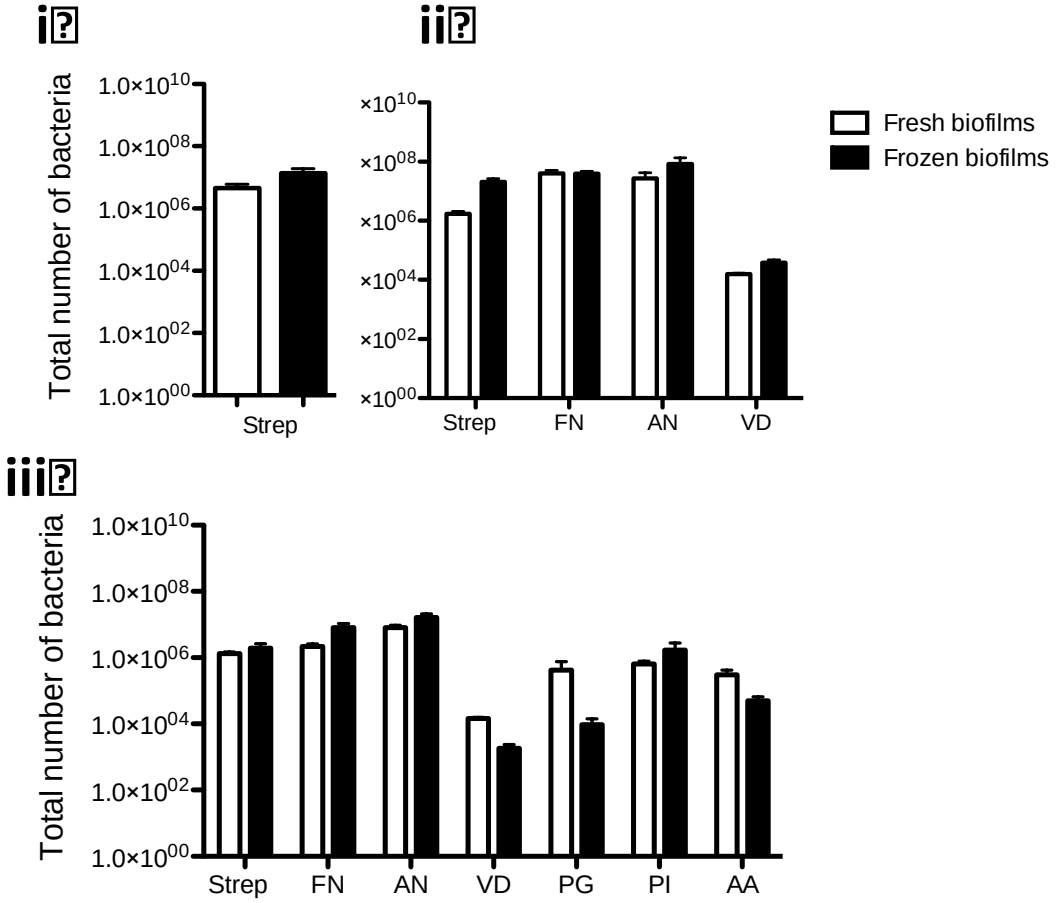


Figure 4.8: Quantification of Fresh and Frozen Biofilms

Fresh (□) and frozen (■) 3 [i], 7 [ii] and 10 [iii] species biofilms were sonicated to detach the biofilm from the coverslip. Bacterial DNA from the 3, 7 and 10 species biofilms was extracted using the Masterpure Gram positive DNA extraction kit and each species quantified using SYBR® GreenER based qPCR. Data shown are mean ±SD of 3 independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed unpaired t-test.

4.3.4 Quantification of biofilm in culture conditions

The process of growing multi-species biofilms and then experimentally using in co-culture requires biofilms to be exposed to a variety of growth conditions, so the composition of biofilms following culture in these conditions was investigated. Mature biofilms, some of which contain obligate anaerobes, are co-cultured with human cells in 5% CO_2 therefore 3, 7 and 10 species biofilms were

cultured in AS for 4 and 24 hours at 37°C in 5% CO_2 and compared with biofilms cultured in anaerobic conditions in AS (Figure 4.9). No significant differences were observed in the total number of each bacterial species in either 3, 7 or 10 species biofilms.

Three species biofilms were not significantly different between 5% CO_2 and anaerobic culture, with total *Streptococcus* species quantified at 7.7 x 10^7 total bacteria in 5% CO_2 and 5.1 x 10^7 bacteria in anaerobic cultures (Figure 4.9 i). Additionally, 7 species biofilms showed no significant differences between 5% CO_2 and anaerobic cultures, with total *Streptococcus* species quantified at 3.5 x 10^7 and 1.6 x 10^7; *F. nucleatum* at 9.9 x 10^5 and 1.4 x 10^7; *A. naeslundii* at 2.0 x 10^7 and 1.2 x 10^7 and *V. dispar* at 8.6 x 10^4 and 2.5 x 10^6 for 5% CO_2 and anaerobic cultures respectively (Figure 4.9 ii). Finally, 10 species biofilms also showed no significant differences between 5% CO_2 and anaerobic cultures with total *Streptococcus* species quantified at 4.3 x 10^6 and 1.0 x 10^7; *F. nucleatum* at 1.2 x 10^6 and 1.4 x 10^7; *A. naeslundii* at 1.2 x 10^7 and 2.4 x 10^7; *V. dispar* at 7.0 x 10^6 and 3.2 x 10^6; *P. gingivalis* at 1.6 x 10^4 and 2.6 x 10^4; *P. intermedia* at 3.9 x 10^6 and 2.9 x 10^6 and *A. actinomycetemcomitans* at 4.9 x 10^4 and 1.7 x 10^6 for fresh and frozen biofilms, respectively (Figure 4.9 iii).

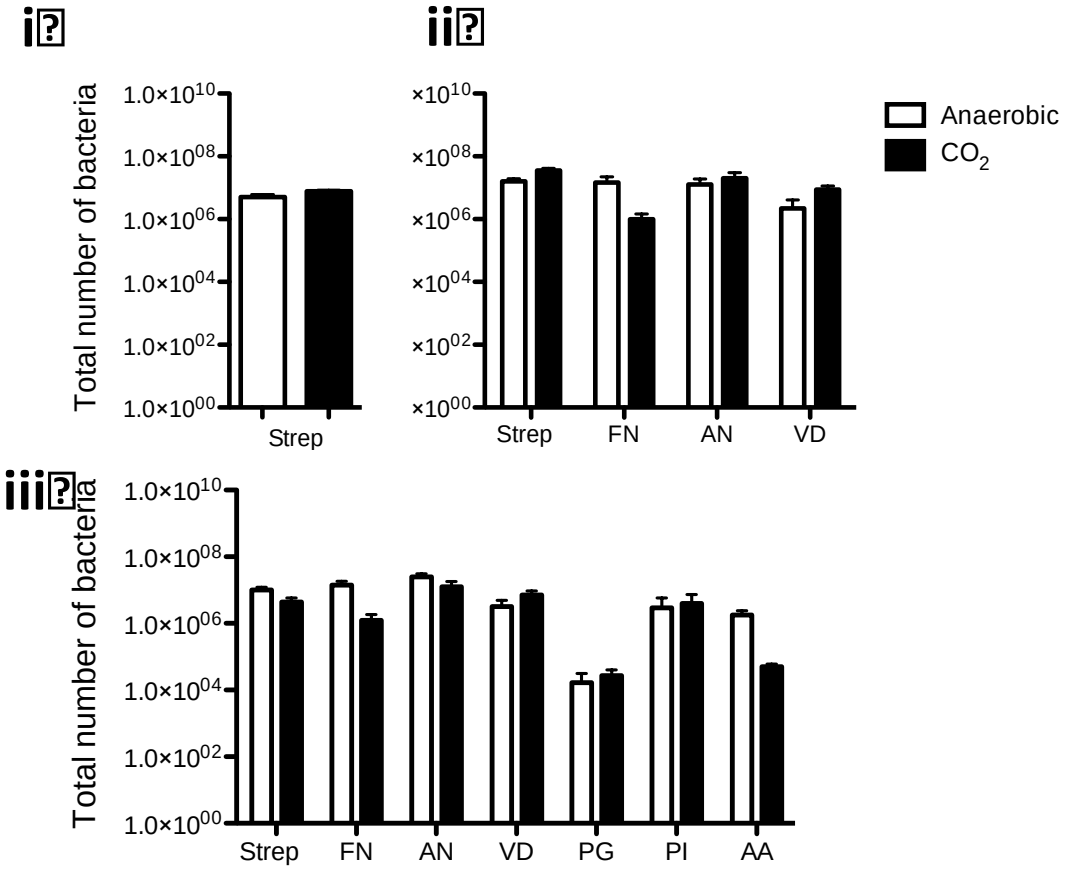


Figure 4.9: Quantification of Biofilms in AS (CO_2)

Three [i], 7 [ii] and 10 [iii] species biofilms were incubated for 24 hours in AS at 37ºC in either anaerobic (□) or 5% CO_2 (■) before sonication to detach the biofilm from the coverslip. Total bacteria DNA was extracted from each biofilm using Masterpure Gram positive DNA extraction kit. The total number of each species was quantified using SYBR® GreenER based qPCR from previously calculated standards of each bacterial species. Data shown are mean ±SD of 3 experiments performed in triplicate. Statistical analysis was performed using a two-tailed unpaired t-test.

The previous data showed that biofilm composition was not affected by culture in 5% CO_2 compared with anaerobic conditions. Next the composition of biofilms was then investigated when cultured in d-KSFM over time a cell culture media used in co-culture experiments (Figure 4.10). Mature 3, 7 and 10 species biofilm cultured in d-KSFM in 5% CO_2 for both 4 and 24 hours showed no significant

difference in composition compared to freshly grown biofilms and no significant difference in biofilm composition between each time point.

Three species biofilms were not significantly different when cultured in d-KSFM between 4 and 24 hours, with total *Streptococcus* species quantified at 1.1 x 10^6 total bacteria at 4 hours and 3.7 x 10^6 bacteria at 24 hours (Figure 4.10 i). Additionally, 7 species biofilms showed no significant differences each time point, with total *Streptococcus* species quantified at 1.0 x 10^8 and 2.8 x 10^7; *F. nucleatum* at 7.2 x 10^7 and 2.6 x 10^6; *A. naeslundii* at 1.3 x 10^7 and 1.5 x 10^7 and *V. dispar* at 6.0 x 10^7 and 5.2 x 10^7 at 4 and 24 hours respectively (Figure 4.10 ii). Finally,10 species biofilms also showed no significant differences between time points with total *Streptococcus* species quantified at 2.3 x 10^7 and 7.2 x 10^7; *F. nucleatum* at 7.2 x 10^6 and 8.2 x 10^7; *A. naeslundii* at 2.4 x 10^7 and 1.3 x 10^7; *V. dispar* at 4.5 x 10^7 and 3.1 x 10^7; *P. gingivalis* at 7.7 x 10^4 and 1.6 x 10^4; *P. intermedia* at 3.6 x 10^6 and 3.7 x 10^5 and *A. actinomycetemcomitans* at 3.5 x 10^5 and 1.6 x 10^5 for fresh and frozen biofilms, respectively (Figure 4.10 iii).

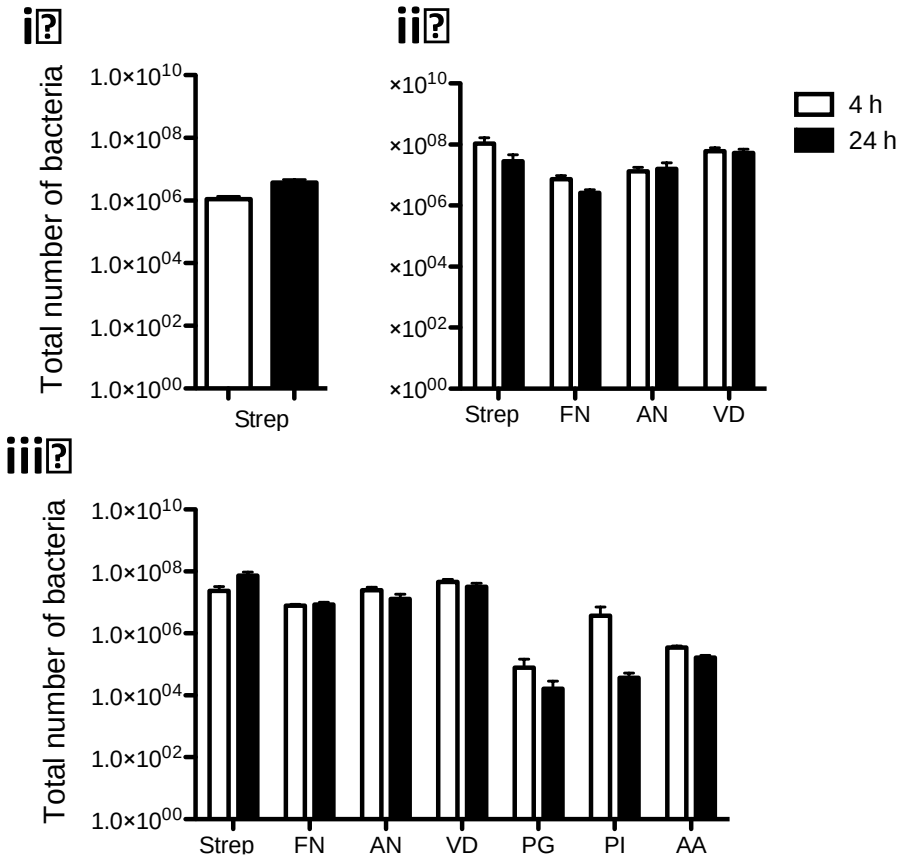


Figure 4.10: Quantification of biofilms grown in d-KSFM

Three [i], 7 [ii] and 10 [iii] species biofilms were cultured for 4 (☐) and 24 (■) hours in d-KSFM in 5% CO2 before biofilms were removed and sonicated. Bacterial DNA was then extracted from each biofilm using the Masterpure gram positive DNA kit. The total number of each species was quantified using SYBR® GreenER based qPCR from previously calculated standards of each bacterial species. Data shown are mean ±SD of 3 independent experiments performed in triplicate Statistical analysis was performed using a two-tailed unpaired t-test.

Next, the composition of the biofilms following experimental use was investigated. Biofilms were co-cultured with oral epithelial cells in d-KSFM at 37ºC in 5% CO_2 for 4 and 24 hours before DNA extraction and bacterial composition quantified (Figure 4.11). As with the previous conditions no significant difference between any of the biofilms compared with freshly grown biofilms was observed. Additionally no significant differences were observed when comparing each bacterial species at 4 and 24 hours.

Three species biofilms were not significantly different when co-cultured with oral epithelial cells between 4 and 24 hours, with total *Streptococcus* species quantified at 1.6 x 10^6 total bacteria at 4 hours and 3.9 x 10^6 bacteria at 24 hours (Figure 4.11 i). Additionally, 7 species biofilms showed no significant differences each time point, with total *Streptococcus* species quantified at 6.2 x 10^7 and 3.5 x 10^7; *F. nucleatum* at 1.3 x 10^7 and 9.9 x 10^6; *A. naeslundii* at 3.7 x 10^7 and 2.3 x 10^7 and *V. dispar* at 2.2 x 10^7 and 8.6 x 10^6 at 4 and 24 hours respectively (Figure 4.11 ii). Finally,10 species biofilms also showed no significant differences between time points with total *Streptococcus* species quantified at 4.0 x 10^7 and 4.3 x 10^6; *F. nucleatum* at 1.9 x 10^7 and 1.2 x 10^6; *A. naeslundii* at 6.9 x 10^7 and 1.2 x 10^7; *V. dispar* at 4.1 x 10^7 and 7.0 x 10^6; *P. gingivalis* at 6.8 x 10^4 and 2.6 x 10^4; *P. intermedia* at 4.6 x 10^6 and 3.9 x 10^5 and *A. actinomycetemcomitans* at 1.3 x 10^5 and 4.9 x 10^4 for fresh and frozen biofilms, respectively (Figure 4.11 iii).

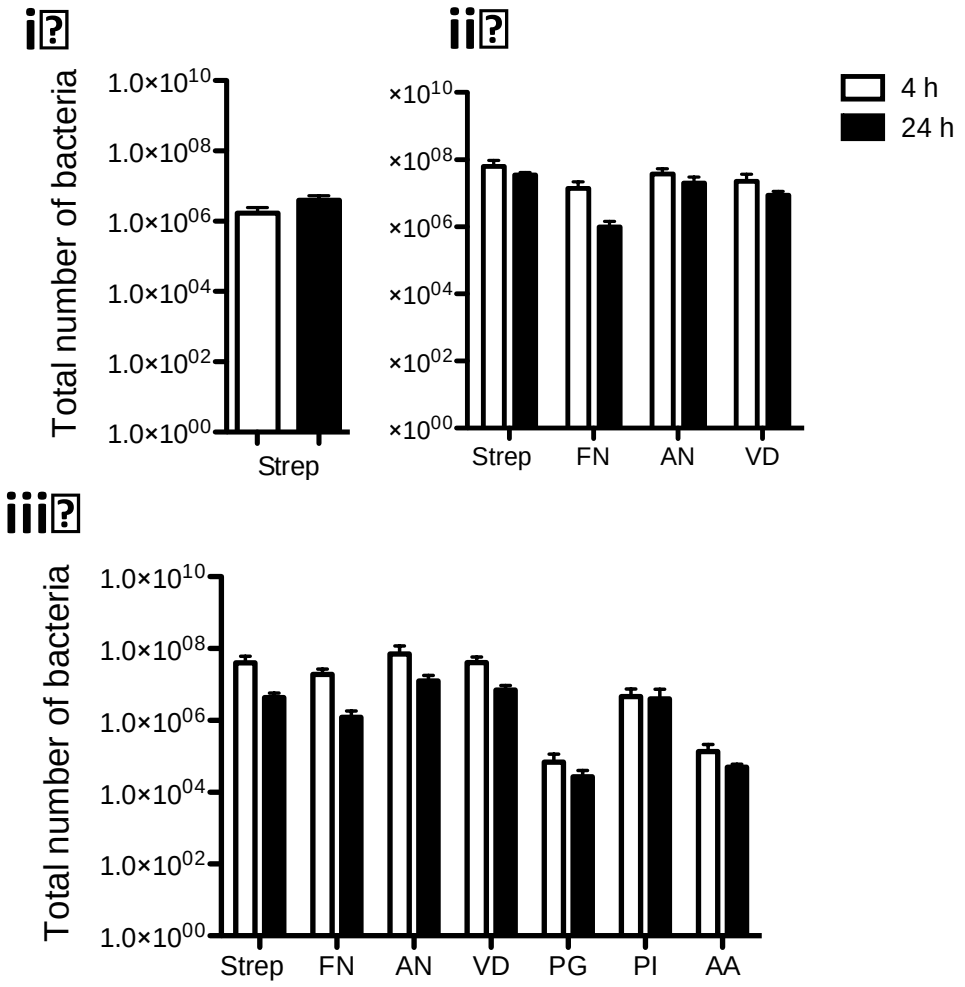


Figure 4.11: Quantification of biofilms grown in co-culture

OKF6-TERT2 oral epithelial cells were grown to confluence and seeded at 1 x 10^5 cells /mL in 24 well plates. Three [i], 7 [ii] and 10 [iii] species biofilms were co-cultured with OKF6-TERT2 cells for both 4 (□) and 24 (■) hours in d-KSFM in 5% CO_2. Following co-culture biofilms were removed and sonicated. Bacterial DNA was then extracted from each biofilm using the Masterpure Gram positive DNA kit. The total number of each species was quantified using SYBR® GreenER based qPCR from previously calculated standards of each bacterial species. Data shown are mean ±SD of 3 independent experiments performed in triplicate. Statistical analysis was performed using a two-tailed unpaired t-test.

Finally, the number of live bacteria from biofilms co-cultured with oral epithelial cells at 4 and 24 hours was quantified using the live SYBR® GreenER based qPCR method (Figure 4.12). No significant differences were observed in the number of live bacteria compared with the total number of bacteria in 3 species biofilms at either time point (Figure 4.12 i-ii). Co-culture of 7 species biofilms with oral epithelial cells resulted in significant differences at 4 hours in live and total numbers of bacteria, with a 1.86 ($p<0.05$) log difference of *A. naeslundii* and 1.55 ($p<0.05$) log difference of *V. dispar* (Figure 4.12 iii). At 24 hours a 2.04 ($p<0.001$) log difference between the live and total number of *Streptococcus* species was observed in 7 species biofilm co-cultures (Figure 4.12 iv). In the 10 species co-cultures with oral epithelial cells *A. naeslundii* showed significant differences between the live and total number of bacteria, with 2.17 ($p<0.05$) and 1.69 ($p<0.05$) log difference at 4 and 24 hours respectively (Figure 4.12 v-vi). Additionally, no significant differences were observed when comparing the number of live bacteria present in fresh biofilms with those which had been co-cultured in d-KSFM 5% CO_2 for 24 hours (Table 4.1).

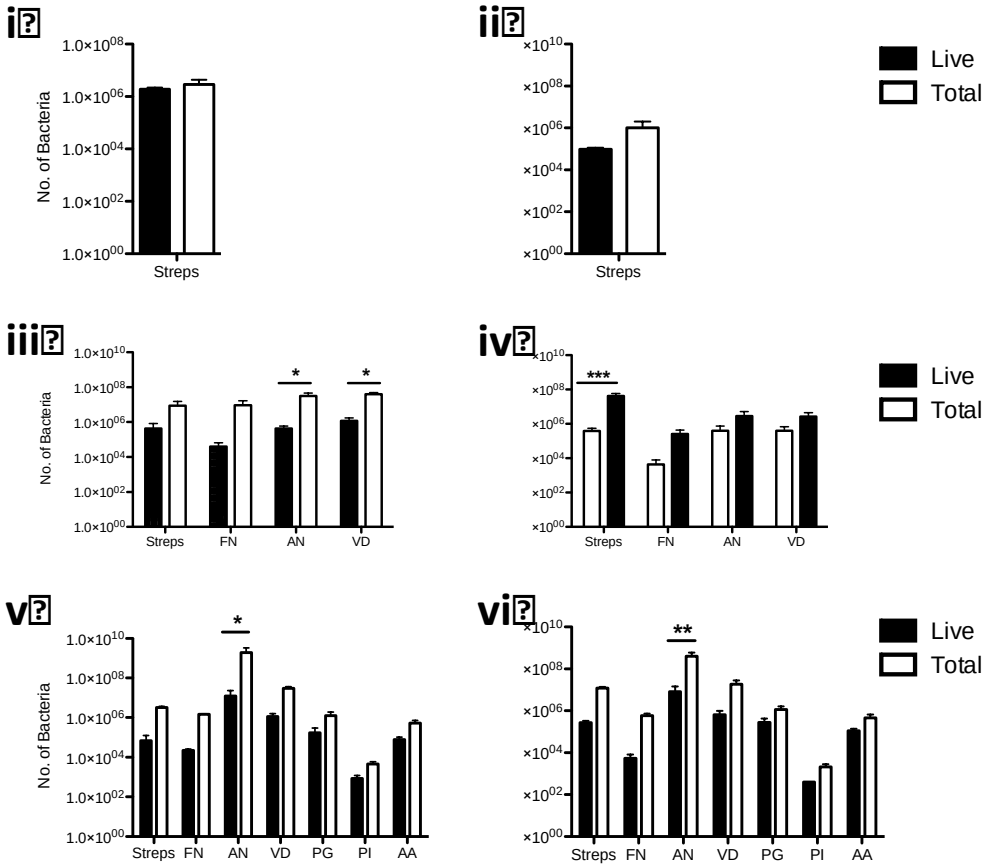


Figure 4.12: Live and total bacteria quantification of biofilms grown in co-culture

Three [I, ii], 7 [iii, iv] and 10 [v, vi] species biofilms were co-cultured with OKF6-TERT2 cells for both 4 [i, iii, v] and 24 [ii, iv, vi] hours in d-KSFM in 5% CO2. Following co-culture biofilms were removed and sonicated. Half the samples were treated with 5 µL of 10 mM of propidium monoazide, incubated for 10 minutes in the dark before a 5 minute exposure to a 650W halogen light to determine the proportion of live bacteria (■). Control biofilms (total □) were not treated with propidium monoazide. The total number of each species was quantified using SYBR® GreenER based qPCR from previously calculated standards of each bacterial species. Data shown are mean ±SD of 3 independent experiments performed in triplicate Statistical analysis was performed using a two-tailed unpaired t test (*p<0.05, ** p<0.01, *** p<0.001).

Table 4.1: Comparison of live bacterial species from fresh biofilms or following 24 hour co-culture with oral epithelial cells

	Fresh	±SD	24 h co-culture	±SD
3 species biofilm				
SM	1.9×10^5	1.2×10^5	9.6×10^4	2.9×10^4
7 species biofilm				
SM	9.5×10^5	1.2×10^5	3.8×10^6	2.7×10^5
FN	5.1×10^3	2.4×10^3	4.3×10^3	5.2×10^3
AN	4.5×10^4	2.7×10^4	4.0×10^5	6.1×10^5
VD	3.0×10^5	1.1×10^5	4.0×10^5	4.8×10^5
10 species biofilm				
SM	9.6×10^5	5.8×10^5	2.7×10^5	1.0×10^5
FN	7.2×10^3	5.4×10^3	5.3×10^3	4.9×10^3
AN	5.2×10^4	3.1×10^4	8.1×10^6	1.1×10^6
VD	3.6×10^5	1.8×10^5	6.5×10^5	5.8×10^5
PG	8.7×10^2	6.1×10^2	2.8×10^4	2.5×10^5
PI	4.6×10^2	1.4×10^2	4.6×10^2	1.9×10^2
AA	5.0×10^6	3.1×10^6	1.1×10^5	5.0×10^4

4.4 Discussion

Bacteria exist in the oral cavity as multi-species biofilms, where compositional changes of specific bacteria can alter the dynamics of the micro-community, classifying biofilms as health-associated or disease-associated. In this chapter three multi-species biofilm models have been developed and validated for further downstream analyses.

In this study, each of the biofilm models differed both in composition and architecture when analysed by SEM. The three species biofilm containing *S. mitis*, *S. intermedius* and *S. oralis* biofilms, while less complex than the other models due to less bacterial species, successfully formed biofilms that were evenly distributed over the surface. This correlated with other studies using *Streptococcus* species, where biofilms were analysed by SEM showing these species forming a confluent biofilm across the entire surface of the various substrates used (Loo et al., 2000, Lonn-Stensrud et al., 2007). Oral streptococci make up 80% of early biofilm species and play an important role in facilitating the addition of other bacterial species to the biofilm (Kreth et al., 2009). Streptococcal species also compete with each other for binding sites on the surface, which in turn can shape the spatial and temporal composition of developing oral biofilms (Nobbs et al., 2007).

The 7 species biofilm model builds on this to allow for the inclusion of *F. nucleatum, F. nucleatum* ssp. *vincentii, A naeslundii* and *V. dispar* to create a more complex biofilm, which while itself is not considered pathogenic *per se*, contains bacterial species which facilitate the addition of anaerobes typically associated with periodontitis. In particular, *F. nucleatum* species are important colonizers and bridging organisms, supporting the inclusion of disease-associated anaerobes to the biofilms. *F. nucleatum* can be present in low numbers in periodontal health; however, are found to increase and are considered a risk factor of PD(Alpagot et al., 1996, Stingu et al., 2012). Due to their ability to co-aggregate with a large number of both aerobes and anaerobes and alter the local environment, *F. nucleatum* are considered to play a key role in the transition of health to disease in oral biofilms (Diaz et al., 2002, Zilm and Rogers, 2007,

Kolenbrander et al., 2002). Additionally, when viewed by SEM the 7 species biofilms showed visible co-aggregation of bacterial species that formed micro-colonies across the surface of the biofilm. This increase in complexity and distribution of bacteria is typically associated with complex biofilm models (Inaba et al., 2013, Blanc et al., 2014).

The final 10 species biofilm model builds on the previous 7 species model, with the addition of the disease-associated bacteria *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* (Feng et al., 2014). Part of Sokransky's 'red complex', these species were chosen due to their strong association with PD (Socransky et al., 1998). Furthermore, these species have been shown to alter the host immune response, which in turn can disrupt homeostasis and contribute to immune deregulation and disease (Palm et al., 2013, Garlet et al., 2006). The SEM analysis of this model showed a far more complex biofilm compared to that of the 7 species biofilm, with the micro colonies of bacteria merging across the entire surface to form a topographically heterogeneous biofilm. This increase in biomass may relate to finding by Hajishengallis *et al* (2011), where the addition of *P. gingivalis* in mice resulted in a significant increase in total bacterial load (Hajishengallis et al., 2011). Additionally, 454-sequencing studies have reported an increase in biomass of periodontal plaque compared to healthy plaque in human subjects and implicated PD-associated bacteria in this process (Abusleme et al., 2013).

Investigating the development of the 10 species biofilm also supported the findings that biofilm attachment of bacterial species occurs in a defined sequential manner (Marsh, 2004). The formation of these biofilms requires initial adherence to oral tissues and teeth, species specific co-aggregation and local environmental changes to allow the biofilm to develop (Kolenbrander et al., 2010a). Previous studies have reported in the absence of early colonizers such as *S. oralis* and *S. anginosus* overall biofilm structure was looser and more dispersed, combined with a significant shift in the relative loads of each bacteria species, with significant decreases in *P. intermedia* and *P. gingivalis* (Ammann et al., 2013a). A study by Periasamy *et. al.* (2009) reported the inability of *F. nucleatum* to form a biofilm with *S. oralis* in the absence of *A. naeslundii*,

highlighting the importance species-specific interactions for biofilm formation (Periasamy et al., 2009).

In this study biofilms were quantified using CFU counts, quantitative PCR, including live vs. dead analysis and SEM. Enumeration of bacteria by CFU is a relatively cheap and quick method which allows for viable cell quantification on species specific agar plates. However, chains or aggregates of bacteria can be viewed as a single colony and species-specific plates can also cause lower counts of the selected bacteria due to the suppressive growth of unwanted species. As a result more recent studies focus on molecular methods to quantify biofilm composition. Quantitative PCR allows for the total DNA of each species to be quantified and enumerated on a standard curve and has been found to correlate well with CFU counts when comparing freshly grown biofilms (Boutaga et al., 2005). However, as the number of dead cells in a biofilm increases, studies have shown results from each method become increasingly different, as qPCR quantifies both live and dead cells and CFU counts viable cells only (Ammann et al., 2013b). To discriminate between live and dead cells by qPCR a recent method using propidium monoazide has been proposed. This method involves the pre-treatment of samples with propidium monoazide which penetrates the membrane of dead cells and binds to double stranded DNA. When photo activated using a bright light the bound propidium monoazide forms a stable covalent bond which prevents DNA amplification of dead cells. This technique has been used to successfully quantify the relative viability of each species in both planktonic cultures and oral biofilms (Alvarez et al., 2013, Loozen et al., 2011). The use of live/dead qPCR is especially useful for studying viability in multi-species biofilms which are cultured for long periods of time and therefore species survival is hard to determine. Studies have also shown that host cells respond differently to live bacteria compared with dead bacteria in co-culture therefore it was important to ensure the biofilms developed in this chapter were alive for co-culture studies and antimicrobial susceptibility testing (Jose, 2013).

These methods are frequently being employed alongside microscopy to gain a further understanding of biofilm formation and bacterial co-aggregation. In this study biofilms were analysed visually by SEM, a method frequently employed to

visualise biofilm architecture. However, there are limitations to using this method for biofilm studies including alteration in biofilm morphology due to the post-processing dehydration of samples and difficulty distinguishing specific bacterial species or viability state based on morphology alone. An alternative to this would be confocal laser scanning microscopy (CLSM), a non-invasive method which has been used successfully to visualise the distribution of live and dead bacteria in oral multi-species biofilm models (Sanchez et al., 2011, Guggenheim et al., 2001a). Furthermore, CLSM can be combined with multiplex fluorescent *in situ* hybridization (M-FISH) to evaluate the spatial distribution of specific bacterial species within the biofilm without disruption of the biofilm structure (Thurnheer et al., 2004, Karygianni et al., 2014).

Overall, a number of groups have developed *in vitro* multi-species oral biofilms to study spatial distribution, biofilm architecture and potential antimicrobial compounds (Shapiro et al., 2002, Foster and Kolenbrander, 2004, Ammann et al., 2012, Guggenheim et al., 2001a). However, the explicit purpose of this study was to investigate host-pathogen interactions using three different biofilm models. Therefore it was important to evaluate how each biofilm responded to cell culture conditions including 5% CO_2 and cell culture media over a 24 hour period. Interestingly, none of the biofilms showed any significant changes in composition in any of the cell culture variables tested when compared with freshly grown biofilms. This was the same for the total number of live cells following 24 hour co-culture in 5% CO_2 in d-KSFM with oral epithelial cells compared with fresh biofilms. It has been shown that oral biofilms provide microenvironments for bacteria, which allow them to survive and make them less susceptible to antimicrobial compounds and environmental changes (Periasamy and Kolenbrander, 2009, Park et al., 2014). In particular Diaz *et. al.* (2002) reported the ability of *P. gingivalis*, a strict anaerobe, to survive aerobic conditions when in a biofilm containing *F. nucleatum* (Diaz et al., 2002). Additionally, the multi-species biofilms in this study survive freeze-thawing with no apparent effect on the composition, allowing biofilms to be grown in batches, improving the process of culturing and reducing variation between single biofilms. Guggenheim *et. al.* (2009) also froze their 9 species subgingival biofilm model at -80^{o} C following culture; however, it was not reported if this method

significantly alters the biofilm composition (Guggenheim et al., 2001a). Many groups have investigated host-pathogen interactions using multi-species oral biofilms; however none have reported previous testing validating the model for use in co-culture with host cells (Belibasakis et al., 2011a, Peyyala et al., 2013). Guggenheim *et. al.* (2009) investigated the composition of multi-species biofilms during growth and reported qualitative differences compared to biofilms grown in saliva and serum (Guggenheim et al., 2009). The group also studied biofilm composition following culture in KSFM in an oxygen containing atmosphere and reported that although variations were observed in the biofilm the number of anaerobic bacteria which survived in this environment was comparable to pre exposure levels, which is similar to the data found in this chapter.

In conclusion, this component of the thesis has developed three *in vitro* multi-species biofilm models representing the transition from health to disease in the oral cavity, and investigated the composition of these mature biofilms in culture conditions for further use in co-culture studies with different host cell types.

CHAPTER FINDINGS

Three multi-species biofilm models have been developed to reflect health, the transition from health to disease, and disease plaque in the oral cavity.

Each biofilm can be frozen for later use and thawed without significantly affecting the composition.

Biofilm composition is not affected by changes to culture conditions, including 5% CO_2, d-KSFM and co-culture with oral epithelial cells over time.

5 The inflammatory response of oral epithelial cells to multi-species biofilms *in vitro*

5.1 Introduction

The gingival sulcus is lined by a non-keratinized, stratified squamous epithelium which includes both the sulcular epithelium and junctional epithelium and surrounds the tooth structure in the oral cavity (Figure 5.1). At this site epithelial cells are in constant contact with bacteria and their products and are integral to maintaining oral health and immune homeostasis (Dale, 2002). These cells provide the first line of defence against invasion of oral microorganisms. The epithelium provides a physical barrier, as well as playing an active role in innate host defence by releasing soluble mediators such as cytokines and antimicrobial peptides (Hans and Madaan Hans, 2014). In the absence of meticulous oral hygiene, bacterial biofilms accumulate at the gingival margin and cause gingivitis; however, only in a proportion of individuals does this reversible inflammation progress to PD (Loe et al., 1986). This implicates not only the bacteria within the biofilm, but also the host immune response in disease pathogenesis. Therefore, investigating the interactions between oral bacteria and the host immune system is paramount to understanding the aetiology of PD.

