MECHANISTIC LINKS BETWEEN PERIODONTITIS AND DIABETES

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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iii. Abbreviations

$(NH_4)_2SO_4$ – Ammonium sulphate

ACTB – β-actin gene

AGE – Advanced glycation end product

AGER – Advanced glycation end product receptor gene

AMP – Adenosine monophosphate

ANOVA – Analysis of variance

APC – Allophycocyanin

ATP – Adenosine triphosphate

BHI – Brain heart infusion

bp – Base pair

BP1 – Bactericidal/permeability-increasing protein 1

BSA – Bovine serum albumin

BspA – *Bacteroides* surface protein A

CBA – Columbia blood agar

cDNA – Complementary DNA

CFAT - Cadmium sulphate fluoride acridine trypticase agar

CFU – Colony forming unit

CML – N-carboxymethyl lysine

CO_2 – Carbon dioxide

CRP – C-reactive protein

DC – Dendritic cells

DCF – 2',7'-dichlorofluorescein

Del-1 – developmental endothelial locus-1

DHR 123 – Dihydrochodamine

DIAMOND – Double index alignment of next-generation sequencing data

DNA – Deoxyribonucleic acid

ds DNA – Double stranded DNA

ECM – Extracellular matrix

EDTA – Ethylenediaminetetraacetic acid

EggNOG – Evolutionary genealogy of genes: non-supervised orthologous groups

ELISA – Enzyme linked immunosorbent assay

EMBL – European molecular biology laboratory

ERK – Extracellular signal-regulated kinase

FACs - Fluorescence-activated cell sorting

FBS – Foetal bovine serum

FFAs – Free-fatty acids

FITC - Fluorescein isothiocyanate

fMLP - N-formyl-methionyl-leucyl-phenylalanine

FSC – Forward scatter

GATA3 – GATA-binding protein 3

GCF – Gingival crevicular fluid

Gcp – Glycopeptidase

GM-CSF – Granulocyte monocyte-colony stimulating factor

H_2DCFDA – 2',7'-dichlorodihydrofluorescein diacetate

H_2SO_4 – Sulphuric acid

HA – Hydroxyapatite

HbA1c – Haemoglobin A1c

HMGB1 – High mobility gggg box 1

HMP – Human microbiome project

HOCl – Hypochlorous acid

HOMD – Human oral microbiome database

HPC – High performance computing

HPLC – High performance liquid chromatography

HPRT1 – Hypoxyanthine phosphoribosyltransferase gene

HRP – Horseradish peroxidase

HSA – Human serum albumin

ICAM-1 – Intracellular adhesion molecule

IFN – Interferon

Ig – Immunoglobulin

IL – Interleukin

iNOS - Inducible nitric oxide synthase

JAK2 - Janus kinase 2

JE – Junctional epithelium

K_2HPO_4 – Dipotassium phosphate

KCl – Potassium chloride

KH_2PO_4 – Monopotassium phosphate

LAP – Localised aggressive periodontitis

LDH – Lactate dehydrogenase

LPS – Lipopolysaccharide

LRP – Leukocyte-rich-plasma

LRR – Leucine rich repeat

MAMPs – Microbial-associated molecular patterns

MAPK – Mitogen-activated protein kinase

MCP – Monocyte chemoattractant protein

MEGAN – METaGenome ANalyzer

MFI – Mean fluorescence intensity

MG – Methylglyoxal

$MgSO_4$ – Magnesium sulphate

MIP – Macrophage inflammatory protein

MMP – Matrix metalloproteases

MPO – Myeloperoxidase

mRNA – Messenger RNA

Na_2CO_3 – Sodium carbonate

Na_2HPO_4 – Disodium hydrogen phosphate

NaCl – Sodium chloride

NADPH – Nicotinamide adenine dinucleotide phosphate

$NaHCO_3$ – Sodium bicarbonate

NALP3 - Nacht domain-, LRR-, and PYD-containing protein 3

NCBI – National center for biotechnology information

NET – Neutrophil extracellular trap

NF-κB – Nucleotide factor κB

NGAL – Neutrophil gelatinase-associated lipocalin

NGS – Next generation sequencing

NK – Natural killer cells

NLRP2 – NACT, LRR and PYD domains-containing protein 2

NO – Nitric oxide

NOD – Nucleotide oligomerization domain receptor

OBL – Osteoblast

OCL – Osteoclast

OD – Optical density

OPG – Osteoprotegerin

OTU – Operational taxonomic unit

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PCoA - Principle coordinate analysis

PCR – Polymerase chain reaction

PE – Phycoerythrin

PGE_2 – Prostaglandin E_2

PMA – Phorbol 12-myristate 13-acetate

PMT – Photomultiplier tube

PRR – Pattern recognition receptor

PYD – Pyrin domain

PYR – Pyrraline

QIIME – Quantitative insights into microbial ecology

qRT-PCR – Quantitative real time – polymerase chain reaction

R 123 – Rhodamine 123

RAGE – Receptor for advance glycation end product

RANK - Receptor activator of NF-κB

RANKL – Receptor activator of NF-κB ligand

rDNA – Ribosomal DNA

RhoA – Ras homolog gene family, member A

RIPA – Radioimmunoprecipitation assay buffer

RNA – Ribonucleic acid

ROR-γ – Retinoic acid receptor related orphan receptor γ

ROS – Reactive oxygen species

rpm – Rotation per minute

rRNA – Ribosomal RNA

RTF – Reduced transport fluid

SD – Standard deviation

SDS – Sodium dodecyl sulphate

SSC – Side scatter

STAT - Signal transduction and activation of transcription

Strep HRP – Streptavidin horseradish peroxidase

TBS-T – Tris-buffered saline-tween

TGF – Transforming growth factor

TGS – Tri-Glycine SDS

Th – T-helper cells

TIGK – Telomerase immortalised gingival keratinocytes

TIMP – Tissue inhibitor of metalloproteinase

TLR – Toll-like receptor

TMB – 3,3’,5,5’-Tetramethylbenzidine

TNF – Tumour necrosis factor

TYK2 - Tyrosine kinase 2

WCL – Whole cell lysate

iv. Abstract

Introduction: A complex two-way link between periodontitis and diabetes is widely accepted. Although the mechanisms underlying this link have not been fully elucidated, disruption of host immunity (neutrophil function, cytokine release), tissue biochemistry, accumulation of advanced glycation end products (AGEs), matrix metalloproteases (MMPs) levels and the oral microbiota have been implicated.

Aims and objectives: This project aims to investigate potential mechanisms through analysis of the impact of AGEs (a consequence of hyperglycaemia), on biofilm composition and telomerase immortalised gingival keratinocytes (TIGK). In addition, presented here is a preliminary feasibility study for comparison of oral biofilm composition and functionality, neutrophil function, and saliva and gingival crevicular fluid (GCF) cytokine and MMP profiles from healthy and periodontitis individuals.

Methods: The study characterised receptor for AGE (RAGE) expression in TIGK cells and the consequent immune response initiated by AGE/RAGE interactions in these cells. Model oral biofilms (comprising five periodontal species) grown in the presence and absence of AGE were characterised using culture dependent methods. Complex biofilms, derived from combined saliva/tongue/plaque inocula, grown with AGE concentrations representative of hyperglycaemia or health were analysed using next generation sequencing. In a preliminary study, biofilms were also co-cultured with TIGK cells in media supplemented with high AGE concentrations to determine changes in inflammatory responses.

Individuals with periodontitis and healthy controls were recruited through DenTCRU for the preliminary clinical study. Neutrophil migration, phagocytosis and respiratory burst in these individuals were analysed. Plaque, GCF and saliva were collected for analysis of cytokine and MMP expression and oral microbiome profiles.

Results: The expression of RAGE by TIGK cells, at either the mRNA or protein level, did not change with varying concentrations of AGE. The addition of AGE to model five species biofilms encouraged the growth of *A. naeslundii* while reducing the proportion of *P. gingivalis* in the biofilms. Analysis of complex biofilms indicated enrichment of genera including *Prevotella*, *Streptococcus* and *Veillonella* and decreases in *Fusobacterium*, *Campylobacter* and *Bacteroides* amongst others.

The preliminary clinical study indicated feasibility of analysing neutrophil function and cytokine and MMP profiles from saliva and GCF. While impairment of neutrophil functions, increase in MMP8 and MMP9, changes in biofilm compositions and increases in cytokines in saliva (IL-8, IL-1β and MCP-1), plasma (IL-8 and IL-1β) and GCF (IL-8,

IL-1β and MCP-1) were observed in periodontitis, further analysis using a larger cohort of individual's ± periodontitis is required for these to reach significance.

Conclusion: Together the results suggest AGEs can alter the composition of biofilms, appearing to encourage the growth of health associated genera. Preliminary co-culture experiments demonstrate co-culture of TIGK cells with complex biofilms decreases IL-8 and IL-6 release.

1. Introduction

The oral cavity is in a delicate balance between the microorganisms that colonise it and the host immune response. The following, reviews the current understanding of microbiology and immunological processes involved in the maintenance of oral health and how dysregulations can lead to oral diseases. A particular interest is paid to periodontitis; understanding the aetiology of periodontitis can help our understanding of the mechanisms that link periodontitis to systemic diseases. This review and the research presented in this thesis examines the relationship between periodontitis and diabetes. The two diseases have long been considered interlinked, but the processes involved are relatively unknown.

The research presented here focusses on the immune responses of periodontitis and how hyperglycaemic conditions in diabetes can influence both the bacterial composition of the oral cavity microbiota and the inflammatory responses of oral cells.

1.1. The oral microbiome

The multitude of microorganisms colonising the human body are commonly reported to outnumber human cells 10:1, providing functions which are essential for survival (Bryan *et al*., 2017). Although still quoted in the literature, this ratio was recently challenged in a study which re-evaluated both the number of bacterial cells and human cells in an average 70 kg body and concluded a 1:1 ratio was more accurate (Sender *et al*., 2016). The microorganisms of the body and microbial communities of various sites are referred to as the 'microbiota' (Lederberg and McCray, 2001). Colonisation of the body begins at birth through the exposure to microorganisms from the environment and other people. Factors, including tissue type, temperature, pH and nutrient availability as well as host age, sex and diet, influence microbial colonisation so that distinct sites within the human body have diverse and characteristic microbial communities. This colonisation leads to the generation of the 'microbiome' which is defined as the collective genomes of all the microbial species at a given site as well as the genomes of the environment they reside in (Marchesi and Ravel, 2015, Abdul-Aziz *et al*., 2016). The microbiota impact, both directly and indirectly, aspects of the body's normal physiology, nutrition and defence. In health, the microbiota has a harmonious relationship with the host but a disturbance or imbalance in the normal composition of the microbiota which causes changes to the host responses (dysbiosis) can lead to a variety of diseases (Morgan *et al*., 2013, Petersen and Round, 2014, Abdul-Aziz *et al*., 2016).

As early as the 1680s, studies into the diversity of the human microbiota have been carried out. Antonie van Leeuwenhoek in 1683 began the comparison of the species present in his oral cavity and faecal matter and comparisons between health and

disease, noting the difference between them (Porter, 1976, Ursell *et al*., 2012). Advancements in both culture dependent and culture-independent molecular techniques have vastly increased our understanding of the human microbiota. With the initial DNA sequencing methods of Sanger sequencing to the now commonly applied high-throughput NGS, a vast amount of data have been collected and analysed, providing insight into the composition and functionality of the human microbiota (Curtis *et al*., 2011, Human Microbiome Project, 2012, Morgan *et al*., 2013, Abdul-Aziz *et al*., 2016).

Although the majority of the 10-100 trillion symbiotic microbes that make up the human microbiota are found as bacteria in the gut, the oral cavity harbours the second largest microbiota within the human body (Lazarevic *et al*., 2010, Ursell *et al*., 2012, Wade, 2013). The oral microbiota is considered to include microbes and biofilms (surface associated, structured microbial community enclosed in an extracellular polymeric substance matrix) originating from various sites within the mouth such as the mucosal surfaces of the tongue, cheeks, gingiva (gums) and tonsils and the harder supragingival or subgingival surfaces of the teeth (Curtis *et al*., 2011, Ursell *et al*., 2012, Proctor and Relman, 2017). The diversity in biofilm structure and composition across these various sites exists partially due to heterogeneity in the topographical anatomy which leads to variations in local chemistry, temperature, moisture, host physiology and immunity (Proctor and Relman, 2017). In line with this, a recent study using human microbiome data showed microbial communities on the exposed tooth surfaces are influenced by both tooth aspect and class of tooth as well as the physical distance separating the sites (Callahan *et al*., 2016).

Furthermore, variations in tissue types and their associated structure also influence microbial communities. The oral cavity consists of both shedding (oral mucosa) and non-shedding (dental enamel) tissue, as well as non-keratinized and keratinized cells (which can further be divided into parakeratinized or orthokeratinized cells), all of which contributes to the differences in microbial colonisation seen across sites in the oral cavity (Proctor and Relman, 2017).

In addition to anatomical and structural differences in the oral cavity, proximity to salivary glands also impacts microbial communities (Proctor and Relman, 2017). For example, microbial differences seen between cheek facing surfaces of teeth and tongue facing surfaces of teeth is partially associated with the dense network of the minor salivary glands. They bathe the glands and surrounding tissues (labial, palatal and buccal mucosa) in viscous, highly proteinaceous secretions with low buffering capacity so that these tissues have distinct microbial communities (Sato *et al*., 2015, Simon-Soro *et al*., 2013, Proctor and Relman, 2017). The major salivary glands have differing salivary

secretory rates and salivary composition, creating gradients in salivary film velocity, oral clearance and pH across teeth surfaces (Proctor and Relman, 2017).

Bacterial communities of the mouth encompass over 1000 different species with individuals harbouring approximately 400 species at any given time (Dewhirst *et al*., 2010, Wade, 2013, Samaranayake and Matsubara, 2017). The advancements in technologies and the completion of the Human Microbiome Project (HMP) have enabled the identification of the dominant phyla of the oral microbiome; *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, *Spirochaetes* and *Fusobacteria* account for 96% of species present (Human Microbiome Project, 2012, Zhu and Kreth, 2012, Wade, 2013, Zaura *et al*., 2014). In addition to the bacterial population, the oral microbiome also includes viruses, archaea, fungi and protozoa (Wade, 2013). Given the high oral bacterial density, viral presence in the mouth is mostly associated with bacteriophages (Pride *et al*., 2012, Wade, 2013). A range of disease-associated viruses (such as upper respiratory infection causing viruses) can also be found in the mouth during acute phases of infection (Wade, 2013). A much smaller number of protozoa are associated with the oral microbiome, mainly *Entamoeba gingivalis* and *Trichomonas tenax,* with an increase in both seen in oral diseases and poor oral hygiene. Although initially this increase implicated the microorganisms as potential pathogens, it is now believed that the lack of oral hygiene causes an increase in food debris and bacteria providing a rich nutritional environment for the protozoa (Wade, 2013). The predominant fungal genera are *Candida, Cladosporium, Aureobasidium, Saccharomycetales, Aspergillus, Fusarium and Cryptococcus. Candida* species, although causing acute and chronic infections, are carried asymptomatically by many individuals (Arendorf and Walker, 1979, Wade, 2013). Archaea in the oral microbiota is limited to select species able to colonise/survive in the environment; *Methanobrevibacter oralis* and two *Methanobrevibacter* phylotypes (*Methanobacterium curvum/congolense* and *Methanosarcina mazeii*). These methanogens are detected in a healthy mouth in small proportions, but an increase is observed in oral diseases (Lepp *et al*., 2004, Matarazzo *et al*., 2011, Wade, 2013).

1.1.1. Establishment of a healthy oral microbiota

The oral cavity, being nutrient-rich and humid with a resting pH of between 6.75 and 7.25, is an ideal environment for microbial colonisation (Rosier *et al*., 2014). The biofilms found in the oral cavity, particularly on the surface of the teeth, are referred to as dental plaque and although found in health, they are also associated with the most common oral diseases (Rosier *et al*., 2014, Samaranayake and Matsubara, 2017, Marsh *et al*., 2015). Data from the HMP have indicated that the oral microbiome has the largest number of commonly shared microbes amongst unrelated individuals, more so than habitats such as the gut or the skin (Human Microbiome Project, 2012, Li *et al*., 2013).

Despite daily physical and chemical challenges the oral microbiome encounters through processes such as food intake and oral hygiene, a long-term stable microbiome is present in healthy mouths. The ability of the oral microbiota to adapt to changes in oxygen availability, temperature, pH, antimicrobials and dietary components and survive mechanical sheer forces (through mastication and brushing), helps establish and maintain health (Wright *et al*., 2013, Zaura *et al*., 2014). Although the processes that contribute to the development of diseases (particularly caries and periodontal diseases) are well documented, the mechanisms that maintain health are relatively poorly understood.

1.1.1.1. Early colonisation

Although the process of oral bacterial colonisation begins immediately following birth, tooth eruption creates novel microhabitats. These new potential colonisation sites can increase the diversity of the microbiota. In addition, complement, phagocytes and other components from the bloodstream begin to enter the oral cavity in minute amounts, providing novel growth substances and immune control of commensal and pathogenic bacteria (Proctor and Relman, 2017). This subsequently changes microbiota composition and is reflected in infants where species such as *Streptococcus salivarius* and *Streptococcus mitis* colonise within days of birth but species such as *Streptococcus sanguinis* are not seen until tooth eruption, due to the ability of *S. sanguinis* to colonise dental enamel but not mucosa (Carlsson *et al*., 1975, Proctor and Relman, 2017).

Dental plaque formation is a continuous process which begins following the selective absorption of salivary glycoproteins to the surface enamel of teeth, forming a protein film called a pellicle (Figure 1.1). The pellicle contains both saliva and gingival crevicular fluid (CGF) proteins including glycoproteins, lysozymes, proline rich peptides, lactoperoxidases, lipids and phosphoproteins (Marsh *et al*., 2016). The acidic proline rich proteins contain binding sites for both hydroxyapatite and bacteria and may provide highly specific attachment sites for early colonising bacteria (Carlen *et al*., 1998, Dodds *et al*., 2005). If the supragingival plaque matures and extends down the tooth to develop into subgingival plaque, an inflammatory response may be initiated leading to the development of gingivitis (Figure 1.1).

Streptococcus and *Actinomyces* species are the predominant early colonisers of tooth surfaces with evidence showing early colonisation by *Actinomyces* following tooth cleaning with relative proportions shifting to favour streptococci (particularly *S. mitis* and *Streptococcus oralis*) between 2 and 6 hours after tooth cleaning (Li *et al*., 2004). Early colonisers recognise molecules which allow adherence to tooth surfaces. For streptococci and *Actinomyces* species these include host derived receptors such as

statherin and proline rich proteins which have absorbed into the pellicle from saliva. Later colonisers are attracted to these via bacterial signals and metabolites and are able to adhere to the early colonisers (co-aggregate) leading to the generation of a more complex biofilm (Kolenbrander *et al*., 2010, Curtis *et al*., 2011). Certain species, such as *Streptococcus gordonii*, can interact with both salivary proteins (which would include those in the pellicle) and other bacteria, and as such make effective early colonisers (Nobbs *et al*., 2009). Other species, such as *Fusobacterium nucleatum*, are particularly strong co-aggregators, with the ability to interact with early and late colonisers and are considered important bridging bacteria in the processes of biofilm formation (Kolenbrander *et al*., 2010).

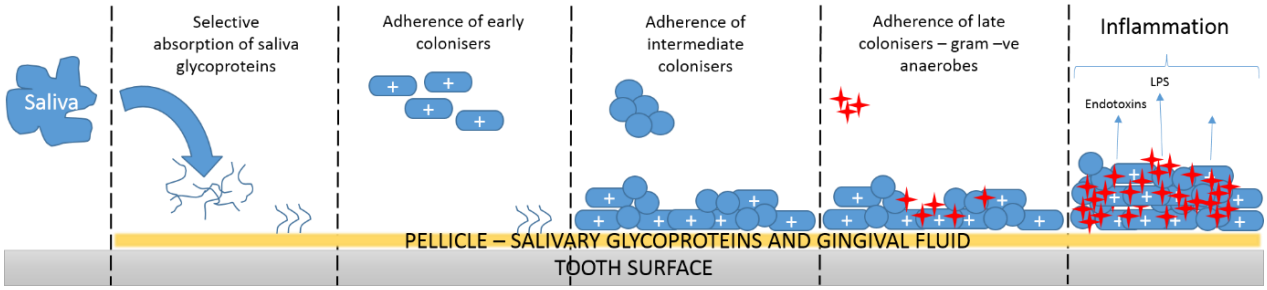


Figure 1.1: Colonisation of oral biofilm leading to the establishment of gingivitis. During the formation of biofilms there is a selection of early, intermediate, and late-colonizing species (Kolenbrander *et al*., 2010, Curtis *et al*., 2011). Gram-positive aerobic and facultative anaerobic early colonisers are able to adhere to the pellicle proteins, and then form binding sites themselves for the addition of more bacteria allowing the development of the biofilm (Murray *et al*., 1992, Ahn *et al*., 2002, Kolenbrander *et al*., 2010, Curtis *et al*., 2011, Wade, 2013). Biofilm maturation can increase the presence of Gram-negative anaerobes which then activate host inflammatory responses.

1.1.1.2. Microbial community interaction and microbial regulation involved in maintaining oral health

Microbial community interactions also play a role in preservation of healthy microbiota. Once formed, dental plaque is structurally highly organised with complex mature biofilms having more resilience than immature biofilms or planktonic bacteria to mechanical removal (through oral hygiene processes) and to host immunity (Wright *et al*., 2013, Xu and Gunsolley, 2014).

In established biofilms, specific bacteria are often found near each other or as a mixed population to create specific structures which encourage growth or adherence (Kuramitsu *et al*., 2007, Xu and Gunsolley, 2014). For example, *Actinomyces naeslundii* and *S. oralis* cannot effectively colonise saliva coated surfaces alone but together can produce biofilms on these surfaces due to the combined utilisation of salivary metabolites (Palmer *et al*., 2001, Kuramitsu *et al*., 2007). Bacterial co-operation also exists in biofilms where multiple species are involved in the metabolism of complex host molecules (such as glycoproteins) which cannot be metabolised by one species alone (Marsh *et al*., 2015). Indeed, as early as 1992 it was documented that *Streptococcus mutans* and *Streptococcus sobrinus* independently metabolise bovine serum albumin (BSA) with low efficiency but combined with *S. oralis* or *F. nucleatum*, higher BSA degradation was observed (Homer and Beighton, 1992).

Commensal bacterial communities have established mechanisms to prevent pathogenic colonisation which include competition for nutrients, neutralization of virulence factors, production of antagonists to inhibit growth and interference in growth impacting signalling mechanisms (Kuramitsu *et al*., 2007, Zaura *et al*., 2014). An example of direct control of colonisation by pathogens is seen in *S. salivarius* K12*,* which has been shown to inhibit the growth of periodontitis- and halitosis- associated Gram-negative bacteria through the production of the bacteriocins; salivaricin A2 and salivaricin B (Burton *et al*., 2006, Masdea *et al*., 2012, Wade, 2013). *S. gordonii*, exemplifies the ability of some bacterial species to interfere with growth mechanisms of other bacteria. In biofilms and to a lesser effect in broth, *S. gordonii* has been demonstrated to antagonize several quorum sensing-dependent mechanisms (particularly the production of bacteriocins) of *S. mutans*. This process is reliant on the inactivation of the *S. mutans* competence-stimulating peptide (a quorum sensing mediator) by *S. gordonii* (Wang and Kuramitsu, 2005).

Conversely, some bacteria aid colonisation and growth of other bacterial species (often commensals). For instance, *A. naeslundii* is able to cleave sialic acid residues found in oligosaccharide side chains on epithelial cells and in the enamel pellicle through the

action of a neuraminidase. This then exposes galactosyl sugar residues that can be utilised by bacteria expressing galactosyl-binding lectins for attachment (Gibbons *et al*., 1990, Marsh *et al*., 2016).

Community members of a commensal oral biofilm not only hinder pathogen colonisation but also influence other members of the community via synergistic mechanisms that stimulate the growth/survival of other members. Furthermore, inter-species bacterial communication is important to the integrity of the biofilm with inter-microbial adhesion, cell signalling through cell-cell contact, metabolic interactions and quorum sensing all playing crucial roles (Kuramitsu *et al*., 2007, Wright *et al*., 2013, Zaura *et al*., 2014).

Commensal bacteria aid in preventing a heavily colonised site from entering a damaging state of inflammation by regulating host responses. Commensal bacteria from subgingival plaque can influence neutrophil deployment by regulating expression levels of intracellular adhesion molecule I (ICAM-1), E-selectin and cytokines (Dixon *et al*., 2004, Devine *et al*., 2015). Cytokines such as interleukin-8 (IL-8, also known as CXCL8), CXCL1 and CXCL2 secreted from gingival cells and found in gingival tissues, saliva and gingival crevicular fluid (GCF) establish gradients for neutrophil recruitment (Devine *et al*., 2015). Neutrophils (as discussed in detail in section 1.1.1.3) are important in the maintenance of healthy microbiota. Neutrophil recruitment is regulated by cytokines/chemokines, expression of neutrophil cytokine/chemokine receptors and via their effects on expression of the adhesion molecules (detailed in section 1.1.1.3). Thus, altering neutrophil recruitment through cytokine release of gingival cells may be a method by which commensal bacteria establish a health-associated inflammatory status (Devine *et al*., 2015). In fact, a study has demonstrated that mouse colonisation by commensal bacteria increased CXCL2 expression and consequently increased neutrophil recruitment (Zenobia *et al*., 2013). While both CXCL1 and CXCL2 were expressed in periodontal tissues of germ-free mice, the CXCL2 up-regulation was only observed in mice with commensal colonisation.

Control of cytokine levels by commensal bacteria is of much interest considering the role cytokines play in both innate and adaptive immunity in the oral cavity (discussed in more detail in section 1.2.4). In cases where changes in cytokines have been explored, commensal bacteria often demonstrate inhibition or down-regulation of cytokine release. Illustrating this is a study showing *S. salivarius* K12 inhibition of epithelial cell IL-8 secretion. *S. salivarius* has also been shown to prevent normal IL-8 responses from epithelial cells stimulated by LL37, flagellin and *Pseudomonas aeruginosa* (Cosseau *et al*., 2008). In addition, K12 has been implicated in regulating immune responses through cytokine independent mechanisms, these include production of antimicrobial peptides (salivarin A2 and B), up-regulation of hepcidin (antimicrobial and iron regulating peptide)

and activation of type I and type II interferon responses (Hyink *et al*., 2007, Cosseau *et al*., 2008, Devine *et al*., 2015). Furthermore, a study using oral administration of the probiotic lactobacillus, *Lactobacillus reuteri*, to individuals with mild gingival inflammation was associated with a decrease in GCF IL-8 and tumour necrosis factor-α (TNF-α) concentrations (Twetman *et al*., 2009). This provides more evidence for the role of commensal bacteria in the regulation of immune responses in the oral cavity.

There is also evidence suggesting that bacteria found in oral health can up-regulate cytokine production. A transcriptomic analysis of *S. gordonii* and *F. nucleatum* co-cultured with gingival epithelial cells, demonstrated that while both bacteria altered the epithelial transcriptome to a lesser extent than pathogens, they did alter MAPK (mitogen-activated protein kinase) and TLR (toll-like receptor) expression (Hasegawa *et al*., 2007); both MAPK and TLR can impact cytokine production, suggesting bacterial immunomodulatory roles. Indeed, *S. gordonii* was shown to inhibit IL-8 and IL-6 secretion while *F. nucleatum* caused an increase (Hasegawa *et al*., 2007). This suggested that a delicate balance in immune regulation is achieved by oral commensals to ensure the gingival tissues do not enter a detrimental inflammatory state.

1.1.1.3. Microbial recognition and immune regulation by the oral mucosa

Following colonisation and the establishment of the stable microbiota, a complex, bi-directional interaction between host and microbiota maintains health. Site-specific and continual interactions between microbes and the host immune system mean microbiota of mucosal surfaces (including those in the oral cavity and gut) are influenced by both innate and adaptive immune responses (Zaura *et al*., 2014).

The oral mucosa plays an important role in health through immune activation in response to the constant exposure to microbes, environmental antigens (diet derived and airborne) and damage through mastication and oral hygiene. In health, the oral cavity must maintain effective immune surveillance and clearance of pathogenic or excessive bacteria without mounting uncontrolled or disproportionate immune responses (Moutsopoulos and Konkel, 2017). To aid in this, the oral mucosa is composed of both oral epithelial cells and immune cells (including cells such as macrophages, dendritic cells (DCs), natural killer (NK) cells and polymorphonuclear neutrophils), which contribute to the plethora of immunomodulatory cytokines found in the oral cavity. Oral keratinocytes further contribute to immune regulation through the production of biological mediators such as antimicrobial peptides (Feller *et al*., 2013).

The specifics involved in health and immune homeostasis maintenance, however, are not well understood. While immune tolerance to commensal bacteria at other barrier surfaces (such as skin) in the human body are relatively well explored, immune control

in oral health is not (Moutsopoulos and Konkel, 2017). Although specific local immunity in oral health initiated by commensal microbes is not fully understood, Dutzan *et al* (2016) has shown lymphocytes in healthy oral tissue have a high proportion of T-cells and a network of antigen presenting cells, suggesting priming of the immune cells to local antigens. There is also evidence, however, to suggest that homeostasis is not only dependent on host-microbial interactions but also microbe independent regulation. For example, a study in germ-free mice showed no changes in gingival Th17 (T-helper 17) cell number compared with controls, indicating gingival Th17 cell accumulation was not dependent on commensal colonisation (Dutzan *et al*., 2017). The role of Th17 cells in the oral cavity is discussed in more detail in section 1.2.2.3. Contrastingly, a different study in germ-free mice demonstrated microbial colonisation may have distinct effect of innate immune cells by showing an increase in neutrophil recruitment in commensal colonised mice compared with the germ-free mice (Zenobia *et al*., 2013).

Much like other barriers in the human body (skin/gut), the oral mucosa's first line of defence is a physical barrier in the form of keratinized epithelia. The oral mucosa consists of a variety of epithelial cells of varying keratinization. The gingival crevice epithelium is a non-keratinized squamous epithelium lining the inside of the gingivae (gingival sulcus) and towards the base of the sulcus (where the gingiva meets the tooth) becomes incompletely differentiated epithelium, the junctional epithelium (JE). The JE and lack of keratinization makes the gingival crevice epithelium particularly vulnerable to bacterial and mechanical damage. These JE are attached to the tooth by hemi-desmosomes to provide a permeable connection for the movement of GCF. Furthermore, JE cells are important to the transmigration of neutrophils (Groeger and Meyle, 2015, Moutsopoulos and Konkel, 2017). Indeed, histological investigation showed an accumulation of inflammatory cells at a steady state in the gingival crevice (Moskow and Polson, 1991).

In addition to the physical properties, gingival epithelial cells are also important in the recognition of bacterial products. Microorganism recognition by cells is mainly accomplished through members of the pattern recognition receptor (PRR) family and opsonins. While a variety of receptors are associated with PRRs to enable recognition of viral, fungal, protozoal and bacterial MAMPs (microbial-associated molecular patterns), TLRs and nucleotide oligomerization domain receptors (NODs) are the most prevalent and well-studied in oral cells (Ross and Herzberg, 2016, Feller *et al*., 2013). Oral and gingival epithelial cells have been shown to express TLRs 1 – 9 with variable and inducible expression and localisation (Sugawara *et al*., 2006, Beklen *et al*., 2007, McClure and Massari, 2014).

Upon activation, TLRs initiate inflammatory responses via signalling cascades (NF-κB, MAPK and interferon regulatory factors) that lead to the transcription of cytokines.

Although many of the cytokines produced are pro-inflammatory and, as such, are involved in the recruitment of immune cells, immune cell-independent responses are also induced in oral keratinocytes. This includes production of antimicrobial peptides (LL-37 and β-defensins) which are immunomodulatory molecules and can directly kill microorganisms (Ross and Herzberg, 2016). Epithelial release of IL-1α in response to microbial stimulation leading to the transcription of the antimicrobial protein calprotectin is an example of immune cell-independent responses (Sorenson *et al*., 2012). The study suggested this was achieved through IL-1 receptor interactions on adjacent or the same epithelial cell. Furthermore, increased resistance to bacterial invasion was also observed (Sorenson *et al*., 2012). The activation of anti-microbial mechanisms such as these, highlight potential roles of gingival epithelial cells in maintaining health and immune homeostasis.

TLR activation in the gingival epithelium initiates the release of proinflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8 and TNF-α, which are discussed in detail in section 1.2.4 (Meyle and Chapple, 2015, Groeger and Meyle, 2015). The release of these cytokines initiates inflammatory responses that are aimed at clearance of microorganisms. Regulation by negative feedback loops ensures resolution is achieved upon clearance of the stimulus, helping maintain the balance between effective microbial control and host damage (Figure 1.2). TLR specificity controls the magnitude and duration as well as type of adaptive immune response. For example, TLR2 recognises MAMPs associated with Gram-negative and Gram-positive bacteria (including lipoproteins and peptidoglycan) while TLR4 recognises bacterial components such as Gram-negative bacterial lipopolysaccharide: LPS. The clearance of pathogens is achieved through activation of phagocytosis by immune cells, release of antimicrobial peptides/inflammatory mediators and activation of inflammasomes (Feller *et al*., 2013, Kramer and Genco, 2017).

In addition to release of cytokines, epithelial detection of microbes has also been linked to activation of the autophagosome and inflammasome as well as release of reactive oxygen species (ROS) and components of the complement system (Feller *et al*., 2013, Ross and Herzberg, 2016). The complement system is comprised of a cascade of interacting soluble and cell surface bound molecules including pattern recognition molecules, proteases, regulators and signalling receptors. Together these work to initiate and regulate the inflammatory response and are involved in direct microbe killing (Ricklin *et al*., 2010). For example, C3a and C5a recruit inflammatory cells while C3b functions as a microbial opsonin and C5b-C9 attacks membranes of susceptible microbes (Hajishengallis *et al*., 2013). Both complement and TLRs are involved in early defence mechanisms and responses against microbes, acting rapidly once activated to mediate

the link between innate and adaptive immunity (Hajishengallis and Lambris, 2016). Interestingly, microbial products typically associated with TLR activation (such as LPS, zymosan and CpG DNA) have also been demonstrated to initiate complement cascades (Zhang *et al*., 2007, Mangsbo *et al*., 2009). Recently, Hajishengallis and Lambris (2016) reviewed in depth, the studies linking TLR and complement activation and highlighted the importance of the interactions between the two systems, which can act synergistically (to enhance immune responses) or antagonistically (to regulate immune responses and maintain homeostasis).

DCs are antigen presenting cells and are resident members of the oral mucosa. They act as regulators of immune tolerance and protection and play an important role in health. Antigen capture and presentation by DCs leads to the expression of cytokines and molecules required for presentation of antigens to B- and T-cells and the subsequent activation of adaptive immunity. In addition to mounting defences against pathogenic bacteria, DCs can also inhibit immune responses against commensal bacteria via complex mechanisms that involve deletions of T-cells through apoptosis and functional inactivation of T-cells (Santoro *et al*., 2005). Furthermore, they play a role in the development of antigen specific T-regulatory cells (T-regs). These multifactorial roles highlight their importance in homeostasis maintenance. In the gut, DCs can suppress inflammation and promote immunological tolerance of commensal bacteria (Iliev *et al*., 2007). However, when pathogen load is high, non-immunosuppressive DCs are recruited to promote protective immune responses. This mechanism may also take place in the oral cavity (Santoro *et al*., 2005, Zaura *et al*., 2014).

Polymorphonuclear neutrophils perhaps play the biggest role in maintaining oral health and immune homeostasis especially when considering they account for approximately 95% of leukocytes in the oral mucosa and have been shown to be increased during inflammation (Dutzan *et al*., 2016). They are constantly extravasated from the circulation into the gingival tissue via the JE following chemokine gradients established by oral epithelial cells and other immune cells. Their exact role in oral health maintenance is not fully understood (Kinane *et al*., 2017). However, recently a cross-sectional clinical study looking at both oral and systemic polymorphonuclear neutrophils did demonstrate higher neutrophil activation in oral samples compared with systemic neutrophils even in the absence of periodontal disease indicating a role in oral health maintenance (Rijkschroeff *et al*., 2016). A more in-depth discussion of polymorphonuclear neutrophils and in particular their role in periodontitis progression is discussed in section 1.2.1.

1.1.1.4. Role of saliva and gingival crevicular fluid in establishing healthy microbiota

Saliva, being protein rich and continuously bathing the surfaces of the oral cavity, is important in oral health. Components from both saliva and GCF contribute to health associated immunity. The GCF is a serum transudate which flows continuously into the gingival sulcus. It is composed of a variety of variable substances including immunoglobulins, albumin, immune modulators, antimicrobial peptides, tissue breakdown products and microbes from surrounding plaque. It resembles serum and in health is found in small quantities (Khurshid *et al*., 2017, Sonnenschein and Meyle, 2015). Saliva, much like GCF, contains a variety of complex molecules including glycoproteins, inorganic ions, proline-rich peptides, host-defence peptides, mucins and amylase (Marsh *et al*., 2016).

As described previously (section 1.1.1.1) salivary and GCF components play an important role in the generation of the pellicle and subsequent bacterial adherence. In addition, certain salivary proteins act only when in fluid saliva. For example, salivary agglutinin (also known as gp340) and the salivary mucin MUC7 are involved in aggregating planktonic bacteria, leading to their removal through swallowing and preventing their attachment to the surfaces of the oral cavity (Marsh *et al*., 2016, Madsen *et al*., 2010). While this is beneficial in situations where the bacteria could be detrimental to health, it could potentially hinder the establishment of a commensal microbiota. To overcome this, certain proteins found in saliva are equipped to select for particular bacteria. For example, proline rich peptides found in saliva contain bacterial attachment protein segments which are only exposed when the protein is absorbed into the pellicle and bound to the hydroxyapatite on the surface of teeth. This then allows commensal bacteria to avoid aggregation and removal but still bind to the pellicle (Marsh *et al*., 2015). Salivary agglutinins also have the additional function of activating the complement system via the classical pathway (Leito *et al*., 2011). An early study demonstrated interaction of salivary agglutinin with the C1q receptor of complement (the first component of the classical pathway) in freshly isolated human serum (Boackle *et al*., 1993). Under normal conditions, these two proteins would not interact, but under inflammatory conditions where blood or GCF may leak into the saliva their potential for interaction increases (Madsen *et al*., 2010). More recent studies have demonstrated finer control of complement activation by salivary agglutinin, whereby activation of complement is achieved only when agglutinin is surface bound and inhibition is observed when it is in solution (Leito *et al*., 2011, Reichhardt *et al*., 2012). Together these findings suggest that agglutinins may be important in regulating immune responses as well as impacting the microbiota.

Other immunomodulatory molecules in saliva and GCF are the antibodies, secretory immunoglobulin A (sIgA) and to a lesser extent immunoglobulin G (IgG). The exact role of these in health, beyond antimicrobial immune response activities (such as neutralisation and aggregation), are not fully understood (Dawes *et al*., 2015). However, IgA proteases are well known virulence factors of pathogens such as *Neisseria meningitidis* and *Streptococcus pneumoniae* but are also produced by commensal bacteria such as *S. mitis*, *S. oralis* and *S. sanguinis*, all of which are primary colonisers of the oral cavity and are found as the major colonisers of oral mucosal sites of adults (Marsh *et al*., 2009, Zaura *et al*., 2014). This suggests a role in both prevention of colonisation and survival of particular species.

Although the exact role of all the proteins in health are not fully understood, Table 1.1 highlights important health maintenance roles of some of the many proteins found in saliva.

1.1.2. Periodontal diseases

The periodontal diseases gingivitis and periodontitis are two of the most common human diseases (Preshaw *et al*., 2012, Xu and Gunsolley, 2014). Periodontitis is a common condition that affects approximately 10-15% of adults in its severe form and 40-60% in the moderate form (Petersen and Ogawa, 2012, Preshaw *et al*., 2012, Kinane *et al*., 2017). Although not fatal periodontal diseases can have a substantial impact on health services and a patient's quality of life.

Periodontitis predominantly falls into two categories – chronic and aggressive (American Academy of Periodontology, 2015). Chronic periodontitis progresses from untreated gingivitis and is associated with chronic inflammation leading to irreversible tissue damage (Kinane *et al*., 2017). Aggressive periodontitis is often more severe, has an earlier onset and faster progression rate (Armitage, 1999, Wade, 2013). More rare categories of periodontitis include necrotizing ulcerative periodontitis and syndromic chronic periodontitis. While necrotizing ulcerative periodontitis is an acute form of the disease with rapid progression, syndromic chronic periodontitis is associated with systemic diseases (such as Chediak-Higashi syndrome and Papillon-Lefèvre syndrome) which impact host immune responses (Kinane *et al*., 2017). The inflammatory responses associated with periodontitis can lead to the formation of the characteristic periodontal pocket and eventual loss of attachment between teeth and gingivae (Figure 1.2 and Figure 1.3). Periodontal tissue destruction, although slow to progress, is in the main irreversible (Darveau, 2010, Preshaw *et al*., 2012, Scannapieco, 2013, Wade, 2013).

Table 1.1: The role of some salivary components in oral health maintenance

Component	Function in oral health maintenance
α-amylase	Inhibits growth of pathogenic bacteria *Porphyromonas gingivalis* (Ochiai *et al*., 2014).
Histatins	Inhibits growth of opportunistic oral fungus *Candida albicans* (Oppenheim *et al*., 1988).
Statherin	Inhibits growth on anaerobic bacteria (Gorr, 2009). Acts in a buffering capacity and limits damage associated with pH changes caused by bacterial metabolism (Zaura *et al*., 2014) Provides attachment sites in the pellicle for oral colonisers (Dodds *et al*., 2005).
Glycans	Acts as decoys to prevent adherence of *C. albicans* (Everest-Dass *et al*., 2012). Involved in toxin interaction and neutralization (Dawes *et al*., 2015).
Lactoperoxidase	Catalyses the conversion of the bacterial metabolite hydrogen peroxidase and saliva-secreted thiocynate into hypothiocyanite. Hypothiocyanite acts as an antimicrobial and inhibits bacterial glycolysis (Kilian *et al*., 2016).
Lysozyme	Cationic protein that causes bacterial cell wall damage (Dawes *et al*., 2015).
Lactoferrin	Acts as iron chelator. Removal of iron can interfere with metabolism of some pathogens (Dawes *et al*., 2015).

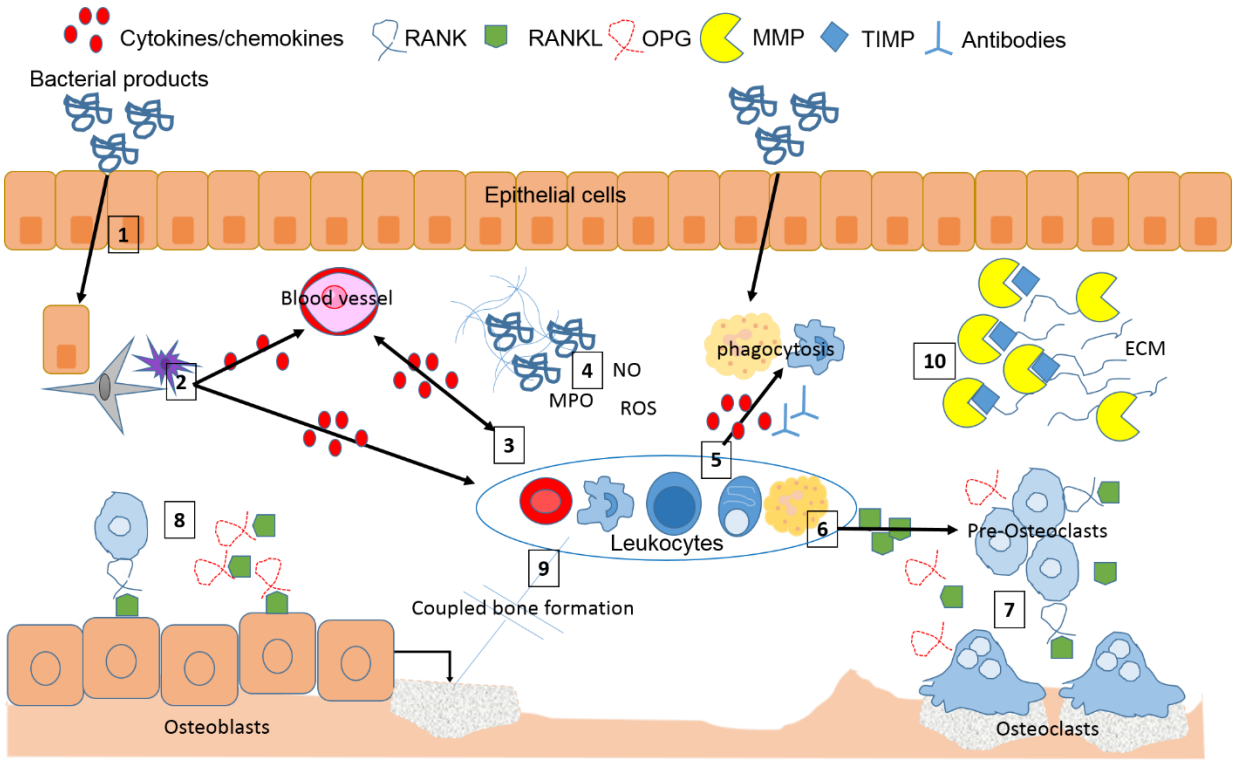


Figure 1.2: Host immune responses and the impact on periodontitis progression. Bacterial products are recognised by cells in the periodontal environment via toll like receptors (TLRs) and nucleotide oligomerization domain receptors (NODs) (1) causing intracellular signalling cascades which result in the release of inflammatory cytokines and chemokines by residential cells (epithelial cells/fibroblasts/dendritic cell) (2). These act on blood-vessels and circulating leukocytes to selectively recruit leukocytes to the periodontal space causing a further release of pro-inflammatory cytokines and chemokines (3). Recruited leukocytes, upon recognition of bacterial products (via TLRs and NODs) become activated to release antimicrobial compounds including myeloperoxidase (MPO) and nitric oxide (NO) and neutrophil extracellular traps (NETS) (4). Maintenance of periodontal health is also aided by the release of cytokines and antibodies that initiate phagocytosis (5). In periodontitis leukocytes also produce or induce production, by resident cells, of RANKL (receptor activator of nuclear factor-κB ligand) (6). An increase in RANKL and a disruption in levels of its inhibitor, OPG (osteoprotegerin) (7) increases bone resorption through activation of osteoclasts (OCLs). This causes a disruption in the bone formation (8) and bone resorption balance. Inflammatory mediators produced by the leukocytes also inhibit coupled bone formation (9) in an attempt to counteract the increased OCL activity. The local chronic inflammatory state results in an imbalance of MMP (matrix metalloproteases) and TIMP (tissue inhibitors of metalloproteinase) ratio leading to increase in extracellular matrix (ECM) destruction of periodontal tissues (10). Periodontal pathogens also activate antimicrobial compounds such as lactoferrin, α- and β- defensins and LL-37. Adapted from Garlet (2010).

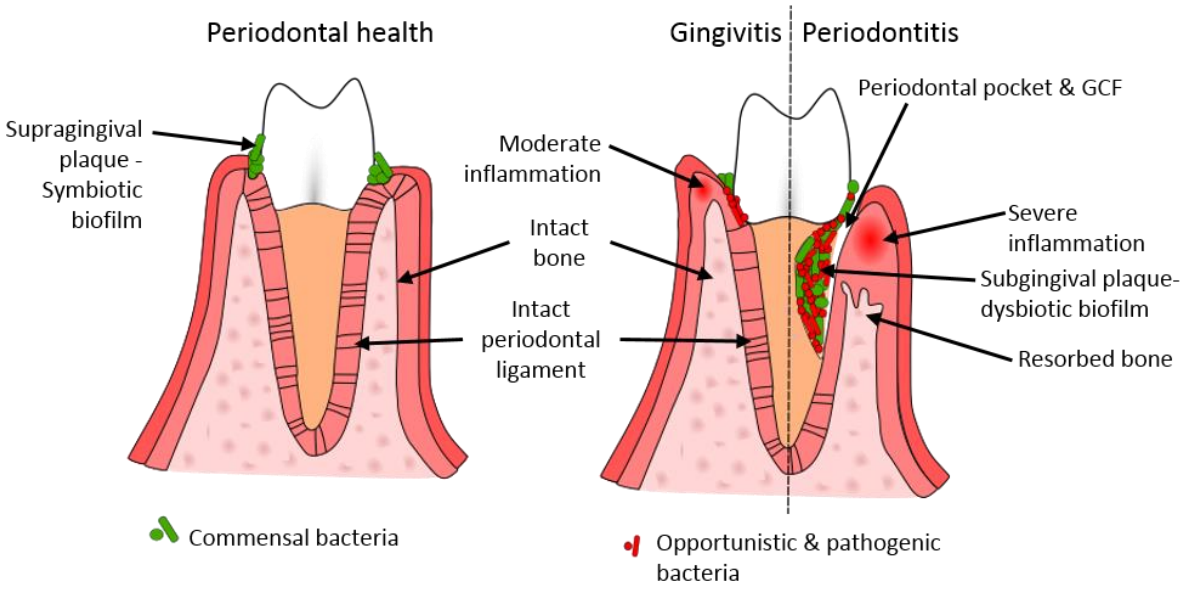


Figure 1.3: Progression of gingivitis and periodontitis (adapted from Hajishengallis, 2015). The accumulation of bacteria at the gingival crevice leads to the initiation of gingivitis. A subsequent shift in bacterial composition from Gram-positive aerobes and facultative anaerobes to predominantly Gram-negative anaerobes disrupts inflammatory responses and activates the progression to periodontitis and the formation of a periodontal pocket. This leads to eventual destruction of the collagen fibres of the periodontal ligament, bone resorption and attachment loss (Preshaw *et al*., 2012, Scannapieco, 2013).

Our understanding of oral diseases has progressed substantially over the years. It is now understood that gingivitis and periodontitis are characterised by a combination of pathogenic bacterial accumulation and subsequent detrimental host inflammatory responses, but the exact cause of periodontal disease onset is continually under review (Preshaw *et al*., 2012, Ramamurthy *et al*., 2014, Xu and Gunsolley, 2014). Initially periodontitis was considered to be caused by an accumulation of microorganisms within the dental plaque and the subsequent host response (Schultz-Haudt *et al*., 1954, Macdonald *et al*., 1956). This hypothesis was followed, in 1986, by a theory based on studies utilising culture dependent methods to identify a complex biofilm of over 200 species in the absence of brushing, with an increase in bacterial load and shifts in proportions in periodontitis but no identification of a single causative species. Thus, the theory of multiple responsible species was put forward (Theilade, 1986). In 1994 the ecological plaque hypothesis was put forward by Marsh, who postulated that shifts in microbial composition as a consequence of changes in the environment led to flourishing of bacterial species and development of pathogenic bacterial consortia which could cause host infection (Marsh, 1994). A more recent theory based on the ecological plaque hypothesis is the polymicrobial synergy and dysbiosis hypothesis, which proposes that oral diseases are initiated and maintained by synergistic and dysbiotic microbial communities (rather than specific oral pathogens) so that they represent a specific genetic fingerprint to work together to stabilise and grow the disease provoking biofilm (Hajishengallis and Lamont, 2012, Hajishengallis *et al*., 2012).

Dental plaque maturation contributes to the progression of gingivitis and periodontitis. If kept in check through regular tooth cleaning, the dental plaque remains present only in small amounts and the activation of detrimental inflammatory processes can be avoided (Socransky, 1977, Wade, 2013). When plaque matures however, there is an accumulation of bacteria and an increase in the presence of bacteria with pathogenic potential (including opportunistic pathogens which are damaging to the host under certain conditions). This accumulation of bacteria triggers the onset of gingivitis where the gingivae become inflamed through the activation of a defensive inflammatory response. It is now widely accepted that this accumulation of bacteria alone is not sufficient to trigger the advancement to periodontitis, but rather a series of complex interactions between the gingivitis associated biofilm and the host immune response is required for progression (Figure 1.1 and Figure 1.2) (Kilian *et al*., 2016, Zijnge *et al*., 2010, Wade, 2013).

In gingivitis, the GCF changes from a transudate to a microbial and host-derived substance rich exudate. The host derived components include pro-inflammatory cytokines, immune cells and enzymes such as proteases (Kilian *et al*., 2016,

Sonnenschein and Meyle, 2015, Lamster, 1997). This change in composition added to an increased flow of GCF and accompanied local gingival bleeding leads to disruption in the microenvironment. This then causes the main bacterial load in the gingival crevice to shift from predominantly Gram-positive aerobes and facultative anaerobes towards Gram-negative anaerobes (Figure 1.1). Increase in bacterial load and subsequent oxygen utilisation in addition to deepening of the periodontal pocket creates an increasingly anaerobic and reduced environment (Figure 1.2 and Figure 1.3), which further enhances the shift from health to dysbiosis by encouraging the growth of an obligate anaerobic community in the subgingival plaque (Hajishengallis, 2015).

In addition to the formation of the periodontal pocket, inflammation can cause bleeding in the gingival crevice which increases the haemoglobin and transferrin content of the environment. This produces conditions ideal for the growth of the periodontitis associated pathogens such as *Porphyromonas gingivalis* (Kuramitsu *et al*., 2007, Meyle and Chapple, 2015, Kilian *et al*., 2016).The metabolic processes of the now favoured bacteria, increases pH but also contributes to making the environment more anaerobic further encouraging growth of the bacteria adapted to these environments such as *P. gingivalis* and *Tannerella forsythia*. These proteolytic bacteria not only directly damage host tissue, but also impact host immunity through degradation of immunoregulatory proteins (Marsh *et al*., 2015).

The progression of gingivitis to periodontitis occurs in individuals when gingivitis-associated inflammation is no longer proportionate or self-resolving but rather becomes exaggerated and ineffective, particularly in the connective tissue supporting the teeth. This can be perpetuated by external factors which increase susceptibility such as tobacco consumption and poorly-controlled diabetes (Kilian *et al*., 2016). The shift in the biofilm composition causes the destruction of periodontal tissue through exacerbated and dysregulated inflammation (Abdul-Sater *et al*., 2009, Garlet, 2010, Scannapieco, 2013).

The destruction of periodontal ligaments through the breakdown of collagen fibres can cause the deepening of the periodontal pocket (Figure 1.2 and Figure 1.3). Tissue breakdown also provides further nutrients for pathogenic bacterial growth, promoting further inflammation, tissue destruction and dysbiosis (Kilian *et al*., 2016). Colonisation of the periodontal pocket, a prime location for anaerobic bacteria, also triggers downstream alveolar bone resorption leading to attachment loss (Figure 1.2 and Figure 1.3) (Preshaw *et al*., 2012).

Unlike in gingivitis, there are several species of bacteria which are associated with periodontitis. Socransky *et al* in 1998 proposed a model which distinguished bacteria into

coloured complexes based on their involvement in periodontitis (Figure 1.4) (Socransky *et al*., 1998). Based on data from over 13,000 subgingival plaque samples, analysed for the presence of 40 culturable species (using DNA-DNA hybridization) it was concluded that the bacteria associated with the blue, green, purple and yellow complexes were involved in initial colonisation of tooth surfaces. The Orange complex bacteria showed increased prevalence in moderate disease and were described as being associated in the progression of periodontitis. Red complex bacteria had increased prevalence in diseased sites and are associated with established and severe periodontitis (Figure 1.4) (Socransky and Haffajee, 2002). Although this nomenclature is still used today, the advancement in culture independent bacterial identification has improved microbial profiling so that bacteria associated with each of these colour complexes now includes non-culturable species not described in the original model (Hiranmayi *et al*., 2017).

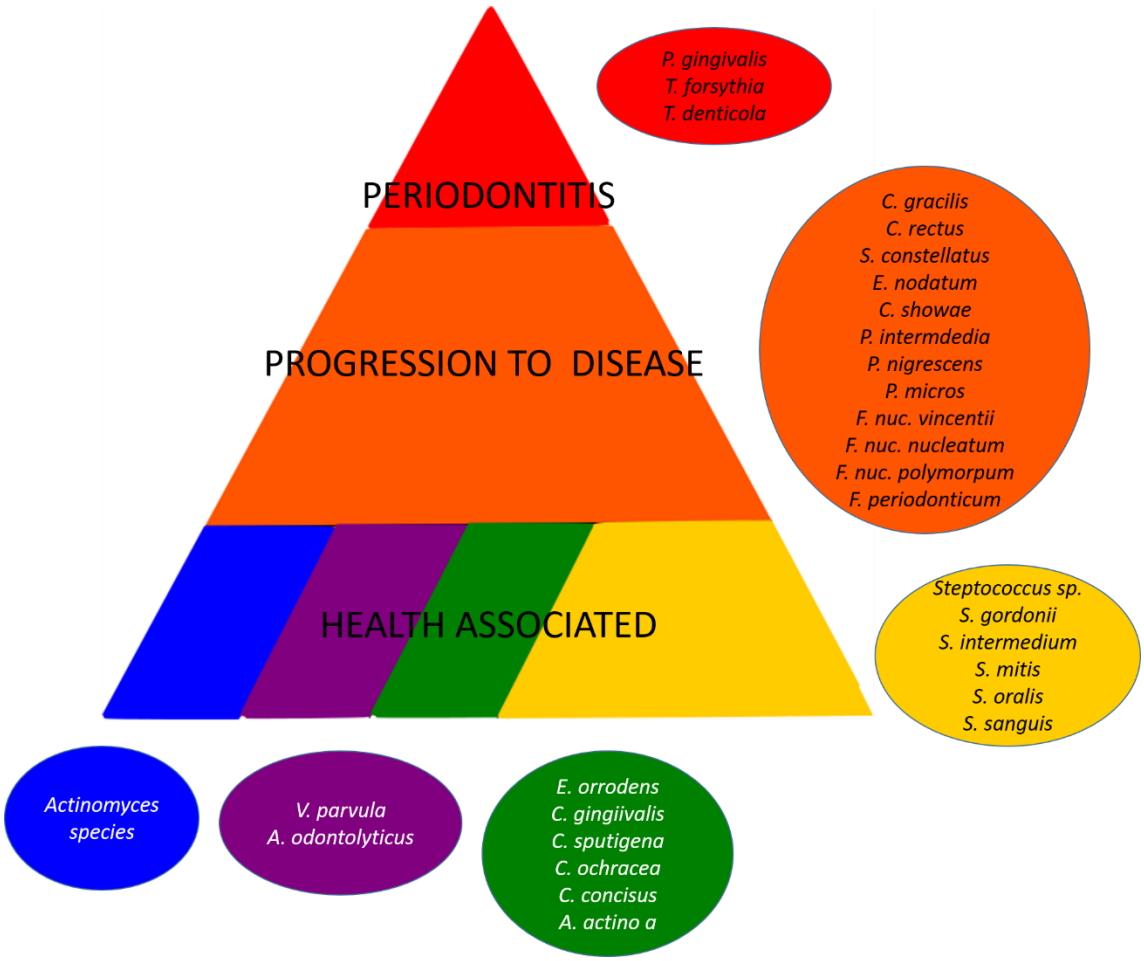


Figure 1.4: Representation of the colour complexes defined using the checkerboard technique of culturable bacteria associated with periodontal health and disease. The base of the pyramid represents health associated bacteria which are early colonisers of tooth surfaces. Upon plaque maturation the orange complex bacteria increase in predominance and bridge the colonisation of red complex bacteria which are found to be more prevalent in periodontitis (adapted from Socransky and Haffajee, 2002).

Disease associated bacterial communities persist in the periodontal pocket without invading the periodontal tissue. The immune system is unable to effectively eliminate the microorganisms causing a chronic inflammatory state such that the prolonged host responses lead to tissue destruction (Okada and Murakami, 1998). Despite the association of these bacteria with periodontitis, it remains unclear if any particular species is involved in disease onset or if it is the shift in biofilm composition leading to the formation of the pocket and subsequent chronic inflammation which produces optimal conditions for these bacteria to flourish (Curtis *et al*., 2011, Wade, 2013).

The role of viruses in periodontitis has also recently been investigated. In particular herpesviruses have been implicated in gingival infections, periodontal diseases and mucosal ulceration (Silva *et al*., 2015). Herpesviruses often have latent persistence in a variety of cells including immune cells and can modulate immune responses. Although little is known about the exact mechanisms linking viral load to progression of periodontitis, these viruses have been shown to have a higher prevalence in periodontal tissues in patients with periodontitis (Slots and Contreras, 2000, Silva *et al*., 2015). It is proposed that local immunomodulatory effects caused by herpesvirus infection could enable the increase in bacterial load and virulence in periodontitis. In addition, herpesvirus infection could induce the release of cytokines/chemokines contributing to the excessive immune response associated with periodontitis (Silva *et al*., 2015, Meyle and Chapple, 2015, Slots and Contreras, 2000, Wright *et al*., 2008).

1.2. Molecular mechanisms involved in the progression of periodontitis

With the advancement of technology, our understanding of the immune response associated with periodontitis has evolved. Since it was first suggested in 1976 by Page and Schroeder, the idea that bacterial presence is essential but not sufficient for the progression of periodontitis is still valid. Active periodontitis could be characterised by the changes in inflammatory cells (including polymorphonuclear neutrophils, DCs, T-cells and macrophages) as well alterations in immunomodulatory molecules (including cytokines and complement) with alveolar bone resorption and gingival tissue damage as a consequence of prolonged immune responses. Below is a brief description of some aspects of both the innate and adaptive immune responses that have been shown to be involved in periodontitis.

1.2.1. Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (referred to as neutrophils from this point) as previously alluded to (section 1.1.1.3) are significant players in the establishment and maintenance of a healthy periodontium. In the absence of inflammation, the number of leukocytes migrating through the JE amount to 3000 leukocytes/min into the periodontal pocket

(Schiott and Loe, 1970). These are predominantly neutrophils and an increase in numbers has been observed in periodontitis. Interestingly an increase in systemic numbers of neutrophils is also observed in both localised and generalised periodontitis (Loos *et al*., 2000), suggesting an exacerbated systemic innate immune response stemming from the increased inflammation in the oral cavity associated with periodontitis. Furthermore, treatment of patients with generalised periodontitis led to a decrease in systemic neutrophil numbers (Christan *et al*., 2002). These studies suggest a mechanism by which local infection controls neutrophil generation.

Once in the periodontal pocket, neutrophils form a barrier between microorganisms and the host cells, providing early lines of immune defence (Nicu and Loos, 2016). Neutrophils function following a two-step processes. The first step, priming, results from the initial exposure to activating factors (cytokines and bacterial products) (Summers *et al*., 2010). Priming ensures maximal neutrophil degranulation and oxidative burst by making neutrophils more responsive to activating agents and able to survive longer through delayed apoptosis (Guthrie *et al*., 1984, Colotta *et al*., 1992, Nicu and Loos, 2016). Priming is followed by activation and subsequent removal of activating agent (Summers *et al*., 2010). Following is a brief overview of neutrophil activation, the mechanisms employed by neutrophils for bacterial control and the roles played by neutrophils in periodontitis progression and associated tissue damage.

Prior to activation, neutrophils act in a searching capacity, circulating in blood to find potential targets or areas of inflammation to which they may be recruited. These targets include the cytokines produced by epithelial cells in response to microorganisms or tissue damage (section 1.2.4), which signal the presence of pathogens (Nicu and Loos, 2016). In addition to release of chemokines (such as IL-8, C5a and prostaglandin E_2: PGE_2) by DCs, macrophages, mast cells, endothelial and epithelial cells, response to local infection also includes an up-regulation of adhesion molecules on local blood vessel endothelium. Together these changes direct and aid the migration of neutrophils from the blood into the infected tissue (Delima and Van Dyke, 2003, Phillipson and Kubes, 2011, Amulic *et al*., 2012, Hajishengallis and Chavakis, 2013). In the JE, IL-8 production is paramount to the generation of a chemotactic gradient for recruitment of neutrophils into the periodontal pocket (Curtis *et al*., 2011, Uriarte *et al*., 2016). The role of IL-8 in periodontitis is discussed in more detail in section 1.2.4.1.