Until recently, many *in vitro* studies investigating the host-pathogen relationship in the oral cavity focused on using bacteria derived soluble or secreted molecules, such as LPS or proteases, or planktonic single species co-cultured with human primary cells or cell lines. These reports identified the specific role of molecules, receptors and ligands as well as response patterns to specific bacteria (Madianos et al., 2005). Studies investigating human gingival epithelial cells (HGEC) challenged with live or heat killed planktonic bacteria, demonstrated minimal release of cytokines, such as IL-6, IL-8 and IL-1ß, in response to early colonizers such as *S. gordonii*. However, cytokine release was significantly elevated in response to disease-associated species, such as *F. nucleatum* (Hasegawa et al., 2007, Stathopoulou et al., 2010). Co-culture studies using planktonic *P. gingivalis* and epithelial cells report the ability of *P. gingivalis* to degrade cytokines and invade host cells (Kinane et al., 2012, Stathopoulou et al., 2009). While useful for preliminary studies, the use of planktonic bacteria or bacterial components is far removed from the conditions

in vivo where bacteria exist as complex multi-species biofilms (Costerton et al., 1987).

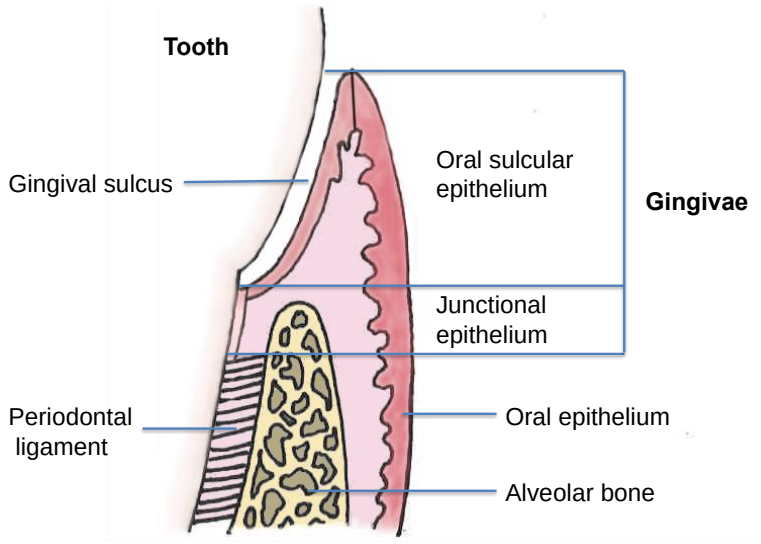


Figure 5.1: Anatomy of the gingival sulcus and surrounding tissues
Gingival epithelium surrounds the teeth in the oral cavity and is made up of three distinct sections. The **oral epithelium** is a keratinised, squamous epithelium, which forms the external surface of the oral mucosa and extends from the mucogingival junction to the gingival margin. The **oral sulcular epithelium** is a non-keratinised, squamous epithelium that lines the surface of the gingival sulcus. The **junctional epithelium** forms at the end of the gingival sulcus and attaches to the tooth enamel of the tooth and connective tissue of the periodontal ligament. Illustrated by Emma Millhouse.

There are few studies investigating the epithelial cell response to multi-species oral biofilms *in vitro*. *Guggenheim* et al. (2009) developed a nine species subgingival biofilm model, the 'Zurich biofilm model,' which was co-cultured with primary HGECs from periodontally healthy patients (Guggenheim et al., 2009). In co-culture with the biofilm, HGECs produced significantly increased levels of IL-8 and IL-6 protein and significant apoptosis was observed, with up to 85% cells showing signs of apoptosis at 24 hours compared with 0% in the unchallenged cell only control. Studies by Peyyala *et al* in 2012 and 2013 investigated the response of the immortalized epithelial cell line OKF4 to single species planktonic or biofilm cultures of 6 orally relevant bacteria, and three multi-species biofilms mimicking health and disease (Peyyala et al., 2013,

Peyyala et al., 2012). These studies demonstrated a clear hierarchy in the bacterial induction of inflammatory cytokines, with multispecies biofilms stimulating the greatest cytokine release, followed by mono-species biofilms, followed by planktonic bacteria. However, *P. gingivalis* abrogated inflammatory cytokine concentrations, even within multi-species biofilm containing species such as *F. nucleatum*.

These studies suggest that the epithelial cell response to oral bacteria is influenced by the state and composition of bacteria present in co-culture. The main question which remains to be answered is the extent to which multi-species biofilm composition can modulate host cell responses which may in turn contribute to PD pathology. The studies by Peyyala *et al* in 2013 and 2014 are the only reported work to investigate the role biofilm composition has on epithelial cell inflammatory protein release in co-culture at the time of writing. However, these studies only use 3 species within each of their biofilms and only investigate protein release. To further explore the role of biofilm composition on epithelial cells inflammatory responses, this chapter investigates changes in oral epithelial cell inflammatory gene and protein expression in response to the three compositionally different multi-species biofilm models developed in the previous chapter.

5.2 Aims

The aim of this study was to investigate the inflammatory response of oral epithelial cells (OKF6-TERT2 epithelial cells and primary epithelial cells) co-cultured with the 'health-associated' 3 species, 'intermediate' 7 species and 'disease-associated' 10 species biofilms.

The study sought to define the inflammatory response of the epithelial cells by assessing cytokine mRNA gene expression and protein release after 4 and 24 hours of co-culture with biofilms, and to determine differences between the responses to each biofilm co-culture; and the response to the biofilms cultures compared with cells cultured with media only ('cells only control').

5.3 Results

To investigate the effect of multi-species biofilms on epithelial cells the immortalized oral epithelial cell line OKF6-TERT2, derived from human oral mucosal keratinocytes was chosen. This cell line was selected as it resembles primary oral keratinocytes in studies of cytokine expression and cytotoxicity (Dongari-Bagtzoglou and Kashleva, 2003). Furthermore, this particular cell line has been used extensively in oral biology studies (Dickson et al., 2000, Wang et al., 2013, Volk et al., 2012).

5.3.1 Epithelial cell protein release is not influenced by orientation of the biofilms in the co-culture model

The epithelial cell: multi-species biofilm co-culture model used in this study involves hanging the biofilm over the monolayer of cells using a Millicell® cell culture insert as described in section 2.2.3. In the oral cavity biofilms grow on the surface of the tooth parallel to the gingivae and while some bacteria may detach from the biofilm surface *in vivo,* and they are under some small pressure from the flow of GCF, they are presumably not exposed to the same constant gravitational forces as the *in vitro* model. Studies have shown the presence of both supragingival and subgingival bacterial species in GCF, which correlates with the bacterial diversity of the biofilm and probability of disease at the sampling site (Asikainen et al., 2010). Therefore, it was important to investigate to what extent the epithelial cells were responding to bacteria that simply dropped off the biofilm in the inverted model system.

To test this, the model was inverted and OKF6-TERT2 cells were grown on Thermanox® coverslips, attached to the Millicell® cell culture inserts, added to 24 well plates where the multi-species biofilms were grown on Thermanox® coverslips on the bottom and cultured in 5% CO_2 as usual as described in section 2.2.4. The release of IL-8 protein following co-culture for 4 and 24 hours was compared to the original model (Figure 5.2). There were no significant differences between the inverted and normal models when measuring the epithelial cell IL-8 protein released following co-culture with each biofilm, suggesting that the responses observed in this chapter in co-culture models are not due simply to bacteria dropping off the biofilms.

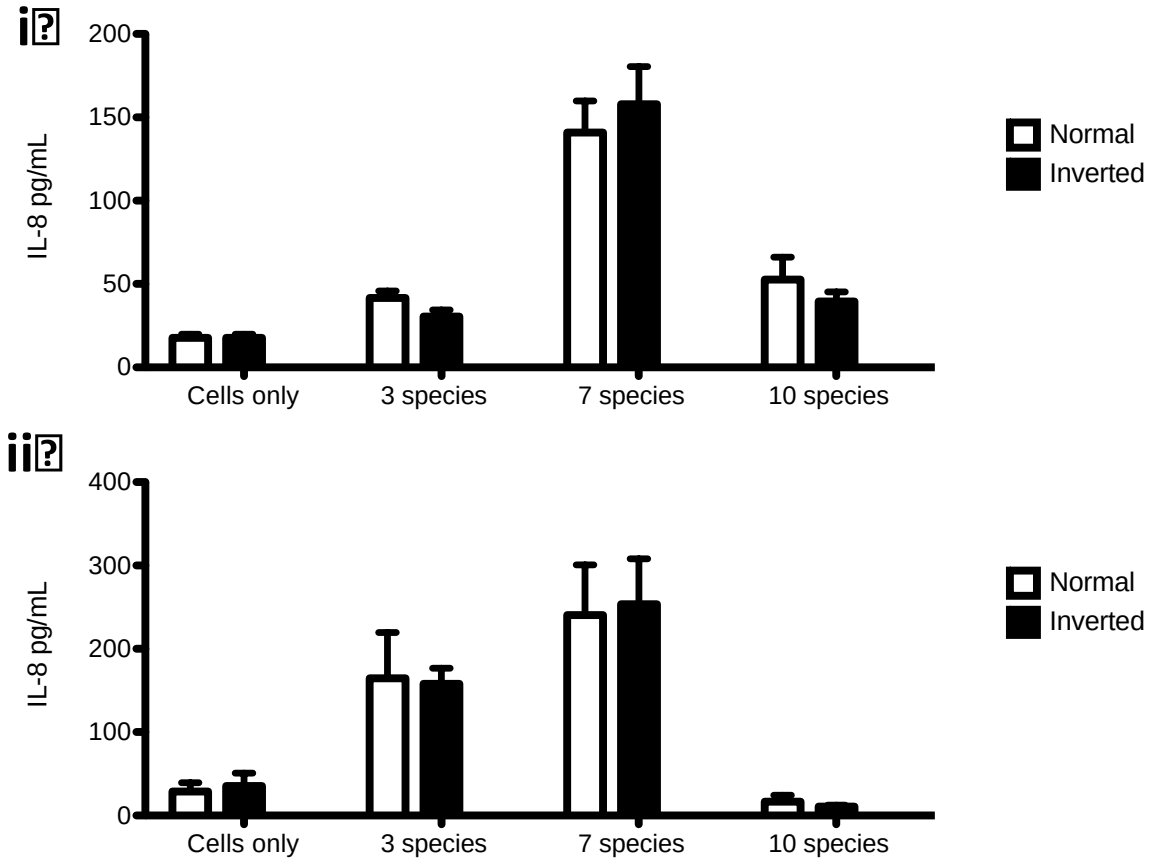


Figure 5.2: Epithelial cell IL-8 protein release in biofilm co-culture

Three, 7 and 10 species biofilms were co-cultured with epithelial cells for 4 (i) and 24 (ii) hours in 5% CO_2. For inverted co-culture culture, OKF6-TERT2 oral epithelial cells were grown on Thermanox® coverslips, inverted on Millicell® inserts and co-cultured with 3, 7 and 10 species biofilms which were grown directly in 24 well plates. Epithelial cells incubated for 4 and 24 hours without a biofilm were used as a cells only control. Following co-culture, supernatants were removed and IL-8 protein measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD. Statistical analysis was performed using a paired Students t-test to compare inverted vs. normal co-cultures each at each time point. No statistical significance was observed.

5.3.2 Multi-species biofilms are increasingly cytotoxic to epithelial cells over time

Prior to investigating the responses of oral epithelial cells to biofilms, the viability of the host cells following co-culture was determined (Figure 5.3). Epithelial cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours and cell viability quantified using AlamarBlue®. The percentage reduction of the AlamarBlue® compound was calculated according the manufacturers instructions using media only, without any cells, as a 0% baseline. Cells cultured in media only were used as a cells only control and used to compare cell viability in co-culture with biofilms at each time point. At 4 hours, the viability of the cells only control was 77.6%. Co-culture with the 10 species biofilm resulted in a significant decrease in epithelial cell viability to 53.2% ($p<0.05$). No significant differences in cell viability were observed compared to the cells only control in co-cultures containing 3 or 7 species biofilms at 4 hours, with observed viability of 78.1% and 62.2%, respectively. Following 24 hour culture the viability of the cells only control was 60%. At this time point co-cultures with any of the multi-species biofilms resulted in significant decreases in cell viability compared to the control, with cell viability observed at 29.4% ($p<0.05$), 25.1% ($p<0.01$) and 18.7% ($p<0.001$) when cultured with 3, 7 and 10 species biofilms, respectively.

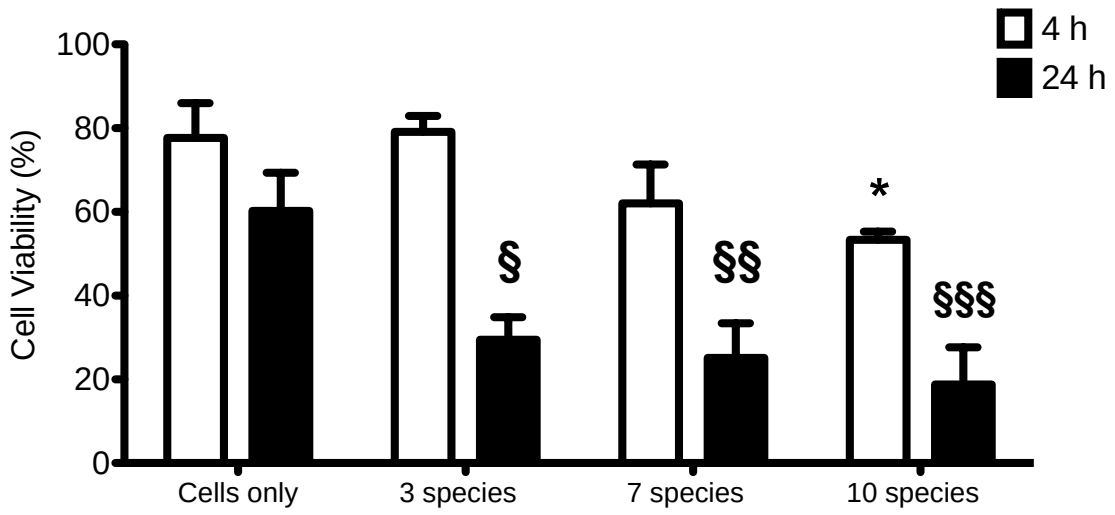


Figure 5.3: Epithelial cell viability following co-culture

Three, 7 and 10 species biofilms were co-cultured with OKF6-TERT2 epithelial cells for 4 (white bars) or 24 hours (black bars), and cell viability measured by addition of AlamarBlue® for the final 4 hours of culture. Epithelial cells incubated for 4 and 24 hours without a biofilm were used as a cells only control. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups with the cells only control at each time point (4 hours - * $p<0.05$) (24 hours - § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$).

To further investigate the nature of the observed reduction in epithelial cell viability, apoptosis was assessed by caspase-3 ELISA (Figure 5.4). Caspase-3 is part of the cysteine-dependent aspartate-specific protease family, which plays a central role in the apoptotic signalling network of cells and is activated during cell apoptosis. The active caspase-3 ELISA is an antibody based detection assay that detects the active heterodimer form of caspase-3, which is cleaved at Asp175/Ser176 during apoptosis. Oral epithelial cells were cultured with 3, 7 and 10 species biofilms for 4 and 24 hours. Cells cultured in media only (cells only) or cells treated for 30 minutes with 0.2% v/v chlorhexidine (CHX), a compound highly toxic to cell lines, were used as controls. Following 4 hour culture, the cells only control contained 0.02 ng/mL active caspase-3, compared with cells treated with CHX which contained 0.29 ng/mL ($p<0.001$). Cells co-cultured with

10 species biofilms contained 0.26 ng/mL ($p<0.01$) active caspase-3. Co-cultures containing 3 and 7 species biofilms showed no significant difference compared to the cells only control. After 4 hours, only the 10 species biofilm caused significant release of caspase-3. At 24 hours the cells only control contained 0.02 ng/mL caspase-3 compared with the CHX treated cells, which released 0.87 ng/mL ($p<0.001$). Co-cultures with 7 and 10 species biofilms showed elevated caspase concentrations in the supernatant compared with the cells only control, releasing 0.15 ng/mL ($p<0.05$) and 0.86 ng/mL ($p<0.001$) respectively. Caspase 3 release in co-culture with 3 species biofilms remained similar to the cells only control. This data shows that biofilms cause significant cell death over time; however, the composition of the biofilm appears to be an indicator of the time it will take for significant cell death to be reached, with disease-associated biofilms causing significant decreases in cell viability earlier than other multi-species biofilms.

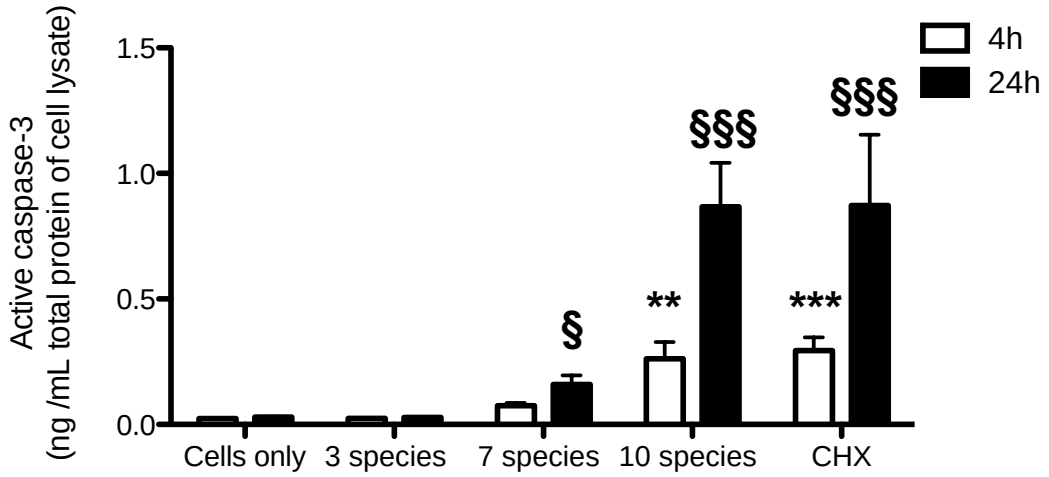


Figure 5.4: Active caspase-3 release from epithelial cells following co-culture
Three, 7 and 10 species biofilms were co-cultured with OKF6-TERT2 epithelial cells for 4 (white bars) and 24 (black bars) hours in 5% CO_2. Epithelial cells incubated without a biofilm were used as a cells only control and cells pre-treated with 0.2% v/v CHX for 30 minutes used as a positive control. Apoptosis was measured using an active caspase-3 ELISA. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups with the cells only control at each time point (4 hours - * $p<0.05$) (24 hours - § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$).

5.3.3 Multi-species biofilms differentially modulate the inflammatory gene and protein response of oral epithelial cells in co-culture

To assess the response of the epithelial cells to the biofilms, gene expression was measured in oral epithelial cells co-cultured with the oral biofilms. First, epithelial cell IL-8 mRNA expression, normalised to the housekeeping gene *GAPDH*, was measured. Cells cultured in media only were used as a cells only control and compared with cells in co-culture with biofilms at each time point. Co-cultures with biofilms were compared against each other at each time point. The data are expressed as percentage IL-8 expression relative to the housekeeping gene *GAPDH* to allow for comparisons between the cells only controls and multi-species biofilms at both time points. At 4 hours IL-8 gene expression was 0.2% in the cells only control; 11.1% in the 3 species co-culture; 118.4% in the 7 species co-culture and 59.7% in the 10 species co-culture. Thus, 4 hours after exposure to each of the biofilms, there was significant increases in IL-8 gene expression in 7 species co-cultures compared with the cells only control ($p<0.001$) and 3 species biofilm ($p<0.01$) (Figure 5.5). At 24 hours IL-8 gene expression was 0.91% in the cells only control; 16.1% in the 3 species co-culture; 47.1% in the 7 species co-culture, and 28.5% in the 10 species co-culture. IL-8 gene expression from 7 species biofilm co-cultures was significantly higher than both the cells only control ($p<0.001$) and 3 species co-culture ($p<0.01$). Co-cultures with 10 species biofilm also demonstrated significantly increased levels of IL-8 gene expression compared with the cells only control ($p<0.05$) (Figure 5.5).

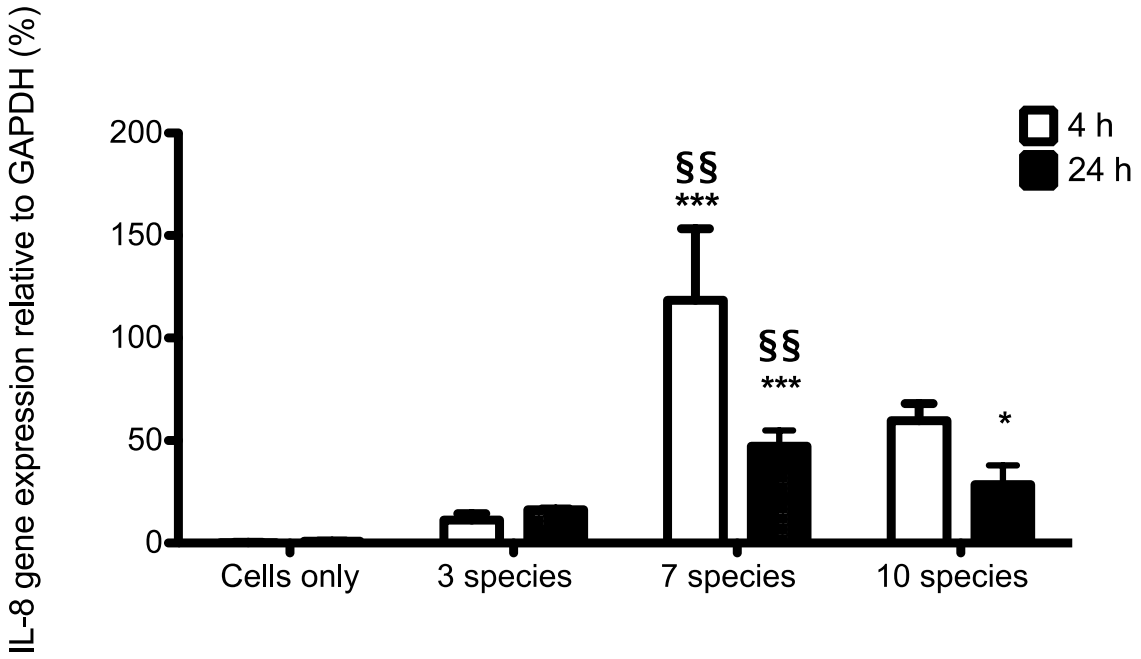


Figure 5.5: Epithelial cell IL-8 gene expression in multi-species biofilm co-culture

Three, 7 and 10 species biofilms were co-cultured with OKF6-TERT2 epithelial cells for 4 (white bars) and 24 (black bars) hours in 5% CO_2. Epithelial cells incubated without a biofilm were used as a cells only control. Following co-culture IL-8 mRNA expression was measured by qPCR. Experiments were carried out with each condition in triplicate, on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups at each time point (Comparison with cells only control * $p<0.05$, *** $p<0.001$; comparison with 3 species biofilm §§¤$p<0.01$).

To investigate whether other genes associated with the inflammatory response of oral epithelial cells were modulated by co-culture with multi-species oral biofilms, a multiplex qPCR platform was used to evaluate 11 different pro-inflammatory genes simultaneously, and the data used to determine gene expression relative to the housekeeping gene *GAPDH,* which was then expressed relative to the cells only control, which contained no biofilm (Figure 5.6). The genes selected for use in the multiplex assay were based on previous work

conducted on epithelial cell responses to 4 species oral biofilms in the Glasgow Dental School.

At 4 hours, co-culture with 3, 7 and 10 species biofilm induced increased gene expression of all genes measured relative to the cells only control (Figure 5.6 i). Notably, there was a 37.4 fold increase in IL-8 gene expression by epithelial cells co-cultured with 3 species biofilms; 302.7 ($p<0.05$) fold increase in IL-8 gene expression in 7 species co-culture and 167.3 ($p<0.05$) fold increase in IL-8 gene expression in 10 species co-culture. Hence, the 7 and 10 species biofilms induced significantly more IL-8 gene expression than the 3 species biofilm. Additionally, co-culture of epithelial cells with 7 species biofilms resulted in significantly higher levels of CXCL1 ($p<0.05$) and CSF2 ($p<0.01$) epithelial gene expression compared with 3 species co-cultures. At 24 hours, co-culture with 3, 7 an 10 species biofilms caused an increase in gene expression of all genes measured relative to the cells only control with the exception of CXCL5. In co-culture with 3 species biofilms there was an apparent down regulation of CXCL5 gene expression relative to the cells only control. At this time point no significant differences in gene expression were observed between 3, 7 and 10 species biofilm co-cultures (Figure 5.6 ii).

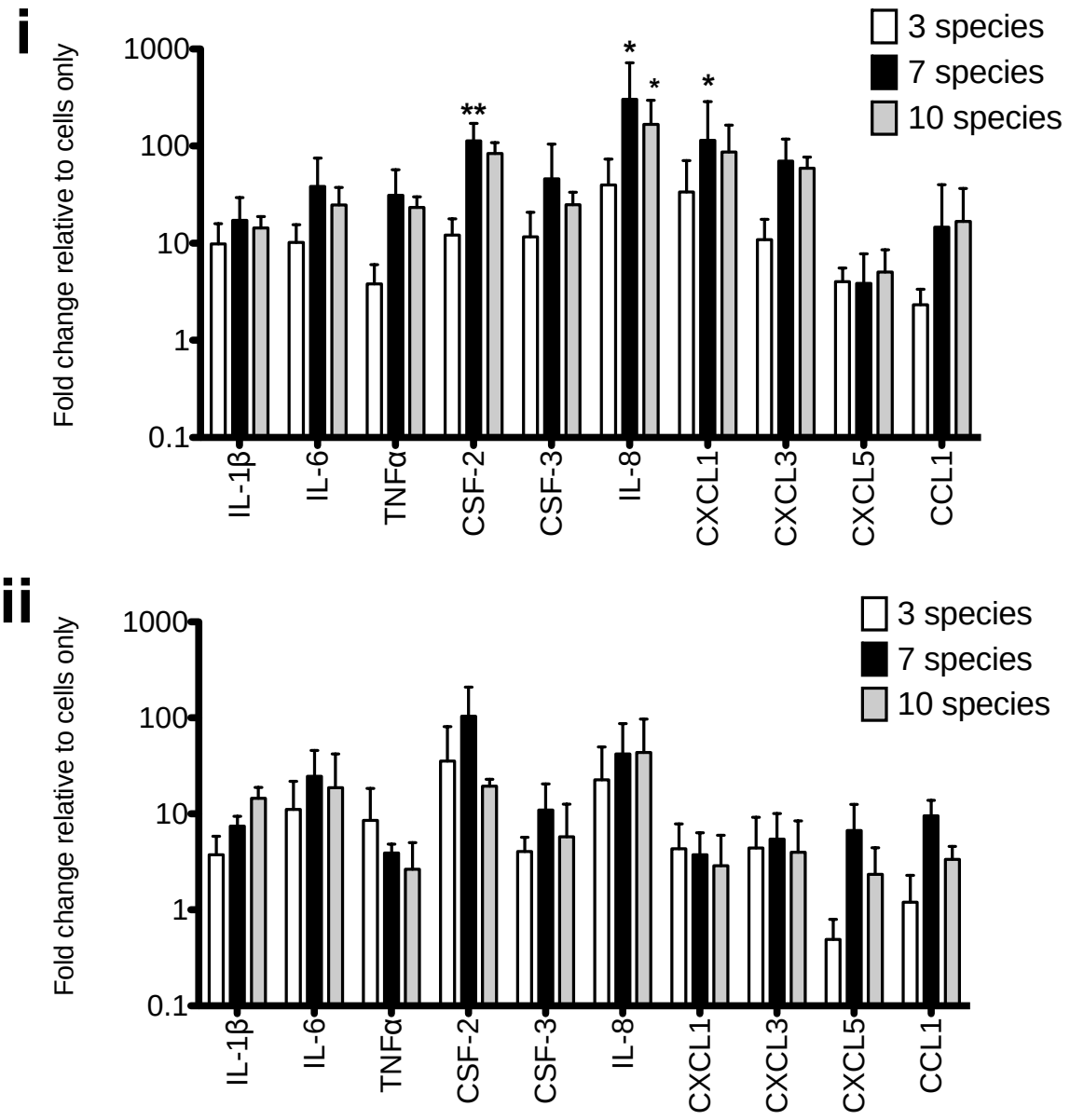


Figure 5.6: Epithelial cell pro-inflammatory gene expression in biofilm co-culture

Three, 7 and 10 species biofilms were co-cultured with epithelial cells for 4 (i) and 24 (ii) hours in 5% CO_2. Epithelial cells incubated without a biofilm were used as a cells only control. Gene expression was assessed my multiplex qPCR. All groups were assayed in duplicate on three separate occasions. Data presented are normalised to the housekeeping gene *GAPDH* and relative to the cells only control. Data represents mean ± SD fold change, relative to the cells only control. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test to compare all groups at each time point (Comparison 3 species biofilm * $p<0.05$, ** $p<0.01$).

The previous data showed that co-cultures of epithelial cells with biofilms differentially altered gene expression. For this reason protein release was examined. Initially, IL-8 protein in culture supernatants following co-culture was measured (Figure 5.7). Epithelial cells incubated without a biofilm were used as a cells only control. At 4 hours, 104.1 pg/mL of IL-8 was present in the cells only control supernatant with significant increases in IL-8 protein release from epithelial cells co-cultured with 7 and 10 species biofilms, containing 875.7 pg/mL ($p<0.05$) and 1052 pg/mL ($p<0.001$), respectively. At 24 hours, 145.1 pg/mL IL-8 protein was present in the cells only control supernatants and IL-8 protein release in co-cultures was similar to the cells only control at this time point.

Due to the apparent differences between IL-8 protein release at 4 and 24 hours a Bonferroni post-test was used to compared each co-culture over time. Notably, there was significantly less IL-8 in the supernatant of co-cultures with 10 species biofilms at 24 hours compared with 4 hours ($p<0.001$).

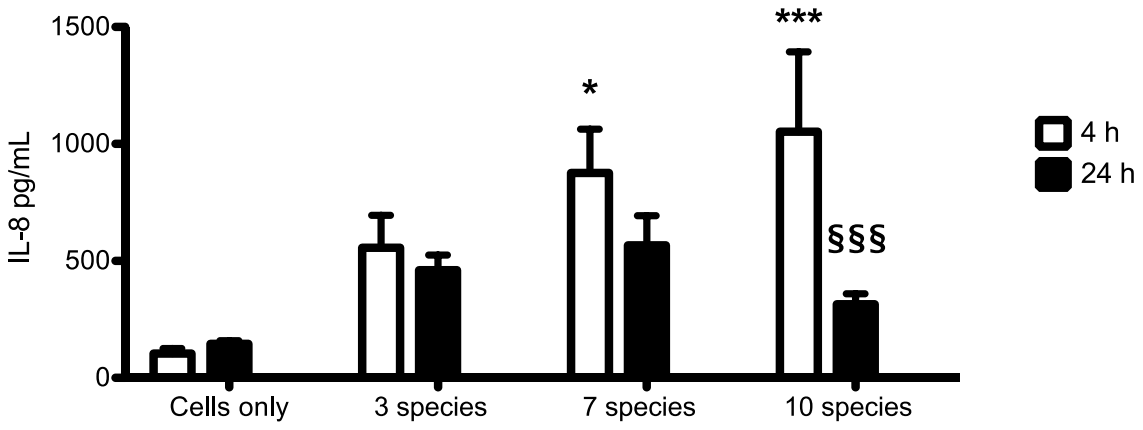


Figure 5.7: Epithelial cell IL-8 protein release in biofilm co-culture

Three, 7 and 10 species biofilms were co-cultured with epithelial cells for 4 and 24 hours in 5% CO_2. Epithelial cells incubated without a biofilm were used as a cells only control. IL-8 protein in co-culture supernatants was measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a two-way ANOVA with Bonferroni's multiple comparison post-test to compare all groups (Comparison to cells only * $p<0.05$, ** $p<0.01$)(4 hours vs. 24 hours §§§ $p<0.001$).

As with gene expression, protein expression was further examined by analysing a panel of cytokines following co-culture at both 4 and 24 hours (Figure 5.8). Epithelial cells incubated without a biofilm were used as a cells only control and data are presented as fold change relative to this control. There were unexpected variations in the standards used, and this variation was subsequently attributed to the results of machine error. Hence, comparisons of the protein release from epithelial cells in response to multi-species biofilms at each time point were carried out using fluorescent units.

Co-culture of 3, 7 and 10 species biofilms with epithelial cells resulted in increased levels of pro-inflammatory cytokines present in the supernatant at 4 hours compared with the cells only control. At 4 hours, there was a 3.7 fold increase in TNFα protein in supernatants from the 3 species biofilm co-culture; 7.92 fold increase in the 7 species co-culture and 4.3 fold increase in the 10 species co-culture relative to the cells only control. TNFα protein release was significantly higher in 7 species co-culture than both 3 species ($p<0.01$) and 10 species ($p<0.05$) co-cultures. At this time point there was also a 1.7 fold increase in IL-8 protein in supernatants from the 3 species biofilm co-culture; 5.1 fold increase from 7 species co-culture and 3.09 fold increase from 10 species co-culture relative to the cells only control. IL-8 protein release was significantly higher in the 7 species co-culture compared to the 3 species co-culture ($p<0.05$). The protein release from the IL-8 ELISA also showed significant increases in the 7 species co-culture at 4 hours; however, co-culture with 10 species biofilms also caused significant increases in IL-8 protein release. Luminex assays are believed to be more sensitive than traditional ELISAs and this may account for the variation observed. At 24 hours, co-culture of 3, 7 and 10 species biofilms with epithelial cells resulted in increased levels of IL-1β, IL-6, CSF-2 and IL-8 present in the supernatant at 4 hours compared with the cells only control with the exception of TNFα which was not detectable at 24 hours under any condition. At 24 hours, there was a 7.5 fold increase in CSF-2 protein in supernatants from the 3 species co-culture; 10.4 fold increase from the 7 species co-culture and 2.1 fold increase from the 10 species co-culture compared with the cells only control. Hence, co-cultures with 10 species biofilms contained significantly less CSF-2 protein than 3 species co-cultures ($p<0.001$) and 7 species co-cultures ($p<0.001$).

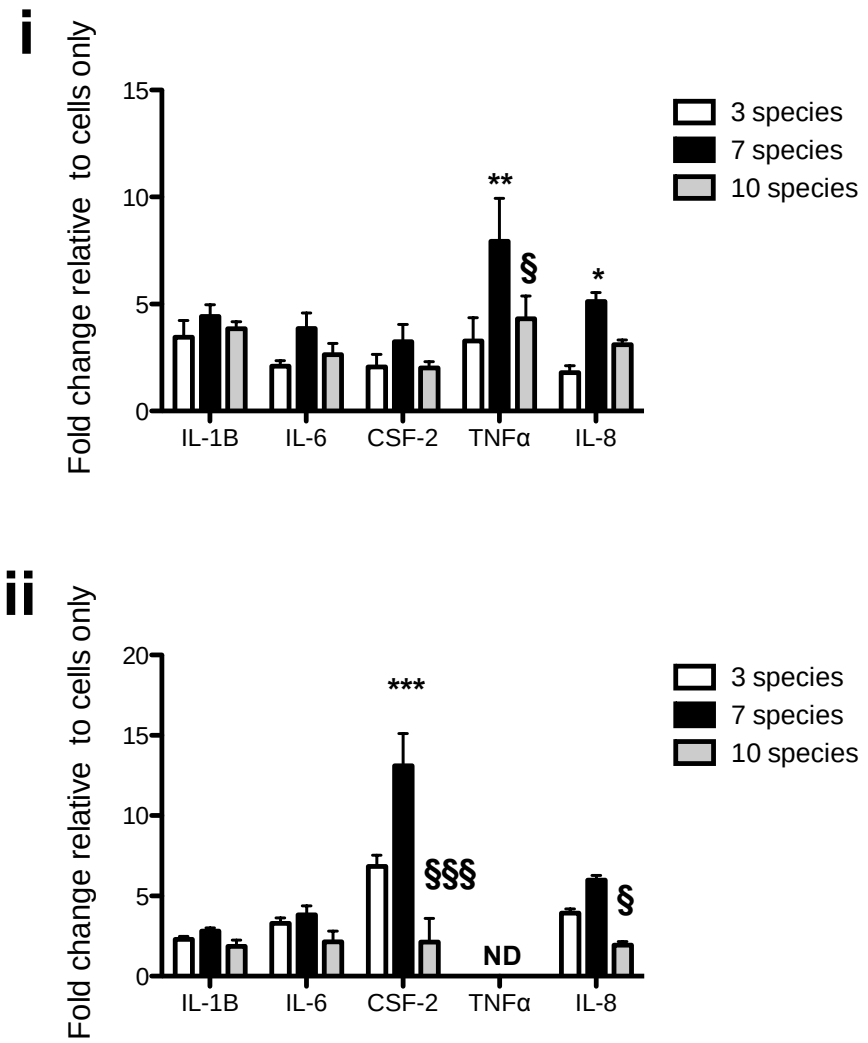


Figure 5.8: Epithelial cell pro-inflammatory protein release in biofilm co-culture

Three, 7 and 10 species biofilms were co-cultured with epithelial cells for 4 (i) and 24 (ii) hours. Epithelial cells incubated without a biofilm were used as a cells only control. Following co-culture biofilms supernatants were removed and cytokines measured by Luminex®. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD fold change relative to the cells only control. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups (Comparison to 3 species co-culture * $p<0.05$, ** $p<0.01$ *** $p<0.001$)(Comparison to 7 species co-culture § $p<0.05$, §§§ $p<0.001$).

The data using the immortalized oral epithelial cell line OKF6-TERT2 suggests that the composition of biofilms is able to differentially modulate epithelial cell inflammatory gene and protein responses in co-culture. However, these data were obtained using an immortalized cell line. Therefore it was important to investigate if these findings were replicated using a primary oral epithelial cells. This would allow comparisons between co-cultures using OKF6-TERT2 cells or primary epithelial cells and determine the validity of the findings observed using the OKF6-TERT2 cell line. Primary oral epithelial cells derived from human gingivae were used for the remainder of this study unless specified.

5.3.4 Multi-species oral biofilms show similar trends in cell death in co-culture with immortalised epithelial cells and primary epithelial cells

Primary epithelial cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours to assess cell viability following co-culture (Figure 5.9 i) and compared with viability of the OKF6-TERT2 cells following co-culture (Figure 5.9 ii). Due to the limited lifespan and limited cell number of primary cells a viability assay was selected to allow use of the minimum possible volume of cell culture supernatant to maximise availability for other experiments. An LDH cytotoxicity assay was therefore used to measure the cytotoxicity by quantifying the lactate dehydrogenase, which is effective in very small volumes of culture medium. As this is a different viability assay than used in previous work the viability of both the primary cells and the OKF6-TERT2 cells was assessed and compared. Percentage cytotoxicity of co-cultures and cells only controls was quantified relative to a positive control containing 100% dead cells generated by exposing either OKF6-TERT2 or primary epithelial cells to 0.2% chlorhexidine for 1 hour.

At 4 hours, the 10 species biofilms caused primary cell cytotoxicity, with 21.67% cytotoxicity, which was significantly higher than the cells only control ($p<0.05$). At 24 hours, primary cells co-cultured with 7 and 10 species biofilms showed an increase in cell cytotoxicity with 53.7% ($p<0.001$) and 30.63% ($p<0.01$) respectively compared with the cells only control. In co-cultures using the OKF6-

TERT2 cell line, all co-cultures at 4 hours showed no significant increases in cell cytotoxicity compared with the cells only control. Co-cultures at 24 hours showed a significant increase in cytotoxicity compared with the cells only control with both 7 and 10 species biofilm co-cultures containing 15.63% ($p<0.05$) and 14.55% ($p<0.05$) cytotoxicity, respectively.

Comparing the cytotoxicity at 4 hours in primary epithelial cell co-cultures to the OKF6-TERT2 epithelial cell line co-cultures, the primary cell line co-cultured with 10 species biofilms showed significantly more cell death than the epithelial cell line co-culture ($p<0.001$). At 24 hours, primary cells showed significant increases in percentage cytotoxicity than the epithelial cell line when co-cultured with 7 species biofilms ($p<0.001$). Overall, the primary cells appeared more susceptible to the cytotoxic effects of the biofilms.

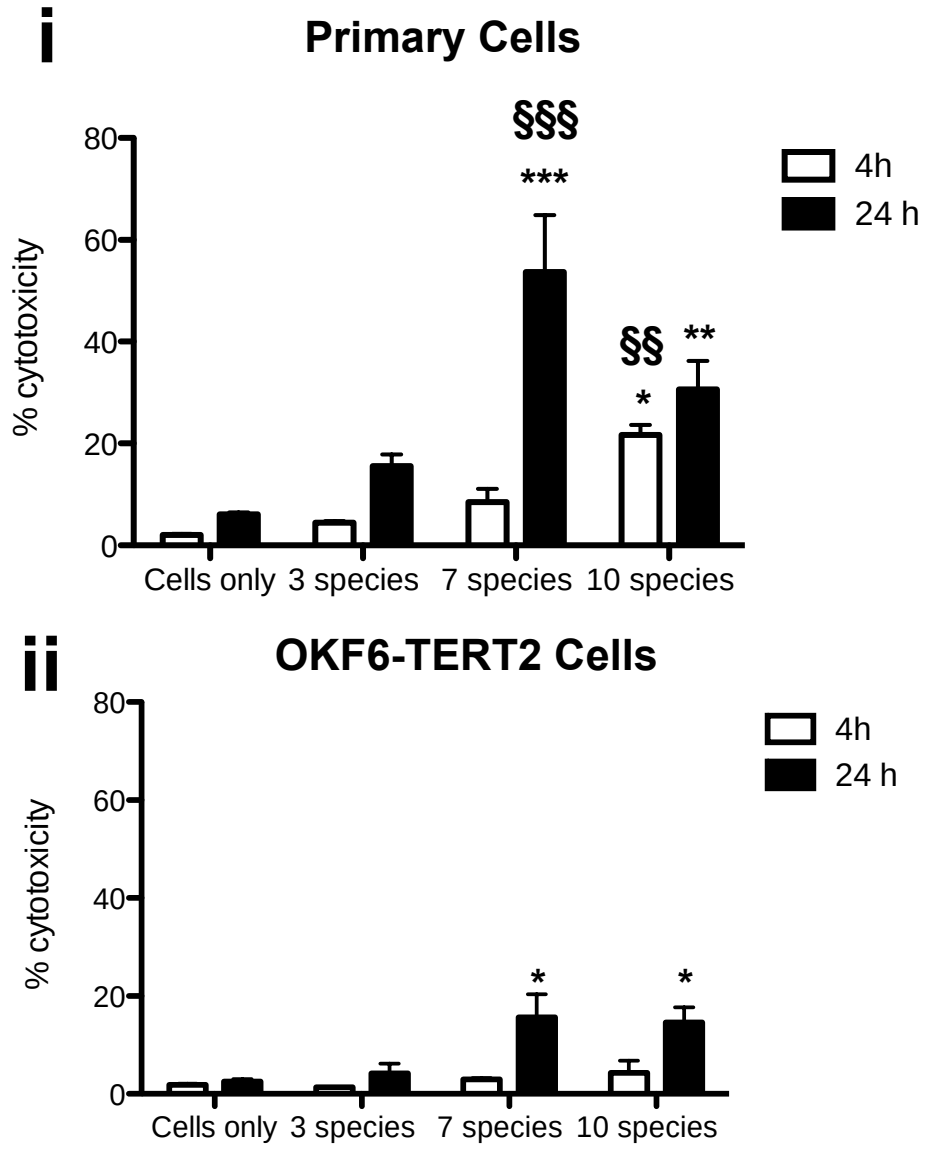


Figure 5.9: Primary cell and OKF6-TERT2 cell viability following co-culture assessed by lactate dehydrogenase release

Primary human oral epithelial cells (i) and OKF6-TERT2 epithelial cell line (ii) were co-cultured with 3, 7 and 10 species biofilms for both 4 and 24 hours. Cells incubated without a biofilm were used as a cells only control. Percentage cytotoxicity was measured by LDH cytotoxicity assay. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups with the cells only control at each time point (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)(Primary cells vs. OKF6-TERT2 cells §§ $p<0.01$, §§§ $p<0.001$).

5.3.5 Multi-species biofilms differentially modulate the inflammatory gene and protein response of primary epithelial cells in co-culture

The IL-8 gene expression from primary epithelial cells following co-culture was measured (Figure 5.10). At 4 hours, IL-8 gene expression was 0.05% in the cells only control; 0.01% in 3 species co-culture; 0.66% in the 7 species co-culture and 1.06% in the 10 species co-culture. Hence, primary cells co-cultured with 10 species biofilms had significantly more IL-8 gene expression than the cells only control ($p<0.05$) and 3 species biofilm co-cultures ($p<0.01$). At 24 hours, IL-8 gene expression was 0.017% in the cells only control; 0.019% in the 3 species co-culture; 0.11% in the 7 species co-culture and 0.17% in the 10 species co-culture. At this time point, cells co-cultured with 10 species biofilms demonstrated significantly more IL-8 gene expression than the cells only control ($p<0.05$) and 3 species co-cultures ($p<0.05$). Due to the apparent differences between IL-8 gene expression at 4 and 24 hours a Bonferroni post-test was used to compare each co-culture over time. This demonstrated a significant decrease in IL-8 gene expression between 4 and 24 hours when primary epithelial cells were co-cultured with 10 species biofilms ($p<0.01$).

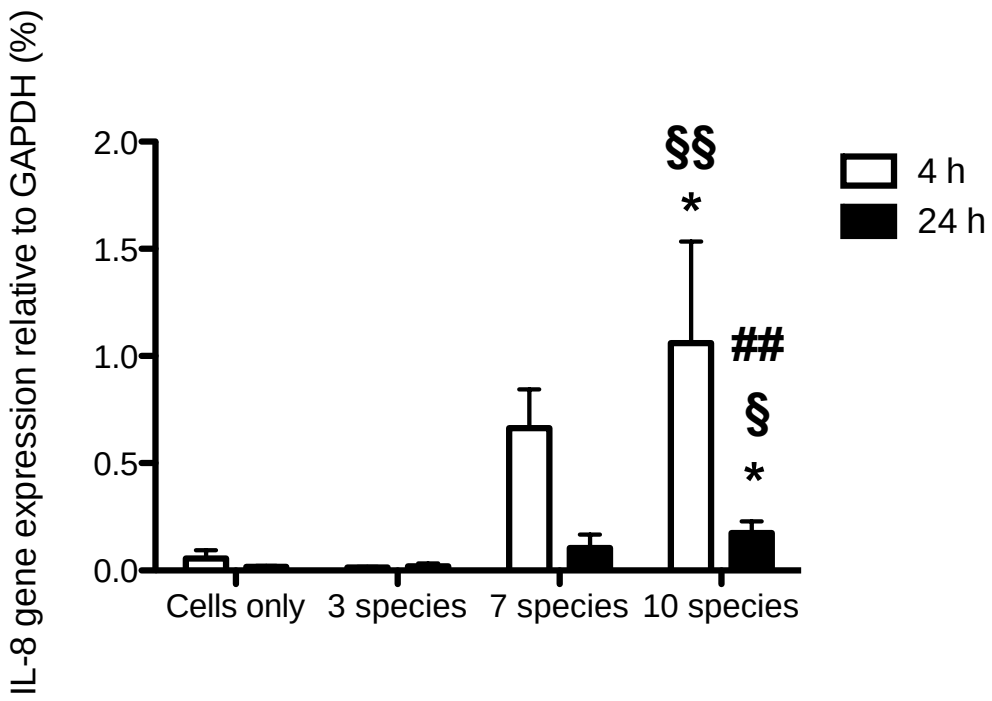


Figure 5.10: Primary epithelial cell IL-8 gene expression in multi-species biofilm co-culture

Primary human oral epithelial cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours. Primary epithelial cells incubated without a biofilm were used as a cells only control. IL-8 gene expression was assessed by qPCR and gene expression calculated relative to the housekeeping gene *GAPDH*. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a two-way ANOVA with Bonferroni's multiple comparison post-test to compare all groups (Comparison with cells only control * $p<0.05$) (Comparison with 3 species biofilm §□$p<0.05$, §§ $p<0.01$) (Comparison between 4 and 24 hours ## $p<0.01$).

To further investigate if primary epithelial cells differentially respond to multi-species biofilms, the expression of a panel of pro-inflammatory genes was assessed following co-culture of primary oral epithelial cells with multi-species biofilms (Figure 5.11). In this experiment gene expression was calculated relative to the housekeeping gene *GAPDH* and expressed relative to the media control to compare expression of each gene between multi-species biofilm co-cultures. At 4 hours, in co-cultures with 7 species biofilms, primary cell gene expression of IL-6, CSF2 and IL-8 increased by 193.3 fold ($p<0.05$), 208.5 fold

($p<0.01$) and 219 fold ($p<0.01$), respectively, compared to cultures with the 3 species biofilm. In co-cultures containing 10 species biofilms, expression of IL-6 was 206.1 fold higher ($p<0.01$) and CSF2 was 134.2 fold higher ($p<0.05$) than co-cultures with 3 species biofilms (Figure 5.11 i). At 24 hours, there was a trend to enhanced primary cell pro-inflammatory gene expression as the complexity of biofilms in the co-culture increased, although this did not reach statistical significance (Figure 5.11 ii). Additionally, although there appeared to be reduced gene expression of some of the cytokines following co-culture with 3 species biofilms, none of these reached statistical significance.

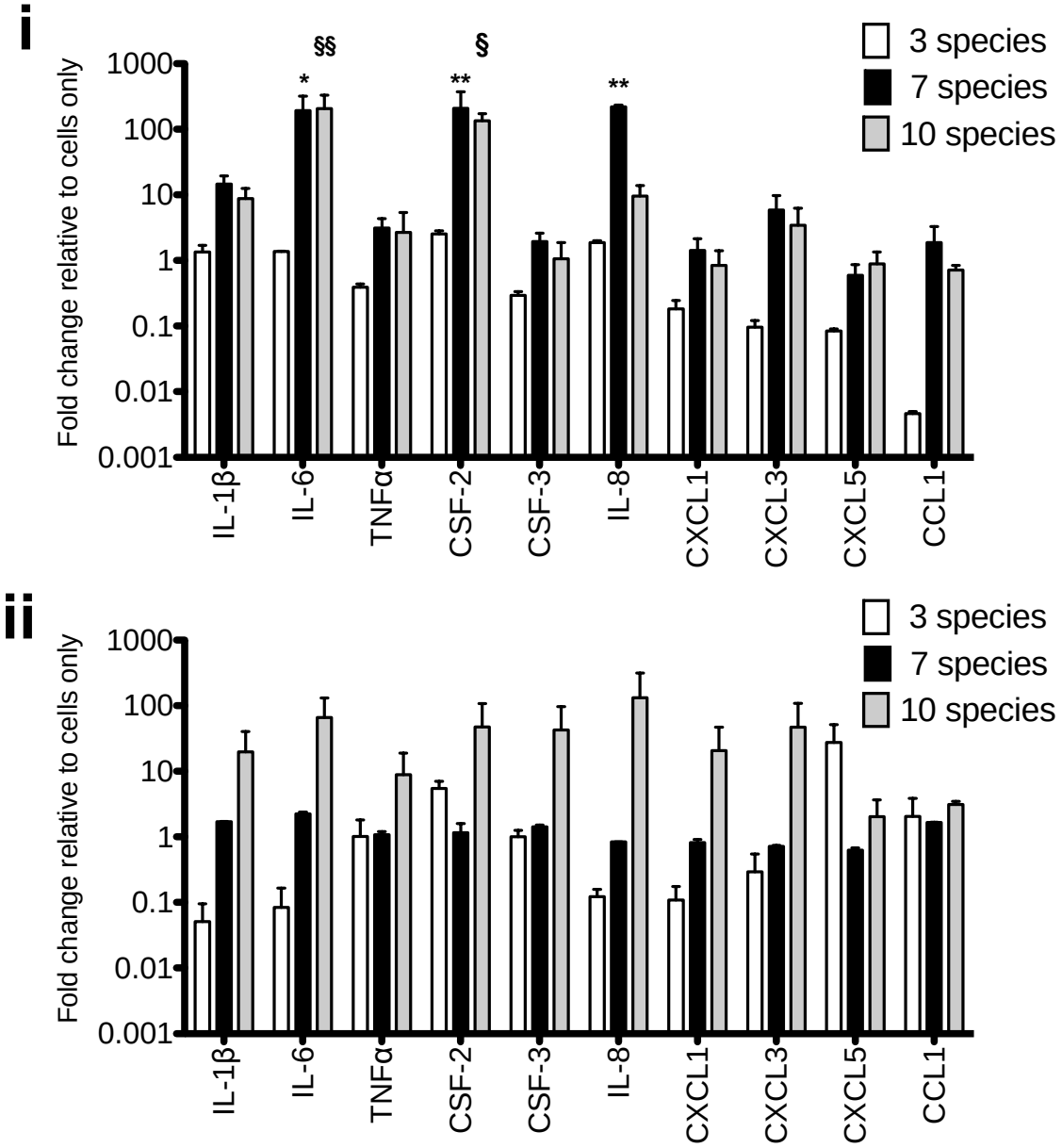


Figure 5.11: Primary epithelial cell pro-inflammatory gene expression in biofilm co-culture

Primary human oral epithelial cells were co-cultured with 3, 7 and 10 species for both 4 (i) and 24 (ii) hours. Primary epithelial cells incubated without a biofilm were used as a cells only control. Gene expression was assessed my multiplex qPCR. All groups were assayed in duplicate on three separate occasions. Gene expression was normalised to the housekeeping gene *GAPDH*. Data presented are gene expression relative to the cells only control. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test to compare all groups at each time point (Comparison with 3 species biofilm * $p<0.05$, ** $p<0.01$, § $p<0.05$, §§ $p<0.01$).

IL-8 protein release from primary epithelial cells following co-culture was measured (Figure 5.12). At 4 hours, there was 127.1 pg/mL IL-8 protein in supernatants from cells only control; 87.42 pg/mL in supernatants from 3 species co-culture; 262 pg/mL in supernatants from 7 species co-culture; and 309.6 pg/mL in supernatants from 10 species biofilm co-culture. Hence, the 7 and 10 species biofilms induced significantly more IL-8 release than both the cells only control and 3 species biofilm. At 24 hours there was 244.6 pg/mL of IL-8 protein in the cells only control; 432.9 pg/mL in the 3 species co-cultures; 1565.6 pg/mL in the 7 species co-culture and was below the level of detection in 10 species co-cultures. At this time point 7 species biofilms caused significant increase in IL-8 protein release in co-culture compared with the cells only control ($p<0.001$) an 3 species biofilm co-cultures ($p<0.01$). Unfortunately, due to the limited volume of samples and financial restrictions, Luminex® was unable to be performed on co-cultures with primary epithelial cells.

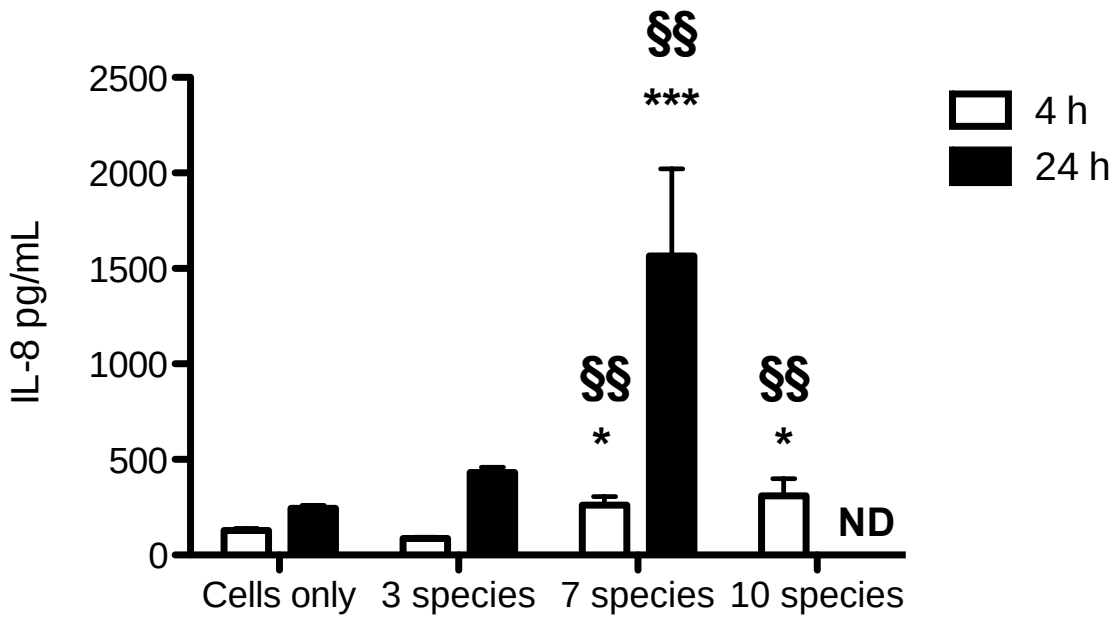


Figure 5.12: Primary cell IL-8 protein in biofilm co-culture supernatants
Primary human oral epithelial cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours in 5% CO_2. Primary cells incubated without a biofilm were used as a cells only control. IL-8 protein release in cell culture supernatants was measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups at each time point (Comparison to cells only * $p<0.05$, ** $p<0.01$)(Comparison to 3 species co-culture §§ $p<0.01$).

5.4 Discussion

The main finding in this chapter was that both primary epithelial cells and an epithelial cell line produced a distinct cytokine gene and protein expression signatures in response to each of the multi-species biofilms. This result demonstrated immune function consequences of differing biofilm composition, which apparently relates to whether the species present are associated with health, transitioning from health to disease, or disease. Furthermore, these inflammatory profiles arguably mirror the inflammatory profiles found in the oral cavity, where mild controlled inflammation is seen in health and elevated chronic and dysregulated inflammation observed PDs.

In both primary cell and OKF6-TERT2 cell co-cultures, biofilms were shown to affect cell viability. The variety of assays used, including AlamarBlue®, active-caspase 3 ELISA and LDH cytotoxicity assays, evaluated different aspects of viability; AlamarBlue® measures viability through metabolic activity; caspase-3 ELISA measures cell death through apoptosis pathways and the LDH assay measures cell death through the rupturing of the cell membrane and release of LDH into the surrounding media. Similar patterns of cell death were observed using each method. Taken together, these assays suggest that epithelial cell death when exposed to multi-species biofilms is the result of a combination of apoptosis and necrosis. The data suggest that the more complex biofilms cause significant epithelial cell death more rapidly. Guggenheim et al (2009) observed that HGEC co-cultured with their 'subgingival' 'Zurich' 9 species biofilm model underwent apoptosis in a time dependant manner at 4 and 24 hours (Guggenheim et al., 2009). This study found ~85% apoptotic cells after 24 hour co-culture, which was similar to the 82% reduction in OKF6-TERT2 cell viability observed following 24 hour co-culture with 10 species biofilms reported in this chapter. Eberhard et al (2009) co-cultured HGECs for 2 hours with biofilms from patients with no discernable PD and observed no differences in viability between untreated and treated cells, a result which may be due to the short co-culture time tested (Eberhard et al., 2009). Studies by *Peyyala et. al.* investigated co-culture of multi-species oral biofilms and OKF4 cells but do not report cell viability (Peyyala et al., 2012, Peyyala et al., 2013). The model used by this group is co-cultured anaerobically to mimic the conditions of the gingival pocket

and although the decrease in the metabolic activity of the epithelial cells was found to be negligible in anaerobic conditions when measured using the WST-1 cell viability reagent, the additional cytotoxic effect of biofilms was not considered (Peyyala et al., 2011).

In host-pathogen co-culture models understanding the relationship between biofilms and cell viability is key due to the implications on further cellular analysis, in particular gene and protein data. Interestingly, primary epithelial cells appeared to show minimal cell death or inflammatory gene or protein response to 3 species biofilms, suggesting primary epithelial cells are less susceptible to 3 species biofilms than the OKF6-TERT2 cell line. It is important to note the difference in nature of these cell types, immortalized cell lines, characteristics can be altered such as the loss of TLRs due to repeated passage, whereas primary cells have a very limited lifespan for use *in vitro*. Importantly for this work, these primary cells have previously been exposed to oral bacterial biofilms *in vitro*, unlike the epithelial cell line. This could imply primary epithelial cells have acquired tolerance to commensal species and may account for the lack of response seen in primary cell co-culture with 3 species biofilms and the enhanced response in co-culture with 7 and 10 species biofilms when comparing the primary cell to OKF6-TERT2 cell response. In the gut it is believed that intestinal epithelial cells show tolerance to commensal bacteria after birth, with studies showing a lack of bacterial LPS responsiveness by intestinal epithelial cells, which has been associated with post-translational down regulation of IL-1 receptor associated kinase, which is required for TLR4 signalling and increase of cytokines that protect the gut including IL-6 and TNF (Lotz et al., 2006, Rakoff-Nahoum et al., 2004). Furthermore the interactions of commensal bacteria with TLRs have been suggested to be essential in maintaining tolerance and host homeostasis, with TLR4 deficient mice showing increased epithelial cell injury, bleeding and Gram-negative bacterial translocation in a mouse colitis model (Fukata et al., 2005). While these studies do not appear to have been replicated in the oral cavity the interactions between commensal bacteria and epithelial cells at these sites may occur in a similar manner to ensure host homeostasis. For example, the oral bacteria *S. salivarius* has been shown to modulate innate inflammatory gene expression of

bronchial epithelial cells following co-culture through the down-regulation of the NF-κB pathway (Cosseau et al., 2008).

The work in this chapter was performed *in vitro,* with no *in vivo* validation, using a monolayer of epithelial cells that is different from gingivae in the oral cavity. This consists of multiple layers of epithelial cells in close relation to the underlying connective tissue. The differences in cell viability have been highlighted in studies using skin models, which demonstrated that monolayers were more susceptible to cytotoxic agents than 3D culture models (Sun et al., 2006). Furthermore, studies using gingival tissue biopsies have shown increased levels of apoptosis in periodontitis samples compared with healthy controls suggesting that tissue destruction by apoptosis plays a role in the pathogenesis of periodontitis (Gamonal et al., 2001).

The innate immune response of the epithelial cells appears to be dependent on the type of bacterial challenge. These data document that the composition of multi-species biofilms plays a key role in dictating the epithelial cell immune response. Studies using bacteria in planktonic culture or a single species biofilms studies using commensal species such as *S. oralis*, intermediate species such as *F. nucleatum* or pathogenic species such as *A. actinomycetemcomitans* cause species specific responses in oral epithelial cells. Intermediate and pathogenic planktonic bacteria stimulated increased protein levels of pro-inflammatory cytokines such as IL-6 and IL-8 and IL-1β (Stathopoulou et al., 2010, Peyyala et al., 2012). The maturation state of oral cavity derived biofilms has been shown to result in differential expression of IL-8 and antimicrobial peptides by epithelial cells, with mature biofilms being more pro-inflammatory than less complex biofilms (Eberhard et al., 2008). The inflammatory gene expression and protein release following co-culture using 3 species biofilms was indistinguishable from the media control cultures, suggesting a lack of response by host cells to this biofilm. This is in agreement with reports using planktonic, mono-species *Streptococcus* biofilms or multi-species biofilms containing bacteria classed as 'early colonizers' (Peyyala et al., 2013, Peyyala et al., 2012, Stathopoulou et al., 2010). The greatest increases in gene expression and cytokine detection were elicited by 7 species co-cultures. This biofilm contains *F. nucleatum*, which acts as an intermediate between early and late colonizer

species, and has been shown to cause a significant pro-inflammatory profile in epithelial cells during planktonic and mono-species biofilm co-culture (Stathopoulou et al., 2010, Peyyala et al., 2012). OKF6-TERT2 and primary epithelial cells demonstrated elevated gene expression of pro-inflammatory cytokines such as IL-6, IL-8, CSF-2, CXCL1 following co-culture with 7 and 10 species biofilms. Previous similar studies have reported increased Gro1α, IL-1α, IL-6, IL-8 TNFα, fractalkine, MIP-1α and IP-10 in epithelial cell co-cultures following exposure to oral biofilms, including the 9 species 'Zurich' model and multi-species biofilm models containing *S. gordonii, S. oralis, S. sanguinis, F. nucleatum, A. naeslundii* and *P. gingivalis* (Peyyala et al., 2013, Guggenheim et al., 2009). Gene expression by epithelial cells was comparable between 7 and 10 species co-cultures; however, protein expression was different, with a time dependent reduction in the concentrations of cytokines detectable following co-culture with 10 species biofilms. This suggests that the epithelial cells are responding to these biofilms in a similar manner but there is post-translational host modification by the 10 species biofilm. The data in this chapter demonstrated reduced cytokine protein in the supernatant was reduced at 24 hours compared with 4 hours in co-cultures of 10 species biofilms with epithelial cells. Studies using multi-species oral biofilm models have reported similar findings when investigating protein expression and attributed this to cytokine degradation by *P. gingivalis,* observing reduction of host cell IL-8 in supernatant following co-culture only when *P. gingivalis* was present in the biofilm (Guggenheim et al., 2009, Peyyala et al., 2013, Stathopoulou et al., 2009). Therefore, *P. gingivalis* is suspected to play a vital role in modulating host defences by degrading pro-inflammatory cytokines (Hajishengallis et al., 2011, Bao et al., 2014b). One method to test this would have been to methanol fix the *P. gingivalis* before addition to the multi-species biofilms, which would make it unable to degrade the cytokines. However, once the bacteria are fixed they are dead and most likely unable to integrate into the biofilm and in the same way. Alternatively, gingipains deficient mutants could be used, where differences in the pro-inflammatory cytokine profile have been shown in planktonic co-culture with epithelial cells compared to wild type strains (Stathopoulou et al., 2009). This method also has limitations as *P. gingivalis* gingipains mutants have been shown to qualitatively and quantitatively affect multi-species biofilms including

increasing biofilm thickness and affecting other 'red complex' species induction into the biofilm (Bao et al., 2014b, Takasaki et al., 2013).

In summary, the work in this chapter shows the ability of biofilms to differentially modulate the epithelial cell immune response based on their composition. Both the oral epithelial cell line OKF6-TERT2 and primary human epithelial cells from gingivae showed similar trends in pro-inflammatory gene and protein response. This work has established an *in vitro* co-culture model using three multi-species biofilms which each induce three distinct inflammatory profiles in epithelial cells. The hope for the future for this model is for use with a variety of host tissues in co-culture with biofilms and potential for use in identifying potential novel therapeutic targets for PD.

CHAPTER FINDINGS

Biofilms induce increased epithelial cell death in a biofilm-composition dependant and time dependant manner

There is increased expression of pro-inflammatory cytokines in epithelial cells in response to 7 and 10 species biofilms

Disease-associated 10 species biofilms associate with a time dependent reduction in cytokine protein present in supernatant following co-culture

Both primary epithelial cells and epithelial cell lines demonstrated similar trends in gene and protein expression following co-culture with multi-species biofilms

6 The innate immune response to oral biofilms *in vitro*

6.1 Introduction

In the oral cavity microbial biofilms are necessary but not sufficient to cause PD and the host plays a pivotal role in disease pathogenesis through the dysregulated immune response to oral biofilms. In health, the gingival tissues are policed by neutrophils and a small number of other phagocytes capable of antigen presentation, as well as T cells, B cells and mast cells. Histological studies have observed that the inflammation associated with increased plaque in gingivitis is associated with increased numbers of both lymphocytes and other mononuclear cells in the early lesion and plasma cells in the established lesion (Page and Schroeder, 1976). Therefore, potentially the bacteria themselves and also the products released by the gingival epithelium can mediate this response, and if the host response malfunctions then periodontitis is believed to ensue (Younes et al., 2009).

Neutrophils are a first line of defence during infection and represent up to 95% of the leukocytes recruited into the gingival crevice in response to oral biofilms (Delima and Van Dyke, 2003). These cells play an essential role in clearing pathogens by phagocytosis, degranulation, the release of neutrophil extracellular traps (NETs), and recruit and activate other immune cells through the production of pro-inflammatory cytokines including TNFα, IL-6, IL-8 and IFNγ. Animal studies comparing the histology of germ-free and wild type mice observed similar neutrophil infiltration in the junctional epithelium in both groups suggesting genetic programming of cells to this site regardless of microbial challenge (Heymann et al., 2001). Furthermore, patients with leukocyte adhesion deficiency (LAD), who have deficient neutrophil adhesion and migration into tissues, typically have aggressive periodontitis at a young age, which suggests a role for neutrophils in maintaining oral health (Moutsopoulos et al., 2015).

Neutrophils have been implicated as a critical link between the innate and adaptive immune response in chronic inflammation by producing chemokines, such as CCL2, which promote chemotaxis of T_h1 and T_h17 cells to the site of infection (Pelletier et al., 2010). Some oral bacteria, notably Sokransky's 'red complex' species, possess immune evasion mechanisms that can directly and

indirectly impair neutrophil functions. The keystone pathogen *P. gingivalis* has been shown to impair neutrophil recruitment by altering expression of pro-inflammatory cytokines, such as IL-8 and TNFα (Darveau, 2010). In patients with chronic periodontitis, leukocytes show changes in mRNA expression to increase survival and decrease apoptosis (Gamonal et al., 2003, Lucas et al., 2010, Lakschevitz et al., 2013). Some neutrophils recovered from the GCF of patients with PD also show histone citrullination and advanced stages of NET formation (Vitkov et al., 2010).

In PD monocytes and macrophages have been shown to comprise of 5-30% of inflammatory cells in periodontal lesions (Berglundh et al., 2011). These cells clear pathogens and dying cells by phagocytosis, release inflammatory mediators such as IL-1, TNFα, IL-6 and PGE2 to enhance immune cell activity and can present antigen to T cells (Dennison and Van Dyke, 1997). Macrophages are considered a flexible cell type, with ability to polarize to specific subsets in response to local mediators (Mosser, 2003). RANKL and TNFα can stimulate macrophage differentiation into osteoclasts. Macrophages from periodontitis patients are primed and particularly susceptible to osteoclastogenic stimuli, therefore potentially playing a critical role in alveolar bone resorption (Lam et al., 2000, Herrera et al., 2014). In patients with periodontitis, increases in the numbers of monocytes and macrophages has been associated with greater levels of MMPs and increased collagen breakdown compared with healthy controls (Seguier et al., 2001). Periodontal pathogens can modulate monocyte and macrophage activity by decreasing phagocytic and bactericidal functions (Carneiro et al., 2012). *P. gingivalis* impairs iNOS dependant killing of intracellular bacteria in macrophages by altering C5a-TLR2 signalling (Wang et al., 2010).