Upon reaching a site of infection, neutrophils undergo a series of processes to transmigrate through the blood vessels into the tissue. Chemokines produced by host cells and microbial components (LPS, lipoteichoic acid or N-formyl-methionyl-leucyl-phenylalanine: fMLP) initiate this process (Nicu and Loos, 2016). The release of histamine and complement components (C3a and C5a) cause vasodilation, slowing of

blood flow and increased vascular permeability thus allowing the neutrophils to successfully undergo the tethering process (Meyle and Chapple, 2015). The increase in vascular permeability also increases serum release into the periodontal pocket. In health, serum, being rich in inflammatory mediators (such as antibodies and complement components), aids the clearance of pathogenic bacteria (Meyle and Chapple, 2015). In periodontitis, however, the increase in the GCF serum content can provide nutrients to enhance the growth of pathogenic communities. Indeed, *P. gingivalis* has been shown to activate C5a through cysteine protease (gingipain) associated conversion of C5. This potentially causes an increase in migration and retention of neutrophils into tissues and activates macrophages to prolong inflammation, leading to an increase in potential growth substances (Wingrove *et al*., 1992, Meyle and Chapple, 2015). The increase in local C5a levels induced by *P. gingivalis* also interferes with the interactions between TLR2 and C5a such that IL-12p70 is inhibited and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) are increased. In an *in vivo* mouse model, this mechanism presents as evasion of immune clearance and inflammatory bone loss which is not found in either C5a receptor or TLR2 deficient mice (Liang *et al*., 2011). Furthermore, C5a receptor-TLR cross talk has also been shown to be exploited by *P. gingivalis* to evade macrophage associated killing which could be recovered by addition of a C5a receptor antagonist (Wang *et al*., 2010). Complement associated manipulations by *P. gingivalis* are associated with a host of other mechanisms including impairment of iNOS (inducible nitric oxide synthase) dependent killing of pathogens, gingipain proteolytic cleavage of C3 thus prevention of complement activation, activation of TLR2 and subsequent IL-12 down-regulation and cleavage of complement regulatory protein (CD46) from host cell surfaces (Hajishengallis, 2010, Olsen *et al*., 2017).

The up-regulation of adhesion molecules on local blood vessels further aid the initial tethering of neutrophils to endothelial cells and slows their movement to allow rolling. Rolling is dependent on interactions between neutrophil cell surface molecules such as L-selectin (neutrophil cell surface), P-selectin (endothelium), E-selectin (endothelium) and endothelial cell receptor P-selectin glycoprotein ligand-1 (endothelium and neutrophils) (Ley *et al*., 2007). Platelets also express a high level of P-selectin and, given decreases observed in neutrophil rolling and adhesion in response to anti-platelet antibodies, it has been proposed platelets act as bridges between neutrophils and endothelial cells to aid adherence of neutrophils (Carvalho-Tavares *et al*., 2000). Interestingly, untreated periodontitis has been associated with increased platelet P-selectin expression and adhesion of platelets to neutrophils in response to bacterial challenge and increased systemic platelet activation (Nicu *et al*., 2009, Papapanagiotou *et al*., 2009). Furthermore, greater phagocytosis responses have been observed with

neutrophil-platelet complexes compared with neutrophils alone in both periodontitis and periodontally healthy patients (Nicu *et al*., 2009).

The process of rolling allows binding between integrins on neutrophil cell surfaces (such as lymphocyte function-associated antigen and macrophage-1 antigen) to their receptors on activated endothelium (including intercellular adhesion molecule 1 and 2) for firm adhesion. Adhesion requires the binding of chemokines to their neutrophil receptors (achieved during neutrophil rolling) to activate neutrophils and lead to the integrin confirmation changes needed for receptor binding (Nicu and Loos, 2016). In addition, binding of chemokines to neutrophils initiates cytosolic actin reorganisation and development of cellular polarity, both of which aid in contact between neutrophils and the endothelial cells (Meyle and Chapple, 2015). Once adhered to the endothelial surface, neutrophils begin the process of transmigration after crawling along the endothelial lining to find an appropriate place. Transmigration is a complicated process reliant on a host of molecules including; junctional adhesion molecules, platelet endothelial cell adhesion molecule-1, integrins and vascular endothelial cadherin (reviewed in detail in Schmidt *et al.*, 2011 and Muller, 2013).

Neutrophils are characterised by both their lobed nuclei and the high granulation of their cytoplasm. These granules contain a large variety of antimicrobial peptides and enzymes and are divided into four categories; azurophilic, specific, gelatinase and secretory vesicles (Uriarte *et al*., 2016). A brief description of some of the contents of the azurophilic, specific and gelatinases is outlined in Table 1.2 (Faurschou and Borregaard, 2003) with the addition of lysozyme which is found in all three types of granules and functions by cleaving peptidoglycan polymers of bacterial cell walls and bind LPS to reduce associated cytokine release (Faurschou and Borregaard, 2003, Wiesner and Vilcinskas, 2010).

Table 1.2: Neutrophil granular content and function. Predominant antimicrobial protein functions within each granule (Faurschou and Borregaard, 2003).

Granule	Antimicrobial protein	Main function
Azurophilic (primary)	Myeloperoxidase	Lead to the formation of microbicidal molecules (hydrochlorous acid, N-chloramines, tyrosine radicals and reactive nitrogen intermediates) which attack the surface membrane of microorganisms. Formed via interactions with hydrogen peroxide.
	Serine proteases	Involved in hydrolysis of extracellular matrix. Includes elastases, cathepsin G, azurocidin and proteinase 3.
	Defensins	Forms multimeric transmembrane pores. Small cationic antimicrobial and cytotoxic peptides.
	Bactericidal/permeability-increasing protein (BP1)	Cationic protein which binds to lipopolysaccharide of Gram-negative bacteria leading to the rearrangement of outer membrane lipids, growth inhibition and inner membrane damage. Promotes bacterial attachment to neutrophils and monocytes.
Specific (secondary)	Lactoferrin	Sequesters iron to inhibit bacterial growth. Binds to bacteria cell membranes causing irreversible damage and cell lysis
	Human cathelicidin	Antimicrobial protein whose C-terminal (LL-37) has antimicrobial activity against Gram-negative and Gram-positive bacteria and acts as a chemoattractant for neutrophils, T cells and monocytes.
	Lipocalin neutrophil gelatinase-associated lipocalin (NGAL)	Bacteriostatic through sequestering of ferric-siderophore complexes.
	Matrix metalloproteinase 8 (MMP-8)	Collagenase
Gelatinase (tertiary)	Matrix metalloproteinase 9 (MMP-9)	Gelatinases
	Macrophage-1 antigen	Firm adhesion of neutrophils to endothelial cells
	Nicotinamide adenine dinucleotide phosphate oxidase component (cytochrome b558)	Involved in the generation of ROS.
	Natural resistance-associated macrophage protein 1	Deprives microorganism in the phagosome of essential metals such as iron, magnesium and zinc.

Mobilisation and degranulation in neutrophils is hierarchical, such that neutrophil activation favours mobilisation and degranulation of gelatinase granules over specific granules which are favoured over azurophilic granules (Nauseef and Borregaard, 2014). This process allows the neutrophil to transform from a sentinel cell into a potent active innate immune cell (Faurschou and Borregaard, 2003). Secretory vesicles are mobilised during the initial activation of neutrophils (during rolling) and function via their membranes to provide neutrophils with additional receptors (including integrins, macrophage-1 antigen, LPS/lipoteichoic acid-receptor, CD14, immunoglobulin receptors, formylated bacteria peptide receptors and matrix metalloproteinase (MMP)/ /leukolysin receptors). This mobilisation aids in priming neutrophils for transmigration, making them integrin presenting cells which are highly responsive (Nicu and Loos, 2016).

Once neutrophils transmigrate through the endothelial cells, they move through the vascular basement membrane to exit the vessel and enter the infected tissue. This process requires the release of proteases such as MMPs, elastases and proteinase-3 (Nussbaum and Shapira, 2011, Kuckleburg *et al*., 2012). Neutrophils contain three main MMPs types: collagenases, gelatinases and leukolysin. Prior to degranulation these are stored as inactive pro-forms mainly in gelatinase and specific granules (Faurschou and Borregaard, 2003).

Neutrophil activation occurs through binding of TLRs of which neutrophils express most, excluding TLR3 and TLR7. Once these bind their ligands, neutrophils are activated and phagocytosis, superoxide generation and production of cytokines/chemokines is initiated (Prince *et al*., 2011). Phagocytosis is the most characteristic mechanism by which neutrophils clear activating agents. The process is facilitated by opsonization through which activating agents are coated in host derived molecules such as complement factors, antibodies and mannose-binding lectin. These opsonins have receptors on the surface of neutrophils (including C3b receptors and antibody/immunoglobulin receptors) which, once bound, lead to the invagination of the neutrophil membrane and internalisation of the activating agent into a phagosome (Nicu and Loos, 2016). Killing within the phagosome is achieved through two main mechanisms: ROS generation and release of granular proteases (Roos *et al*., 2003). Furthermore, hydrogen peroxide (one of the ROS generated) is also a substrate for myeloperoxidase (MPO) for the formation of a chlorinated derivative which is toxic to many microbes (Table 1.2, Faurschou and Borregaard, 2003).

In addition to phagocytosis, neutrophil extracellular traps (NETs) have more recently been indicated as important in neutrophil killing (Brinkmann *et al*., 2004). These structures are assembled intracellularly following the loss of nuclear integrity and the combining of double stranded DNA, histones, cathelicidin (LL-37), elastases and MPO.

Release of NETs is achieved following plasma membrane perforation or cell lysis in a ROS release-dependent process known as NETosis (Fuchs *et al*., 2007). In chronic periodontitis NETs have been observed in periodontal pockets and GCF (Vitkov *et al*., 2009). Interestingly, *P. gingivalis*, *T. forsythia*, *F. nucleatum* and *Prevotella intermedia*, have been shown to have DNAse activity and can degrade NETs under certain growth conditions, thus, avoiding NET-associated killing (Palmer *et al*., 2012).

Following removal of the neutrophil activating agent, resolution is achieved through apoptosis of neutrophils (Nussbaum and Shapira, 2011). Achieving this requires suppression of pro-inflammatory cytokine production and induction of anti-inflammatory cytokine (such as IL-10 and TGF-β) production by phagocytic macrophages (Kennedy and DeLeo, 2009). Disposal of unused neutrophil granules is carried out following anti-inflammatory cytokine-associated recruitment of anti-inflammatory macrophages. This minimises the risk of excessive tissue damage (Savill *et al*., 1989). A distortion in the apoptosis and clearance of neutrophil products can prolong tissue damage and inflammation. Apoptosis resistant neutrophils have been found in the GCF of patients with periodontitis. These patients showed a decrease in granulocyte monocyte-colony stimulating factor (GM-CSF) which can increase mitochondrial stability and reduce caspase 3 activity to reduce neutrophil apoptosis (Gamonal *et al*., 2003). Furthermore, there is evidence for inhibition of apoptosis in periodontal neutrophils by LPS and a consequent increase in necrosis (Turina *et al*., 2005). This in turn may cause the release of neutrophil enzymes and ROS into the periodontium thus exacerbating the tissue damage associated with periodontitis.

Although neutrophils are generally effective at clearing bacteria that pass through the oral epithelial barrier, some periodontal pathogens (*Aggregatibacter actinomycetemcomitans* and *P. gingivalis* being two of the most well studied) have developed methods to diminish or avoid neutrophil clearance. *A. actinomycetemcomitans* has been shown to produce a leukotoxin which can lead to loss of effectiveness in neutrophils (Berthold *et al*., 1992, Kolodrubetz, 1996, Lally *et al*., 1996, Dennison and Van Dyke, 1997, Delima and Van Dyke, 2003). *P. gingivalis*, on the other hand, secretes a serine protease which has been shown to inhibit the synthesis of IL-8 by epithelial cells and thus interferes with recruitment of neutrophils (Darveau *et al*., 1998, Bainbridge *et al*., 2010). As well as IL-8 secretion inhibition, the decrease of intracellular adhesion molecule-1 has also been demonstrated, further suggesting *P. gingivalis* interferes with neutrophil recruitment (Madianos *et al*., 1997). In addition, *P. gingivalis* has a dense capsule which can hinder phagocytosis (Delima and Van Dyke, 2003).

While neutrophils are imperative to control of microbial growth in the oral cavity, their function may also contribute to the pathogenesis of periodontitis. The release of ROS and MMPs required for neutrophil transmigration and killing can diffuse through the JE into supporting tissues (such as the periodontal ligament) causing damage and potential attachment loss (Nicu and Loos, 2016).

1.2.2. Adaptive immune cells

Alongside the innate immune system, a profile of adaptive immune cells and cytokines has also been described in association with periodontitis pathogenesis. This aspect of the immune response in periodontitis is controlled by T-cells and B-cells. T-cells mediate the activation of polyclonal B-cells and subsequent production of specific antibodies (Gonzales, 2015). In periodontitis, B-cells are associated with pathogenesis due to their RANKL (receptor activator for nuclear factor κB ligand) positive status (Kawai *et al*., 2006). As discussed in detail below (section 1.2.4.4), RANKL plays an important role in periodontitis associated bone resorption.

Of the adaptive immune cells, T-cells predominate over others in gingival tissues with the majority being accounted for by $CD4^+$ T-cells (Dutzan *et al*., 2016). They are subdivided based on their cytokine production, specific functions and gene-expression patterns. Of these subsets T-helper 1 (Th1), T-helper 2 (Th2), Th17 and T-regs are those mostly associated with the progression of periodontitis (Silva *et al*., 2015). The process of naïve T-cell differentiation is initiated by T-cell receptor-mediated activation followed by the activation of specific transcription factors for each lineage. The presence of specific patterns of cytokines polarises cell differentiation towards that of a particular subset, where upon cells will express a specific set of chemokine receptors to aid in the leukocyte migration towards infection sites (Gonzales *et al*., 2012, Gonzales, 2015, Meyle and Chapple, 2015).

In the 1990s, Baker *et al* carried out multiple studies which implicated the adaptive immune response in the destructive pathogenesis of periodontitis. Using a *P. gingivalis* induced periodontitis mouse model these studies demonstrated that mice lacking both T- and B-cells had decreased periodontitis-associate bone loss compared with control mice (Baker *et al*., 1999, Baker *et al*., 2002). The group also demonstrated a specific role for $CD4^+$ cells, showing a decrease in periodontitis bone loss in $CD4^+$ cell deficient mice compared with $CD8^+$ deficient mice and controls (Baker *et al*., 1994). A more recent study also demonstrated that in transplant patients receiving cyclosporin A treatment (prevents T-cell activation) and in animal studies using cyclosporin A treatment, there was no periodontal disease progression or periodontitis associated bone loss despite loss of T-cells (Pejcic *et al*., 2014, da Silva Peralta *et al*., 2015). A brief description of the most

studied subsets of $CD4^+$ cells, their activation profile and subsequent impact on periodontitis follows below.

1.2.2.1. T-helper 1 cells

Th1 cell differentiation is predominantly activated through the action of IL-12 sourced from innate immune cells such as antigen presenting DCs (Hajishengallis and Korostoff, 2017). Binding of IL-12 to its $CD4^+$ cell receptor initiates signalling through janus kinase 2 (JAK2) and tyrosine kinase 2 (TYK2). This leads to the subsequent phosphorylation of signal transduce and activation of transcription (STAT) 4 which promotes the expression of the T-bet transcription factor. These IL-12 activated signalling cascades initiate the transcription of cytokines characteristic of Th1 cells (Oestreich and Weinmann, 2012). Interferon γ (IFN-γ) also activates T-bet and has been shown to increase IL-12 receptor activation, further encouraging Th1 differentiation (Murphy and Reiner, 2002, Appay *et al*., 2008). Combined STAT4 and T-bet activity have been shown to be crucial to the regulation of IFN-γ in Th1 cells where T-bet alone was unable to activate transcription required for the Th1 linage (Thieu *et al*., 2008). Th1 cells are characterised by expression of IFN-γ, IL-2 and TNF-α (Campbell *et al*., 2016). In addition, they are involved in production of IgG2 opsonising and complement-fixing antibodies, activation of macrophages and antibody-dependent cell cytotoxicity (Gonzales, 2015). As such their role can be considered as one which aids phagocytic host responses.

The role for Th1 in periodontitis progression is not entirely clear. While in some cases T-cells in periodontitis were associated with the Th1 subsets based on their chemokine receptor profiles and associated increases in Th1 chemokines (such as IL-5), other studies indicated a predominance of Th2 cells in patients with early onset or advance periodontitis (Manhart *et al*., 1994, Gamonal *et al*., 2001, Lappin *et al*., 2001, Taubman and Kawai, 2001). More recently, an increased expression of T-bet has been shown in active lesions of periodontitis compared with inactive lesions, with the increase correlating with increases in IL-1β and IFN-γ (Dutzan *et al*., 2009a). This suggests that $CD4^+$ differentiation in periodontitis is shifted towards Th1, correlating with early studies where Th2 was found less frequently than Th1 in periodontitis gingival tissues (Seymour *et al*., 1993).

The cytokine release profiles of Th1 cells have been associated with infectious inflammatory bone destruction, indicating a role in alveolar bone resorption in periodontitis (Silva *et al*., 2015). In addition, studies have demonstrated upregulations in RANKL expression through the action of IFN-γ and subsequent increase in TNF-α and IL-1β, as well as chemoattraction of RANKL positive cells to increase osteoclast (OCL)

activation and bone resorption initiation (Figure 1.3) (Honda *et al*., 2006, Gao *et al*., 2007, Garlet *et al*., 2008, Repeke *et al*., 2010).

1.2.2.2. T-helper 2 cells

Opposing the bone destruction associated with Th1, Th2 cell cytokine release is associated with decreasing bone loss. Th2 differentiation is dependent on IL-4 (from B-cells and naïve $CD4^+$ T-cells) which leads to the downstream phosphorylation and activation of STAT3 and subsequent induction of the GATA3 transcription factor (Gilmour and Lavender, 2008, Hosoya *et al*., 2010). Activation of GATA3 promotes increased transcription of IL-4 and additional anti-inflammatory cytokines (including IL-5 and IL-13) (Mjosberg *et al*., 2012). Th2 differentiation also inhibits the expression of the IL-12 receptor β2 subunit, thus, further preventing Th1 differentiation (Ouyang *et al*., 1998). Th2 cells have also been shown to undergo histone modifications which inhibit the transcription IFN-γ and encourage commitment to the Th2 linage (Chang and Aune, 2007). In addition to the production of IL-4, IL-5 and IL-13, Th2 cells have been demonstrated to produce IL-10. Knockout mice studies have shown that the production of IL-10 by Th2 was enhanced by an additional transcription factor – Etv5 (Koh *et al*., 2017). Evidence suggests that both IL-4 and IL-10 impair macrophage killing activity through inhibition of NO synthesis (Cenci *et al*., 1993). In addition, Th2 cells have been associated with IgE and IgG1 switching, mucosal immunity through mast cell production, growth and differentiation induction of eosinophils (Hajishengallis and Korostoff, 2017). Together these functions suggest Th2 aid in phagocytosis-independent host responses.

IL-4 could potentially inhibit the function of MMPs and an upregulation of TIMP2 (tissue inhibitor of metalloproteinase 2) has been demonstrated as a response to IL-4 (Ihn *et al*., 2002). In periodontitis this could provide protection from MMP-associated tissue destruction. Indeed, increased concentrations of IL-4 have been observed in GCF in periodontal health compared with periodontitis (Pradeep *et al*., 2008). Furthermore, Th2 cells have been implicated in inhibition of osteoclastogenesis by acting on precursor cells through the action of secreted IL-4. This is highlighted in mouse *P. gingivalis* periodontitis models where Th1 predominant mice showed higher infiltrations of OCLs compared with Th2 predominant and control mice which showed almost none (Stashenko *et al*., 2007).

1.2.2.3. T-helper 17 cells

Although originally T-cells were divided into the Th1 and Th2 subsets, it is now understood additional subsets also exist with an amount of plasticity between them (Cosmi *et al*., 2014). In periodontal health and disease, Th17 have been implicated in addition to Th1 and Th2 cells. Th17 cells are characterised, as the name suggests, by their production of IL-17 and undergo differentiation in a manner distinct from that of Th1

and Th2 cells (Harrington *et al*., 2005, Park *et al*., 2005, Wilson *et al*., 2007). Their polarisation down this cell lineage is initiated by a complex cytokine profile involving IL-6, IL-1β, IL-23 and TGF-β which, upon receptor binding, initiate phosphorylation of STAT3 leading to the activation of the transcription factor ROR-γ (Ivanov *et al*., 2006, Annunziato *et al*., 2012). The importance of STAT3 was highlighted in a mouse study where deletion of the STAT3 inhibited Th17 differentiation, which could be recovered following retroviral introduction of STAT3 (Yang *et al*., 2007).

Th-17 cells mediate a variety of immune response through secretion of IL-17A, IL-17F and IL-23. Interesting, Th17 cells have also been shown to secrete IL-8, thereby showing neutrophil chemo-attractive properties, as well as production of TNF-α and GM-CSF which could contribute to activation and survival of neutrophils (Pelletier *et al*., 2010). Furthermore, Th17 cells have also been demonstrated to produce IL-10 in an antigen-dependent matter, such that Th17 cells produced IL-10 and IL-17 when stimulated with *S. aureus* but produced IL-17 and IFN-γ in response to *Candida albicans* (Zielinski *et al*., 2012).

Activation of ROR-γ leads to induction of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) so that increased Th17 and the subsequent release of cytokines could be part of the non-resolving inflammatory response seen in periodontitis. Exemplifying this, an increase in pro-inflammatory cytokines such as IL-17, TGF-β, IL-1β, IL-6 and IL-23 was observed in gingival tissues of periodontitis patients (Takahashi *et al*., 2005, Cardoso *et al*., 2009, Ohyama *et al*., 2009).

Furthermore, IL-17, IL-1β and TNF-α were observed in periodontitis with an increased production of MMP1 and MMP3. The same study showed that while IL-17 did not induce MMPs to the same extent as IL-1β and TNF-α, it induced further IL-1β and TNF-α production by macrophages as well as IL-6 and IL-8 from gingival fibroblasts (Beklen *et al*., 2007). There is also evidence suggesting that the Th17 associated cytokine release is important in neutrophil recruitment; mice lacking the IL-17 receptor (IL-17RA-dificient mice) showed increased periodontal bone resorption in response to *P. gingivalis*, reduced chemokine levels and reduced neutrophil migration (Yu *et al*., 2007). Like Th1 cells, Th17 cells have also been associated with bone resorption. Using primary OCLs, it has been demonstrated that IL-17 induced the expression of OCL differentiation factor, encouraging the formation of OCLs (Kotake *et al*., 1999). Together these studies indicate Th17 cells play a role in the progression of periodontitis and the associated alveolar bone reabsorption.

The role of Th cells in periodontitis is evident but controversy still exists regarding the responses associated with progression. Adding to this controversy is the difficulty in

determining which of the T-cell subsets are found in periodontal lesions as well as distinguishing roles of their cytokine profiles in destructive and protective processes. The presence of T-regulatory cells, the associated release of anti-inflammatory cytokines (IL-10 and TGF-β) and their potential role in RANKL regulation and subsequent periodontitis protection further complicates the role of T-cells in periodontitis pathogenesis (Steinsvoll *et al*., 1999, Dutzan *et al*., 2009b).

1.2.3. Matrix metalloproteinases

Another important aspect of periodontitis progression, is the extracellular matrix (ECM) and collagen breakdown associated with MMPs, which leads to destruction of periodontal tissues leading to eventual permanent attachment loss (Silva *et al*., 2015).To date 23 human MMPs have been identified and are zinc-dependent endopeptidases (Franco *et al*., 2017). Given the destructive potential of MMPs, regulation of activity is tightly controlled. This is achieved through gene activation, proenzyme activation, and inhibition. In humans, MMPs are inhibited by four TIMPs with differing specificity (Arpino *et al*., 2015).

Periodontal inflammation and the persistence of pathogens, as described in previous sections, is associated with pro-inflammatory mediators (such as pro-inflammatory cytokines), generation of ROS, increased numbers of neutrophils and an increase in bacterial proteases; many of which can lead to the increase in prevalence and activity of MMPs. Classically, MMPs are linked to the degradation of ECM components and basement membranes, characteristics that are associated with periodontal tissue destruction in periodontitis (Silva *et al*., 2015). They, however, are also involved in regulatory functions which include: chemokine processing and the subsequent neutrophil regulation, proteolysis of receptors and their ligands, enzyme modulation and release of bioactive molecules from the ECM (Franco *et al*., 2017).

Due to the high levels of collagen I in the ECM of soft and hard periodontal tissues, studies to date have predominately concentrated on the functions of collagenases (MMP8 and MMP13) and gelatinases (MMP2 and MMP9) in periodontitis (Sorsa *et al*., 2010). Of the MMPs with collagenase activities found in the GCF and gingival tissues, MMP8 is the most predominant. In the case of gelatinases, two are detected in the oral cavity and of these MMP9 is the most prevalent (Sorsa *et al*., 2016). When considering the high prevalence of neutrophils in periodontal tissues in both health and disease, and the presence of MMP8 and MMP9 in neutrophil granules (section 1.2.1) it is unsurprising that these MMPs predominate in gingival tissues and GCF. Given this, a brief description of the association of MMP8 and MMP9 with periodontitis follows. Smaller amounts of

other MMPs (including 1, 2, 3, 7, 12, 13, 14, 25 and 26), TIMP1 and TIMP2 have also been demonstrated to be present in GCF and saliva (Buduneli and Kinane, 2011).

Production of MMP8 takes place in a range of cells (resident and inflammatory) but it is found mainly to be produced by neutrophils in periodontal tissues. On the other hand, MMP9 is produced by a limited number of cells types and is found mainly to be produced by immune cells (Silva *et al*., 2015).

As discussed in previous sections, MPO release as a result of neutrophil activation can lead to the production of oxidant hypochlorous acid (section 1.2.1) which has antimicrobial activity and is also involved in the oxidative, non-proteolytic activation of MMPs (Alfakry *et al*., 2016). ROS are key mediators of tissue degradation of MMPs through their ability to directly oxidise the enzymes and hypochlorous acid has been shown to activate both MMP8 and MMP9 (Weiss *et al*., 1985, Peppin and Weiss, 1986, Saari *et al*., 1990). This suggests that the increase in neutrophils in periodontitis can also lead to an increase in MMP activation and, thereby, an exacerbation of periodontitis pathogenesis. Indeed, a longitudinal study of GCF samples from periodontitis patients showed high levels of both MMP8 and MPO prior to periodontal treatment in both active and inactive sites, with decreases in MPO levels in active sites following treatment (Hernandez *et al*., 2010). An increase in glutathione peroxidase, lactoferrin, MPO and IL-1β in periodontitis has also been observed, which could contribute to the activation of MMPs (Wei *et al*., 2004). Furthermore, concentrations/levels of MMP8, TIMP2, MPO and MMP9 were found to be higher in GCF of patients with periodontitis compared with controls but no differences were observed in TIMP1. A decrease in MMP8, TIMP2, MPO and MMP9 was observed following periodontal treatment (Marcaccini *et al*., 2010). There is also evidence for ROS-dependent MMP9 activation in periodontal fibroblast cells (Osorio *et al*., 2015).

Many cytokines/chemokines are targets for MMP cleavage, including periodontitis upregulated Th1 pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6), anti-inflammatory cytokines (such as TGF-β1 and IL-10) and chemokines (such as IL-8) (Franco *et al*., 2017). This suggests that MMPs may regulate cytokine/chemokine expression and consequently impact periodontal health. For example, upregulation of IL-6 and MMP9 (levels and activity) in periodontal ligament fibroblasts stimulated with ROS was observed, while inhibition of MMP activity led to higher IL-6 levels suggesting MMP degradation of IL-6 (Cavalla *et al*., 2015).

Emerging evidence also suggests that MMP13 plays a role in the tissue damage associated with periodontitis. In addition to MMP8 and MPO, MMP13 was also higher in the GCF of patients with periodontitis compared with those with gingivitis and controls

(Leppilahti *et al*., 2014). MMP13 has also been implicated in activation of MMPs through a proteolytic cascade in which MMP13 has been shown to induce MMP9 in gingival cells from periodontitis patients (Hernandez Rios *et al*., 2009, Franco *et al*., 2017). MMP13 dependent regulation of OCL activation has also been reported whereby MMP13 activated MMP9 cleaves the osteoclastogenesis inhibitor galectin-3 (Pivetta *et al*., 2011). Furthermore, MMP13 can regulate the RANKL/OPG (osteoprotegerin) (discussed in section 1.2.4.4) axis to favour RANKL and as such encourage bone reabsorption (Nannuru *et al*., 2010).

In addition to changes in MMP levels, there have also been studies indicating changes in TIMP levels suggesting, perhaps, periodontitis progression is dependent on shifts in the ratios of MMPs and their inhibitors, particularly when considering MMPs are present even in the healthy oral cavity (Makela *et al*., 1994, Hernandez *et al*., 2007, Meschiari *et al*., 2013, Reddy *et al*., 2014, Sapna *et al*., 2014, Noack *et al*., 2017). In GCF of periodontitis patients, periodontitis progression correlated with an increase in MMP1 concentration and a decrease in TIMP1 concentration which was reversed following treatment (Ghodpage *et al*., 2014, Popat *et al*., 2014).

1.2.4. Cytokine/chemokines

As indicated above, cytokines play a crucial role in the modulation of the immune system in the oral cavity and are involved in the progression of periodontitis. Many cytokine concentrations and activities have been shown to be dysregulated in periodontitis. There is evidence for cross-regulation of cytokine/chemokine release by gingival cells. For example, gingival fibroblasts (which are highly responsive to LPS that can induce the production of pro-inflammatory cytokines) have an exacerbated IL-8 response to LPS following prior exposure to IFN-γ (Tamai, 2002). IL-1β is not only able to induce IL-6, IL-8 and TNF-α expression in gingival fibroblasts but can also encourage the expression of more IL-1β in an autocrine manner (Agarwal *et al*., 1995, Chae *et al*., 2005). A synergistic activation of IL-8 and IL-6 secretion by IL-1β and TNF-α was also observed in gingival fibroblasts which was supressed by IFN-γ (Takigawa *et al*., 1994, Kida *et al*., 2005).

Below is a brief description of selected cytokines and their involvement in periodontitis. Particular interest is paid to IL-8 (due to its potent neutrophil chemotactic ability and the high numbers of neutrophils in gingival tissue and GCF), IL-1β (due to its pivotal role in cytokine/chemokine regulation and initiation of inflammatory response) and IL-6 (due to its impact on T-cells and central role in immune regulation). The importance of IL-1β and IL-6 is highlighted by Kinane and Preshaw, who described these cytokines as being involved in first responses in both innate and adaptive immune responses (Kinane *et al*., 2011).

1.2.4.1. IL-8

IL-8, a chemotactic factor for leukocytes and neutrophils, has been demonstrated to be vital in oral health (section 1.1.1.3 and 1.2.1) and has been implicated in the progression of periodontitis. IL-8 was the first chemokine of the CXC chemokine family to be described. In 1987 Yoshimura identified a protein called monocyte-derived neutrophil chemotactic factor or neutrophil activating peptide as a chemoattractant for neutrophils but not monocytes. Shortly after, this protein was also shown to attract T-cells and was renamed IL-8 (Larsen *et al*., 1989). Since then our understanding of IL-8 has vastly increased and its importance in immune responses has been highlighted.

IL-8 is a member of the CXC chemokine family and in humans is initially produced as a 99-amino acid peptide (Palomino and Marti, 2015). Secretion of IL-8 is induced in many cell types including monocytes, fibroblasts, lymphocytes, mesenchymal cells and endothelial cells following stimulation by inflammatory mediators such as IL-1β and TNF-α (Okada and Murakami, 1998, Sonnenschein and Meyle, 2015). As discussed previously (section 1.2.1), in the periodontium, IL-8 acts as a chemoattractant for neutrophils. Neutrophils express receptors (CXCR1 and CXCR2) which are able to bind IL-8 (Holmes *et al*., 1991, Murphy and Tiffany, 1991, Zeilhofer and Schorr, 2000). Activation of these G-coupled receptors can initiate an increase in free intracellular Ca^{2+} which leads to the subsequent activation of β2 integrins, cytoskeletal rearrangement, phagosome-lysosome fusion and exocytosis of granule proteins (Jaconi *et al*., 1990, Kernen *et al*., 1991, Lawson and Maxfield, 1995, Van Kooyk *et al*., 2009, Palomino and Marti, 2015). In addition, IL-8 may regulate ROS generation by neutrophils through the upregulation of complement receptor 1 and, consequently, an upregulation of complement-dependent responses (Paccaud *et al*., 1990). In addition to having chemotactic functions, IL-8 has also been shown to initiate monocyte differentiation into OCLs, indicating IL-8 may regulate osteoclastgenosis (Bendre *et al*., 2003). Furthermore, osteoblasts (OBLs) have been shown to express CXCR1, suggesting a role for IL-8 in modulation of OBL activity and subsequently a role in bone resorption (Silva *et al*., 2007).

Given these chemoattractive and regulatory roles, it is unsurprising that IL-8 is found in healthy periodontal tissues and is associated with periodontitis progression. Indeed, an increase in neutrophils associated with an increase in IL-8, ICAM-1, IL-1β and TNF-α has been observed in the gingival tissues of periodontitis patients (Liu *et al*., 2001). Evidence suggests that the periodontal pathogen *P. gingivalis* increases IL-8 (in addition to IL-1α, Il-1β, IL-6 and IL-12) secretion by oral epithelial cells via TLR2 activation (Sandros *et al*., 2000, Kusumoto *et al*., 2004). In contrast, there is also evidence to suggest that while the bacteria, *F. nucleatum*, *Neisseria flavescens* and *Haemophilus*

parainfluenzae initiated IL-8 secretion by oral epithelial cells, their co-inoculation with *P. gingivalis* prevented this. Furthermore, *P. gingivalis* was also shown to degrade IL-8 accumulated in gingival epithelial cells in response to the other bacteria (Darveau *et al*., 1998, Huang *et al*., 2001). This proteolytic activity of *P. gingivalis* on IL-8 is associated with gingipains. Interestingly, gingipains have been shown to stimulate IL-8 and IL-6 secretion in monocytes through protease-activated receptor signalling (Uehara *et al*., 2008). Although gingipains can penetrate gingival epithelial and fibroblast cells and stimulate IL-8 secretion, higher concentrations have been shown to cause a decrease in IL-8 (O'Brien-Simpson *et al*., 2009).

It appears different bacteria initiate different IL-8 responses in gingival cells. *T. denticola* (a periodontal pathogen), for example, did not induce IL-8 or IL-6 secretion by gingival epithelial cells (Brissette *et al*., 2008). *A. actinomycetemocomitans* and *F. nucleatum*, on the other hand, increased IL-8 (and IL-1β with *A. actinomycetemocomitans)* in gingival epithelial cells while a commensal bacterium (*S. gordonii*) produced no significant differences in cytokine production (Uchida *et al*., 2001, Stathopoulou *et al*., 2010). Furthermore, BspA (*Bacteroides* surface protein A) from *T. forsythia* stimulated IL-8 secretion from gingival epithelial cells (Onishi *et al*., 2008). Decreases in IL-8 secretion could perhaps aid in increasing periodontal colonisation by decreasing neutrophil recruitment while increases in IL-8 could initiate exacerbated neutrophil recruitment, which could in-turn initiate the periodontal tissue damage observed in periodontitis.

In addition to chemotactic roles of IL-8 in periodontitis there is also evidence for its potential role as an immunomodulator. For instance, IL-8 has been demonstrated to prevent apoptosis of CXCR1 and CXCR2 expressing endothelial cells, enhancing their survival, in addition to enhancing the production of MMP2 and MMP9 (Li *et al*., 2003). These roles in the periodontium could enhance periodontal tissue damage and prolong inflammation.

While studies investigating the responses of cells to individual bacteria or simple mixed cultures are informative, they do not take into account the complexities of a biofilm and its interaction with the host. To get a better understanding of changes in IL-8 associated with periodontitis clinical studies are required. To date, such studies looking at IL-8 levels in gingival tissues, GCF and saliva are contradictory. In GCF, IL-8 analysis was predominantly carried out by ELISAs with results indicating both an increase in IL-8 (Gamonal *et al*., 2001, Giannopoulou *et al*., 2003, Holzhausen *et al*., 2010, Konopka *et al*., 2012, Ertugrul *et al*., 2013, Khalaf *et al*., 2014, Lutfioglu *et al*., 2016) and a decrease (Jin *et al*., 2000, Luo *et al*., 2011) in periodontitis. In addition, checkerboard immunoblotting and Luminex immunoassays have also been applied to measure IL-8 in GCF and in both cases increased IL-8 was observed in periodontitis (Teles *et al*., 2009,

Tymkiw *et al*., 2011). In gingival tissues and saliva, results were more consistent and IL-8 was observed at higher concentrations in periodontitis conditions (Cesar-Neto *et al*., 2007, Michiels *et al*., 2009, Venza *et al*., 2010, Lisa Cheng *et al*., 2014, Khalaf *et al*., 2014, Souto *et al*., 2014).

1.2.4.2. IL-1

IL-1 was first described in 1972 and has been widely researched in variety of inflammatory disorders (Horton *et al*., 1972). Although originally classified as an OCL activating factor, it is understood to exist in two isoforms (Dewhirst *et al*., 1985, March *et al*., 1985). These isoforms, IL-1α and IL-1β, only share 27% homology at the amino acid level but have similar biological activities. IL-1α remains cell bound whereas IL-1β is secreted, but both bind to the same receptors (IL-1 receptor 1 and IL-1 receptor 2) found on nearly all cell types (Weber *et al*., 2010). While both play a role in the periodontium, IL-1β has been shown to be consistently detected at higher levels in GCF of patients with periodontal diseases with decreases observed following treatment and as such has been the target of many studies (Preshaw and Taylor, 2011). As described in previous sections, IL-1β impacts periodontal colonisation, host immunity in the periodontium, neutrophils, Th cells, tissue breakdown and tissue homeostasis and is thought to play an important role in the pathogenesis of periodontitis. Indeed, some studies discussed in the previous section observed not only increases in IL-8 in gingival tissues in response to periodontal bacteria but also increases in IL-1α and/or IL-1β (section 1.2.4.1).

Once stimulated, many cell types in the periodontium produce IL-1 including macrophages, monocytes, lymphocytes, epithelial cells, endothelial cells and fibroblasts (Preshaw and Taylor, 2011, Garlanda *et al*., 2013). In periodontitis, IL-1 is mainly produced by macrophages but production can be induced in monocytes by LPS, lipoteichoic acid and fimbriae, potentially accounting for the increase seen in periodontitis (Okada and Murakami, 1998, Sonnenschein and Meyle, 2015).

Cellular secretion of IL-1β requires the activation of TLR-4 to initiate the transcription of the pro-IL-1β. Further processing of pro-IL-1β is dependent on an additional signal to the activated cell (such as extracellular ATP, extracellular DNA or ROS) and the activation of the inflammasome. This leads to caspase-1 mediated activation of IL-1β (Latz, 2010, Yilmaz and Lee, 2015). IL-1β processing is associated with the NALP3 (nacht domain-, leucine-rich repeat-, and pyrin domain (PYD)-containing protein 3) inflammasome complex. NALP3 is regulated by an effector molecule (apoptosis associated speck-like protein) and antagonised by NLRP2, both of which have been shown to be down-regulated in monocytes in response to *P. gingivalis* with an associated increase in NALP3 (Bostanci *et al*., 2009). The same study also demonstrated an increase in NALP3

in chronic and aggressive periodontitis patients, with an associated increase in IL-1β and IL-18. Activation of the TLR4 in gingival cells is often shown to induce release of IL-1β, as well as IL-6 and TNF-α (Jotwani and Cutler, 2004, Diya *et al*., 2008, Hajishengallis *et al*., 2009, Sahingur *et al*., 2010). In addition, there is evidence suggesting gingival fibroblasts from patients with periodontitis not only produce more IL-1 but are more responsive to *P. gingivalis* and as such produce more IL-1 compared with healthy tissues stimulated with *P. gingivalis* (Kent *et al*., 1999).

Although *P. gingivalis* and *A. actinomycetemcomitans* growth was not impacted when cultured with IL-1β and IL-6, *P. gingivalis* does appear to degrade IL-1β, IL-6 and IL-1Ra (the IL-1 receptor antagonists). When considering the role IL-1β has on modulating immune responses, this suggests *P. gingivalis* can alter IL-1β and potentially dampen immune responses (Fletcher *et al*., 1997). Despite this, there is evidence that IL-1β is found at higher concentration in gingival tissues and GCF of patients with periodontitis compared with healthy controls (Giannopoulou *et al*., 2003, Holzhausen *et al*., 2010, Luo *et al*., 2011, Tymkiw *et al*., 2011, Konopka *et al*., 2012, Ertugrul *et al*., 2013). Similarly, salivary levels of IL-1β are also increased in patients with periodontitis (Miller *et al*., 2006, Tobon-Arroyave *et al*., 2008, Gursoy *et al*., 2009, Mirrielees *et al*., 2010, Kaushik *et al*., 2011, Ebersole *et al*., 2013, Rathnayake *et al*., 2013). These levels of IL-1β in saliva correlated with disease severity and decreased with treatment (Ng *et al*., 2007, Scannapieco *et al*., 2007, Kaushik *et al*., 2011, Kinney *et al*., 2011, Sexton *et al*., 2011, Rathnayake *et al*., 2013).

IL-1 promotes increases in type 1 procollagen, collagenase, hylauronate, PGE_2 and fibronectin, and is therefore important for periodontal tissue homeostasis (Okada and Murakami, 1998, Sonnenschein and Meyle, 2015). Studies have shown an increase in levels in GCF and gingival tissue from periodontitis diseased sites compared with healthy sites, with a decrease in levels following periodontitis treatment (Masada *et al*., 1990, Irwin and Myrillas, 1998, Sonnenschein and Meyle, 2015). Furthermore, IL-1β has been linked to OCL activity, with activation of OCLs achieved through the IL-1R1 and subsequent NF-κB activation, thus, suggesting a role for IL-1β in periodontitis-associated bone resorption (Kim *et al*., 2009). Indeed, local and systemic inhibition of IL-1β in animal models of periodontitis have led to decreases in alveolar bone loss (Assuma *et al*., 1998, Graves *et al*., 1998, Delima *et al*., 2001).

In human gingival fibroblasts, IL-1β with TNF-α has been shown to increase PGE_2 which resulted in an increase in IL-6 (Czuszak *et al*., 1996, Palmqvist *et al*., 2008). The IL-1β-associated increase in IL-6 was associated with the activation of p38, MAPK and NF-κB signalling pathways (Chae *et al*., 2005). In addition, IL-1 is a potent inducer of bone resorption and an inhibitor of bone formation. Although both isoforms mediate effects on

bone, studies have shown that IL-1β is more potent than IL-1α (and TNF-α) with higher levels of IL-1β in GCF from periodontitis sites (Gowen and Mundy, 1986, Irwin and Myrillas, 1998, Okada and Murakami, 1998). The diverse roles of IL-1β have been highlighted by Steinberg *et al* (2006), who showed an upregulation in genes in human gingival fibrocytes related to inflammatory cytokines (such as IL-6 and TNF-α), the NF-κB pathway, chemokines (such as IL-8, CXCL1, CXCL2 and CCL2), MMPs (such MMP3 and MMP12) and adhesion molecules (such as ICAM-1, CD44 and vascular cell adhesion moleculre-1) in response to IL-1β treatment (Steinberg *et al*., 2006b, Vardar-Sengul *et al*., 2009).

1.2.4.3. IL-6

IL-6 is a multifunctional cytokine produced by many different cell types (including B-cells, T-cells, monocytes, macrophages, keratinocytes, endothelial cells and fibroblasts) with secretion dependent on stimulation by bacterial LPS or other cytokines such as IL-1 and TNF-α (Tanaka *et al*., 2014, Sonnenschein and Meyle, 2015).

Many of the immunomodulatory roles of IL-6 have been discussed in previous sections. In a protective capacity, IL-6 can play an important role on the maintenance of oral health. For instance, IL-6 (in combination with TGF-β) is required for differentiation of Th17 cells and can also inhibit T-reg differentiation (Bettelli *et al*., 2006, Korn *et al*., 2009). As discussed previously, differentiation of Th cells down the Th17 lineage, while playing a role in health, can also be damaging to the periodontium so that an increase in IL-6 could cause an exacerbation of periodontitis through dysregulation of the Th cell differentiation. In addition to T-cell differentiation modulation, IL-6 has also been implicated in B-cell differentiation into antibody-producing plasma cells which in the oral cavity can play a regulatory role in controlling bacterial colonisation but when found in excess can lead to destructive hypergammaglobulinemia and autoantibody production (Tanaka *et al*., 2014).

In health, IL-6 acts as a pro-inflammatory mediator in an attempt to control infection and therefore can act in a protective capacity. In disease this role becomes dysregulated and there is evidence to suggest oral pathogens can initiate this dysregulation and potentially prolong the inflammatory response. For example, *P. gingivalis* gingipains have been shown to stimulate IL-6 secretion in epithelial cells, monocytes and fibroblast cells (Lourbakos *et al*., 2001, Uehara *et al*., 2008, O'Brien-Simpson *et al*., 2009). Furthermore, gingival fibroblasts increased IL-8 and IL-6 secretion in response to *A. actinomycetemcomitans* and *Campylobacter rectus* (Dongari-Bagtzoglou and Ebersole, 1996). Periodontal ligaments displayed a similar trend, with increases in IL-6, IL-1β, IL-8 and TNF-α in response to *P. gingivalis* and *P. intermedia* (Yamamoto *et al*., 2006).

Furthering the destructive capability of IL-6 is the ability to impact MMP levels. Co-culture of fibroblasts with macrophages led to an increase in MMP1 and was enhanced by fibroblast released IL-6 (Sundararaj *et al*., 2009). In gingival fibroblasts, an IL-6 dose dependent increase was observed in MMP1 (Irwin *et al*, 2002). Contrastingly, IL-6 has a protective role, highlighted by the ability of L-6 to induce antagonistic effects against IL-1 receptors and TNF-α soluble receptors. Given that both IL-1 and TNF-α induce production of IL-6, these antagonistic effects suggest a role in balancing pro-inflammatory effects (Irwin and Myrillas, 1998).

Furthermore, in response to bone resorbing agents such as IL-1 and TNF-α, OBLs secrete IL-6, further enhancing cell differentiation into OCLs and indicating the potential role of IL-6 in promoting bone resorption (Teng, 2006, Preshaw and Taylor, 2011). IL-6 has also been demonstrated to initiate bone resorption in mouse models in response to pro-inflammatory cytokines which initiate an increase in RANKL and decrease in OPG (Nakashima *et al*., 2000). IL-6 concentrations and resulting bone remodelling activities in periodontal tissues differ across studies; some studies have shown no resorptive activity induced by IL-6 while others suggest high concentrations of IL-6 (>100 ng ml^{-1}) act against IL-1- and TNF-α-induced OCL bone resorption (Poli *et al*., 1994, Flanagan *et al*., 1995, Irwin and Myrillas, 1998, Okada and Murakami, 1998, Sonnenschein and Meyle, 2015).

Although there is a significant amount of IL-6 detected in healthy GCF and saliva, studies regarding expression levels in periodontitis have been contradictory. The majority of studies indicate an increase in the concentration of IL-6 from periodontitis patients compared with healthy controls, with increased levels correlating with the severity of the disease (Cesar-Neto *et al*., 2007, Costa *et al*., 2010, Holzhausen *et al*., 2010, Venza *et al*., 2010, Tymkiw *et al*., 2011, Prakasam and Srinivasan, 2014). In other cases, no significant differences were observed (Rathnayake *et al*., 2013, Gursoy *et al*., 2009). In some clinical studies no correlation between IL-6 and alveolar bone reabsorption in periodontitis was observed (Ng *et al*., 2007, Scannapieco *et al*., 2007). Gingival mononuclear cells from periodontitis patients have also been shown to have in increase in expression of IL-6 (mRNA and protein) which was not seen in peripheral blood mononuclear cells, suggesting local IL-6 production (Fujihashi *et al*., 1993, Irwin and Myrillas, 1998). Other studies, however, have shown an increase in serum levels of IL-6 in people with periodontitis indicating a more systemic response (Loos, 2005, Paraskevas *et al*., 2008).

1.2.4.4. RANKL

As indicated above, RANKL plays an important role in the bone resorption associated with periodontitis. RANKL belongs to the tumour necrosis factor and ligand superfamily along-side its receptor (receptor activator for nuclear factor κ, RANK) and its inhibitor, the soluble decoy receptor, OPG. RANK binding of RANKL is able to induce osteoclastogenesis, while OPG binding of RANKL prevents this activation (Simonet *et al*., 1997, Lacey *et al*., 1998, Nakagawa *et al*., 1998). RANKL/RANK interactions initiate NF-κB and MAPK signalling cascades to trigger cellular cytoskeletal reorganisation and cellular polarization (Yavropoulou and Yovos, 2008). Maintaining a balance between bone forming OBLs and bone-resorbing OCLs is important in ensuring excessive bone resorption (osteoporosis) or excessive gain in bone mass (osteopetrosis) is avoided (Nagy and Penninger, 2015). The balance of OBLs and OCLs is in a tightly regulated homeostatic state, and with the observation of T-cells expressing RANKL, connections between chronic inflammation, dysregulation of the RANKL/OPG axis and bone loss were established (Anderson *et al*., 1997, Wong *et al*., 1997, Kong *et al*., 1999a, Kong *et al*., 1999b).

As described above, activation of Th cells and their subsequent cytokine release profiles are important in maintaining homeostatic levels of RANKL and modulating their action. The release of Th1 and Th17 associated cytokines (including IL-1β, IL-17 and TNF-α) can stimulate the expression of RANKL on the surfaces of periodontal OBLs as well as Th17 cells (Chabaud *et al*., 1999, Dong, 2008). Furthermore, patients with active periodontitis had higher levels of GCF RANKL compared with patients without periodontitis. This was associated with an increase in IL-17 production by gingival Th17 cells alongside a down regulation of IL-10 and TGF-β (Vernal *et al*., 2004, Takahashi *et al*., 2005, Vernal *et al*., 2005, Silva *et al*., 2008, Dutzan *et al*., 2009a). Although exact concentrations of RANKL in GCF appear to vary between studies, a general trend is seen with higher concentrations of RANKL observed in periodontitis patients compared with healthy controls (Silva *et al*., 2015).

1.3. The oral microbiome and its impact on general health

The relationship between the oral microbiome and systemic health is complex but evidence suggests both positive impacts of a healthy microbiota and negative impacts of dysbiotic microbiota to systemic health. Changes associated with periodontitis have been linked to systemic diseases such as diabetes, rheumatoid arthritis and cardiovascular disease (Bartold and Mariotti, 2017). Altered oral microbiomes are observed not only during systemic disease but also when body homeostasis is altered, such as during pregnancy and lactation (Scannapieco, 2005, Scannapieco, 2013). Our understanding of the potential impact of the oral microbiome on systemic diseases is

expanding. Its role in systemic health, however, is still not fully defined beyond the role of pathogen colonisation prevention (section 1.1.1.2).

An example of health associated consequences of the oral microbiome is the impact on nitrate metabolism. Although the majority of ingested nitrate is excreted in urine, a portion is actively taken up by salivary glands, where it is concentrated (20-fold) and reduced (Lundberg and Govoni, 2004, Bryan *et al*., 2017). Mammals lack an effective method for reduction of nitrate and so a complex bacterial community interaction is relied upon for the reduction to nitrite, with commensal facultative anaerobic bacteria undertaking this task via nitrate reductases (Bryan *et al*., 2017). The nitrite can then be taken up by the blood stream via gastric absorption and converted to NO. NO is essential for vascular health (impacting suppleness and pliancy) as well as providing anti-hypertensive effects via vasodilation (Wade, 2013, Bryan *et al*., 2017). This is illustrated in a study which demonstrated a decrease in salivary nitrite when antibacterial mouthwash was used prior to sodium nitrate consumption. Furthermore, there was no difference in nitrate accumulation in saliva, suggesting reduction in commensal bacteria impacted the conversion of nitrate to nitrite (Govoni *et al*., 2008).

Periodontitis progression can lead to an increase in the gingival wound surface (deepening of periodontal pocket) which increases the likelihood of bacterial translocation from the wound area into the bloodstream resulting in bacteraemia. Dental treatments such as tooth extractions and subgingival scaling in periodontitis patients also has the potential to lead to bacteraemia (Larsen and Fiehn, 2017). Bacteria translocation from the periodontal pocket often results in an infected root canal, from where the bacteria can enter the blood stream and spread into surrounding tissues. Once in blood, oral bacteria can spread and infect a variety of organs and have been associated with infectious endocarditis as well as brain and liver abscesses (Mylonakis and Calderwood, 2001, Marques da Silva *et al*., 2004, Wade, 2013). Oral bacteria have also been shown in atherosclerotic plaques. *P. gingivalis* and *S. sanguinis* have both been shown to aggregate platelets causing the formation of atherosclerotic plaques (Herzberg and Meyer, 1996, Herzberg and Weyer, 1998). Evidence also suggests a link between *P. gingivalis* translocation and rheumatoid arthritis, with higher prevalence of *P. gingivalis* DNA in synovial tissues and synovial fluid of patients with rheumatoid arthritis (Farquharson *et al*., 2012, Totaro *et al*., 2013).

Since the establishment of the HMP, studies have shown a decrease in species diversity at particular sites (alpha diversity) in diseased states when compared with health (Kramer and Genco, 2017). Such changes have been observed in oral squamous cell carcinoma where *Streptococcus* species dominate in tumour site microbiota compared with non-tumour sites in the same individuals (Pushalkar *et al*., 2012). In other cancers,

a dysbiosis of the oral microbiota is observed; for example, in saliva samples of patients with squamous cell carcinoma and adenocarcinoma, a higher abundance of *Capnocytophaga*, *Selenomonas* and *Veillonella* and a lower abundance of the commensal *Neisseria* was observed (Yan *et al*., 2015).

In addition to bacterial changes, the increased inflammatory burden associated with periodontitis and the potential increase in systemic inflammation is thought to play a central role in linking periodontitis to chronic inflammatory diseases such as rheumatoid arthritis, osteoporosis and diabetes (Bartold and Mariotti, 2017). Inflammatory mediators associated with periodontitis, including IL-6, endotoxins, LPS and neutrophils are increased systemically in patients with periodontitis (Loos, 2005, Pussinen *et al*., 2007, Shaddox *et al*., 2011). In addition, an increase in C-reactive protein (CRP), an indicator of acute phase response, has also been observed at increased systemic levels in periodontitis (Slade *et al*., 2000, Noack *et al*., 2001). While periodontitis was initially considered a consequence of risk factors such as diabetes and smoking, in 1998 Page suggested a bidirectional relationship between oral health and systemic health, whereby periodontitis could exacerbate systemic disease in addition to systemic disease impacting on periodontal health (Page, 1998, Mark Bartold and Mariotti, 2017). One of the diseases believed to have a two-way relationship with periodontitis is diabetes which is explored in detail below.

1.4. Periodontitis and diabetes

Diabetes mellitus (a group of metabolic disorders) are common chronic diseases which according to the World Health Organization are increasing in prevalence worldwide. Two main types of diabetes exist: type 1 and type 2. Although both are associated with hyperglycaemia (elevated blood sugar) the mechanisms leading to the hyperglycaemic state differ. Type 1 diabetes is considered an autoimmune disease accounting for 5 – 10% of diabetics. This form of diabetes is caused by the immune destruction of pancreatic β cells (Kharroubi and Darwish, 2015). Type 2 diabetes, on the other hand, is caused by insulin resistance in peripheral tissues as well as eventual defects in β cell functions, leading to their inability to meet increased insulin demand (Kharroubi and Darwish, 2015). The characteristic hyperglycaemia of diabetics has been implicated in activation of pathways that lead to an increase in inflammation, oxidative stress and apoptosis (Preshaw *et al*., 2012).

Initially periodontitis was thought to be a commonly occurring consequence of diabetes (Loe, 1993, Wilson, 1989). Epidemiological studies demonstrated susceptibility to periodontitis increases in diabetics and a positive correlation between the degree of hyperglycaemia and severity of periodontitis (increased bone loss and attachment loss)

has been observed (Emrich *et al*., 1991, Shlossman *et al*., 1990, Taylor *et al*., 1998). Furthermore, there is evidence to suggest hyperglycaemia plays a significant role in increased susceptibility to periodontitis, as patients with poorly controlled diabetes had a higher prevalence of periodontitis compared with healthy people but those with well controlled diabetes showed no significant differences in periodontitis prevalence (Tsai *et al*., 2002). Now it is understood that a more complex and two-way relationship exists between the two diseases, so that not only is diabetes a risk factor for periodontitis but that periodontitis has negative impacts on glycaemic control (Preshaw *et al*., 2012, Scannapieco, 2013, Casanova *et al*., 2014). Although the majority of studies regarding diabetes and periodontitis have concentrated on type 2 diabetes, it is important to note that type 1 diabetes also has an increased risk of periodontitis due to the hyperglycaemia associated with both forms of diabetes (Cianciola *et al*., 1982, Lalla *et al*., 2007).

The systemic pro-inflammatory cytokine increase in diabetes can influence local host responses in periodontal tissues (Lalla and Papapanou, 2011, Sonnenschein and Meyle, 2015). This leads to the hypothesis that diabetes effects the host responses to bacteria so that immune and tissue biochemistry modulators (for example cytokines and MMPs) in diabetic GCF, saliva and gingival tissues are different to those in systemically healthy individuals (Lalla and Papapanou, 2011, Sonnenschein and Meyle, 2015). Thus, hyperglycaemia-induced exacerbation of periodontal immune response and tissue degradation could contribute to periodontal disease and attachment loss (Iwamoto and Hioki, 2001, Kiran *et al*., 2005, Scannapieco, 2013). This increased immune response to bacterial stimulation was indicated in a study that identified an up-regulation of adhesion molecules, cytokines and chemokines in cardiovascular tissue of mice following subcutaneous inoculation of LPS. The up-regulation was seen to be more rapid and pronounced in the diabetic mouse compared with the non-diabetic mouse (Graves *et al*., 2006). Furthermore, diabetes can increase immune responses in periodontal tissues, thus, exacerbating periodontitis. This is highlighted by the observation of higher levels of IL-1β in the GCF of diabetic patients with periodontitis compared with patients with the same extent of periodontitis but no diabetes (Salvi *et al*., 1997b). In addition, poor glycaemic control appears to be the driving force behind this increase, with the increase being observed in diabetics with poor glycaemic control but not those with good glycaemic control (Engebretson *et al*., 2004).

On the other hand, an increase in systemic pro-inflammatory cytokines as a consequence of periodontitis could impair intracellular insulin signalling leading to an exacerbation of diabetes and potentially linking the two diseases (Iwamoto and Hioki, 2001, Rotter *et al*., 2003, Paraskevas *et al*., 2008, Lalla and Papapanou, 2011, Preshaw *et al*., 2012, Sonnenschein and Meyle, 2015). The potential for periodontitis-associated

increases in systemic inflammation is supported by studies which show that therapeutic approaches against periodontitis reduce systemic inflammation (as seen by a reduction in mediators such as TNF-α, CRP and IL-6) and potentially improve glycaemic control (as indicated by a reduced haemoglobin A1c: HbA1c) (Iwamoto *et al*., 2001, D'Aiuto *et al*., 2004, Kiran *et al*., 2005, Marcaccini *et al*., 2009). Epidemiological studies suggest periodontitis is associated with poor glycaemic control (regardless of diabetic state) (Taylor *et al*., 1996, Saito *et al*., 2004, Demmer *et al*., 2010, Morita *et al*., 2010). This indicates the increase in insulin resistance associated with periodontitis can exacerbate diabetes and suggests a higher risk of developing diabetes-associated complications (Borgnakke *et al*., 2013, Scannapieco, 2013). This is exemplified by increases in incidence of neuropathy and end-stage renal disease in diabetics with positive correlations to severity of periodontitis (Shultis *et al*., 2007).

The triggering of an acute phase response by the liver, thought to be a consequence of bacteraemia caused by periodontitis, can cause a release in acute-phase proteins (the majority of which are CRP, serum amyloid P component and serum amyloid A protein) (Steel and Whitehead, 1994). Pro-inflammatory cytokines (including IL-1, IL-6 and TNF-α) have been implicated in stimulation of hepatocytes to initiate the release of CRP (Gruys *et al*., 2005, Polepalle *et al*., 2015). As described previously, pro-inflammatory cytokines are key in the progression of periodontitis and have also been observed at increased systemic levels in periodontitis (sections 1.2 and 1.4). Given this, it is unsurprising that increased systemic and GCF levels of CRP have been observed in periodontitis with periodontitis therapy leading to a decrease (Slade *et al*., 2000, Fitzsimmons *et al*., 2010, Megson *et al*., 2010, Leite *et al*., 2014). Increased CRP has also been associated with increased insulin resistance (Nesto, 2004). Although the exact mechanism by which CRP impacts insulin resistance is unclear, a study in Fcγ receptor IIB knockout mice demonstrated a protection to CRP associated insulin resistance. The Fcγ receptor IIB is involved in glucose delivery and, thus, the study proposes a role for CRP in inhibiting Fcγ receptor IIB associated glucose delivery (Tanigaki *et al*., 2013). Another study, indicated a role for ERK1/2 by demonstrating that inhibition of the MAPK/ERK1/2 pathway reversed CRP induced insulin resistance in rat hepatocytes (Xi *et al*., 2011). The increased potential for bacteraemia in periodontitis patients and the subsequent increases in pro-inflammatory cytokines and acute-phase proteins could be a potential linking mechanism between diabetes and periodontitis (Hasturk and Kantarci, 2015, Meyle and Chapple, 2015).

As technology advances, our understanding of microbial involvement in linking diabetes and periodontitis has increased. Culture dependent methods for periodontal bacterial recovery in an early study indicated that the levels of periodontal pathogens (including

A. actinomycetemcomitans, *F. nucleatum* and *P. intermedia)* were similar in diabetics and non-diabetics but *P. gingivalis* was higher in diabetics (Thorstensson *et al*., 1995). Later culture dependent studies demonstrated changes in oral microbial composition in the supragingival plaque of diabetics with increased prevalence of periodontitis associated pathogens (Figure 1.4) such as *T. denticola*, and *P. nigrescens* (Hintao *et al*., 2007). An increase in the periodontitis associated bacteria *Eubacterium nodatum* in the subgingival plaque of diabetics was also demonstrated (Lalla *et al*., 2006). Studies utilising DNA checkerboard hybridization demonstrated increased abundances of *P. gingivalis*, *A. actinomycetemcomitans* and *Campylobacter* spp. in periodontitis sites of diabetics compared with periodontitis sites of those without diabetes (Ebersole *et al*., 2008). A recent study utilising culture independent methods (16s rRNA sequencing) contradicts these previous studies and demonstrated periodontitis subgingival plaque of diabetic patients with poor glycaemic control had higher abundances of some genera (such as *Neisseria*, *Actinomyces*, *Capnocytophaga*, *Fusobacterium*, *Veillonella*, *Aggregatibacter*, *Selenomonas* and *Streptococcus*) and lower abundances of others (including *Synergistetes*, *Tannerella, Porphorymonas* and *Eubacterium*) (Casarin *et al*., 2013). Furthermore, Xiao *et al* (2017) used a diabetic mouse model to demonstrate a shift in bacterial composition as a consequence of diabetes, with an increase in *Enterobacteriaceae*, *Aerococcus*, *Enterococcus* and *Staphylococcus* as well as a decrease microbial alpha diversity in diabetic mice but did not describe any differences in bacteria typically associated with periodontitis. The study, did however, demonstrate pathogen- and periodontitis- associated bone loss could be initiated following microbial transfer of microbiota from diabetic mice to germ free mice. Transfer from diabetic mice showed a significantly higher level of bone loss compared with transfer from non-diabetic mice. These inconclusive results highlight the necessity for further study in relationships between the oral microbiota and diabetes, particularly considering the changes in gut microbiota associated with diabetes (as reviewed in Tilg and Moschen , 2017).

Diabetes mellitus and periodontitis are both associated with increased levels of inflammation. The most promising immune response-associated linking mechanisms are discussed in more detail below and outlined in Figure 1.5.

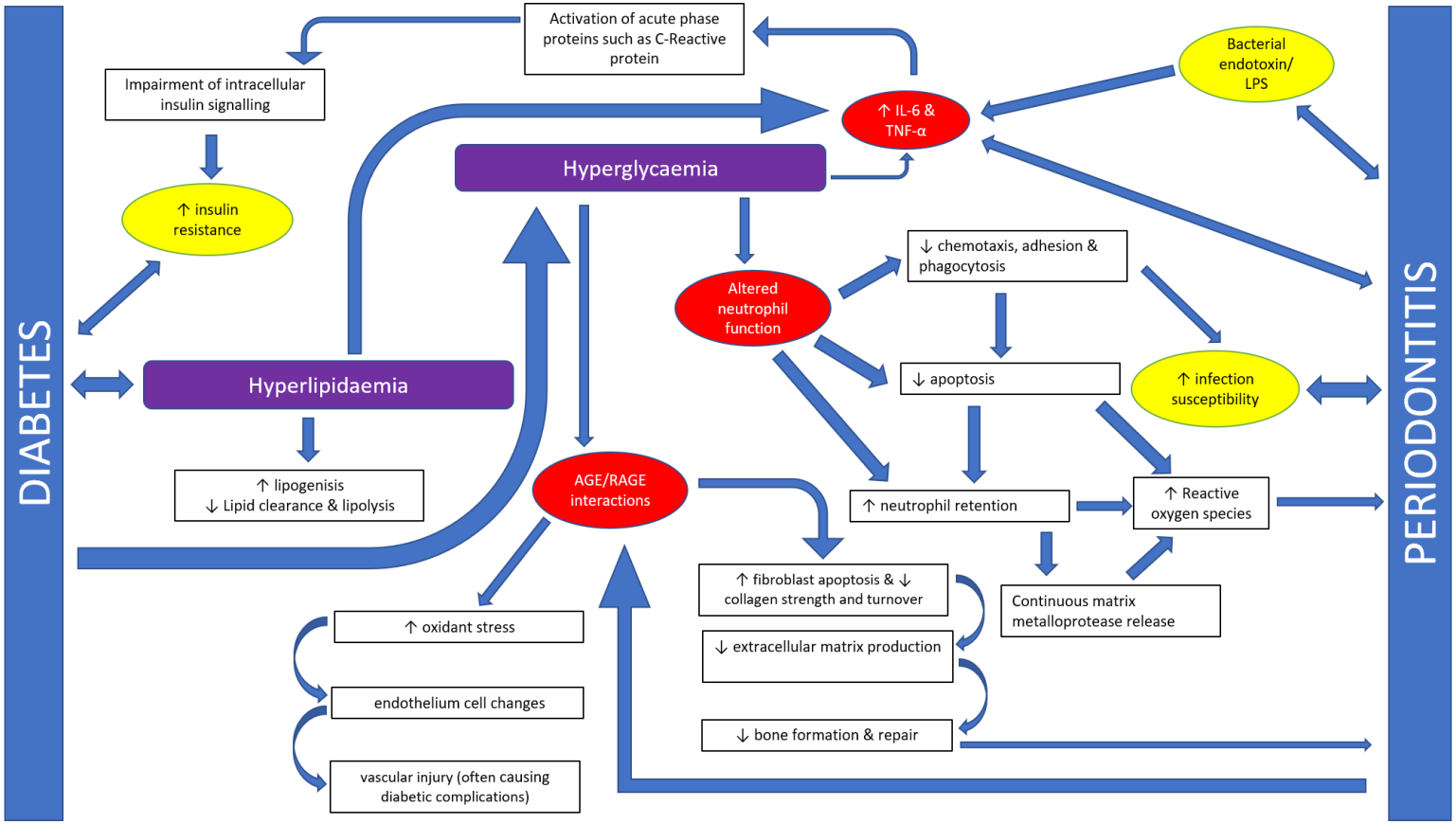


Figure 1.5: Potential mechanistic links between diabetes and periodontitis. Schematic highlighting some of the immune responses implicated in the complex relationships between periodontitis and diabetes. Red indicates aspects of the immune response which impact periodontitis and/or diabetes. Yellow indicates host responses which trigger downstream cascades that can exacerbate periodontitis and/or diabetes. Purple boxes indicate consequences of diabetes. AGE: advanced glycation end product, RAGE: receptor for advanced glycation end products, LPS: lipopolysaccharide, IL-6: interleukin-6, TNF-α; tumour necrosis factor-α.

1.4.1. Cytokine/chemokine networks linking diabetes and periodontitis

As eluded to in the above section (1.4), changes in local (saliva, GFC and gingival tissues) cytokine profiles play a crucial role in periodontitis progression and can lead to changes in systemic cytokine/chemokine levels. As such, these changes have been implicated in linking diabetes to periodontitis. A few of these potential cytokines/chemokines and their roles in both diseases are briefly described below.

Diabetes is characterised by an abnormal metabolism and impaired transport of glucose, which is associated with defects in insulin production and increases in tissue insulin resistance. As a consequence hyperglycaemia is observed which can lead to increases in free fatty-acids (FFAs) and pro-inflammatory cytokines (Boni-Schnetzler *et al*., 2009). Pro-inflammatory cytokines have been reported to negatively affect pancreatic β-cells. For example, IL-1β has been demonstrated to cause β-cell decreases in docking and fusion of insulin granules to β-cell membrane which cause a decrease in β-cell response to glucose (Ohara-Imaizumi *et al*., 2004). Prolonged exposure to IL-1β and IL-1β in combination with IFN-γ, and the associated β-cell functional impairment, have been reported to initiate apoptosis of β-cells via NF-κB or STAT1 activation (Giannoukakis *et al*., 2000, Eizirik and Mandrup-Poulsen, 2001, Heimberg *et al*., 2001). IL-1β has also been demonstrated to induce NO production dependent on iNOS as demonstrated by a lack of NO in iNOS negative β-cells (Andersson *et al*., 2001). In the same study IL1-β and IL-1β/IFN-γ were also reported to impair glucose-stimulated insulin release, decrease the overall insulin in the β-cell, decrease IL-1β/IFN-γ oxidation rates and increase cell death. Contrastingly, while IL1-β impaired insulin release in iNOS negative cells, IL-1β/IFN-γ in combination did not impact cell viability or oxidation rates. Together these findings suggest a role for IL1-β and IL-1β/IFN-γ in impairment of insulin release independent of NO and cell death. Moreover, the results suggest that combined IL-1β/IFN-γ-associated cell death was dependent on NO (Andersson *et al*., 2001). Furthermore, FFAs release associated with hyperglycaemia can also induce release of IL-1β, IL-6 and IL-8, thus, exacerbating the diabetic condition (Boni-Schnetzler *et al*., 2009). TNF-α has also been detected at higher concentrations in diabetics (Pickup *et al*., 2000, Chen *et al*., 2007). TNF-α is associated with an increase in insulin resistance via activation of protein phosphatase 2C which leads to inhibition of 5'AMP-activated protein kinase and subsequently causes a decrease in glucose uptake and an increase in insulin resistance (Steinberg *et al*., 2006a). As discussed previously (section 1.2.4), systemic pro-inflammatory cytokines are increased in periodontitis and it is possible these increases can exacerbate the immune disruption of function and destruction of β-cells via these mechanisms. On the other side of the two-way relationship, it is possible that

these systemic increases associated with diabetes can increase cytokine-mediated pathogenesis of periodontitis.

Elevation in systemic IL-6 has also been observed in patients with diabetes (Kado *et al*., 1999, Pickup *et al*., 2000, Mirza *et al*., 2012, Ryba-Stanislawowska *et al*., 2013). As described previously (section 1.2.4.3) IL-6 plays an important role in T-cell differentiation and in diabetes the increase in IL-6 has been associated with an increase in peripheral Th17 cells and a decrease in T-reg cells compared with healthy individuals (Ryba-Stanislawowska *et al*., 2013). The increase in Th17 is associated with an increase in pro-inflammatory cytokines (section 1.2.2.3) which in turn can cause the progression of diabetes. Given the Th17 role in periodontitis, a diabetes-associated systemic increase could potentially exacerbate periodontitis.

When determining impact of diabetes on cytokine/chemokine associated periodontitis progression, the GCF has been the main focus of investigations. While some studies have demonstrated an increase in IL-1β concentrations in GCF of patients with periodontitis and diabetes compared with those without diabetes or healthy controls (Bulut *et al*., 2001, Duarte *et al*., 2007b, Duarte *et al*., 2007a, Salvi *et al*., 2010, Bastos *et al*., 2012), others have indicated no differences in GCF IL-1β levels associated with diabetes (Navarro-Sanchez *et al*., 2007, Correa *et al*., 2008, Kardesler *et al*., 2008). Although some studies showed no differences in IL-1β concentrations between diabetics with periodontitis and systemically healthy participants with periodontitis, Navarro-Sanchez *et al* (2007) demonstrated lower HbA_{1C} in diabetics following non-surgical periodontitis treatment, thus, suggesting improvement of periodontitis (and the subsequent decrease in inflammatory mediators such as IL-1β and TNF-α) impacts metabolic control.

An increased concentration of TNF-α in the GCF of diabetic individuals with periodontitis has been shown compared with systemically healthy individuals but no significant differences were observed in periodontal/gingival tissue (Duarte *et al*., 2014). In a similar manner to IL-1β, other studies have shown no significant differences in TNF-α concentrations between periodontitis patients with and without diabetes (Takeda *et al*., 2006, Navarro-Sanchez *et al*., 2007, Cole *et al*., 2008, Santos *et al*., 2010). Interestingly, one study demonstrated a difference in IL-1β and TNF-α GCF levels between type 1 and type 2 diabetics with periodontitis, with higher concentrations observed in type 1. This suggests type 1 diabetes is more detrimental to periodontal health than type 2 diabetes (Aspriello *et al*., 2011).

Differences in IL-6 concentrations between patients with periodontitis and diabetes and those with periodontitis alone are also contradictory. Some studies have shown an

increase in IL-6 CGF levels (Duarte *et al*., 2007b, Cole *et al*., 2008, Ross *et al*., 2010, Kardesler *et al*., 2011, Bastos *et al*., 2012, Duarte *et al*., 2014) and others demonstrated no significant differences (Cutler *et al*., 1999, Camargo *et al*., 2013). IL-6 was also reported to be decreased in GCF of diabetic patients with periodontitis following treatment (Kardesler *et al*., 2011).

Studies into the changes in IL-8 concentration are comparably sparse. While some studies indicated no differences in IL-8 concentrations between periodontitis patients with and without diabetes (Engebretson *et al*., 2004, Duarte *et al*., 2007a) others demonstrated a decrease in IL-8 in gingival tissues and GCF (Duarte *et al*., 2007b, Engebretson *et al*., 2006).

In addition to cytokine analysis in patients, mice models have also provided an insight into the relationship between diabetes and periodontitis. For example, Pacios *et al* (2012) demonstrated prolonged inflammation in a diabetic mouse model with ligature-induced periodontitis, which was inhibited by the treatment of mice with a TNF-α inhibitor. Furthermore, inhibition of TNF-α in the model also reduced mRNA levels of cytokines, including IL-1β and IFN-γ (Pacios *et al*., 2012). Takano *et al* (2010) also demonstrated reversal of *P. gingivalis* induced increases in serum cytokines (IL-6 and TNF-α) in diabetic mice treated with a TNF-α inhibitor. Nishihara *et al* (2009) demonstrated increased serum levels of TNF-α and IL-6 in diabetic mice in response to *P. gingivalis* with a corresponding increase in mRNA of these cytokines in liver and visceral adipose tissue.

1.4.2. Polymorphonuclear neutrophils in diabetes and periodontitis

Neutrophils are also an important factor influencing the relationship between diabetes and periodontitis. Diabetic patients have been shown to have impaired neutrophil phagocytosis, apoptosis, chemotaxis and anti-microbial functions due to the metabolic changes associated with diabetes (Manouchehr-Pour *et al*., 1981, Salvi *et al*., 1997b, Alba-Loureiro *et al*., 2006, Graves *et al*., 2006, Alba-Loureiro *et al*., 2007, Preshaw *et al*., 2012).

A study investigating peripheral blood neutrophil apoptosis demonstrated a delayed apoptosis response in diabetics. A significant decrease in apoptosis, caspase-3 and caspase-8 was observed in neutrophils from patients with diabetes and diabetes with periodontitis (Manosudprasit *et al*., 2017). In addition to changes in apoptosis, neutrophils in diabetes have also been reported to have altered metabolism. In a diabetic rat model, phagocytosis, measured as phorbol myristate acetate (PMA)-stimulated H_2O_2 production, was decreased in diabetic rats compared with healthy controls. A decrease in glucose-6-phosphate dehydrogenase and glutaminase (enzymes involved in

glucose/glutamine metabolism) and increases in phosphofructokinase were observed in neutrophils from diabetic rats. Following treatment with insulin, neutrophil changes were abolished. These results indicate an alteration in neutrophil metabolism in diabetics may lead to changing in functions such as phagocytosis and oxidative burst (Alba-Loureiro *et al*., 2006). In addition to changes in apoptosis and metabolism, studies have also demonstrated impaired chemotaxis, phagocytosis and bactericidal activity in diabetes (Cutler *et al*., 1991, Lecube *et al*., 2011). Despite these decreases, other aspects of neutrophils in diabetes appear to be exacerbated. This is highlighted in a recent study which reported a higher basal level of ROS in diabetic neutrophils which, following neutrophil stimulation with PMA, remained consistently high, in contrast with healthy neutrophils in which ROS levels decreased over time (Ridzuan *et al*., 2016). Furthermore, increased pro-inflammatory cytokines/chemokines (IL-1β, IL-8 and TNF-α) were released from neutrophils of diabetics both under unstimulated and LPS-stimulated conditions (Hatanaka *et al*., 2006). In contrast, Gupta *et al* (2017) saw no differences in cytokine levels (including IL-6, IL-8, IL-1β and TNF-α) in neutrophils from diabetics with both poor and good glycaemic control and with and without complications. They did, however, report increase mRNA of IL-6, IFN-γ and TNF-α in neutrophils of patients with good and poor glycaemic control but with no complications compared with systemically healthy participants (Gupta *et al*., 2017). As described previously (section 1.2.1) NETs are important aspects of neutrophil associated protection and are involved in neutrophil-associated periodontal destruction. Recently, a study reported basal levels of NETs in diabetics were higher compared with healthy controls both *in vitro* and *in vivo* (using blood nucleosomes and HNE-DNA complexes as an indirect measurement of NET formation) (Carestia *et al*., 2016). Together these alterations in neutrophils as a consequence of diabetes have the potential to exacerbate periodontitis. Impaired apoptosis in diabetics could increase neutrophil retention in the periodontium thus prolonging their destructive properties (for example MMP release), with increases in ROS and pro-inflammatory cytokines in response to stimuli (hyper-responsive neutrophils) adding to tissue destruction.

The study of neutrophils in diabetics has predominantly concentrated on peripheral cells and *ex vivo* assay with limited studies examining the GCF neutrophils in patients with periodontitis and diabetes (Sonnenschein and Meyle, 2015). Peripheral neutrophil studies have demonstrated changes in neutrophils as a consequence of diabetes. For example, Gursoy *et al*. (2008) indicated a decrease in neutrophil chemotaxis in diabetics with an increase in periodontal pocket depth. However, they observed no difference in phagocytosis or intracellular killing between periodontitis patients with and without diabetes. Shetty *et al*. (2008), on the other hand, did detect significant decreases in

phagocytosis and *P. gingivalis* killing by neutrophils from diabetics with periodontitis in addition to decrease in chemotaxis. Furthermore, peripheral neutrophils from patients with periodontitis were more reactive to periodontal pathogen extracts (*P. gingivalis* and *A. actinomycetemcomitans)* compared with healthy individuals. This hyper-reactivity could indicate the role of periodontitis in the tissue destruction associated with diabetes complications such as neuropathy (Restaino *et al*., 2007, Silva *et al*., 2015).

While studies in human GCF neutrophils are limited, animal model studies have provided an insight into local changes associated with diabetes in periodontitis. For example, diabetic mice were shown to have increased gingival vessel permeability, increased numbers of rolling leukocytes and increased leukocyte expression of P-selectin glycoprotein ligand-1. These results indicate a higher potential for neutrophil migration and translocation into gingival tissues in diabetes compared with health (Sima *et al*., 2010). A similar observation was made by Gyurko *et al*. (2006) who also showed enhanced leukocyte rolling and attachment to gingival vessels in diabetic mice in addition to decreased chemotaxis and increased ROS release by neutrophils from diabetic mice.

The role of MMPs in periodontitis has been discussed previously and as mentioned their regulation is dependent on neutrophils. Given the dysregulation of neutrophils in both diabetes and periodontitis, a consequential dysregulation in MMP levels can also be suggested which could contribute to the attachment loss. Indeed, evidence suggests increased MMP8 and MMP9 levels in periodontal tissues, GCF and saliva from periodontitis patients with diabetes (Kumar *et al*., 2006, Correa *et al*., 2008, Costa *et al*., 2010, Hardy *et al*., 2012). Furthermore, increases in TIMP2 and TIMP4 have also been observed in gingival tissues of diabetics with periodontitis compared with systemically healthy participants (Shin *et al*., 2010, Kim *et al*., 2011, Jung *et al*., 2013). This perhaps suggests a role for both MMPs and their inhibitors in the progression of periodontitis in diabetics.

1.4.3. Advanced glycation end products

Another major inflammation-associated element playing a role in increased periodontal inflammation in diabetic individuals is the increased prevalence of advanced glycation end product (AGEs) and their 35 kDA transmembrane receptor RAGE (receptor for advanced glycation end products).

The formation of AGEs is a non-enzymatic protein modification which results in protein structure change. The post-translational modification occurs when amino groups on proteins (particularly lysine, arginine and histidine) condense with the carbonyl group of reducing sugars. Generation of AGEs occurs mainly through the Maillard reaction which transitions from reversible Schiff base adducts to protein bound Amadori products that

undergo a series of irreversible oxidation and dehydration reactions to form a broad range of heterogeneous products (Figure 1.6) (Ahmad *et al*., 2017). The resulting AGEs are mostly fluorescent nitrogen- and oxygen- containing heterocyclic compounds (Younessi and Yoonessi, 2011, Tsutsui *et al*., 2016, Ott *et al*., 2014). During the conversion of the Schiff base into Amadori products, superoxides are formed while during later stages (conversion of Amadori products to AGEs) hydroxyls are formed (Ahmad *et al*., 2017). This generation of ROS during the production of AGEs could contribute to complications associated with diabetes (microvascular complications) and potentially exacerbate local tissue damage in periodontitis (Brownlee, 2005, Chilelli *et al*., 2013). Structural protein changes associated with the glycation process have been implicated in diseases such as Alzheimer's, atherosclerosis, nephropathy, retinopathy and diabetes (Zheng *et al*., 2002, Lin *et al*., 2003, Semba *et al*., 2010, Wang *et al*., 2012). In particular, proteins with longer half-lives that are directly exposed to high extracellular glucose levels for prolonged periods of time (such as albumin, haemoglobin, collagen and alkaline phosphatases) are susceptible to glycation (Singh *et al*., 2001).

Although the full extent of AGE involvement in diabetes is not known, studies have implicated AGEs in cellular insensitivities to insulin in diabetes as a consequence of AGE associated overproduction of ROS, impairment in proteasomal activities and dysregulation of inflammatory responses (Berbaum *et al*., 2008, Younessi and Yoonessi, 2011). Glycated proteins often form aggregates and so are more resistant to degradation and proteasomal activity. This causes a retention of cross-linked and AGE modified proteins in cells and body tissues impacting their normal function. For example, AGE modified collagen aggregates are responsible for ECM stiffening which can lead to organ and vessel dysfunction, both of which can exacerbate diabetic complications (Badenhorst *et al*., 2003). Furthermore, a recent study showed that although AGE modified albumins were rapidly captured by scavenger cells, the proteins and protein fragments accumulated in the liver and showed hindered excretion. This increase in AGE proteins in the liver can affect normal liver excretion pathways and may play an important role in liver complications associated with diabetes (Tsutsui *et al*., 2016).

In addition to structural changes caused by the formation of AGEs, interaction of AGEs with RAGE have been shown to cause inflammation and oxidative stress both of which are important in the progression of periodontitis and diabetes. RAGE is constitutively or inducibly expressed in a variety of cell types, including neurones, immune cells (neutrophils, monocytes, macrophages, lymphocytes and dendritic cells), smooth muscle cells and vascular endothelial cells (Younessi and Yoonessi, 2011, Chuah *et al*., 2013). It contains a short cytoplasmic domain, a single transmembrane domain and an extracellular domain, composed of a variable domain and two constant domains. The

protein belongs to the immunoglobulin super family and has the rare characteristic of having the potential to bind multiple ligands from different families. RAGE is thus considered a PRR (Younessi and Yoonessi, 2011, Ott *et al*., 2014).

Given the high prevalence of neutrophils in periodontitis and indeed the dysregulation of neutrophil recruitment and function associated with both diabetes and periodontitis (as discussed previously; 1.2.1 and 1.4), AGE/RAGE interactions on these cells have the potential to cause increased local inflammatory responses. Upon AGE binding to RAGE, expression of inflammatory mediators (such as IL-1β, TNF-α and IL-6) are upregulated (Lalla *et al*., 2000, Graves *et al*., 2006). Furthermore, AGE is associated with increased levels of ROS and oxidative stress (causing endothelial cell changes and vascular injury), impairment of bone formation and repair (potentially impacting bone resorption in periodontitis), a decrease in ECM production and decrease in collagen strength and turnover, all of which may impact periodontal health and the progression of diabetic complications (Lalla *et al*., 2001, Vlassara, 2001, Cortizo *et al*., 2003, Santana *et al*., 2003, Wong *et al*., 2003, Preshaw *et al*., 2012).

Linking diabetes to periodontitis, increased serum AGE in diabetics with a positive correlation to increased periodontitis-associated attachment loss has been demonstrated (Takeda *et al*., 2006). Furthermore, immunohistochemical analysis of gingival tissues from diabetics indicated a higher percentage of AGE on cells (epithelium, blood vessels and fibroblasts) in diabetes with periodontitis compared with healthy tissues (Zizzi *et al*., 2013). On the other hand, studies have also demonstrated increased RAGE expression in gingival tissues of diabetics with periodontitis in both human (Katz *et al*., 2005) and animal studies (Chang *et al*., 2013). Ren *et al*. (2009) showed gingival fibroblasts exposed to AGE modified human serum albumin had decreased cell viability and impairment of intracellular collagen I and collagen III synthesis and expression. This suggests AGE dysregulates collagen turnover which may exacerbate periodontitis tissue destruction.

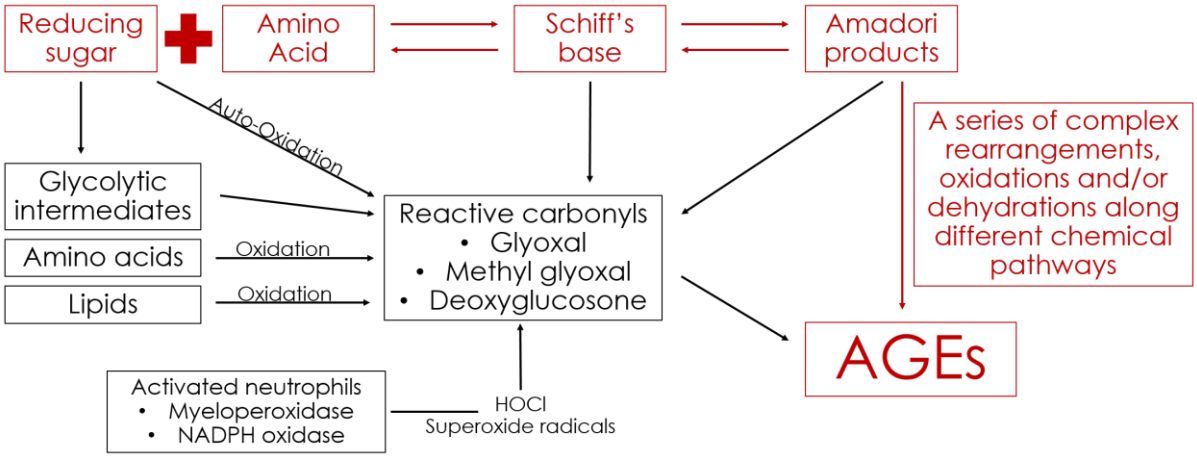


Figure 1.6: Formation of advanced glycation end products through various pathways. In addition to the Maillard reaction (red), various other pathways lead to the formation of AGEs. These include lipid and amino acid degradation and cleavage of dicarbonyl compounds from glycolytic intermediates as well as the formation of carbonyl compounds following auto-oxidation of monosaccharides such as glucose, ribose, fructose and glyceraldehyde. AGE: advanced glycation end product, HOCl: hypochlorous acid, NADPH: nicotinamide adenine dinucleotide phosphate.

1.4.4. Other potential linking mechanisms

As discussed previously (section 1.1.1.4), saliva is important in the maintenance of a healthy oral microbiome. In diabetes, salivary flow rate and composition have been shown to be altered (Harrison and Bowen, 1987, Thorstensson *et al*., 1989, Dodds *et al*., 2005). Dodds *et al*. (2005) showed a reduction in stimulated and unstimulated submandibular/sublingual saliva outputs. Diabetic salivary proteins were also demonstrated to have increases in lactoferrin, MPO and salivary peroxidase in stimulated parotid and increased levels of secretory IgA, albumin and lactoferrin in stimulated submandibular/sublingual saliva. Although the significance of these changes is yet to be fully understood, upregulated salivary proteins in diabetes could encourage dysbiosis of the oral microbiota. For instance, changes in IgA levels could potential effecting colonisation. Furthermore, increases in salivary albumin could cause an increase in AGE modified albumin in diabetics with hyperglycaemia, thus an increase in AGE associated immune responses which could contribute to the progression of periodontitis (Dodds *et al*., 2005).

Impairment of apoptosis has also been suggested as a potential link between diabetes and destruction of tissue in periodontitis. In diabetic mice, a higher incidence of fibroblast apoptosis was observed when compared with non-diabetic mice upon induction of tissue injury through *P. gingivalis* inoculation (Liu *et al*., 2004). An increase in apoptosis of these matrix-producing cells could indicate an impairment in inflamed tissue repair which in periodontitis could contribute to attachment loss (Dodds *et al*., 2005).

1.5. Conclusions

Periodontitis is a complex disease involving both the oral microbiome and the host responses. Periodontitis is considered an inflammatory disease, with the innate immune response playing crucial roles in periodontal tissue destruction. Diabetes is similarly associated with dysregulated immune responses. While the two diseases have been shown through epidemiological studies to be linked in a two-way relationship the exact mechanisms are unclear. Some potential links have been discussed above but others (such as OPG/RANKL levels, TLR expression, T-cells and B-cells) have also been suggested as potential links and have been reviewed in detail elsewhere (Sonnenschein and Meyle, 2015).

In addition to host responses, the microbiome of the oral cavity has also been implicated in the progression of periodontitis. While studies regarding the differences in the oral microbiome between systemically healthy and diabetic individuals with periodontitis are contradictory and sparse, it is well established that changes are observed in microbiota composition in periodontitis so that periodontitis bacteria (for example *P. gingivalis* and

T. forsythia) are favoured. With advances in technology, the role of the microbiota in linking diabetes and periodontitis can be better understood.

Although many potential linking mechanisms have been postulated for the two-way relationship between periodontitis and diabetes, current evidence is unclear and often contradictory indicating a need for further investigations.

1.6. Aims and objectives

It is now widely accepted that a two-way relationship exists between periodontitis and diabetes. To date, studies comparing the changes associated with the two diseases have produced contradictory results. The aim of this study is to provide evidence for potential linking mechanisms between diabetes and periodontitis.

It is the objectives of this project to determine if increased AGEs (a consequence of hyperglycaemia) increase AGE-RAGE interactions to change inflammatory responses (measured as changes in IL-8, IL-1β and IL-6) in gingival keratinocytes. Furthermore, this study utilises an in-house developed biofilm model to ascertain if AGEs influence biofilm bacterial composition (using both culturing methods and NGS) as well as how biofilms grown with concentration of AGEs that are commensurate with diabetes and health impact inflammatory responses (IL-1β, IL-8, IL-6 and ROS changes) when co-cultured with gingival keratinocytes.

In addition to *in vitro* studies, a preliminary clinical study was undertaken to uncover differences in biofilm composition and functionality in systemically healthy individuals with and without periodontitis. This was achieved through next generation sequencing (NGS). In addition to this it is important to determine the local and systemic levels of pro-inflammatory mediators (cytokines/chemokines and MMPs) in these individuals to establish molecular mechanisms involved in periodontitis. The study also looked at systemic neutrophil functions (migration, phagocytosis and respiratory burst) as defective neutrophils have been associated with periodontitis progression. By understanding the systemic changes involved in periodontitis, one could understand the potential effect multiple diseases with distinct pathogenesis, indeed this preliminary clinical study will set the basis for a more complex study involving diabetics with and without periodontitis.

2. Material and Methods

2.1. Solutions and Buffers

Table 2.1: Table of buffers and solutions used

Artificial Saliva	2.5 g/L mucin, 6.52 mM NaCl, 14.94 mM KCl, 5.42 mM KH_2PO_4, 11.35 µM ascorbic acid. Added post sterilisation: 9 mM Urea and 5 mM arginine
Basal medium	10 g/L Proteose-peptone, 5 g/L Trypticase-peptone, 5 g/L yeast extract, 33.53 mM KCl, 4.13 mM cysteine. pH 7.4
Blocking Buffer	5% semi skimmed milk powder dissolved Tris Buffered Saline (Bio-Rad Laboratories Ltd) with 1% (v/v) Tween-20 (TBS-T)
ELISA coating buffer	51.1 mM $NaHCO_3$, 50 mM Na_2CO_3, pH 9.4
ELISA stop buffer	1.8N H_2SO_4
ELISA wash buffer	1.46mM KH_2PO_4, 9.9 mM KH_2PO_4 – $3H_2O$, 1.36 mM EDTA, 5%(v/v) Tween 20, pH 7.4
Reduced transport fluid (RTF) (Rundell *et al*., 1973)	68.11 mM $(NH_4)_2SO_4$, 154 mM NaCl, 28.70 mM K_2HPO_4, 33.07 mM KH_2PO_4, 37.74 mM Na_2CO_3, 13 mM EDTA, 12.97 mM Dithiothreitol, 7.30 mM $MgSO_4$, 0.1% (v/v) Resazurin
RIPA buffer (Radioimmunoprecipitation assay buffer)	150 mM NaCl, 1% (v/v) Triton X, 0.5% (v/v) sodium deoxycholate, 0.1% (v/v) SDS, 50 mM Tris
Serum medium	Basal medium supplemented with 20% (v/v) heat inactivated foetal bovine serum (FBS), 1.45 mM haemin and 1.1 µM menadione
TGS buffer (Bio-Rad Laboratories Ltd)	Tris/Glycine/SDS buffer Diluted to 1x working solution in dH_2O prior to use
Tris EDTA (ethylene diamine tetra acetic acid buffer) (TE Buffer)	10 mM Tris, 1 mM EDTA, pH 8.0
ELISA assay buffer	136.89 mM NaCl, 7.96 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 0.5% (w/v) Bovine Serum Albumin (BSA), 0.1% (v/v) Tween 20, pH 7.4

2.2. Tissue culture

Cells were cultured using standard aseptic techniques. Human telomerase immortalized gingival keratinocytes (TIGK: Moffatt-Jauregui *et al*., 2003) were kindly gifted from Dr Richard Lamont (School of Dentistry, University of Louisville). The TIGK cells were grown in Basal Medium Dermalike K complete kit with supplements (LifeLine® cell technology, USA) and incubated at 37°C, in 5% CO_2. Medium was changed every 2-3 days and cells were passaged when confluency reached 70-80% and used between passages 5 and 15. To passage, the monolayer of cells was washed with phosphate buffered saline (PBS) prior to incubation with 0.25% (v/v) Trypsin-EDTA solution (Sigma-Aldrich, UK), for 5 min or until cells detached at 37°C, 5% CO_2. Upon cell detachment, trypsin was neutralised by the addition of fresh medium at a 1:1 ratio and the suspension centrifuged at 300 *g* for 5 min. The supernatant was discarded, and the resulting cell pellet was re-suspended in an appropriate volume of medium to enable counting using a haemocytometer and seeding of tissue culture flasks or plates at the desired density. All tissue culture was carried out in sterile tissue-culture-treated flat bottom plates or tissue culture flasks (Corning® Costar®).

2.3. Western blot analysis

Cell lysates were generated using standard RIPA (Radioimmunoprecipitation assay buffer) lysis buffer protocols. Briefly, monolayer cells were washed with ice-cold PBS and the cells detached, using a cell scraper, directly into ice-cold RIPA buffer with added protease inhibitor cocktail (1 tablet/10 mL, cOmplete™, Mini protease inhibitor cocktail tablets, Roche, UK). The cell suspension was then incubated on ice for 15 min with agitation and centrifuged at 17000 *g*, 4°C for 20 min. Cell medium supernatant was also harvested and centrifuged at 17000 *g*, 4ºC for 20 min. Resulting supernatants were freeze dried and stored at -20°C for later analysis.

Protein concentrations were quantified prior to Western blot analysis using the Bradford assay (Pierce™ Coomassie (Bradford) Protein Assay Kit, ThermoFisher Scientific, UK) as per manufacturer's instructions for microplate procedures. Briefly, 5 µL of BSA standards (100-1500 µg/mL) or 5 µL sample was mixed with 250 µL Coomassie in a 96 well plate. Following a 10 min incubation at room temperature (RT), the absorbance was read at 595 nm in a microplate reader (Varioskan Flash, Thermo scientific) and a four-parameter standard curve generated to extrapolate sample protein concentrations.

Mini-PROTEAN TGX Stain Free Gels (4-15%, Bio-Rad Laboratories Ltd, UK) were used for western blotting. 10 µg total protein, with a 1:1 ratio of 2x Laemmli sample buffer (Bio-Rad Laboratories Ltd, UK) and supplemented with 100 mM β-mercaptoethanol was boiled for 5 min at 95°C prior to gel loading and the gel run for approximately 75 min at

120 V in TGS buffer. In addition to samples, Precision Plus Protein WesternC standards (Bio-Rad Laboratories Ltd, UK) were also loaded onto gels (10 – 250 kDa). Gels were then transferred to a PVDF (polyvinylidene difluoride) membrane (Trans-Blot Turbo transfer pack, 7 x8.5 cm, Bio-Rad Laboratories Ltd, UK) using a Trans-Blot ® Turbo™ transfer system (Bio-Rad Laboratories Ltd). Following transfer, membranes were rocked at RT in blocking buffer for a minimum of 1 h to reduce background binding of primary antibodies. Blocking buffer was discarded and membranes incubated with primary antibody diluted in 0.25% (w/v) milk in TBS-T, overnight at 4°C on a rocking platform (Table 2.2). This was followed by 3x 5 min washes in TBS-T with rocking at RT. Membranes were then blocked again to reduce non-specific secondary antibody binding followed by 3x 5 min washes in TBS-T with rocking at RT and incubation for 1 h at RT with the secondary antibody and a Precision Protein ™ StrepTactin-horseradish peroxidase (HRP) conjugate (Bio-Rad Laboratories Ltd) in 0.25% (w/v) milk in TBS-T (Table 2.2). The StrepTactin-HRP was added to enhance visualisation of standards. Unbound antibody was removed with 3x 5 min washes and the antibody staining revealed by 5 min incubation of membranes in SuperSignal™ WestFemto Maximum Sensitivity Substrate (Fisher Scientific Ltd, UK) for chemiluminescence exposure and visualised using the ChemiDoc™ MP imaging system (Bio-Rad).

For multiple antibody probing, membranes were rocked in Restore PLUS Western Blot Stripping buffer (Thermo Fisher Scientific, Life Technologies, UK) for 30 min at RT followed by 3x 5 min washes in TBS-T with rocking at RT and the process above repeated from the first blocking step.

Table 2.2: Antibody details for Western Blotting. HRP; Horseradish peroxidase, RAGE; receptor for advanced glycation end products, IgG; Immunoglobulin G.

Primary Antibody	**Secondary Antibody**
Antibody: Mouse monoclonal to human RAGE Dilution: 1:1000 dilution Final concentration: 0.5 µg/mL Abcam (MM0520-8D11)	Antibody: Rabbit polyclonal antibody to mouse IgG (HRP) Dilution: 1:10000 dilution Final concentration: 2 µg/mL Abcam
Antibody: Rabbit polyclonal to human β-Actin Dilution: 1:1000 dilution Final concentration: 0.5 µg/mL Abcam	Antibody: Goat polyclonal antibody to rabbit IgG (HRP) Dilution: 1:20000 dilution Final concentration: 1 µg/mL Abcam

2.4. Enzyme-linked immunoassay

Enzyme linked immunoassays (ELISAs) were carried out with the antibodies reconstituted according to manufacturer's instructions. Briefly a 96 well flat bottomed plate (Greiner bio-one) was coated with monoclonal capture antibody (Table 2.3) and incubated at RT overnight. The plate was then washed (x3) in wash buffer and dried prior to blocking with assay buffer at RT for 1 to 2 h. The wash step was repeated before samples and standards were added to the plate and incubated at RT for 2 h. Standards were added to each assay plate (with ranges as indicated Table 2.3) in addition to a negative control of assay buffer alone. The wash step was repeated and assay buffer containing biotinylated detection antibody (Table 2.3) was added to each well and the plate incubated at RT for 2 h. Plates were washed again prior to the addition of Streptavidin horseradish peroxidase (Strep-HRP) (Table 2.3) and then incubated at RT for 20 min. Following a final wash, 200 µL of 3,3',5,5' - tetramethylbenzidine (stabilized chromogen TMB, Invitrogen, UK) was added as a substrate. Plates with TMB added were incubated at RT with protection from light for approximately 20 min. Once the appropriate amount of colour development was achieved a stop solution was added producing a yellow colour which was detectable at 450 nm. The absorbance of the wells were read and recorded, within 15 min of adding the stop solution, using a plate reader (Varioskan Flash, Thermo scientific) set to measure photometric absorbance at a wavelength of 450 nm.

Sample absorbance readings were converted to analyte concentrations (pg/mL) using the curve of the line equation generated from the standard curve produced for each plate. Analyte concentrations were calculated with correction for background levels of absorbance.

Table 2.3: Antibody details used for ELISAs. IgG: Immunoglobulin G, IL: Interleukin.

Analyte	**Antibody Manufacturer**	**Capture antibody concentration (Host/Clone/ Diluent)**	**Detection antibody concentration (Host/ Clone)**	**Standards range (pg/mL)**	**Strep HRP concentration**
IL-8	R&D systems	1 µg/mL (Mouse IgG/ 6217/ Coating buffer)	1 µg/mL (Goat IgG/ polyclonal)	15.625 – 1000	1:5000 dilution, R&D systems
IL-6	BD Pharmingen	1 µg/ml (Rat IgG/ MQ2-13A5/ Coating buffer)	2 µg/ml (Rat IgG2a/ MQ2-39C3)	15.625 – 1000	1:5000 dilution, R&D systems
IL-1β	R&D systems – Duoset	4 µg/ml (-/-/PBS)	12 µg/mL (n/a)	3.91 – 250	1:40 dilution, R&D Duoset
RAGE	R&D systems – Duoset	1 µg/ml (-/-/PBS)	100 ng/mL (n/a)	62.5 – 4000	1:200 dilution, R&D Duoset

2.5. Next generation sequencing

2.5.1. Bacterial DNA extraction and quantification

DNA was extracted from samples using a DNA extraction kit (UltraClean® Microbial DNA Isolation kit, MoBio Laboratories, Inc., UK) following the manufacturer's instructions. Briefly, microbial cell pellets were re-suspended in MicroBead solution, gently vortexed and transferred to a MicroBead tube followed by heating at 65°C for 10 min and the addition of lysis solution. The MicroBead tubes were then vortexed for 10 min on a horizontal vortex. The specialised beads and bead beating tube in combination with the heating and addition of lysing solution uses chemical and mechanical mechanisms for microbial lysis. Following microbial lysis, released DNA was bound to spin filters, washed and eluted in DNA-free Tris buffer. Extracted DNA was stored at -20°C for further analysis.

DNA concentration was determined using a Quant-iT™ PicoGreen™ double stranded (ds) DNA assay (ThermoFisher Scientific, UK) according to manufacturer's instruction. Briefly, 1 µL of extracted DNA was added to wells of a 96 well plate containing 99 µL of TE (Tris EDTA) buffer. DNA standards were run in conjunction with samples with a range of 1000 ng/mL to 10 ng/mL (high range quantitation) or 50 ng/mL to 1 ng/mL (low range quantitation). PicoGreen (Quant-iT™ PicoGreen™ ds DNA assay, ThermoFisher Scientific, UK) was added to each well (100 µL) and the plate incubated, protected from light, for 2 to 5 min at RT. Fluorescence was read at 480 nm excitation, 520 nm emission and DNA concentration of samples calculated using the curve of the line equation generated from the standards.

2.5.2. DNA shearing and size distribution electrophoresis

Extracted bacterial DNA was sheared using Covaris microTUBEs (Covaris, Inc.) to 200 base pairs (bp) fragments using the pre-set 'Microtubes 200 bp' program on the sonicator (Covaris s220 high performance ultrasonicator, Covaris, Inc.). The fragment size of the sheared DNA was immediately checked using the Agilent 2200 TapeStation system (Agilent). Briefly, 2 µL of DNA sample was added to the well of a V-bottom 96 well plate (Agilent, UK) with an equal volume of loading buffer (High sensitivity D1000 sample buffer, Agilent, UK). Samples were run along-side a High sensitivity D1000 ladder (Agilent, UK) and loaded onto High sensitivity D1000 ScreenTapes (Agilent, UK). Following size verification (Figure 2.1) sheared DNA was immediately used for library preparation or stored at -20ºC for later processing.

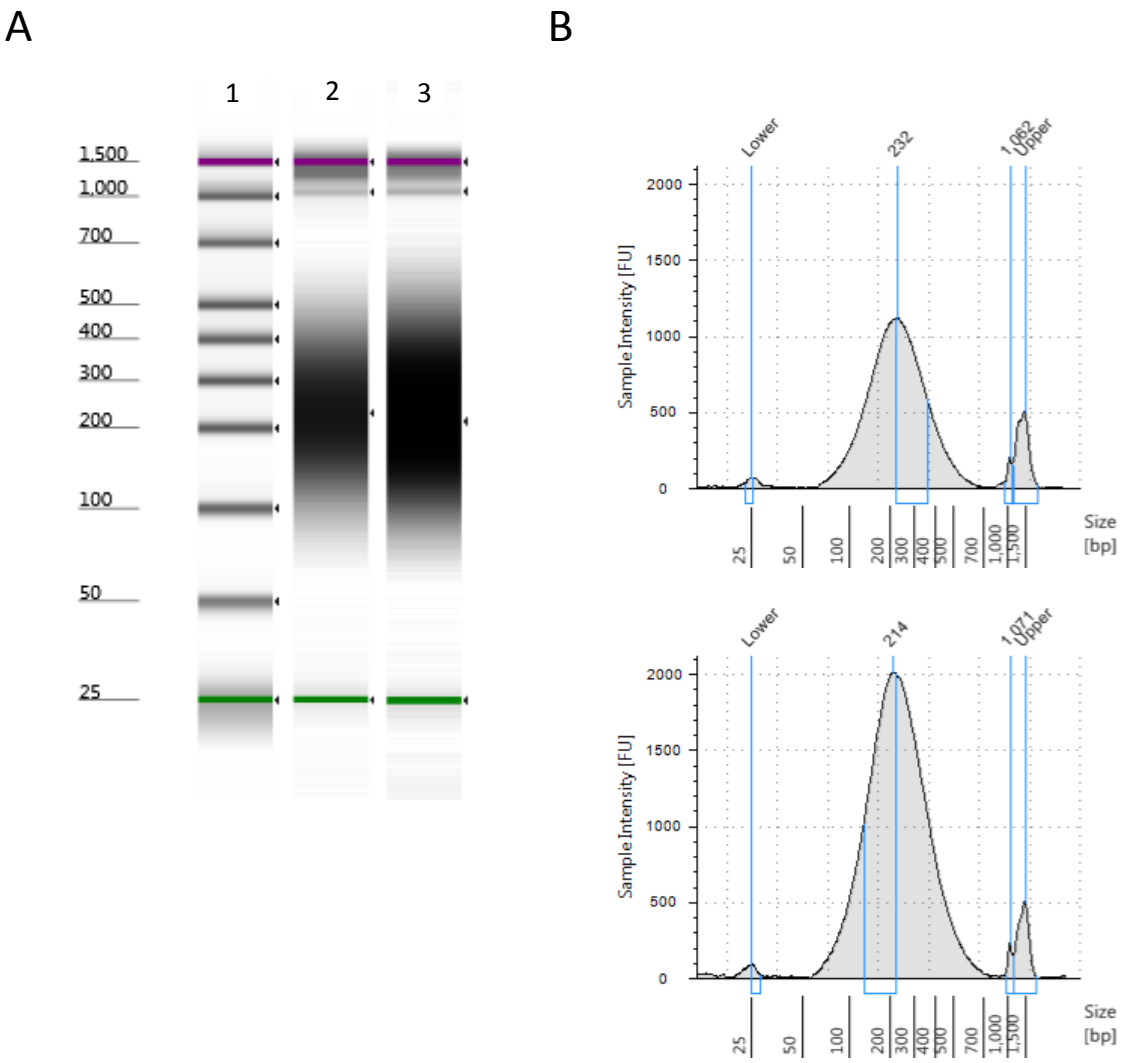


Figure 2.1: Example of tape station results post DNA shearing. (A) Example of electrophoresis tape image. Lane 1 shows ladder while lane 2 & 3 show sheared DNA extracted from saliva. (B) Example of size distribution of sheared samples. Sheared samples show a bell curve with a peak at approximately 200 bp indicating successful shearing.

2.5.3. Next generation sequencing library preps

Libraries for Next Generation Sequencing (NGS) were prepared using the NEBNext Ultra (for samples with extracted DNA 5 ng – 1 µg) or NEBNext Ultra II (for DNA <5ng) library preparation kit for Illumina (New England BioLabs, USA). Libraries were prepared following the manufacturer's instructions as outlined in Figure 2.2 . NEBNext Multiplex adaptors (set 1 (E7335) and set 2 (E7500), New England Biolabs, USA) were used for multiplexing library preparations.

Resulting libraries were then checked for quality using an Agilent TapeStation as described above. In cases where starting DNA concentrations were low, adaptor contamination could occur (Figure 2.3A). To remove adapter contamination a clean-up step using 0.8x beads was carried out. DNA concentrations of resulting libraries was established using Quant-iT™ PicoGreen™ ds DNA assay (section 2.5.1) and an equimolar concentration of each sample was added to a pool (24 samples with different index primers per pool maximum) and the samples sequenced on one lane of the Illumina HiSeq3000 available at the NGS facility at the University of Leeds (http://dna.leeds.ac.uk/genomics/).

2.6. Next generation sequencing data analysis

Sequencing data was received de-multiplexed from the NGS facility and analysed using the high performance computing (HPC) system. These data were processed as outlined in Figure 2.4 utilising QIIME (Caporaso *et al*., 2010b), SortMeRNA (Kopylova *et al*., 2012), DIAMOND (Buchfink *et al*., 2015) and MEGAN (Huson *et al*., 2007) for filtering, mapping, operational taxonomic unit (OTU) picking and functional analysis. SortMeRNA allows the sorting of reads into rDNA reads and non-rDNA reads using a reference database. During this analysis the HOMD was utilised for rDNA sorting (http://www.homd.org/index.php?name=seqDownload&file&type=R). The rDNA and DNA sorted reads were mapped to reference databases using QIIME and DIAMOND/MEGAN, respectively.

OTU count tables were loaded in R (https://www.R-project.org/) through the phyloseq package (McMurdie and Holmes, 2013). For clinical sample analysis, the OTU count table, metadata mapping file and phylogenetic tree were loaded into phyloseq to generate a phyloseq object for analysis of sample bacterial composition. Graphs were generated using the ggplot package in R (Wickham, 2009). For analysis of complex biofilm NGS data, phyloseq objects of OTU counts and functional counts were used in DESeq2 for statistical analysis of bacterial composition. Differential abundance was determined using the Wald test to determine significance ($p < 0.1$).

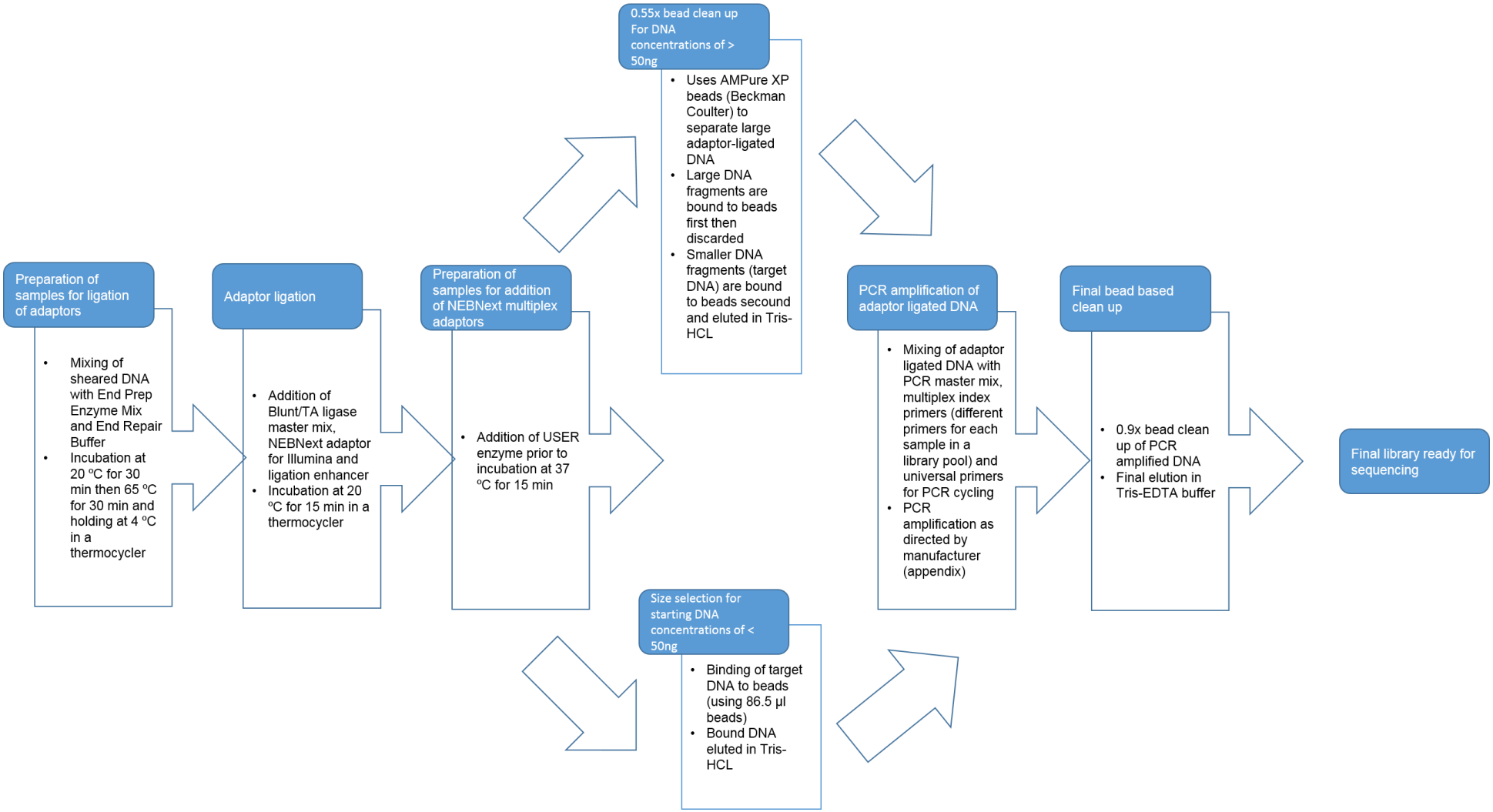
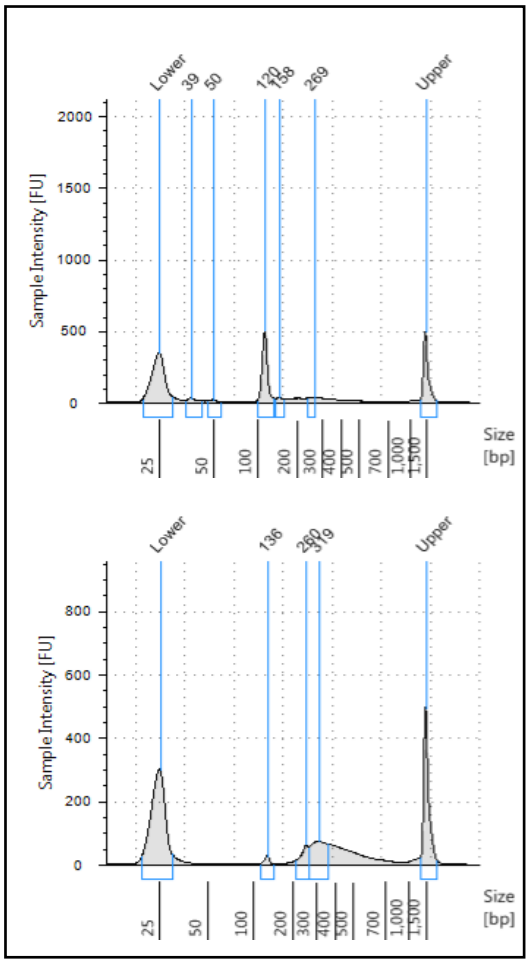


Figure 2.2: Schematic outlining the process for next generation sequencing library preparation using NEBNex library prep kits for Illumina.

A



B

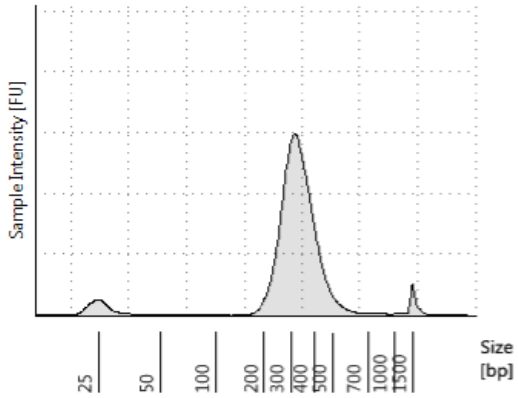


Figure 2.3: TapeStation analysis of next generation sequencing library preparations. (A) Example of library preparation with adapter contamination. Adapters at 120 bp (top) were removed using 0.8x bead clean-up so that the library peak (at approximately 319 bp) was larger than any remaining adapter contamination (bottom). (B) Example of library preparation without any adaptor contamination

Table 2.4: Example of a metadata file used in analysis pipeline.

#SampleID	BarcodeSequence	linkerPrimerSequence	InputFileName	SampleType	SampleSite	Description
p001p	ATCACG	p001plaque	p001pinterleaved.fna	Periodontitis	Plaque	p001pperio
p002p	CGATGT	p002plaque	p002pinterleaved.fna	Periodontitis	Plaque	p002pperio
p003p	TTAGGC	p003plaque	p003pinterleaved.fna	Healthy	Plaque	p003phealth
p004p	TGACCA	p004plaque	p004pinterleaved.fna	Periodontitis	Plaque	p004pperio
p005p	ACAGTG	p005plaque	p005pinterleaved.fna	Periodontitis	Plaque	p005pperio
p006p	GCCAAT	p006plaque	p006pinterleaved.fna	Periodontitis	Plaque	p006pperio
p007p	CAGATC	p007plaque	p007pinterleaved.fna	Healthy	Plaque	p007phealth
p008p	ACTTGA	p008plaque	p008pinterleaved.fna	Healthy	Plaque	p008phealth
p009p	GATCAG	p009plaque	p009pinterleaved.fna	Healthy	Plaque	p009phealth
p010p	TAGCTT	p010plaque	p010pinterleaved.fna	Healthy	Plaque	p010phealth
p011p	GGCTAC	p011plaque	p011pinterleaved.fna	Healthy	Plaque	p011phealth

2.6.1. Taxonomic analysis based on rDNA reads

As outlined in Figure 2.4 raw read files (as .fastq files) were first processed for the removal of adaptors using Cutadapt (Martin, 2011) and trimmed for removal of low quality reads using Sickle (Joshi and Fass, 2011). The paired end reads were then interleaved using SortMeRNA so that forward and reverse reads for each sample were in one file. Interleaved read files were converted to fasta for use with QIIME. The interleaved files for each sample were combined into a single file containing all sample reads with labels corresponding to sample identity using a metadata mapping file which provides information on each file (see Figure 2.4 for example). These reads were then sorted into rDNA and non-rDNA following comparison to the HOMD database (reads provided as .fastq file).

Representative sequences for each OTU were then picked from rDNA reads for taxonomic assignment. Although achieved through QIIME scripts, taxonomic assignment used an uclust algorithm to cluster nucleotides based on sequence similarities, via the RDP Classifier 2.2. (Wang *et al*., 2007, Edgar, 2010).

Taxonomic classification was followed by generation of OTU count tables for downstream analysis. In addition to OTU count table generation, representative OTU sequences were used for phylogenetic tree building using QIIME, PyNast (Caporaso *et al*., 2010a) and FastTree 2.1.3. (Price *et al*., 2010) to align representative sequences to existing alignments, Greengenes core reference alignment, (DeSantis *et al*., 2006) and tree generation.

Principle coordinate analysis (PCoA) with Bray Curtis dissimilarity plots were generated using MEGAN to visualise sample relationships based on taxonomy. OTU tables were loaded into MEGAN (as .biom files) with NCBI taxonomy assignments and PCoA analysis carried out based on absolute counts.

2.6.2. Taxonomic and functional analysis based on DNA contigs

Raw reads were processed as outline in Figure 2.5. Following sorting of quality trimmed reads using SortMeRNA (Figure 2.5 – steps 1-4), non-rDNA short reads in each sample were assembled *de novo* using MEGAHIT (Li *et al*., 2015). The resulting contigs were aligned to the non-redundant (nr) NCBI protein database using DIAMOND. DIAMOND generated files, containing DNA contig based counts for each alignment labelled according to NCBI accession numbers, were imported into MEGAN.

MEGAN allows interactive visualisation of metagenomics data. DIAMOND files were collectively loaded into MEGAN for assignment of accession numbers to taxonomy and function (as described in Figure 2.5). Taxonomical assignment was based on the NCBI protein database. Functional assignment was based on EggNOG (evolutionary genealogy of genes: non-supervised orthologous groups, Huerta-cepas *et al*, 2015) and SEED (Overbeek *et al*., 2005). Both databases are used to assign protein functions to genes. EggNOG is EMBL (European molecular biology laboratory) hosted database which provides information about orthologous groups of proteins at various taxonomical levels. SEED, contrastingly, assigns gene functions using homologous genes from complete genomes and presents them in functional categories.

Taxonomy OTU counts and functional counts were exported from MEGAN and important into the phyloseq package of R with corresponding mapping files for further analysis (section 2.6).

2.7. Preliminary study of clinical samples

2.7.1. Sample Collection

Saliva, blood, plaque and GCF was collected from participants with periodontitis (diseased) and healthy controls. Participants were recruited and samples collected at the Leeds Dental Translational and Clinical Research Unit, following ethical approval (Leeds East Research Ethics Committee) and informed consent (14/YH/0010). For all participants, samples from periodontally diseased sites were taken from participants with periodontitis, and samples from periodontally healthy sites only were taken from healthy participants. The periodontitis group were identified by research dental practitioners as having periodontal pockets with a probing depth of 5 mm or greater with bleeding on probing. For the periodontally healthy groups, sites were identified as having sulcus depths of 3 mm or less with no bleeding on probing and no clinical signs of inflammation.

2.7.1.1. Saliva collection

Participants were asked to provide a minimum of 2 mL of stimulated saliva (by chewing on sterile wax). Samples were kept on ice and centrifuged at 1500 *g* and 4°C for 15 min. The supernatant and resulting pellet were frozen at -80°C for future analysis.

2.7.1.2. Blood collection and plasma separation

Blood was collected from participants by venepuncture into both EDTA and lithium heparin coated anti-coagulant tubes. Whole blood (with lithium heparin anti-coagulant) was used immediately for neutrophil analysis (2.7.2, 0 and 2.7.4 for methodology). To

separate cell free plasma, samples were centrifuged at 1500 *g* at RT for 10 min with the brake disabled. Resulting plasma was stored at -80°C for future analysis.

2.7.1.3. GCF collection and processing

GCF was collected from 5 sites from each participant using Perio Paper strips (Oraflow Inc, New York). In all cases accessible sites were selected where moisture contamination could be controlled, with sites where bleeding on probing was extensive being rejected. Prior to collection, supragingival plaque was removed using a sterile cotton wool pledget. A strip was then inserted into the gingival crevice for 10 sec, with two strips being used per site. The strips from each site were placed into a single polypropylene tube before freezing at -80°C for later analysis.

For analysis, immediately prior to assay, GCF was processed as previously described (Awang *et al*., 2014). Briefly each strip was thawed on ice before the addition of 200 µl ice cold phosphate buffered saline (PBS) supplemented with 1% (w/v) BSA and rotated for 60 min at 4°C. Following removal of the strips, the eluted GCF was centrifuged at 350 *g* for 60 min at 4°C and then at 13,000 *g* for 3 min at 4°C and the resultant supernatant from the 10 strips from each patient pooled and used immediately for analysis.

2.7.1.4. Plaque collection and processing

Subgingival plaque was collected using sterile paper points (Maillefer Pro Taper Paper Points F3, BF Mullholland Ltd., UK). Prior to insertion of paper points into the gingival sulcus, collection sites were isolated and dried using sterile gauze and supragingival plaque removed with a sterile cotton pledget. A total of 5 sites were sampled with 3 paper points used for each site. Following collection paper points were stored at -80°C for further analysis.

For plaque extraction a sterile glass bead was added to each tube containing 3 paper points from a single site. The tubes were vortexed vigorously for a minimum of 5 mins. Bacterial DNA was extracted (section 2.5.1) with the following adaptation: 300 µL of the microbead solution was added directly to a tube containing paper points from one site and a sterile glass bead. The tube was then vortexed and the solution transferred to a tube containing paper points from a different site from the same participant and a sterile glass bead. The process was repeated and in this manner plaque from the 5 sites from each participant was pooled prior to DNA extraction.

Figure 2.4: Pipeline for analysis of rDNA reads from metagenomics data. Blue text represents code.