Numerous studies have implied that host immune systems ability to mount an appropriate inflammatory response is critical to maintaining tissue homeostasis and host-microbial symbiosis. However, there are still many gaps in the knowledge of the interactions between innate immune cells and oral biofilms, which is critical to understanding the dysbiosis observed in PD. The work in this chapter will attempt to expand on current knowledge by investigating how the composition of microbial biofilms may modulate innate immune inflammatory

responses at a gene and protein level, which may play a role in the chronic inflammation observed in PD.

6.2 Aims

The aim of this chapter was to investigate if the three multi-species biofilms, developed in chapter 5, differentially modulated the innate immune response. The following key questions were investigated:

- Does biofilm composition differentially modulate neutrophil inflammatory gene expression, protein release and NET formation?

- Does biofilm composition differentially modulate pro-monocyte activation, differentiation, inflammatory gene expression and protein release?

- Do differentiated monocytes differentially respond to direct co-culture with biofilms or with biofilm or epithelial cell conditioned media?

6.3 Results

6.3.1 Multi-species biofilms differentially modulate neutrophil gene expression, protein release and NET formation

Neutrophils were co-cultured with 3, 7 and 10 species biofilms for 4 and 16 hours at which time inflammatory gene and protein expression was assessed. At 4 hours, TNFα gene expression was 0.03% in the cells only control; 0.21% in the PMA positive control; 0.07% in the 3 species; 1.05% in the 7 species and 2.21% in the 10 species; hence, co-culture with 10 species biofilm resulted in significant increases in TNFα gene expression compared with the 3 species co-culture ($p<0.05$) and cells only control ($p<0.05$). IL-1β gene expression was 0.003% in the cells only control; 0.09% in the PMA positive control; 0.07% in the 3 species; 0.06% in the 7 species and 1.08% in the 10 species. Therefore, IL-1β was significantly increased in 10 species co-cultures compared with all other conditions ($p<0.05$). At 16 hours the RNA quality following co-culture was insufficient for gene expression studies (data not shown).

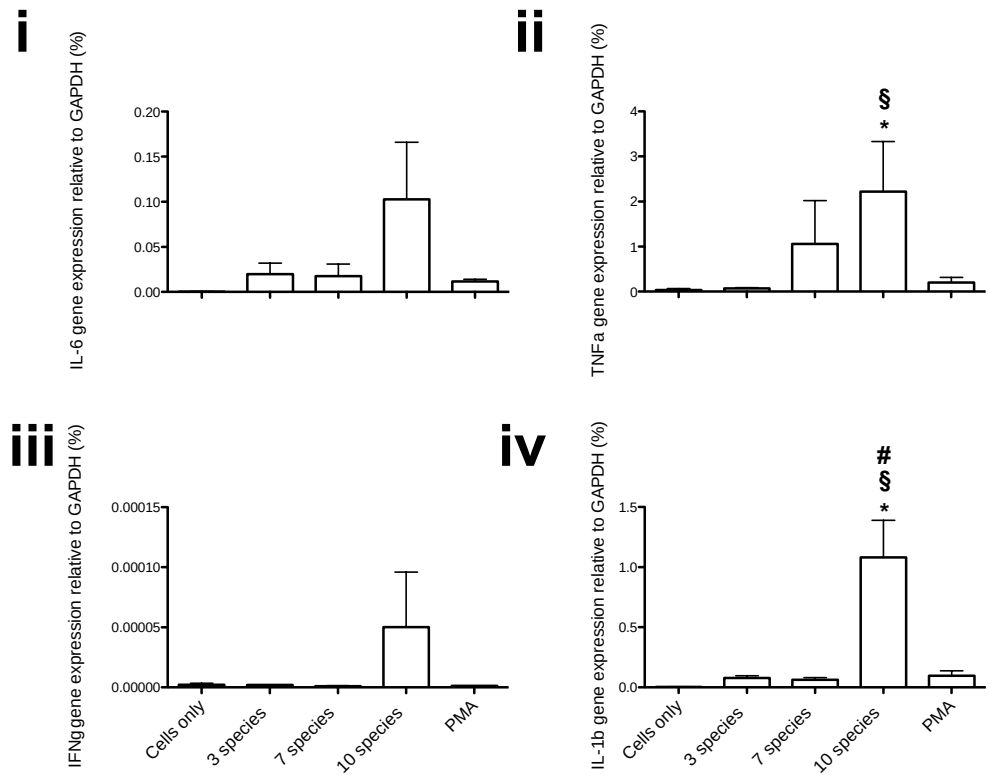


Figure 6.1: Neutrophil pro-inflammatory gene expression following co-culture Bone marrow derived neutrophils co-cultured with 3, 7 and 10 species biofilms for 4 hours. IL-6 [i], TNFα [ii], IFNγ [iii] and IL-1ß [iv] gene expression was assessed by qPCR. Cell stimulated with 600 nM of PMA or media only were used as controls. Data presented are normalised to the housekeeping gene *GAPDH*. All groups were assayed in duplicate on three separate occasions. Data represent mean ± SD (compared to cell only * $p<0.05$; compared to 3 species co-culture § $p<0.05$). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test to compare gene expression under each condition.

Next the inflammatory protein release by neutrophils following 4 and 16 hour co-culture was examined (Figure 6.2). At 4 hours, neutrophils in the cells only control released 81.6 pg/mL of TNFα; 3 species co-cultures released 55.3 pg/mL; 7 species co-cultures released 367.9 pg/mL; 10 species co-cultures released 302.8 pg/mL and the PMA positive control released 72.9 pg/mL. At 16 hours, neutrophils cultured in the cells only control released 177.3 pg/mL TNFα; 3 species co-cultures released 324.4 pg/mL; 7 species co-cultures released 495.7 pg/mL; 10 species co-cultures released 556.4 pg/mL and the PMA positive

control released 248.4 pg/mL. Thus, at both 4 and 24 hours, 7 and 10 species biofilms caused significantly increased release of TNFα compared with media control ($p<0.05$, Figure 6.2i). KC, the murine homolog of human IL-8, was also measured in these co-cultures. Low concentrations of KC were detected in media and PMA controls. KC was undetectable following 16 hour co-culture with 7 and 10 species biofilms (Figure 6.2 ii).

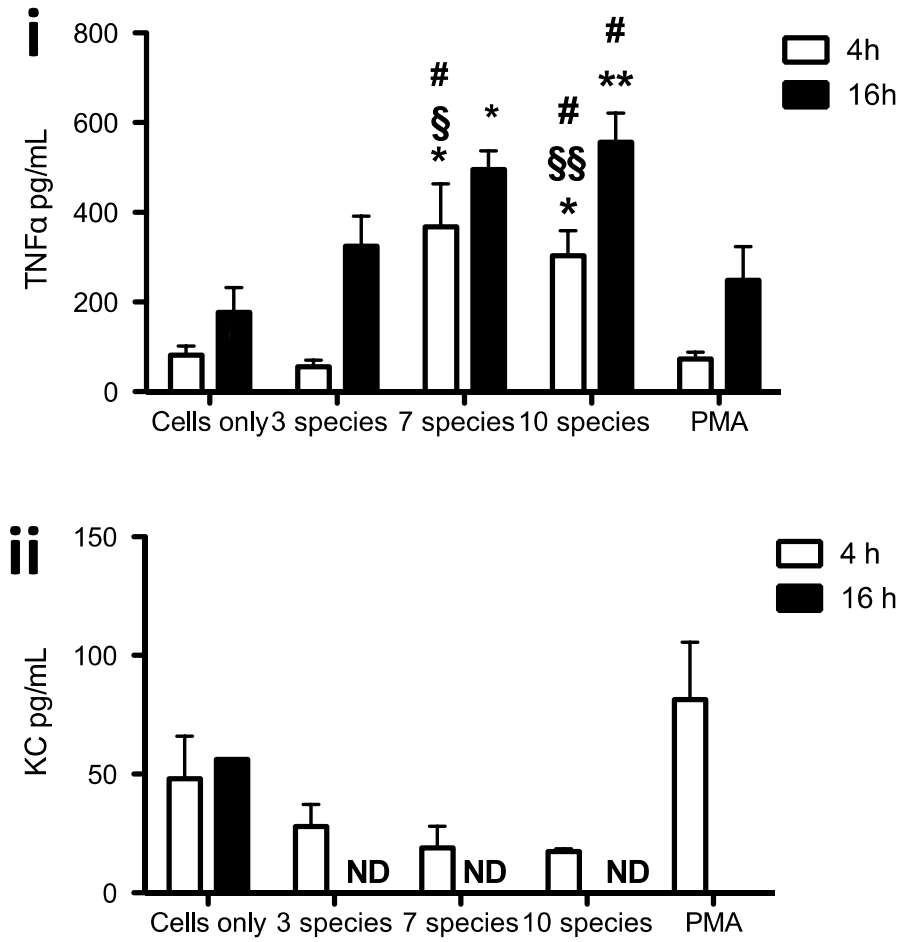


Figure 6.2: Neutrophil pro-inflammatory protein expression following co-culture

Bone marrow derived neutrophils co-cultured for 4 and 16 hours with 3, 7 and 10 species biofilms. Cell stimulated with 600 nM of PMA or media only were used as controls. Protein release was measured by ELISA for TNFα [i] and Luminex® for KC [ii]. All groups were assayed in duplicate on three separate occasions. Data represents mean ± SD (compared to cell only * $p<0.05$, ** $p<0.01$)(compared to 3 species co-culture § $p<0.05$, §§ $p<0.01$)(compared to PMA # $p<0.05$). Statistical analysis was performed using a one-way ANOVA with Tukey's post test.

Neutrophil extracellular trap (NET) formation was investigated following 16 hours co-culture with multi-species biofilms. NETs are defined as extracellular networks primarily composed of DNA, elastase and citrullinated histones that bind and kill microorganisms (Brinkmann et al., 2004). To detect NETs following co-culture, neutrophils were stained to highlight neutrophil elastase (shown in red), anti-histone H3 (shown in green) and DNA (shown in blue) and visualised by confocal microscopy. Neutrophils cultured for 16 hours in complete RPMI without stimulus showed intracellular DNA staining and minimal elastase staining inside the cell with no apparent NET formation (Figure 6.3 i). The positive control of neutrophils stimulated with 600 nM of PMA showed increased levels of neutrophil elastase at the cell surface and release of DNA shown as long branching strands of DNA (Figure 6.2 ii). In the co-culture of neutrophils with 3 species biofilms, clustered neutrophils show increased levels of neutrophil elastase being released and visible DNA release is observed where cells are spread further apart, although this appears as diffuse staining rather than the clear strands in 6.3 ii (Figure 6.3 iii). Co-culture of neutrophils with 7 species biofilms appears to show clustering of neutrophils, there is some staining of elastase and release of DNA into a matrix which encapsulates the neutrophils and neutrophil elastase underneath (Figure 6.3 iv). Neutrophils co-cultured with 10 species biofilms show large amounts of DNA present. Interestingly, in this co-culture there are large quantities of neutrophil elastase present; however the majority of this appear to still be inside the neutrophils. Some elastase appears extracellular, although as this does not clearly associate with defined strands of DNA; necrotic cell death seems a more likely source of this released elastase (Figure 6.3v). Anti-histone H3 was not observed in any of the cultures.

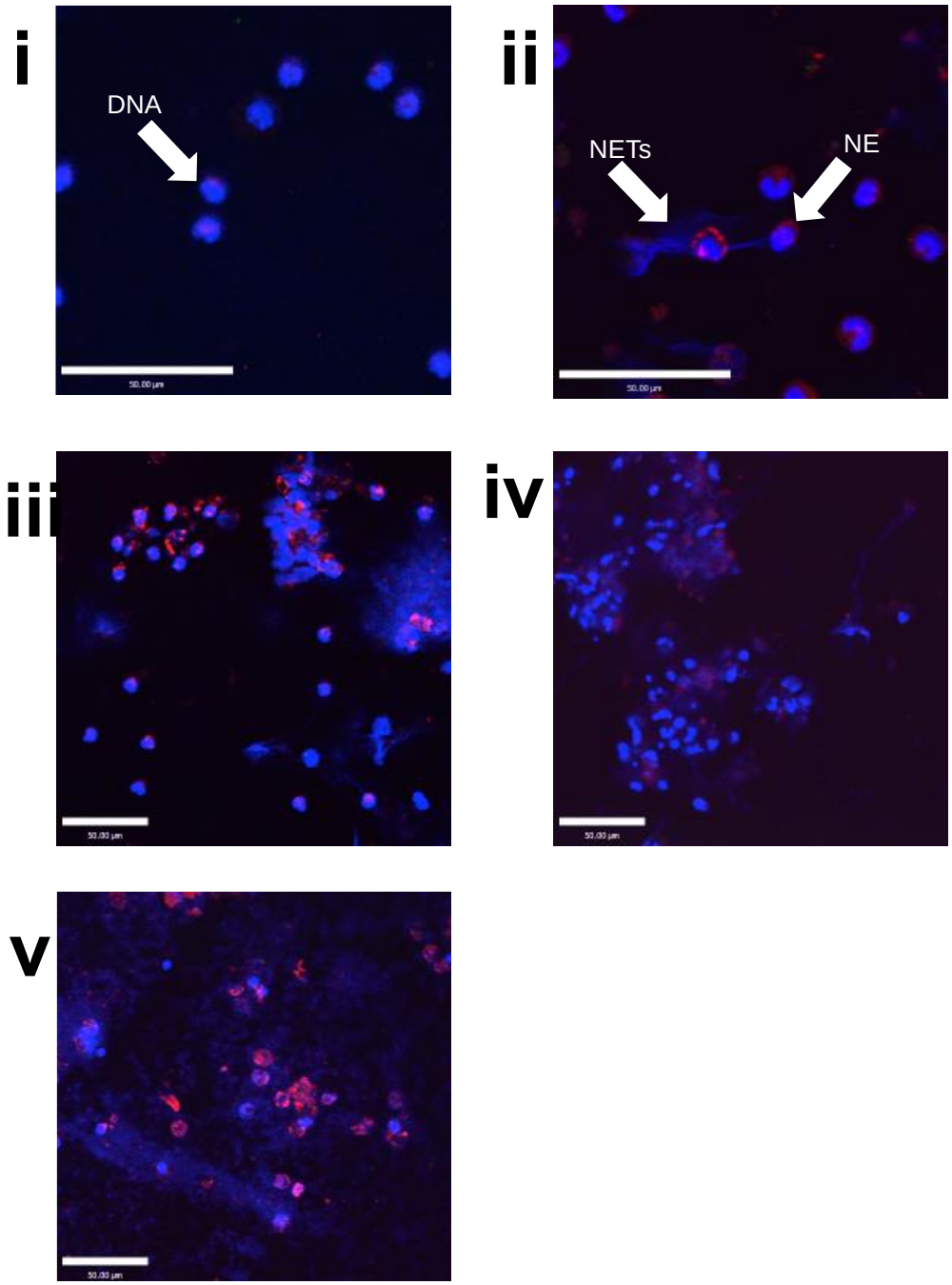


Figure 6.3: Neutrophil NET formation following co-culture with multi-species biofilms

Bone marrow derived neutrophils co-cultured for 16 hours with 3 [iii], 7 [iv] and 10 [v] species biofilms. Cell stimulated with 600 nM of PMA [ii] or media only [i] were used as controls. Neutrophils were removed and stained for neutrophil elastase (red), anti-histone H3 (green) and DNA (blue) and imaged on a confocal microscope. The images are representative of three independent experiments using duplicate samples. Scale bar represents 50 μm.

6.3.2 Biofilms modulate differentiation and inflammatory protein response of undifferentiated THP-1 cells in co-culture

Monocytes and macrophages can comprise up to 30% of the cells in the periodontal lesion. Monocytes and macrophages respond and interact with their local surroundings responding to mediators produced by both local tissue and immune cells. This includes both host immune mediators such as inflammatory markers produced by gingival epithelial cell at the gingival margin and bacterial components produced by biofilms, such as bacterial LPS or gingipains produced by *P. gingivalis*, which can alter the local environment. To further understand the role the local environment plays on monocyte and macrophage cytokine profiles, PMA and vitamin D_3 treated THP-1 cells were co-cultured with either conditioned media from biofilm: epithelial cell co-cultures or conditioned media from mature biofilms. THP-1 cells are a human pro-monocytic cell line derived from a myeloid leukaemia patient and has been widely used to study immune responses and cell function while cells are in the monocyte, differentiated macrophage-like and dendritic-like states (Tsuchiya et al., 1980, Schwende et al., 1996). This cell line was selected as it has been shown to closely resemble peripheral mononuclear blood monocytes when comparing cell functions, markers and immune responses (Gao et al., 2000, Chanput et al., 2014). The THP-1 cells line has also been extensively used to investigate the function of cells of the monocytic lineage in oral biology studies (Nahid et al., 2011, Gokyu et al., 2014).

The ability of multi-species biofilms to differentiate THP-1 cells from pro-monocytes into monocytes or macrophages was investigated by quantifying cell adherence after co-culture with biofilms. Positive controls of naïve THP-1 cells stimulated with PMA or vitamin D_3 were used as these stimuli have been shown to differentiate the cells towards a monocytic and macrophage-like phenotype respectively (Rovera et al., 1979, Collins, 1987). Following co-culture, THP-1 cells were stained with CFSE and adherence measured using fluorescent intensity and visualised by microscopy (Figure 6.4). At 4 hours no significant difference in THP-1 cell adherence between co-cultures with biofilms and controls was observed; however, following 24 hour co-culture, THP-1 cells stimulated with vitamin D3 and PMA showed significantly increased adherence compared to all

other biofilm stimulated cells or media controls (Figure 6.4i). Notably, THP-1 cells co-cultured with 10 species biofilms also showed increased cell adhesion compared with the cells only ($p<0.05$) control and 3 species biofilms ($p<0.05$) at this time point. These finding were confirmed visually using fluorescence microscopy (Figure 6.4 ii-xiii). At both time points the fluorescent microscopy showed the number of THP-1 cells adhering to the coverslip increasing as biofilm complexity increased. The cells only control showed few adherent cells and both the PMA and vitamin D_3 showed the largest number of adherent cells. The number of adherent cells in all conditions increased between 4 and 24 hours.

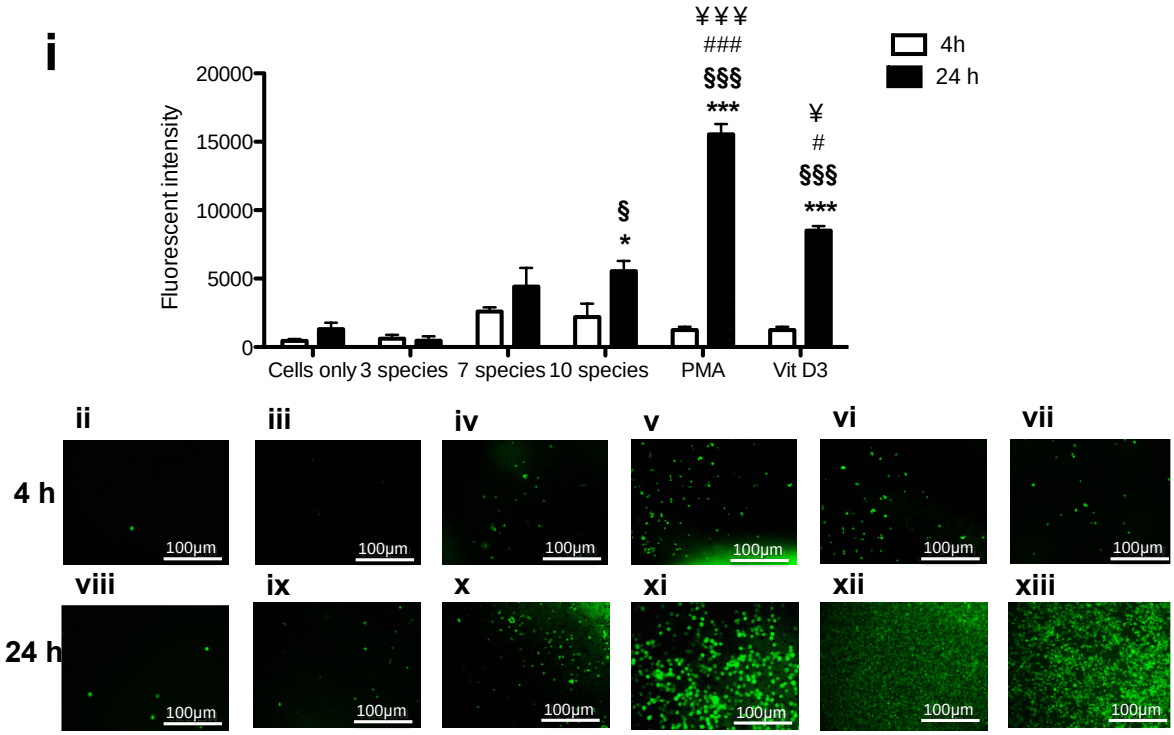
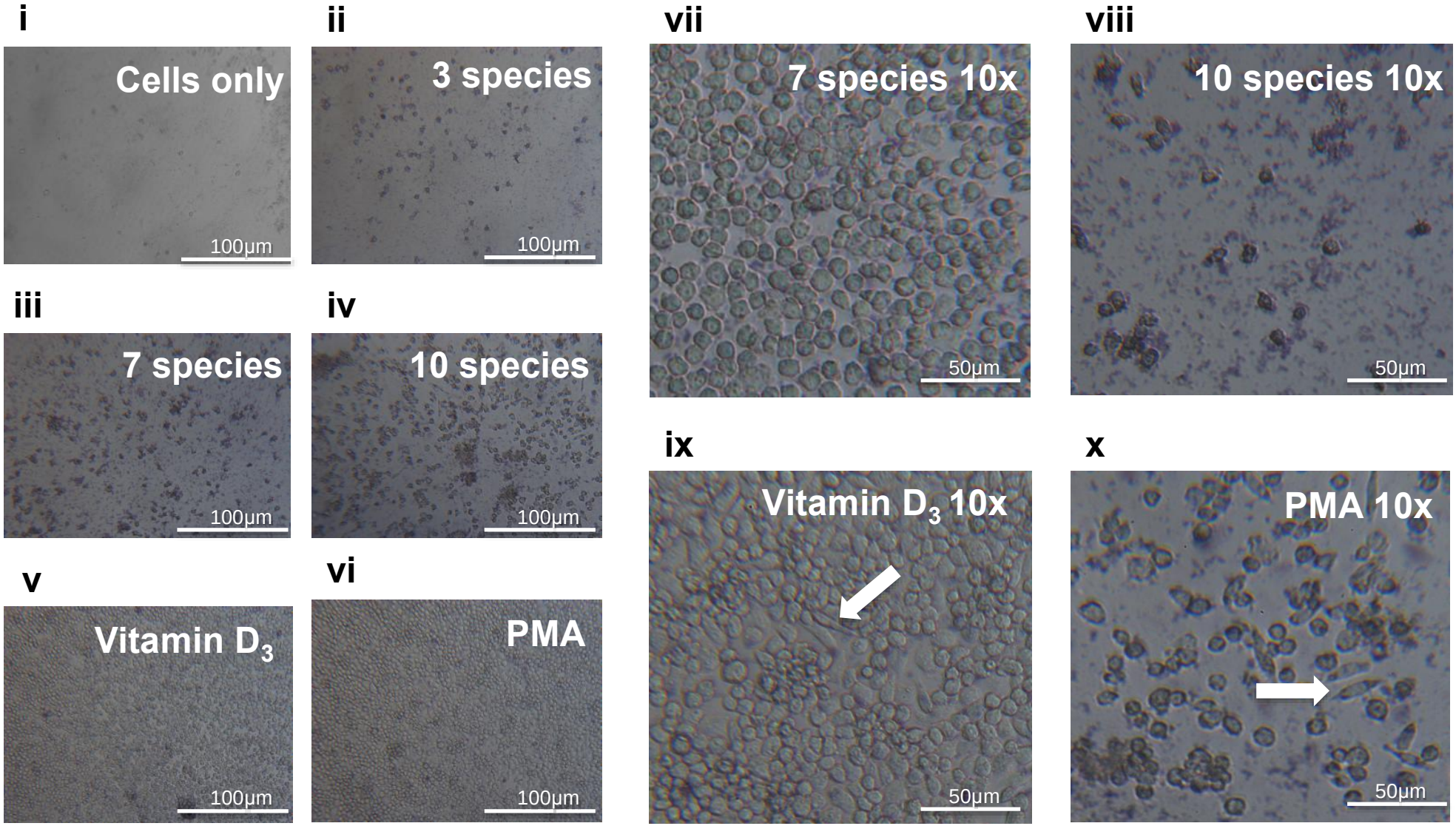


Figure 6.4: Pro-monocyte differentiation in response to biofilm co-culture

THP-1 cells were stained with CFSE prior to co-culture with 3, 7 and 10 species biofilms for 4 and 24 hours. Cells cultured with media only (cells only) or stimulated with 100 nM vitamin D_3 or 200 nM PMA were used as controls. Cell adhesion was quantified by fluorescence intensity using a plate reader [i]. Adhesion of THP-1 cultured with cells only [ii, viii], 3 species [iii, ix], 7 species [iv, x] and 10 species biofilms [v, xi], PMA [vi, xii] and vitamin D_3 [vii, xiii] was also visualized by fluorescence microscopy (40x magnification) at 4 and 24 hours. All groups were assayed in duplicate on three separate occasions. (i) Data represents mean ± SD (compared to cells only * $p<0.05$, *** $p<0.001$; compared to 3 species co-culture § $p<0.05$, §§§ $p<0.001$; compared to 7 species # $p<0.05$ ### $p<0.001$; compared to 10 species ∞ $p<0.05$ ∞∞∞ $p<0.001$). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test to compare all groups at each time point.

THP-1 cells showed differential adhesion patterns following co-culture with biofilm and controls therefore the morphology of THP-1 cells following 24 hour co-culture was also investigated using light microscopy (Figure 6.5). Notably, similarities between the 7 species co-culture and vitamin D_3 stimulation were observed with adherent cells maintaining a round morphology (Figure 6.5 vii-viii). Furthermore, THP-1 cells cultured with 10 species biofilms or stimulated with PMA showed greatest cell adherence with most cells attached to the coverslip and cells spreading on the surface (Figure 6.5 ix-x).

Figure 6.5: Morphology of undifferentiated THP-1 cells following co-culture
(Below) Undifferentiated THP-1 cells co-cultured with 3 [ii], 7 [iii, viii] and 10 [iv, x] species biofilms for 24 hours. Cells cultured with media only (cells only)(i) or stimulated with 100 nM vitamin D_3 [v, vii] or 200 nM PMA [vi, ix] were used as controls. Cell adhesion and morphology were visualized by light microscopy at 40x [i-vi] and 100x [vii-x] magnification. THP-1 cell spreading following 10 species co-culture and PMA stimulation highlighted by arrows.


i
Cells only
100µm
ii
3 species
100µm
iii
7 species
100µm
iv
10 species
100µm
v
Vitamin D_3
100µm
vi
PMA
100µm
vii
7 species 10x
50µm
viii
10 species 10x
50µm
ix
Vitamin D_3 10x
50µm
x
PMA 10x
50µm

Co-culture with multi-species biofilms caused increased THP-1 cells adherence and altered morphology. Next, the release of IL-8 in response to the biofilms was investigated (Figure 6.6). At 4 hours THP-1 cells released 9.9 pg/mL IL-8 in the cells only control; 413.6 pg/mL in 3 species co-culture; 1345.6 pg/mL in 7 species co-culture and 1145.7 pg/mL in 10 species co-culture. Following 24 hours co-culture THP-1 cells released 7.8 pg/mL IL-8 in the cells only control, 320.7 pg/mL in the 3 species co-cultures; 1076.1 pg/mL in 7 species co-cultures and 919.0 pg/mL in 10 species co-cultures. Overall, at both 4 and 24 hours, IL-8 release was significantly higher in 7 and 10 species co-cultures than both the cells only control ($p<0.05$) and 3 species co-cultures ($p<0.05$). There was significant, albeit relatively modest, IL-8 release from 3 species co-cultures ($p<0.05$). These data shows biofilms co-cultured with undifferentiated THP-1 cells cause differential IL-8 release.

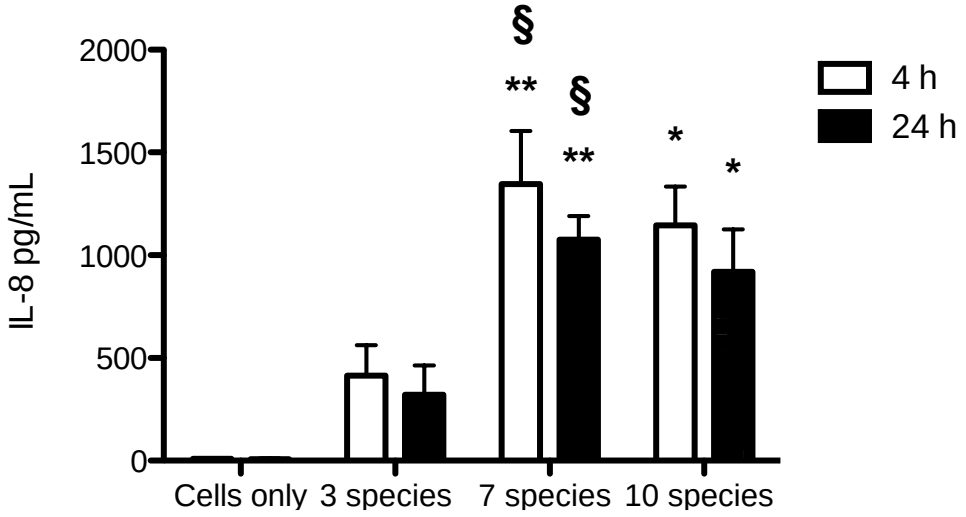


Figure 6.6: IL-8 protein response by undifferentiated THP-1 cells following co-culture

THP-1 cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours. Cells cultured with media only were used as a cells only control. IL-8 protein release was measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represent mean ± SD of three independent experiments performed in triplicate (compared with cells only * $p<0.05$, ** $p<0.01$; compared with 3 species biofilm § $p<0.05$). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test to compare all groups at each time point.

6.3.3 THP-1 cells up-regulate differentiation markers following co-culture with multi-species biofilms

The previous data show co-culture with multi-species biofilms causes differential adherence, changes to cell morphology and inflammatory protein release in undifferentiated pro-monocytes. However, pro-monocytes are typically found in the bone marrow and the cells of that lineage present in the oral cavity are usually monocytes, macrophages and dendritic cells. Therefore it was important to investigate if co-culture with multi-species biofilms induced differential differentiation of THP-1 cells. Studies also frequently use PMA and vitamin D_3 to differentiate the THP-1 cells into macrophages and monocytes respectively. Flow cytometry was used as a sensitive method to quantify the phenotype of live cells to determine if co-culture with biofilms, PMA or vitamin D_3 differentiated THP-1 cells into monocytes or macrophages (Figure 6.7a and b). To quantify THP-1 cell differentiation CD14, a marker on both monocytes and macrophages; CD40, a macrophage maker; and CD69, a monocyte marker, were used. Viability was measured using eFluor450® which binds to dead cells to allow live cells to be gated.

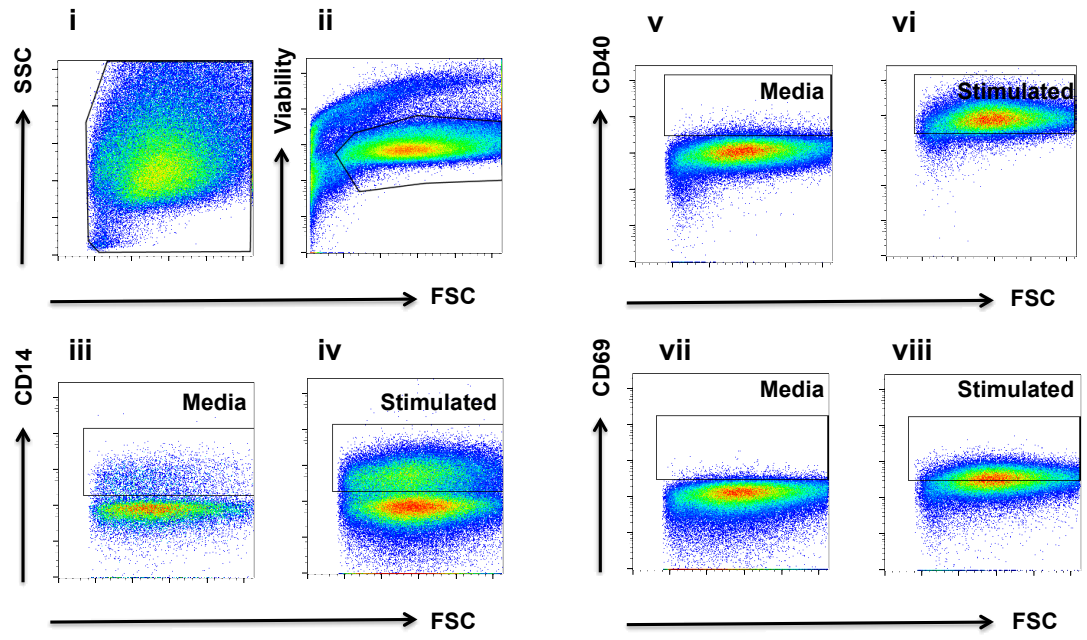
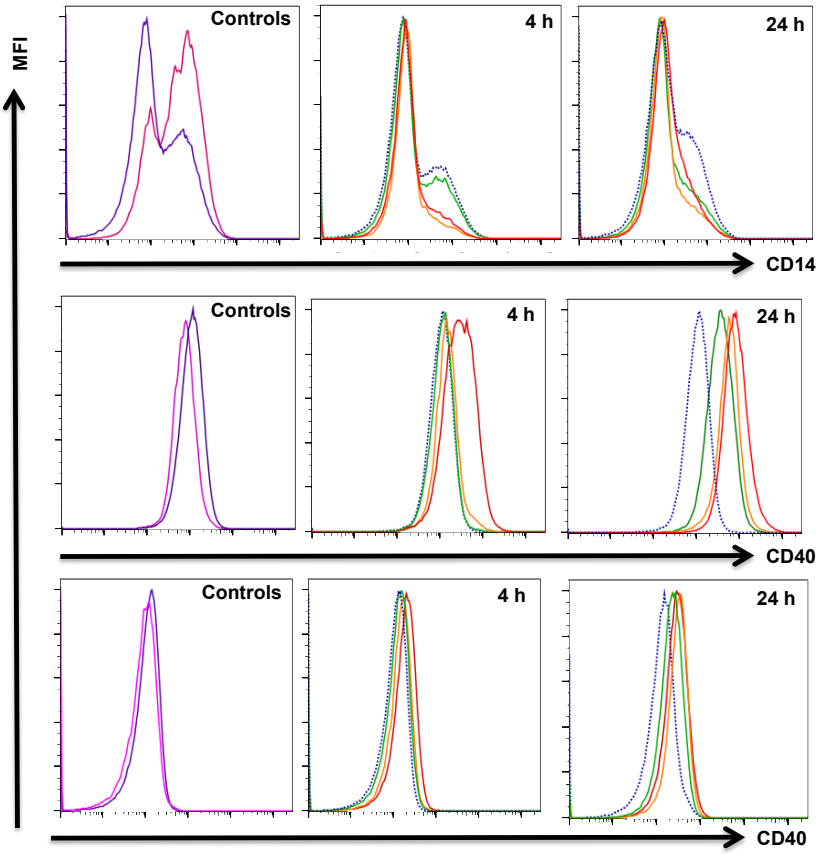


Figure 6.7a: Example flow cytometry plots and gating strategy of THP-1 cells following culture

THP-1 cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours or stimulated with 100 nM vitamin D_3 or 200 nM PMA. Cells cultured in media alone were used as a cells only control. Following culture, cells were analysed by flow cytometry to investigate changes cell surface expression of CD14, CD40 and CD69. Cells were gated based on their forward and side scatter [i] and from this viable cells were then gated [ii]. Viable cells were quantified using an e450 viability dye, which labels dead cells allowing, hence a 'live cell gate' was placed on the e450 negative cells. The proportion of gated viable THP-1 cells that were CD14+ (monocytes and macrophages)[iii-iv], CD40+ (macrophages)[v-vi] and CD69+ (monocytes)[vii-viii] was analysed. Data represent examples of staining on undifferentiated 'media' cells [i, iii, v, vii]) and staining on cells differentiated with PMA or vitamin D_3 'stimulated' [ii, iv, vi, viii]) for CD69, CD40 and CD14.