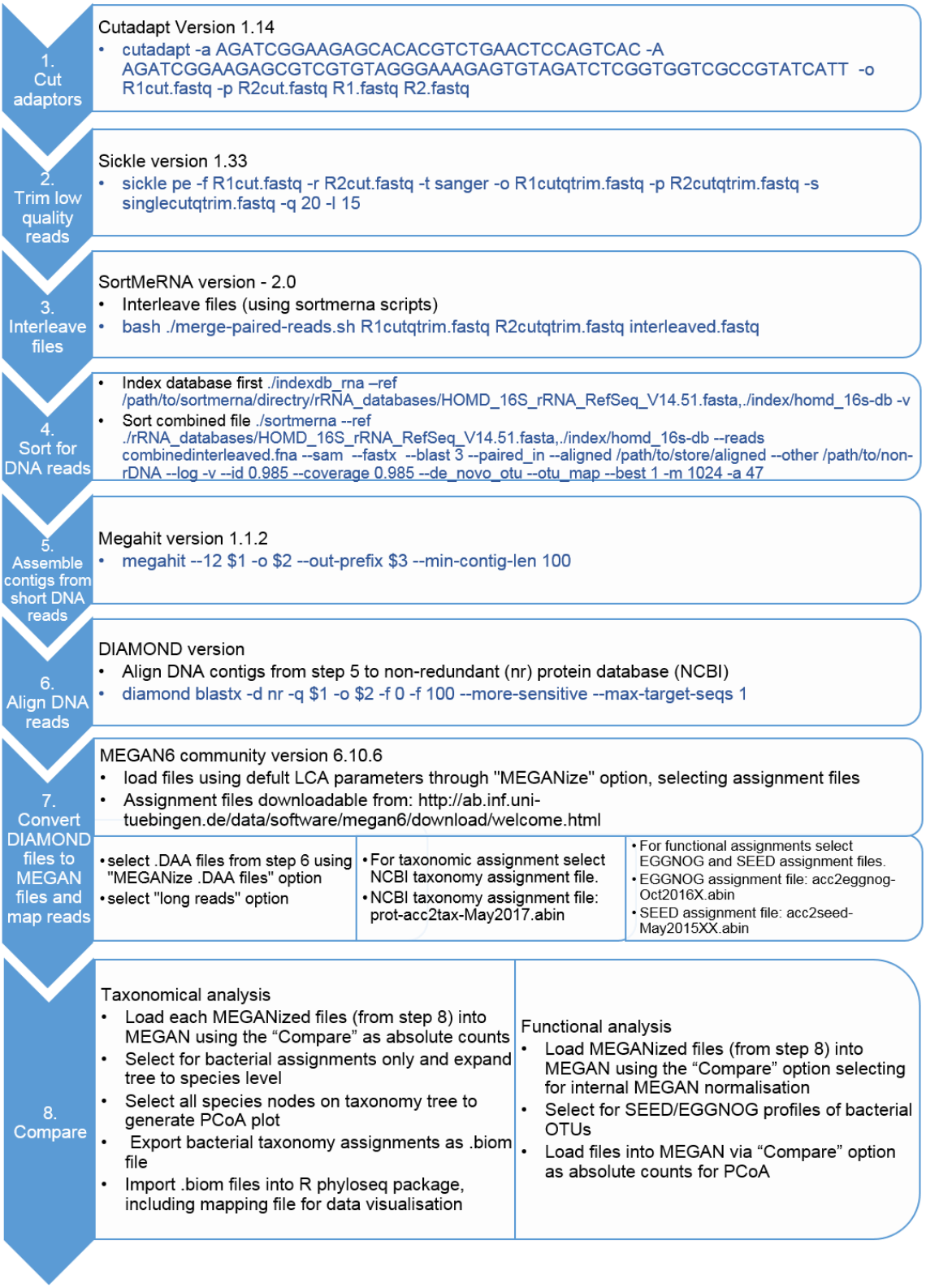


Figure 2.5: Pipeline for analysis of rDNA reads from metagenomics data. Blue text represents code.

2.7.2. Quantifying migration of leukocytes

The Migratest assay (Glycotope Biotechnology) allows the quantitative analysis of leukocyte chemotaxis. The numbers of cells migrating through cell culture inserts (3 µm pore size) towards a concentration gradient of the chemo-attractant N-formylmethionine-leucyl-phenylalanine (fMLP) were quantitated against counting beads whilst changes in cell shape and the down regulation of the cell adhesion molecule L-selectin, were determined to monitor cell activation. The Migratest assay was performed as per manufacturer's instructions with blood samples assayed within the recommended 24 h of collection, typically within 6 to 14 h.

Leukocyte-rich-plasma (LRP) was generated by overlaying 1 mL of whole blood on top of the supplied leukocyte separation medium for 40 min at RT. The upper phase was collected and placed in cell culture inserts pre-positioned in wells (24 well plate) containing 350 µL fMLP (0.05 µM) in the supplied incubation buffer (Reagent B) or buffer alone for negative controls (Figure 2.6). Each sample was carried out in triplicate (referred to hereafter as technical repeats). Plates with inserts within wells were incubated in a 37 °C water bath for 30 mins. Cell suspensions were then removed from the wells and transferred to polystyrene FACS (fluorescence-activated cell sorting) tubes (BD Biosciences) and placed on ice. Cell suspensions remaining within the control inserts (20 µL) were also removed to provide comparison with un-migrated cells. 20 µL of Reagent D (containing counting bead suspension and monoclonal anti-L-selectin-FITC (fluorescein isothiocyanate)) was added to each sample, vortexed and incubated on ice for 10 min protected from light. Finally, 20 µL of 1x Reagent E (DNA stain) was added to each tube, the samples mixed and incubated on ice in the dark for 5 min.

The data were acquired using a LSRII flow cytometer equipped with a 488 nm argon-ion laser (BD Bioscience) and BD Diva software within 120 min of protocol completion. The fluorescence from the counting beads and DNA stain were detected at 488 nm (within PerCP-Cy5-5-A labelled channel) and used to set an initial gate which identified leukocytes and counting beads (Figure 2.7A). A second gate was then set around the counting beads using a SSC (side scatter) vs FSC (forward scatter) contour plot to distinguish between the counting beads and leukocytes (Figure 2.7B). Data were acquired until 2000 events were recorded in the counting bead gate. This method standardised the volume analysed per data file and therefore ensured the amount of leukocytes within each sample was comparable. Leukocytes were quantitated and also analysed for surface L-selectin expression (FITC mean fluorescence intensity; MFI), a reduction in which indicates cellular activation by fMLP (Figure 2.7C). In addition, the median FSC was used as an indication of changes in cell size. The sample of cells taken

from the remainder within the insert were used as a comparison for these indicators of activation.

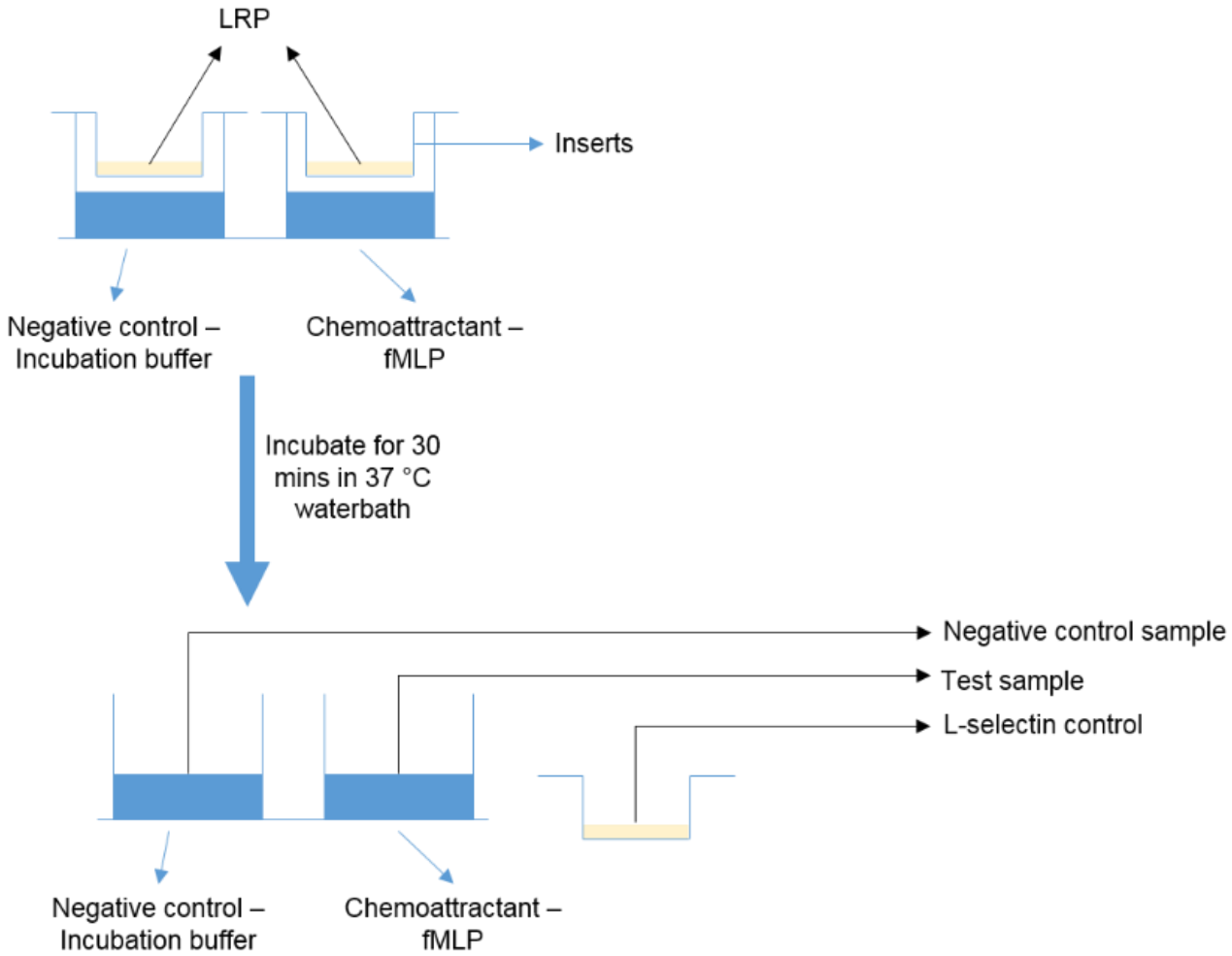


Figure 2.6: Schematic of sample stimulation for analysis of leukocyte chemotaxis. Sample leukocyte-rich-plasma was loaded onto cell culture inserts and placed in 24 well plates above wells containing either incubation buffer or fMLP (N-formylmethionine-leucyl-phenylalanine) as a chemoattractant. Following 30 min incubation, inserts were removed and samples taken from each well for fluorescence-activated cell sorting.

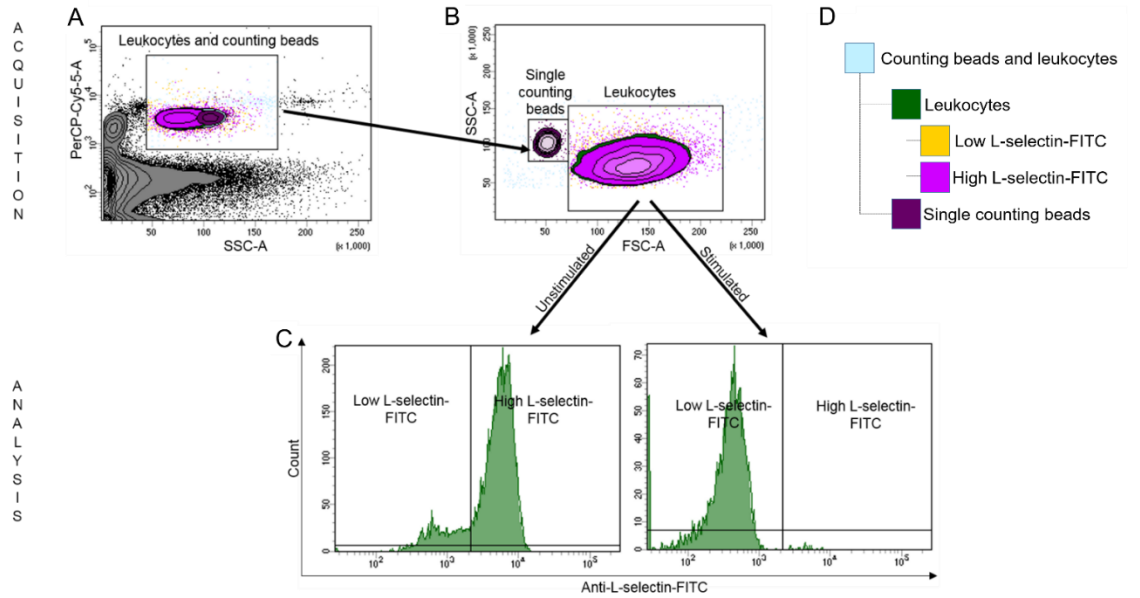


Figure 2.7: Gating strategy for FACs acquisition and downstream analysis of neutrophil migration. (A) Initial acquisition gate was used to identify counting bead and leukocyte population. (B) Gates were drawn to distinguish between counting beads and leukocytes and 2000 events were collected within the counting bead region. (C) Gates set on FITC histogram were used to distinguish between low and high L-selectin expression. The unstimulated histogram shows samples with high-L-selectin-FITC indicating non-migrated cells. The stimulated histogram show samples with low-L-selectin-FITC indicating migrated cells. (D) Population hierarchy and colour key for contour plots.

2.7.3. Quantifying phagocytosis by neutrophils

The Phagotest (Glycotope Biotechnology) assay allows the quantitative analysis of phagocytosis. The assay detects and quantitates the ingestion of opsonised fluorescent (FITC-labelled) *E. coli*, allowing for the calculation of both the percentage of phagocytically active neutrophils and the extent of that activity (amount of bacteria per cell).

The Phagotest protocol was carried out with adaptations to the manufacturer's instructions. Briefly, 100 µL of heparinized whole blood (per test) was cooled on ice for 10 min prior to the addition of either 20 µL ($2 \times 10^7 - 4 \times 10^7$), 5 µL ($5 \times 10^6 - 1 \times 10^7$) or 2 µL ($2 \times 10^6 - 4 \times 10^6$) of the supplied and pre-cooled *E. coli* preparation (Reagent B). The control samples remained on ice to prevent phagocytosis, while test samples were incubated in a 37°C water bath for 10 min. Samples were immediately placed on ice to prevent further phagocytosis. 100 µL of quenching solution (Reagent C) was added to each sample to eliminate fluorescence from *E. coli* adhering to external cell surfaces (fluorescence from internalised *E. coli* remains unaltered). Each sample was washed twice with 3 mL of the wash solution (Reagent A) and centrifuged at 250 *g* at 4°C for 5 min. The resulting cell pellet was resuspended in 200 µL of pre-warmed (RT) lysing solution (1 x Reagent D) and incubated for 20 min in the dark at RT. Following lysis, the samples were centrifuged for 5 min at 250 *g* and 4°C and the wash process repeated prior to resuspension of the pellet in 200 µL DNA stain (Reagent E).

Samples remained on ice until data acquisition (minimum of 10 min). Data were acquired using a LSRII flow cytometer equipped with 488 nm argon-ion laser (BD Bioscience, UK) and BD Diva software within 120 min of protocol completion. The fluorescence from the DNA stain (detected at 488 nm - within PE labelled channel) was used to set an initial gate to distinguish between bacterial aggregates and leukocytes (Figure 2.8A). 10000 events per data file were collected within this gate.

Analysis was carried out using the Diva software (BD Bioscience). An initial analysis gate was set on the population representing neutrophils (Figure 2.8B) as defined by FSC vs SSC. The FITC fluorescence of this gated population was analysed to calculate the percentage of cells that had performed phagocytosis (percentage of cells within the FITC positive gate in Figure 2.8C) and the amount of ingested bacteria (where mean fluorescence correlated to bacteria per leukocyte). For this purpose the negative controls were used to set the background level of FITC fluorescence shown as 'FITC negative' in Figure 2.8C. Any fluorescence above this marker was considered 'FITC positive' and indicated phagocytically active cells.

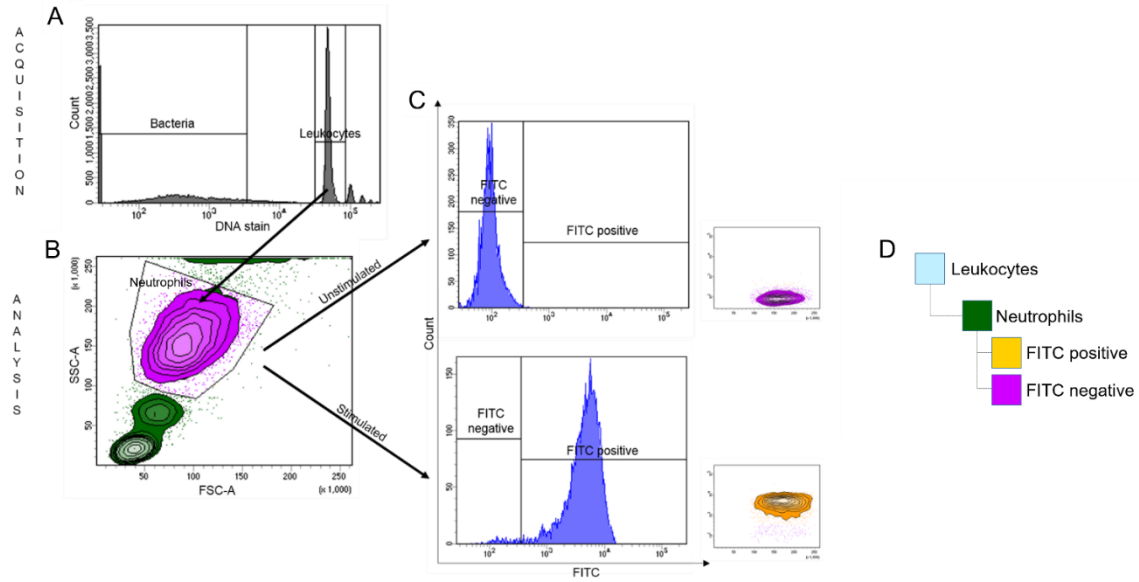


Figure 2.8: Gating strategy for FACs acquisition and downstream analysis of neutrophil phagocytosis. (A) DNA staining was used to distinguish between bacterial cells and Leukocytes. 10000 events were collected within the leukocyte gate. (B) The neutrophil gate was set to distinguish neutrophil populations of interest during analysis. (C) FITC histograms were gated to identify FITC positive and FITC negative cells within in the neutrophil population. Unstimulated cells remained within the FITC negative region while stimulated cells shifted into the FITC positive region. The FITC shift can also been seen in FITC vs SSC contour plots. (D) Population hierarchy and colour key for contour plots.

2.7.4. Quantifying respiratory burst of neutrophils

Phagoburst (Glycotope Biotechnology) allows the quantitative analysis of neutrophil oxidative burst. The assay utilizes opsonised *E. coli*, phorbol 12-myristate 13-acetate (PMA) and fMLP as phagocytosis stimulants with dihydrochodamine (DHR) 123 as the fluorogenic substrate. The production of reactive oxidants following phagocytosis, oxidises DHR 123 to R 123 (rhodamine 123), the fluorescence of the latter can be measured to determine the percentage of cells which have produced ROS and their enzymatic activity (mean fluorescence intensity correlates to amount of converted DHR 123).

The Phagoburst assay was carried out with adaptations to the manufacturer's instructions. Heparinised whole blood (100 µL per test) was cooled on ice for 10 mins prior to the addition of either the wash solution (negative control), fMLP (weak stimulant), PMA (strong stimulant) or the supplied pre-cooled *E. coli* (Table 2.5). The samples were then mixed and incubated for 10 min in a 37°C water bath. Following incubation, 20 µL of the DHR 123 substrate (Reagent E) was added to each sample, the sample vortexed and incubated for a further 10 min in a 37°C water bath. Red blood cells were then lysed through the addition of 2 mL pre-warmed (RT) lysing solution (1 x Reagent F), vortexing and incubation at RT for 20 min with protection from light. Lysed blood was centrifuged for 5 min at 250 *g* and 4°C and the supernatant discarded. Resulting pellets were washed with 3 mL of the wash solution (1 x Reagent A) and centrifuged again for 5 min at 250 *g* and 4°C. The final pellet was re-suspended in 200 µL of DNA staining solution (Reagent G), the tubes mixed and incubated for a minimum of 10 min on ice prior to acquisition.

Data were acquired using an LSRII flow cytometer (equipped with 488 nm argon-ion laser) (BD Bioscience) and BD Diva software within 30 mins of final incubation. The fluorescence from the DNA stain (detected at 488 nm - within the PE fluorescence channel) was used to set an initial gate to distinguish between bacterial aggregates and leukocytes (Figure 2.9A). 10000 events per data file were collected within this gate.

Analysis was carried out using BD Diva software. An initial analysis gate was set around the population of cells representing neutrophils (Figure 2.9B) using a FSC vs SSC contour plot. FITC histograms of this gated population were then used to calculate the percentage of cells which had undergone respiratory burst and their amount of ROS generated (where mean fluorescence correlated to the amount of R 123 product generated). For this purpose the negative controls were utilised to identify and gate the population considered 'FITC negative' (Figure 2.9C). Anything above this marker was considered 'FITC positive' and indicated cells which had undergone respiratory burst.

Table 2.5: Concentration of stimulants used in Phagoburst assay to initiate respiratory burst in neutrophils. PMA; Phorbol 12-myristate 13-acetate, fMLP; N-formylmethionine-leucyl-phenylalanine.

Test	**Reagent**	**Volume**	**Final concentration**
Control	Wash buffer Reagent A	20 µL	N/A
Test 1	*E. coli* Reagent B	20 µL	$2 \times 10^7 – 4 \times 10^7$
Test 2	*E. coli* Reagent B	5 µL	$5 \times 10^6 – 1 \times 10^7$
Test 3	*E. coli* Reagent B	2 µL	$2 \times 10^6 – 4 \times 10^6$
Weak stimulant	fMLP Reagent C	20 µL	0.834 µM
Strong stimulant	PMA Reagent D	20 µL	1.35 µM

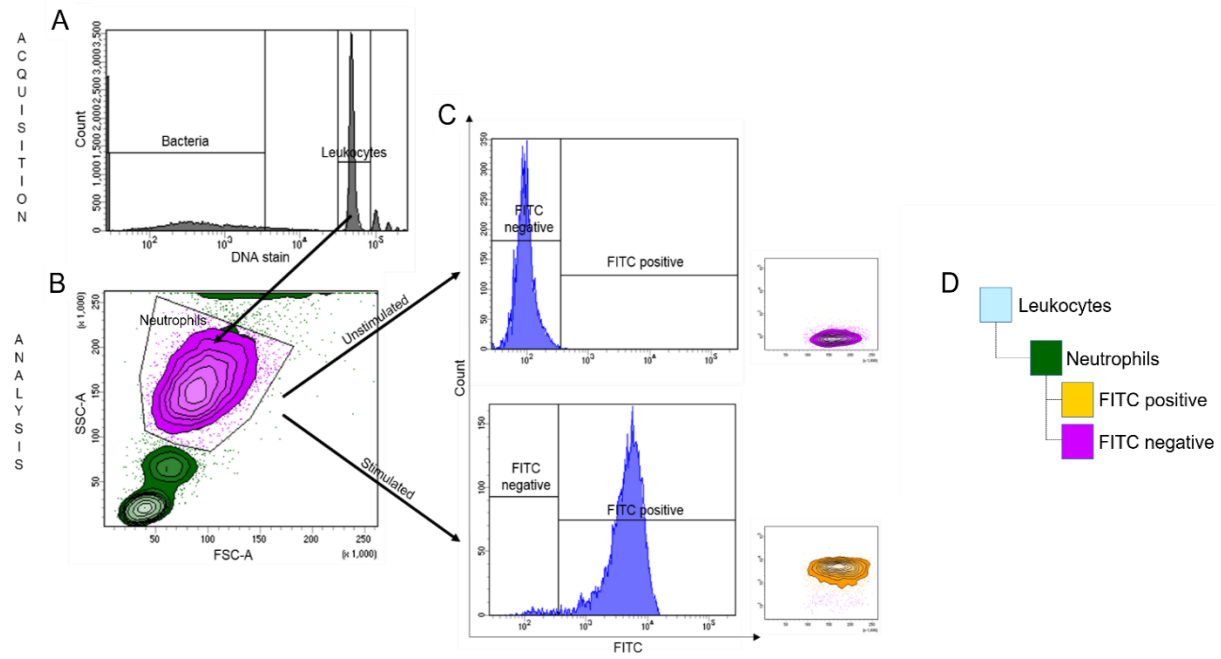


Figure 2.9: Gating strategy for FACs acquisition and downstream analysis of neutrophil respiratory burst. (A) DNA staining was used to distinguish between bacterial cells and leukocytes. 10000 events were collected within the leukocyte gate. (B) A gate was set to identify neutrophil populations of interest during analysis. (C) FITC histograms were gated to identify FITC positive and FITC negative cells within the neutrophil population. Unstimulated cells show cells within the FITC negative region while stimulated cells shift into the FITC positive region. (D) Population hierarchy and colour key for contour plots.

2.7.5. Analysis of inflammatory mediators

The inflammatory profile of clinical samples (saliva, GCF and plasma) was investigated using the multiplex LEGENDplex™ Multi-Analyte Flow Assay kit (Biolegend). The custom panel of human inflammatory mediators and markers included IL-8, IL-1β, IL-6, MCP-1, MIP-1α, TNF-α, IL-17A, IL-17F and IL-23. The LEGENplex™ system allows detection and quantitation of multiple analytes in a single sample through use of beads conjugated with antibodies raised to analytes of interest. Sample exposure to capture beads is followed by the addition of biotinylated detection antibodies which can then be detected using the fluorescence generated by the addition and binding of Streptavidin-phycoerythrin (Strep-PE), where the fluorescent signal intensity is proportional to the amount of bound analyte. The assay utilises two sets of beads with unique sizes and analyte specific APC (allophycocyanin) fluorescence to allow the distinction between different analytes.

Prior to data acquisition, parameters were set using the provided set-up beads. FSC and SSC parameters were adjusted to distinguish between the two bead populations which were of different sizes (Figure 2.10) and the setting for APC and FITC adjusted until the bead signals were visible within the expected range (Figure 2.10B). In addition, PMT (photomultiplier tube) voltage of the classification channel APC reporter channel PE and the FITC channel were adjusted for ideal separation of bead populations. The APC channel enables distinguishing between beads associated with different analytes. These settings were then applied for data acquisition.

Plasma, saliva and eluted GFC samples were all diluted 2-fold in assay buffer prior to analysis and the assay carried out according to the manufacturer's assay instructions for analysis of plasma samples in a V-bottom 96 well plate (provided, Biolegend). Briefly, 25 µL of standards were mixed with 25 µL of matrix buffer while diluted samples were mixed with assay buffer. Solutions were then mixed with an equal volume of capture beads prior to incubation for 2 h at RT, protected from light with shaking at 800 rotations per min (rpm) (plate shaker). Following incubation, the plate was centrifuged at 250 *g*, RT for 5 min in a swing out rotor equipped with plate adaptor. The supernatant was discarded, and wash step carried out with 200 µL wash buffer, followed by shaking at 800 rpm, RT for 1 min and centrifugation at 250 *g*, RT for 5 min. After removal of supernatant 25 µL of the detection antibody was added and the samples incubated for 1 h at RT, protected from light with shaking at 800 rpm. Immediately after incubation 25 µL Strep-PE was added and samples incubated for a further 30 min at RT, protected from light with shaking at 800 rpm. Following a final wash, resulting bead pellets were re-

suspended in 150 µL wash buffer for analysis. All standards and samples were run in duplicate.

Data were acquired using a LSRII flow cytometer equipped with 488 nm argon-ion laser (BD Bioscience) and BD Diva software. 3000 events were collected per well using a high throughput 96 well plate autosampler (Figure 2.10). Analysis was carried out using the LEGENDplex™ Data Analysis Software. Initial gates were set to differentiate between the two bead populations, followed by assignment of the multiple APC intensities to their appropriate analyte (Figure 2.10C and D). The APC fluorescence of the standards were then used to generate a standard curve for each analyte, which was used to calculate analyte concentrations in the samples.

2.7.6. Quantification of MMP8, MMP9 and TIMP1 in saliva, plasma and GCF

Saliva, plasma and GCF samples were analysed for MMP8, MMP9 and TIMP1 concentrations using Quantikine® ELISA kits (R&D systems). Saliva and plasma assays were performed following the recommended manufacturer's instructions and dilutions, while GCF was analysed undiluted. Briefly, assay diluent (100 µL for MMP9 and TIMP1, 150 µL for MMP8) was added to the wells of the supplied capture antibody pre-coated plate. Samples and standards (100 µL for MMP9 and TIMP1, 50 µL for MMP8) were added to the appropriate wells prior to 2 h incubation at RT on a horizontal orbital microplate shaker at 500 ± 50 rpm. Wells were aspirated, and washed by addition of 400 µL wash buffer followed by complete removal of wash buffer. The wash was repeated a total of 4 times and the plate blotted to ensure complete removal of liquid. 200 µL of conjugate was then added to each well, the plates incubated for 1 h (MMP9, TIMP1) or 2 h (MMP8) at RT with shaking followed by a wash step. TMB substrate solution (200 µL) was then added to each well and the plates incubated protected from light, at RT, for 30 min. A stop solution was added (50 µL) to prevent further colour development and the optical density read within 30 min using a plate reader (Varioskan Flash, Thermo scientific) set to measure photometric absorbance at a wavelength of 450 nm. Sample absorbance readings were converted to analyte concentrations (pg/mL) using the curve of the line equation generated from the standard curve produced for each plate.

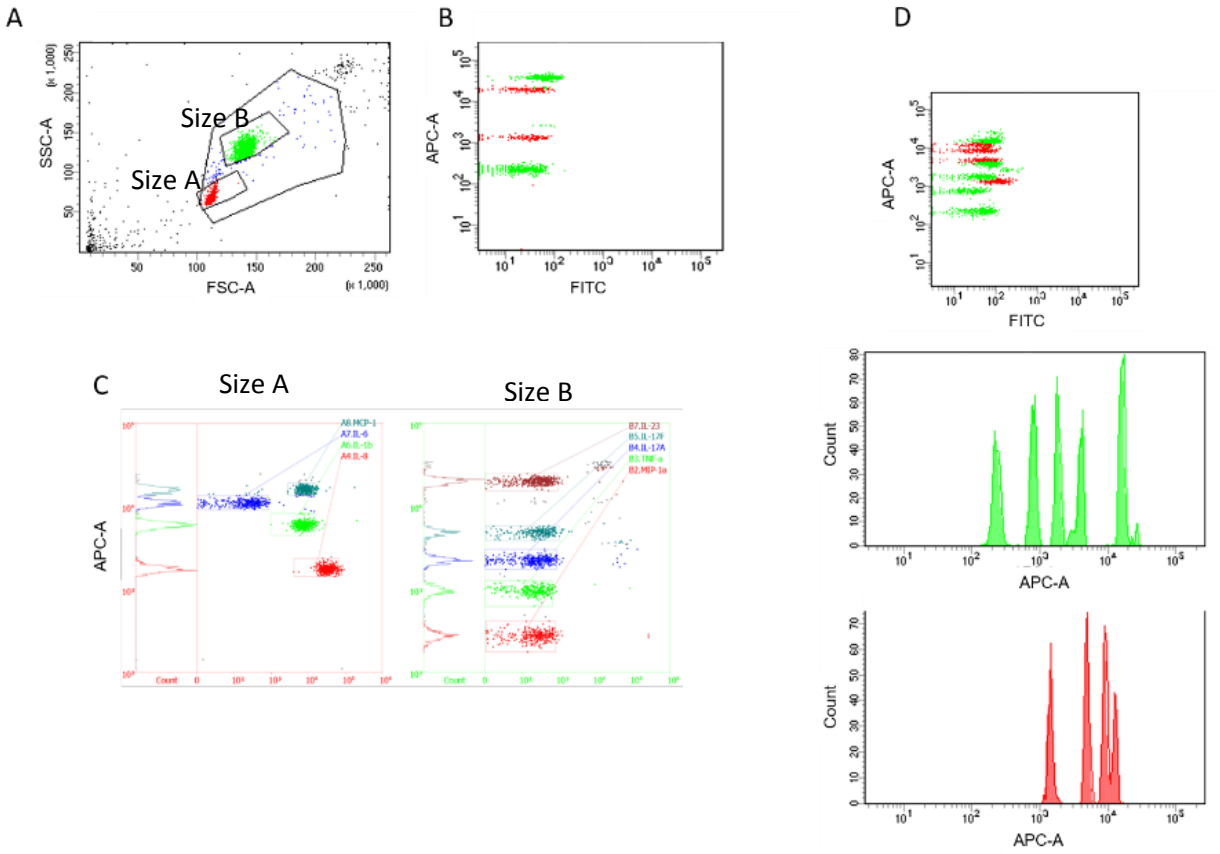


Figure 2.10: Gating strategy for LEGENDplex™ bead array. (A) Gates around the two bead populations were generated using set-up beads. (B) Dot plot indicating ranges of FITC and APC for set-up beads. (C) Assignment of APC intensities to analytes for generation of standard curves during experiments. (D) Example dotplot (top) of FITC intensities in test samples and example histograms of samples for the two bead populations (middle and bottom).

2.8. Generation of Advanced Glycation End product modified Human Serum Albumin (HSA-AGE)

Human serum albumin (HSA) was modified as previously described (Liu *et al*., 2009). Briefly, a sterile solution of 1.75 g/L HSA (A5843, Sigma-Aldrich, UK) and 0.1M D-glucose (G8769, Sigma-Aldrich, UK) in PBS was made and incubated at 37 ºC for 8 weeks. Every 2 weeks, the pH of the solution was checked and adjusted to 7.4 and aliquots of solution removed to track progress of AGE generation. Generation of a control HSA solution (HSA-C) followed the procedure as above but without the addition of D-glucose.

Following 8 weeks incubation, samples were dialysed for removal of excess glucose (Slide-A-Lyzer dialysis cassettes, molecular weight cut off 2 kDa, ThermoFisher Scientific). Solutions were dialysed against sterile PBS for 5 days at RT with stirring. PBS was changed daily under sterile conditions. Resulting solutions were aliquoted and stored at -80ºC for later use.

Presence of AGE was confirmed through fluorescence reading (Munch *et al*, 1997). Protein concentrations were determined using the Pierce™ Coomassie (Bradford) Protein Assay Kit as described previously (2.3) and fluorescence of 1 mg/mL total protein at Excitation/Emission 360/430 nm was determined (Varioskan Flash, Thermo scientific).

Solution endotoxin levels were also tested using the *Limulus* Amebocyte Lysate (LAL) chromogenic Endotoxin quantitation kit (Pierce™, ThermoFisher Scientific, UK) as per manufacturer's instructions. Briefly, a sterile endotoxin free 96 well plate (Corning® Costar®) was heated to 37ºC for 10 min, following which 50 µL of samples or standards were added to wells while the plate remained at 37ºC. The plate was covered and incubated for a further 5 min, prior to addition of 50 µL LAL reagent. Following further covered incubation for 10 min, 100 µL of substrate solution was added to each well, the plate vortexed and incubated at 37ºC for 6 min. The reaction was stopped by addition of 50 µL of 25% acetic acid to each well and the photometric absorbance at 405 nm measured (Varioskan Flash, Thermo scientific). Endotoxin levels were calculated from the standard curve and samples were considered endotoxin free as defined by the manufacturer (< 0.1 EU/mL).

2.9. TIGK viability in the presence of HSA-AGE

TIGK cell viability was determined in the presence of HSA-AGE using the cell proliferation reagent WST-1 (Roche) as per manufacturer's instructions. The WST-1 tetrazolium salt is cleaved to soluble formazan through the glycolytic production of NADPH (nicotinamide adenine dinucleotide phosphate) in viable cells. The conversion

initiates a colour change which can be measured photometrically. Briefly, TIGK cells were seeded in 96 well plates to a density of 1 x 10^4 as previously described (section 2.2). Following a 48 h incubation at 37°C, 5% CO_2 medium was removed and fresh medium supplemented with HSA-AGE or HSA-C was added to the wells. Cells were then incubated for a further 8, 24 or 36 h. At each time point WST-1 was added to the wells at a 1:10 dilution, the plates incubated at 37°C, 5% CO_2 for 1 h and the photometric absorbance at 420 nm read using a microplate reader (Varioskan Flash, Thermo scientific). For the 0 h time point, analysis was carried out immediately following the medium change.

2.10. Characterisation of RAGE expression on TIGK cells

2.10.1. Cell culture and exposure to AGE

TIGK cells were seeded in 6 well plates at a density of 3 x 10^5 or in 24 well plates at a density of 1 x 10^5 and cell growth was maintained for 48 h until cells reached approximately 80% confluency (section 2.2). Medium was replaced supplemented with HSA-AGE or HSA-C. Cells were incubated at 37°C, in 5% CO_2 and harvested at 0 (immediately) 8, 24 and 36 h.

For Western blot and ELISA analysis, cell lysates and supernatants were generated and immediately analysed as previously described (section 2.3) or frozen at -80ºC prior to analysis. For RNA extraction or for assessment of gene expression using quantitative real time polymerase chain reaction (qRT-PCR), cells were detached using trypsin (section 2.2) and RNA extracted as described below (section 2.11).

2.11. Real time PCR

2.11.1. RNA Extraction and cDNA synthesis

Total RNA was isolated from detached cells using the RNeasy kit (Qiagen, UK) as per manufacturer's instructions. Briefly, 600 µL buffer RLT was added to the harvested cell pellets in addition to 600 µL of 70% (v/v) ethanol. Samples were mixed and transferred to an RNeasy Mini spin column prior to centrifuging for 15 sec at 10,000 *g*. The flow-through was discarded and buffer RW1 (700 µL) was added to the column, the column centrifuged again for 15 sec at 10,000 *g* and the flow-through discarded. Columns were then washed by addition of 500 µL of buffer RPE to the columns. The column was centrifuged for 15 sec at 10,000 *g* and the flow-through discarded. Columns were washed again and a drying step was carried out (columns centrifuged for 2 min at 10,000 *g*). RNA was eluted by addition of 30 µL RNase-free water to the membrane followed by centrifuging for 1 min at 10,000 *g*.

Prior to cDNA synthesis, 1 µg RNA was treated with Amplification Grade DNAse I (Invitrogen) as per manufacturer's instructions. Briefly, 1 µg RNA was incubated for 15 min at RT with 1 µL 10X DNAse I reaction buffer and 1 U/µL DNase I. Following incubation, DNase was inactivated through addition of 1 µL of 25 mM EDTA and heating for 10 min at 65ºC. The resulting RNA was used immediately for cDNA synthesis or stored at -20ºC for downstream analysis.

Reversed transcribed cDNA was synthesised using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems) as per manufacturer's instructions. Briefly, 1 µg of DNase treated RNA was mixed with 2 x RT buffer, 20 x RT enzyme mix and a sufficient volume of RNase free water for a final reaction volume of 20 µl. Samples were then loaded into a thermocycler with conditions outlined in Table 2.6. Resulting cDNA was stored at -20ºC for downstream analysis.

2.11.2. Analysis of RNA expression in cells

Following cDNA synthesis, gene expression was analysed using TaqMan Gene Expression assays (Applied Bioscience, UK) as per manufacturer's instructions. Briefly, 1 µL synthesised cDNA was mixed with 2x TaqMan Gene Expression Master Mix, 20 x TaqMan Gene expression Assay (Table 2.7) and RNase-free water to a final volume of 20 µL in a 96 well plate (V-bottom LightCycler® 96 well plates, Roche). Plates were processed in a LightCycler (LightCycler 480, Roche) using a software pre-loaded protocol (Mono Color Hydrolysis Probe – UPL probe 96-II). The LightCycler 480 software was also used to automatically set thresholds for each plate and calculate C_T values for each sample.

Gene expression was determined as described by Schmittgen and Livak (2008). Technical replicates of 3 for each cDNA sample were averaged for use with the equations below. Final results were obtained by averaging biological replicate results obtained from four individual experiments.

Absolute expression: 2^{-C_T}

Expression relative to internal control: $2^{-\Delta C_T}$

Where $\Delta C_T = C_T$ gene of interest – C_T internal control

Expression relative to control samples and normalised to internal control: $2^{-\Delta\Delta C_T}$

Where $\Delta\Delta C_T = (C_T$ gene of interest - C_T internal control) test sample – (C_T gene of interest - C_T internal control) control sample

Table 2.6: High capacity RNA to cDNA thermocycler conditions.

	Step 1	Step 2	Step 3
Temperature (ºC)	37	95	4
Time	60 min	5 min	Final hold

Table 2.7: Assay identifications for genes analysed using TaqMan based quantitative real time polymerase chain reaction. *AGER*: advanced glycation end product receptor gene, *HPRT1*: hypoxyanthine phosphoribosyltransferase gene, *ACTB*: β-actin.

Gene	**Assay ID**
AGER	Hs00542584_g1
HPRT1	Hs02800695_m1
ACTB	Hs01060665_g1

2.12. Generation of five species biofilm model

2.12.1. Solutions, growth media and buffers

All solutions, media and buffers were made using Milli-Q water, under sterile conditions, using aseptic techniques. Sterilisation was accomplished through standard autoclaving procedures (121 °C for 15 min) unless otherwise stated and resulting solutions stored at 4°C until required.

All solid growth media were made as described in Table 2.8. Solutions were sterilised through autoclaving (unless otherwise stated), allowed to cool to approximately 50°C, blood/antibodies added (if required) and distributed into 5 mL petri dishes/plates. Antibiotics were sterilised by filtering using 0.2 µm-pore-size syringe filters (Sigma-Aldrich, UK) prior to addition to media. Once set, plates were stored at 4°C and used within 2 weeks.

Sterilized saliva was generated as previously described (Palmer *et al*, 2001; Sanchez *et al*, 2011). Briefly, stimulated saliva (stimulated by chewing on sterile parafilm) was collected 1.5 hours after drinking, eating, or brushing teeth. Saliva was collected over several days, held on ice during collection and processing and stored at -20°C. Pooled saliva was treated with 2.5 mM DL-Dithiothreitol, stirred for 10 min and clarified by centrifuging at 27, 000 *g* for 20 min, 4°C. The resulting supernatant was diluted 1:1 in sterile PBS, filtered through 0.22 µm-pore-size low-protein binding filter (Corning Inc., UK), aliquoted and stored at -20°C for later use. Sterility was ensured though plating onto CBA plates and incubation both anaerobically and in 10 % CO_2 at 37°C (Table 2.8).

Table 2.8: Composition, selectivity and incubation conditions for growth on various solid growth media

Media	Composition	Selective for	Incubation conditions
Columbia Blood agar (CBA)	39 g/L CBA base (Oxoid), 5% (v/v) horse blood (Oxoid). Standard autoclaving sterilisation.	Facultative anaerobes and aerobes	10 % CO_2, 37 °C 2-5 days
		Facultative anaerobes	10 % H_2, 10% CO_2, 80% N_2, 37 °C 5-7 days
Columbia Blood agar (CBA) + Vancomycin	39 g/L CBA base (Oxoid), 5% (v/v) horse blood (Oxoid), 7.5 mg/L vancomycin. Standard autoclaving sterilisation.	Gram negative anaerobes	10 % H_2, 10% CO_2, 80% N_2, 37 °C 5-7 days
Cadmium sulphate fluoride acridine trypticase agar (CFAT)	30 g/L trypticase soy broth (Sigma-Aldrich), 5 g/L glucose, 15 g/L agar, 13 mg/L cadmium sulphate, 80 mg/L sodium fluoride, 1.2 mg/L neutral acriflavin, 2.5 mg/L potassium tellurite, 0.25 mg/L basic fuchsine, 5% (v/v) horse blood, pH 7.3. Standard autoclaving sterilisation.	*Actinomyces*	10 % H_2, 10% CO_2, 80% N_2, 37 °C 5-7 days
Rogosa agar	82 g/L Rogosa agar (Oxoid), dissolve by boiling before addition of 1.32 ml/L glacial acetic acid. Sterilised by heating to 90-100 °C with stirring until fully dissolved.	Lactobacilli	10 % CO_2, 37 °C 2-5 days
Mitis Salivarius agar	90 g/L Mitis Salivarius Agar (Sigma-Aldrich, Uk), 1% (v/v) potassium tellurite solution (Sigma-Aldrich, UK) added post sterilisation. Standard autoclaving sterilisation.	Streptococci	10 % CO_2, 37 °C 2-5 days

2.12.2. Growth of five species periodontitis biofilm model for determination of HSA-AGE impact on biofilm composition

5 species biofilms, modelling the composition of biofilms associated with periodontitis (Figure 2.11), were grown on hydroxyapatite (HA) coated pegs in a Calgary device (MBEC Assays, Innovotech) following a sequential inoculation method of *S. salivarius* (clinical isolate ULCP97), *A. naeslundii* (clinical isolate), *P. intermedia* (OMZ 248), *F. nucleatum* (ATCC 10953) and *P. gingivalis* (W83) as established in-house by Naginyté (2017, unpublished). All biofilm growth was carried out in an anaerobic cabinet at 37°C (Don Whitley Scientific) with all growth media, solutions and buffers being pre-reduced by introduction to the anaerobic cabinet a minimum of 24 hours prior to use. All facultative aerobic bacteria were grown at 37°C, in air with 10% CO_2.

Prior to initial inoculation of HA-pegs, bacteria for biofilm growth were revived from glycerol stocks (stored at -80 °C) onto solid growth media (Table 2.9 and Figure 2.11). All five species were then subcultured into brain heart infusion (BHI) broth and grown under anaerobic conditions. Biofilms were grown as outlined in Figure 2.11. HA-pegs were pre-conditioned with 200 µL/peg of sterilised saliva by incubation for a minimum of 5 h at 37 °C with shaking at 65 rmp.

Early coloniser inoculation was accomplished by addition of 1.58 µL/mL (OD_{600} 0.2) *S. salivarius* (ULCP97) and 4.375 µL/mL (OD_{600} 0.2) *A. naeslundii* (clinical isolate) to serum medium. This inoculated serum medium was added to a 96 well plate (175 µL/well); the plastic lid containing the HA coated pegs was replaced and the plate incubated for a minimum of 12 h under anaerobic conditions. Following the 12 h incubation, the HA-peg lid was transferred to a 96 well plate containing pre-reduced, uninoculated serum medium and incubated overnight under anaerobic conditions.

HA-pegs were then inoculated with late colonisers by the addition of 12.5 µL/mL (OD_{600} 0.2) *F. nucleatum* (ATCC 10953), 25 µL/mL (OD_{600} 0.2) *P. intermedia* (OMZ 248) and 25 µL/mL (OD_{600} 0.2) *P. gingivalis* (W83) to pre-reduced serum medium, the inoculated serum medium added to a 96 well plate (175 µL/well) and the lid transferred to the fully inoculated plate. The plates were incubated under anaerobic conditions overnight, following which the late coloniser inoculation was repeated as described above. Subsequent to the second late coloniser inoculation, the lid was transferred to a 96 well plate containing fresh, sterile pre-reduced serum medium every day for the duration of the experiment. The impact of HSA-AGE or HSA-C on biofilms was tested by the introduction of serum medium supplemented with HSA-AGE or HSA-C at day 7 until day 10 after which the medium was replaced daily (without HSA-AGE or HSA-C) for a recovery period of 3 days (Figure 2.11).

A total of 4-6 biofilms for each condition were harvested at day 10 and again at day 14. Each HA-peg was removed from the plastic lid using sterile pliers to snap the peg at the base, washed to remove loosely bound bacteria by dipping the entire peg 3 times consecutively into PBS. Biofilms were harvested in the anaerobic cabinet, by scraping the biofilm into 500 µL PBS, followed by vortex mixing for 1 min. 100 µL of the resulting suspension was serially diluted in PBS to a final dilution of $1:10^6$ with various dilutions plated on CBA vancomycin plates for anaerobic growth and CBA plates for aerobic growth. Anaerobic plates were incubated for a minimum of 7 days, while aerobic plates were incubated for a minimum of 3 days. Following incubation, colonies on plates from each biofilm were counted.

Table 2.9: Species, strains and growth conditions of the five species used in the generation of a biofilm modelling periodontitis. CBA; Columbia blood agar.

Bacteria	**Strain details**	**Growth conditions**	**Solid growth medium**
Streptococcus salivarius	ULCP97 Clinical isolate	Aerobic (10 % CO_2) and anaerobic 37°C	CBA
Actinomyces naeslundii	Clinical isolate	Aerobic (10 % CO_2) and anaerobic 37°C	CBA
Fusobacterium nucleatum	ATCC 10953	Anaerobic 37°C	CBA + Vancomycin
Prevotella intermedia	OMZ 248	Anaerobic 37°C	CBA + Vancomycin
Porphyromonas gingivalis	W83	Anaerobic 37°C	CBA + Vancomycin

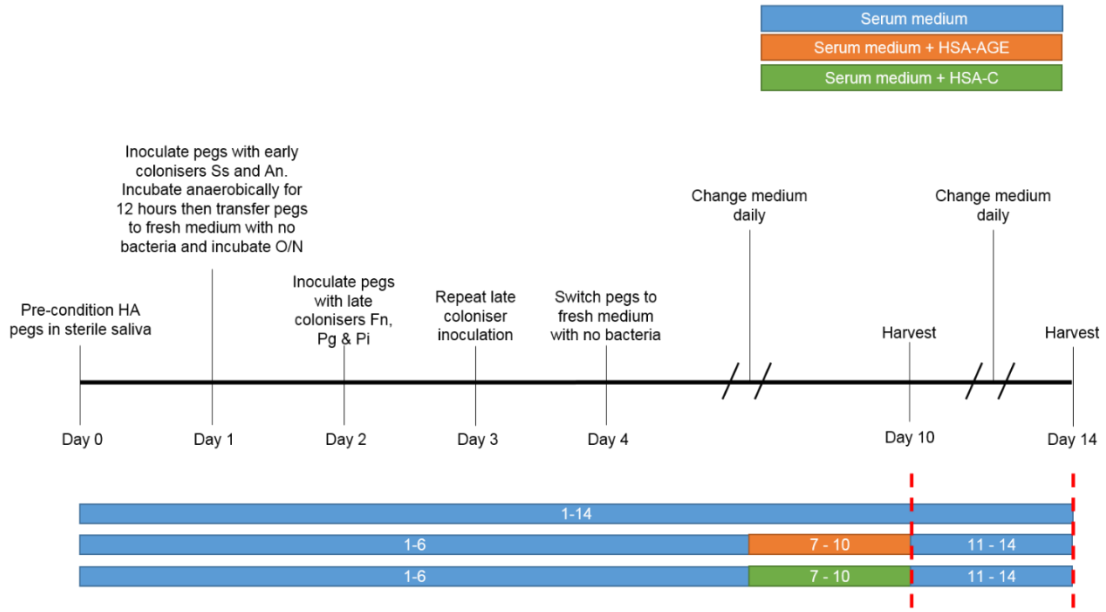


Figure 2.11*:* **Timeline for growth and harvesting of the five species periodontitis model**. Ss; *S. salivarius*, An; *A. naeslundii*¸ Fn; *F. nucleatum*, Pi; *P. intermedia*, Pg; *P. gingivalis*, HSA-AGE; human serum albumin-advanced glycation end product, HSA-C; human serum albumin-control. Dotted red lines represent biofilm harvests.

2.13. Generation of complex inoculum biofilms for determining the consequences of HSA-AGE addition to colonisation and composition

2.13.1. Sample collection and preparation of inocula

Saliva, tongue scraping and supragingival plaque samples were collected from eight volunteers following ethical approval by University of Leeds Dental Research Ethics Committee (DREC: 020915/MN/175). Volunteers were asked to refrain from brushing their teeth eating or drinking on the day of collection until collection was complete. For saliva collection, volunteers were asked to produce a minimum of 5 mL parafilm wax stimulated saliva. Sterile tongue depressors were used to collect tongue scrapings, with volunteers asked to scrape all aspects of the tongue and place the tongue depressor directly into 6 mL ice-cold serum medium. Volunteers were also asked to use sterile toothpicks to scrape plaque from as close to the gum as possible from all tooth surfaces and place the toothpicks directly into 6 ml serum medium. All samples were held on ice until processing.

Salivary bacteria were pelleted by centrifuging pooled saliva samples at 27, 000 *g* for 20 min at 4°C. The resulting supernatant was discarded, and the bacterial pellet resuspended in serum medium. Tongue scrapings and supragingival plaque were harvested into serum medium by vigorous vortexing and the toothpicks/tongue depressors discarded. The resulting tongue scrapings, supragingival plaque and saliva were used as the inoculum for the production of biofilms for co-culture experiments (section 2.14) under conditions outlined in Table 2.10. For bacterial composition analysis, these pooled samples were separated into 3 and used as 3 individual inocula.

For analysis of inoculum composition, each suspension used as the inoculum was serially diluted (final dilution $1x10^6$) and several dilutions plated on solid selective media and incubated as indicated in Table 2.8. Following HA-peg inoculation, the remaining inoculum was propidium monoazide treated to select for DNA only from viable organisms prior to DNA extraction as described previously (section 2.5.1). Briefly, suspensions of bacteria were treated with 50 µM of propidium monoazide, incubated in the dark for 5 min with mixing followed by light exposure for 2 min. Following photo-induced cross linking, samples were centrifuged at 5000 *g* for 5 min. The resulting supernatants were used for DNA extraction (section 2.5.1).

2.13.2. Complex biofilm growth

Complex biofilms were grown on HA coated pegs on a Calgary device in the anaerobic cabinet at 37 °C. HA-pegs were first pre-conditioned as described previously (section 2.13) with sterile saliva supplemented with various concentrations of HSA-AGE/HSA-C to represent health and disease (Table 2.10). Following pre-conditioning HA-peg lids

were transferred to 96 well plates containing 175 µL/well complex inoculum and incubated for 24 h under anaerobic conditions. After 24 h lids were transferred to fresh serum medium supplemented as described in Table 2.10. HA-pegs were transferred to fresh medium every 2-3 days. At day 2, day 7 and day 14, 4-6 biofilms for each condition were harvested. Biofilms were harvested in the anaerobic cabinet, by scraping the HA-peg into 500 µL reduced transport fluid (RTF), followed by vortex mixing. Harvested biofilms from HA-pegs for each condition were pooled, and 100 µL of the resulting bacterial suspension was serially diluted in RTF to a final dilution of $1x10^6$. Various dilutions were plated on selective media and incubated as described in Table 2.8. The remainder of the harvested biofilm was propidium monoazide treated (section 2.13.2) and DNA harvested (section 2.5.1). DNA from biofilms harvested at day 2 and day 14 as well as inoculum DNA were used to generate NGS libraries and sequenced as described previously (section 2.5). Individual plates were set up for different harvest time points to minimise biofilm disturbance and minimise contamination during harvests.

2.14. Immune responses of TIGK cells in response to co-culture with complex biofilms with HSA-AGE co-stimulation

2.14.1. Cytokine profiles of TIGK cells following co-culture with complex biofilms and HSA-AGE

TIGK cells were seeded in 96 well plates, as described previously (section 2.2) to a density of $1x10^4$ cells/well. Cells were grown for 48 hours to approximately 80% confluency and culture medium replaced immediately prior to co-culture. Following complex inoculum biofilm growth, mature biofilms (at day 15) grown under various conditions (Table 2.10) were co-cultured with TIGK cells and co-stimulated with 100 µg/mL HSA-AGE or 100 µg/mL HSA-C. Biofilms were first washed in PBS (3 times) and introduced to the cells either by transferring the HA-peg containing lids directly into the 96 well plate, or by detaching the HA-pegs from the lid and placing them into the wells on top of the cells. Co-cultures were incubated for 8 hours in 10% CO_2 at 37 °C.

After 8 hours, culture medium was harvested. Media from the same conditions (8 biofilm/cell co-culture wells) were pooled and stored at -20°C until analysed for levels of cytokines IL-6, IL-8 and IL-1β via ELISA (section 2.4).

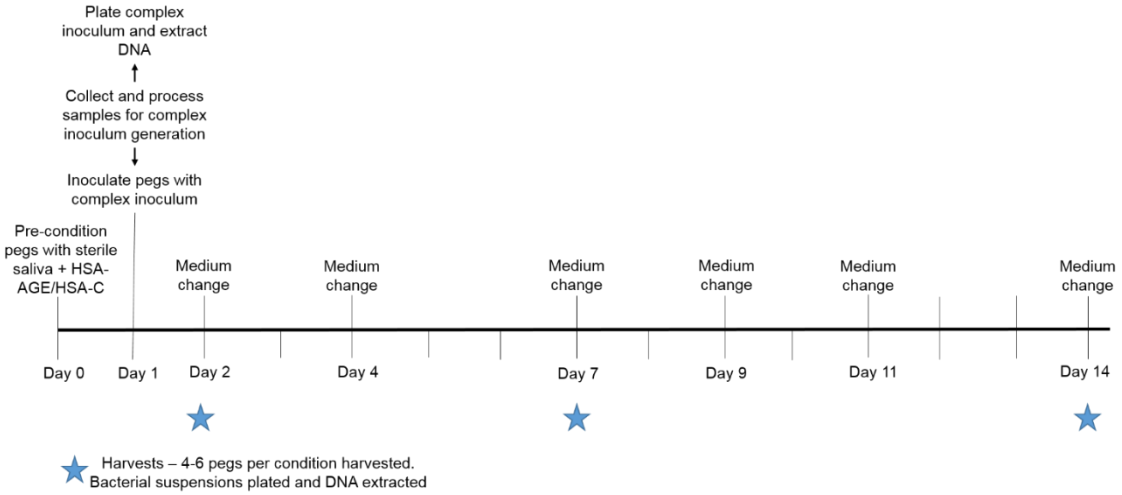


Figure 2.12: Timeline for growth and harvesting of the complex inoculum biofilms. HSA-AGE; human serum albumin-advanced glycation end product, HSA-C; human serum albumin-control.

Table 2.10: Hydroxyapatite peg pre-conditioning and complex inocula biofilm growth conditions for next generation sequencing and analysis of cytokine release following co-culture with TIGK cells. HSA-AGE; Human serum albumin-advanced glycation end product. HSA-C; human serum albumin-control. HA; hydroxyapatite.

HA peg pre-conditioning	**Growth medium**	**Modelling systemic health or disease**
Sterile saliva + 1 µg/mL HSA-AGE and 99 µg/mL HSA-C	Serum medium	Health pre-conditioning – medium control growth
	Serum medium + 1 µg/mL HSA-AGE and 99 µg/mL HSA-C	Health
	Serum medium + 100 µg/mL HSA-C	Health pre-conditioning – HSA control growth
Sterile saliva + 100 µg/ml HSA-AGE	Serum medium	Disease pre-conditioning – medium control growth
	Serum medium + 100 µg/mL HSA-AGE	Disease
	Serum medium + 100 µg/mL HSA-C	Disease pre-conditioning – HSA control growth
Sterile saliva + 100 µg/ml HSA-C	Serum medium	HSA control
	Serum medium + 1 µg/mL HSA-AGE and 99 µg/mL HSA-C	HSA control
	Serum medium + 100 µg/mL HSA-AGE	HSA control
	Serum medium + 100 µg/mL HSA-C	HSA control

2.14.2. Reactive oxygen species generation by TIGK cells following co-culture with complex biofilms and HSA-AGE

Saliva, tongue scrapings and supragingival plaque were collected from three volunteers (DREC: 020915/MN/175) and processed to generate a single complex inoculum for growth of complex biofilms modelling systemic health, systemic disease or a HSA-C control (Table 2.10) (section 2.13.1) and subsequently co-cultured with TIGK cells (section 2.14.1.).

The impact of co-culture on cellular generation of ROS was determined using the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Life technologies, ThermoFisher Scientific, UK). Upon entering the cells, intercellular esterase cleaves the acetate group to form an intermediate, which is converted to the fluorescent compound 2',7'-dichlorofluorescein (DCF) on exposure to ROS and can be detected at Ex/Em 485/535 nm.

Immediately prior to use, the H_2DCFDA was reconstituted in 100% (v/v) ethanol as per manufacturer's instructions. Prior to introduction of biofilms to cells, cells were washed with PBS, 10 µM of H_2DCFDA in culture media added to each well and the cells incubated at 37°C in 5% CO_2 for 30 min. Following the 30 min incubation, medium was aspirated and replaced by fresh medium or medium supplemented with 100 µg/mL HSA-AGE or HSA-C. Complex biofilms were then introduced to the cells and the co-cultures incubated for 7 hours at 37°C (section 2.14.1, Figure 2.13). ROS release was measured directly in the cell culture plates using a microplate reader (Varioskan Flash, Thermo scientific) set to measure fluorescence at Ex/Em 485/535. Prior to fluorescence readings, pegs that had been in direct contact with TIGK cells were removed and discarded.

In parallel to cellular ROS generation, cellular death was also measured by determining LDH release using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, UK). After the 7 h initial incubation, 10x lysis solution (CytoTox 96 assay kit) was added to the control wells to generate 100% LDH release (positive control) and the co-cultures incubated for a further 1 h. Following this, 100 µL medium was removed from each well, clarified by centrifuging at 17,000 *g* for 5 min at 4°C (to remove bacteria and cell debris) and the resulting supernatant immediately analysed for LDH release, as per manufacturer's instructions. Briefly, 50 µL sample was mixed with 50 µL of CytoTox 96 reagent in a 96 well plate (Corning® Costar®) and incubated at RT for 30 min before 50 µL stop solution was added. LDH release was measured using a microplate reader (Varioskan Flash, Thermo scientific) at photometric absorbance 490 nm. LDH release from each sample was calculated as a percentage of maximum LDH release.

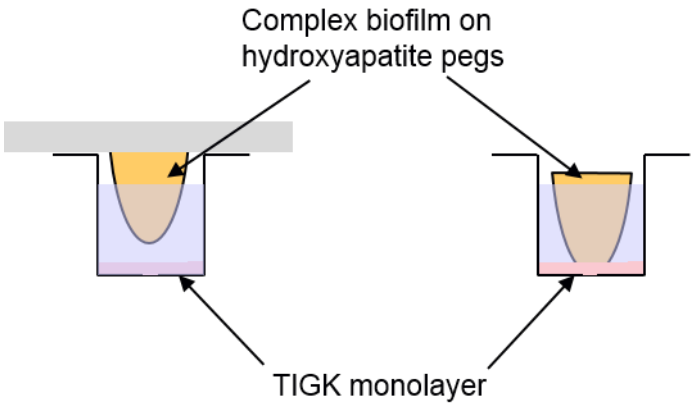


Figure 2.13: Schematic for biofilm-TIGK cell co-culture. TIGK cells were co-cultured with complex inoculum biofilms in the presence of HSA-AGE or HSA-C. Biofilms (3 biofilms for each condition) were either placed into wells while still attached to the lid to prevent direct contact (left) or removed from lid and placed directly on top of TIGK monolayer (right) for direct biofilm-cell contact.

Table 2.11: Hydroxyapatite peg pre-conditioning and complex inocula biofilm growth conditions for analysis of reactive oxygen species release and cell viability following co-cultures with TIGK cells. HSA-AGE; Human serum albumin-advanced glycation end product. HSA-C; human serum albumin-control. HA; hydroxyapatite.

HA peg pre-conditioning	**Growth medium**	**Modelling systemic health or disease**
Sterile saliva + 1 µg/mL HSA-AGE and 99 µg/mL HSA-C	Serum medium + 1 µg/mL HSA-AGE and 99 µg/mL HSA-C	Health
Sterile saliva + 100 µg/ml HSA-AGE	Serum medium + 100 µg/mL HSA-AGE	Disease
Sterile saliva + 100 µg/ml HSA-C	Serum medium + 100 µg/mL HSA-AGE	HSA control

3. Evaluation of telomerase immortalised gingival keratinocyte expression of RAGE for use as a model system to examine the potential of AGE-RAGE interactions in linking diabetes to periodontitis

3.1. Introduction

The presence of AGEs has been linked to the progression of diabetes and its complications. Indeed, measurement of blood haemoglobin A1c or glycated haemoglobin is used as an indication of diabetic control and glucose regulation (Wautier *et al*., 2017). As described previously (section 1.4.3), diabetes-associated hyperglycaemia can lead to the accumulation of AGEs and subsequent chronic inflammatory responses, which have been suggested to play an important role in the progression of diabetic complications (Hiroshima *et al*., 2018). Of these complications, increased periodontitis severity has been correlated to hyperglycaemia (Shlossman *et al*., 1990, Emrich *et al*., 1991, Taylor *et al*., 1998, Tsai *et al*., 2002). As described in more detail in section 1.4.3, this suggests the hyperglycaemic accumulation of AGE in diabetes could impact the progression of periodontitis.

Previous studies have shown that the increase in serum levels of AGEs from diabetic patients correlated with an increase in periodontitis-linked attachment loss as well as increased immunoreactivity of AGEs in gingival tissues (Takeda *et al*., 2006, Zizzi *et al*., 2013, Hiroshima *et al*., 2018). Furthermore, the receptor for AGE, RAGE, has been shown to be highly expressed in gingival tissue of patients with periodontitis and diabetic periodontitis patients. In particular, high expression was shown in gingival epithelial cells and circulating leukocytes. This contrasted with healthy gingiva, where RAGE expression was limited (Abbass *et al*., 2012).

RAGE, a 45-50 kDa molecule, is encoded by a gene found on locus 6p21.3, next to the major histocompatibility complex (MHC) class III protein family (Wautier *et al*., 2017, Ott *et al*., 2014). Although it is now understood that AGE associated downstream signalling cascades are mediated by a variety of cell surface receptors (including AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3), RAGE is the most well studied (Araki *et al*., 1995, Vlassara *et al*., 1995, Li *et al*., 1996, Ohgami *et al*., 2001a, Ohgami *et al*., 2001b, Ohgami *et al*., 2001c, Jono *et al*., 2002, Tamura *et al*., 2003). The multiple ligand receptor (pattern recognition receptor) belongs to the immunoglobulin superfamily and consists of a large extracellular domain (variable or V domain and two constant, C1 and C2, domains), a single transmembrane domain-spanning helix and a short cytoplasmic domain in its full length form (Ott *et al*., 2014, Wautier *et al*., 2017). To date 20 major splicing variants have been identified including full-length RAGE, N-truncated RAGE (lacking the ligand binding V-domain) and soluble RAGE (lacking the C-terminal domain).

In addition to the mRNA encoded soluble RAGE, which is secreted, proteolytic cleavage of full-length RAGE (by metalloproteinases ADAM10 and MMP9) also forms soluble RAGE (Yonekura *et al*., 2003, Hudson *et al*., 2008, Kalea *et al*., 2009). Soluble RAGE is considered a decoy receptor to prevent excessive RAGE activated immune responses (Ott *et al*., 2014, Wautier *et al*., 2017, Leung *et al*., 2016) .

As discussed in detail previously (section 1.4.3), interactions between AGE and RAGE are linked to generation of ROS and initiation of pro-inflammatory responses (Younessi and Yoonessi, 2011). The binding of AGE to RAGE is thought to induce the activation of downstream NFκB, MAPK and other signalling pathways (Figure 3.1) (Schmidt *et al*., 2000, Ishihara *et al*., 2003, Kokkola *et al*., 2005, Mallidis *et al*., 2007).

This study aimed to determine if TIGK cells were appropriate for use as a model system to examine the impact of AGE/RAGE interactions of oral epithelial cells. The objectives of the following experiments were to determine if TIGK cells expressed RAGE and whether expression was inducible or increased following addition of AGE to the cell culture media. Following characterisation of TIGK cell RAGE expression, we investigated cytokine release (IL-1β, IL-6 and IL-8) as a consequence of AGE/RAGE interactions.

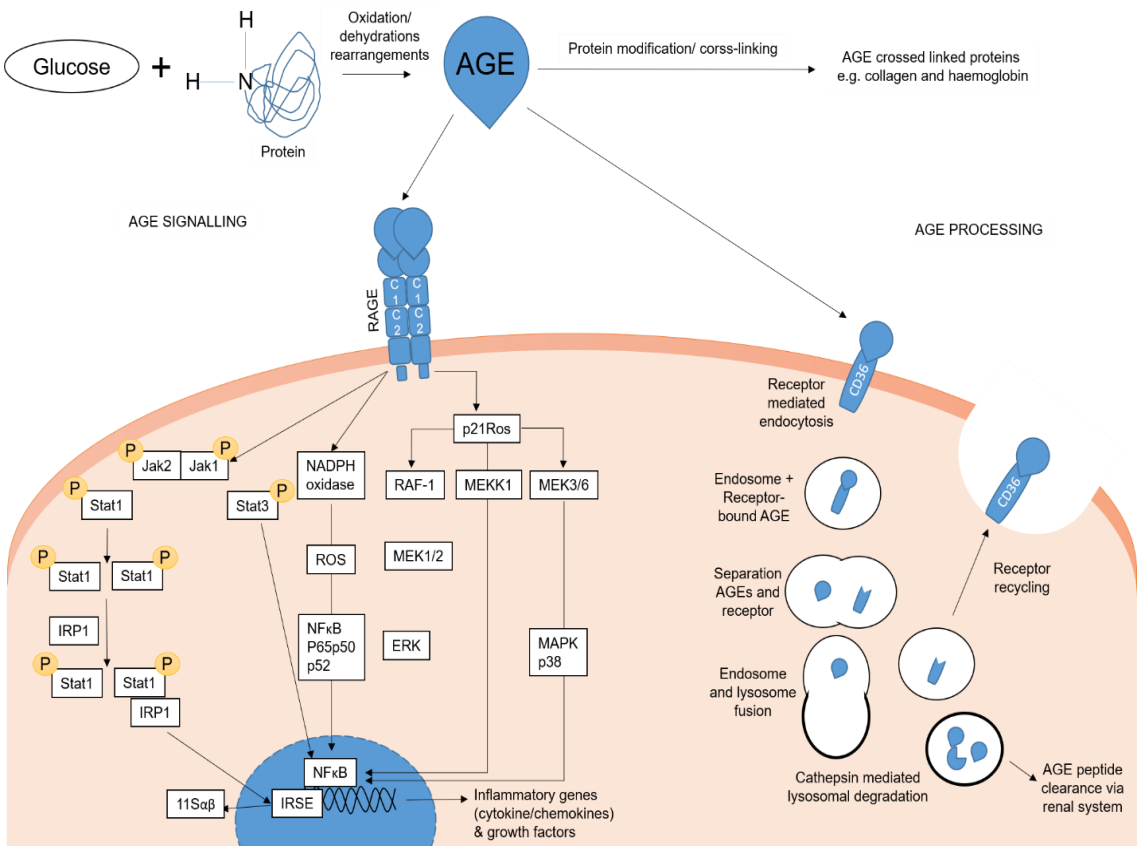


Figure 3.1: AGE related signalling and processing. The non-enzymatic modification of proteins following condensation of carbonyl groups of reducing sugars (such as glucose) and amino groups on proteins leads to the generation of an advance glycation end product (AGE). AGEs impact protein structure through cross linking or interact with a variety of receptors such as RAGE (receptor for advanced glycation end product). AGE/RAGE interactions initiate downstream signalling cascades leading to transcription of various inflammatory genes and growth factors. AGEs are processed and cleared following CD36 receptor mediated endocytosis (adapted from Ott *et al*. (2014)).

3.2. Results

3.2.1. Generation of AGE modified human serum albumin for characterisation of TIGK cell RAGE expression and AGE/RAGE interactions

Considering the increase in GCF volume in periodontitis patients compared with healthy and the associated increase in GCF albumin concentrations, human serum albumin (HSA) was deemed an appropriate choice for the generation of AGE modified HSA (HSA-AGE) for use in a periodontitis-diabetes model (Carneiro *et al*., 2014). HSA was incubated with or without glucose as previously described (section 2.8) to generate HSA-AGE and HSA-C (HSA-control) respectively.

As described previously (section 1.4.3) AGEs are fluorescent proteins and so fluorescence was measured (section 2.8) over the incubation period to track accumulation of AGE modified HSA (Figure 3.2). After an 8 week incubation period, HSA and glucose solutions had a higher fluorescence (Ex/Em 360/430 nm) in comparison with HSA solutions (11.44 nm ± 0.75 and 0.94 nm ± 0.43 respectively).

After 8 weeks incubations, dialysis was carried out for removal of excess glucose and samples stored at -80°C for later use (section 2.8). Prior to use, protein concentrations of dialysed samples were quantified (as described in section 2.3) and fluorescence measured to ensure preservation of AGE modified proteins following dialyses and thawing of frozen samples.

3.2.2. Viability of TIGK cells grown with HSA-AGE or HSA-C

TIGK cells were treated with varying concentrations of the HSA-AGE preparation in cell culture and any impact on cell viability was assessed using a WST-1 assay as described previously (section 2.9). Conversion of the WST-1 tetrazolium salt to formazan and the resulting colour change is measured photometrically, so that absorbance correlates directly with the number of metabolically active cells.

As indicated in Figure 3.3, the viability of TIGK cells appeared to be enhanced following incubation with all three concentrations of HSA-C compared with untreated cells and cells incubated with HSA-AGE. However, these differences were not significant (n=3, Two-way ANOVA, Tukey post-hoc, $p > 0.05$) and could be a consequence of cell proliferation. This indicates no markedly detrimental effects on cell viability over the time frame needed for further experiments with TIGK cells.

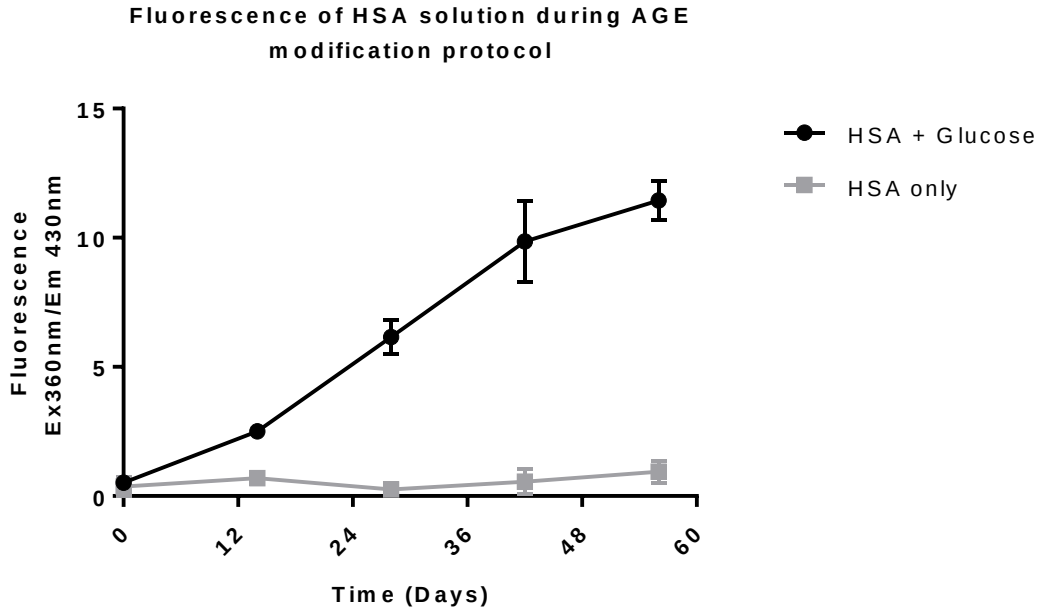


Figure 3.2: Fluorescence of HSA solutions during the incubation period for AGE modification. HSA was incubated with glucose for 8 weeks at 37ºC for the non-enzymatic modification of HSA with AGE. In parallel HSA was incubated under the same conditions without glucose for use as a control. Fluorescence (± standard deviation, SD) was measured as an indication of AGE modification throughout the 8 week incubation period. AGE; advanced glycation end products, HSA; human serum albumin.

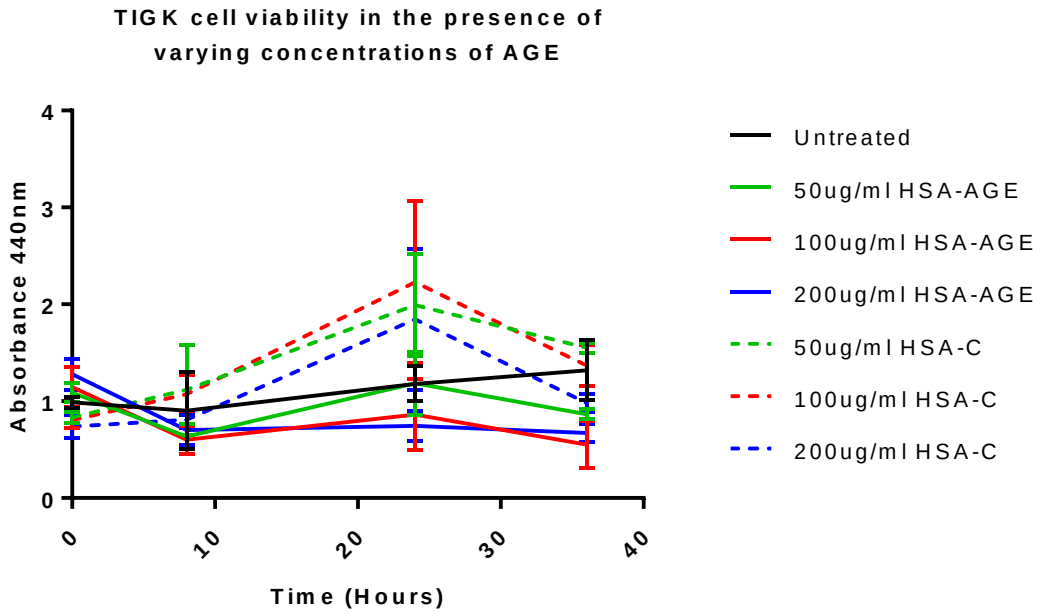


Figure 3.3: TIGK cell viability following exposure to HSA-AGE or HSA-C for 36 hours. TIGK cells were grown with varying concentrations of HSA-AGE or HSA-C for 0, 8, 24 and 36 hours prior to analysis of cell viability using a WST-1 assay. Conversion of WST-1 and the resulting colour change was measured as absorbance to indicate cell viability. Results are presented as means ± SD, n=3. HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control.

3.2.3. Characterisation of RAGE expression of TIGK cells

3.2.3.1. RAGE protein expression

Prior to analysis of RAGE expression by TIGK cells, anti-RAGE antibody (Abcam, MM0520-8D11) was tested against recombinant RAGE (rRAGE) (Abcam, ab63271). In addition, rRAGE was incubated at 37°C with 100 µg/mL HSA-AGE or HSA-C for 8 hours to determine if interactions between AGE and RAGE interfere with anti-RAGE antibody binding (Figure 3.4). Western blot protein analysis showed the detection of a band at approximately 45 kDa following probing of the membrane with an anti-RAGE antibody, and intensity increased with increasing amounts of loaded rRAGE. This correlates with the expected size of rRAGE which is stated to be 45 kDa. Additionally, no difference in size was observed in samples incubated with either HSA-AGE or HSA-C, indicating addition of receptor ligands does not impact size of band correlating to RAGE during protein detection using western blotting methods.

Following confirmation that HSA-AGE and HSA-C are non-toxic to TIGK cells and detection of rRAGE through western blotting, RAGE expression on TIGK cells was characterised. TIGK cells were grown with various concentrations of HSA-AGE for 8, 24 and 36 hours to determine if RAGE was constitutively expressed and if presence of AGE induced RAGE expression (as described previously in section 2.10). TIGK cells were treated with 200 µg/mL, 100 µg/mL or 50 µg/mL HSA-AGE or HSA-C prior to western blot protein analysis of both cell surface bound RAGE and secreted RAGE. Experiments were repeated 3 times and representative blots presented (Figure 3.5).

Western blot analysis of RAGE resulted in bands of approximately 52 - 55 kDa (Figure 3.5). While this was higher than the predicted molecular weight of RAGE (48 kDa), the previous detection of rRAGE with the anti-RAGE antibody suggests bands visualised were RAGE. The differences in size could be a consequence of post-translational modification of RAGE. Equal amounts of whole cell lysate (WCL) protein were loaded into each well with visualisation of β-actin as confirmation. Although no appropriate protein loading control is available for cell culture supernatant and analyses of secreted proteins, RAGE protein levels were normalised through loading of equal amounts of total protein.

In WCL generated from untreated cells, RAGE was detected with maximum protein expression following 8 hours of cell culture (Figure 3.5A). Cell culture supernatant was freeze dried and reconstituted and loaded onto SDS-PAGE gels (10 µg total protein per well) for analyses of secreted RAGE (section 2.3). In culture supernatant of untreated cells, secreted RAGE levels were consistent over the 36 hours. Together the results

suggest RAGE secretion was unaltered over time while WCL RAGE levels were at their highest following 8 hours of cell culture with decreased levels after 24 and 36 hours.

When cells were treated with 50 µg/mL of HSA-AGE the expression of RAGE in WCL and culture supernatant appeared to remain consistent over 36 hours (Figure 3.5B). A similar pattern was observed when cells were treated with 50 µg/mL HSA-C, thus, indicating 50 µg/mL HSA-AGE does not impact TIGK RAGE protein expression (Figure 3.5C).

Upon TIGK cell treatment with 100 µg/mL HSA-AGE there appeared to be maximum WCL protein expression of RAGE following 8 hour of cell culture, with a decrease after 24 and 36 hours (Figure 3.5D). In addition, a lower expression of secreted RAGE in culture supernatant following 8 hours of culture with an increase after 24 and 36 hours was observed (Figure 3.5E). There appeared to be an increase in secreted RAGE expression at the later time points with 100 µg/mL HSA-AGE (Figure 3.5D) compared with 50 µg/mL HSA-AGE (Figure 3.5B) or untreated cells (Figure 3.5A) . When TIGK cells were treated with 100 µg/mL HSA-C, levels of secreted RAGE and WCL RAGE appeared to remain relatively consistent over time (Figure 3.5E).

Finally, when cells were treated with 200 µg/ml HSA-AGE RAGE, protein levels remained relatively consistent over time in WCL (Figure 3.5F). Following cell treatment with 200 µg/mL HSA-AGE, levels of secreted RAGE were similar to the those observed with 100 µg/mL HSA-AGE (Figure 3.5G). Treatment of TIGK cells with 200 µg/mL HSA-C resulted in no discernible differences in WCL RAGE or secreted RAGE levels.

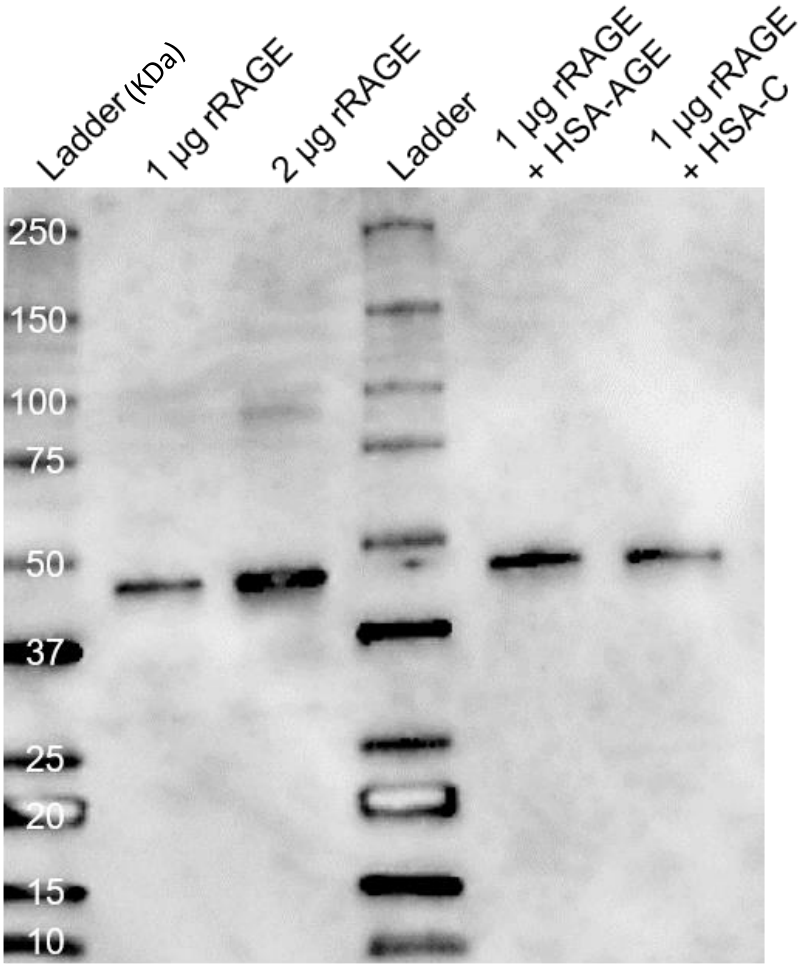


Figure 3.4: Representative western blot analysis of recombinant RAGE in the presence of absence of HSA-AGE or HSA-C. Recombinant RAGE (rRAGE) was detected via western blot using an anti-RAGE antibody. Recombinant RAGE was also incubated with advanced glycation end product modified human serum albumin (HSA-AGE) or control HSA (HSA-C) to determine if AGE-RAGE interactions interfered with detection of RAGE (n=3). HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control, RAGE; receptor for advanced glycation end products.

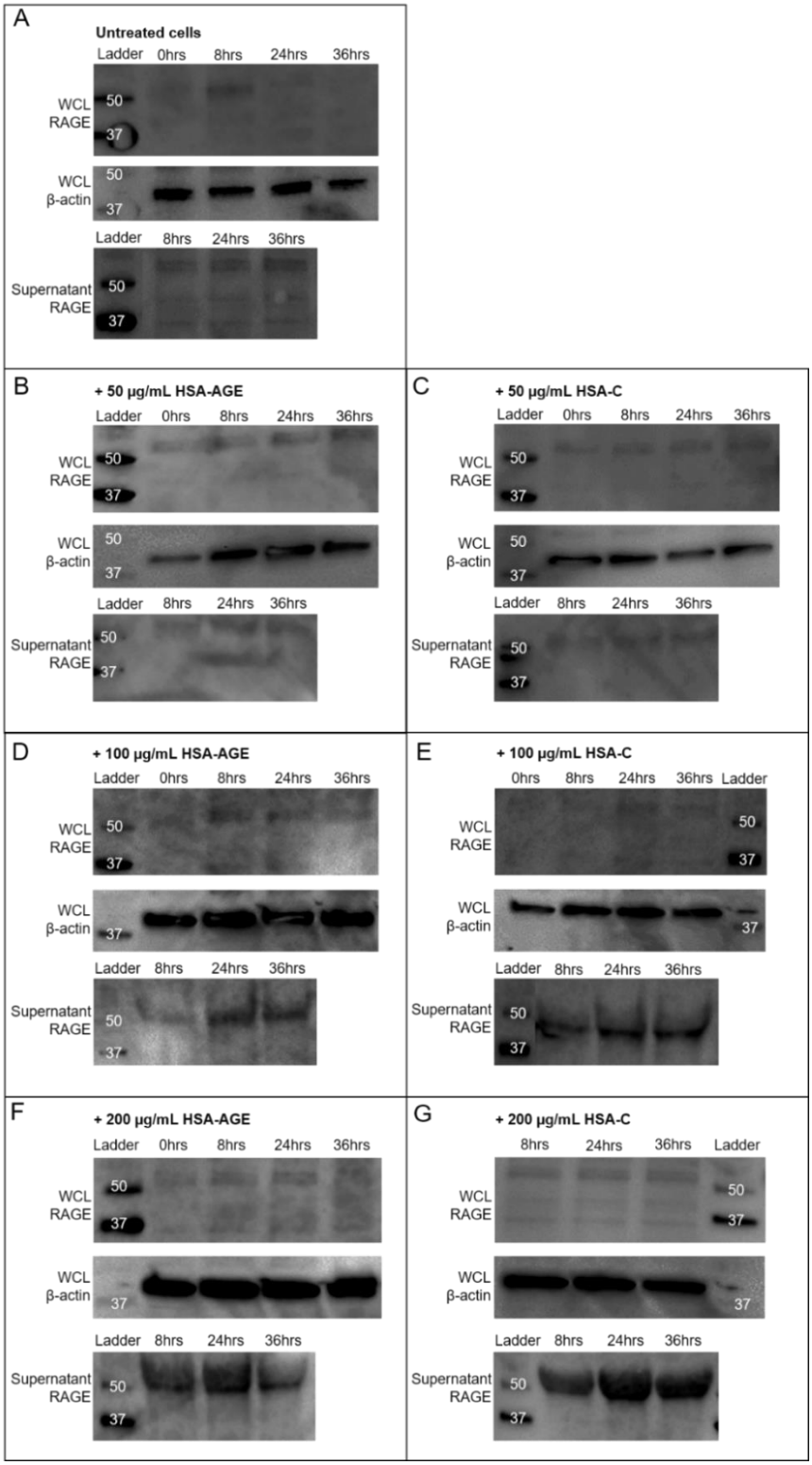


Figure 3.5: Representative western blot analysis of RAGE expression in TIGK cells. Cells were treated with various concentrations of HSA-AGE or HSA-C, whole cell lysate (WCL) and cell culture supernatant were harvested, and 10 µg total protein loaded onto each well. WCL RAGE was used as an indication of cell surface bound RAGE and supernatant RAGE was an indication of secreted RAGE (n=3). HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control, RAGE; receptor for advanced glycation end products.

3.2.3.2. RAGE mRNA expression

The impact of AGE on the expression of the advanced glycation end product receptor (*AGER*) gene in TIGK cells was analysed using qRT-PCR (section 2.10 and section 2.11). Prior to qRT-PCR analysis for *AGER*, housekeeping genes were screened to ensure no changes in mRNA expression occurred over the duration of the experiment in all conditions tested. Both β-actin (*ACTB*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) mRNA expression were analysed in TIGK cells over 36 hours under treatment with 100 µg/mL HSA-AGE or HSA-C as described previously (section 2.10 and section 2.11). There was a significant difference (n=4, two-way ANOVA, Tukey, $p < 0.05$) in *ACTB* mRNA expression following treatment of cells for 36 hours with both HSA-AGE and HSA-C (Figure 3.6A) whereas no significant difference (n=4, two-way-way ANOVA, Tukey, $p > 0.05$) was observed in mRNA expression of *HPRT1* over 36 hours. Furthermore, qRT-PCR analysis indicated no significant difference in *HPRT1* mRNA expression in untreated cells compared with cells treated with 100 µg/mL HSA-AGE or HSA-C (Figure 3.6B). *HPRT1* was confirmed as an appropriate choice for use as a housekeeping gene for analysis of *AGER* expression.

The impact of AGE on expression of the *AGER* in TIGK cells was analysed by the quantification of *AGER* mRNA following exposure to 100 µg/mL HSA-AGE and 100 µg/mL HSA-C over 36 hours (section 2.10 and 2.11). There was no significant difference in the fold change of *AGER* mRNA expression (Figure 3.7) relative to untreated cells over time regardless of treatment with HSA-AGE or HSA-C (n=4, two-way ANOVA, Tukey, $p > 0.05$).

Considering *AGER* expression did not increase beyond the 8 hour timepoint, dose dependent AGE induced changes in *HPRT1* and *AGER* expression were analysed following culture of TIGK cells with 50, 100 or 200 µg/mL HSA-AGE or HSA-C for 8 hours (Figure 3.8). In a similar manner to *AGER* fold changes in TIGK cells treated with 100 µg/ml HSA-AGE or HSA-C, no significant differences were observed in fold changes of *AGER* expression relative to untreated cells regardless of treatment (Figure 3.8B – n=4, one-way ANOVA, Tukey, $p > 0.05$).

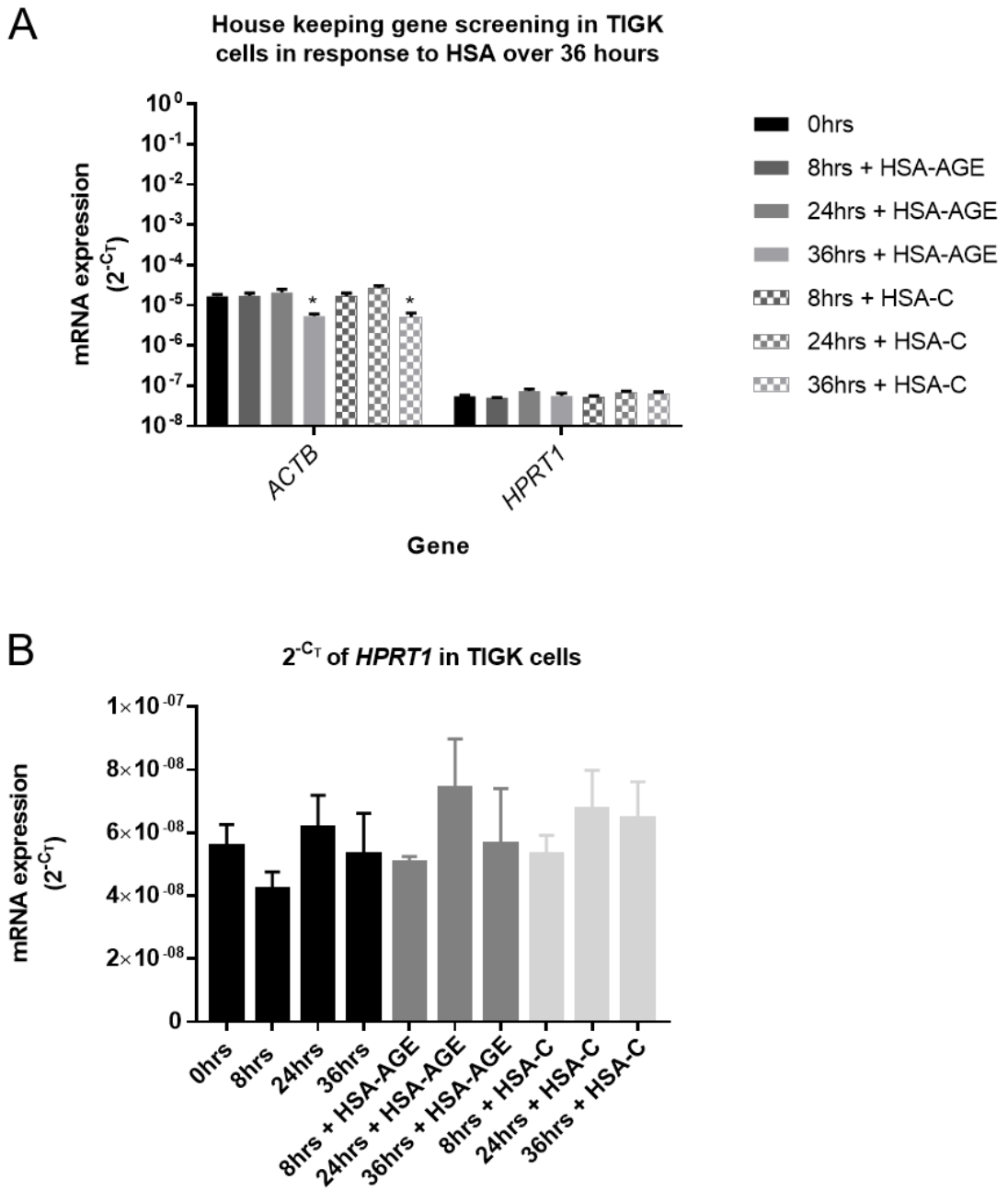


Figure 3.6: Housekeeping gene screening for downstream qRT-PCR analysis. (A) screening of *ACTB* and *HPRT1* housekeeping genes for qRT-PCR in TIGK cells cultured with 100 µg/mL HSA-AGE or HSA-C for 8, 24 or 36 hours. (B) Expression of *HPRT1* in TIGK cells cultured untreated or treated with 100 µg/mL HSA-AGE or HSA-C. Results are presented as means ± SD, n=4. Symbol (*) indicates significant differences in gene expression (two-way ANOVA, Tukey, $p < 0.05$). HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control.

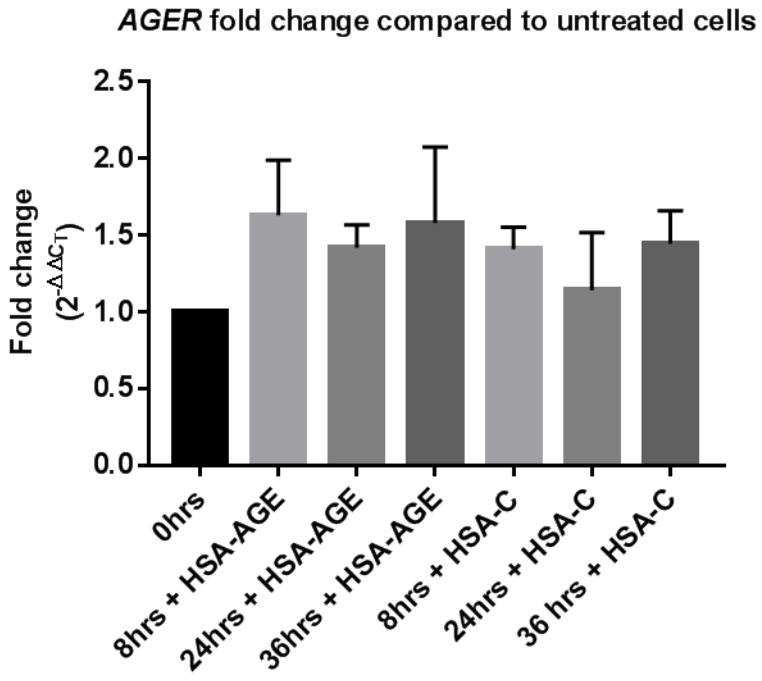


Figure 3.7: *AGER* gene expression of TIGK cells in response to HSA-AGE/HSA-C treatment. Expression of *AGER* in TIGK cells in response to treatment with 100 µg/mL HSA-AGE or HSA-C, analysed using qRT-PCR with expression normalised to *HPRT1* and relative to untreated cells. Results are presented as means ± SD, n=4. HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control, *AGER*; advanced glycation end products receptor gene.

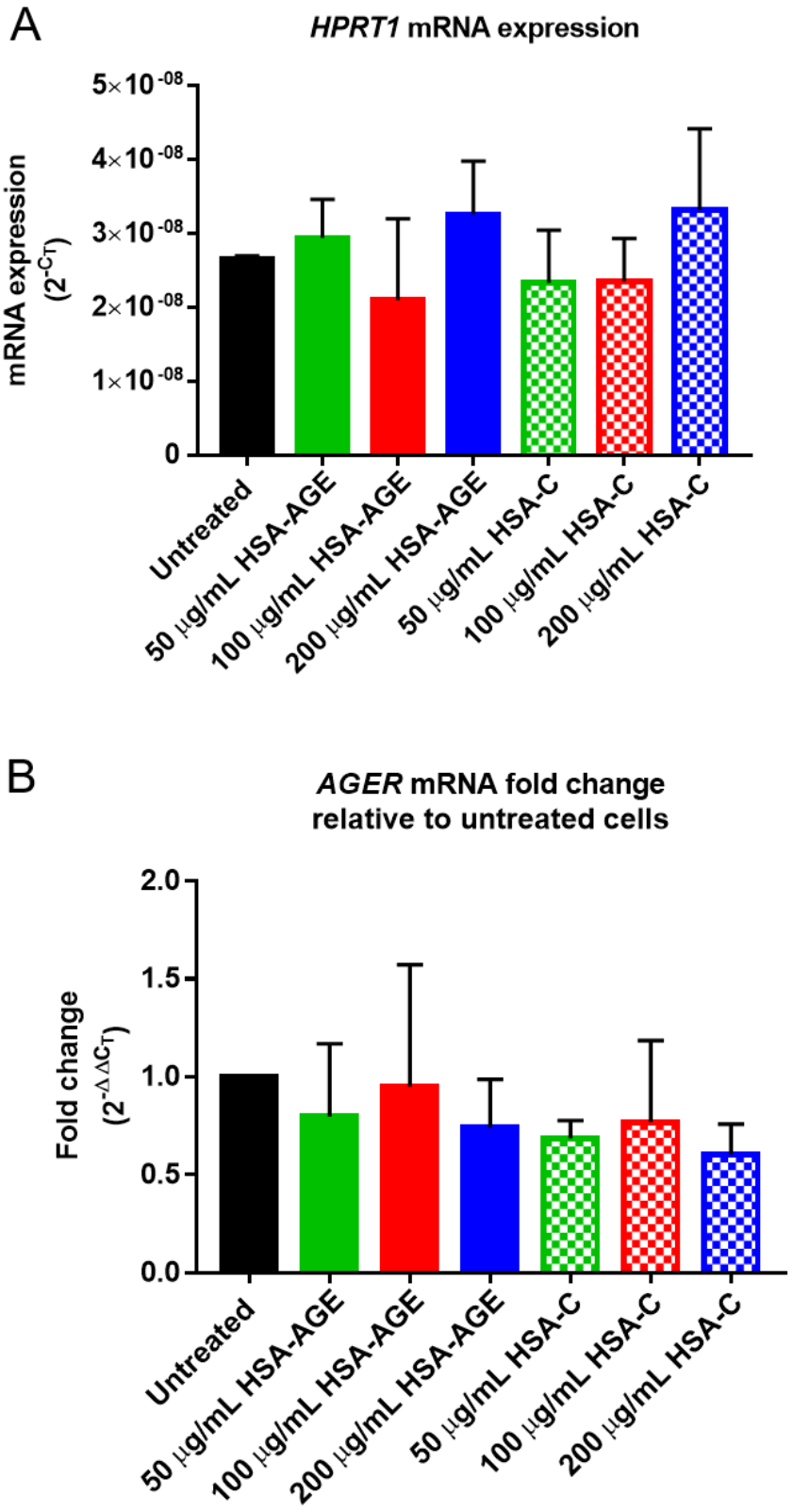


Figure 3.8: *HPRT1* and *AGER* gene expression of TIGK cells following treatment with various concentrations of HSA-AGE/HSA-C. (A) Expression of the housekeeping gene *HPRT1* following treatment with 50, 100 or 200 µg/mL HSA-AGE or HSA-C for 8 hours (n=4). (B) Fold change of *AGER* in TIGK cells in response to treatment with 50, 100 or 200 µg/mL HSA-AGE or HSA-C, analysed using qRT-PCR with expression normalised to *HPRT1* and relative to untreated cells. Results are presented as means ± SD, n=4. HSA-AGE; Human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control, *AGER*; advanced glycation end products receptor gene.

3.2.4. Inflammatory response of TIGK cells to HSA-AGE and HSA-C

Following confirmation of the expression of *AGER* by TIGK cells, inflammatory responses of cells were analysed after treatment with varying concentrations of HSA-AGE or HSA-C. TIGK cells were cultured and treated with 50, 100 or 200 µg/mL HSA-AGE or HSA-C for 8 hours, the supernatant harvested and analysed for cytokine release using ELISAs as previously described (section 2.4).

Of the three cytokines analysed, IL-8 was expressed at the highest concentrations. Despite an apparent trend whereby concentrations appeared to be lower in untreated cells (105.7 ± 85 pg/mL), compared with cells treated with either HSA-AGE or HSA-C (maximum mean of 359 ± 273.5 pg/mL) no significant differences were observed when comparing untreated with treated. This is possibly due to the variations observed between biological replicates across all treatment groups. Furthermore, no significant differences were seen between TIGK treatment with HSA-AGE or HSA-C (Figure 3.9A – n=3, one-way ANOVA, Tukey, $p > 0.05$).

In a similar manner, IL-6 concentrations on average stayed consistent across the different treatment groups. In addition, IL-6 concentrations in untreated TIGK cells (2.43 ± 1.75 pg/ml) were lower than average concentrations of the different treatment groups (maximum mean of 16.6 ± 8.62 pg/mL) but still no significant difference was observed (Figure 3.9B – n=3, one-way ANOVA, Tukey, $p > 0.05$). IL-6 concentrations, in contrast to IL-8 concentrations, were generally lower and had less variation in concentrations comparing different biological replicates across the different treatment groups (0.62 – 24.73 pg/mL).

Out of the three cytokines analysed, IL-1β had the lowest detectable concentrations ranging from 0.04 to 7.6 pg/mL. Similar to IL-8 and IL-6, IL-1β concentrations were not significantly different across the different treatment groups, although this is possibly due to the concentrations observed being below or at the lower end of the detectable range of the assay and as such are less reliable (Figure 3.9C – n=3, one-way ANOVA, Tukey, $p > 0.05$).

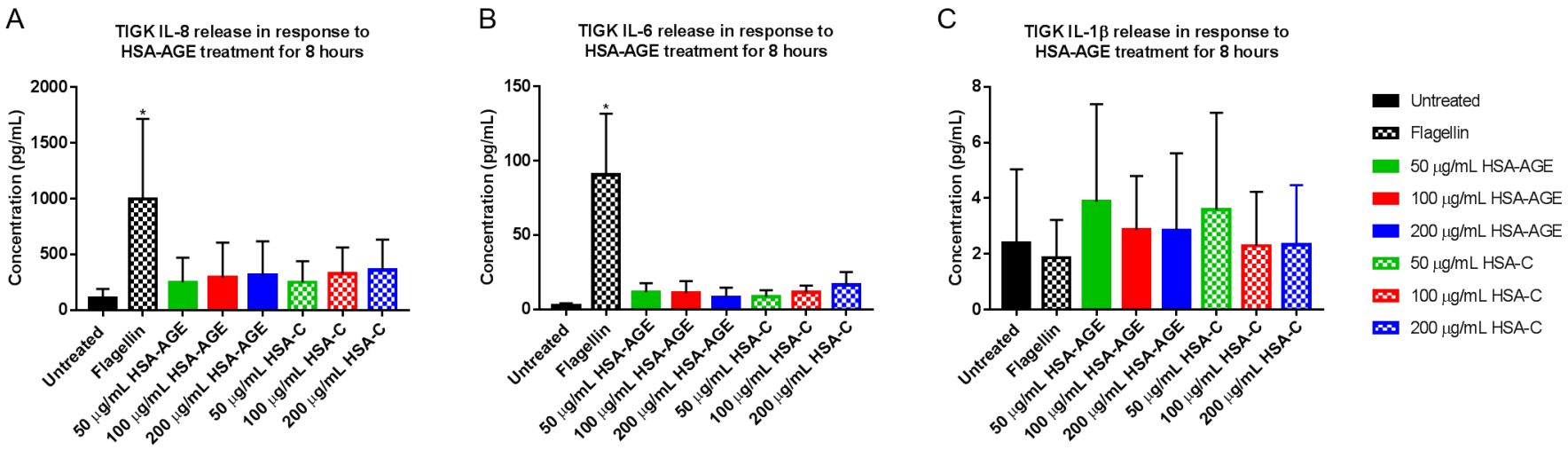


Figure 3.9: Immune responses initiated by TIGK cells in response to treatment with HSA-AGE or HSA-C. (A) IL-8 cytokine, (B) IL-6 cytokine and (C) IL-1β cytokine release measured in cell culture supernatant following 8 hour treatment of TIGK cells with 50, 100 or 200 µg/mL HSA-AGE or HSA-C. Concentrations were measured using ELISAs. Significance was calculated using a one-way ANOVA followed by a Tukey post-hoc (*, $p < 0.05$, n=3). HSA-AGE; human serum albumin-advanced glycation end products, HSA-C; Human serum albumin-control,

3.3. Discussion

Periodontitis has long been associated with diabetes, but the underlying mechanism linking the two diseases is still uncertain. The interactions between AGE and RAGE and the subsequent immune responses have been marked as a potential linking mechanism (Preshaw *et al*., 2012). Studies have previously shown an increased prevalence of RAGE in gingival tissues of individuals with periodontitis and more so in individuals with both periodontitis and diabetes (Abbass *et al*., 2012).

Previous studies have shown an upregulation of RAGE in response to AGE in a variety of different cell types including immune cells, endothelial cells and epithelial cells, although the majority of studies concentrated on bronchial epithelial cells and immune cells (such as macrophages) (Schmidt *et al*., 2000, Ott *et al*., 2014, Wautier *et al*., 2017, Xu *et al*., 2016). However, recently, Hiroshima *et al*. (2018) characterised RAGE expression of OBA-9 (Simian virus-40 antigen immortalized human gingival epithelial cells) and showed an increase in *AGER* (mRNA) and RAGE protein in response to AGE (500 µg/mL AGE). Interestingly, our results did not correlate with this, and although they showed the expression of *AGER* and RAGE by TIGKs, mRNA expression of *AGER* was unaffected by AGE regardless of concentration or exposure time. These observed differences in AGE response are potentially due to differences in cell types. Hiroshima *et al*. (2018) also demonstrated a significant increase in RAGE expression following co-stimulation with AGE and *P. gingivalis* LPS compared with unstimulated and stimulation with AGE alone. This perhaps suggests that co-stimulation is required for alterations in RAGE expression.

It is possible that the lack of significant differences in *AGER* mRNA expression observed here was a consequence of the choice of *AGER* primer. The qRT-PCR carried out would most likely amplify all variants of *AGER*. To elucidate whether the lack of significant differences in *AGER* mRNA expression is indeed accurate or if it is rather a shift in the ratio of isoforms expressed, mRNA analysis for specific isoforms is required, especially considering our results do suggest a difference in protein expression of secreted *AGER* in response to AGE. A study demonstrating a positive correlation between secreted RAGE and MCP-1 and TNF-α in type 2 diabetics, further highlights the necessity to analyse isoforms independently (Nakamura *et al*., 2008).

Recent studies have shown that different *in vivo* AGE products (of which methylglyoxal HSA (HSA-MG) and N-carboxymethyl lysine HSA (HSA-CML) are the most predominant) differentially regulate *AGER* isoform expression despite binding to the same receptor. While HSA-MG stimulates the expression of full-length, N-truncated and secreted isoforms in endothelial cells, HSA-CML upregulates only full-length and N-truncated

(Grossin *et al*., 2009, Wautier *et al*., 2017). In addition, due to the various methods for protein modification with AGE, and the heterogeneous nature of AGEs produced, it is difficult to ascertain what proportion (if any) of the generated HSA-AGE is HSA-MG and what proportion is HSA-CML, and indeed how that compares to previous studies. To fully understand the impact of the various HSA-AGEs on *AGER* expression in TIGK cells, the HSA-AGE composition generated needs to be established through methods such as mass-spectrometry. Alternatively, more defined and commercially available ligands (such as CML, S100 proteins and HMGB1) could be utilised for clarification of RAGE expression and activation in TIGK cells.

However, our results also indicate high levels of secreted RAGE as a response to 200 µg/mL HSA-C. This perhaps indicates that RAGE secretion is a consequence of the addition of exogenous proteins as opposed to a direct consequence of HSA-AGE, particularly when considering under normal conditions TIGK cells are cultured using serum free media. Presence of AGE in the HSA-C preparation was determined through analysis of fluorescence. Although fluorescence in HSA-C preparations were lower than that seen in HSA-AGE preparations there is potential that the HSA used (indicated as ≥ 96% pure by manufacturers) contained low amounts of AGE modified protein prior to HSA-C generation. This could explain why higher levels of secreted RAGE were observed with higher concentrations of HSA-C.

The western blot analysis, however, is semi quantitative and to more accurately understand changes at a protein level quantitation of RAGE is required. To do this ELISAs were carried out (as described previously in section 2.4 and according to manufacturer's instructions) but concentrations were below the detectable range by this method, despite attempts to concentrate samples (data not shown). Due to time limitations, however, this analysis could not be optimised.

Interaction of AGE with RAGE is known to lead to the activation of signalling cascades and downstream activation of inflammatory responses (Ott *et al*., 2014, Wautier *et al*., 2017). In gingival epithelial cells specifically, an increase in IL-6 mRNA expression was observed in response to AGE (Hiroshima *et al*., 2018). Our results, however, showed no induction in IL-6, IL-8 or IL-1β expression by AGE. This is potentially due to the lack of increase in RAGE expression (as described above) such that no associated increase in cytokines is observed. There is also the possibility that the consistent level of cytokine expression is a consequence of sequestering of AGE by secreted RAGE, thus, preventing activation of membrane bound RAGE and downstream signalling cascades. This can be associated with the potential increase in secreted RAGE observed.

Although RAGE is the most studied receptor for AGE, various others have also been identified; AGE clearance receptor complex (AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3) and members of the scavenger receptor family (SR-A, SR-B, CD36, SR-B1, SR-E, LOX-1, FEEL-1, FEEL-2). Prior to the elimination of AGE modified proteins, an intracellular processing of AGE proteins into AGE modified peptides must occur. These alternative receptors are thought to be involved in this first step of the removal process by binding extracellular AGEs to allow the uptake via receptor mediated endocytosis. Given their high affinity for AGEs, it is possible that these receptors compete against RAGE, inhibiting AGE-RAGE interactions and, thus, preventing downstream signalling (Sourris *et al*., 2009, Ott *et al*., 2014). However, to determine if these cells express these receptors and if AGE interacts with these receptors in TIGK cells requires further analysis.

Finally, due to the complex immunoregulatory mechanisms employed by cells in an attempt to prevent excessive immune responses (particularly in the densely colonised oral cavity) it is possible that TIGK cellular responses to AGE require co-stimulation of other receptors with pathogens. Indeed, a recent study has shown increased RAGE and IL-6 expression in gingival epithelial cells in response to AGEs and *P. gingivalis* LPS compared with AGE alone (Hiroshima *et al*., 2018). The study also demonstrated AGE alone did not change IL-8 mRNA expression but co-stimulation of RAGE with AGE and *P. gingivallis* LPS decreased IL-8 mRNA and protein levels (Hiroshima *et al*., 2018). In addition, as mentioned previously, studies in patients with periodontitis alone and periodontitis as well as diabetes, showed higher RAGE expression in tissues of the latter (Abbass *et al*., 2012).

To fully understand RAGE expression and immune responses in TIGK cells further analysis is required. Studies including higher concentrations of AGEs to overcome competition by other receptors, use of alternative, better defined AGE products, co-stimulation of cells and analysis of RAGE isoforms in TIGK cells would increase our understanding of AGE-RAGE interactions in oral epithelial cells.

4. Changes in a five species biofilm model for periodontitis in response to HSA-AGE

4.1. Introduction

In mammalian cells and tissues, AGEs are relatively well studied, with investigations into aspects of AGE generation, metabolism and impact during disease. We now understand AGEs accumulate, both intracellularly and extracellularly, over time and more so in certain diseases (such as diabetes and Alzheimer's) (Cohen-Or *et al*., 2013). Given this accumulation it is possible AGEs could affect bacterial cells, particularly in densely colonised areas of the host such as the gut and oral cavity.

To date, few studies have investigated the role of AGEs or AGE associated cross-linked proteins in bacterial adherence. Furthermore, studies examining the bacterial metabolism of AGEs and consequences of AGE breakdown products in terms of effects on the microbiota are sparse and generally limited to single species experiments. A series of studies have demonstrated AGEs produced by *E. coli* K12 are metabolised intracellularly by metalloproteases and secreted as low-molecular weight AGE peptides into the growth media (Katz *et al*., 2010, Cohen-Or *et al*., 2011, Cohen-Or *et al*., 2013). While this group demonstrated the potential for bacterial metabolism of AGEs, their studies are limited to intracellular bacterial AGEs and did not investigate bacterial uptake of extracellular/mammalian AGEs and subsequent metabolism.

Studies examining the relationship between AGEs and bacteria are, to date, limited to the gut, with evidence suggesting potential AGE metabolism and utilisation: human and rat studies have indicated only partial recovery of AGEs in faecal matter or urine following oral administration (Delgado-Andrade *et al*., 2012, Alamir *et al*., 2013, Hellwig *et al*., 2015). Hellwig *et al*. (2015), showed the ability of human colonic microbiota to metabolise three different AGE proteins (the early Amadori product N-ε-fructodyllysine and the irreversible final AGE products N^{ε}-carboxymethyl lysine (CML) and Pyrraline (PYR). The different AGE proteins were incubated with faecal suspensions and the resulting metabolites measured using high performance liquid chromatography (HPLC) (Hellwig *et al*., 2015).

In addition to studies examining bacterial AGE metabolism, there is evidence of AGE modified proteins passing through the colon in individuals with ulcerative colitis changing the composition of the gut microbiota (Mills *et al*., 2008). Using faecal inoculations from ulcerative colitis patients in a continuous flow culture model, bacterial composition in medium containing AGE-modified BSA as a growth substrate was shown to have higher abundances of *Clostridium*, *Bacteroides* and sulphate-reducing bacteria and decreases in the prevalence of *Eubacterium* and *Bifidobacterium*, when compared with bacterial

compositions of cultures in un-modified BSA. However, these changes as a response to AGE modified BSA were not observed when using faecal samples from healthy individuals (Mills *et al*., 2008). Other studies have shown consumption of diets high in AGEs inhibit the growth of lactobacilli in the colonic microbiota (Hernandez-Hernandez *et al*., 2011, Corzo-Martínez *et al*., 2013, Seiquer *et al*., 2014, Hellwig *et al*., 2015). These studies suggest that AGE associated changes in the microbiota are a consequence of both presence of AGE and host disease state, highlighting the complex relationship between AGEs and the microbiota.

While the above described studies have demonstrated changes in gut microbiota as a consequence of AGEs, to date no studies have linked AGEs to changes in the oral microbiota. Investigating the role of AGEs on the oral microbiota is important when considering the present organisms are likely the first to encounter ingested dietary AGEs. Furthermore, there is evidence of an increased prevalence of HSA-AGE in the GCF of individuals with diabetes and periodontitis compared with those with periodontitis or diabetes alone (Kajiura *et al*., 2014). The evidence from studies of the gut microbiota and the increased prevalence of HSA-AGE in the GCF of individuals with both diabetes and periodontitis could suggest that the association between periodontitis and diabetes may be, in part, the result of changes induced in the oral microbiota by the increased abundance of AGEs in the subgingival environment.

Given the evidence of AGE associated changes in gut microbiota, the following study utilises a five species biofilm (to model periodontitis) which was developed in our laboratory by Naginyté (2017, unpublished) to investigate the effect of HSA-AGE on biofilm composition. The model was developed to use HA coated pegs, pre-conditioned with salivary proteins, to represent tooth surfaces. The pegs are inoculated sequentially with early (*A. naeslundii* and *S. salivarius*) and late (*F. nucleatum*, P*. intermedia* and *P. gingivalis*) colonisers to generate a stable biofilm. In the study presented here, the bacterial growth protein rich medium is supplemented with 20% (v/v) FBS (serum medium; SM) to select for pathogenic/late colonisers and produce a biofilm representative of periodontitis. The effect of HSA-AGE addition to the SM of a 6 day old biofilm was investigated to determine if AGEs (as a representative of diabetic hyperglycaemia) alter biofilm composition. I hypothesise that the addition of HSA-AGE selects for the growth of periodontitis associated pathogens and could provide evidence for linking diabetes and periodontitis.

4.2. Results

4.2.1. HSA-AGE associated changes in species composition of five species biofilms

4.2.1.1. Composition of day 6 biofilms grown in serum medium to select for periodontitis associated pathogens

Five species model biofilms were grown on HA coated pegs to model periodontitis (as described previously in section 2.12). Biofilms harvested at day 6 were analysed for relative abundance of each species based on selective media CFU counts (section 2.12.2) (Figure 4.1). Six to eight pegs each from three individual experiments (minimum 18 pegs) were harvested, CFUs counted and used to calculate the percentage abundance of each species in individual biofilms relative to total biofilm CFU.

Both *A. naeslundi* and *S. salivarius* accounted for the majority of the biofilm mass and were significantly more prevalent than *F.nucleatum*, *P. intermedia*, *P. gingivalis* (black lines, Figure 4.1). However, significantly less *A. naeslundi* was observed compared with *S. salivarius* (*,Figure 4.1). This difference is unsurprising given the later HA peg inoculation with *F. nucleatum*, *P. intermedia* and *P. gingivalis* (section 2.12) and the slower growth rate of these species. Importantly, the results indicated that all five species were present in the day 6 biofilm, such that any changes in biofilm composition upon addition of HSA-AGE or HSA-C reflect the impact of the addition as opposed to absence of particular species in the day 6 biofilm.

4.2.1.2. Impact on day 6 biofilms following HSA-AGE addition to growth medium

After 6 days, varying concentrations of HSA-AGE or HSA-C were added to the biofilm growth SM which was changed daily until day 10 (section 2.12). Day 10 biofilms were harvested and subsequent biofilm compositions analysed (Figure 4.2 and Table 4.1). In biofilms grown in SM without the addition of any HSA-AGE or HSA-C (baseline biofilms), *P. gingivalis* accounted for the bulk of the biofilm (mean abundance of 49.00 ± 21.19% of total viable counts) followed by *S. salivarius* (mean abundance of 22.55 ± 11.32%) and *P. intermedia* (mean abundance of 12.97 ± 9.94%) (Table 4.1). *A. naeslundii* and *F. nucleatum* accounted for similar percentages of the bacteria in the biofilm (mean abundance of 8.29 ± 8.93% and 7.00 ± 4.54% respectively), although more variability was observed in the percentage of *A. naeslundii* compared with *F. nucleatum.* Given the predominance of *P. gingivalis* and *P. intermedia* (periodontitis associated bacteria) in biofilms grown with SM for 10 days, the results confirm the periodontitis associated pathogens behave in the model biofilm in a manner that reflect their increased prevalence in periodontitis (Figure 4.1 and Table 4.1).

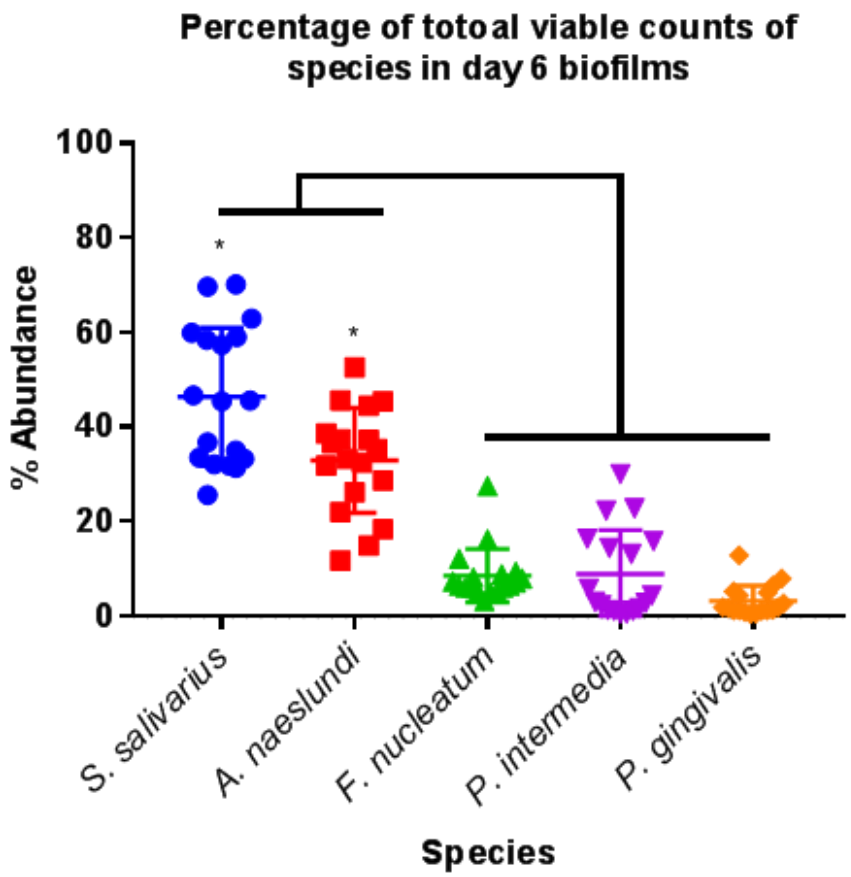


Figure 4.1: Bacterial composition of day 6 biofilms. Biofilms were grown following sequential inoculation of hydroxyapatite pegs with *S. salivarius*, *A. naeslundii*, *F. nucleatum*, *P. intermedia* and *P. gingivalis*. Following growth for 6 days under anaerobic conditions at 37°C in protein rich medium supplemented with 20% FBS (serum medium), 6-8 biofilms were harvested for determining CFU (colony forming unit) counts on selective media and calculation of percentage abundances of total viable counts. Lines and * indicate significant differences (One-way ANOVA, Tukey, $p < 0.05$, n=3).

Significant decreases in mean relative abundance of *A. naeslundii* and *S. salivarius* were observed when comparing HSA-AGE biofilms with their corresponding HSA-C biofilms (Table 4.1). Although no significant differences (two way ANOVA, Tukey, $p > 0.05$) in the early coloniser abundances were observed between the different HSA-C biofilms, a significantly higher average percentages of *A. naeslundii* were observed in all HSA-C biofilms compared with baseline biofilms. This potentially indicates HSA is partially responsible for the increase in *A. naeslundii* growth in HSA-AGE biofilms. This increase in *A. naeslundii* corresponded with a decrease in mean *P. gingivalis* abundance (such that mean percentages were significantly lower in all HSA-C biofilms compared with baseline biofilms but not completely abolished as seen in HSA-AGE biofilms). In addition, no significant difference (two-way ANOVA, Tukey, $p > 0.05$) in mean abundances were observed in the different concentrations of HSA-C (Table 4.1).

Growth media supplemented with HSA-AGE appeared to inhibit all three late colonisers. While these decreases are significant in *P. gingivalis* and *P. intermedia,* changes in mean abundance of *F. nucleatum* were not significant (two-way ANOVA, Tukey, $p > 0.05$), potentially due to the low percentages observed in baseline and HSA-C biofilms. *P. intermedia* abundances did not appear to be significantly different when comparing HSA-AGE biofilms to their matched HSA-C biofilms (two-way ANOVA, Tukey, $p > 0.05$) but the decrease in HSA-AGE biofilms was significant compared with baseline biofilms (Table 4.1).