Colour	Sample
Blue	Untreated control
Green	3 species
Orange	7 species
Red	10 species
Pink	PMA
Purple	Vitamin D_3

Figure 6.7b: Example MFI THP-1 cells following culture

THP-1 cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours or stimulated with 100 nM vitamin D_3 or 200 nM PMA. Cells cultured in media alone were used as a cells only control. Following culture, cells were analysed by flow cytometry to investigate changes cell surface expression of CD14, CD40 and CD69. Cells were gated based on their forward and side scatter and from this viable cells were then gated. Examples of mean fluorescent intensity (MFI) of CD14 positive, CD40 positive and CD69 positive gates are included to show differences between each species.

To understand if biofilm composition can alter innate cell responses the ability of multi-species biofilms to differentiate THP-1 cells in co-culture for 4 and 24 hours was assessed (Figure 6.8). Undifferentiated THP-1 cells were also stimulated with either PMA or vitamin D_3 for 3 and 8 days, respectively, and viability and differentiation markers assessed to confirm if cells had differentiated as expected from the literature (Figure 6.9).

Viability of THP-1 cells was similar between PMA and vitamin D_3 stimulated groups, with 53.3% and 63.9% (Figure 6.9 i). THP-1 cell viability in the cells only control was 80% and 70.35% at 4 and 24 hours respectively. After 4 hours co-culture with 7 species biofilms, THP-1 cell viability was 24.55%, significantly lower than the cells only control ($p<0.05$). At 24 hours, co-culture with 10 species biofilms reduced cell viability to 3.87%, which was significantly reduced compared to the cells only control (Figure 6.8 i).

CD14+ expression following co-culture was measured on THP-1 cells following co-culture with biofilms (Figure 6.8 ii) and on PMA and vitamin D_3 differentiated cells (Figure 6.9 ii). Of the viable cells, 65.3% of PMA stimulated and 50.5% of vitamin D_3 stimulated THP-1 cells were positive for CD14 (Figure 6.9 ii). At 4 hours the proportion of CD14+ was 15.88% in the cells only control; 15.75% in 3 species co-culture; 18.25% in 7 species co-culture and 21.55% in 10 species co-culture. At 24 hours, the proportion of CD14+ THP-1 cells was 34.7% in the cells only control; 23.8% in 3 species co-culture; 7.0% in 7 species co-culture and 29.3% in 10 species co-culture. At 24 hours the proportion of CD14+ cell in 7 species biofilm co-culture was significantly lower than the cells only control ($p<0.01$), 3 species biofilm ($p<0.05$) and 10 species biofilm ($p<0.05$).

The macrophage marker CD40 was measured on THP-1 cells following co-culture with biofilms (Figure 6.8 iii) and on PMA and vitamin D_3 differentiated cells (Figure 6.9 iii). CD40 was present on 2.62% of PMA stimulated cells and 2.67% of vitamin D_3 simulated cells. At 4 hours the proportion of CD40+ THP-1 was 6.5% in the cells only control; 3.03% in the 3 species co-culture; 8.8% in the 7 species co-culture and 34.3% in the 10 species co-culture. Hence, co-culture with 10 species biofilm significantly increases the CD40+ population of THP-1 cells compared with the cells only control ($p<0.05$). At 24 hours the proportion of CD40+ THP-1 cells was 1.28% in the cells only control; 61.3% in 3 species co-culture; 80.85% in 7 species co-culture and 87.89% in 10 species co-culture. At this time point there was significant increases in the CD40+ populations in 3 ($p<0.01$), 7 ($p<0.001$) and 10 ($p<0.001$) species co-culture compared with the cells only control. Additionally, the CD40+ population in 10 species co-culture was significantly increased compared to 3 species co-culture at 24 hours ($p<0.05$).

The monocyte marker CD69 was measured following THP-1 cell co-culture with biofilms (Figure 6.8 iv) and on PMA and vitamin D_3 differentiated cells (Figure 6.9 iv). CD69 was present on 2.18% of PMA stimulated cells and 0.85% vitamin D_3 stimulated cells. At 4 hours the proportion of CD69+ THP-1 cells was 0.8% in the cells only control; 5.0% in 3 species co-culture; 3.17% in 7 species co-culture and 21.2% in 10 species co-culture. At 24 hours the proportion of CD69+ THP-1 cells was 0.3% in the cells only control; 10.6% in 3 species co-culture; 30.9% in 7 species co-culture and 26.5% in 10 species co-culture. At 24 hours, the CD69+ population was significantly increased in 3 ($p<0.05$), 7 ($p<0.01$) and 10 ($p<0.01$) species co-cultures compared with the cells only control. Additionally, the CD69+ population in 7 ($p<0.01$) and 10 ($p<0.01$) species co-cultures was significantly increased compared with 3 species co-cultures.

Collectively these data suggests that the co-culture with biofilms reduces undifferentiated THP-1 cell viability and up-regulates differentiation markers compared with the cells only control. Furthermore the mean fluorescent intensity (MFI) was also measured but so significant differences were observed. Additionally, data from the PMA and vitamin D_3 stimulated THP-1 cells suggest the THP-1 cells have differentiated from pro-monocytes following PMA or vitamin D_3 stimulation due to the CD14+ positive population; however, the lack of clear CD40 or CD69 positive populations within these groups does not allow definitive answers if PMA and vitamin D_3 promote a macrophage vs. monocyte phenotype.

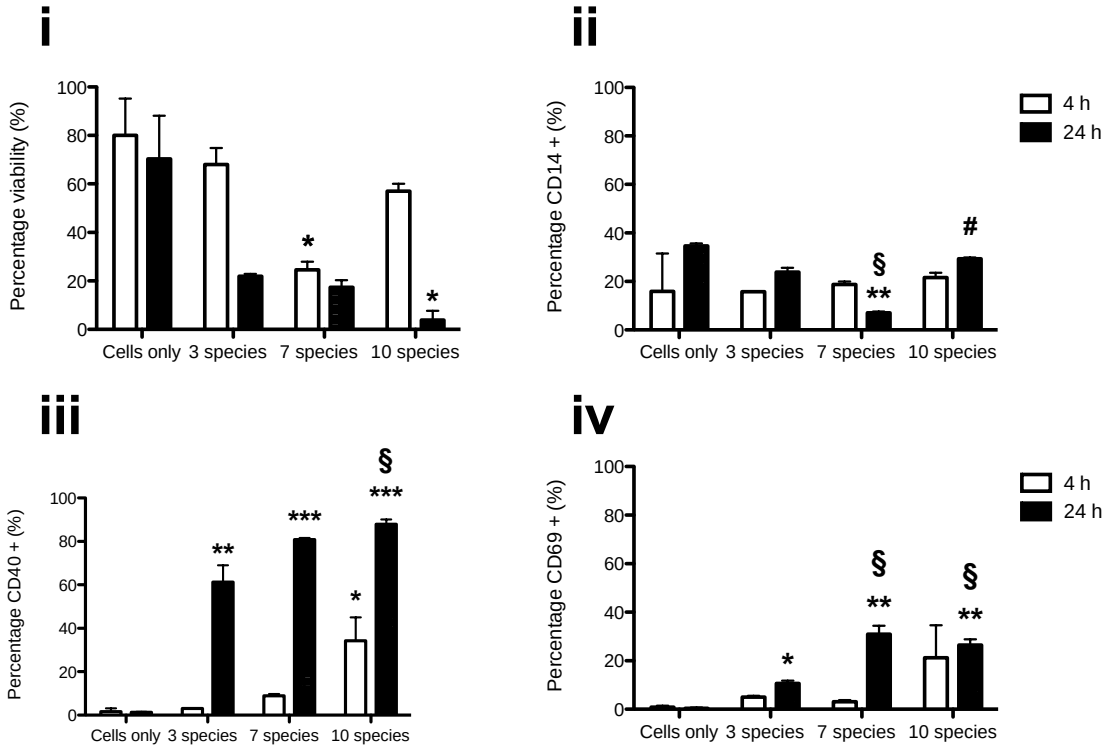


Figure 6.8: Cell viability and differentiation marker expression in THP-1 cells following culture

THP-1 cells were co-cultured with 3, 7 and 10 species biofilms for 4 and 24 hours. Cells cultured in media alone were used as a cells only control. Following culture cells were analysed by flow cytometry to investigate changes in cell viability [i], and cell differentiation using CD14 [ii], CD40 [iii] and CD69 [iv]. Data represent mean ± SD of two independent experiments performed in duplicate (compared with cells only * $p<0.05$, ** $p<0.01$ *** $p<0.001$; compared with 3 species biofilm § $p<0.05$; compared with 7 species biofilm # $p<0.05$). Statistical analysis was performed using a one-way ANOVA with Tukey's post-test to compare all groups at each time point.

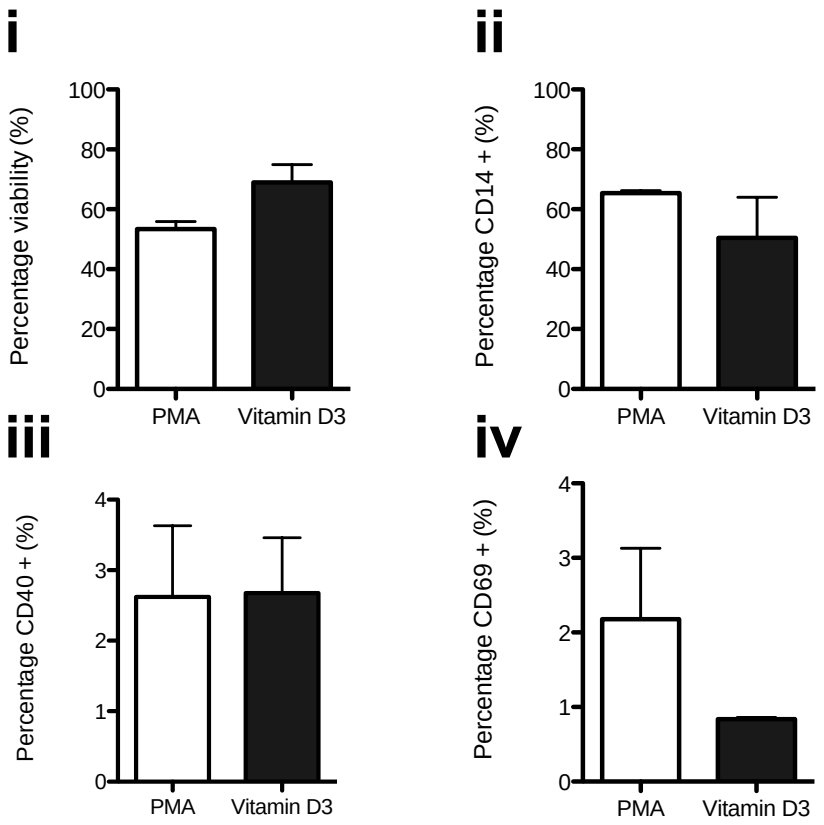


Figure 6.9: Cell viability and differentiation marker expression in THP-1 cells following PMA and Vitamin D_3 stimulation

THP-1 cells were stimulated with 100 nM vitamin D_3 or 200 nM PMA. Following culture cells were analysed by flow cytometry to investigate changes is cell viability [i], and cell differentiation using CD14 [ii], CD40 [iii] and CD69 [iv]. Data represent mean ± SD of two independent experiments performed in duplicate.

6.3.4 Differentiated THP-1 cells show distinct inflammatory gene and protein responses following direct co-culture

The previous data show that stimulation of undifferentiated THP-1 cells with oral biofilms, PMA or vitamin D_3 resulted in differential differentiation of the cells. Next, THP-1 cells differentiated with PMA or vitamin D_3 and subsequently co-cultured with biofilms were investigated to determine if biofilm composition modulates inflammatory gene expression or protein release in differentiated THP-1 cells.

THP-1 cells were differentiated with either PMA or vitamin D_3 before co-culture for 4 and 24 hours with 3, 7 and 10 species biofilms or in media alone as a cells only control. Following this, gene expression and protein release of IL-8, IL-1ß, CXCL5 and TNFα was measured (Figure 6.10). The results show biofilms differentially modulate THP-1 cell gene and protein response with significant differences occurring at both gene and protein level (Figure 6.11). However, the inflammatory gene and protein profiles of THP-1 cells stimulated with either PMA or vitamin D_3 were quite different.

At 4 hours, PMA differentiated THP-1 cell IL-8 protein release was 3.2 pg/mL in the cells only control; 542.1 pg/mL in the 3 species co-culture; 2132.7 pg/mL in 7 species co-culture and 838.01 pg/mL in the 10 species co-culture. Thus, after 4 hour exposure to biofilms there was significant increases in IL-8 protein expression following 7 ($p<0.001$) and 10 species ($p<0.05$) co-culture compared with the cells only control. At this time point IL-1ß protein release was 9.4 pg/mL in the cells only control; 22.8 pg/mL in 3 species co-culture, 94.1 in 7 species co-culture and 134.8 pg/mL in 10 species co-culture. IL-1ß protein release was significantly increased in 10 species co-culture compared with the cells only control ($p<0.05$) and 3 species co-culture ($p<0.05$).

At 24 hours, PMA differentiated THP-1 cell IL-8 protein release was 29.9 pg/mL in the cells only control; 736.45 pg/mL in 3 species co-culture; 3544.59 pg/mL in 7 species co-culture and 983.11 pg/mL in 10 species co-culture. At this time point only co-culture with 7 species biofilm caused significant IL-8 protein release compared with the cells only control ($p<0.001$). IL-1ß protein release was 6.8 pg/mL in the cells only control; 6.1 pg/mL in 3 species co-culture; 240.83 pg/mL in 7 species co-culture and 33.42 in 10 species co-culture. IL-1ß protein release was significantly increased in 7 species co-culture compared with the cells only control ($p<0.01$) and 3 species co-culture ($p<0.01$). At this time point IL-8 gene expression was 0.23% in the cell only control; 274.2% in 3 species co-culture; 862.2% in 7 species co-culture and 704.2% in 10 species co-culture. Thus, IL-8 gene expression was significantly increased in 7 species co-cultures compared with the cells only control ($p<0.05$).

At 4 hours, vitamin D_3 differentiated THP-1 cell IL-8 protein release was 21.7 pg/mL in the cells only control; 1148.8 pg/mL in 3 species co-culture; 1243.9 pg/mL in 7 species co-culture and 750.6 pg/mL in 10 species co-culture. Thus, the IL-8 protein release in 3 species ($p<0.001$), 7 species ($p<0.001$) and 10 species ($p<0.05$) co-cultures was significantly higher than the cells only control. At this time point IL-1ß protein release was 1.1 pg/mL in the cells only control; 129.8 pg/mL in 3 species co-culture, 1094.6 in 7 species co-culture and 376.5 pg/mL in 10 species co-culture. IL-1ß protein release was significantly increased in both 7 and 10 species co-cultures compared with the cells only control ($p<0.001$) and 3 species co-culture ($p<0.001$). IL-1ß protein release was also significantly increased in 7 species co-cultures compared with 10 species co-cultures ($p<0.001$). TNFα protein release was 12.9 pg/mL in the cells only control; 336.6 pg/mL in 3 species co-culture; 723.3 pg/mL in 7 species co-culture and 224.7 pg/mL in 10 species co-culture. CXCL5 protein release was significantly increased in 7 species co-cultures compared with the cells only control ($p<0.05$).

At 24 hours, vitamin D_3 differentiated THP-1 cell IL-8 protein release was 20.7 pg/mL in the cells only control; 1160.6 pg/mL in 3 species co-culture; 991.6 pg/mL in 7 species co-culture and 218.7 pg/mL in 10 species co-culture. Thus, the IL-8 protein release in 3 species ($p<0.001$) and 7 species ($p<0.001$) co-cultures was significantly higher than the cells only control. At this time point IL-1ß protein release was 1.0 pg/mL in the cells only control; 226.2 pg/mL in 3 species co-culture, 1165.8 in 7 species co-culture and 280.9 pg/mL in 10 species co-culture. IL-1ß protein release was significantly increased in both 7 and 10 species co-cultures compared with the cells only control ($p<0.001$) and 3 species co-culture ($p<0.001$). IL-1ß protein release was also significantly increased in 7 species co-cultures compared with 10 species co-cultures ($p<0.001$). CXCL5 protein release following co-culture at this time point was 1.88 pg/mL in the cells only control; 21.2 pg/ml in 3 species co-culture; 1.29 pg/mL in 7 species co-culture and 3.1 pg/mL in 10 species co-culture. Thus, CXCL5 protein release was significantly increased in co-culture with 3 species biofilms compared to all other conditions ($p<0.05$). TNFα protein release was 13.6 pg/mL in the cells only control; 211.9 pg/mL in 3 species co-culture; 130.5 pg/mL in 7 species co-culture and 83.3 pg/mL in 10 species co-culture. TNFα protein release was

significantly increased in 3 species co-cultures compared with the cells only control ($p<0.05$).

When measuring inflammatory gene expression of vitamin D_3 differentiated THP-1 cells, at 4 hours TNFα gene expression was 0.29% in the cells only control; 53.6% in 3 species; 174.6% in 7 species and 102.7% in 10 species co-culture. TNFα gene expression was significantly increased in 7 species co-cultures compared with the cells only control ($p<0.05$).

When measuring inflammatory gene expression of vitamin D_3 differentiated THP-1 cells, at 24 hours IL-8 gene expression was 1.19% in the cells only control; 619.2% in the 3 species; 1219.4 in 7 species and 3376.6% in 10 species co-culture. IL-8 gene expression was significantly increased in the 10 species co-culture compared with the cells only control ($p<0.001$). At 24 hours, IL-1ß gene expression was 1.9% in the cells only control; 237.5% in the 3 species co-culture; 566.7% in 7 species co-culture and 1036.7% in 10 species co-culture. IL-1ß gene expression was significantly increased in the 10 species co-culture compared with the cells only control ($p<0.01$) and 3 species co-culture ($p<0.01$). At this time point, CXCL5 gene expression was 1.32% in the cells only control; 1.88 pg/mL in 3 species; 14.4 pg/mL in 7 species and 13.2 in 10 species co-cultures. Thus significant increases in CXCL5 gene expression were observed in 7 ($p<0.05$) and 10 ($p<0.05$) species co-cultures compared with the cells only control. Additionally CXCL5 gene expression was significantly increases in 7 species co-cultures compared to 3 species co-cultures ($p<0.05$).

In an attempt to provide some overall context to the inflammatory gene expression and protein release of differentiated THP-1 cells following co-culture with biofilms the data at 4 and 24 hours have been presented as spider diagrams (Figure 6.11). These profiles show that the responses observed following 3, 7 and 10 species co-cultures are different, with a visible shift from the profile of the cells only controls. This representation shows the differences in inflammatory profiles between time points, with levels of inflammation typically increasing over time. Finally, these data shows different inflammatory profiles between PMA differentiated and vitamin D_3 differentiated THP-1 cells, highlighting that

the cells in different states of differentiation may respond differently to each biofilm.

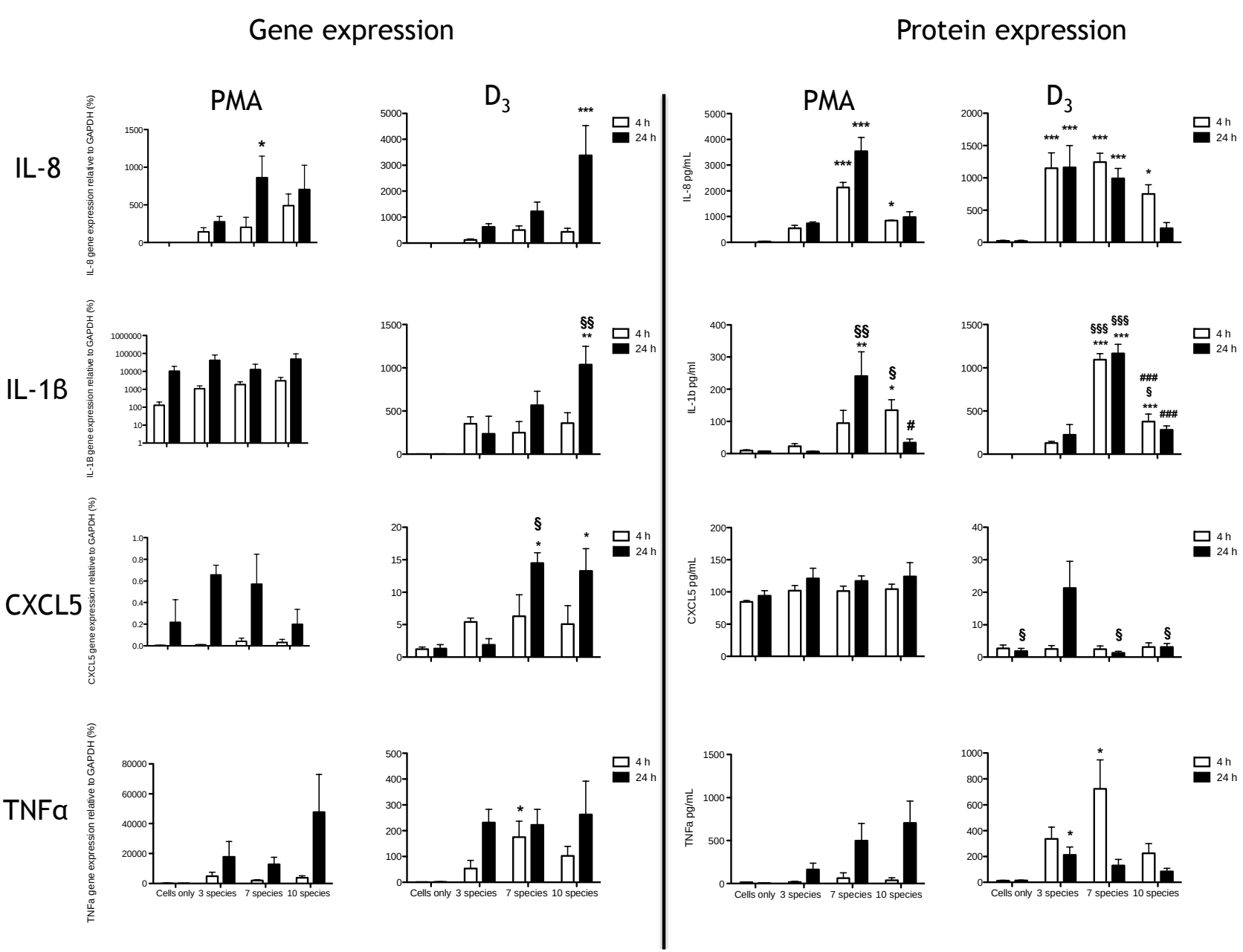
Collectively these data shows the biofilms differentially modulate THP-1 inflammatory gene and protein responses. Furthermore, the differentiation of THP-1 cells with either PMA or vitamin D_3 also plays a role in the inflammatory response observed following co-culture.

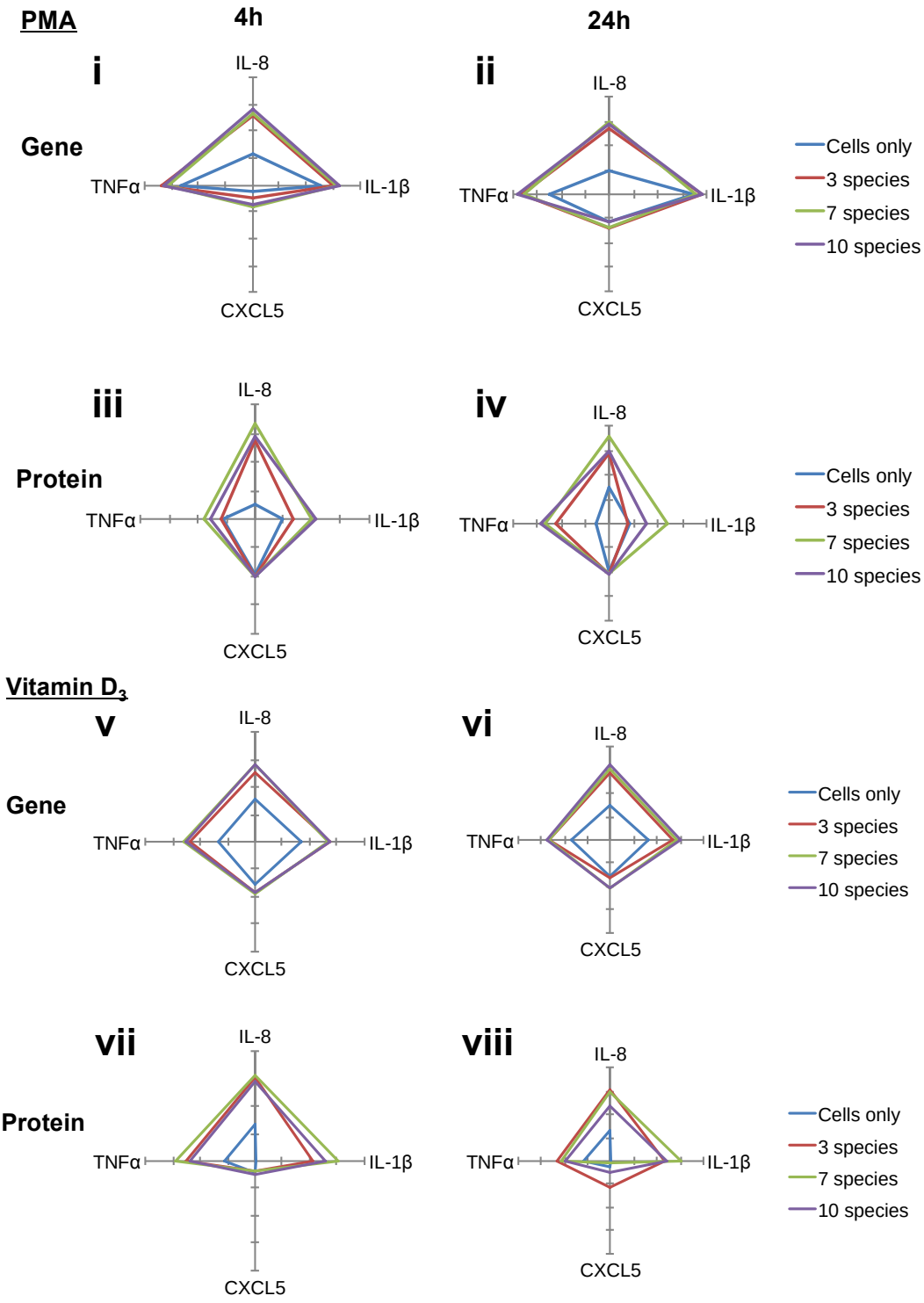
Figure 6.10: Inflammatory gene and protein expression of differentiated THP-1 cells following biofilm co-culture

THP-1 cells were differentiated using either PMA or vitamin D_3 and co-cultured for 4 and 24 hours with 3, 7 or 10 species biofilms. Cells cultured media only were used as cells only controls. IL-8, IL-1ß, CXCL5 and TNFα gene expression measured using SYBR® GreenER™ based qPCR relative to the housekeeping gene *GAPDH* (left panel). Protein release was measured by ELISA (right panel). Data represents mean ± SD of three independent experiments performed in duplicate (compared with cells only * $p<0.05$, ** $p<0.01$ *** $p<0.001$)(compared with 3 species biofilm § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$)(compared with 7 species # $p<0.05$, ## $p<0.01$, ### $p<0.001$). Statistical analysis was performed using a one-way ANOVA with Tukey's post test to compare all groups at each time point.

Figure 6.11: Spider diagram representation of the inflammatory profile of differentiated THP-1 cells co-cultured with multi-species biofilms

THP-1 cells were differentiated as in figure 6.10 using either PMA [i-iv] or vitamin D_3[v-viii] as then co-cultured for 4 [i, iii, iv, vii] and 24 [ii, iv, vi, viii] hours with 3, 7 or 10 species biofilms. Cells cultured in media only were used as cells only controls. IL-8, IL-1ß, CXCL5 and TNFα gene expression measured using SYBR® GreenER™ based qPCR relative to the housekeeping gene *GAPDH* [i, ii, v, vi]. Supernatants were also removed and protein release measured by ELISA [iii, iv, vii, viii]. Data represent mean of three independent experiments performed in duplicate.


Gene expression
Protein expression
PMA
D3
IL-8
IL-1β
CXCL5
TNFα
IL-8 gene expression relative to GAPDH (%)
IL-1B gene expression relative to GAPDH (%)
CXCL5 gene expression relative to GAPDH (%)
TNFa gene expression relative to GAPDH (%)
IL-8 pg/mL
IL-1b pg/ml
CXCL5 pg/mL
TNFa pg/mL
4 h
24 h
Cells only
3 species
7 species
10 species


PMA
4h
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i
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Gene
IL-8
TNFα
IL-1β
CXCL5
Cells only
3 species
7 species
10 species
iii
iv
Protein
Vitamin D3
v
vi
vii
viii

6.4 Discussion

PD results from the dysbiosis of interactions between oral microbial biofilms and the host immune system. In this chapter the interactions between multi-species biofilms and innate immune cells have been investigated in an attempt to further understand the response to biofilms and how biofilm composition may alter the immune response.

The main finding in this chapter is that biofilm composition can differentially influence a variety of activation and pro-inflammatory factors of neutrophils and cells of the monocytic lineage. Overall, the changes in pro-inflammatory gene and protein expression followed similar patterns to those observed in the previous chapter using epithelial cells. As biofilm complexity increases so too does inflammatory gene expression in neutrophils and monocytes in addition to monocyte cell adhesion, neutrophil NET formation and monocyte differentiation. The inflammatory profiles observed in response to each biofilm may have local and downstream affects on the immune system, particularly the adaptive immune response, which may ultimately dictate the overall inflammatory state observed in gingival health and PD.

The data in this chapter showed differential inflammatory gene and protein profiles by neutrophils in response to 3, 7 and 10 species biofilm in co-culture. Neutrophil IL-1β and TNFα gene expression was significantly increased in co-culture with 10 species biofilms and significantly increased levels of TNFα protein were present in co-cultures with 7 and 10 species biofilms. TNFα has been shown to have conflicting effects on neutrophil viability by promoting survival or enhancing apoptosis and it has been suggested TNFα related neutrophil survival is promoted through presence of inflammatory cytokines such as IL-8 (Walmsley et al., 2004, Cross et al., 2008). This may relate to the increased pro-survival phenotype of neutrophils found in periodontitis tissues (Lakschevitz et al., 2013). Furthermore, TNFα production triggers superoxide production from neutrophils which plays an important role in local tissue destruction a feature observed in the loss of gingival tissue in periodontitis patients (Kantarci et al., 2003). It would be of interest in future studies to investigate the impact of biofilms and their products on neutrophil superoxide

production. The cytokine IL-1β has shown to be present at higher levels in the gingivae and GCF of patients with PD than healthy controls (Engebretson et al., 2002). IL-1β has been shown to be an important factor in neutrophil recruitment into periodontal tissues and osteoclast bone resorption, a hallmark of periodontitis (Graves et al., 1998, Miller et al., 2007). Like TNFα, increases in IL-1β expression have also been proposed to promote neutrophil survival in PD which again may relate to the increased IL-1β gene expression observed when neutrophils were co-cultured with 10 species biofilms (Lakschevitz et al., 2013). The observations of neutrophils producing mediators such as IL-1β and TNFα in response to 7 and 10 species co-culture correlates with clinical studies which find numerous activated neutrophils in the gingivae in periodontitis, and the neutrophils appear to be associated with attachment loss and advanced periodontal destruction and have been postulated to directly contributed to the severity of tissue destruction and inflammation (Liu et al., 2001).

NETs are a recently discovered addition to the defensive capabilities of neutrophils (Brinkmann et al., 2004). The production of NETs occurs through a controlled apoptosis mechanism called NETosis and this has been observed in the periodontal pockets of patients with chronic periodontitis (Vitkov et al., 2009). Neutrophils co-cultured with multi-species biofilms appeared to cause differential formation of NET-like structures, with increasing levels of DNA release from the neutrophils as biofilm complexity increases. No histones were observed in any of the neutrophil cultures in this study. This may be due to inadequate staining or there may be a lack of true NETs in response to co-culture. Time limitations prevented extensive further optimisation of staining and sample preparation methods, which would be required to confirm the findings. Interestingly, increasing levels of neutrophil elastase was observed outside the cells in 3 and 7 species co-cultures with 10 species biofilms showed high levels on neutrophil elastase within the cells and some visible release in what appears to be neutrophil necrosis. This finding may be due to the time point at which the images were taken and to fully understand NET formation in response to biofilm co-cultures time-course imaging, and imaging specifically for different types of cell death, may be beneficial. It has been speculated that DNAse producing bacteria including *F. nucleatum, P. gingivalis* and *P. intermedia* are able to degrade the chromatin NET backbone and thus evade

killing neutrophil killing (Palmer et al., 2012). Additionally, the inability to distinguish bacterial DNA from neutrophil DNA is problematic, as oral biofilms are known to contain large amounts of extracellular DNA, which is visualised along with NETs during microscopy. Repeating the studies herein would allow further understanding of the interactions between oral biofilms and NET formation. Importantly, quantifying the viability of neutrophils following co-culture would allow us to determine if some of the DNA release observed during microscopy was due to necrosis. Differential inflammatory gene and protein responses were also observed following co-culture of neutrophils with biofilms. Cytokines including TNFα and IL-1ß have been shown to increase NET formation in neutrophils (Keshari et al., 2012). It would therefore be useful in future studies to investigate how cytokine release following co-culture with different biofilms can affect NET formation and if biofilm modulation can alter this response.

The main limitation of this work investigating the differential responses of neutrophils to oral biofilms is that the neutrophils used for this study are mouse bone marrow derived cells. These cells were readily available to our studies, and are frequently used for studies investigating neutrophil responses to oral pathogens due to the limited number of cells recovered from circulation (approximately 10-25% compared with 65-75% in human circulation); however the bone marrow contains neutrophils at different stages of maturation and hence may respond differently compared with mature, in vivo activated and differentiated neutrophils. For example, mouse bone marrow derived neutrophils require approximately 16 hours stimulation for 30% of the total cells to form shorter and more compact NETs, compared with human peripheral blood neutrophils that require 3-4 hours of stimulation for 80% of total cells to form NETS (Ermert et al., 2009).

The work in this chapter co-culturing THP-1 derived monocytes and macrophages with multi-species biofilms show differential gene and protein response when looking at each cell type with each biofilm. THP-1 cells are a pro-monocytic cell line derived from an acute myeloid leukaemia patient and therefore have been used extensively for studies of both monocyte and macrophage function *in vivo* due to the ease of acquisition compared to use of primary tissue macrophages

which do not easily expand in culture. Furthermore, there are numerous studies documenting differentiation of these cells down either macrophage or monocyte lineages studies use PMA and vitamin D_3 respectively (Schwende et al., 1996, Daigneault et al., 2010). During differentiation THP-1 cells become adherent to cell surfaces and increase their cytoplasmic cell ratio. In this chapter THP-1 cell adherence following stimulation following co-culture with biofilms was observed, with cell adhesion increasing as the complexity of the biofilm increased. Both PMA and vitamin D_3 were strong promoters of cell adhesion, with significant increases in cell adhesion at 24 hours, and the cells showed different morphological characteristics, with PMA promoted cell spreading on the surface while vitamin D_3 cells were still round in morphology when visualised by microscopy. Notably, THP-1 cells co-cultured with 10 species biofilms showed similar morphology to PMA treated cells and 7 species biofilms with cell stimulated with vitamin D_3. Studies have shown similar findings in the differences in THP-1 cell adhesion following exposure to vitamin D_3 or PMA which is associated with greater cell differentiation (Daigneault et al., 2010). Although at the time of writing no studies investigating THP-1 differentiation to oral biofilms have been undertaken it has been shown that stimulation of naïve THP-1 cells with *P. gingivalis* and *F. nucleatum* LPS causes up regulation of activation marker CD11b, CD11c and the MHC class II cell surface receptor HLA-DR over time compared with the cells only control (Baqui et al., 1999).