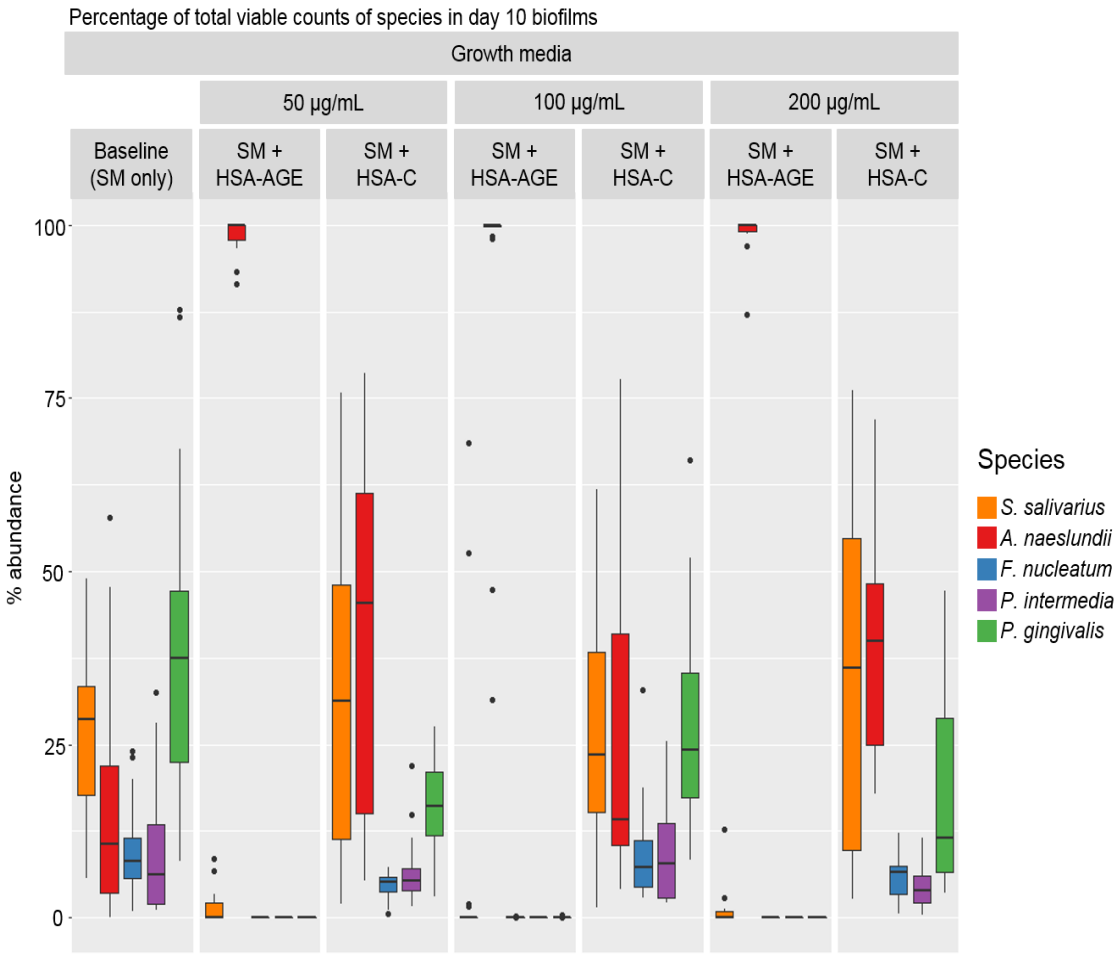


Figure 4.2: Bacterial composition of day 10 biofilms. Biofilms were cultivated for 6 days in SM prior to the introduction of HSA-AGE/HSA-C to the SM until day 10. On day 10 biofilms were harvested and the relative abundances of each species in individual biofilms were calculated and presented as medians with maximum and minimum percentages. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium.

Table 4.1: Bacterial composition of day 10 biofilms. Biofilms were cultivated for 6 days in SM prior to the introduction of HSA-AGE/HSA-C to the SM until day 10. On day 10 biofilms were harvested and the relative abundances of each species in individual biofilms were calculated and presented as mean % ± SD. Results represent 3 experiments with 6-8 biofilms harvested per experiment. Bold indicates significant differences compared with basal biofilms. Underlined indicates significant differences when comparing different concentrations of HSA-AGE or HSA-C. Colours indicate significant differences between HSA-AGE and HSA-C. All significance was calculated using a two-way ANOVA followed by a Tukey post-hoc ($p < 0.05$). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium

	Percentage of total viable counts of biofilms grown in:													
	Basal (SM only)		SM + 50 µg/mL HSA-AGE		SM + 50 µg/mL HSA-C		SM + 100 µg/mL HSA-AGE		SM + 100 µg/mL HSA-C		SM + 200 µg/mL HSA-AGE		SM + 200 µg/mL HSA-C	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
S. salivarius	22.55	11.32	**1.44**	2.55	32.87	23.66	**0.29**	0.68	21.64	12.65	**1.11**	3.03	33.64	25.00
A. naeslundii	8.29	8.93	**98.56**	2.55	**39.69**	25.44	**99.67**	0.67	**31.14**	24.15	**98.89**	3.03	**39.09**	15.13
F. nucleatum	7.00	4.54	0.00	0.00	4.81	1.83	0.01	0.03	10.43	7.26	0.00	0.00	5.75	3.06
P. intermedia	12.97	9.94	**0.00**	0.00	6.66	4.94	**0.00**	0.00	7.92	6.33	**0.00**	0.00	4.37	2.93
P. gingivalis	49.18	21.19	**0.00**	0.00	**15.96**	7.01	**0.02**	0.08	**28.86**	16.39	**0.00**	0.00	**17.15**	13.51

4.2.1.3. Analysis of biofilms following change of medium from HSA-AGE/HSA-C supplemented to non-supplemented serum medium

Following growth of biofilms in HSA-AGE or HSA-C supplemented medium (from day 6 to 10), growth medium was replaced with unsupplemented SM until day 14. At day 14, biofilms were harvested and subsequent percentage abundances calculated. The results indicated that at day 14, baseline biofilms maintained the higher prevalence of periodontitis associated bacteria (Figure 4.3 and Table 4.2). These biofilms favoured the growth of *P. gingivalis* and *P. intermedia* with abundances of *S. salivarius*, *A. naeslundii* and *F. nucleatum* only accounting for a small percentage of the total biofilm. Furthermore, the relatively high percentage abundance of *S. salivarius* observed at day 10 (Table 4.1) decreased significantly at day 14 (Table 4.2), in addition to significant decreases in *A. naeslundii* and *F. nucleatum* (two-way ANOVA, Tukey, $p < 0.05$). Together this suggests the maturation and preservation, over 14 days, of a biofilm that represents the increased prevalence of bacteria generally associated with periodontitis, which in this model is seen as higher percentage abundances of *P. gingivalis* and *P. intermedia*.

Biofilms exposed to HSA-AGE supplemented growth medium prior to changing to SM showed significant recovery in the abundance of *S. salivarius* but no significant recovery in any of the late colonisers (two-way ANOVA, Tukey, $P > 0.05$), regardless of the HSA-AGE concentration. Interestingly, the inability to recover late coloniser growth allowed the *S. salivarius* to grow to significantly higher abundances on average than in baseline and HSA-C biofilms. The recovery of *S. salivarius* was not significantly different across the different HSA-AGE concentrations (two-way ANOVA, Tukey, $p > 0.05$). In HSA-C exposed biofilms, no significant differences were observed in abundance of *S. salivarius* at day 14 in comparison with day 14 baseline biofilms (two-way ANOVA, Tukey, $p > 0.05$, Table 4.1).

In HSA-AGE recovery biofilms, *S. salivarius* abundance increases correlate with a significant decrease in *A.* naeslundii. Despite the *A. naeslundii* decrease, the abundance in HSA-AGE biofilms was significantly higher when compared with baseline biofilms and correlating HSA-C biofilms. The higher abundance of *A. naeslundii* was consistent across all concentrations of HSA-AGE that biofilms were exposed to prior to the recovery period. In addition, *A. naeslundii* abundances in HSA-C recovery biofilms showed no significant difference compared with baseline biofilms (two-way ANOVA, Tukey, $p > 0.05$), indicating changes observed in *A. naeslundii* in recovery HSA-AGE biofilms were a direct consequence of the AGE supplemented growth medium as opposed to changes associated with the addition of HSA (Figure 4.3 and Table 4.1)

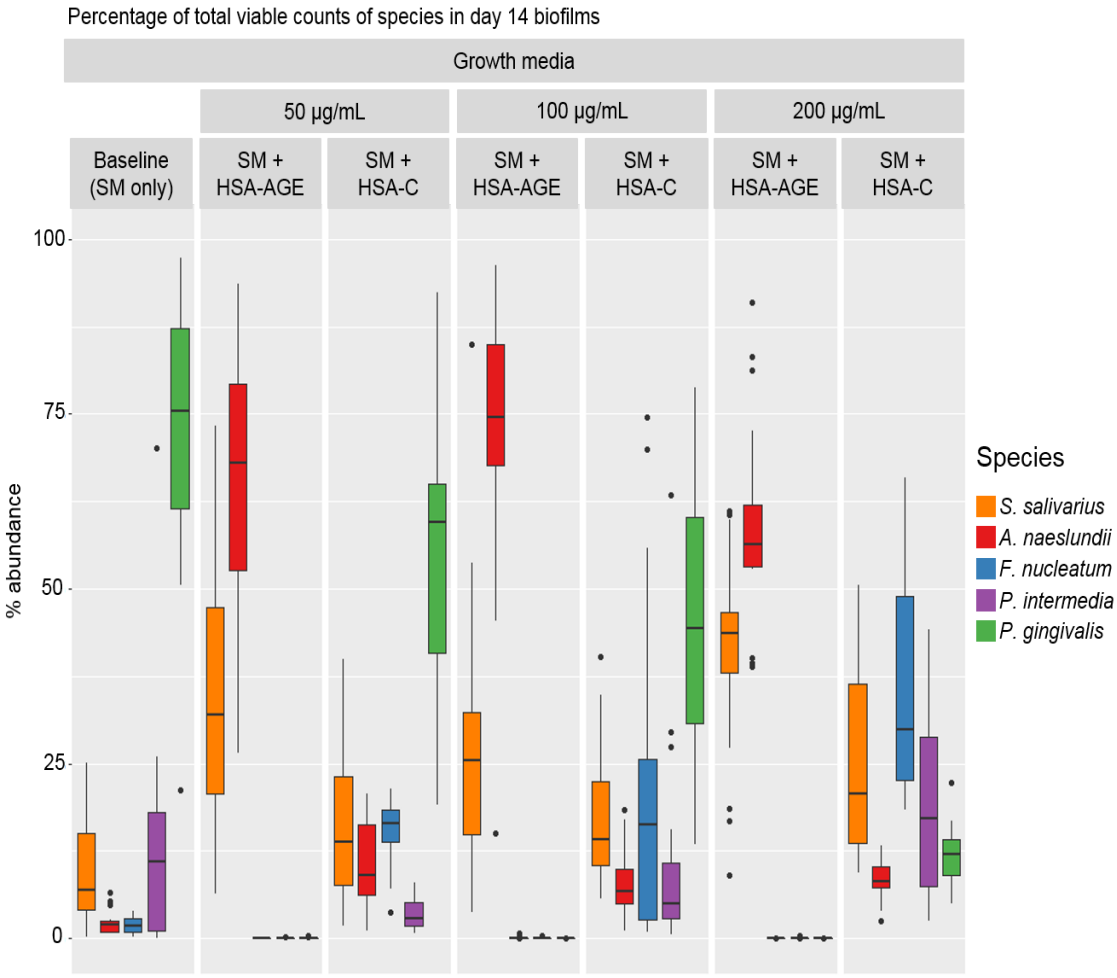


Figure 4.3: Bacterial composition of day 14 biofilms. Biofilms were cultured for 6 days in SM prior to introduction of HSA-AGE or HSA-C to growth media until day 10 after which media were changed to SM (day 10 to day 14). Relative abundances of each species in individual biofilms were calculated and presented as medians with maximum and minimum percentages. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium.

Table 4.2: Bacterial composition of day 14 biofilms. Biofilms were cultured for 6 days in SM prior to introduction of HSA-AGE or HSA-C to growth media until day 10 after which media were changed to SM (day 10 to day 14). On day 14 biofilms were harvested and the relative abundances of each species in individual biofilms were calculated and presented as mean ± SD. Results represent 3 experiments with 6-8 biofilms harvested per experiment. Bold indicates significant differences compared with basal biofilms Bold indicates significant differences compared with basal biofilms. Underlined indicates significant differences when comparing different concentrations of HSA-AGE or HSA-C. In the case of *P. gingivalis*, double underlined specifies 50 µg/mL and 100 µg/mL HSA-C are significantly different to 200 µg/mL. Colours indicate significant differences between HSA-AGE and HSA-C. All significance was calculated using a two-way ANOVA followed by a Tukey post-hoc ($p < 0.05$). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium.

	Percentage of total viable counts of biofilms grown in:													
	Basal (SM only)		SM + 50 µg/mL HSA-AGE		SM + 50 µg/mL HSA-C		SM + 100 µg/mL HSA-AGE		SM + 100 µg/mL HSA-C		SM + 200 µg/mL HSA-AGE		SM + 200 µg/mL HSA-C	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
S. salivarius	10.00	8.16	**34.38**	19.00	15.67	9.90	**32.03**	17.54	12.61	4.92	**40.78**	14.61	24.64	12.60
A. naeslundii	2.27	1.72	**65.62**	19.00	10.56	6.46	**67.87**	17.62	7.45	4.17	**59.18**	14.60	8.35	2.84
F. nucleatum	1.92	1.16	0.03	0.03	**15.70**	4.72	0.08	0.21	**20.65**	26.28	0.01	0.02	**35.40**	16.15
P. intermedia	13.28	16.68	0.04	0.05	3.36	2.22	0.02	0.10	11.96	15.32	0.03	0.08	19.89	14.25
P. gingivalis	72.52	19.14	**0.06**	0.09	**54.70**	19.08	**0.01**	0.02	**47.33**	20.74	**0.00**	0.01	**11.72**	4.25

In contrast to day 10, day 14 HSA-C biofilm *A. naeslundii* abundances were not significantly different to baseline biofilms (two-way ANOVA, Tukey, $p > 0.05$). The decrease in *A. naeslundii* abundance at day 14 in HSA-C recovery biofilms correlated with a significant increase in *F. nucleatum* (Figure 4.3 and Table 4.2). Thus it appeared that the presence of HSA-C encouraged the growth of *A. naeslundii*, which is outgrown by *F. nucleatum* following withdrawal of HSA-C. The significant decrease in *F. nucleatum* of baseline biofilms over time and the significant, dose dependent increase in *F. nucleatum* abundance in HSA-C recovery biofilms could suggest that the addition of HSA-C and subsequent increase in *A. naeslundii* abundances produces metabolic by-products, which upon withdrawal of HSA-C encourages *F. nucleatum* growth to a point where its growth surpasses *A. naeslundii.*

In addition, a significant dose dependent decrease in *P. gingivalis* mean abundance in all HSA-C recovery biofilms compared with baseline biofilms was observed. Similarly, average *P. intermedia* prevalence in recovery biofilms was lower than in baseline biofilms, however this was not significant (two-way ANOVA, Tukey, $p > 0.05$). *P. intermedia* average abundances in HSA-C biofilms also demonstrate a dose dependent increase, reaching significance between 100 and 200 µg/mL HSA-C (Table 4.2).

4.2.1.4. Analysis of pH changes in biofilm growth media

To evaluate changes in pH as a consequence of bacterial metabolism, pH of the biofilm growth medium was recorded every 24 hours for 14 days. This was achieved through use of a pH probe capable of measuring pH directly in wells of 96 well plates. I hypothesised changes in pH as a result of the metabolism of AGEs by *A, naeslundii* could be indicative of changes in pH causing late coloniser death.

There was a daily drop in pH to approximately 6 over the first 6 days, prior to addition of HSA-AGE/HSA-C. In baseline and HSA-C biofilms pH changes remained relatively consistent over the course of the experiment, with pH not decreasing below 6 (Figure 4.4). However, upon addition of various concentrations of HSA-AGE to growth media, a pH of approximately 4.5 was observed. Despite correction of pH by media replacement every 24 hours, the acidic environment created following the addition of HSA-AGE could account for the complete inhibition of late colonisers and the inability to revive them following a recovery period. Following removal of HSA-AGE, the pH of the environment reverted to pH 6 – 7 within 24 hours. The changes in pH were consistent across all HSA-AGE concentrations which reflects the limited differences observed in biofilm composition between HSA-AGE biofilms (Figure 4.4).

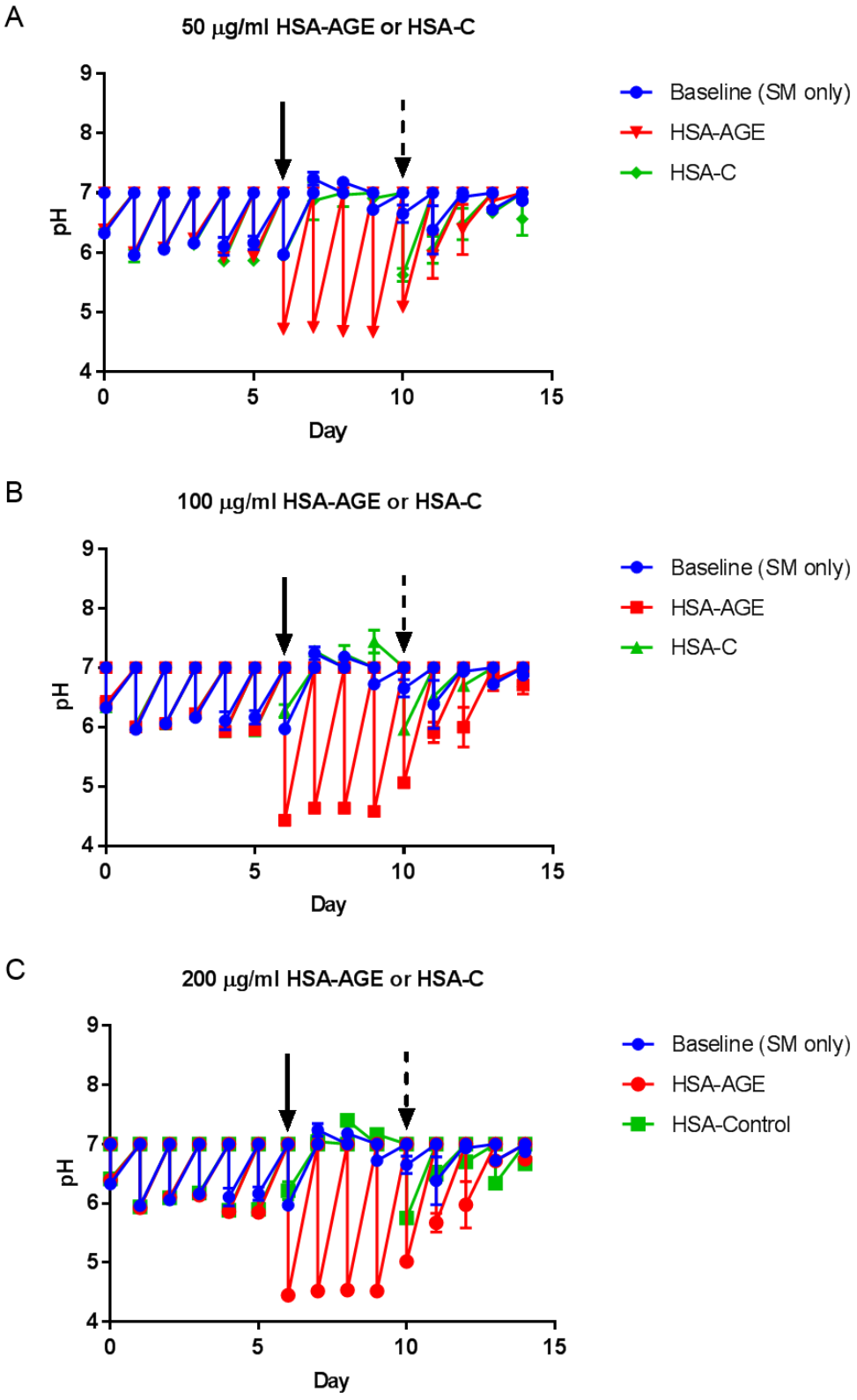


Figure 4.4: pH measurements of culture media following 24 hour incubation of biofilms with daily media changes accounting for pH correction to 7. Changes in pH measurements in biofilm growth with 50 (A), 100 (B) and 200 (C) µg/ml HSA-AGE or HSA-C. Solid arrow indicates introduction of medium supplemented with HSA-AGE or HSA-C, with daily changes until day 10. Dotted arrow indicates start of recovery period where medium was reverted to SM until day 14. Results represent 3 experiments with pH measured for 6-8 biofilms per experiment (mean ± SD). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium.

4.2.2. Single species growth analysis to determine species responsible for changes observed in biofilms

In addition to analysis of pH changes during the course of the five species biofilm growth, the bacteria were grown individually in SM and SM supplemented with 100 µg/mL HSA-AGE or 100 µg/mL HSA-C. Media were inoculated with each species at the same proportion as used for HA peg inoculation and incubated anaerobically for 24 hours. The resulting bacterial suspension was diluted and plated as previously described for calculation of CFU. The pH of each suspension was also measured.

In unsupplemented SM, CFU counts of *P. gingivalis* and *P. intermedia* were significantly lower than those of *S. salivarius*, *A. naeslundii* and *F. nucleatum* (Figure 4.5A). This suggests a slower growth rate of *P. gingivalis* and *P. intermedia* in SM compared with the other species. Contrastingly, in medium supplemented with HSA-AGE, *P. intermedia* and *A. naeslundii* both had significantly higher CFU counts compared with SM and HSA-C. The increase in *A. naeslundii* grown with medium supplemented with HSA-AGE reflects the increase in growth observed in the five species HSA-AGE biofilms (Figure 4.5A).

F. nucleatum CFU counts in HSA-AGE media were significantly lower compared with SM but higher compared with HSA-C. *P. gingivalis* growth, on the other hand, was significantly higher in HSA-C media compared with both HSA-AGE and SM. Furthermore, no significant difference in *P. gingivalis* CFUs following growth in HSA-AGE medium and SM was observed (one-way ANOVA, Tukey, $p > 0.05$), perhaps suggesting HSA encourages *P. gingivalis* growth but modified HSA does not.

After a 24 hour incubation, *S. salivarius* CFUs were significantly lower in HSA-AGE supplemented growth medium compared with SM but significantly higher in HSA-C supplemented medium. This is partially reflected in the biofilm model where growth of *S. salivarius* is decreased in the presence of HSA-AGE.

Comparison of different bacterial suspensions under different growth conditions indicated a significantly lower pH following *A. naeslundii* growth in SM compared with both *S. salivarius* and *P. gingivalis* but no significant differences when comparing to *F. nucleatum* and *P. intermedia* (one-way ANOVA, Tukey, $p > 0.05$). This indicates that in SM, *A. naeslundii* and to a lesser extent *F. nucleatum* and *P. intermedia* reduced the pH of the media while *S. salivarius* and *P. gingivalis* maintain a neutral pH (Figure 4.5B).

In contrast, growth of *S. salivarius* and *A. naeslundii* in HSA-AGE supplemented SM caused a decrease in the pH of the culture. For *S. salivarius* this decrease was significant compared with growth in HSA-C and SM. In general the pH profiles of bacteria grown in

HSA-C and SM are the same and only S. salivarius growth in HSA-AGE caused a significant decrease in the pH compared with the two control groups.

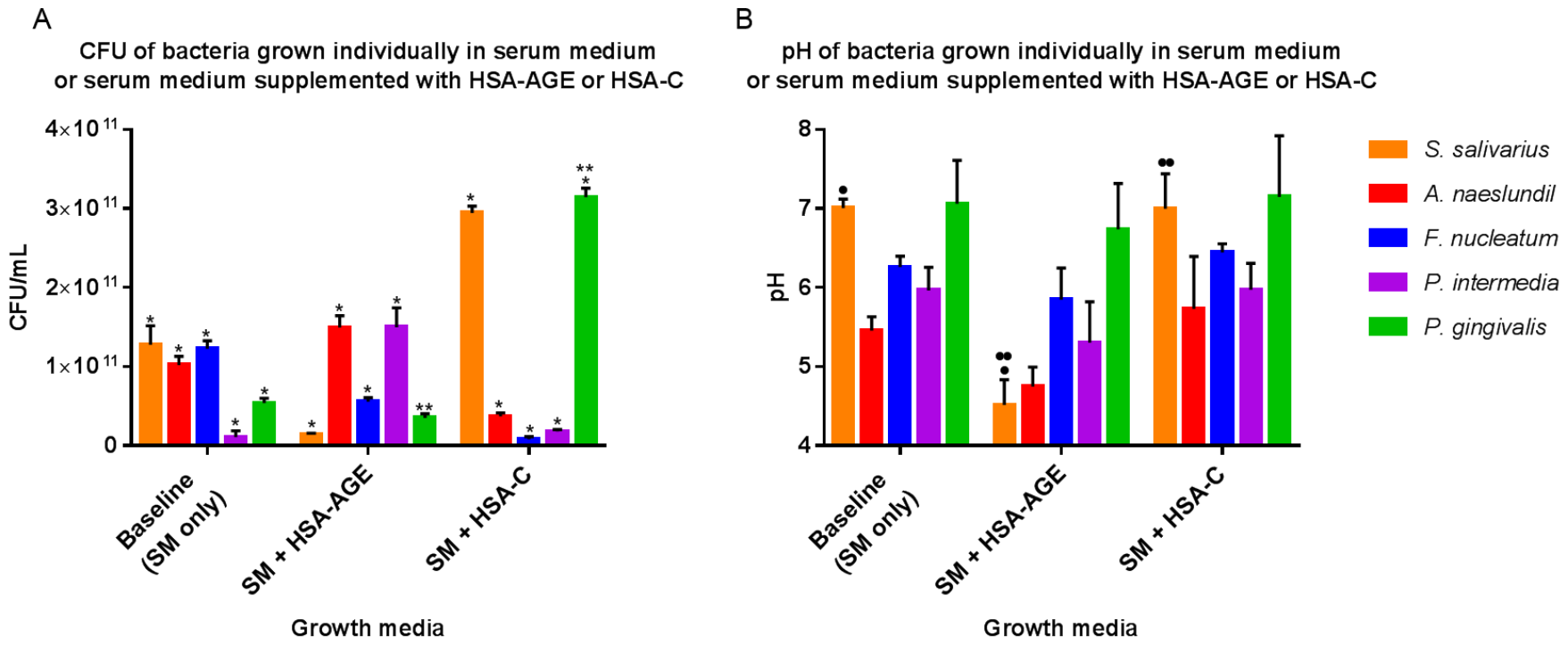


Figure 4.5: Growth and pH changes associated with *S. salivarius*¸ *A. naeslundii*, *F. nucleatum*, *P. intermedia* and *P. gingivalis* grown as single species cultures in SM supplemented with HSA-AGE or HSA-C. (A) CFU counts and (B) pH changes of bacteria cultured for 24 hours in SM or SM supplemented with 100 µg/mL HSA-AGE or 100 µg/mL HSA-C. Matching symbols indicate significant differences in CFU counts (*, **) or pH (•, ••) of each species when grown in different media (One-way ANOVA, Tukey, $p < 0.05$ n=3). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, SM: serum medium.

4.3. Discussion

The roles of AGEs in mammalian cells and tissues are relatively well studied, with particular attention paid to the effects of AGEs on inflammation and diseases such as diabetes and Alzheimer's. The presence of AGEs has been shown to initiate inflammatory responses and alter protein structures and functions. However, to date, little is known about the influence of AGEs in determining the composition of the human microbiome.

The aim of this study was to determine if HSA-AGE can cause changes in biofilm composition when using a five species model. The results demonstrated that in the presence of HSA-AGE, within the concentration range tested, the composition of the biofilm altered so that *A. naeslundii* outgrew *S. salivarius*, *F. nucleatum*, *P. intermedia* and *P. gingivalis*. This increased abundance of *A. naeslundii* was accompanied by the inhibition of *P. gingivalis*, *P. intermedia* and *F nucleatum* growth which could not be revived following withdrawal of HSA-AGE. Analysis of the growth medium every 24 hours prior to medium change and resulting pH correction, demonstrated the development of an acidic environment when a 6 day biofilm was incubated in HSA-AGE supplemented medium. This drop in pH over the HSA-AGE growth period could account for the lack of *P. gingivalis*, *P. intermedia* and *F. nucleatum* growth. The prolonged exposure to the acidic environment may have caused bacterial death of these species and could explain the inability to revive them following withdrawal of HSA-AGE. The detrimental effects of acid environments on *P. gingivalis*, *P. intermedia* and *F. nucleatum* has been demonstrated in previous studies which indicate intolerance of these species to low pH. *P. gingivalis* has the lowest tolerance and cannot survive below a pH of 6, while *P. intermedia* and *F. nucleatum* can tolerate pH 5 and pH 5.5, respectively (Takahashi *et al*., 1997).

Results from the single species experiments further supports the effect of pH on bacterial growth. When cultured in SM or SM supplemented with HSA-AGE/HSA-C, *A. naeslundii* lowered the pH of the environment. *S. salivarius*, on the other hand, only caused a significant decrease in pH when cultured with HSA-AGE. This suggests that the pH lowering ability of *S. salivarius* is based on its ability to metabolise AGEs resulting in acidic by-products. Given that the pH decrease associated with *A. naeslundii* was seen across all single species growth conditions but a *S. salivarius* associated decrease was only observed in HSA-AGE growth conditions, it is possible that in the five species model the decrease in pH which shifts the composition is a consequence of *S. salivarius* metabolism of HSA-AGE. To confirm this hypothesis further analysis is required. This can be achieved through NGS of *S. salivarius* cultures and investigation of metabolic

pathways, validated using culture dependent methods which utilise gene knockouts of identified proteins potentially involved in HSA-AGE metabolism. Furthermore, protein analysis (western blot, HPLC etc) of culture medium could help elucidate extent of HSA-AGE metabolism.

Interestingly, in the presence of HSA-C, an increase in *P. gingivalis* growth was observed. This could be indicative of the ability of *P. gingivalis* to utilise HSA as a carbon and nitrogen source through gingipain activity (Grenier *et al*., 2001). This was however not reflected in the model biofilm and could indicate that *S. salivarius* outcompete *P. gingivalis* for utilisation of HSA. The increase in *S. salivarius* growth in HSA-C cultures further validates this possibility. However, given the decrease in growth of *A. naeslundii* in HSA-C growth conditions compared with SM in single species experiments but an increase in abundance in the five species model it is evident that a more complex relationship exists. *S. salivarius* potentially metabolises HSA, producing products that *A. naeslundii* can utilise for growth when part of a biofilm.

It is clear that analysis of AGE metabolism by these bacterial species is required to fully understand their growth under the conditions of these experiments and elucidate the complex relationships in biofilms. This is particularly important when considering the potential changes in biofilm composition are a consequence of HSA-AGE metabolism. To date, few studies have investigated bacterial AGE metabolism and of these none have examined bacterial response to external AGE. Katz *et al*. (2010), identified a glycopeptidase (Gcp) involved in the prevention of AGE accumulation in *E. coli*. Using a Gcp depleted *E. coli* phenotype, they demonstrated a 60% increase in AGE accumulation through fluorescence analysis of the bacterial lysate. Although Gcp was demonstrated to be involved in AGE metabolism, the group also detected an increase in Amadori products (an intermediate of the Maillard reaction) and therefore suggested Gcp is involved directly in the metabolism of Amadori products. They proposed that the accumulation of Amadori products following Gcp depletion increases the potential for AGE development (Katz *et al*., 2010). A search of *E. coli* homologues and potential Gcp proteins (using the NCBI database) did identify the presence of Gcp in *S. salivarius*, however *in silico* protein analysis (using SignalP) showed lack of a signal peptide indicating the inability of Gcp to metabolise extracellular proteins. Further analysis is required in the organisms used in this study to determine if AGEs are transported into the cells and if the intracellular Gcp is involved in AGE metabolism. It is also possible that proteins with similar functions to Gcp which are secreted into the environment are present in the five species investigated in this study.

There is also the potential for AGEs to interact with specific receptors on the surface of bacteria to initiate endocytosis (such as CD36) or toxic responses (RAGE), similar to those seen in mammalian cells (Ott *et al*., 2014, section 3.1). However, an *in silico* protein search (using the NCBI database) for bacterial equivalents of RAGE and CD36 yielded no results. This suggests that if AGE metabolism is initiated following receptor activation, the bacterial receptors are distinct from those found on mammalian cells.

I propose that in the five species biofilm model, when HSA-AGE is introduced to the growth medium, both early colonisers (*A. naeslundii* and *S. salivarius*) metabolise the AGE lowering the pH to such an extent that the environment becomes too acidic for the growth of *P. ginigivalis, P. intermedia* and *F. nucleatum* (which have a pH tolerance of 6, 5.5 and 5, respectively). In SM and HSA-C conditions, the pH changes associated with *A. naeslundii* alone can potentially be negated by the action of *P. gingivalis*, *P. intermedia* and *F. nucleatum* (as all three have previously been shown to neutralise acids during growth) (Takahashi *et al*., 1997). However, in the presence of HSA-AGE the pH is lowered (through the combined action of *A. naeslundii* and *S. salivarius*) at a rate or to a pH where acid neutralisation is no longer effective and the late colonisers are killed. The acidic environment allows the *A. naeslundii* to flourish and outgrow but not entirely kill *S. salivarius*. Or perhaps the acidic pH as a direct consequence of AGE metabolism inhibits *S. salivarius* growth as indicated by a decrease or inhibition of growth in the single species experiments.

This simple model highlights the potential of HSA-AGE in influencing bacterial growth and provides an insight into how HSA-AGE metabolism may change pH and, as a consequence, biofilm composition. Further work is required to elucidate the pathways through which HSA-AGE is metabolised (perhaps through NGS) and the by-products of this metabolism (through methods such as mass spectrometry analysis). To fully understand the role of HSA-AGE in influencing the composition of the oral microbiota a more complex model is required, as not only does this model not entirely represent the complex dense microbiota of the mouth but also lacks representation of host-microbiota interactions and complex biochemical and immune changes associated with diabetes and periodontitis.

5. Complex biofilm

5.1. Introduction

Periodontitis and diabetes have long been understood to have a two-way relationship but the mechanisms linking these two diseases are complex and not fully understood. Of the many potential links (Figure 1.5 and section 1.4), the increased presence of AGEs in diabetes have been postulated to be involved in increased periodontal inflammation leading to increased infection susceptibility and the periodontitis immune associated tissue destruction (section 1.4.3). Although the effect of AGEs on mammalian cells is reasonably well studied (section 1.4.3 and 3.1), their influence on the colonisation and maturation of biofilms in the oral cavity is unknown. There are, however, studies demonstrating changes in the gut microbiota as a consequence of excessive AGEs and microbial metabolism of AGEs in the gut (Mills *et al*., 2008, Seiquer *et al*., 2014, Hellwig *et al*., 2015) or metabolism of AGEs in single species cultures (Chalova *et al*., 2012).

Considering the increased prevalence of HSA-AGE in the GCF of diabetics with periodontitis (Kajiura *et al*., 2014) and the results from the five species biofilm model (described in section 4) indicating a change in biofilm composition as a consequence of HSA-AGE addition to growth medium, it was hypothesised that HSA-AGE will also change the composition of a complex oral biofilm generated on a Calgary device using a physiological inoculum. This was tested in experiments described in this chapter. A proof of principle study was carried out to determine the use of this model as a method to investigate the role of HSA-AGE on the colonisation of HA coated pegs by pre-conditioning the pegs with various concentrations of HSA-AGE. This was based on the possibility of AGE adsorption onto the HA peg alongside other salivary proteins (section 1.1.1) thus potentially altering bacterial adherence and colonisation of the pegs. In addition, these preliminary experiments also aimed to establish the validity of the model to examine the changes in biofilm composition in response to HSA-AGE.

In addition to analysis of bacterial composition, inflammatory responses of TIGK cells initiated by the complex biofilms were also assessed. Previous studies have implicated excessive AGE concentrations in increased inflammatory responses of a variety of cell types (including oral epithelial and immune cells). In the oral cavity, the high AGE concentration conditions can potentially lead to uncontrolled inflammatory responses (including cytokine/chemokine release and ROS generation) which are implicated in exacerbation of periodontitis (Lalla *et al*., 2001, Vlassara, 2001, Wong *et al*., 2003, Santana *et al*., 2003, Cortizo *et al*., 2003, Preshaw and Taylor, 2011, Preshaw *et al*., 2012).

Although TIGK cells were shown to express RAGE (section 3), the previous experiments demonstrated no change in RAGE expression as a consequence of AGE and no changes in concentration of cytokines released following TIGK culture with varying concentrations of AGE (section 3.2.3). The presence of AGE has been correlated with increased cytokine release (section 1.4.3 and 3.1). These increases in cytokine concentrations have also recently been demonstrated to be further increased when coupled with bacterial co-stimulation (Hiroshima *et al*., 2018).Considering this, the generated biofilms were also assessed as a model to test the hypothesis that co-stimulation of TIGK cells with HSA-AGE and complex oral biofilms could lead to a rise in cytokine release and an increase in cellular ROS generation. As a preliminary measure in this study the biofilm-TIGK co-cultures were carried out in HSA-AGE supplemented cell culture media, at concentrations representing those found in the GCF of diabetics by Kajiura *et al* (2014).

5.2. Results

5.2.1. HSA-AGE associated changes on colonisation and mature biofilm composition using a complex biofilm model, analysed using selective media

5.2.1.1. Bacterial composition of inocula and colonisation (day 2) biofilms

Complex biofilms were grown on HA coated pegs using pooled saliva, plaque and tongue scraping inocula as described previously (section 2.13). Prior to inoculation of HA pegs, pooled inoculum was analysed on various selective media to determine its bacterial composition. The results indicated the physiological inoculum contained *Actinomyces*, *Streptococcus*, facultative anaerobes, anaerobes and Gram-negative anaerobes at approximately same abundances while counts of lactobacilli were observed to be 10^4 fold lower (Figure 5.1).

Prior to inoculation, HA pegs were pre-conditioned with artificial saliva containing concentrations of HSA-AGE to represent the consequence of diabetes-associated hyperglycaemia and health. Artificial saliva was supplemented with 100 µg/mL HSA-AGE to model the higher concentrations associated with diabetes and shown to be present in the GCF of diabetics (Kajiura *et al*., 2014). To simulate health, 1 µg/mL HSA-AGE (and 99 µl/mL HSA-C to account for protein differences) was added to the artificial saliva, as low levels of HSA-AGE are observed in the GCF of patients without hyperglycaemia (Kajiura *et al*., 2014). Pre-conditioned HA pegs were inoculated with the complex inoculum and incubated to determine the effects of HSA-AGE on colonisation (pegs harvested after 2 days) and mature biofilm composition (pegs harvested after 7 and 14 days).

Following incubation of the inoculum with HA pegs under anaerobic conditions for 2 days, growth of bacteria on selective media decreased from approximate CFUs of 10^9 – 10^{10} in the inocula to 10^7 – 10^8 in the biofilms, with the exception of Lactobacilli where growth decreased to below detectable levels (Figure 5.2). This suggests not all the bacteria (in the groups investigated) colonised and survived the two-day incubation. This loss of diversity is perhaps due to the limitations associated with the available binding sites (for colonisation) and changes in environmental conditions (such as nutrient availability and pH changes). Comparisons of HA pegs pre-conditioned with different HSA-AGE concentrations, indicated no significant differences in the viable counts of early biofilms (Figure 5.2). This suggests that pre-conditioning using HSA-AGE has limited effect of the colonisation of bacteria to HA pegs as assessed by culture on selective media.

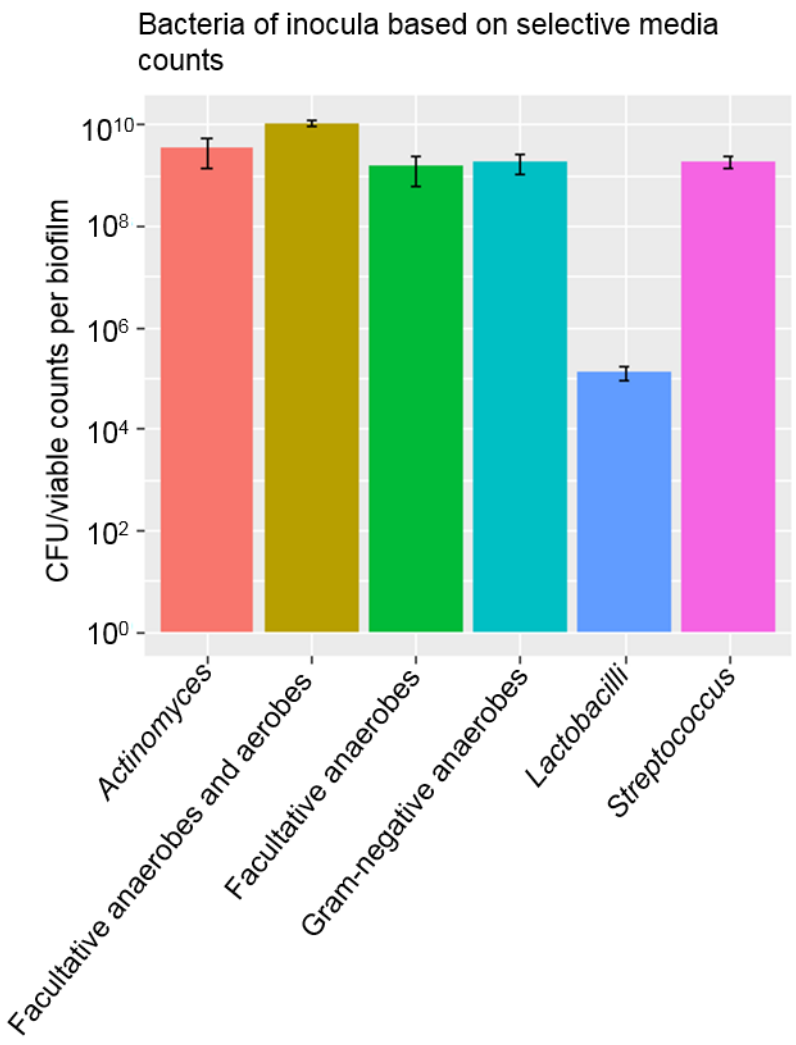


Figure 5.1: Proportion of selected genera and groups of bacteria in inoculum used for growth of complex biofilm. Colony forming units (CFUs) were determined based on viable counts using selective media. The Inoculum was generated using pooled saliva, plaque and tongue scrapings from 7 volunteers. Results are presented as average counts ± standard deviation and represent the inocula used for 3 experiments.

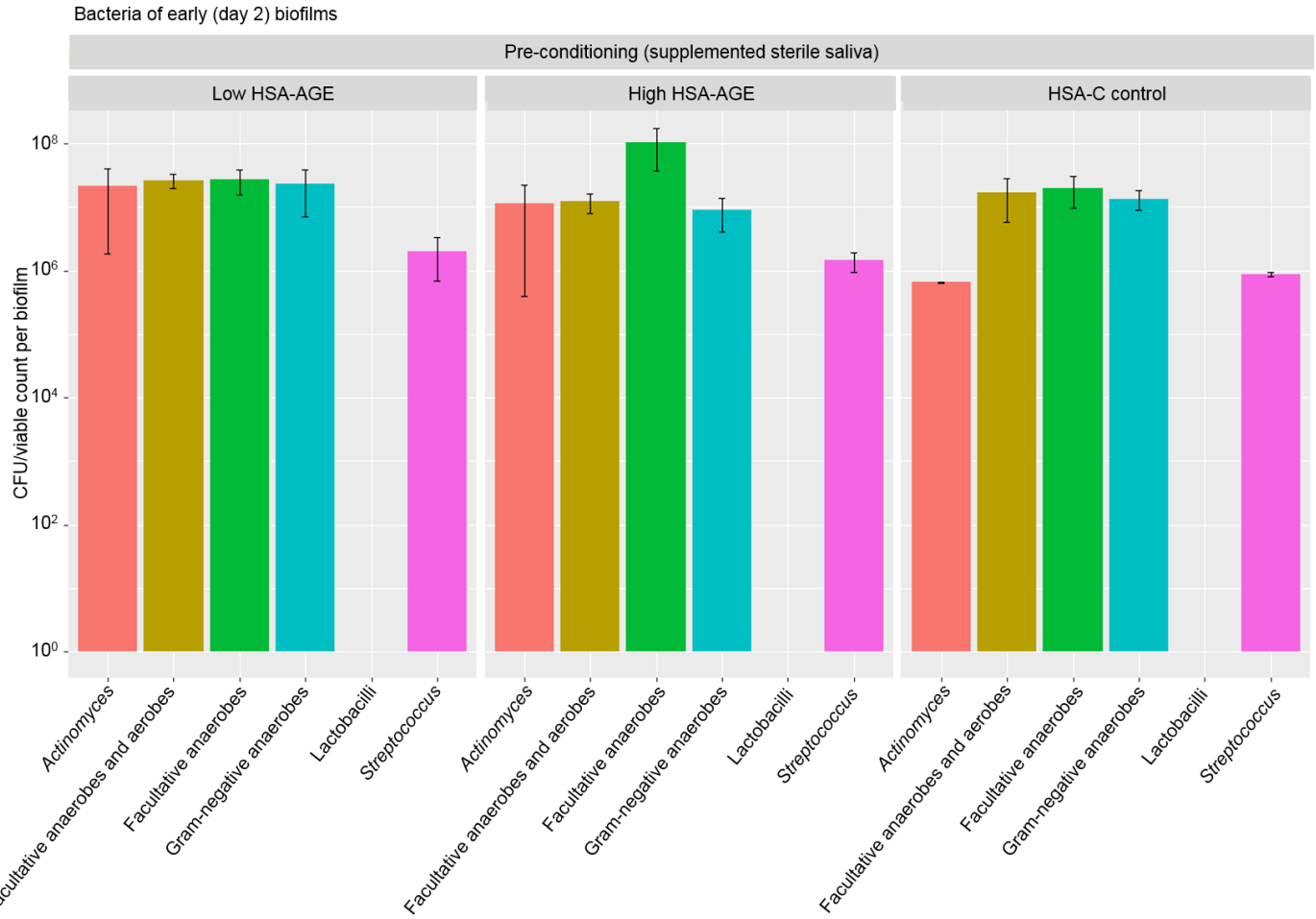


Figure 5.2: Proportion of selected genera and groups of bacteria in early (day 2) biofilms. Colony forming units (CFUs) on selective media for biofilms grown anaerobically following incubation of HA pegs with complex inoculum for 2 days. Results are presented as average counts ± SD and represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control.

5.2.1.2. Bacterial composition of mature (day 7 and day 14) biofilms

Medium for biofilm growth was supplemented with 100 µg/mL HSA-AGE and 1 µg/mL HSA-AGE with 99 µg/mL HSA-C. Serum medium (SM), as described previously (section 2.1), was growth medium supplemented with serum to reflect increased serum conditions associated with periodontitis. This allowed for the representation of periodontitis conditions in the absence of diabetes related HSA-AGE concentrations (low HSA-AGE pre-conditioning and low HSA-AGE supplemented growth medium – and Figure 5.4 , P). In addition, biofilms were grown to represent periodontitis conditions with the hyperglycaemic associated AGE increases observed in diabetes (high HSA-AGE pre-conditioning and high HSA-AGE supplemented growth media) as well as HSA-C control biofilms (and Figure 5.4 –HP and C respectively).

Biofilms were maintained as described previously and pegs harvested at days 7 and 14 (section 2.13). Following day 7 harvests, biofilms contained *Actinomyces*, *Streptococcus*, anaerobes, facultative anaerobes and Gram-negative anaerobes under all conditions. Lactobacilli were only within detectable ranges under some conditions, particularly in biofilms grown following peg pre-conditioning with low HSA-AGE concentrations ().

When comparing the prevalence of each bacterial group in the biofilms as a consequence of pre-conditioning, a significant decrease in facultative anaerobe counts was observed in pegs pre-conditioned with high HSA-AGE concentrations and grown in medium supplemented with 100 µg/mL HSA-AGE or HSA-C compared with unsupplemented SM (, row 2). This suggest that the combination of pre-conditioning with high HSA-AGE and the presence of HSA-AGE in the growth media decreases facultative aerobe growth. When pegs were pre-conditioned with low HSA-AGE concentrations a significant decrease in facultative aerobes was observed in biofilms grown with medium supplemented with HSA-C compared with low HSA-AGE and SM (, row 1). This suggests that when HA pegs are pre-conditioned with health associated HSA-AGE concentrations, the addition of HSA-C to the growth media hinders facultative anaerobe growth. Contrastingly, pegs pre-conditioned with HSA-C control, showed no difference in biofilm bacterial profiles regardless of growth medium conditions (, row 3). This suggests that at day 7, the pre-conditioning of HA pegs with HSA-AGE (both at health and hyperglycaemia mimicking concentrations) may impact long term biofilm composition and in particular alter the abundance of facultative anaerobes and perhaps Lactobacilli.

To determine the impact growth medium had on day 7 biofilm composition, changes in viable counts as a consequence of growth media were compared over different pre-conditioning (, columns). The only differences observed were in abundances of

facultative anaerobes. A dose dependent increase was observed in facultative anaerobes of biofilms grown on HA pegs pre-conditioned with HSA-AGE and grown in SM (*, , column 1). This suggests pre-conditioning of pegs with HSA-AGE selects for facultative anaerobes in day 7 biofilms. However, a significant decrease was observed in facultative anaerobe viable counts in biofilms grown on HSA-AGE pre-conditioned pegs compared with HSA-C control pre-conditioned pegs grown in media supplemented with high concentrations of HSA-AGE (*, , column 3).

Biofilms harvested at day 14 (Figure 5.4) showed a general increase, across all conditions, in viable counts of *Actinomyces*, anaerobes and facultative anaerobes compared with biofilms harvested at day 7 (). In addition, Lactobacilli were observed in biofilms grown under more conditions at day 14 compared with day 7 (Figure 5.4 and). When comparing abundances of the different bacterial groups across different conditions, significant changes were only observed in facultative anaerobes. Different growth media only affected facultative anaerobe growth in biofilms cultivated on low HSA-AGE pre-conditioned pegs (Figure 5.4, row 1). Comparisons of day 14 biofilms from HA pegs with different pre-conditionings resulted in differences only in facultative anaerobes; significantly higher in biofilms grown on pegs pre-conditioned with high HSA-AGE and grown under high HSA-AGE conditions (*, Figure 5.4).

Although pre-conditioning of pegs with HSA-AGE (both high HSA-AGE and low HSA-AGE at day 7, and low HSA-AGE at day 14) appears to impact facultative anaerobe growth in biofilms it is difficult to ascertain the role the growth medium has on these changes. Particularly when considering comparisons of biofilms representing consequences of hyperglycaemia and increased serum levels associated with periodontitis (HP) to those representing periodontitis serum levels alone (P) and controls (C) at day 7 and day 14 showed no significant differences in any bacterial group. This is possibly due to the limited nature of using selective media counts to determine biofilm composition.

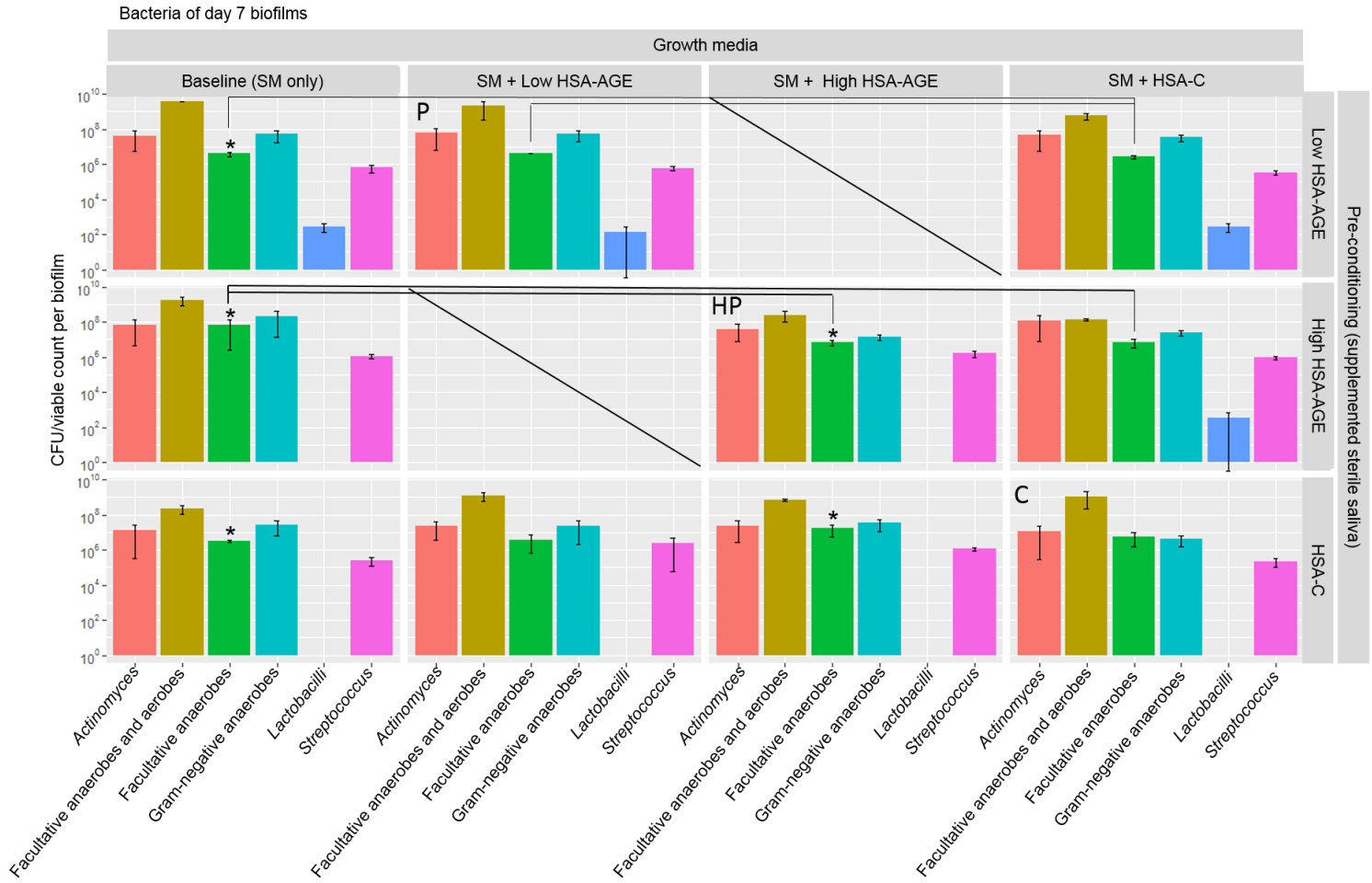


Figure 5.3: Proportion of selected genera and groups of bacteria in day 7 biofilms. Colony forming units (CFUs) on selective media for biofilms grown anaerobically following incubation of HA pegs with pooled saliva, plaque and tongue scrapings inoculum for 7 days. Results are presented as average counts ± standard deviation and represent 3 experiments with 6-8 biofilms harvested per experiment. Symbols represent significance in changes associated with pre-conditioning, lines represent significance associated with growth media (two-way ANOVA, Tukey, $p < 0.05$). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

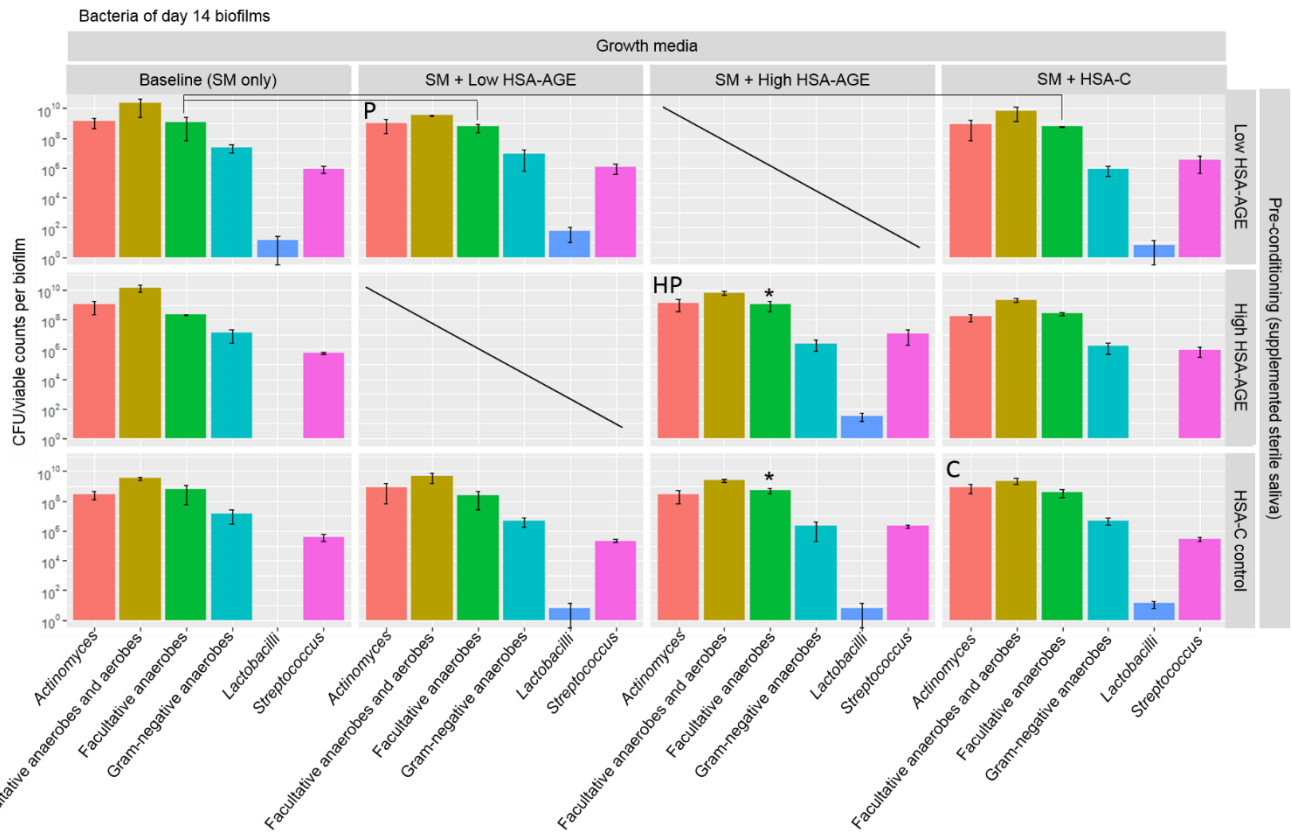


Figure 5.4: Proportion of selected genera and groups of bacteria in day 14 biofilms. Colony forming units (CFUs) on selective media for biofilms grown anaerobically following incubation of HA pegs with pooled saliva, plaque and tongue scrapings inoculum for 14 days. Results are presented as average counts ± SD and represent 3 experiments with 6-8 biofilms harvested per experiment. Symbols represent significance in changes associated with pre-conditioning, lines represent significance associated with growth media (two-way ANOVA, Tukey, $p < 0.05$). HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, P: biofilm representing periodontitis, HP: biofilm representing periodontitis and hyperglycaemia.

5.2.2. Next generation sequencing analysis of complex biofilms

5.2.2.1. Taxonomical analysis of complex biofilms

5.2.2.1.1. Sample richness, sample relatedness and biofilm composition at a phylum level

In addition to analysis of biofilm composition using culture methods, a more comprehensive analysis of inocula, early (day 2) and mature (day 14) biofilms, was carried out using NGS (section 2.5). Results were pre-processed (section 2.6) prior to separation of reads into rDNA and DNA. rDNA reads were analysed following the QIIME pipeline (section 2.6.1) while short DNA reads were *de novo*-assembled to form DNA contigs and analysed used MEGAN (section 2.6.2). All analysis was limited to bacterial reads only. Taxonomic composition of the biofilms was analysed using the operational taxonomic unit (OTU) counts based on alignments against the human oral microbiome database (HOMD) for rDNA reads and NCBI non-redundant protein databases for DNA contigs (section 2.6.1 and 2.6.2).

As an initial analysis step, alpha diversity of samples was carried out to determine if differences in richness (or the number of taxa in each sample) changed over time and under different growth conditions (Figure 5.5). Inoculum and early (day 2) biofilms were richer in taxa compared with mature biofilms (day 14). This is particularly evident when rDNA reads were used for alpha diversity measures (Figure 5.5A). When using DNA contigs for calculations, the results indicate a similar trend but with more variation in the diversity of mature biofilms (Figure 5.5B). The higher richness in early (day 2) biofilms compared with mature (day 14) biofilms is likely to be due to inocula including samples from many aspects of the mouth (including hard and soft oral tissues) and as such containing a plethora of bacteria, that although initially may colonise the HA pegs or have the potential to colonise, do not survive the growth conditions (pH, nutrient availability, oxygen levels etc) imposed in this system.

Alpha diversity measures based on DNA contigs from (day 2) biofilm samples are higher using some calculation methods (Shannon, Simpson and Inverse Simpson) for HSA-C control biofilms but this did not reach significance (two-way ANOVA, Tukey, $p > 0.05$) (Figure 5.5B). This suggests pre-conditioning of HA pegs did not alter the early colonisation. Similar results were observed when using rDNA reads for alpha diversity measures (Figure 5.5A). In mature biofilms (day 14) no pre-conditioning/growth medium dependent patterns were evident in richness of biofilms.

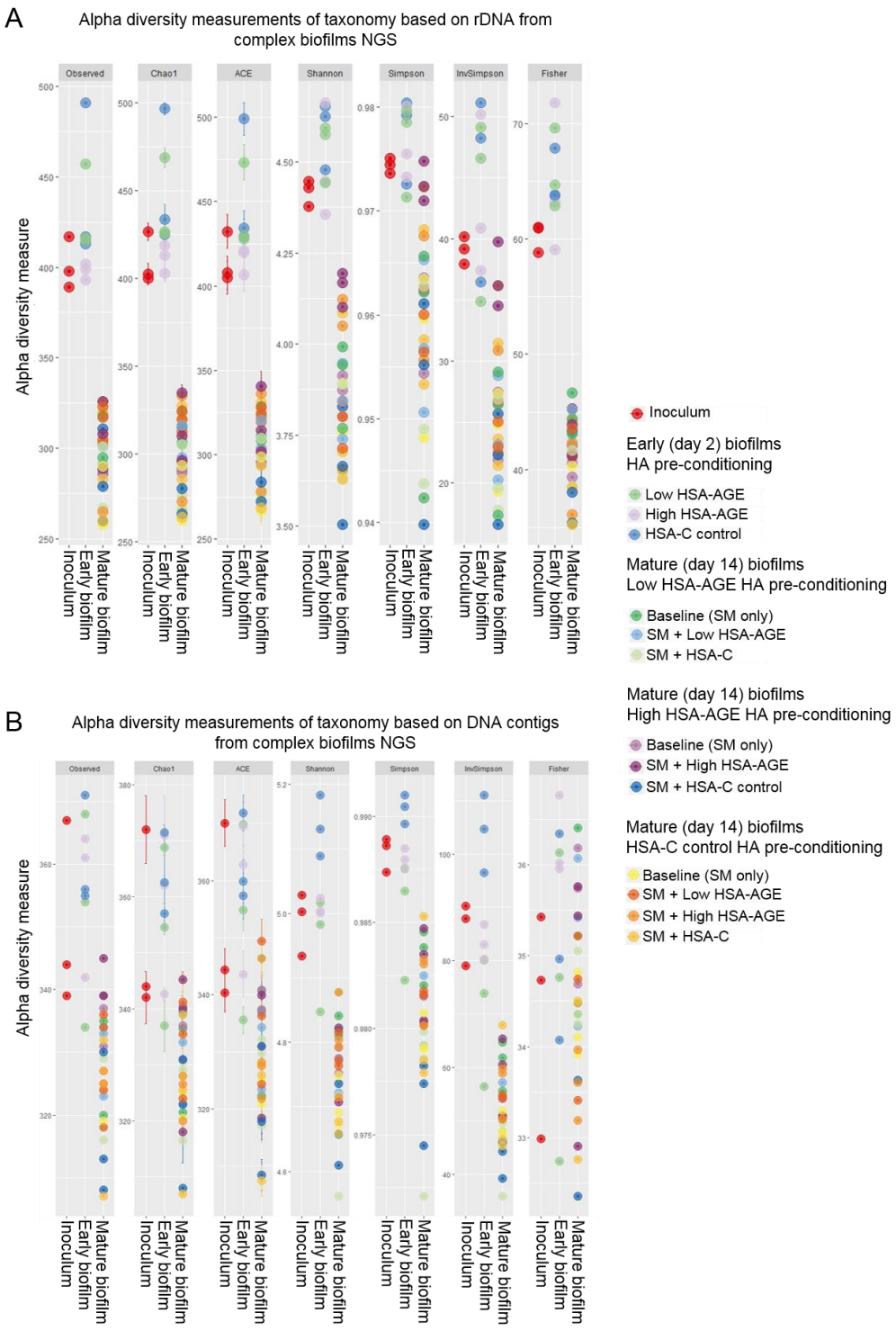


Figure 5.5: Alpha diversity of inoculum for complex biofilms, early biofilms (day 2) and mature biofilms (day 14). (A) Alpha diversity measures calculated based on rDNA alignments. (B) Alpha diversity measures calculated based on DNA contig alignments. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, SM: serum medium.

Principle coordinate analysis (PCoA) with Bray-Curtis dissimilarity plots were generated for comparison of sample relatedness. These results indicate that bacteria colonising pegs pre-conditioned with low HSA-AGE concentrations clustered loosely together and independently of bacteria colonising pegs pre-conditioned with high HSA-AGE concentrations and HSA-C (Figure 5.6). In addition, inoculum, early (day 2) biofilms and mature (day 14) biofilms all clustered independently of each other. This clustering was observed for both rDNA and DNA contig based analysis. Together these data suggest that communities from the inoculum, those able to colonise and those able to survive in a mature biofilm are all relatively different to each other.

Following analysis of sample relatedness, taxonomic composition at a phylum level using OTU counts from both rDNA and DNA contigs were analysed. Results are presented as percentage relative abundances (abundance of each OTU relative to all OTUs in each sample) to allow for a more accurate comparison between samples (Figure 5.7).

Relative percentage abundances based on rDNA reads indicate inocula consisted predominantly of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* with a smaller percentage of *Actinobacteria* and *Fusobacteria* accounting for a large portion of the remainder of the biofilm (Figure 5.7A). Following the two-day incubation of inoculum with HA pegs the predominance of *Proteobacteria* increased with a parallel decrease in *Bacteroidetes* when pegs were pre-conditioned with low HSA-AGE. However, pegs pre-conditioned with high HSA-AGE or HSA-C resulted in phylum profiles similar to inoculum profiles. In mature biofilms (day 14) percentage of *Proteobacteria* and *Fusobacteria* decreased while percentage of *Synergistetes* increased. This was regardless of pre-conditioning and growth media.

Analysis of OTUs based on DNA contigs had similar trends to that described for rDNA (Figure 5.7B). Although similar in patterns of relative abundances, a higher prevalence of *Actinobacteria* and *Fusobacteria* was observed. *Spirochaetes,* which were almost undetectable when using rDNA were observed at higher percentages when using DNA contigs, but only in mature biofilms (day 14). In addition, lower abundances of *Proteobacteria* were observed in analyses of inoculum and early biofilms (day 2) using DNA contigs, but higher percentages were seen in mature biofilms (day 14) compared with analysis based on rDNA reads. As well as the changes in the most abundant phyla, DNA contig based analysis also allowed the detection of less prevalent phyla such as *Chlamydiae*, *Cynobacteria*, *Tenericutes* and others.

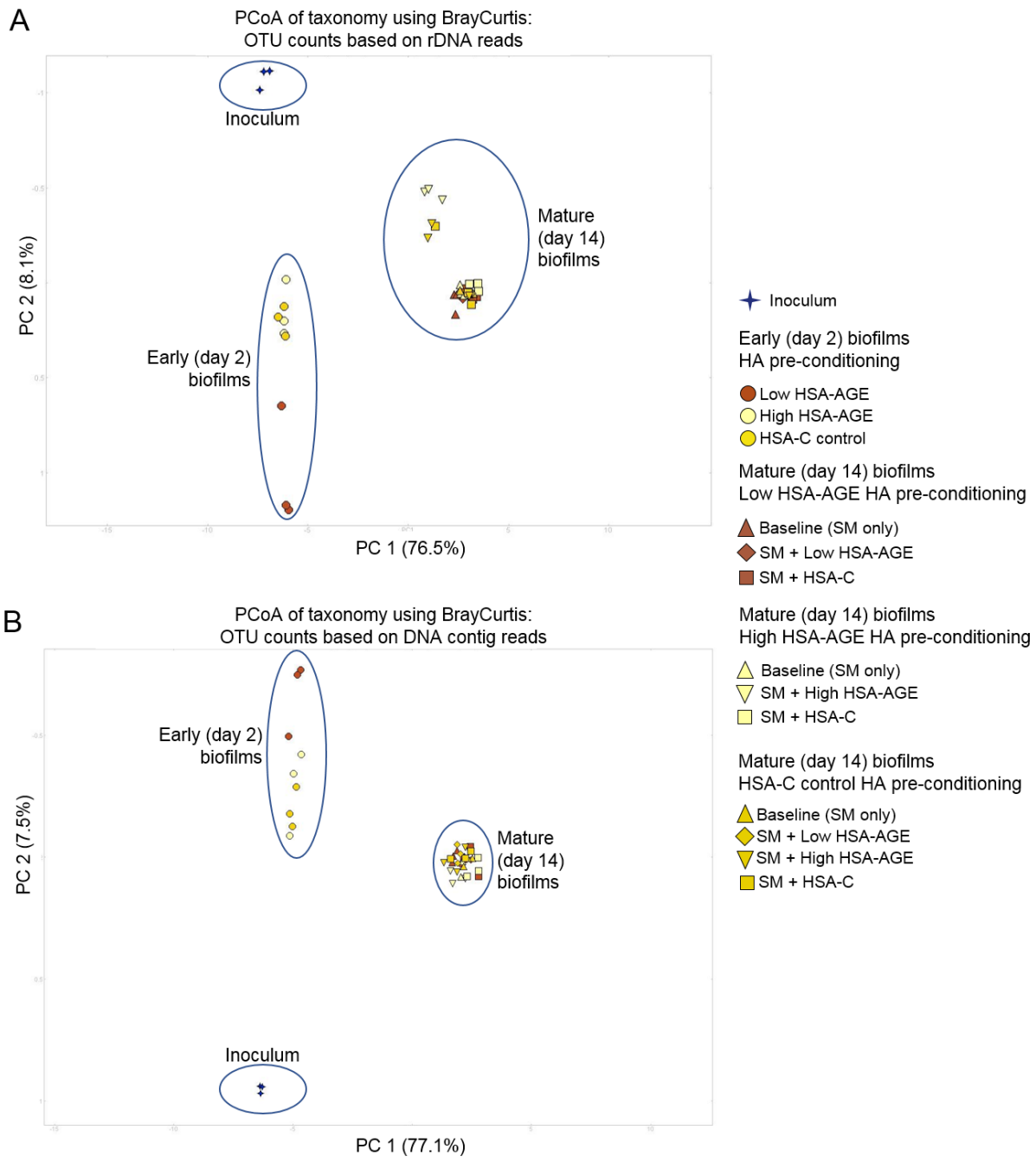


Figure 5.6: Principal component of analysis inoculum for complex biofilms, colonisation biofilms (day 2) and mature biofilms (day 14). (A) Plot for taxonomic clustering of inoculum/biofilms at a species level based on rDNA reads. (B) Plot for taxonomic clustering of inoculum/biofilms at a species level based on DNA contigs. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, SM: serum medium.

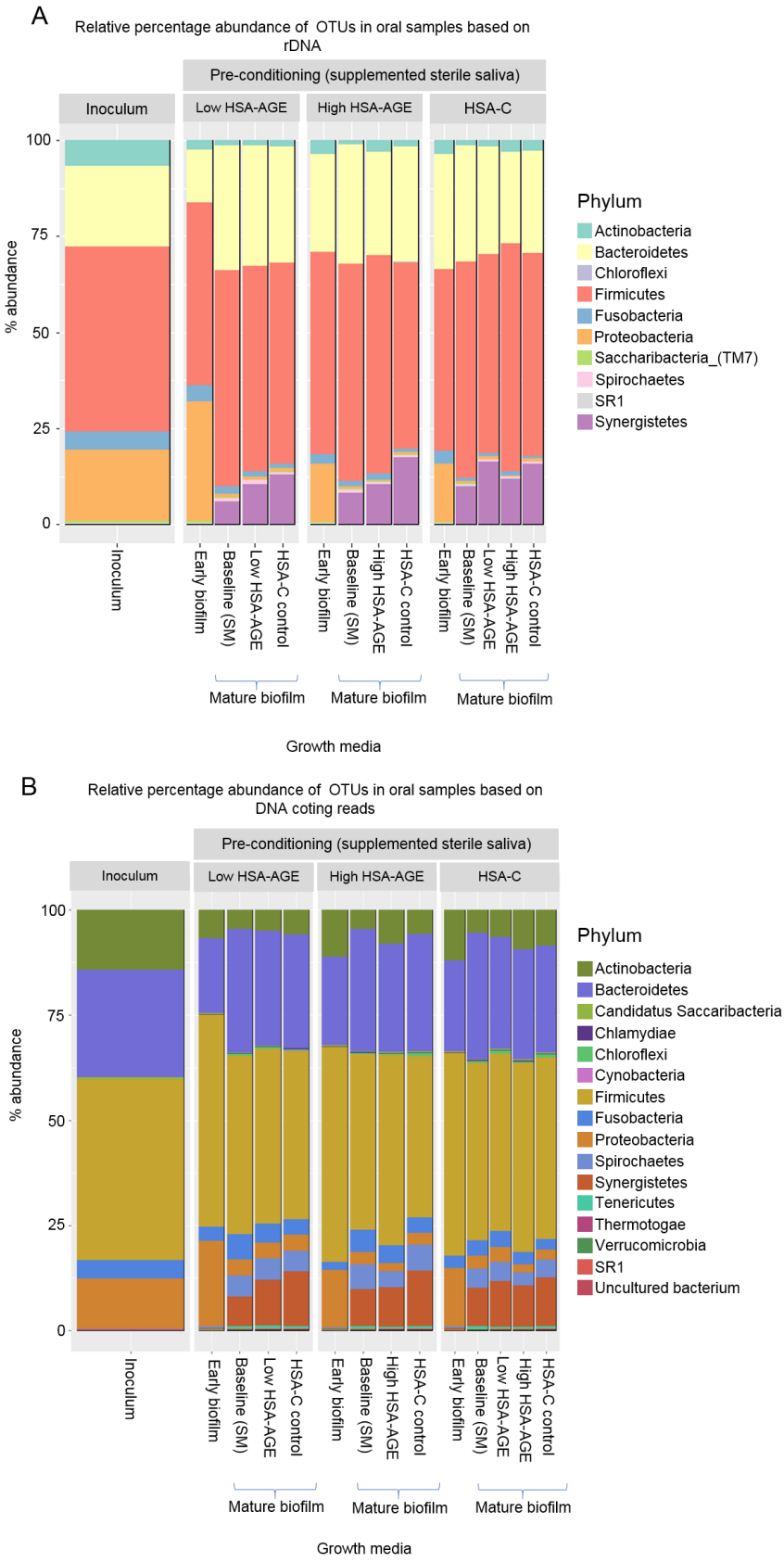


Figure 5.7: Relative percentage abundance of OTUs in inoculum, colonisation and mature biofilms. (A) Taxonomic percentage abundances based on rDNA reads. (B) Taxonomic percentage abundances based on DNA contigs. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

Considering comparisons between the various biofilms remained relatively unchanged between rDNA and DNA contig based taxonomical analysis, the detection of more phyla using the DNA contig method, and increased accuracy in using DNA based alignment methods (rather than rDNA/16S alignment methods) for metagenomics, DNA contigs were used for a more in-depth analysis of the taxonomy.

To get a clearer understanding of the changes in biofilm composition associated with pre-conditioning, OTU counts for both the inoculum and early (day 2) biofilms were analysed at a genera level, limited to the top 50 most abundant and excluding any unclassified taxa (Figure 5.8). The results indicated that *Streptococcus* accounted for the majority of the inoculum, followed by *Prevotella*, *Veillonella* and *Actinomyces* (Figure 5.8A).

Although *Streptococcus* was the most prevalent genus found in the inoculum accounting for approximately 17% of the genera present, following colonisation and growth on control pegs (pre-conditioned with HSA-C) and low HSA-AGE pegs, only between 9.5 and 11% of the early (day 2) biofilm were composed of *Streptococcus* (Figure 5.8A and Figure 5.8B). While prevalence of *Streptococcus* decreased in early (day 2) biofilms grown on high HSA-AGE pegs compared with the inoculum (decreasing to approximately 15%), the decrease was less than that seen under low HSA-AGE and control conditions. This perhaps suggests that in biofilms cultivated on high HSA-AGE pre-conditioned pegs, *Streptococcus* was able to colonise and grow more efficiently than under low HSA-AGE and control conditions. *Prevotella* appeared to follow a similar trend, but with lower abundances in all conditions compared with *Streptococcus*.

Prevalence of *Veillonella* decreased from approximately 8% in the inoculum to approximately 6% of control and high HSA-AGE early (day 2) biofilms (Figure 5.8A and Figure 5.8B). Interestingly, under low HSA-AGE conditions, early (day 2) biofilms had a decrease in *Veillonella* to approximately 3%. This perhaps suggests that while high concentrations of HSA-AGE do not impact colonisation compared with the control, low concentrations of HSA-AGE hinder *Veillonella* adherence to pegs pre-conditioned with health associated concentration of HSA-AGE (Figure 5.8B). In addition to the potential direct effects of HSA-AGE, it is possible that low HSA-AGE concentrations encourage the adherence or growth of other bacterial species which in turn hinder the growth of *Veillonella.* In contrast, *Actinomyces* appeared to be unaffected by pre-conditioning and decreased to approximately the same relative abundance in all three pre-conditioning groups compared with the inoculum. Similarly, *Fusobacterium* was observed at comparable percentages in low HSA-AGE early (day 2) biofilms and control early (day

2) biofilms to those seen in the inoculum, but growth appeared to be hindered by higher concentrations of HSA-AGE.

While the abundances of some genera decreased in early (day 2) biofilms compared with inocula, an increase in abundances was observed with other genera (Figure 5.8B). For example, relative percentage abundance of *Neisseria* increased from approximately 2.5% seen in the inoculum to approximately 5% in biofilms grown on pegs pre-conditioned with HSA-AGE and 3.5% in control pegs. This perhaps suggests HSA-AGE encourages colonisation of *Neisseria* in both HSA-AGE concentrations.

In some cases, genera not observed at all in the top 50 genera of the inoculum were detected in early (day 2) biofilms (Figure 5.8B). These include *Selenomonas*, *Campylobacter*, *Catonella* and *Oribacterium*. In all four cases, abundances were higher in low HSA-AGE early (day 2) biofilms with few differences observed between HSA-C and high HSA-AGE early (day 2) biofilms perhaps suggesting low HSA-AGE concentrations produced conditions to encourage early colonisers either directly (producing binding sites or nutrients) or indirectly (encouraging binding/growth of bacteria which in turn produce conditions for binding or growth).

To determine if changes observed reached significance, differential abundances of genera and species were plotted as log2 fold change between groups, with significance determined using a Wald test ($p < 0.1$) and Benjamini-Hochberg adjusted p-value (section 2.6). The plots show genera in low HSA-AGE early (day 2) biofilms (Figure 5.9) and high HSA-AGE early (day 2) biofilms (Figure 5.9B) which are found at higher or lower abundances compared with HSA-C. Differential abundances of genera in high HSA-AGE early biofilms compared with low HSA-AGE early (day 2) biofilms were also plotted (Figure 5.9C). As described previously, a lower abundance of *Streptococcus*, *Veillonella* and *Prevotella* was observed in low HSA-AGE early (day 2) biofilms compared with control (Figure 5.8B and Figure 5.9A). Interestingly, despite a lack of obvious difference in overall *Actinomyces* genera percentages (Figure 5.8A), certain species were found to have significantly lower abundance in low HSA-AGE early (day 2) biofilms compared with HSA-C control biofilms. In addition, a series of lower abundant genera (not in the top 50) were shown to be significantly decreased in low HSA-AGE early (day 2) biofilms compared with control early (day 2) biofilms. The results also indicated a significant increase in species from *Neisseria*, *Catonella*, *Campylobacter* and *Fusobacterium* (Figure 5.9A).

When looking at genera in high HSA-AGE early (day2) biofilms compared with control biofilms fewer significant differences were observed (Figure 5.9B). Interestingly, species

from the *Treponema* genus were shown to be significantly decreased in high HSA-AGE early biofilms in comparison to control early biofilms. This decrease was also seen with *T. denticola*; a species generally associated with periodontitis. Contrastingly, *P. intermedia* (also associated with periodontitis) appeared to be increased in prevalence.

The differences in biofilm composition of low HSA-AGE early (day 2) biofilms to high HSA-AGE early (day 2) biofilms were also analysed (Figure 5.9C). There was a significant decrease in the abundance of *Treponema*, *Campylobacter*, *Fusobacterium*, *Haemophilus* and *Neisseria* in biofilms cultivated on pegs pre-conditioned with high HSA-AGE compared with biofilms grown on pegs pre-conditioned with low HSA-AGE, but a significant increase in the abundances of *Veillonella*, *Streptococcus*, *Prevotella* and *Actinomyces*. This was of particular interest given the association of the observed decreased species with periodontitis progression (section 1.1.2) and suggests that high HSA-AGE concentrations select for genera associated with periodontal health (such as *Streptococcus* and *Actinomyces*).

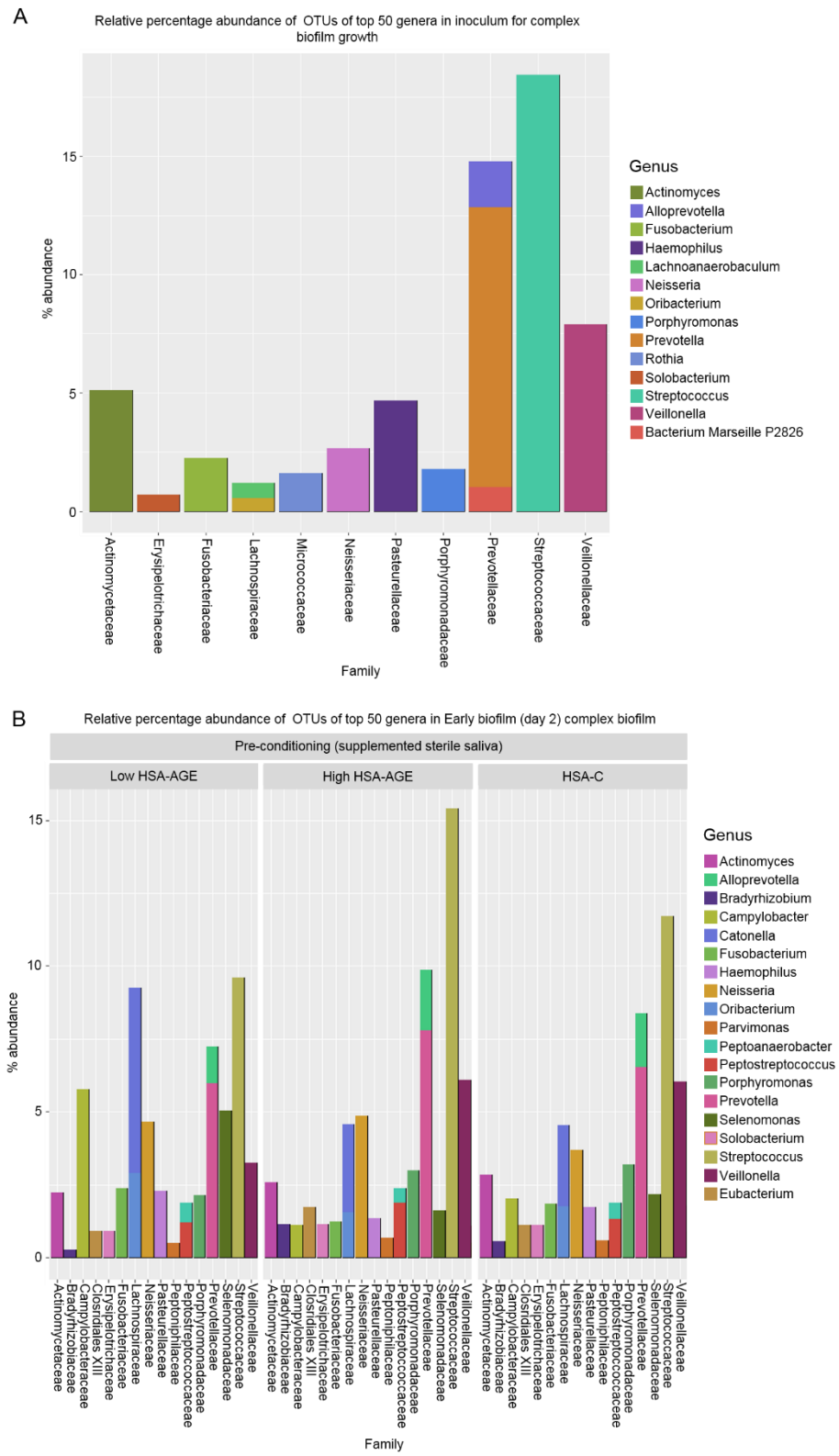


Figure 5.8: Relative percentage abundance of top 50 genera in inoculum (A) for complex biofilms and early (day 2) biofilms (B). Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

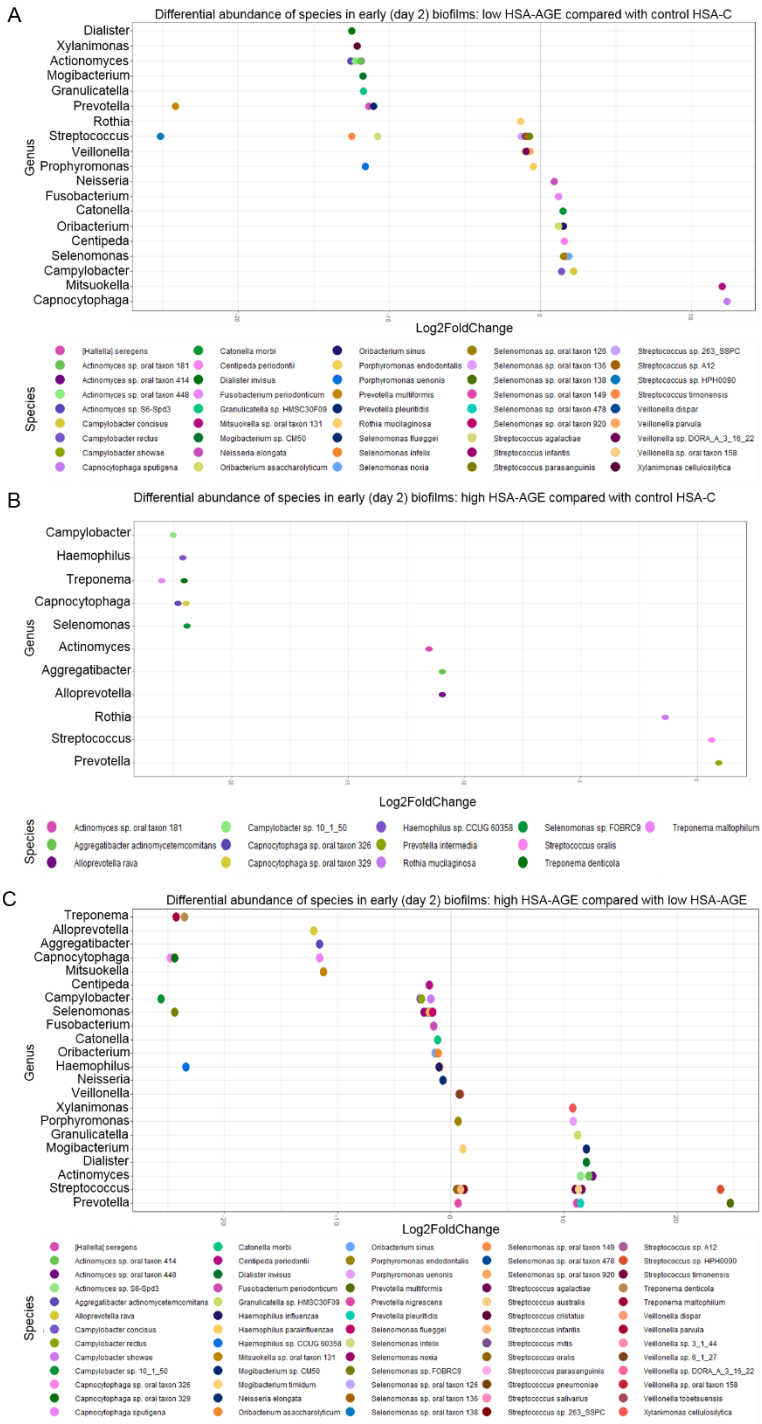


Figure 5.9: Differential abundance of genera in early (day 2) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

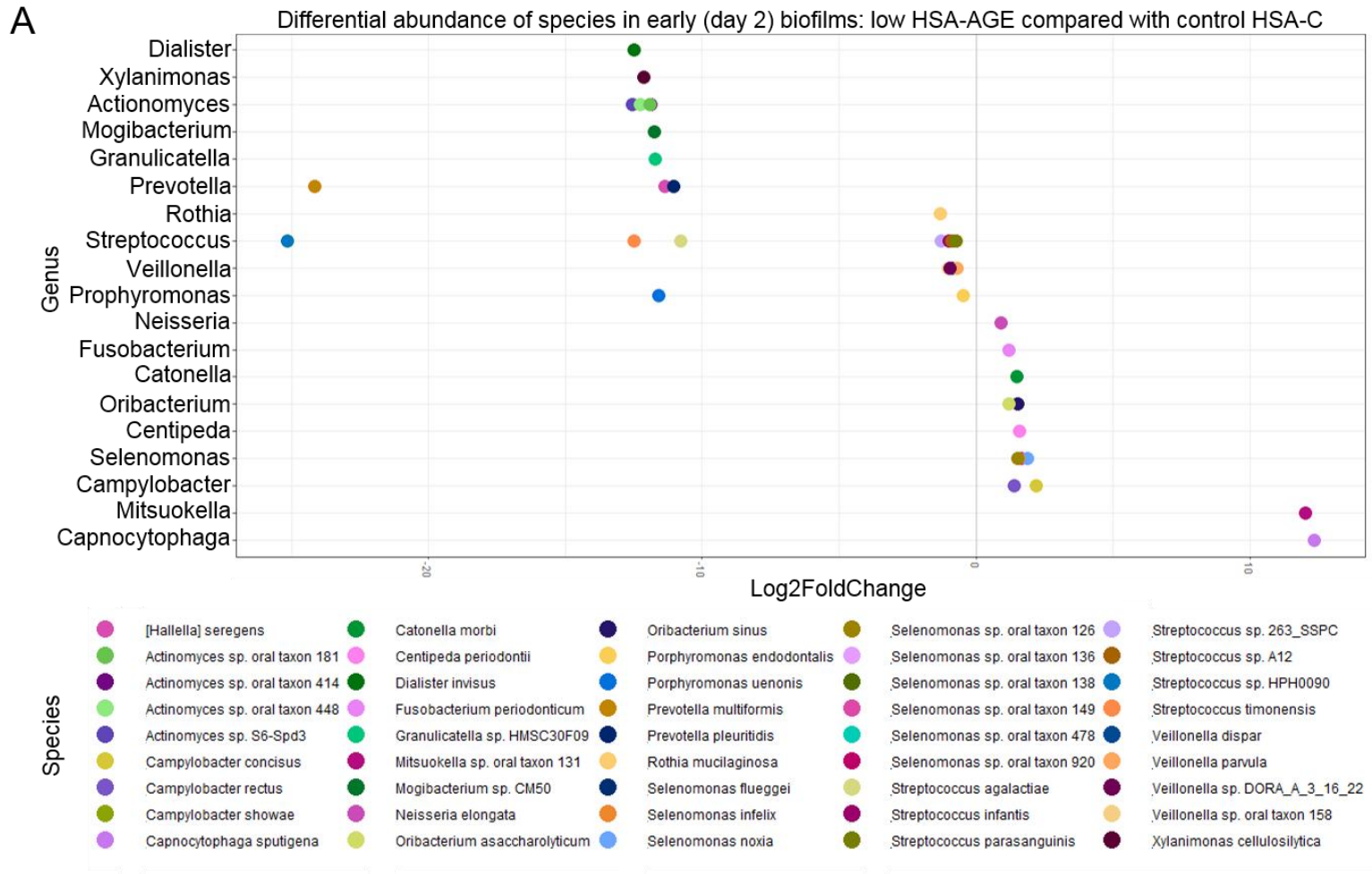


Figure 5.9A: Differential abundance of genera in low HSA-AGE early (day 2) biofilms compared with control HSA-C early (day 2) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

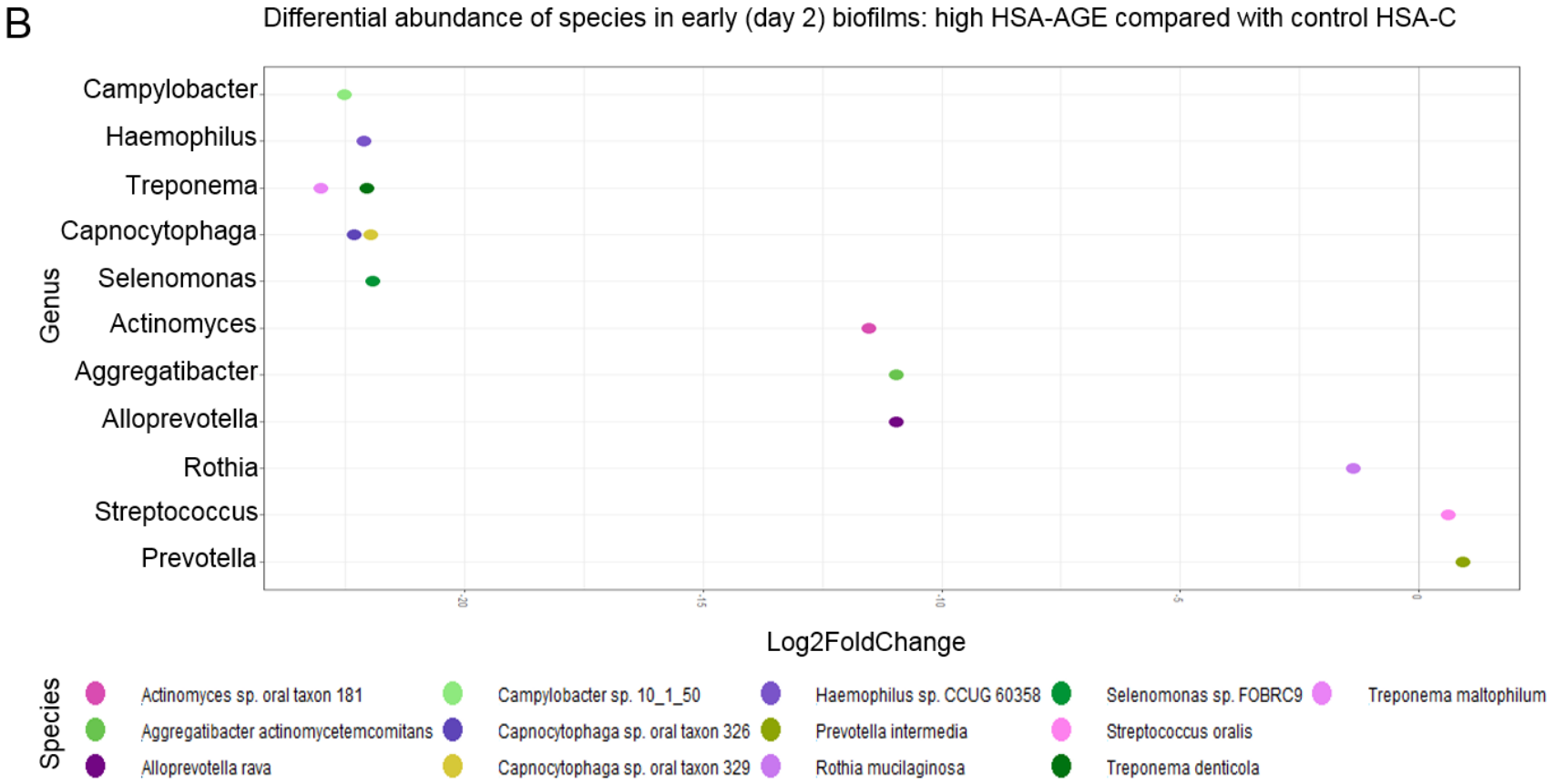


Figure 5.9B: Differential abundance of genera in high HSA-AGE early (day 2) biofilms compared with control HSA-C early (day 2) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

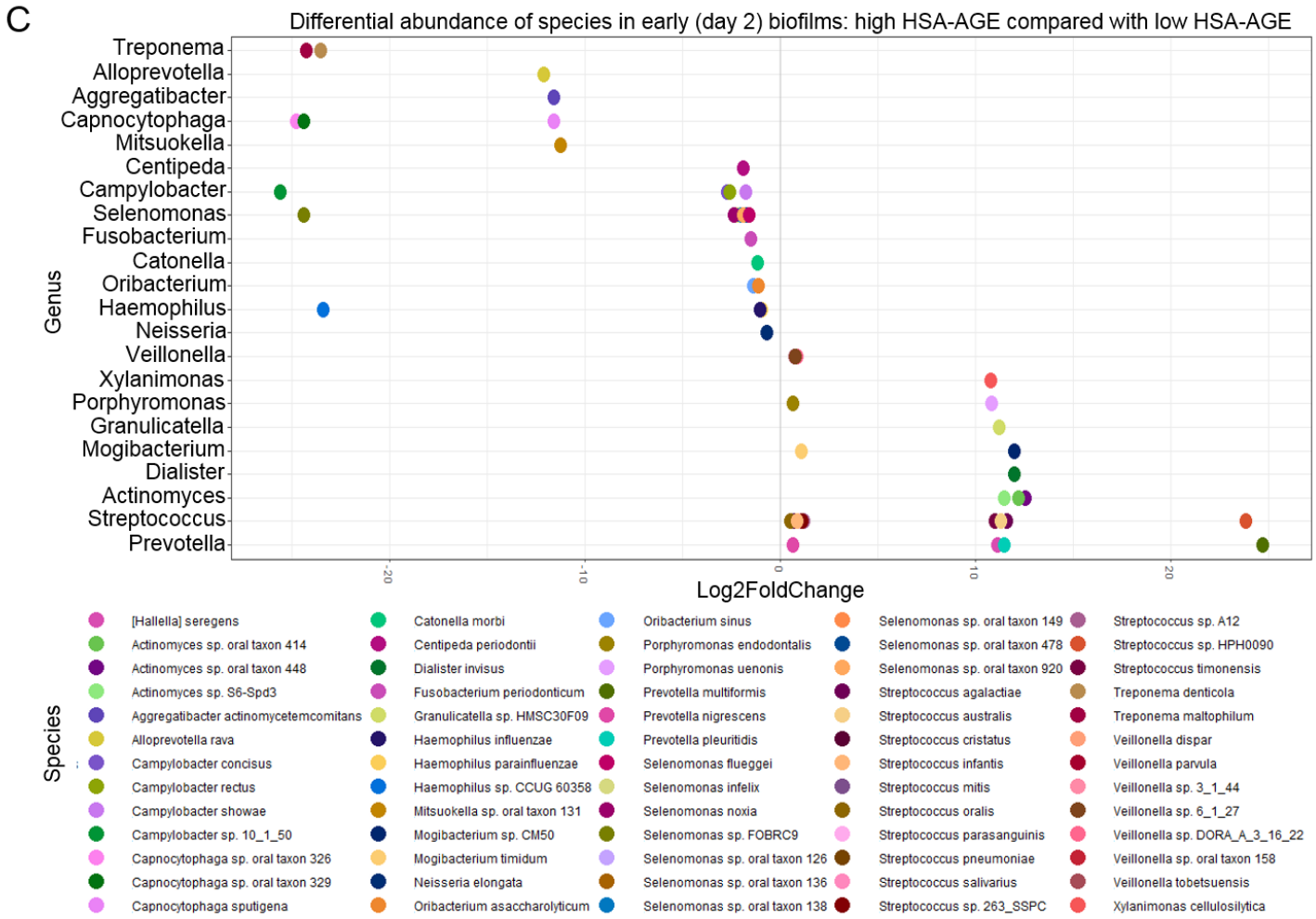


Figure 5.9C: Differential abundance of genera in high HSA-AGE early (day 2) biofilms compared with low HSA-AGE early (day 2) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

5.2.2.1.4. Taxonomic composition of mature (day 14) biofilms

Other biofilms were allowed to reach maturity prior to harvesting at day 14 (mature biofilms) and analysis using NGS. As with early (day 2) biofilms, for clarity, analysis was limited to the top 50 most abundant genera (Figure 5.10). The results appear to show limited differences in mature (day 14) biofilm composition. Differences observed were minor with the most obvious being those seen in abundances of Porphyromonadaceae, Synergistaceae and Peptostreptococcaceae families.

Differential abundances were plotted as described previously to determine significant differences in mature biofilms (section 2.6). Biofilms representing consequences of hyperglycaemia and increased serum levels associated with periodontitis (HP) and biofilms representing periodontitis serum levels alone (P) were compared with control biofilms (C) (Figure 5.10A and Figure 5.10B). In addition, HP biofilms were compared with P mature (day 14) biofilms (Figure 5.10C). Lower concentrations of HSA-AGE encouraged the growth of some periodontitis associated genera (including *Campylobacteria*, *Treponema*, *Porphyromonas* and *Fusobacterium*) when compared with HSA-C control mature (day 14) biofilms and hindered the growth of *Streptococcus* (Figure 5.10A). On the other hand, HP mature (day 14) biofilms showed significantly higher prevalence of *Prevotella salivae* while other members of the *Prevotella* genera (*P. oralis, P. intermedia*) were observed at a lower abundance compared with C mature (day 14) biofilms (Figure 5.10B). In addition, HP biofilms compared with C biofilms indicated that a higher concentration of HSA-AGE significantly hindered the growth of *T. denticola*, *Porphyromonas catoniae*, *Porphorymonas endodontalis* and *Fusobacterium equinum* while encouraging the growth of various *Veillonella* species (genera generally associated with a healthy periodontium), *Campylobacter concisus*, *Porphyromonas sp oral taxon* 279*, F. nucleatum* and *Haemophilus parainfluenzae* (Figure 5.10B).

29 species (including *Fusobacterium periodonticum*, *Haemophilus influenza*, *T. denticola*¸*T. forsythia* and various *Porphyromonas* species) were lower in abundance in HP biofilms and 24 species (including *Veillonella*, *Prevotella* and *Streptoccocus* species) had increased prevalence. As with early (day 2) biofilms, the presence of higher HSA-AGE concentration in the pre-conditioning of pegs and during biofilms growth selected for genera associated with periodontal health (such as *Streptococcus* and *Veillonella*), while hindering growth of species typically associated with severe periodontitis (*T. forsythia* and *T. denticola*).

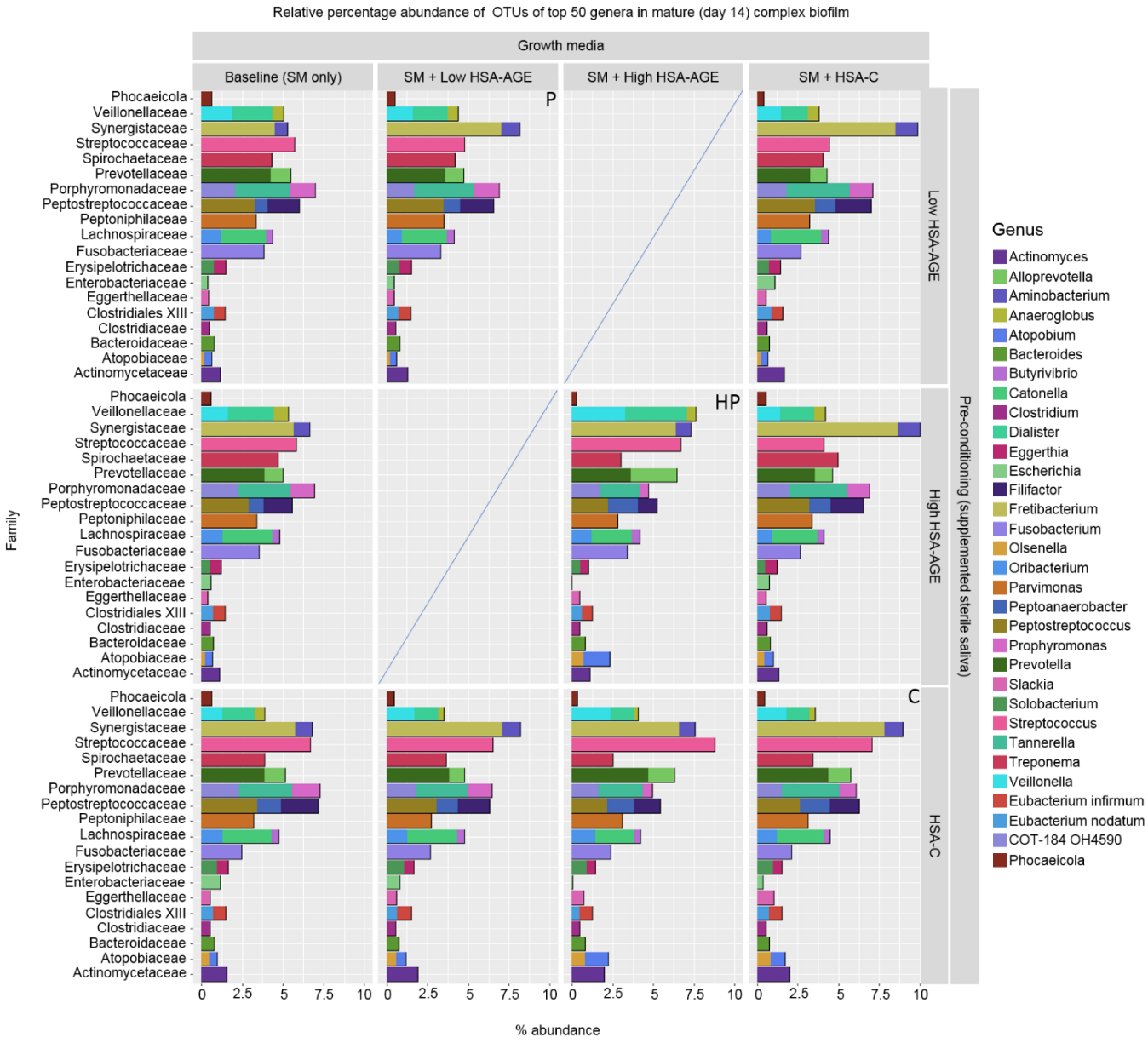


Figure 5.10: Relative percentage abundance of top 50 genera in mature (day 14) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

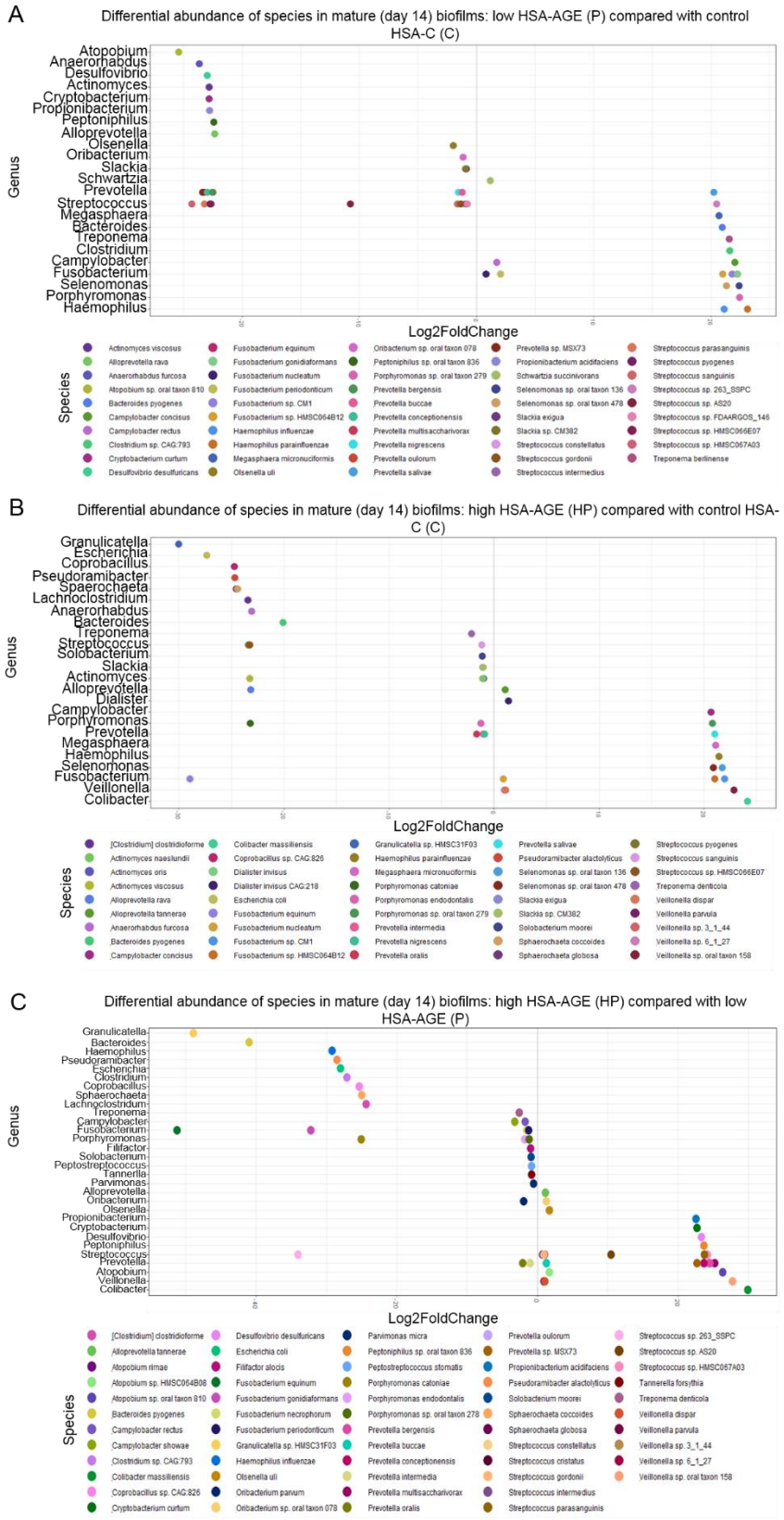


Figure 5.11: Differential abundance of genera in mature biofilms (day 14). Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

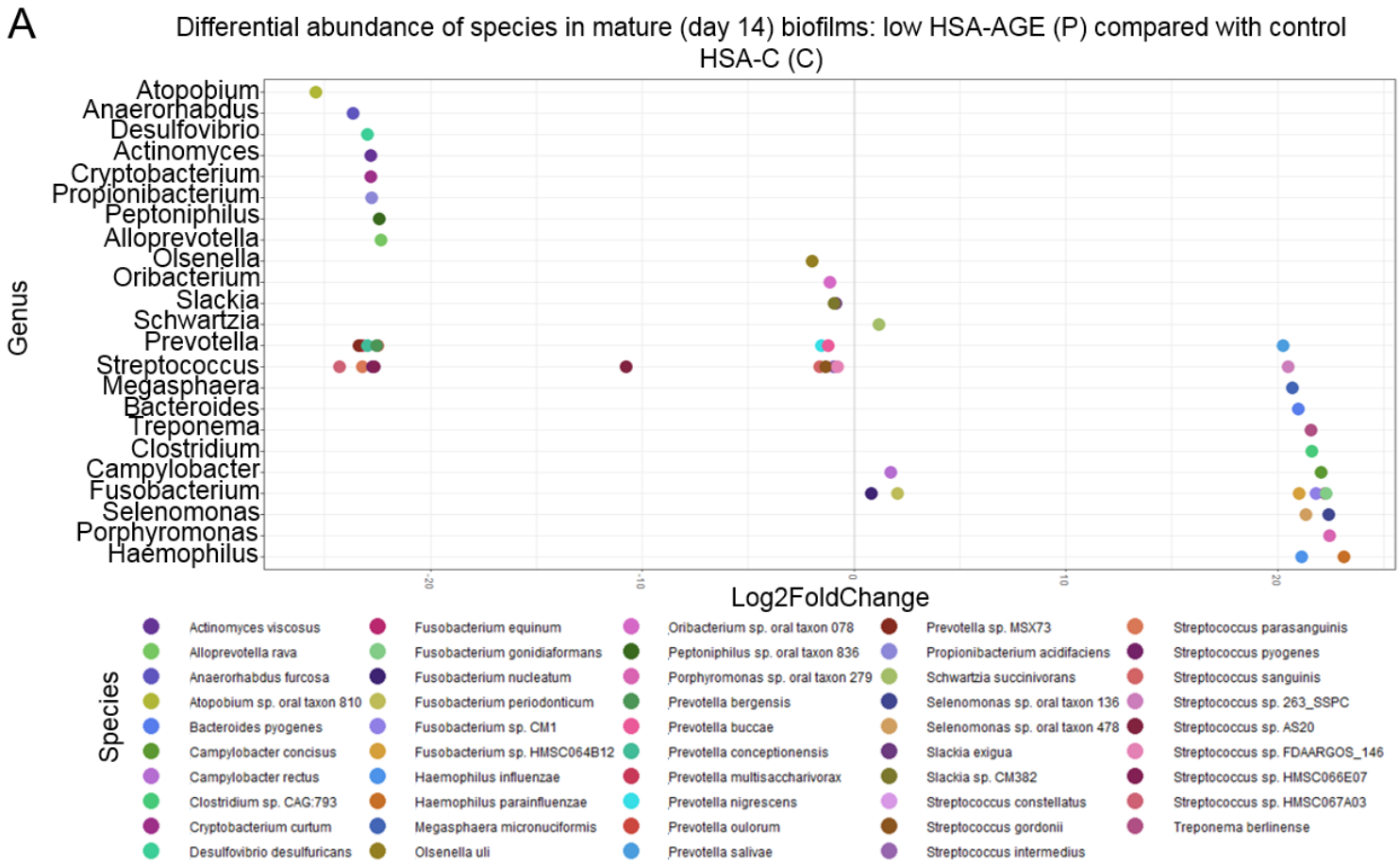


Figure 5.11A: Differential abundance of genera in low HSA-AGE mature (day 14) biofilms compared to control HSA-C mature (day 14) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

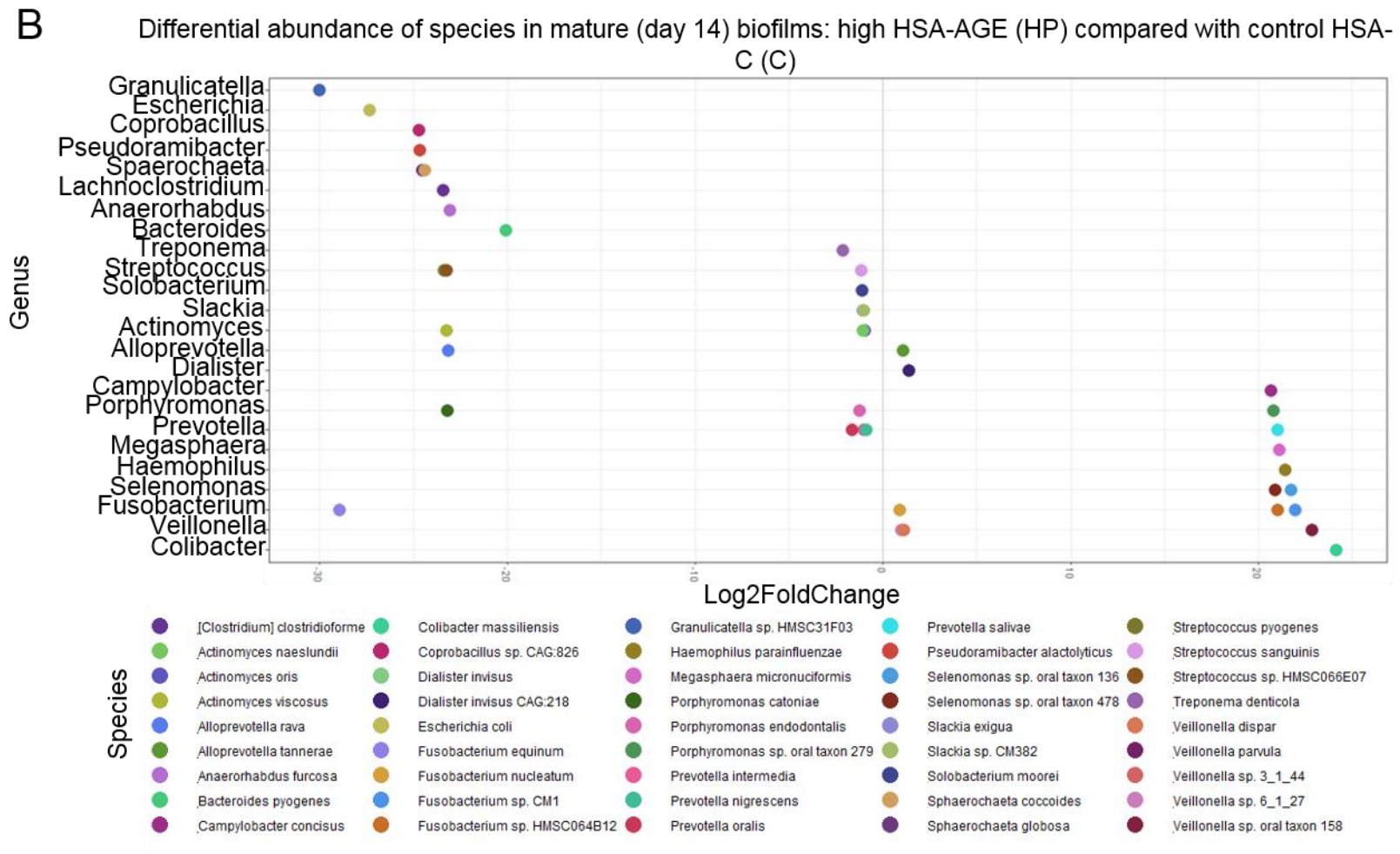


Figure 5.11B: Differential abundance of genera in high HSA-AGE mature (day 14) biofilms compared to control HSA-C mature (day 14) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

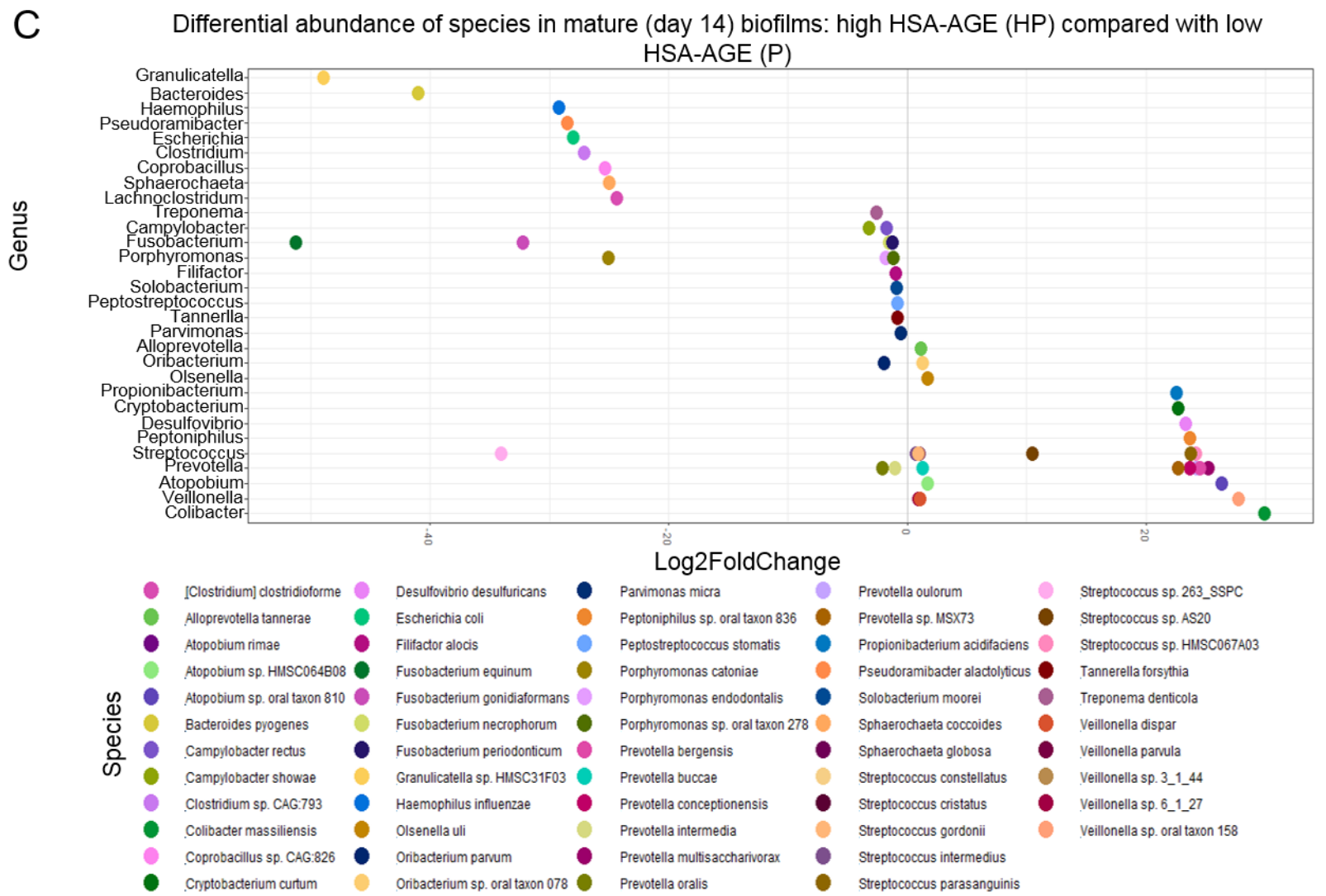


Figure 5.11B: Differential abundance of genera in high HSA-AGE mature (day 14) biofilms compared to low HSA-AGE mature (day 14) biofilms. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

5.2.2.2. Metagenomics functional analysis of complex biofilms

In addition to the above described taxonomic analysis, determination of overall functional profile of the microbiota in these environments was also established. Short DNA reads were assembled into contigs prior to being aligned against the NCBI non-redundant protein database. The resulting protein assignments were then annotated using SEED and EggNOG (section 2.6).

As with taxonomical analysis, a PCoA using the Bray-Curtis distribution was carried out for counts based on both SEED and EGGNOG functions. PCoA using both databases indicate distinct clustering of inocula, early (day 2) biofilms and mature (day 14) biofilms with no discernible patterns between different pre-conditionings and growth media (Figure 5.12). Furthermore, no significant differences in functional profiles (Wald test, $p < 0.1$) were observed between the various early (day 2) biofilms and the mature (day 14) biofilms (Figure 5.14 and Figure 5.13).

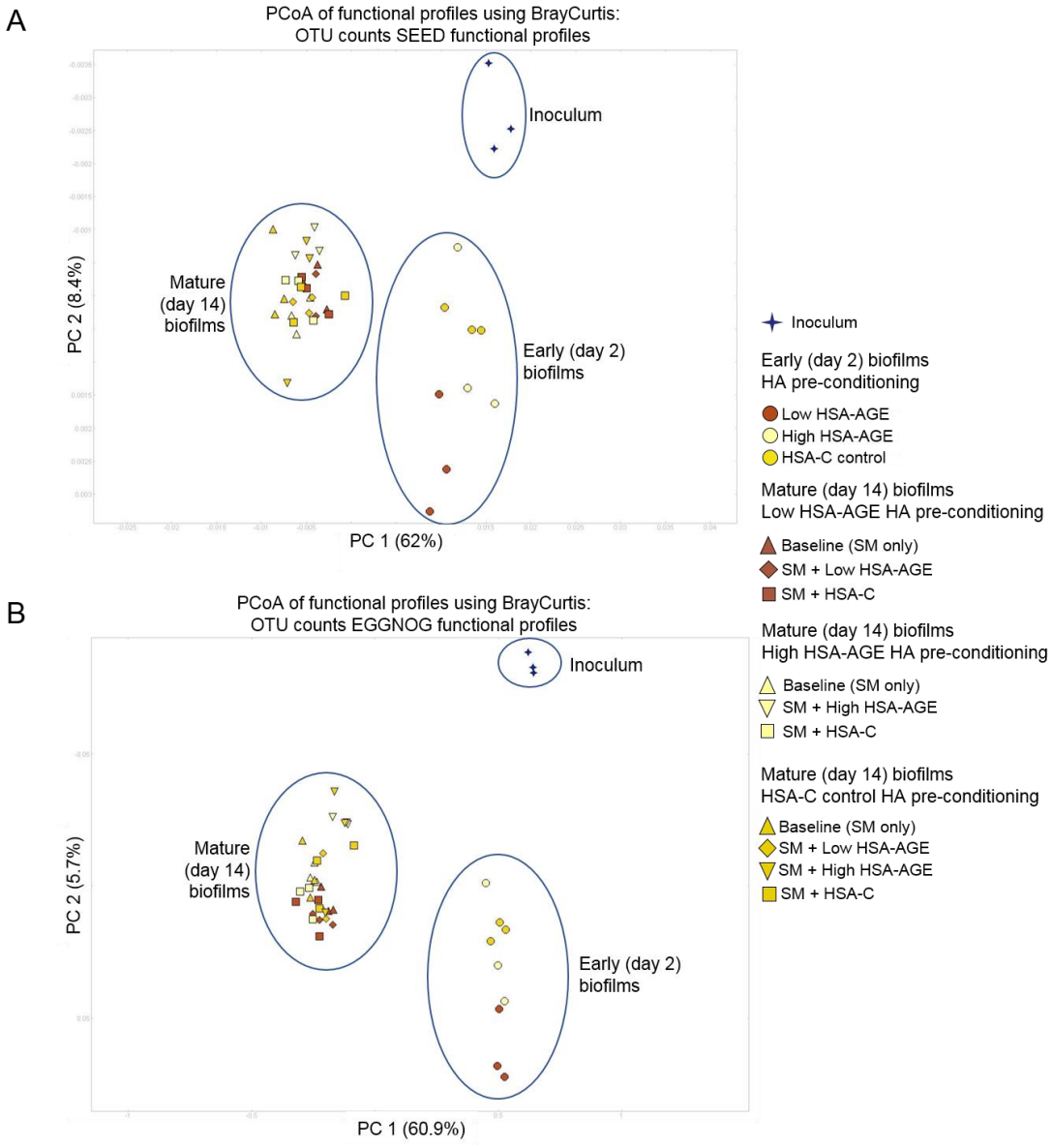


Figure 5.12: Principal component of analysis inoculum for complex biofilms, early biofilms (day 2) and mature biofilms (day 14) based on functional profiles. (A) Plot for functional clustering of inoculum/biofilms based on SEED profiles. (B) Plot for functional clustering of inoculum/biofilms based on EggNOG profiles. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, HA: hydroxyapatite.