To further explore the impact of biofilm co-culture, THP-1 cells were examined by flow cytometry using makers for the monocyte (CD69/CD14) and macrophage (CD40/CD14) lineage. The proportion of THP-1 cells expressing CD14+ was reduced in 7 species co-cultures at 24 hours. CD14 is found on both monocytes and macrophages and is important for the detection of PAMPS such as bacterial LPS via TLR2/4 (Wright, 1995). In periodontitis patients decreased levels of membrane bound CD14 in periodontal pocket tissues and increased levels of soluble CD14 have been observed compared with healthy controls (Nicu et al., 2009, Jin et al., 2004). The reduction of CD14 observed in oral biofilm co-culture may be due to species such as *P. intermedia* and *P. gingivalis* degrading CD14 on the surface of monocytes (Sugawara et al., 2000, Duncan et al., 2004). Alternatively, CD14 may be lost due to the differentiation of these cells to a dendritic cell phenotype (Bullwinkel et al., 2011). To further understand this

finding soluble CD14 could be measured from the supernatants following co-culture.

THP-1 expression of both CD40 and CD69 was increased following co-culture with all biofilms at 24 hours. CD40 is a co-stimulatory molecule found on the surface of antigen presenting cells such as macrophages and dendritic cells and suggests that in oral biofilm co-culture THP-1 cells may be able to present antigen which in future studies could be tested by quantifying MCH class II on the surface of these cells. CD69 is an early activation marker which is predominantly associated with T cell activation, but has also been associated with monocyte and macrophage activation (Marzio et al., 1997, Wobke et al., 2013, Teixeira and Rumjanek, 2014). Collectively, the data suggest that THP-1 cells become activated. However, due to the limited surface markers used for this study no definitive conclusions can be given on the phenotype of THP-1 cells following co-culture, and the exact nature of their differentiation status remains to be determined. THP-1 cells have been shown to differentiate into monocyte-like, macrophage-like and dendritic cell-like states using vitamin D_3, PMA and ionophores respectively (Daigneault et al., 2010, Santegoets et al., 2008). Therefore, future work to examine the phenotype would benefit from additional markers such as MCH Class II (antigen presentation), CD11b (Macrophage phenotype), CD11c (DC phenotype), CD206 (M2 subtype), CD16/32 (M1 subtype) to differentiate monocytes, macrophages and dendritic cell linages and markers to differentiate M1 and M2 macrophages.

Cells of the monocyte lineage also produce inflammatory cytokines which play an essential role in immune cell activation and cell recruitment (Skovbjerg et al., 2010). Co-culture of THP-1 cells differentiated with either PMA or Vitamin with oral biofilms showed differing inflammatory gene and protein profiles in response to 3, 7 and 10 species biofilm co culture when investigating IL-8, IL-1ß, CXCL5 and TNFα. The data in this chapter show that PMA differentiated THP-1 cells increased IL-8 gene and protein expression and IL-1ß gene expression when exposed to 7 species biofilms. Vitamin D_3 stimulated THP-1 cells were much more sensitive to co-culture and showed increased gene expression of all cytokines when co-cultured with 7 and 10 species biofilms as well as increases in protein expression of IL-8, IL-1ß and TNFα. Studies have shown monocytes

produce more IL-1β following LPS stimulation due to the constitutive activation of caspase-1 where as macrophages require a secondary signal for activation (Netea et al., 2009). Additionally, studies using LPS to stimulate monocytes and macrophages and measure subsequent TNFα production observed greater levels of TNFα protein release in monocytes than macrophages, although other studies have observed the opposite effect (Burchett et al., 1988, Gessani et al., 1993). In this study both PMA and vitamin D_3 stimulated cells showed greatest increase in cytokine gene expression when cultured with 10 species biofilm and a greatest increase in protein expression when cultured with 7 species biofilm; which suggests biofilm composition may play a role in the cytokine response to THP-1 cells. Studies have shown human monocytes and tissue macrophages cultured with Gram-positive bacteria produced a more pro-inflammatory profile, high in IL-12, IFNγ and TNFα compared with Gram-negative bacteria which promoted IL-10, IL-6 and IL-8 and PGE_2 (Hessle et al., 2000, Hessle et al., 2003, Skovbjerg et al., 2010). By expanding the investigation monocyte/macrophage cytokines investigated in this chapter a greater understanding of the unique response to each oral biofilm following co-culture would be available. Additionally this work would be a prelude to using THP-1 cells as APCs which could be used for T cell stimulation studies following co-culture. This would allow further understanding of the functional relevance of how biofilm composition differentially activated such cells and the downstream inflammatory profiles produced.

The protocol used in this chapter to differentiate monocytes using PMA was adapted from *Daigneault et. al.* (2010) and their work showed using this differentiation protocol all PMA differentiated cells were classically activated and following stimulation with bacterial LPS produced significantly higher levels of IL-1β and TNFα protein than vitamin D_3 differentiated cells which is in line with the findings in figure 6.10 (Daigneault et al., 2010). Futures studies would seek to further explore the functional relevance of the biofilm impact on THP1 differentiation. The release of a broader range of cytokines would be of interest, for example IL-10 and IL-12. Cytokine regulation may contribute to the failure of macrophages to mount an M1 pro-inflammatory response to oral bacteria such as *P. gingivalis* and *A. actinomycetemcomitans* (Muthukuru et al., 2005, Tanabe and Grenier, 2008).

The work in this chapter is a preliminary study into immune cell and oral biofilm interactions, with a focus on how biofilm composition may differentially modulate the host immune response. The few existing studies which investigate the role of oral biofilm composition on the host inflammatory response, have generally focused on gingival epithelial cells (Peyyala et al., 2013). However, there is great potential for these models to be used to study numerous immune cell types to understand their role in PD. Furthermore, this model allows the generation of conditioned media from cells in co-culture which can be used to stimulate other cell types. It would be interesting to further develop the model into a system which incorporates multiple cells types to investigate the response of both neutrophils and monocytes/macrophages singly and in combinations with different oral biofilms to enhance our understanding or the ability of oral biofilms to modulate the host immune response.

CHAPTER FINDINGS

There is increased expression of pro-inflammatory cytokines by neutrophils in response to 7 and 10 species biofilms

Biofilm composition differentially affects neutrophil NET formation in co-culture

Pro-monocytes increase cell adhesion and IL-8 protein release as biofilm complexity increases in co-culture

There is increased expression of pro-inflammatory cytokines by PMA and vitamin D_3 differentiated pro-monocytes in response to 7 and 10 species biofilms

7 Assessing *in vitro* oral biofilm models for testing potential actives

7.1 Introduction

Due to the complex nature of PD, treatment is difficult. Dentists perform mechanical debridement on teeth to remove both living and calcified bacterial biofilms from the tooth surface in an attempt to lessen the bacterial load to halt the progression of the disease. However, while plaque is necessary, it is not sufficient to cause disease, host: biofilm dysregulation can persist with biofilm re-development beginning within hours of professional cleaning (Teles et al., 2012).

Limiting the accumulation of dental plaque by brushing the teeth daily is the optimal strategy for preventing PD through disruption of the oral biofilm (Lamster, 2006). However, clinical reports and population studies report a large number of the population do not comply sufficiently with this process (Villa et al., 2012, Ciancio, 2003). Additionally, due to illness, having recently undergone surgery, or having fixed orthodontics means some patients are unable to effectively brush their teeth and require supplementary methods to maintain oral health (Pithon et al., 2015, Shi et al., 2013, Burtner et al., 1991). Antimicrobial mouthwashes can supplement oral hygiene and provide an alternative to brushing for those who are unable to (Barnett, 2003). Chlorhexidine (CHX) is widely considered the 'gold standard' due to its superior bactericidal and bacteriostatic properties (Herrera, 2013). Additionally, CHX boasts broad spectrum antimicrobial effects against bacteria, fungi and viruses, and exhibits prolonged substantivity due to its high protein binding onto the pellicle on the surface of teeth (Baqui et al., 2001, Salim et al., 2013, Hannig et al., 2013). Studies have also shown combining scaling and root planning (SRP), the typical treatment for PD, combined with CHX rinsing results in significant improved scores of plaque index, bleeding on probing, probing depth and clinical attachment level compared with SRP alone (Faveri et al., 2006, Feres et al., 2009, Stratul et al., 2010). However, reports have observed that prolonged use of CHX may also be detrimental due to associations with staining of the tooth surface, taste alterations, tongue discolouration and pain of the oral mucosa (Flotra et al., 1971, Najafi et al., 2012, Frank et al., 2001). Additionally various cases of allergic responses including anaphylaxis to CHX have been reported both after single exposure and repeated use (Dyer et al., 2013, Nakonechna et al.,

2014). Due to these adverse reactions many new compounds are tested as alternatives in an attempt to find a compound which has the antimicrobial potency of CHX, but with minimal side effects.

Host modulatory therapy has also been proposed as a treatment for PD due to the role of inflammation in the progression of the disease (Van Dyke, 2008). A variety of compounds have been proposed and tested in experimental periodontal models (Deore et al., 2014, Abe et al., 2012). One such group are polyphenols, which naturally occur in plants such as grapes, have become a focus for oral therapies due to reports of anti-bacterial and anti-inflammatory properties (Palaska et al., 2013). Recent studies have investigated the antimicrobial properties of a range of polyphenols against periodontal bacteria, observing inhibition of planktonic growth and biofilm formation following treatment (Shahzad et al., 2015). Resveratrol (RSV) is a naturally derived polyphenol which has been reported to be anti-inflammatory in periodontitis rat models. Other studies have found RSV to be antimicrobial and able to inhibit microbial and fungal biofilm formation (Casati et al., 2013, Tamaki et al., 2014, Lee et al., 2014). These studies highlight the potential of polyphenols, in particular RSV due to its anti-microbial and anti-inflammatory activities, therefore further work into how such compounds can alter host: biofilm interactions in the oral cavity is worth further investigation.

7.2 Aims

The aims of this chapter were to investigate the potential of the co-culture models for evaluating actives. The following key questions were investigated:

- Can a 4 species co-culture model be used to evaluate both antimicrobial and anti-inflammatory actives?

- Are 3, 7 and 10 species biofilm models differentially affected by antimicrobial agents (CHX)?

- Does treatment of 3, 7, and 10 species biofilm with antimicrobial agents (CHX) subsequently affect oral epithelial pro-inflammatory responses in co-culture?

The data represented in this chapter has been published in:

Millhouse, E., Jose, A., Sherry, L., Lappin, D., Patel, N., Middleton, A., Pratten, J., Culshaw, S. and Ramage, G., 2014. Development of an *in vitro* periodontal biofilm model for assessing antimicrobial and host modulatory effects of bioactive molecules. BMC Oral Health, 14:80

The work in this chapter using the 4 species biofilm model was completed with the help of Leighann Sherry.

7.3 Results

7.3.1 RSV is toxic to oral epithelial cells at high concentrations

When investigating the role of actives as potential therapeutic agents in co-culture it is important to examine the toxicity of these compounds on host cells as well as the oral biofilms themselves to understand their impact on the host inflammatory response measured. To optimise the concentrations of RSV and CHX and treatment times, cytotoxicity tests were undertaken on both oral epithelial cells and biofilms. First, OKF6-TERT2 oral epithelial cells were treated for 0, 2, 10 and 30 minutes with three concentrations of CHX (0.01, 0.05 and 0.2% v/v) and RSV (0.01, 0.05, 0.5% w/v) before washing with PBS and incubating for 4 and 24 hours, after which time cell viability was assessed using the cell viability dye AlamarBlue® as described in section 2.3.12.

The data showed that all treatments (0 - 30 minutes using 0 – 0.2% CHX) with CHX resulted in a significant decrease in epithelial cell viability, with a decrease from 96% in the untreated controls to less than 3% ($p<0.001$) for all treatment times and concentrations at 4 hours. After 24 hours in culture 76% viability in untreated epithelial cells was reduced to 0% ($p<0.001$) following all treatment times and concentrations (Figure 7.1 i-iii). Treatments of 0 – 30 minutes with 0.01% RSV showed no significant decrease at either 4 or 24 hours (Figure 7.1 iv). Treatment with 0.05% RSV caused a significant decrease in cell viability after 4 hours, with cell viability reduced to 56% ($p<0.01$), 58% ($p<0.01$) and 51% ($p<0.01$) at 2, 10 and 30 minute treatment times, respectively (Figure 7.1v). After 24 hour culture, treatment with 0.05% RSV cell viability again significantly reduced to 24% ($p<0.01$), 35% ($p<0.01$) and 41% ($p<0.01$) following 2, 10 and 30 minute treatment times. Finally, treatment of cells with 0.5% RSV resulted in significant decreases in cell viability at all time points ($p<0.001$) (Figure 7.1vi), with viability ranging from 40-33% at 4 hours and 9-12 % at 24 hours. From these data the treatment time taken forward for the reminder of the study was 30 minutes.

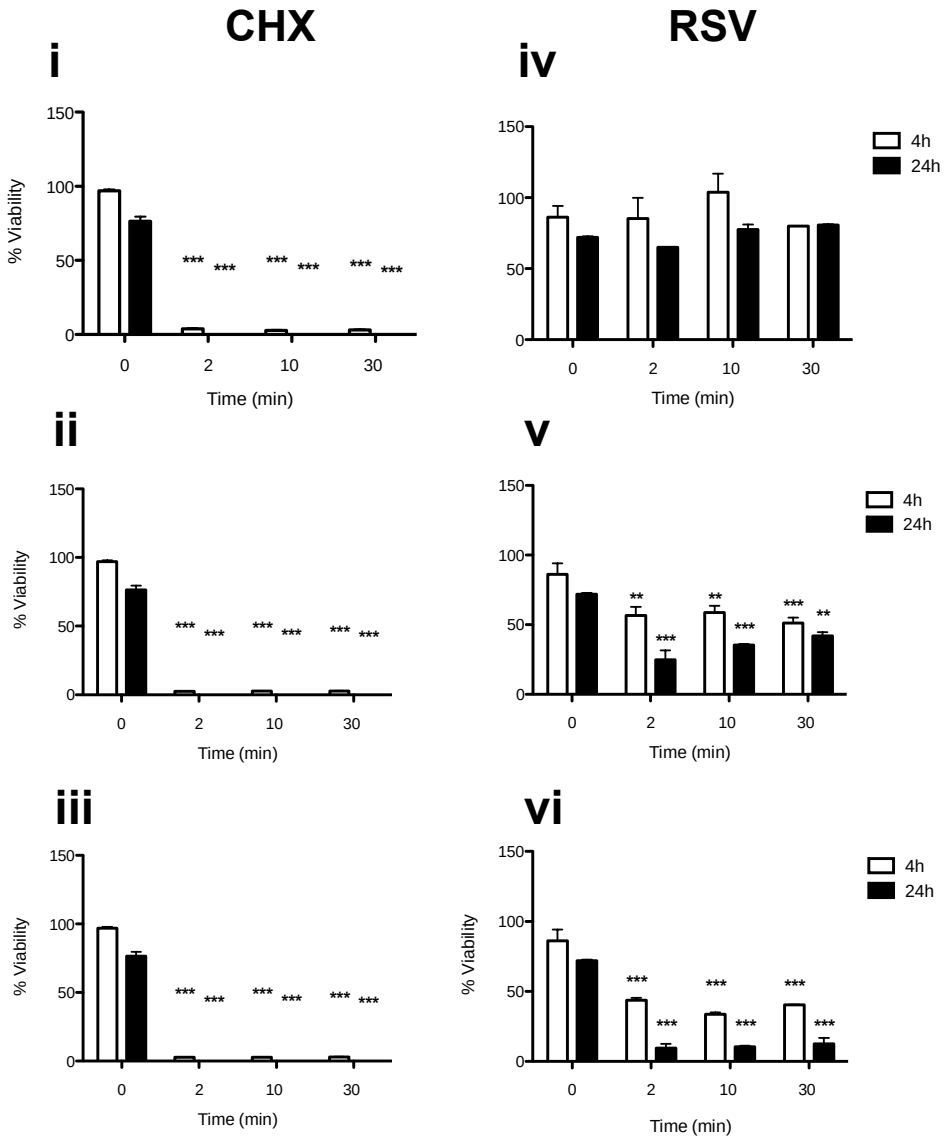


Figure 7.1: Cytotoxicity of CHX and RSV treatment on oral epithelial cells

The oral epithelial cell line OKF6-TERT2 was seeded at 1 x 10^5 cells/mL in 24 well plates for toxicity studies. Cells were treated with concentrations of CHX (0.01 [i], 0.05 [ii] and 0.2% [iii] v/v) and RSV (0.01 [iv], 0.05 [v], 0.5% [vi] w/v) for a range of times (0 - 30 minutes) before washing with PBS. Cells were then returned cultured for a further 4 hours in 5% CO_2 in d-KSFM containing 10% AlamarBlue® before removal of media to be used to measure viability. Cell viability was assessed using the AlamarBlue® assay with absorbance of media read at 570 nm and 600 nm. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (** $p<0.01$, *** $p<0.001$) Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post test to compare all groups with the 0 minute treatment time-point.

7.3.2 RSV treatment does not affect biofilm viability

To measure the antimicrobial properties of the actives, multi-species biofilms containing *S. mitis, F. nucleatum, P. gingivalis* and *A. actinomycetemcomitans* were then treated for 30 minutes with each concentration of CHX and RSV before washing with PBS and viability measured using the metabolic dye AlamarBlue®. RSV caused no significant decrease in biofilm viability at any concentrations (Figure 7.2 i); however, treatment with 0.05 and 0.2% v/v CHX showed significant decreases in biofilm viability compared to the untreated control. Biofilm viability was decreased to 5% and 6% with 0.05% ($p<0.001$) and 0.2% ($p<0.001$) CHX treatment, respectively (Figure 7.2 ii). Based on these data the concentrations taken forward for the remainder of the study were 0.2% v/v CHX and 0.01% RSV.

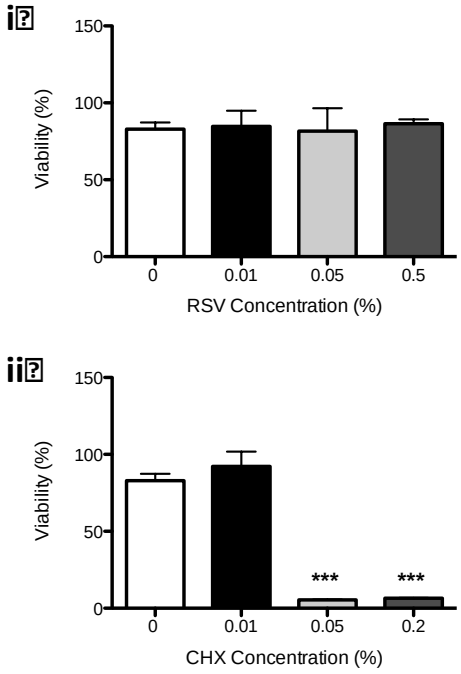


Figure 7.2: Cytotoxicity of CHX and RSV on biofilms
Multi-species biofilms were grown on Thermanox™ coverslips and treated with RSV (0, 0.01, 0.05% w/v) (i) or CHX (0, 0.01, 0.05, 0.2% v/v) (ii) and biofilm viability measured using AlamarBlue®. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (*** $p<0.001$). Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test to compare all groups to the 0% concentration treatment.

7.3.3 RSV does not affect biofilm composition

To determine if treatment with these actives altered the species composition of the biofilm, they were again treated for 30 minutes with either CHX (0.2% v/v) or RSV (0.01% w/v). Following this biofilms were washed in PBS, DNA was extracted and quantification of each species performed by qPCR (Figure 7.3). The data showed no significant change in the composition of the biofilm directly following treatment compared to the untreated biofilm. To further investigate this effect, SEM analysis was performed to examine the impact of each treatment on the architecture of the biofilm (Figure 7.4 i-vi). At both magnifications CHX appeared to destabilise the biofilm, as the complexity of the biofilm was visually reduced (Figure 7.4 ii, v). However, RSV appeared to have no visual effect on the physical architecture (Figure 7.4 iii, vi).

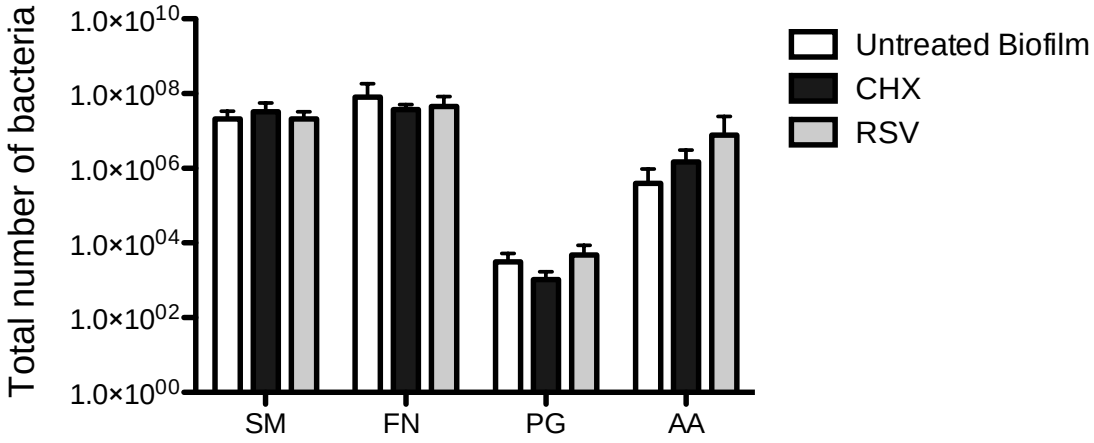


Figure 7.3: Biofilm composition following CHX and RSV treatment
Multi-species biofilms were grown on Thermanox™ coverslips and treated with RSV (0, 0.01, 0.05% w/v) or CHX (0, 0.01, 0.05, 0.2% v/v). Biofilms were washed with PBS and DNA was extracted for quantification of each species using SYBR® GreenER™ based qPCR. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test to compare all treatments to the untreated biofilm.

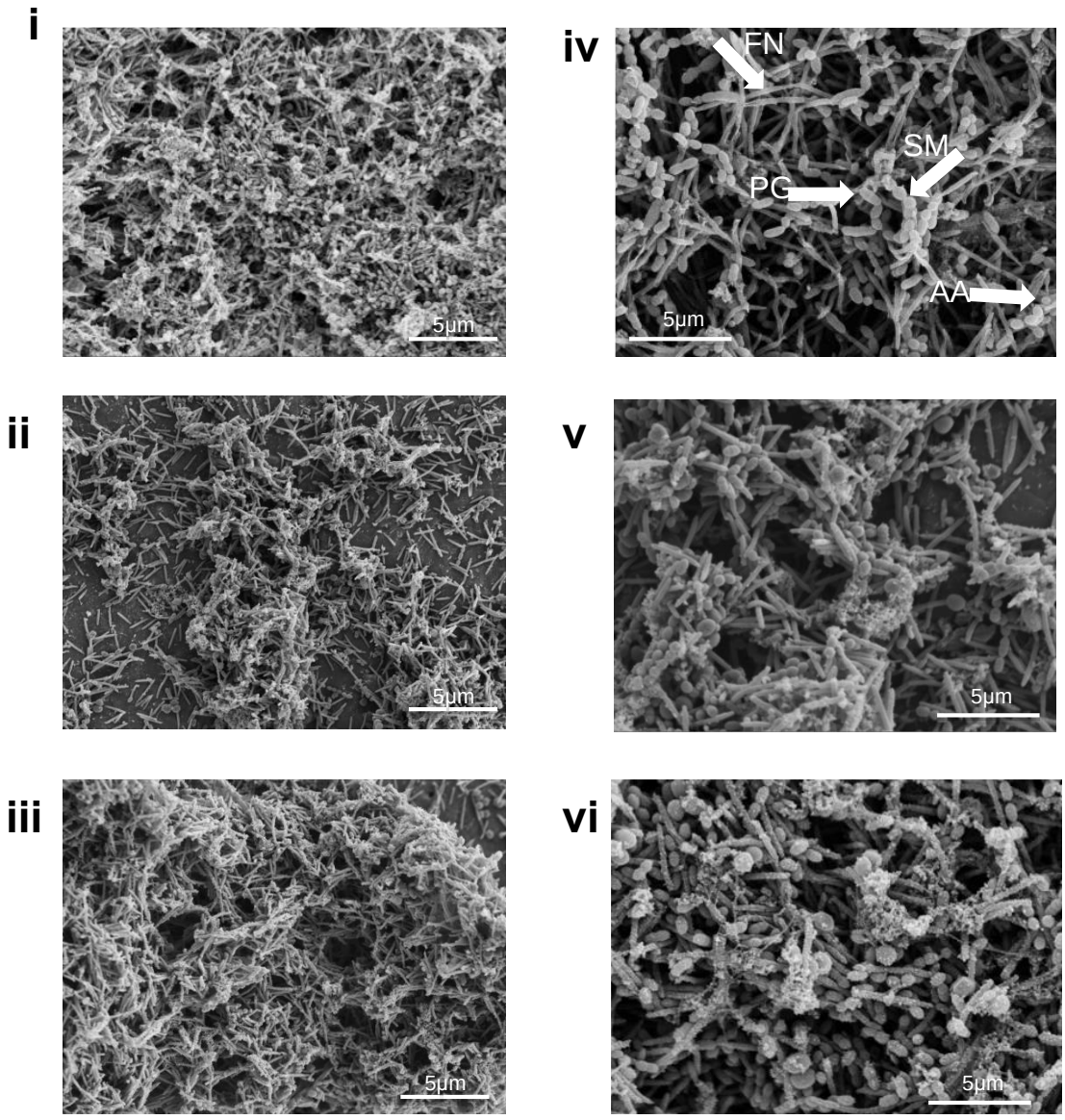


Figure 7.4: SEM analysis of biofilms following CHX and RSV treatment

Biofilms were analysed by SEM at 2000 x (i, ii, iii) and 5000 x (iv, v, vi). Biofilms were treated with either CHX 0.2% v/v (ii, v), RSV 0.01% w/v (iii, vi) and compared to an untreated control (i, iv). *S. mitis* (SM), *F. nucleatum* (FN), *P.gingivalis* (PG) and *A. actinomycetemcomitans* (AA) are annotated on the untreated control. Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope.

7.3.4 RSV alters the expression of pro-inflammatory mediators

To determine whether the actives had additional effects these compounds were tested for their ability to modulate a biological response from the OKF6-TERT2 oral epithelial cell line. This was investigated by measuring changes in pro-inflammatory mediators at the gene and protein level. To quantify this the co-culture model described in section 2.2.3 was used with the simple 4 species biofilm model containing *S. mitis*, *F. nucleatum*, *P. gingivalis* and *A. actinomycetemcomitans* (Sherry et al., 2013). Treatments of either biofilms with 0.2% v/v CHX for 30 minutes or OKF6-TERT2 oral epithelial cells with 0.01% w/v RSV was performed prior to co-culture for 4 or 24 hours.

Initially, IL-8 gene expression was measured by SYBR® GreenER™ based qPCR (Figure 7.5). No significant differences were observed in the IL-8 gene expression of RSV treated OKF6-TERT2 oral epithelial cells when co-cultured for 4 and 24 hours compared with the untreated biofilm control (Figure 7 i). Treatment of the biofilm with CHX prior to co-culture did not affect the subsequent epithelial cell expression of IL-8 expression after 4 hour co-culture, however, significant decreases in the IL-8 measured after 24 hours was observed (Figure 7.5 ii). At 24 hours IL-8 gene percentage expression was significantly reduced from 20% in untreated biofilm co-cultures to 3.6% ($p<0.001$) in co-cultures where biofilms were pre-treated with 0.2 v/v CHX.

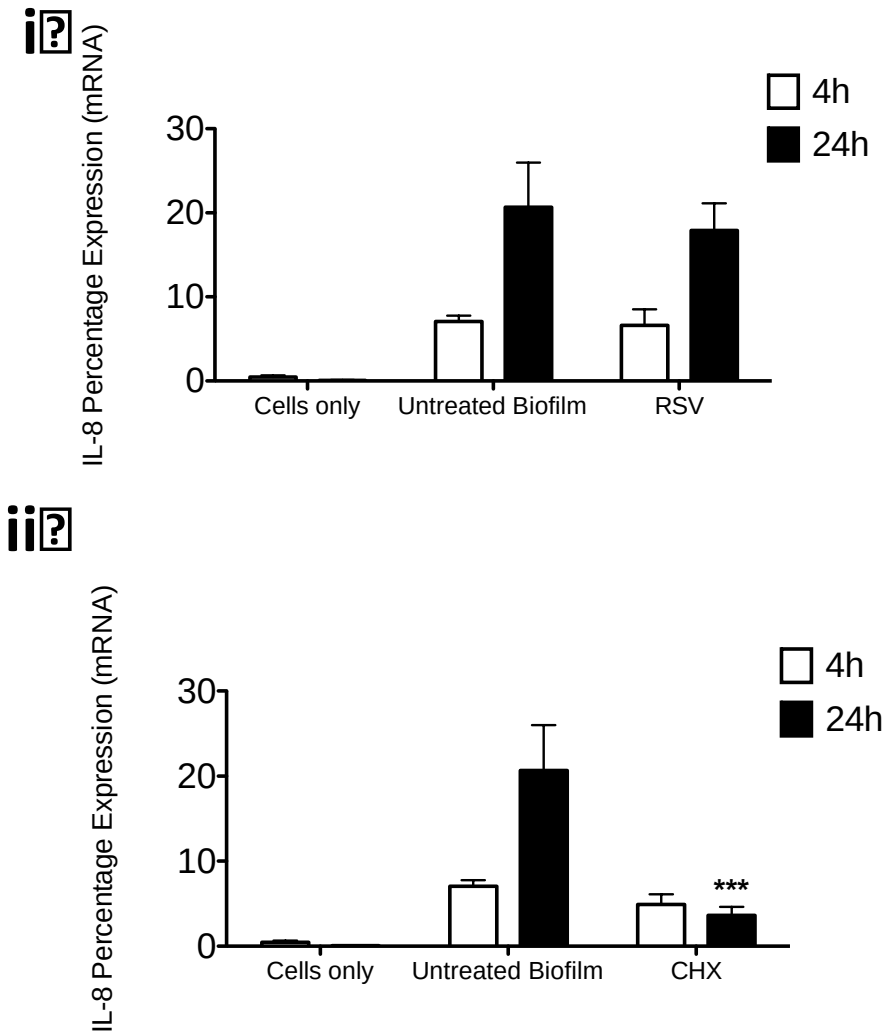


Figure 7.5: IL-8 gene response by OKF6-TERT2 cells in co-culture following treatment with actives

OKF6-TERT2 cells were pre-treated with (i) 0.01% w/v RSV or (ii) multi-species biofilms were pre-treated with 0.2% v/v CHX for 30 minutes before washing with PBS and then co-cultured for 4 and 24 hours with untreated biofilms or cells, respectively. Controls included cells in media only (cells only) and untreated cells in co-culture with an untreated biofilm (untreated biofilm). RNA was extracted from cells at each time point, cDNA was synthesized and IL-8 gene expression measured using SYBR® GreenER™ based qPCR relative to the housekeeping gene *GAPDH*. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (*** $p<0.001$). Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test to compare all groups to each other.

The simultaneous gene expression of multiple pro-inflammatory cytokines produced by OKF6-TERT2 oral epithelial cells was investigated further using the RT^2 Profiler assay (Figure 7.6). After 30 minute pre-treatment of biofilms with either 0.2% v/v CHX or 30 minute pre-treatment of epithelial cells with 0.01% w/v RSV differences in gene expression we measured after 4 hour co-culture. Following treatment of biofilms with CHX a 140.8 ($p<0.05$) fold decrease in epithelial cell IL-8 gene expression was observed in co-culture compared to the untreated controls (Figure 7.6i). No significant differences were observed in any genes following RSV treatment of epithelial cells. (Figure 7.6 ii).

Finally, IL-8 protein expression by OKF6-TERT2 oral epithelial cells in co-culture was investigated (Figure 7.7). As previously described, OKF6-TERT2 cells were treated with 0.01% w/v RSV or biofilms were treated with 0.2% v/v for 30 minutes prior to co-culture for 4 and 24 hours. Treatment of cells with RSV significantly reduced the IL-8 protein measured after co-culture from 534.87 pg/mL in the untreated control to 20.88 pg/mL ($p<0.001$) with treatment at 4 hours and 271.30 pg/mL in the untreated control to 65.59 pg/mL ($p<0.01$) with treatment at 24 hours (Figure 7.6 i). CHX treatment also significantly reduced the IL-8 present after co-culture with 54.26 pg/mL ($p<0.001$) and 20.01 pg/mL ($p<0.001$) measured and 4 and 24 hours, respectively (Figure 7.6 ii).

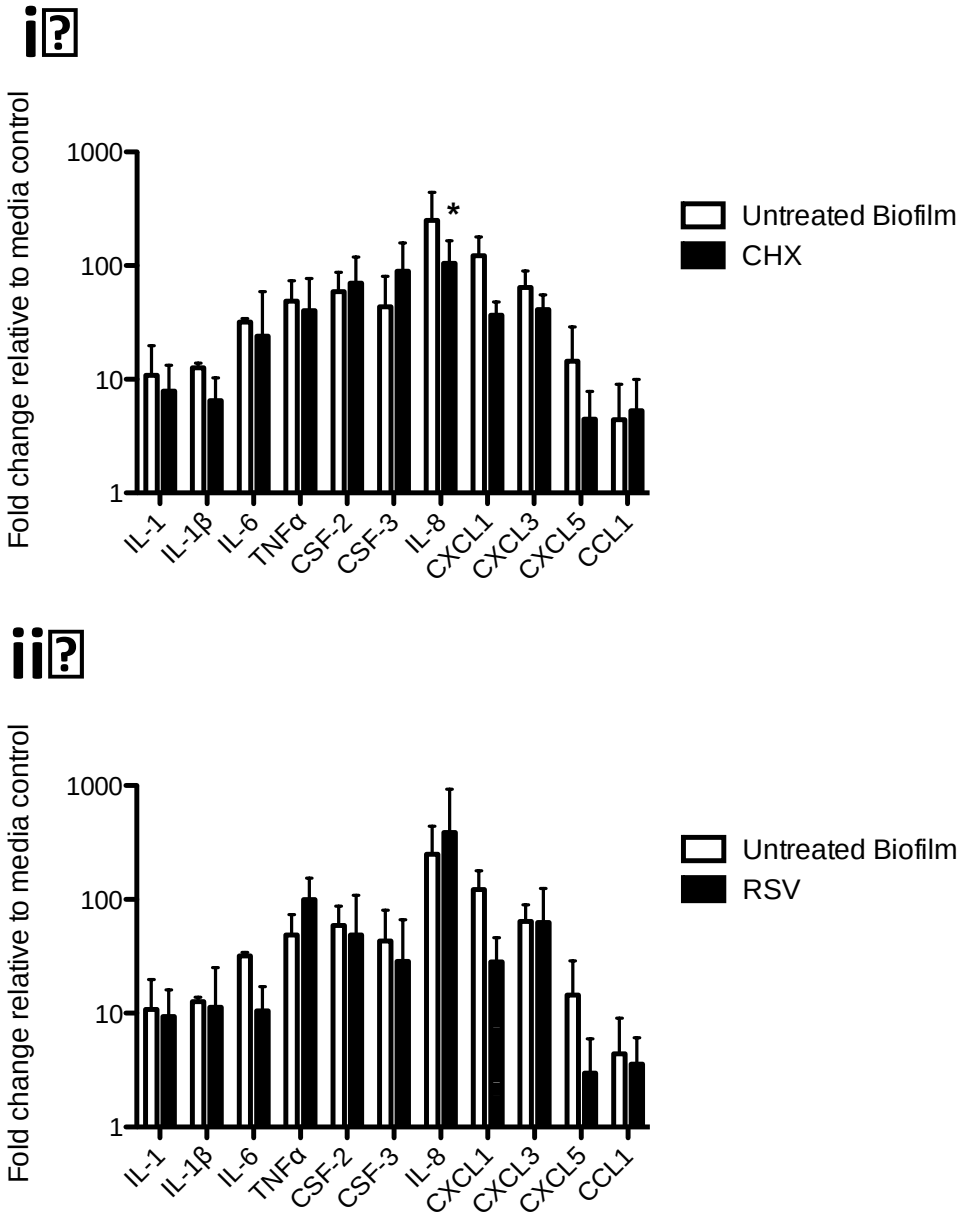


Figure 7.6: Pro-inflammatory gene response by OKF6-TERT2 cells in co-culture following treatment with actives

Multi-species biofilms were pre-treated with 0.2% v/v CHX (i) or OKF6-TERT2 cells were pre-treated with 0.01% w/v RSV (ii) for 30 minutes before washing with PBS and then co-cultured for 4 hours with untreated biofilms or cells, respectively. Untreated controls were included. RNA was extracted from cells at each time point, cDNA was synthesized and pro-inflammatory cytokine gene expression measured using the RT^2 Profiler. Samples are normalised to the housekeeping gene *GAPDH* and relative to the cells only control. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (* $p<0.05$). Statistical analysis was performed using a two-tailed unpaired t test.

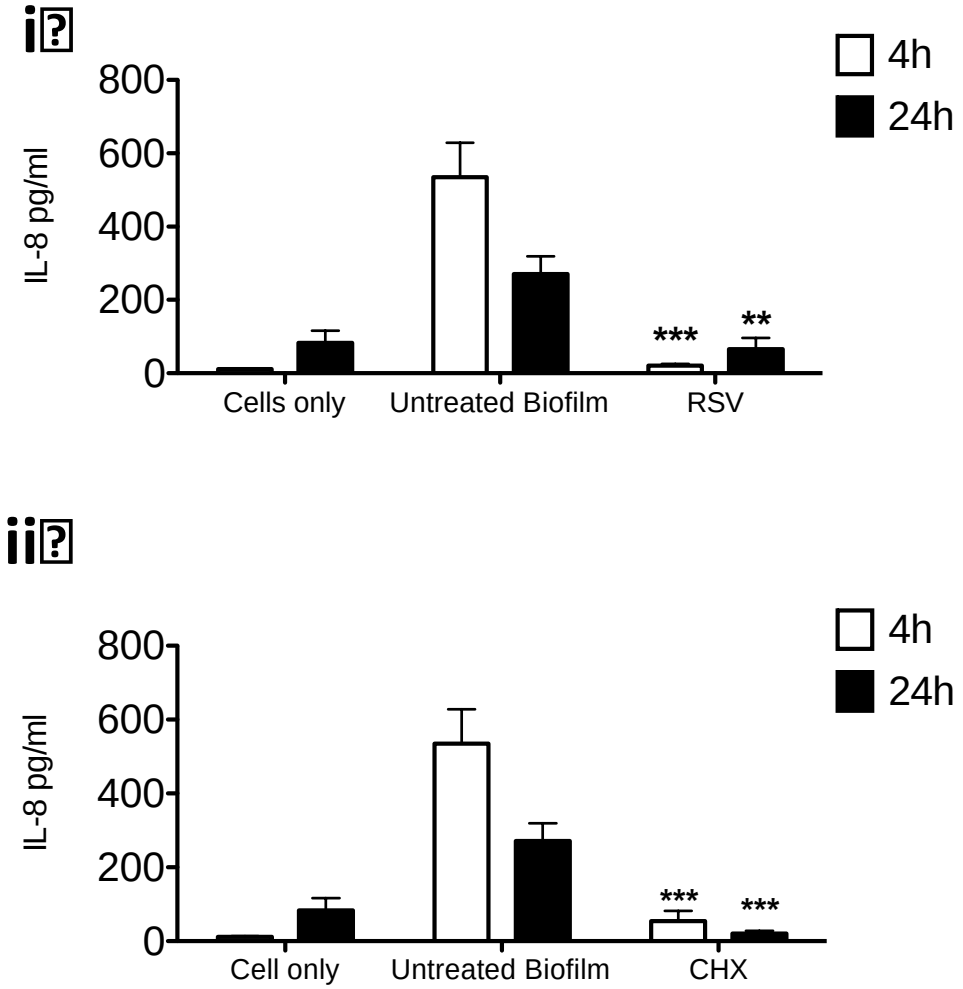


Figure 7.7: IL-8 protein response by OKF6-TERT2 cells in co-culture following treatment with actives

OKF6-TERT2 cells were pre-treated with 0.01% w/v RSV (i) or multi-species biofilms were pre-treated with 0.2% v/v CHX (ii) for 30 minutes before washing with PBS and then co-cultured for 4 and 24 hours with untreated biofilms or cells respectively. Controls included cells in media only (cells only) and untreated cells in co-culture with an untreated biofilm (untreated biofilm). Protein release was measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (** $p<0.01$, *** $p<0.001$). Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test to compare all groups to each other.

7.3.5 CHX treatment affects viability and composition of biofilms

Having confirmed the simple multi-species biofilm model was suitable for use evaluating the potential of actives in PD, the potential of the complex multi-species biofilm models was evaluated. To investigate this 0.2% v/v CHX treatment for 30 minutes on biofilms as performed in the previous work was used for the remainder of this study. CHX toxicity to OKF6-TERT2 oral epithelial cell viability was previously investigated (Figure 7.1). Using 3, 7 and 10 species biofilm models described in section 2.1.5 the viability of each biofilm after 30 minute treatment of 0.2% v/v CHX was investigated (Figure 7.8). The antimicrobial activity of CHX was shown to significantly reduce biofilm viability by 58% ($p<0.001$), 55% ($p<0.001$) and 59% ($p<0.001$) for 3, 7 and 10 species biofilms, respectively, compared to the untreated control biofilms. Additionally significant differences ($p<0.01$) were observed when comparing the viability between the CHX treated biofilms, with an average of 8.6%, 18.6% and 13.5% viability for 3, 7 and 10 species respectively.

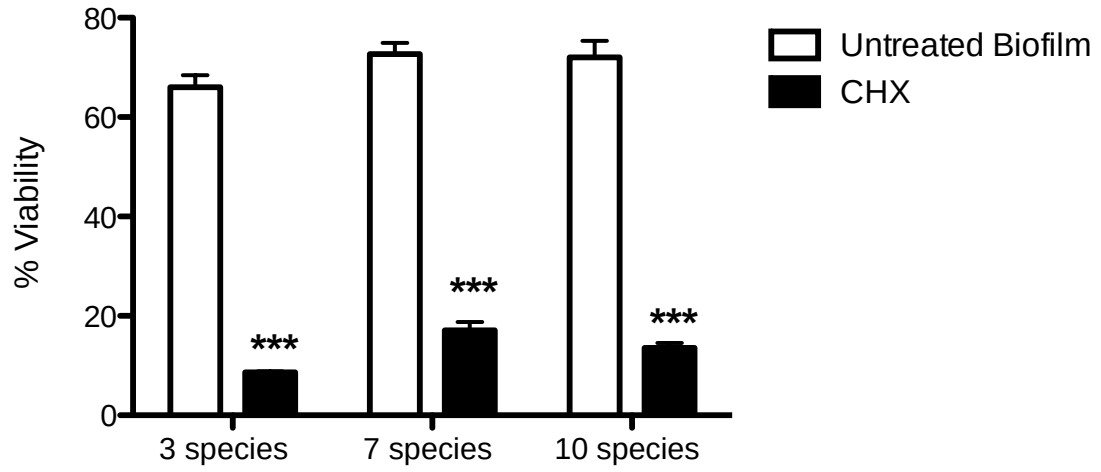


Figure 7.8: Cytotoxicity of CHX on 3, 7 and 10 species biofilms

Mature 3, 7 and 10 species biofilms were treated with 0.2% v/v CHX for 30 minutes and biofilm viability measured using AlamarBlue®. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (*** $p<0.001$). Statistical analysis was performed using a two-tailed unpaired t test.

The composition of each biofilm was also measured after treatment using SYBR® GreenER™ based qPCR (Figure 7.9). A 2.34 log reduction ($p<0.01$) in the total number of streptococci was observed in the CHX treated 3 species biofilm compared to the untreated control (Figure 7.9 i). In the 7 species biofilm, treatment with CHX resulted in a 4.13 log reduction ($p<0.01$) and 4.41 log reduction ($p<0.01$) of *Streptococcus* and *F. nucleatum* species, respectively. A 3.3 log reduction in total *V. dispar* and no significant difference in the total number of *A. naeslundii* when compared to the untreated controls was also observed (Figure 7.9 ii). Finally, significant total bacterial reduction was observed in 10 species biofilm following CHX treatment with a significant 4.08 log reduction ($p<0.05$) of *Streptococcus* species and 3.1 log reduction ($p<0.05$) of *F. nucleatum* species (Figure 7.9 iii). Notably, no *P. intermedia* was recoverable from 10 species biofilms after CHX treatment.

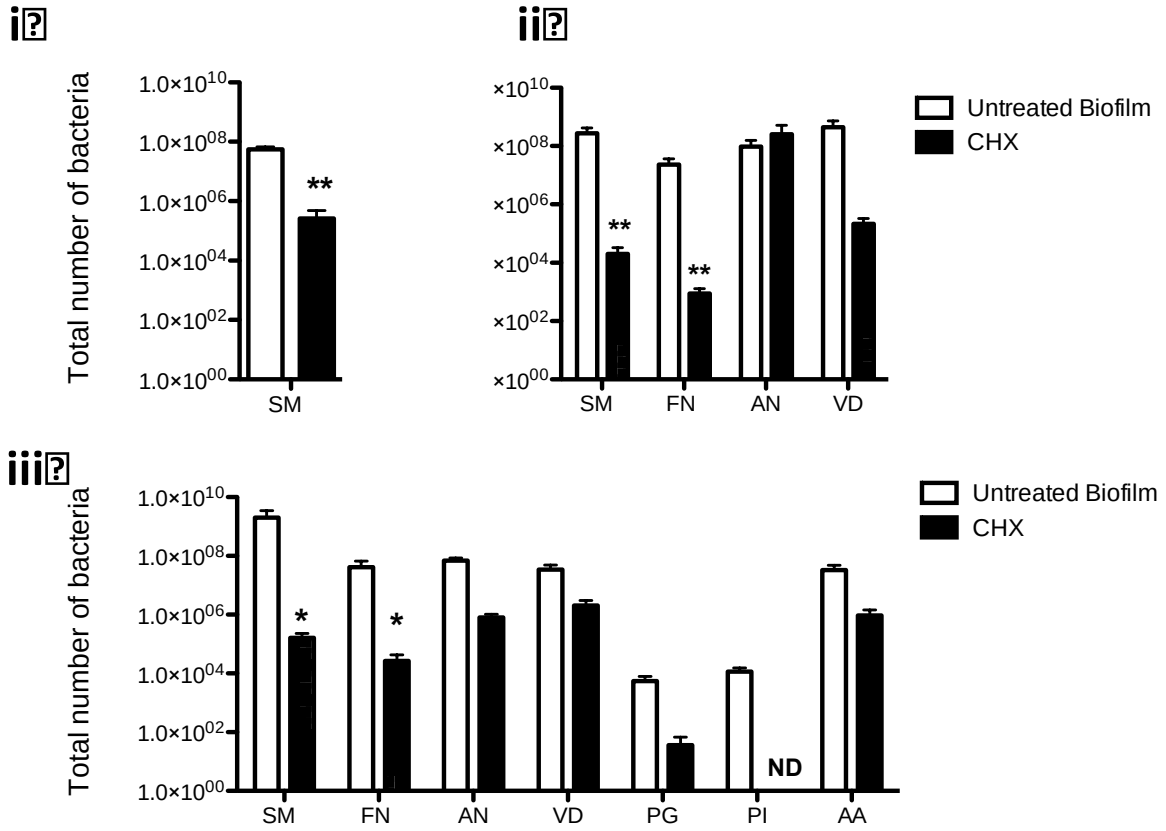


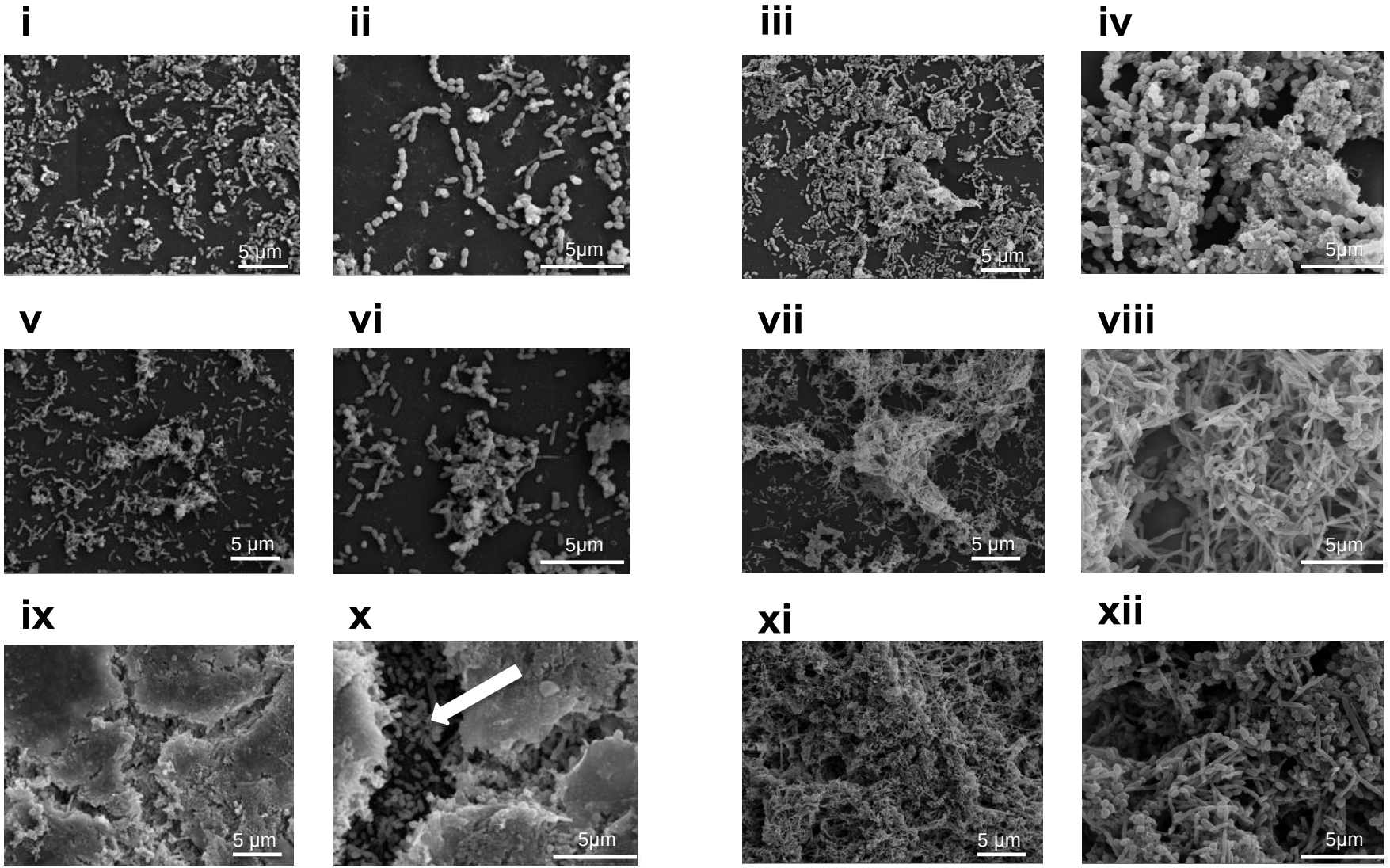
Figure 7.9: Biofilm composition following CHX

Mature 3, 7 and 10 species biofilms were grown on Thermanox™ coverslips and treated CHX (0.2% v/v) for 30 minutes. Biofilms were washed with PBS and DNA was extracted for quantification of each species using SYBR® GreenER™ based qPCR. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (* $p<0.05$, ** $p<0.01$). Statistical analysis was performed using a two-tailed unpaired t test.

The architecture of each biofilm following treatment was also assessed by SEM (Figure 7.10). At both low and high magnifications significant loss of *Streptococcus* species can be seen following CHX treatment of the 3 species biofilm (Figure 7.10 i-ii) compared to the untreated control (Figure 7.10 iii-iv). Similar disaggregation following treatment can be observed in the 7 species biofilms (Figure 7.10 v-vi), where the complex microbial communities observed in the untreated controls (Figure 7.10 vii-viii) have been lost. Unlike the 3 and 7 species biofilms, which lose their complexity following treatment, CHX treatment of 10 species biofilms appears to result in the fusion of the uppermost layer of bacteria (Figure 7.10 ix). At high magnification cracks within this uppermost layer show bacteria appear protected underneath (Figure 7.10 x) although are notably less abundant than the untreated controls (Figure 7.10 xi-xii).

Figure 7.10: SEM analysis of 3, 7 and 10 species biofilms follow CHX treatment

Mature 3 (i, ii, iii, iv), 7 (v, vi, vii, viii), 10 (ix, x, xi, xii) species biofilms were analysed by SEM at both 2000 x (i, iii, v, vii, ix, xi) and 5000 x (ii, iv, vi, viii, x, xii). Biofilms were treated with CHX 0.2% v/v (i, ii, v, vi, ix, x) for 30 minutes and compared to an untreated control (iii, iv, vii, viii, xi, xii). CHX treated biofilms were first compared at low magnification to untreated controls where the architecture of the 3 and 7 species biofilms appears to be lost following treatment. At higher magnifications, 10 species biofilms treated with 0.2% v/v CHX resulted in fusion of the bacteria at the topmost layer of the biofilm however this appears to have protected bacteria underneath as denoted by the arrow. Biofilms were processed and viewed on a JEOL JSM-6400 scanning electron microscope.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7.3.6 CHX treatment differentially alters the expression of pro-inflammatory mediators

Next, the pro-inflammatory gene and protein response to CHX treated 3, 7 and 10 species biofilms in co-culture was investigated and any differential response to treated biofilms noted. After pre-treatment of biofilms with 0.2% v/v CHX for 30 minutes, biofilms were co-cultured with OKF6-TERT2 oral epithelial cells, as previously described in section 2.2.3 for 4 and 24 hours. Following co-culture IL-8 gene expression was quantified using SYBR® GreenER™ based qPCR (Figure 7.11). No significant differences in gene expression were observed when comparing CHX treated biofilms to their untreated controls, nor were there any significant differences in the epithelial cell percentage expression of IL-8 when comparing the CHX treated biofilms to each other.

Gene expression was further investigated by using the RT^2 profiler to measure an array of pro-inflammatory cytokines (Figure 7.12). No significant differences were observed in the gene expression of pro-inflammatory cytokines when comparing co-cultures containing CHX treated 3 species biofilms (Figure 7.12 i) to the untreated control (Figure 7.12 iv). Significant differences were observed when comparing the co-cultures containing CHX treated 7 species biofilms to untreated controls. At 4 hours the CHX treated 7 species biofilm pro-inflammatory gene response of TNFα was increased 10.74 fold ($p<0.001$), CSF3 was increased 61.8 fold ($p<0.001$), CXCL1 was increased 30.7 fold ($p<0.001$) and CXCL3 was increased 19.6 fold ($p<0.05$) compared with the co-culture containing untreated controls (Figure 7.12 ii). At 24 hours IL-6 gene expression was 108.8 fold ($p<0.01$) higher in CHX treated co-cultures than the control. Conversely, CSF2 was 86.1 fold ($p<0.05$) lower in CHX treated co-cultures compared to the untreated control (Figure 7.12 v). In co-cultures with 10 species biofilms at 4 hours IL-6 was increased 199.2 fold ($p<0.01$) compared to the untreated control (Figure 7.12 iii). No significant differences were observed in cytokine gene expression at 24 hours; however, there was a notable trend to increased gene expression of all pro-inflammatory cytokines in CHX treated co-cultures compared with untreated controls (Figure 7.12 vi). Additionally, no significant differences were observed between the gene expression of each cytokine when comparing 3, 7 and 10 species CHX treated co-cultures.

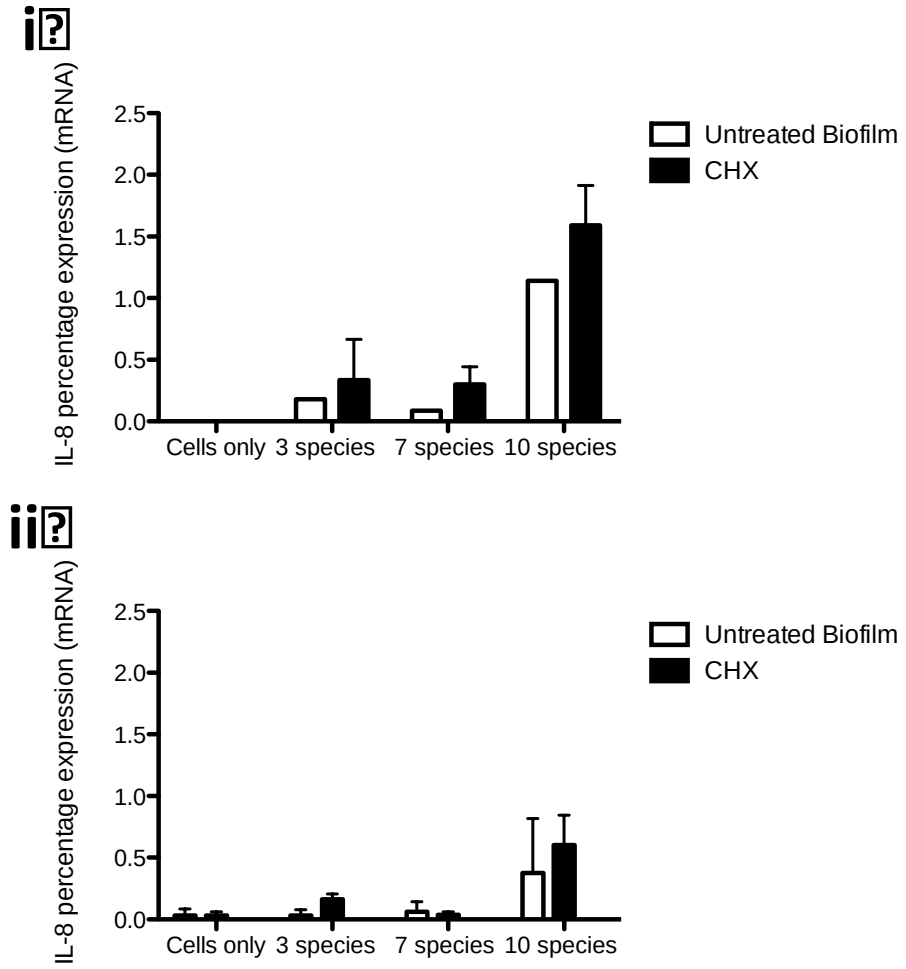


Figure 7.11: IL-8 gene expression by OKF6-TERT2 cells in co-culture following biofilm treatment with CHX

Mature 3, 7 and 10 species biofilms were pre-treated with 0.2% v/v CHX for 30 minutes before washing with PBS and then co-cultured for 4 (i) and 24 (ii) hours with OKF6-TERT2 oral epithelial cells. Controls included untreated cells in media only (cells only) and untreated cells in co-culture with an untreated biofilm (untreated biofilm). RNA was extracted from cells at each time point, cDNA was synthesized and IL-8 gene expression measured using SYBR® GreenER™ based qPCR relative to the housekeeping gene *GAPDH*. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD. Statistical analysis was performed using a two-way ANOVA with Bonferroni post-test to compare all groups to each other.

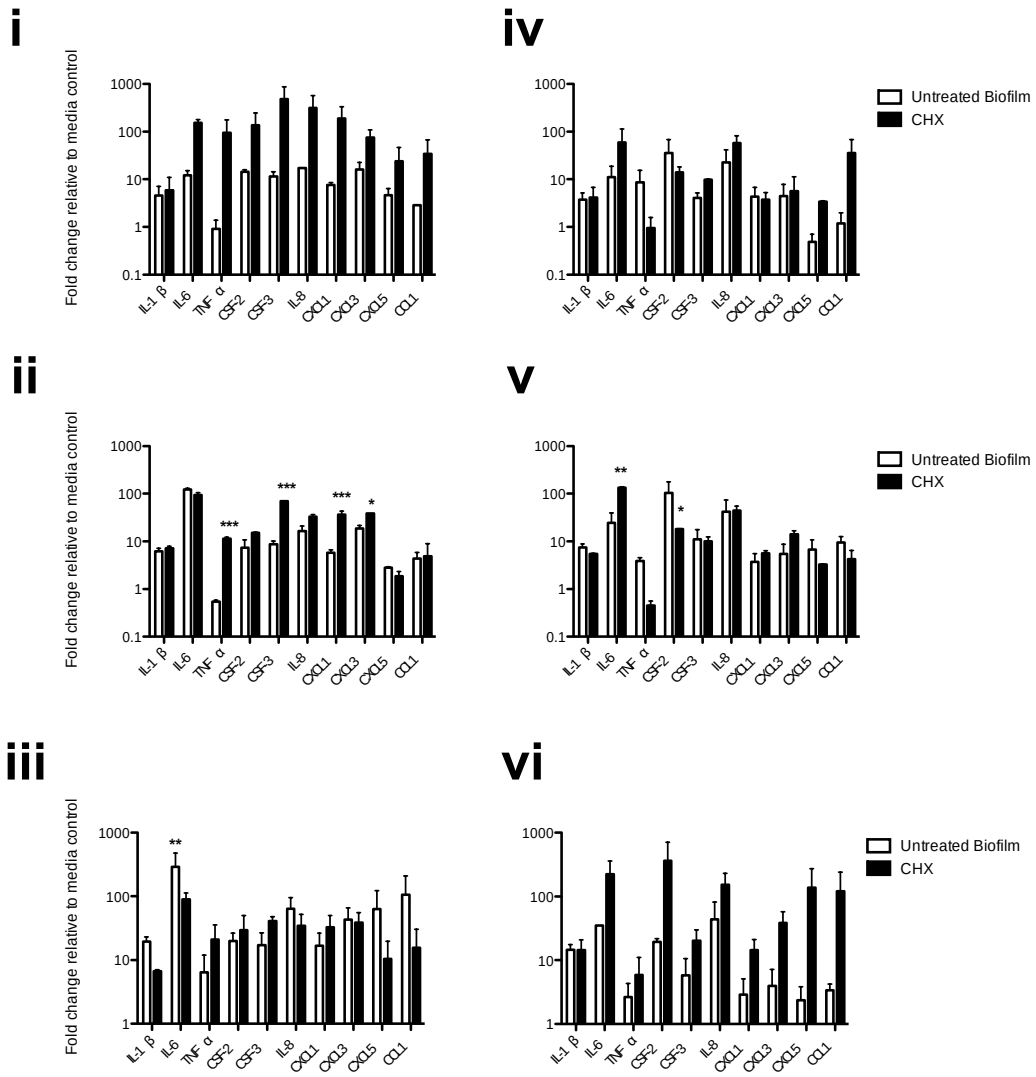


Figure 7.12: Pro-inflammatory gene response by OKF6-TERT2 cells in co-culture following biofilm treatment with CHX

Mature 3 (i, iv), 7 (ii, v) and 10 (iii, vi) species biofilms were pre-treated with 0.2% v/v CHX (ii) for 30 minutes before washing with PBS and then co-cultured for 4 (i, ii, iii) and 24 (iv, v, vi) hours with cells. Controls included untreated cells in media only (cells only) and untreated cells in co-culture with an untreated biofilm (untreated biofilm). RNA was extracted from cells at each time point, cDNA was synthesized and pro-inflammatory cytokine gene expression measured using the RT^2 Profiler. Samples are normalised to the housekeeping gene *GAPDH* and relative to the cells only control. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Statistical analysis was performed using a two-tailed unpaired t test.

Finally, IL-8 protein expression after 4 and 24 hour co-culture was measured by ELISA (Figure 7.13). At 4 hours, significantly less IL-8 protein was measured in the co-culture of CHX treated 7 and 10 species biofilms with a 4.6 fold ($p<0.001$) and 3.1 fold ($p<0.001$) reduction compared with the 7 species and 10 species untreated controls, respectively (Figure 7.13 i). At 24 hours, a 12.43 fold reduction ($p<0.001$) of IL-8 protein was measured when comparing the CHX treated 7 species with the untreated control (Figure 7.13 ii). There was no significant differences in the IL-8 protein response between the CHX treated 3 species biofilms at either time point.

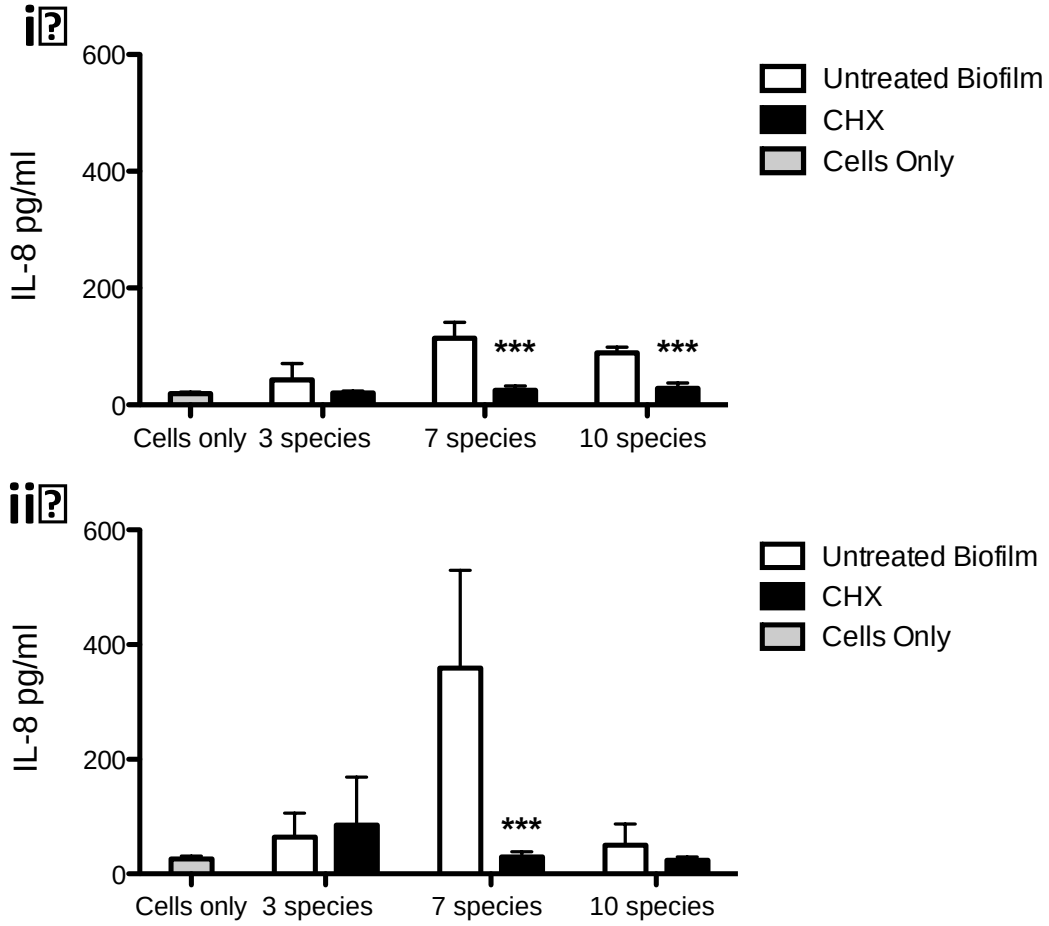


Figure 7.13: IL-8 protein response by OKF6-TERT2 cells in co-culture following biofilm treatment with CHX

Mature 3, 7 and 10 species biofilms were pre-treated with 0.2% v/v CHX for 30 minutes before washing with PBS and then co-cultured for 4 (i) and 24 (ii) hours with OKF6-TERT2 oral epithelial cells. Controls included untreated cells in media only (cells only) and untreated cells in co-culture with an untreated biofilm (untreated biofilm). Protein release was measured by ELISA. All groups were assayed in triplicate on three separate occasions. Data represents mean ± SD (*** $p<0.001$). Statistical analysis was performed using a two-tailed unpaired t test to compare untreated biofilms with CHX treated biofilms.

7.4 Discussion

Daily mechanical disruption (tooth brushing) of oral biofilms is the most important method for prevention of PD. However, biofilms can form over time if patients are not compliant with this process or unable to clean the teeth properly due to illness, surgery, or having fixed orthodontic appliances (Ciancio, 2003). For dentists the 'gold standard' of PD treatment is mechanical debridement of the biofilm. Anti-microbial or anti-inflammatory supplements are only prescribed to patients who are physically unable to clean their teeth (Matthews, 2014). However, many studies have shown benefits of adding antimicrobials in parallel with debridement to augment the outcome of treatment (Herrera et al., 2002, Matesanz-Perez et al., 2013). Recently, studies have also begun to focus on the role of inflammation in periodontitis and investigate anti-inflammatory compounds and their potential in management of the disease (Hasturk et al., 2012). Previous chapters have shown the use of *in vitro* biofilm models to understand the interplay between different microbial biofilms and the host immune system. Furthermore these models can be used to evaluate potential actives' ability to influence the host response as well as understand their basic mode of action. The data presented here show the ability of the models to test anti-microbial and anti-inflammatory compounds as well as the ability to measure differential responses to compounds using a variety of multi-species biofilms within the model.

Firstly, a comparative assessment of CHX and RSV against OKF6-TERT2 oral epithelial cells and a simple 4 species oral biofilm was undertaken. CHX was found to be cytotoxic to OKF6-TERT2 cells at all concentrations and all treatment times used, a finding correlating with other studies on different cell types (Lessa et al., 2010, Li et al., 2014b). When treated with CHX, biofilm viability decreased at concentrations higher than 0.05% v/v and many studies both *in vivo* and *in vitro* confirm the potent antimicrobial properties of this compound (Park et al., 2014, Pilloni et al., 2013). Additionally, microscopy studies of human plaque treated with CHX observed reduction in plaque thickness compared to other treatments and matrix degradation (Vitkov et al., 2005, Jentsch et al., 2013).

RSV did not affect cell viability when used at 0.01% w/v, however, it was found to be cytotoxic to cells at 0.05% and 0.5% w/v regardless of treatment time. Many cancer studies have investigated the role of RSV on cancer cell lines observing dose dependant cytotoxic and anti-proliferative effects following treatment (Berardi et al., 2009, Matic et al., 2010). A recent study by Catania *et al* (2013) also observed IC_{50} of MCF7 breast cancer cells when cultured with 0.05 - 0.13mM of RSV for 72 hours and a study by Joe *et al* (2002) observed cytotoxicity of a variety of human cancer cell lines treated with 0.1 – 0.3% RSV (Catania et al., 2013, Joe et al., 2002). Treatment of biofilms with RSV did not affect biofilm viability or cause any significant changes to the structure when observed by SEM. Thus far no other studies have investigated the effect of RSV on periodontal biofilms; however, RSV treatment has been shown to be inhibit biofilm formation of *Pseudomonas aeruginosa, Escherichia coli* and staphylococcal species (Lee et al., 2014, Moran et al., 2014). It should be noted that these studies did not investigate the ability of RSV to decrease viability of a mature biofilm, which may explain the differences in observations.

The next step was to assess any additional biological characteristics of CHX and RSV when used in a multi-species co-culture model. Using CHX treated 4 species biofilm or RSV treated OKF6-TERT2 oral epithelial cells the inflammatory gene and protein expression were examined after co-culture. Following co-culture with CHX pre-treatment, IL-8 gene expression was significantly reduced with other pro-inflammatory genes being notably decreased, combined with a significant reduction of IL-8 protein expression. Previously it was observed CHX may have anti-inflammatory properties due to its ability to down regulate bacterially induced pro-inflammatory proteins such as TNFα and IFNγ in an *in vivo* mouse chamber model (Houri-Haddad et al., 2008). Additionally, a study by Montecucco *et al* (2009) observed anti-inflammatory effect of CHX when treating human primary neutrophils by destroying toxic compounds produced by these cells (Montecucco et al., 2009). Gingival inflammation scores of patients using CHX also observe reduction of inflammation following treatment as well as significant reductions in plaque scores (Van Strydonck et al., 2012). However, none of these studies assess the potential that the reduction of gene and protein expression may be due to the toxic properties of the compound, so further studies would be required to determine the role cytotoxicity of CHX plays in its

anti-inflammatory properties. Gene expression was not significantly different following RSV compared with co-culture, however, IL-8 protein expression was significantly decreased. A variety of studies investigating the role of RSV and inflammation have found that RSV modulates a variety of cell responses by modulating signal transduction pathways such as p38 MAPK or AMP pathway (Malemud, 2007, Park et al., 2012). With relation to this chapters finding RSV has also been shown to enhance mRNA degradation and modulate pro-inflammatory transcription factors including nuclear factor-κB (NF-κB) and AP-1 (Kundu et al., 2006, Jeong et al., 2011, Bollmann et al., 2014).

The previous data show that a 4 species co-culture model can be used to test both antimicrobial and anti-inflammatory actives. This work was done prior to the development of the 3, 7 and 10 species biofilms used throughout this thesis, therefore it was important to see if these biofilms could also be used to test actives. Using CHX, the 'gold standard' for PD treatment, 3, 7 and 10 species biofilm were pre-treated to investigate if biofilm composition affected antimicrobial activity.

Biofilms pre-treated with CHX showed significant decreases in viability compared with the untreated control which as previously discussed correlates with the known antimicrobial properties of CHX (Herrera, 2013). When quantifying the composition of the CHX treated biofilms, treatment reduced the overall total number of bacteria in all biofilms compared to the untreated controls, with significant reductions in *Streptococcus* species in the 3, 7 and 10 species CHX treated biofilms, and *F. nucleatum* species in the 7 and 10 species CHX treated biofilms. It has been reported that CHX reduces total streptococcal species on tooth-tissue-borne palatal expanders without reducing overall biomass (Maruo et al., 2008). Additionally, Oliveria *et al* (2014) reported differential viability of multi-species biofilm species following CHX treatment, suggesting variation in individual species susceptibility to CHX (Oliveira et al., 2014).

When investigating biofilm architecture following CHX treatment by SEM both 3 and 7 species biofilms had notable disrupted aggregates, appeared less complex and lost more ECM than their untreated controls. Interestingly, CHX treatment of the 10 species biofilms appeared to destroy and degrade the uppermost layer of

the biofilm with bacteria found underneath appearing unaffected. This may be due to the differences in the overall complexity of the 3, 7 and 10 species biofilm models as it is known bacteria in biofilms are more resistant to antimicrobials than those in a planktonic state (Bonez et al., 2013, Park et al., 2014). Additionally, a study by Shen *et al* (2011) investigated the antimicrobial effects of CHX at different stages of biofilm development and found while all the biofilms tested reduced in thickness following CHX treatment, bacteria in mature biofilms were more resistant to CHX killing than less complex biofilms, which may relate to the observations in this chapter (Shen et al., 2011).

As seen in previous chapters, biofilms can differentially modulate the host immune response in co-culture. Therefore, the effect of pre-treating 3, 7 and 10 species biofilms with CHX prior to co-culture was investigated.

The pro-inflammatory gene and protein modulation by 3, 7 and 10 species CHX treated biofilm was measured in co-culture with OKF6-TERT2 oral epithelial cells and compared with untreated co-cultures. No studies investigating the gene response of oral host cells when treated directly with CHX have been reported outwith our laboratory which may be in part due to the potent cytotoxic effects observed when cells are directly treated with CHX as shown in Figure 7.1. However, Breij *et al* (2012) reported a 3-dimensional human skin equivalent used to measure the antimicrobial ability of direct CHX treatment and found no cytotoxic effects and no inflammatory IL-8 and IL-1 mRNA activity. This may be due to the nature of a 3-dimensional skin equivalent containing a stratified top layer, which may protect cells below from the toxicity of CHX in comparison to the monolayer of epithelial cells used in the work in this chapter (de Breij et al., 2012).

The IL-8 protein response by OKF6-TERT2 oral epithelial cells was significantly reduced in CHX treated 7 and 10 species biofilms compared with their untreated controls at 4 hours, while no significant differences were observed between the 3 species biofilms. At 24 hours only the CHX treated 7 species biofilm was significantly different to the untreated control. *In vitro* and *in vivo* studies have reported a reduction in pro-inflammatory protein expression following CHX treatment which may relate to the results observed in this chapter (Rohner et

al., 2014, Turkoglu et al., 2009). However, as previously mentioned the relationship between CHX's potent cytotoxicity and any anti-inflammatory properties it has requires further study to fully understand the mode of action of this potential role of immunomodulation.

Due to the complexity of oral biofilms, using simplified models containing key pathogens in co-culture with host cells *in vitro* provides an attractive starting point for testing potential actives. In this study the co-culture model successfully allows examination of the direct immune response of a given cell type, in this case oral epithelial cells, to a variety of multi-species oral biofilms. However, in the oral cavity the gingival tissue consists of multiple layers of epithelial cells and connective tissue and cells are influenced not only by biofilms directly but other tissue and immune cells. Additionally, host cells are more susceptible to highly cytotoxic compounds such as CHX when cultured in monolayers monolayers. An alternative method to make the host cells in co-culture more similar to the oral cavity *in vitro* would be to use 3D models such as that de Breij *et al* (2012) where the exposed layer of epithelial cells were stratified and conferred protection upon the layers below (de Breij et al., 2012). Investigating the novel active RSV, this study reported anti-inflammatory activity from which a hypothesis of the mode of action could be delineated. In future the potential of this model to test anti-inflammatory compounds could be further validated by use of known anti-inflammatory compounds such as steroids in the co-culture model.

In summary, the co-culture model presented in this study has shown ability to test both antimicrobial and anti-inflammatory compounds in co-culture and can be a valuable asset for pre clinical testing of potential actives. This model allows both studies of the treatment of biofilms and treatment of host cells to investigate interactions, which are observed in the oral cavity.

CHAPTER FINDINGS

Co-culture models using multi-species biofilms can be used to test novel antimicrobial and anti-inflammatory actives

CHX treatment differentially affects composition and cell viability of multi-species biofilms

CHX treatment of biofilms causes differential modulation of inflammatory gene expression and protein release in co-culture with 3, 7 and 10 species biofilms

8 Discussion

8.1 Introduction

PD is a complex disease that involves a multitude of interactions between bacterial species present within complex biofilm consortia and cellular responses from both host tissue and immune cells in the oral cavity. The work described herein addresses the question of how the composition of oral microbial biofilms influences the host response. This has been facilitated through the successful development of three multi-species biofilms that modelled health-associated, intermediate and disease-associate plaque, for use in a co-culture model with appropriate host cells. This model system has demonstrated clear differences in the host response to each of the biofilms, and the utility of this model will allow continued use to ask key questions relating to host: biofilm interactions within the oral cavity and other sites within the body, and how the introduction of biologically active molecules influences these processes.

8.2 Bacterial species variation in health and disease

Prior to the development of the multi-species biofilm models described in this thesis it was important to ensure that bacteria appropriate for the oral disease were used, with particular focus on those associated with periodontitis. *P. gingivalis* has been shown to be strongly associated with periodontitis with approximately 79% of periodontitis patients harbouring the bacteria (Griffen et al., 1998). However, the same study observed that the dental plaque of 25% of periodontally healthy patients also contained *P. gingivalis*. The presence of low numbers of *P. gingivalis* has been shown to alter the commensal oral microflora to promote pathogenicity and alveolar bone loss in mice (Hajishengallis et al., 2011). Therefore, it is imperative to understand why this species, considered the keystone pathogen in periodontitis, can be present with no apparent consequence in periodontally healthy subjects. One explanation proposed for the observations of seemingly non-pathogenic *P. gingivalis* within healthy subjects is that strain variation may play a role in pathogenicity. A key study by Griffen et al. (1999) compared *P. gingivalis* strain diversity in periodontitis patients and those who were periodontally healthy in an attempt to understand this phenomenon (Griffen et al., 1999). In the study 11 different strains of *P.*

gingivalis were detected, of which W83, 49417 and HG1691 were strongly associated with periodontitis, and 23A4, 381 and A7A1 were weakly associated. Healthy subjects were also more likely to harbour multiple strains of *P. gingivalis* leading to the suggestion that in periodontitis, less virulent strains were out competed by more virulent ones, thus helping to promote disease. Similarly, Jandik et al. (2008) observed that *P. gingivalis* strains isolated from diseases sites are more invasive when co-cultured with KB cells than *P. gingivalis* strains isolated from healthy sites (Jandik et al., 2008). In a mouse model of experimental periodontitis strain variation was also shown to alter bone resorption and host immune responses, where *P. gingivalis* W83 and W50 inoculation induced significant alveolar bone loss and high levels of IL-4 protein release compared with no significant alveolar bone loss compared to the control mice and high levels of IL-10 protein release following inoculation with *P. gingivalis* A7A1 (Marchesan et al., 2012). The initial work reported in chapter 3 investigated *P. gingivalis* strain variation as a means of determining the appropriate strain for the disease-associated biofilm, where differences in biofilm formation, cytokine degradation and inflammatory response by epithelial cells in co-culture were observed. These characteristics all have the potential to play a role in pathogenic potential of these strains. In particular, the W83 strain was overall more virulent than W50 and ATCC 33277 strains.

The different strains of *P. gingivalis* demonstrate variable phenotypic expression of virulence factors or genetic variation. Studies characterizing variation of *P. gingivalis* have identified 6 serotypes (K1-6) related to differing antigenicity of the capsule (Laine et al., 1996). Structural variations in these serotypes have been shown to be directly involved in the virulence of *P. gingivalis,* with capsular strains such as W50 and W83 up-regulating IL-1ß, IL-6 and IL-8 gene expression in fibroblasts *in vitro* compared with non-capsular mutants of these strains or naturally non-capsular strains such as ATCC 33277 and 381 (Brunner et al., 2010, Chen et al., 2004). Furthermore, two studies investigating the serotypes of *P. gingivalis* present in periodontitis patients observed prevalence of the K6 serotype in periodontitis patients from Sweden and the Netherlands (Yoshino et al., 2007, Laine et al., 1997). Notably, *P. gingivalis* W83, which is commonly cited as the most virulent of all strains, and frequently used in *in vitro* experimental periodontitis models, is a K1 serotype. This serotype was only

present in a small number of patients within the study, suggesting that there may be other factors that benefit other *P. gingivalis* strains in the oral cavity, such as interactions with other species, to promote pathogenicity. This may be the case as there is growing evidence that polymicrobial interactions synergise the pathogenic potential of one or other microorganism (Stacy et al., 2014). Moreover, this study also suggested that *P. gingivalis* serotype may be an important factor is periodontitis initiation and progression.

While there is still much to be understood about the role of *P. gingivalis* in periodontitis, these studies and this work provide a strong argument that strain variation may play an important role in the virulence and disease associations of the species, so the interpretation of experimental data using characterised laboratory strains should take this into account. A notable example of strain variation playing a role in disease pathogenicity within the oral cavity is the association of the JP2 clone of *A. actinomycetemcomitans* in aggressive periodontitis. *A. actinomycetemcomitans* has been considered for over 30 years to be the most likely etiological agent in aggressive periodontitis, with the 70-90% of patients harbouring the bacteria in subgingival plaque, most commonly Northwest African populations – a population with a higher than average prevalence of aggressive periodontitis (Kononen and Muller, 2014). The JP2 clone belongs to the group b serotype and due to a deletion in the leukotoxin gene has enhanced leukotoxic activity (Brogan et al., 1994). Longitudinal studies to assess the associations of *A. actinomycetemcomitans* and aggressive periodontitis have shown that *A. actinomycetemcomitans* is a risk factor for the initiation of periodontal attachment loss in Moroccan adolescents (Haubek et al., 2008). This study went further to find that adolescents who carried the JP2 clone in plaque were significantly more at risk of periodontal attachment loss than those who carried a non-JP2 strain. Treatment of patients with the JP2 strain of *A. actinomycetemcomitans* appears to be more difficult than those who have a non-JP2 strain. In a study by *Cavalca Cortelli* et al (2009) patients infected with JP2 or non-JP2 strains of *A. actinomycetemcomitans* were treated with mechanical debridement, systemic antibiotic therapy and periodontal surgery (Cortelli et al., 2009). One year later, probing depth, clinical attachment loss and gingival and plaque indexes were measured for each group and found that patients with non-JP2 strains had improved scores compared with

patients with JP2 strains who were more resistant to mechanical and antimicrobial therapies. Furthermore, this study observed that elimination of JP2 strains from patients significantly improved gingival inflammation and suggested that early identification of *A. actinomycetemcomitans* strain may help to predict disease outcome of patients with aggressive periodontitis.

The ability of strain variation to influence disease pathogenicity is true of species within the oral cavity and those at other sites within the body. Studies investigating serotype variation in *S. pneumoniae*, a bacteria which causes invasive pneumococcal disease (IPD), septicaemia and meningitis, observed approximately 20 of the 94 serotypes of *S. pneumoniae* accounted for most of the IPD observed in the UK (Melegaro et al., 2006). While investigating the sequence types and serotypes of *S. pneumoniae* between patients with IPD and healthy nasopharyngeal carriers, significantly more serotype 14 clones in IPD patients and serotype 3 clones in nasopharyngeal carriers were observed, suggesting that serotype rather then genotype is the most successful way of predicting the ability of *S. pneumoniae* to cause disease (Brueggemann et al., 2003). Like *P. gingivalis*, *S. pneumoniae* serotypes are classified based on the capsule composition and this knowledge has been successfully used to guide the development of a conjugate vaccine against the 7 most virulent serotypes of *S. pneumoniae* (Lamb et al., 2014). This has been adapted for immunization using a conjugate vaccine of *P. gingivalis* capsule, which prevented alveolar bone loss in a mouse periodontitis model, highlighting the potential of a carefully designed vaccination against *P. gingivalis,* in the prevention of periodontitis (Gonzalez et al., 2003).

Studies investigating bacterial infections frequently identify a single species as a key player in disease, such as *P. gingivalis* in periodontitis. However, these studies must also consider interspecies variation, which can modulate disease severity. Collectively, the work discussed herein has shown that species can differ in virulence when investigating strain variation, and serotyping and future work investigating the role of *P. gingivalis* as a keystone pathogen in periodontitis may benefit from further characterization of the species. Additionally, care must be used when developing multi-species biofilm models containing *P. gingivalis*, as variation can play a major role in the pathogenicity of the species, and potentially the whole biofilm. Potentially, selection of the

species can affect downstream analysis. Typically avirulent strains such as ATCC 33277 may differentially alter the biofilm composition compared with more virulent strains such as W83. Additionally, *P. gingivalis* W83 has been shown to be more effective at protein degradation than ATCC 33277 in both single and multi-species biofilms. This highlights the considerations required when choosing strains for study and the further applications of the biofilm must be taken into account when developing models.

8.3 Pathogens, commensals, host homeostasis and dysbiosis

During the course of this study, the composition of the multi-species biofilm was shown to affect host immune responses. Health-associated biofilms caused negligible changes in gene and protein response, intermediate biofilms caused a high inflammatory gene and protein response, and disease-associated biofilms caused a high inflammatory gene response, but low levels of pro-inflammatory proteins present in the supernatant following co-culture. This highlights the differential states in which the host interacts with bacterial biofilms; homeostasis with commensal species, and inflammation with pathogen species. Understanding these interactions is key to promoting health and preventing bacterial disease.

Commensal bacteria are present throughout the body acting homeostatically with the host, though dysbiosis of this, through either inclusion of certain species or shift in the microbiome, can lead to diseases such as PD in the oral cavity and inflammatory bowel disease (IBD) in the gut. Many studies investigating the interactions between resident commensal bacteria and the host focus on the gut and have shown that the homeostasic balance is complex and orchestrated by epithelial cells and the innate and adaptive immune system. The default stance of the gut is a pro-inflammatory one, which is balanced through anti-inflammatory and immunosuppressive mechanisms by both immune cells and commensal bacteria. Species like *Bacteroides fragilis* induce T regulatory cell expansion via TLR2 on CD4+ T cells, which produce IL-10 during commensal

colonization and this immunosuppressive process has been shown to prevent and cure experimental colitis animal models (Round and Mazmanian, 2010).

Tissues of the gingivae likely show some similarities to the gut, although it should be noted that tissues of the gingivae are keratinised in some places and lack any mucous layer analogous to that present in gut. Gingival tissues are constitutively inflamed, with studies showing that alveolar bone loss in mice with commensal microflora is a normal component of periodontal tissue homeostasis (Irie et al., 2014). In the oral cavity there are also commensal bacterial species which supress the pro-inflammatory response. Approximately 30-40% of oral streptococci, an early colonizer and commensal species of oral plaque, have been shown to down-regulate cytokine expression by epithelial cells (Devine et al., 2015). The probiotic species *S. salivarius* down regulated IL-8 in cell line and primary oral keratinocytes through NFκB inactivation (Cosseau et al., 2008). Additionally, *S. cristatus* was shown to inhibit IL-8 production by OKF6-TERT2 oral epithelial cells via NF-κB in co-culture (Zhang et al., 2011). Notably, this study documented the ability of *S. cristatus* to attenuate the IL-8 response by epithelial cells to *F. nucleatum* when co-cultured together by degrading IκB-α and subsequently blockading NFκB translocation. While these studies used planktonic bacteria co-cultured with epithelial cells, the work described herein co-culturing the three species biofilm with epithelial cells observed low levels of pro-inflammatory gene expression and protein release, which is consistent with these findings.

The pro-inflammatory response by the gut and oral cavity is regulated by commensal species; however, an excessive anti-inflammatory response is not beneficial to the host as it can facilitate an increase in bacterial load and inability to respond effectively to pathogens. Selective down-regulation of host inflammatory responses is one mechanism that is employed by *P. gingivalis* to promote PD, with gingipains degrading IL-8 thus reducing neutrophil migration into the gingivae. Studies have shown that commensal bacteria up-regulate CXCL2 to promote neutrophil migration into the tissues in SPF mice. Additionally, neutrophils migrate to gingival tissues even in the absence of bacteria, suggesting these cells play an essential role in periodontal health (Zenobia et al., 2013).

The shift from homeostasis between the oral bacteria and host to a destructive dysbiosis resulting is disease is strongly associated with the keystone species *P. gingivalis*. This species has been shown to alter the commensal microflora, even though it represents a very small proportion of the total population. *P. gingivalis* alone cannot induce PD in GF mice, highlighting its need for an established microflora to induce disease (Hajishengallis et al., 2011). In this respect *P. gingivalis* appears to be a bully in the oral cavity; unable to cause a host response alone but able to manipulate and disrupt the other species present to cause disease. The inability of *P. gingivalis* to cause disease alone is similar to the work described in chapter 3, with almost no inflammatory gene or protein response by epithelial cells to single species *P. gingivalis* biofilms in co-culture or as planktonic cells. Similarly, in the gut certain species have been shown to associate with disease, such as *Klebsiella pneumoniae* and *Proteus mirabilis*, which are both strongly associated with colitis in *Tbx21*$^{-/-}$*Rag2*$^{-/-}$ ulcerative colitis (TRUC) mice, i.e. mice that lack an adaptive immune response due to loss of Tbet and RAG2, and SPF mice (Garrett et al., 2007, Garrett et al., 2010). Like *P. gingivalis* in the oral cavity, these studies found inoculation of GF mice with *K. pneumoniae* and *P. mirabilis* does not induce colitis, suggesting their pathogenic effects are dependant on the resident gut microflora. These findings indicate these species may act as keystone pathogens in the gut, as *P. gingivalis* does in the oral cavity. However, the details of how these species modulate normal gut microflora remain to be elucidated.

These studies highlight the dynamic role of bacteria and their interactions with the host, in both health and disease within the oral cavity and beyond. Importantly, they highlight the ability of a few species to shift the commensal microflora from commensals in homeostasis with the host to potential pathogens, which can promote inflammation and disease. Understanding these interactions is difficult; hence the use of multi-species biofilms in co-culture with mammalian cells provides an attractive model to study this.

8.4 The use of *in vitro* models to study host: biofilm interactions in PD

Many different groups have modelled oral biofilms, using both defined and undefined consortia of bacteria, in an attempt to understand biofilm formation, kinetics and bacterial interactions with relation to health and PD (Periasamy and Kolenbrander, 2009, Park et al., 2014, Guggenheim et al., 2001a). Recently these models have included local tissue and immune cells in an attempt to understand how these biofilms interact with the host (Guggenheim et al., 2009, Belibasakis et al., 2013b, Peyyala et al., 2011). These offer many advantages over *in vivo* animal models and human experimental gingivitis studies, most notably being ethical and cost considerations. These models also offer further advantage of being a controlled and reproducible environment, which allows for detailed studies of real time changes in both the biofilm and host responses, and how they interact. A prominent early study by Guggenheim et al (2009) used a 10 species subgingival biofilm model co-cultured with epithelial cells, and this has been emulated by many groups, most recently by Peyyala et al (2012) who used similar biofilms to study how biofilm composition modulates epithelial cell responses (Peyyala et al., 2012, Peyyala et al., 2013, Guggenheim et al., 2009). Collectively, the studies performed by these groups independently show differences in pro-inflammatory cytokine and chemokine gene expression, and protein release to different biofilms and planktonic cells.

It is reported that the oral cavity is host to over 700 different species, which does not account for multiple strains of the same species being present. Hypothetically, there can be 2.44×10^5 potential physical interactions alone between these species, and when including each potential chemical interaction produced by each bacteria, and metabolites produced by mammalian cells, this number increases exponentially. Furthermore, biofilm models which use pooled saliva or pooled plaque typically have problems with reproducibility and species specific analysis, although a potential stable plaque model for testing dental materials was developed (Rudney et al., 2012). Therefore, for this study a reductionist approach was used, growing defined bacterial species on Thermanox® coverslips in AS in a sequential manner. The main advantages of developing the multi-species biofilms in this manner were growth of biofilms in a similar manner to plaque within a biologically relevant media. One disadvantage

of this approach is also the limited number of species used in each model, although this method also allows insights into the role of specific bacteria in modulating inflammatory responses. A particular example of this is the differing inflammatory IL-8 gene expression between 7 and 10 species biofilms. IL-8 gene expression was not significantly different when co-cultured with these biofilms; however, in 10 species co-cultures IL-8 protein in the supernatants was significantly decreased. This finding was consistent with work by Peyyala et al (2013) who observed reduction of IL-8 protein in co-cultures of multi-species biofilms containing *P. gingivalis* with epithelial cells (Peyyala et al., 2013). The results from that study and our own validate the hypothesis that the composition of biofilms directly modulates the host immune response. Simultaneous comprehensive investigation of the three multi-species biofilms described herein offers an advance on the previous work published, which either focused on only pathogenic biofilms, or only investigated protein release and not gene expression, or failed to consider cell viability (Peyyala et al., 2013, Guggenheim et al., 2009).

The use of biofilm models to study health, disease and chemotherapeutics is essential for the oral cavity and beyond. Few bacterial species are found naturally occurring as planktonic cells and many species that cause disease in humans, such as *Pseudomonas aeruginosa* within the lung, are found as biofilms. Growth as biofilms offers a number of advantages for bacterial species, in particular decreased susceptibility to stress factors and antimicrobials. The use of biofilm models for the study of antimicrobials and chemotherapeutics was highlighted when *P. aeruginosa* biofilms showed a 1000-fold reduction in susceptibility to the antibiotic tobramycin compared to planktonic cells (Nickel et al., 1985). This has been shown to be true with oral bacterial species *S. gordonii, F. nucleatum, P. gingivalis* and *A. actinomycetemcomitans*, where the MIC of planktonic cells treated with doxycycline or CHX were 100-10,000 time lower than single species biofilms (Park et al., 2014). This study also observed that the MIC of single species biofilms was 10-100 times lower than multi-species biofilms, highlighting that bacterial interactions within the biofilm can also affect antimicrobial susceptibility. The widespread use of antibiotics in food production, dentistry and medicine generally has resulted in a major increase in the number of antibiotic resistant organisms and highlights the need to find

alternative antimicrobial agents (Gillam and Turner, 2014). The multi-species biofilm models developed herein and within the literature provide an attractive platform for testing novel chemotherapeutics and determining antimicrobial susceptibility. In addition, the use of the co-culture model provides additional insights into the host response to these actives, either being treated directly prior to co-culture or in response to pre-treated biofilms. This was highlighted in our own studies in which epithelial cells treated with RSV showed reduced IL-8 protein release in co-culture with biofilm compared to untreated cells in co-culture with biofilms. The mode of action of RSV in this system remains to be determined. As it is known that the most effective treatment for periodontitis is mechanical debridement, with antibiotics only used occasionally as adjuncts to mechanical debridement, it is unlikely that any novel antimicrobials will entirely supersede this mechanical cleaning (Gillam and Turner, 2014). Nonetheless, further improvements in adjunctive treatment options could be highly beneficial to patients with marked susceptibility to periodontitis, who show limited response to conventional treatment. Moreover, the advantage of developing multi-species biofilm models is the ease with which bacteria can be 'swapped' within the biofilm to easily create biofilms more relevant to different diseases. This could allow testing of antimicrobial actives for use in other areas such as oral candidiasis, or diabetic foot ulcers and understanding how these can affect not only the biofilm but also the epithelial cells within the co-culture model.

8.5 Future work

This body of work has developed three multi-species biofilms, which model health and disease in the oral cavity. These have subsequently been used in co-culture with both host tissue and immune cells to investigate if biofilm composition modulates the host response. This study has shown that the composition of the biofilm has implications for host cell viability, gene expression and protein release that may contribute to disease pathogenesis. The results observed herein show the outcome of interactions between multi-species biofilms and host immune cells. This system would also be useful to evaluate the mechanisms by which biofilms differentially modulate host immune responses with focus either on the biofilm or host cells. For example, the use of defined

mutant species such as gingipains deficient *P. gingivalis* would allow for the understanding of the role of virulence factors in the pathogenesis of periodontitis on a species level. The inclusion or removal of particular oral species in the biofilm to evaluate their contribution to the modulation of host immune responses through the studies described in this work would also be of benefit to understand the role of disease and health-associated bacteria in oral biofilms.

The 3, 7 and 10 multi-species were developed with the intention of co-culture with host cells to investigate the response to different combination of bacteria species and the host. However, these models could also be used to study aspects of biofilm formation, development and interactions between particular bacterial species within the biofilm. It would also be particularly interesting to investigate the differences in the metabolomics of these biofilms as it is suggested that the metabolic outputs such as lipase, protease and glycosidase activity, rather than the bacterial species themselves, define the pathogenicity of oral diseases, due to the interactions between species within the biofilm (Barnes et al., 2011).

In the co-culture model it was observed that biofilm composition influenced epithelial cell responses. Further studies on both epithelial cells and other immune cell types to understand the mechanisms by which these biofilms modulate immune responses would allow better understanding of disease pathogenesis and may provide therapeutic targets. In particular, focus on inflammatory signalling pathways and PAMP receptors would further the understanding of the mechanisms which immune responses are regulated by oral bacteria. This could be achieved using the co-culture model described herein; however, use of a multi-cell model or 3D culture system would enhance studies. This would allow more representative modelling of the *in vivo* situation within the oral cavity such as a multi-cell model using both fibroblasts and epithelial cells to investigate inflammatory biofilm responses. Alternatively, a 3D culture model using epithelial cells and immune cells in co-culture with biofilms could measure chemotaxis to determine if modulation of local host responses alters the ability of immune cells to migrate to the site of inflammation.

In this study preliminary data was generated investigating the differential immunomodulation of cells of the monocyte lineage by multi-species biofilms,

observing differences in activation, gene expression and protein release. There were limitations in characterizing the phenotype of the pro-monocytes following differentiation with biofilms, PMA and vitamin D_3. This work could be taken further with more in-depth analysis of cell phenotype using surface markers. In addition, this would be of particular importance to investigating activation of macrophages to M1 and M2 phenotypes, which can have pro- or anti-inflammatory effects on other immune cell types, which may contribute to disease pathogenicity.

Finally, the work in this thesis using the different biofilm models shows the ability to evaluate how anti-microbial or anti-inflammatory molecules interact with biofilms and host cells. These models could potentially be used in future to understand the mode of action of novel therapeutics.

8.6 Summary

The key findings of the research presented in this thesis are a follows:

Biofilm composition directly affects inflammatory and immune responses by host cells (Figure 8.1)

- Strain variation in *P. gingivalis* can influence epithelial cell responses even in a multi-species biofilm

- Biofilm composition can influence epithelial cell viability, gene expression and protein release in co-culture

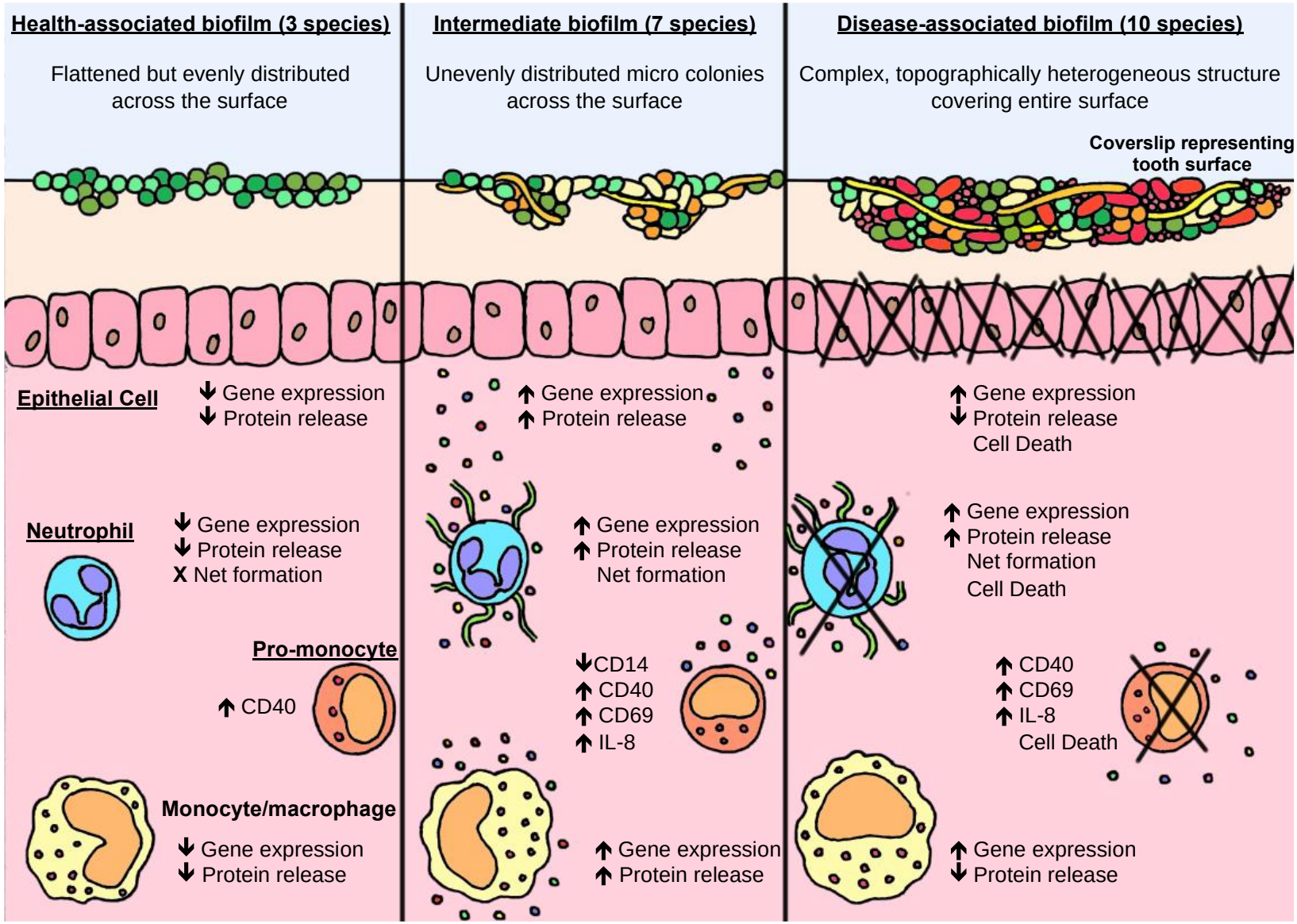
- Biofilm composition can influence neutrophil gene expression, protein release and NET formation in co-culture

- Biofilm composition can influence cells of the monocyte lineage gene expression and protein release both directly and indirectly through conditioned media

- Multi-species co-culture models using biofilms and epithelial cells are a useful tool for studying potential anti-microbial and anti-inflammatory therapeutics

Overall, these findings have increased the understanding of the role of biofilm composition in the modulation of host: pathogen interactions within the oral cavity. The development of the co-culture model system used in this study can allow for future investigation of these interactions using a variety of cell types to allude the mechanisms, which contribute to disease pathogenesis and thereby identify more therapeutic targets for the treatment of PD.

Figure 8.1: Biofilm composition differentially modulates host responses
The three multi-species biofilms showed differences in biofilm architecture, and co-culture with host cells resulted in different host inflammatory gene expression and protein release, cell viability and cell differentiation. Collectively, these data show how biofilm composition can alter host responses providing a greater understanding of the potential mechanisms involved the host: biofilm interactions in oral homeostasis and the pathogenesis of PD. Illustrated by Emma Millhouse.


Health-associated biofilm (3 species)
Flattened but evenly distributed across the surface
Intermediate biofilm (7 species)
Unevenly distributed micro colonies across the surface
Disease-associated biofilm (10 species)
Complex, topographically heterogeneous structure covering entire surface
Coverslip representing tooth surface
Epithelial Cell
↓ Gene expression
↓ Protein release
↑ Gene expression
↑ Protein release
↑ Gene expression
↓ Protein release
Cell Death
Neutrophil
↓ Gene expression
↓ Protein release
X Net formation
↑ Gene expression
↑ Protein release
Net formation
↑ Gene expression
↑ Protein release
Net formation
Cell Death
Pro-monocyte
↑ CD40
↓CD14
↑ CD40
↑ CD69
↑ IL-8
↑ CD40
↑ CD69
↑ IL-8
Cell Death
Monocyte/macrophage
↓ Gene expression
↓ Protein release
↑ Gene expression
↑ Protein release
↑ Gene expression
↓ Protein release

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