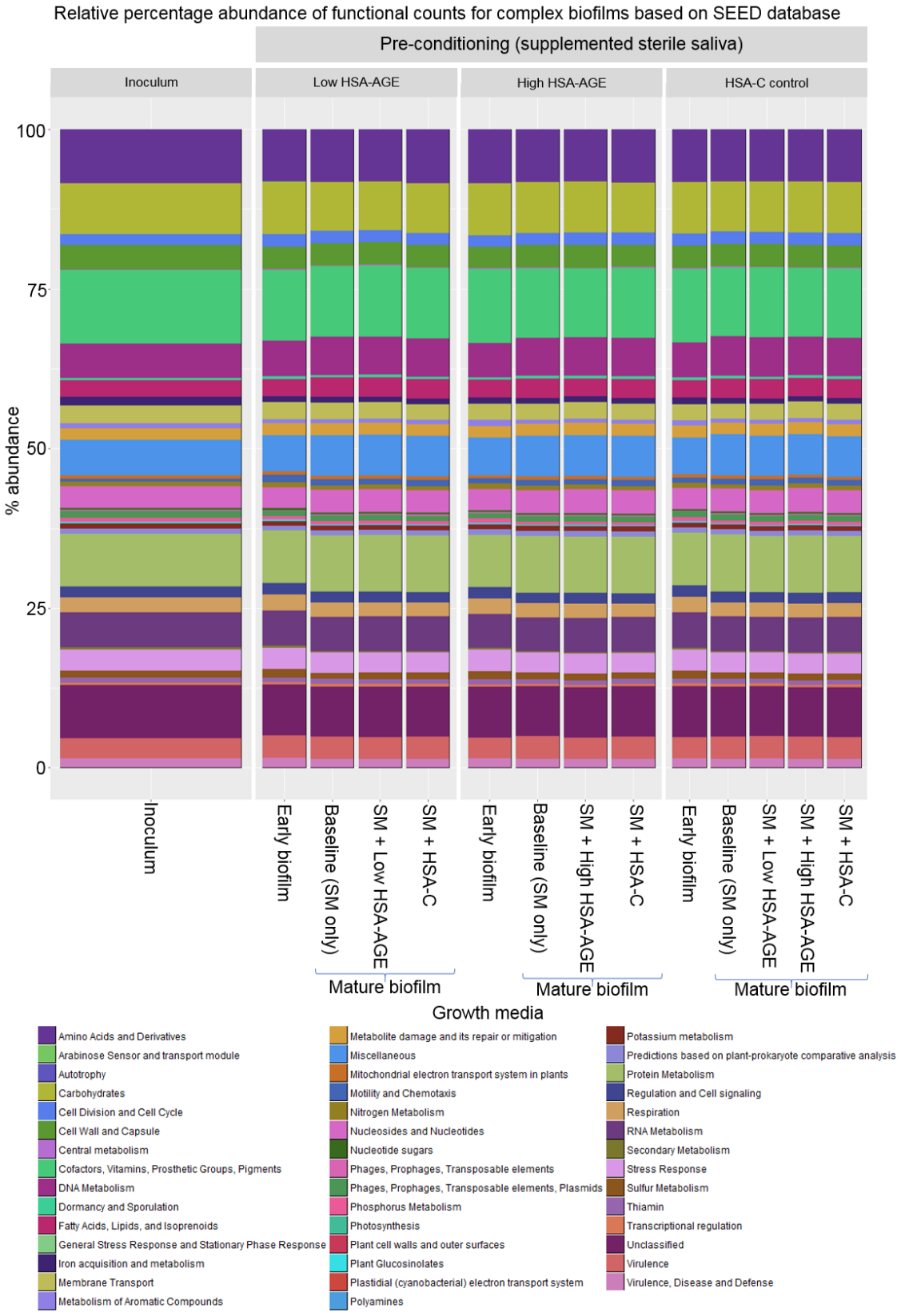


Figure 5.13: Functional analysis of inoculum, colonisation (day 2) and mature (day 14) biofilms using SEED database for annotations. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

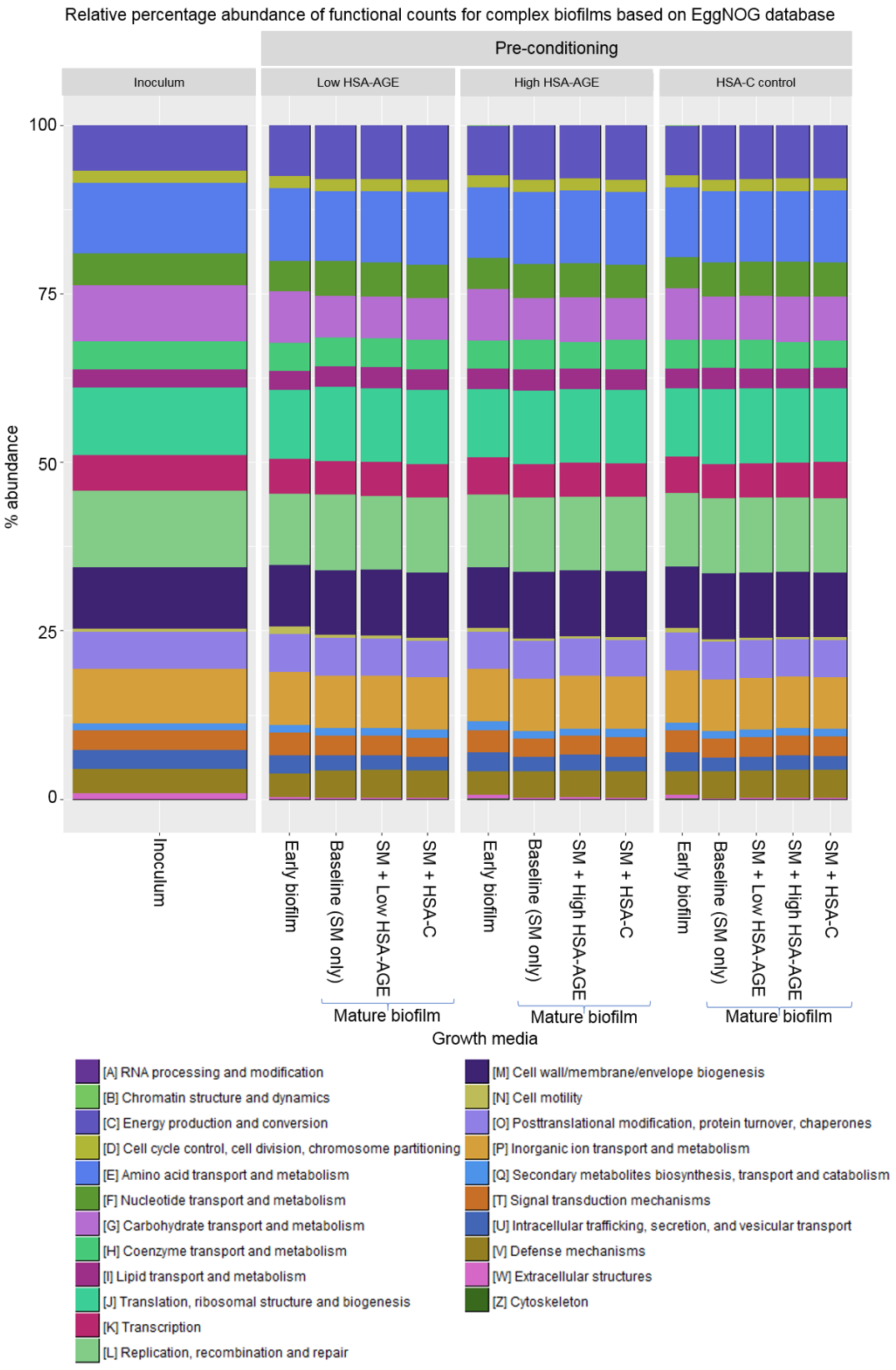


Figure 5.14: Functional analysis of inoculum, colonisation (day 2) and mature (day 14) biofilms using EggNOG database for annotations. Results represent 3 experiments with 6-8 biofilms harvested per experiment. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, C: HSA-C control biofilm.

5.2.3. Co-culture experiments

In addition to investigating the impact of HSA-AGE on complex biofilm models, the inflammatory response of TIGK cells to complex mature (day 14) biofilms was also tested. As a proof of principle study, co-cultures of biofilms with TIGK cells were carried out as described previously (section 2.14.1) and TIGK cell ROS and cytokine/chemokine release measured. The cytotoxic effect of biofilms on TIGK cells was also analysed (section 2.14.2). Biofilms were either suspended above a monolayer of TIGK cells with no direct contact between intact biofilms on the HA peg and TIGK cells (no direct contact) or placed directly on top of TIGK monolayer cells (direct contact) (Figure 2.13).

It is important to note that the proof of principle co-cultures described below, utilise biofilms cultured using a single inoculum and as such only represent one biological replicate. While differences are observed, repeats are required for significance calculations.

5.2.3.1. Analysis of cytokine/chemokine release from TIGK cells in response to co-cultures with biofilms

5.2.3.1.1. Cytokine/chemokine release following uncolonised HA peg-TIGK cell co-cultures

Inocula used to grow biofilms for NGS analysis were used to grow biofilms in parallel to determine use of the complex biofilms in co-culture experiments for the analysis of cytokine/chemokine release from TIGK cells. Biofilms were cultured with TIGK cells, with the addition of either 100 µg/mL HSA-AGE (as a representation of the consequence of hyperglycaemia) or 100 µg/mL HSA-C to TIGK culture media. The subsequent release of IL-8, IL-6 and IL-1β were analysed (section 2.4).

To determine TIGK levels of cytokine/chemokine release in response to HA pegs, uncolonised pegs were co-cultured with TIGK cells with and without direct contact, in the presence of HSA-AGE, HSA-C or unsupplemented culture media (Figure 5.15). IL-8 concentrations were generally increased when cells were cultured with direct HA peg contact compared with cell cultured with no contact (Figure 5.15, row 1). However, no obvious differences were observed in concentrations as a consequence of HSA-AGE or HSA-C addition to the culture media. Interestingly, concentrations of IL-8 following peg-TIGK co-culture with HA-peg contact were more comparable to results from previous experiments examining TIGK IL-8 release in response to varying concentrations of HSA-AGE or HSA-C (section 3.2.4). Similarly, IL-6 was also observed at increased concentrations with direct TIGK-peg contact compared with no contact (Figure 5.15, row 2), however, the differences were less evident and no obvious differences were observed upon supplementation of the culture medium. Concentrations of IL-6 from TIGK cells in

the presence of HA pegs (with or without direct contact) were higher compared with those observed previously in TIGK cultures with HSA-AGE or HSA-C alone (section 3.2.4). Contrastingly, IL-1β appeared decreased in cells with direct peg contact compared with pegs without direct contact (Figure 5.15, row 3). It is important to note that concentrations of IL-1β were below 1 pg/mL and therefore below the accurate detection limit of the ELISA assay. These low IL-1β concentrations were observed previously following exposure of TIGK cells to varying concentrations of HSA-AGE and HSA-C (section 3.2.4).

The concentrations of cytokine/chemokines with HA pegs were considered as blank controls and show the basal level of cellular cytokine/chemokine releases in response to the presence of the pegs (in non-contact conditions) while pegs with direct contact also account for the cellular responses associated with cellular damage caused by the pressure of the peg.

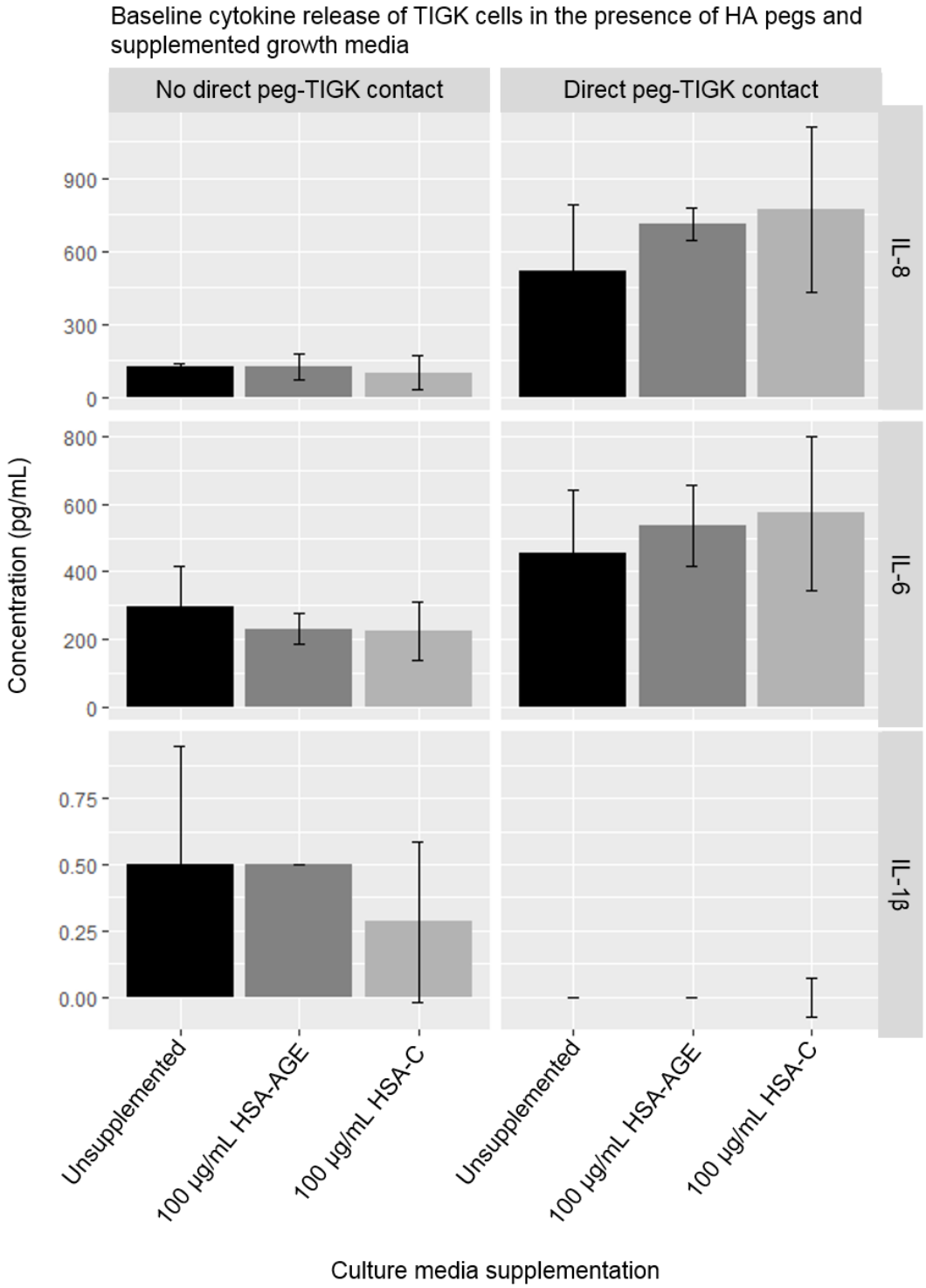


Figure 5.15: Inflammatory response of TIGK cells in response to hydroxyapatite coated pegs. Cytokine/chemokine release from TIGK cells cultured with hydroxyapatite (HA) pegs either suspended above cell monolayer (no direct contact) or placed directly on TIGK monolayer (direct contact). Results are presented as means ± SD and represent 1 experiment and 3-4 harvested biofilms.

5.2.3.1.2. IL-6 release following biofilm-TIGK cell co-cultures

Biofilms were generated as described previously (section 2.13) under different conditions. After 14 days the mature biofilms were co-cultured with TIGK cells in culture media supplemented with 100 µg/mL HSA-AGE or HSA-C (section 2.14) and the resulting cytokine/chemokine release analysed (section 2.14.1).

Biofilms suspended above a monolayer of cells (no direct biofilm-TIGK contact) could potentially decrease IL-6 production, as indicated by a decrease from 225.65 ± 87.19 – 298.86 ± 117.65 pg/mL in HA peg controls (Figure 5.15A, row 2) to below 10 pg/mL (Figure 5.16A) regardless of peg pre-conditioning and biofilm growth conditions. In all biofilm-TIGK co-cultures, concentrations of IL-6 were very low with some co-cultures resulting in undetectable levels of IL-6. While IL-6 was detected following co-culture of TIGK cells with biofilms cultured on pegs pre-conditioned with HSA-C and low HSA-AGE, concentrations were low with large variations (as indicated by SD error bars of technical replicates). As such, IL-6 release following no direct biofilm-TIGK contact appeared to have little differences between the different biofilm types or upon supplementation of culture media with HSA-AGE or HSA-C.

In contrast, co-cultures with direct biofilm-TIGK contact (Figure 5.16B) resulted in higher concentrations of IL-6 than co-cultures without direct biofilm-TIGK contact (Figure 5.16A). However, the decreases observed following both co-culture methods were proportional, owing to the higher basal IL-6 concentrations in co-cultures with direct biofilm-TIGK contact (Figure 5.15, row 2). This suggests that a proportion of IL-6 release following biofilm-TIGK direct contact co-cultures could be due to the pressure and potential cellular damage/stress associated with the contact. Similar to results obtained from co-cultures without any direct biofilm-TIGK contact, IL-6 concentrations did not appear to be altered in the presence of HSA-AGE or HSA-C supplemented TIGK culture media, regardless of the type of biofilm used for biofilm-TIGK co-culture.

Upon comparison of direct contact biofilm-TIGK co-cultures with biofilms representing the consequence of hyperglycaemia and serum condition of periodontitis (HP) to HSA-C control biofilms (C), lower concentrations of IL-6 were observed (Figure 5.16B). Moreover, IL-6 concentration following direct contact biofilm-TIGK co-cultures with biofilms representing serum conditions of periodontitis alone (P) appear lower than those observed in C co-cultures regardless of culture medium supplementation (Figure 5.16B). Comparisons of co-cultures with HP and P biofilms, potentially show higher IL-6 concentrations in HP. These data suggested that P and HP biofilms are able to supress IL-6 release by TIGK cells independently of HSA-AGE or HSA-C in TIGK culture media,

when compared with C biofilm co-cultures and HA peg controls. Furthermore, HP biofilms seem unable to decrease IL-6 concentrations to the same extent as P biofilms.

5.2.3.1.3. IL-8 release following biofilm-TIGK cell co-cultures

Following biofilm-TIGK co-culture without any direct biofilm-TIGK contact, IL-8 release was observed at lower concentrations, with ranges from 0 to 42.93 ± 8.73 pg/mL (Figure 5.17A), in comparison to matched uncolonised HA peg controls (ranging from 100 ± 67.21 – 125.81 ± 10.74 pg/mL, Figure 5.15). The results suggested biofilm-TIGK no contact co-cultures with culture media supplementation have a more pronounced reduction in IL-8 concentrations, although no obvious differences are observed between HSA-AGE and HSA-C. Furthermore, IL-8 concentrations did not appear to be influenced by the different biofilms generated in this system (Figure 5.17A). This suggests that although composition of these biofilms did not alter IL-8 concentrations, co-culturing of TIGK cells with biofilms did decrease concentrations.

In contrast, IL-8 concentrations following biofilm-TIGK co-cultures with direct contact (Figure 5.17B) resulted in a general decrease in IL-8 concentrations in comparison to matched uncolonised HA peg-TIGK co-cultures (Figure 5.15). In addition, no obvious trends were observed between different biofilm types or upon addition of HSA-AGE/HSA-C to the culture media.

5.2.3.1.4. IL-1β release following biofilm-TIGK cell co-cultures

Cellular release of IL-1β following no contact biofilm-TIGK co-cultures generally appeared to increase, with concentrations ranging from 0 to 15.68 ± 1.76 (Figure 5.18), compared with uncolonised HA peg controls (where concentrations ranged from 0 to 0.50 ± 0.45, Figure 5.15). In addition, IL-1β concentrations also differed when comparing concentrations across different culture media supplementation. However, this was also only observed in response to biofilm-TIGK co-culture with certain biofilms and there was no obvious indication as to the cause of the changes (Figure 5.18A).

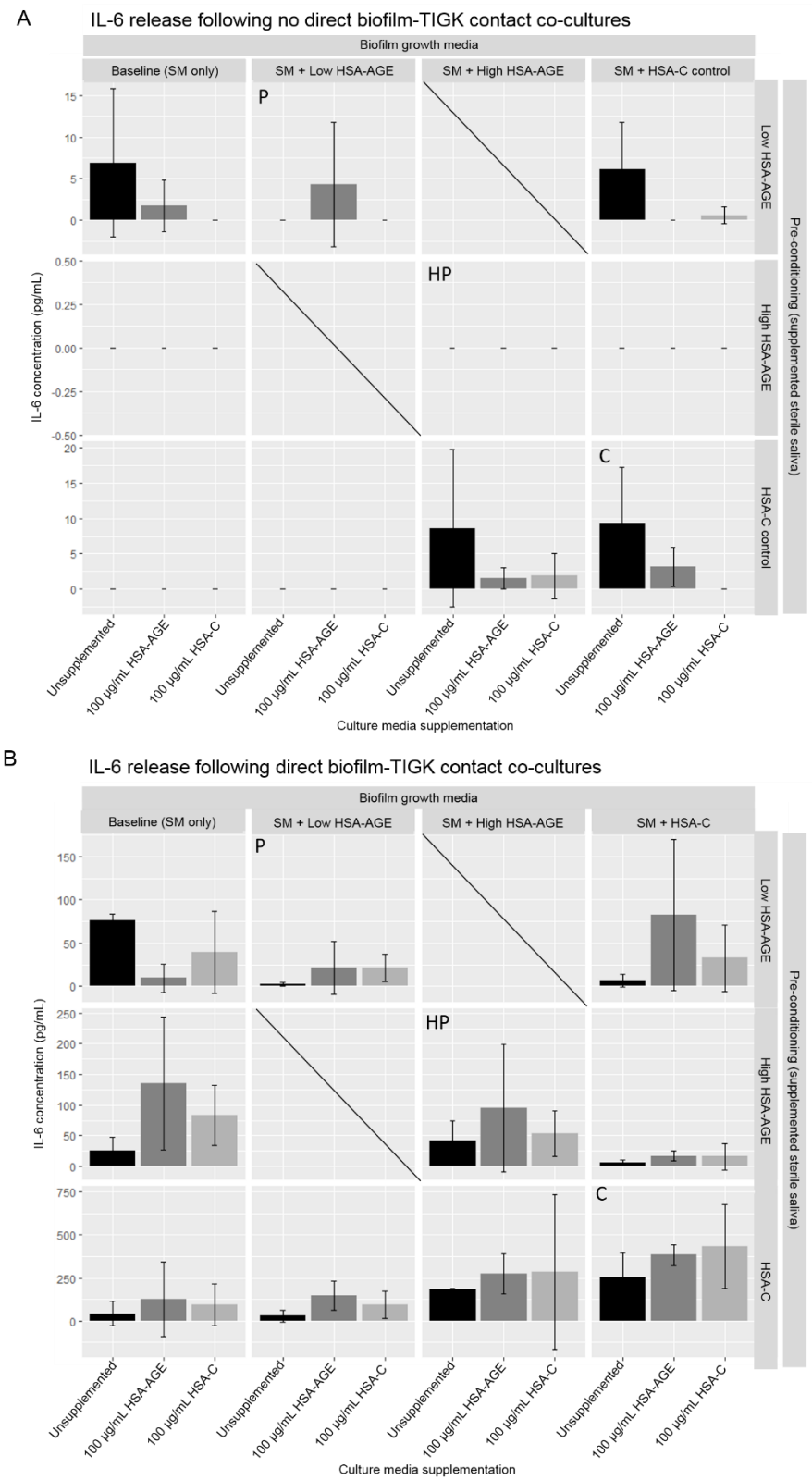


Figure 5.16: IL-6 concentration following biofilm-TIGK cell co-culture. IL-6 release following co-culture with complex biofilms without (A) and with (B) direct biofilm-TIGK contact and supplementation of culture media with 100 µg/mL HSA-AGE or HSA-C. Results are presented as averages ± standard deviation and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control. C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

The results also suggested higher concentration of IL-1β in HP biofilms co-cultured with TIGK cells in the presence of HSA-AGE compared with matched C biofilms following no contact co-cultures (Figure 5.18A), but no obvious differences with HSA-C or unsupplemented culture media. Furthermore, no evident differences were observed between P and HP biofilms. Despite some of the differences, no discernible pattern could be identified. This is perhaps due to the observed concentrations of IL-1β falling at the lower end of the detection limit and thus resulting in more variability and less reliability.

No obvious differences were observed in IL-1β concentrations following biofilm-TIGK co-cultures with direct biofilm-TIGK contact. This was observed when comparing to uncolonised HA peg-TIGK co-cultures, as well as between concentration associated with different biofilm types and the addition of HSA-AGE/HSA-C to the culture media (Figure 5.15 and Figure 5.18B).

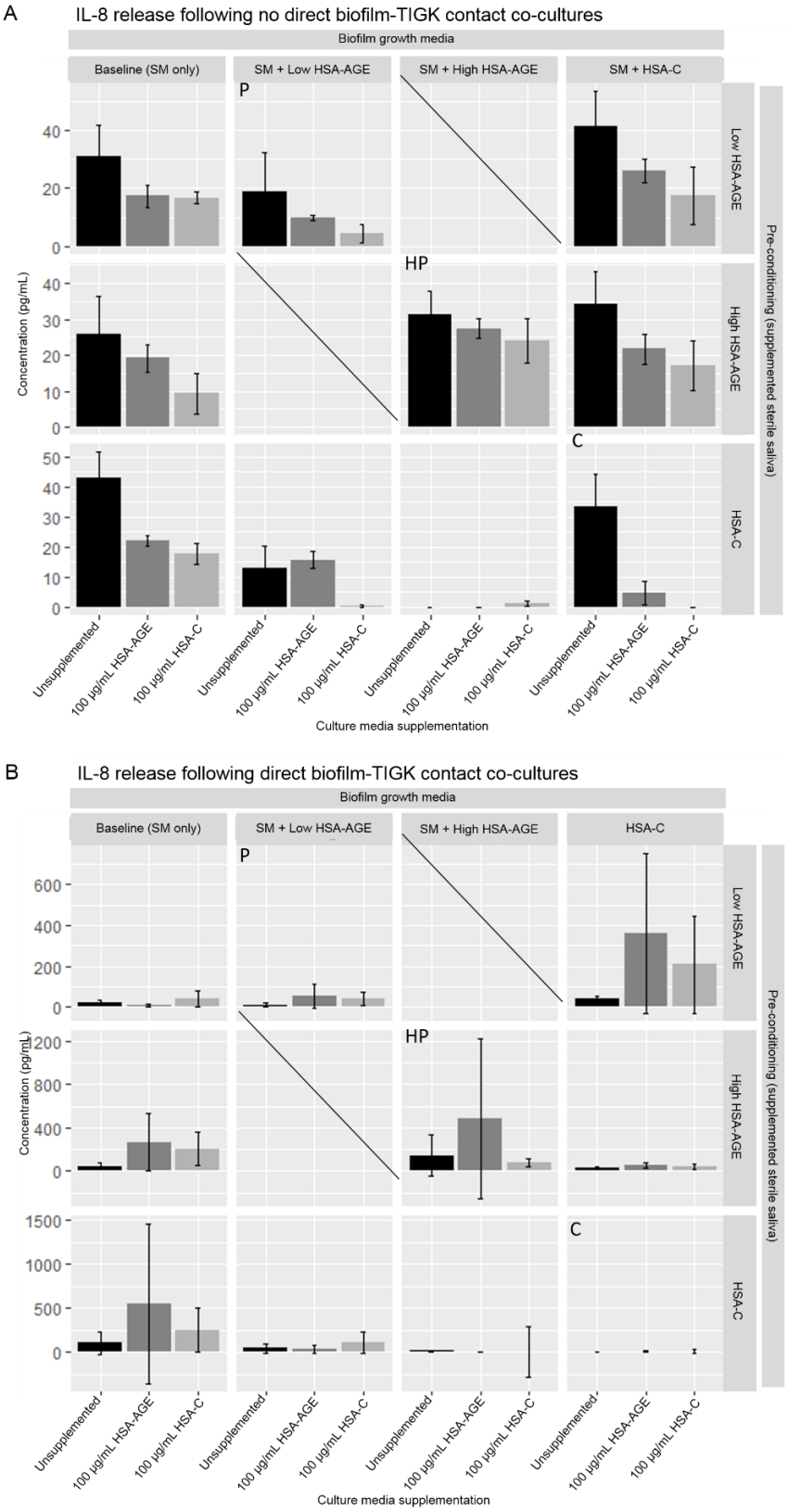


Figure 5.17: IL-8 concentration following biofilm-TIGK cell co-culture. IL-8 release following co-culture with complex biofilms without (A) and with (B) direct biofilm-TIGK contact and supplementation of culture media with 100 µg/mL HSA-AGE or HSA-C. Results are presented as means ± standard deviation and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control. C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

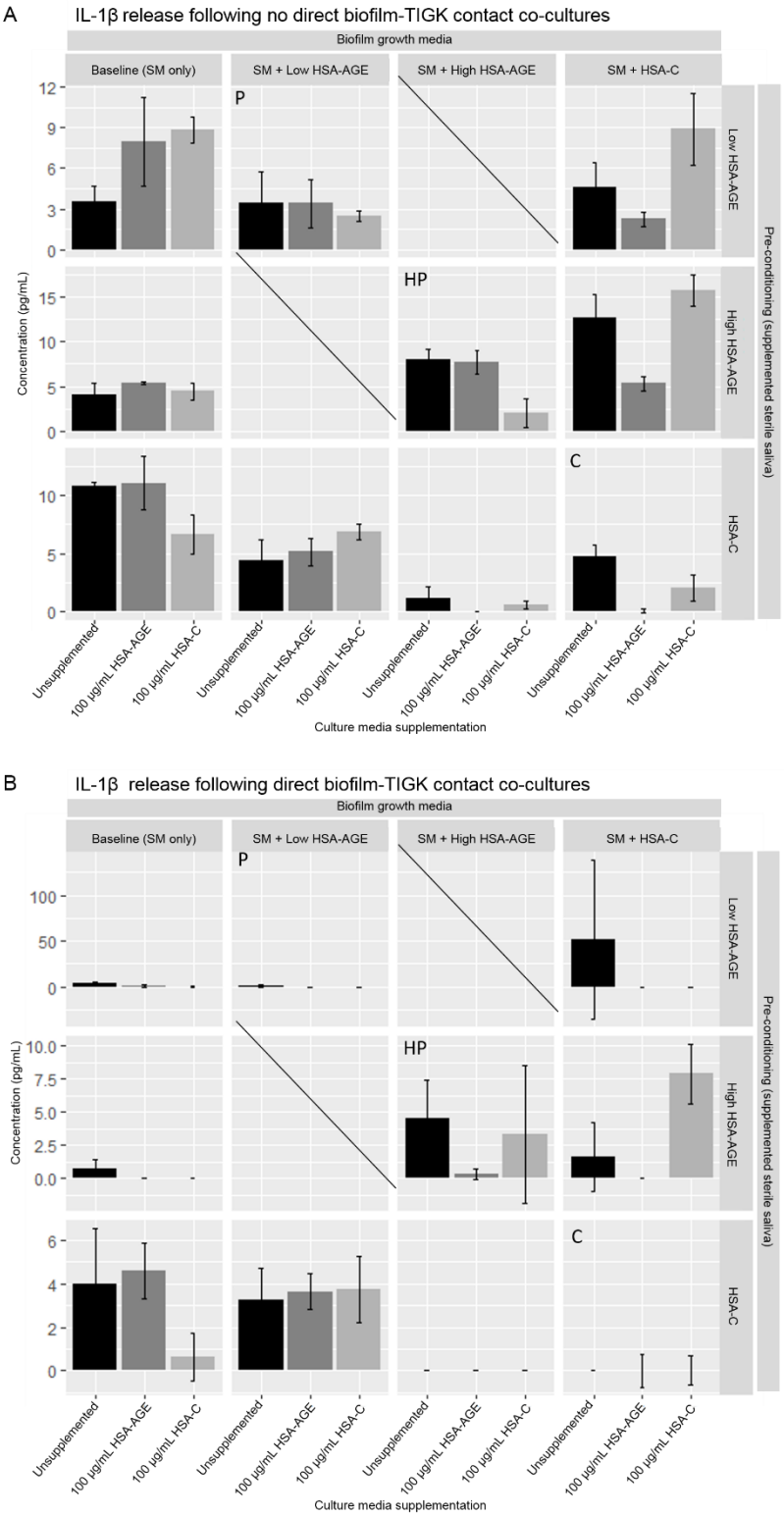


Figure 5.18: IL-1β concentration following biofilm-TIGK cell co-culture. IL-β release following co-culture with complex biofilms without (A) and with (B) direct biofilm-TIGK contact and supplementation of culture media with 100 µg/mL HSA-AGE or HSA-C. Results are presented as means ± standard deviation and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control. C: HSA-C control biofilm, P: biofilm representing increased serum observed in periodontitis with health associated HSA-AGE concentrations, HP: biofilm representing periodontitis and diabetes associated HSA-AGE concentrations.

5.2.3.2. Analysis of cell cytotoxicity and ROS release from TIGK cells in response to co-cultures with biofilms

In addition to changes in cytokine/chemokine profiles in response to TIGK co-culture with complex biofilms cultivated under different conditions, ROS release and cytotoxicity were also analysed. Biofilms were generated using pooled saliva, tongue scrapings and supragingival plaque from three volunteers and grown under conditions representing hyperglycaemia associated accumulation of HSA-AGE and the increased GCF serum associated periodontitis (100 µg/mL HSA-AGE, high HSA-AGE biofilms), high serum levels associated with periodontitis only (1 µg/mL HSA-AGE and 99 µg/mL HSA-C, low HSA-AGE biofilms), and HSA-C control biofilms (100 µg/mL HSA-C (section 2.14.2).

To determine ROS release following introduction of HA pegs to TIGK cells, co-cultures were carried out with and without direct contact (Figure 2.13). As with previous co-cultures, these were grown in culture media supplemented with HSA-AGE or HSA-C. Fluorescence readings were taken as a direct measurement of ROS release (section 2.14.2), with readings corrected using matched unstained cells to eliminate background fluorescence associated with TIGK cells or culture media (Figure 5.19). The results showed that regardless of HSA-AGE presence, basal levels of ROS released from TIGK cells was low, with fluorescence (Ex485/Em535) readings below 5.

In general, higher levels of ROS were observed in co-cultures with direct uncolonised HA peg-TIGK contact. This trend was observed across the different culture media (Figure 5.19). This increase in ROS in direct contact HA peg-TIGK co-cultures could potentially be as a consequence of cell stress associated with the pressure of the peg resting on the TIGK monolayer. Given that results indicate differences in ROS levels of TIGK cells in response to uncolonised HA pegs under different co-culture condition, fluorescence readings following biofilm-TIGK co-cultures were corrected to matched unstained cells and fold change in ROS calculated relative to fluorescence readings induced by matched uncolonised HA pegs

No direct biofilm-TIGK contact co-cultures indicated an overall increase in TIGK ROS release, with ROS levels of between 4.27 ± 0.70 and 15.81 ± 4.76 fold higher (Figure 5.20A) than those observed in uncolonised HA peg-TIGK co-cultures (Figure 5.19). This is perhaps due to the increased stress the TIGK cells are under when in the presence of the biofilm, despite the lack of direct contact between the biofilms and the cells. It is also possible that the biofilms shed bacteria during the duration of the co-culture, so that although the biofilm is suspended over the TIGK monolayer, there is still bacteria-TIGK contact. Indeed, viable bacterial cells were collected from the supernatant following co-culture (data not shown).

The results also suggest high HSA-AGE biofilms had the maximum ROS release when culture media was supplemented with HSA-AGE (Figure 5.20A). There also appears to be increased ROS release in high HSA-AGE biofilm-TIGK co-cultures in HSA-AGE supplemented culture media when compared with all co-cultures in unsupplemented culture media. Together the data suggested that the combination of HSA-AGE supplemented culture media with biofilms grown under high HSA-AGE and serum conditions could increase ROS release.

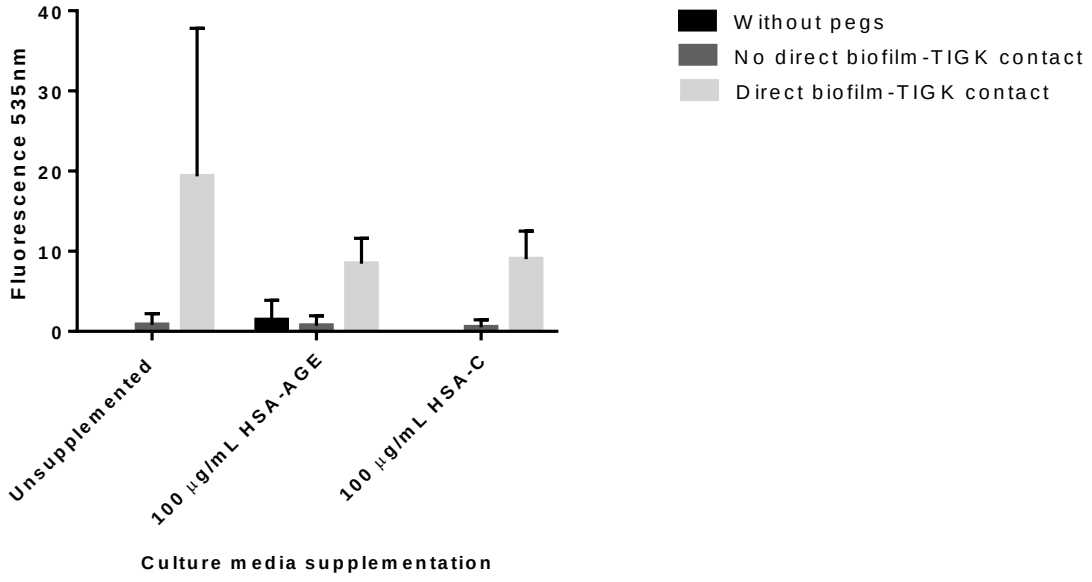


Figure 5.19: ROS release of TIGK cells following incubation with hydroxyapatite pegs and co-stimulated with HSA-AGE and HSA-C. Results are presented as means ± SD and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, HA: hydroxyapatite.

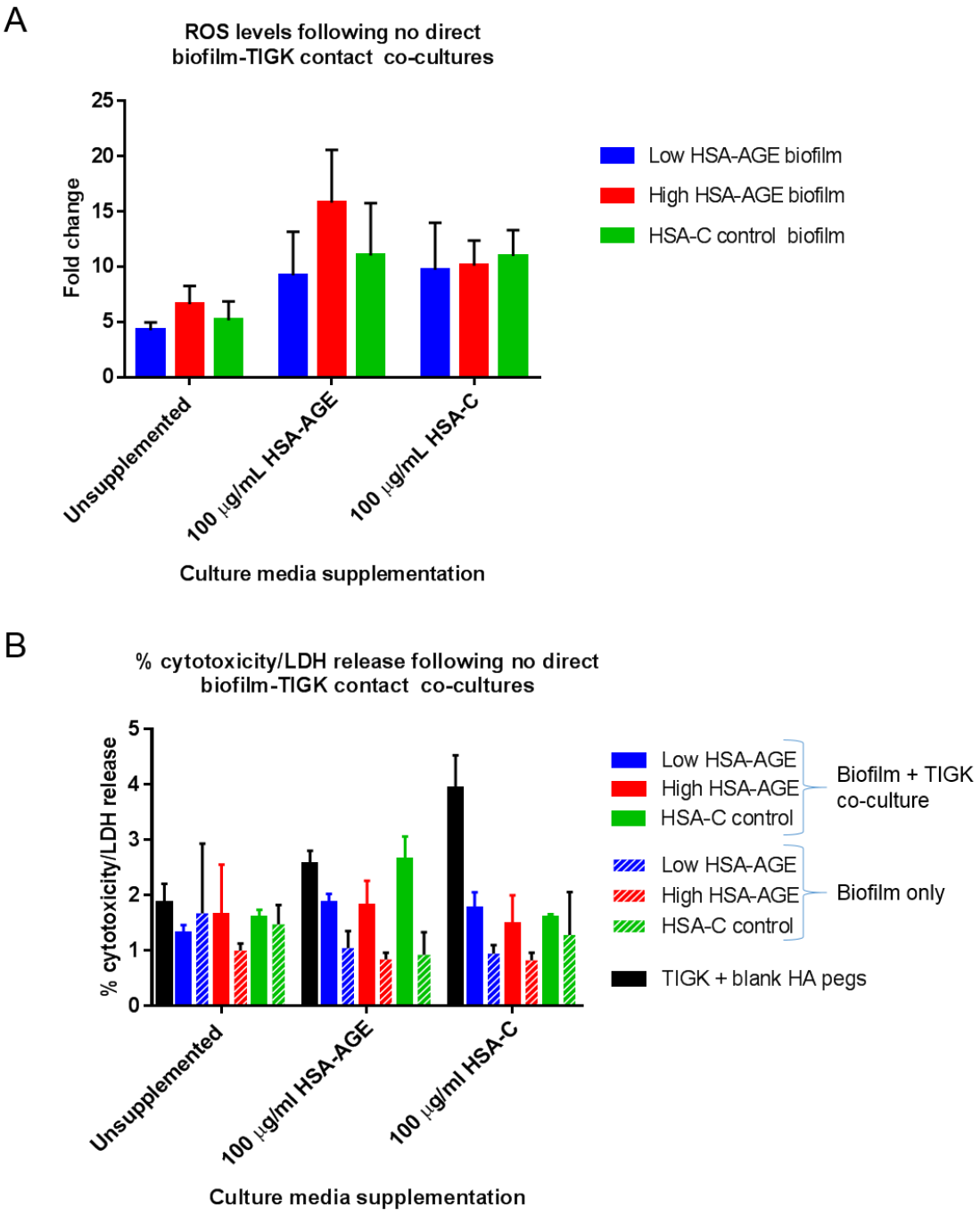


Figure 5.20: ROS levels and % cytotoxicity following no direct biofilm-TIGK contact co-cultures and % LDH release of biofilms. TIGK cells were co-cultured with complex biofilms without any biofilm-TIGK contact in HSA-AGE or HSA-C supplemented culture media. Results are presented as means ± SD and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, HA: hydroxyapatite.

In parallel to measuring ROS release, the cytotoxic effect of biofilms on TIGK cells was also quantified following no contact co-cultures (section 2.14.1). Absorbance readings correlating to LDH release were taken as a measurement of cell death, which were subsequently corrected to matched culture media control readings. Percentage cytotoxicity was calculated relative to matched chemically lysed cell preparations representing 100% cell death. In addition to determining percentage cytotoxicity, LDH release of biofilms in the absence of TIGK cells were also calculated to determine if changes in LDH measurements were associated with biofilms (Figure 5.20B). In general there appears to be no differences in % cytotoxicity/LDH release between biofilms cultured with cells and biofilms cultured without. This suggests that low levels of LDH are potentially released from biofilms. Given the overall low % cytotoxicity/LDH release and the lack of obvious differences between different biofilms and different co-culture media, it can be concluded that these biofilms did not produce a detectable cytotoxic effect in TIGK cells when there was no direct biofilm-TIGK contact during co-culture.

Similarly, both TIGK ROS release and cytotoxicity were analysed following direct contact of biofilm-TIGK co-cultures (Figure 5.21). The results indicated an increase in ROS levels of TIGK cells in response to biofilms, with between 2.06 ± 0.45 and 5.48 ± 2.00 fold increases (Figure 5.21A) relative to uncolonised HA peg-TIGK control co-cultures (Figure 5.19). Interestingly, the increases in ROS levels were lower in TIGK cells co-cultured with direct biofilm-TIGK contact (Figure 5.21A) compared with those without (Figure 5.20A).

Under the direct contact co-culture conditions, ROS levels were more variable with higher ROS observed between co-cultures in HSA-C supplemented culture media compared with unsupplemented culture media (Figure 5.21A). Furthermore, in direct biofilm-cell co-cultures, addition of HSA-C to the culture medium increased ROS in a manner that appeared to be independent of biofilm type.

Cytotoxicity analysis of biofilm-TIGK co-cultures with direct contact resulted in increased cytotoxicity in HSA-C supplemented culture medium (Figure 5.21B). In particular, HSA-C control biofilms appeared to be more cytotoxic to TIGK cells compared with low and high HSA-AGE biofilm co-cultured in HSA-C supplemented culture media (Figure 5.21B). Interestingly, when in HSA-C supplemented culture medium, uncolonised HA pegs appeared to be more cytotoxic to TIGK cells than co-cultures with low or high HSA-AGE biofilms with HSA-C supplemented culture medium (Figure 5.21B), perhaps suggesting in the presence of HSA-C, low and high HSA-AGE biofilms are able to decrease cytotoxicity.

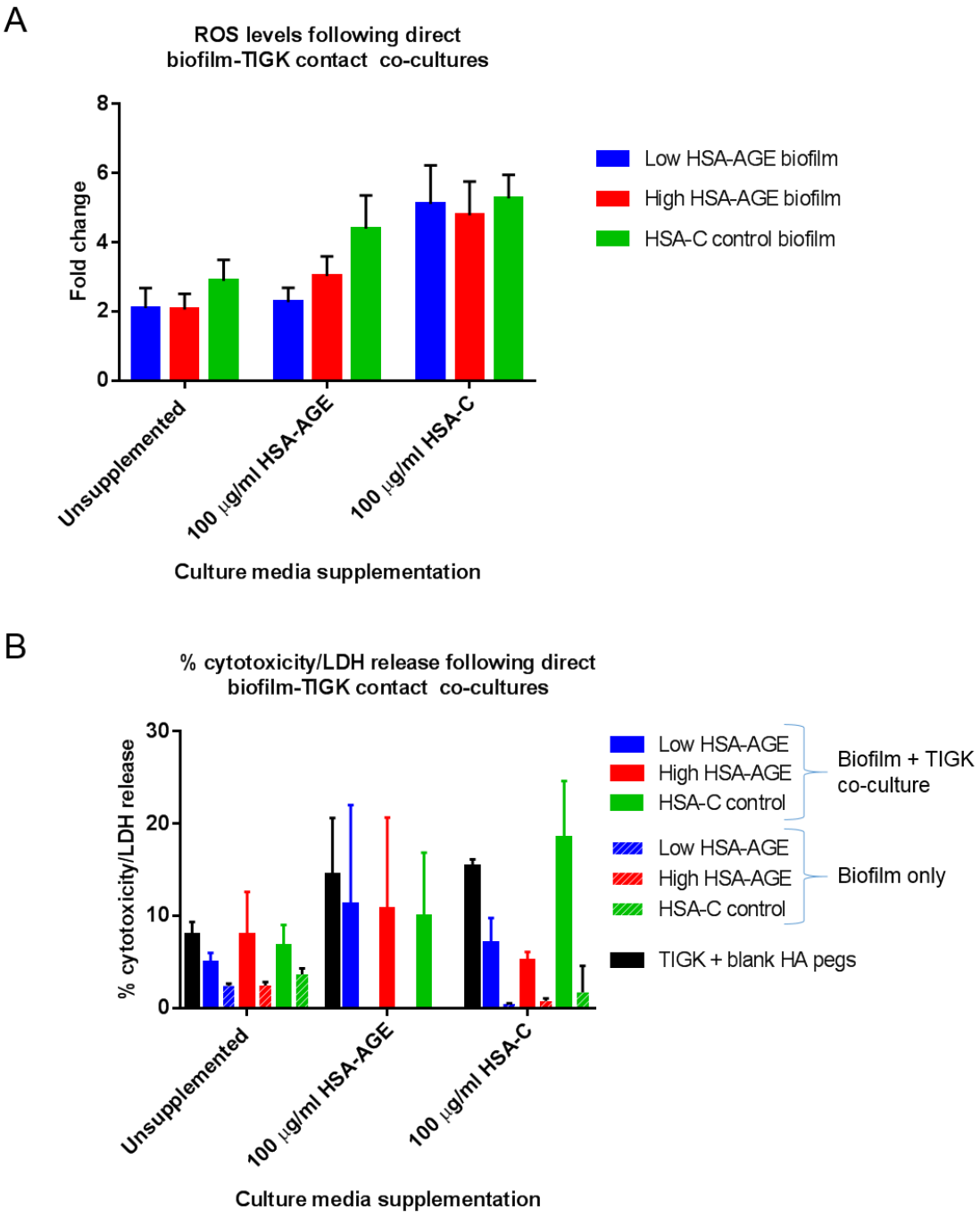


Figure 5.21: ROS levels and % cytotoxicity following no direct biofilm-TIGK contact co-cultures and % LDH release of biofilms. TIGK cells were co-cultured with complex biofilms with direct biofilm-TIGK contact in HSA-AGE or HSA-C supplemented culture media. Results are presented as means ± SD and represent 1 experiment and 3-4 harvested biofilms. HSA-AGE: human serum albumin-advanced glycation end product, HSA-C: human serum albumin-control, HA: hydroxyapatite.

5.3. Discussion

The role of AGEs in inflammatory responses of mammalian cells is reasonably well established but the impact the presence of AGEs has on bacterial growth is still an area of research in its infancy (Ljahdali and Carbonero, 2017). Given that AGEs are found to be increased in the GCF of diabetic patients with further increases observed in participants with diabetes and periodontitis, it is possible AGEs play a role in the progression of periodontitis associated with diabetes (Kajiura *et al*., 2014). The study described in this chapter aimed to determine if the presence of HSA-AGE altered biofilm composition and if these cultured biofilms can be used to analyse changes in inflammatory responses (cytokine/chemokine release and ROS release) when co-cultured with oral epithelial cells (TIGK) in the presence of HSA-AGE concentrations representative of diabetes.

5.3.1. Microbial analysis of complex biofilms

This preliminary study aimed to give an insight into the role of HSA-AGE on the composition of the oral microbiota. The compositional analysis of the inoculum indicated that the collected and combined oral samples, provided a good representation of the oral microbiome. It is well established that *Streptococcus* accounts for the majority of bacteria in most oral habitats (Moon and Lee, 2016). This correlates with the inoculum used for the complex biofilm study described here. Furthermore, HMP data analysis of seven oral sites (buccal mucosa, hard palate, gingiva, saliva, sub- and supra-gingival plaque and tongue scrapings), the predominant phyla in 182-206 healthy individuals were shown to be *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* (Human Microbiome Project, 2012, Zhou *et al*., 2013b, Moon and Lee, 2016). The inoculum isolated for this study was demonstrated to be dominated by these 5 phyla thus indicating their appropriateness as a starting point for the growth of model oral biofilms.

One of the aims of this modelling study was to determine if pre-conditioning of HA pegs with HSA-AGE altered the colonisation of bacteria on to the surface of the pegs. We showed differences in biofilms harvested after 2 days prior to any media changes. Primarily, we demonstrated significant differences in biofilm composition of pegs pre-conditioned with high HSA-AGE compared with those with low HSA-AGE. Interestingly the NGS data for this comparison showed a shift in composition where genera typically associated with periodontitis, such as *Fusobacterium*, *Campylobacter, Treponema* and *Haemophilus*, were less prevalent while genera generally associated with periodontal health (including *Actinomyces*, *Streptococcus* and *Veillonella*) were more prevalent. This suggests that diabetes associated GCF HSA-AGE concentrations actually encourage heath associated bacteria to colonise HA pegs.

Differential abundances comparing high HSA-AGE and high serum (HP) early (day 2) and high serum only early (P) (day 2) biofilms with HSA-C control (C) early biofilms indicated higher concentrations of HSA-AGE select for *Streptococcus* and *Prevotella.* In addition, they reduce colonisation of HA pegs by genera such as *Capnocytophaga* and *Campylobacter.* Contrastingly, low HSA-AGE concentrations appear to selected for *Campylobacter* and *Capnocytophaga* and hindered the colonisation by *Streptococcus* and *Prevotella*. Other bacterial genera (such as *Actinomyces* and *Rothia*) appear to be less prevalent in early biofilms with both health and diabetes associated HSA-AGE concentrations compared with colonisation of pegs with only HSA-C. It is possible this is a consequence of HSA-C (in health and control conditions). To determine if HSA-C affected biofilm composition, colonisation would need to be tested without any HSA-C addition to the sterile saliva during the pre-conditioning step.

In a similar manner, mature P biofilms, appeared to reflect the colonisation patterns, such that a combination of low HSA-AGE (1 µg/mL) with HSA-C (99 µg/mL) in both the pre-conditioning and the growth media resulted in higher abundances of periodontitis associated genera (*Fusobacterium* and *Campylobacter*) when compared with HSA-C control alone. HP biofilms, on the other hand, appeared to significantly encourage the growth of health associated genera and also showed a decrease in the abundance of periodontitis associated genera. Again, this suggests a role for HSA-C in the changes seen in biofilm composition, particularly when combined with low concentrations of HSA-AGE. This is perhaps unsurprising when considering serum, which contains high levels of HSA, that is, as described previously, beneficial to the growth of bacteria such as *P. gingivalis* during periodontitis.

Due to the differences in the initial colonisation of pre-conditioned pegs which would impact the composition of the mature biofilm, there is the potential that the different concentrations of HSA-AGE are adsorbed differently onto the surface of the peg or interact differently with proteins originating from the sterile saliva. This in turn could affect the ability of bacteria to adhere and grow on the surface of the HA pegs. In addition, the protein rich, serum supplemented medium could have selected for species that favour the conditions imposed by the model.

A previous study utilising 16S rRNA sequencing of subgingival plaque from systemically healthy and diabetic patients, indicated an increase in prevalence of *Propionibacteriaceae, Tannerella* and *Capnocytophaga* in diabetes. The study also reported a decrease in *Prevotella* (Zhou *et al*., 2013a). Our model, using HSA-AGE as a representation of hyperglycaemia consequences in diabetes, demonstrated contradictory abundances with increases in *Prevotella* and decreases in *Tannerella* in mature (14 day) biofilms with high HSA-AGE but did show an increase in

Propionibacterium. Casarin *et al*. (2013) demonstrated periodontitis subgingival plaque of diabetic patients with poor glycaemic control had higher abundances of some genera (such as *Neisseria*, *Actinomyces*, *Capnocytophaga*, *Fusobacterium*, *Veillonella*, *Aggregatibacter*, *Selenomonas* and *Streptococcus*) and lower abundances of others (including *Synergistetes*, *Tannerella, Porphorymonas* and *Eubacterium*). The hyperglycaemia consequence models presented here, however, indicates a decrease in *Fusobacterium* and no differences in other genera such as *Actinomyces* and *Neisseria.* The results do, however, reflect the decrease in *Porphyromonas*, *Tanerella* and the increase in *Veillonella*.

It is difficult to directly compare the results from this model to clinical samples, due to the limited nature of the model which does not take into account all the biochemical mechanisms involved in both diabetes and periodontitis. The differences in sequencing methods utilised by different groups (16S rRNA VS shotgun metagenomics) adds to the difficulty in accurately comparing studies. It is also important to note that analysis of biofilms grown in a laboratory also risk the loss of bacteria which cannot be cultured under these conditions, causing shifts in bacterial composition so that clinical samples cannot be accurately compared with model biofilms. The contrasting results, do however highlight the limitations of a model which does not take into account the complex interactions between bacterial and host cells and varying growth conditions found in the oral cavity.

As well as NGS analysis of biofilms, viable counts were carried out. Although differences in facultative anaerobes were observed under different conditions, this method not only groups bacteria so that establishing finer changes is difficult (particularly in facultative aerobes, anaerobes and gram negative anaerobes) but also causes bias when accounting for the broad range of bacteria that cannot be cultured in a laboratory setting or indeed grown independently of a biofilm. Despite this, viable counts do provide some information. For example, Lactobacilli which were observed in the inoculum, were unable to colonise the pegs and only grew in mature biofilms with limited success and in general were not observed under high HSA-AGE concentrations. This correlates with previous studies which have shown inhibition of colonic Lactobacilli growth when diets were high in AGEs (Hernandez *et al*., 2011, Seiquer *et al*., 2014, Hellwig *et al*., 2015).

5.3.2. Inflammatory responses by TIGK cells in response to complex biofilms

The results indicated that, despite the general decrease in TIGK IL-8 and IL-6 concentrations in response to biofilms, limited differences were observed between P and HP co-cultures or in response to the addition of HSA-AGE in the culture media. In both cases, however, it can be concluded that concentrations of cytokines released were

higher when biofilms were in direct contact with the cells compared with biofilms suspended over a TIGK monolayer. Given the correction of results for pressure associated cytokine/chemokine release (uncolonised HA peg-TIGK co-cultures), this suggests direct contact between the biofilm initiates a heightened immune response in TIGK cells. The difference in concentrations following with and without contact co-cultures also suggests bacteria shed from the biofilm cannot initiate the same inflammatory response as bacteria of the intact biofilm.

Although IL-6 concentrations were decreased to the same extent in both co-culture methods relative to their respective uncolonised HA peg controls, the extent by which IL-8 concentrations decreased compared with uncolonised HA peg controls was lower in co-cultures with direct contact compared with those without. This suggests when biofilms are in direct contact with TIGK cells, they decrease IL-6 concentration but do not markedly change IL-8 concentrations compared with uncolonised HA peg controls.

The study hypothesised that co-culturing of biofilms with TIGK cells in HSA-AGE supplemented culture media would lead to altered cytokine concentrations. The results for both IL-8 and IL-6, however, showed minimal differences when co-culturing TIGK cells with biofilms. A trend was observed in direct biofilm-TIGK contact co-cultures, where concentrations of IL-6 were generally higher when HSA-AGE was added to the culture media. In addition, concentrations were higher with diabetes and periodontitis biofilms compared with periodontitis biofilms alone. Together this indicates a potential for higher IL-6 concentrations when the co-culture models high HSA-AGE concentrations throughout (HP biofilm-TIGK co-cultures in HSA-AGE supplemented culture media) compared with the low HSA-AGE model (P biofilm-TIGK co-culture in unsupplemented culture media) with direct biofilm-cell contact. The same trend and observations can be made in concentrations of IL-8 in direct biofilm-TIGK contact co-cultures.

It is important to note, the cytokine analysis was carried out following biofilm growth with a single inoculum and without any replicates on separate Calgary devices and although biofilms were replicated on multiple pegs no true biological replicates were generated. It is possible with more biological replicates differences in cytokine concentrations will be more evident. However, the study does indicate the potential use of the model to analyse changes in cytokines as a consequence of co-cultures using biofilms cultured on HA pegs. In addition, the trends observed highlight the requirement for further analysis.

Furthermore, the cytokine concentrations observed following HSA-C addition to the culture medium and their differences compared with unsupplemented culture media implies HSA-C impacts cytokine release by TIGK cells. It is possible that the lack of serum in TIGK culture media means TIGK cells are sensitive to the presence of the

unmodified HSA. To fully understand if this is the case, cells need to be cultured in serum supplemented medium and perhaps conditioned for long term culturing in serum supplemented media.

Interestingly our studies demonstrated higher ROS levels in no contact HP biofilm-TIGK co-cultures in HSA-AGE supplemented culture media. This suggested under high HSA-AGE conditions (for both biofilm growth and cell culture) cells released more ROS compared with co-cultures in unsupplemented culture media and indeed compared with co-culturing in HSA-AGE culture media but with P or C biofilms. There are, however, no differences between HSA-AGE and HSA-C media supplementation, again suggesting TIGK cells do respond to the presence of HSA-C. This is further confirmed in direct biofilm-TIGK contact co-cultures where cells cultured in the presence of HSA-C release higher levels of ROS compared with both HSA-AGE supplementation and no supplementation of the culture media.

Cytotoxicity analysis indicated, in no contact biofilm-TIGK co-cultures, the changes observed in cytokine release (over-all decreases in IL-8 and IL-6 in response to biofilm presence) and in ROS levels were not due to cell death. In all no contact biofilm-TIGK co-cultures, cytotoxicity was below 5%, thus, biofilms co-cultured suspended above the TIGK monolayer were not toxic to the cells over the time frame tested. When co-cultured with direct biofilm-cell contact there appeared to be higher cellular cytotoxicity suggesting that these co-culture conditions caused more damage to the cells. The increase in cytotoxicity could go towards explaining the overall decrease in ROS release observed in contact co-cultures compared with no contact co-cultures as the ROS assay requires intracellular processing for detection. It could also potentially explain the higher IL-8 and IL-6 concentrations observed in contact co-cultures.

5.3.3. Conclusion

Although differences were observed in biofilm composition in response to HSA-AGE, the lack of differences in functional profiles suggests the need for a transcriptomic approach to fully comprehend the role of HSA-AGE in shaping the biofilm. Transcriptomics could help establish if changes occurring at a protein level in response to HSA-AGE altered the way the biofilm as a whole is functioning at the time of sampling.

In addition, due to cellular inflammatory responses appearing to show limited differences between biofilm types and culture media during co-culturing, biological repeats are required with multiple inocula. It would also be beneficial to test inflammatory responses of immune cells (neutrophils) in biofilm-cell co-cultures particularly when considering their crucial role and high prevalence in the oral cavity (as described in detail previously).

The model presented here uses increase HSA-AGE to reflect long term effects of hyperglycaemia and as such does not fully model diabetes. Despite caveats, the model could give an insight into the contribution of hyperglycaemia/HSA-AGE on oral microbiota and help in establishing the role of AGEs in linking diabetes to periodontitis.

6. Preliminary feasibility study of neutrophils, chemokines/cytokines, MMPs and bacterial profiles in clinical samples from healthy and periodontitis participants

6.1. Introduction

Interactions between the oral microbiota and the host immune responses are in a delicate balance and involve complex mechanisms involving interactions between a variety of cells and molecules including cytokines/chemokines and adhesion molecules (Hajishengallis and Korostoff, 2017). The immune responses central to the maintenance of oral health, the dysregulation of these responses involved in the progression of periodontitis and the potential roles in linking diabetes to periodontitis have been discussed in detail in section 1.1.2.

Of the cells involved in oral health, neutrophils play a crucial role. As described previously (section 1.2.1), neutrophils (a form of terminally differentiated white blood cells) are highly prevalent in the gingival crevice, accounting for more than 95% of leukocytes present and providing the first line of defence against microbial invasion of gingival tissue (Delima and Van Dyke, 2003, Hajishengallis and Hajishengallis, 2014, Dutzan *et al*., 2016, Hajishengallis and Korostoff, 2017). Deficiencies decreasing the number of neutrophils (chronic or cyclic neutropenia), disorders impacting the movement of neutrophils (leukocyte adhesion deficiencies) or disorders compromising antimicrobial properties of neutrophils (such as Papillon Lefevre and morbus Kostman) exemplify the necessity of neutrophil homeostasis and the critical inflammatory regulation they provide as individuals with these conditions are more susceptible to periodontitis (Hajishengallis and Hajishengallis, 2014, Moutsopoulos *et al*., 2014, Hajishengallis and Korostoff, 2017). Although decreases in neutrophils in gingival tissues and the consequent impairment of immune regulation are associated with increased prevalence of periodontitis, neutrophils also play a vital role in periodontal tissue destruction when recruitment to the periodontal tissues is increased through improper regulation or inability to clear/control periodontitis associated microbial challenge (Hajishengallis and Moutsopoulos, 2014, Hajishengallis *et al*., 2015, Hajishengallis and Korostoff, 2017). Some of the central mechanisms involved in neutrophil associated tissue destruction in periodontitis and their potential roles in the diabetes-periodontitis relationship have been discussed in detail previously (section 1.2).

It was, therefore, the aim of this preliminary clinical study to determine the feasibility and validate the methodology for the determination of systemic neutrophil migration, phagocytosis and respiratory burst in systemically healthy participants with and without periodontitis. Following this preliminary study, the long-term aim is to evaluate these

neutrophil functions in diabetics with and without periodontitis to determine their role in diabetes associated periodontitis.

In addition, this study aimed to create a cytokine/chemokine profile based on a panel of nine cytokines/chemokines at both local and systemic levels in these participants. A bespoke panel including IL-8, IL-6, IL-1β, macrophage inflammatory protein 1α (MIP-1α), TNF-α, monocyte chemoattractant protein 1 (MCP-1), IL-17A, IL-17F and IL-23 was designed for this analysis. Some of these chemokines/cytokines (IL-8, IL-6, IL-1β and TNF-α) are central to immune regulation in the oral cavity and their roles in health maintenance, periodontitis progression and the periodontitis-diabetes relationship have been discussed in detail previously (section 1.2.4).

Others (IL-17A, IL-17F and IL-23) are crucial in Th17 mediated immune responses (section 1.2.2) and have been demonstrated to initiate periodontitis associated bone resorption (Kotake *et al*., 1999). A particular interest was paid to IL-17A due to evidence demonstrating higher concentrations of IL-17A in serum, saliva and plaque of periodontitis patients (Awang *et al*., 2014). Furthermore, IL-17A+ and IL-17F+ Th17 cells were both observed at a higher prevalence in chronic periodontitis participants thus, IL-17F was also included in the panel (Luo *et al*., 2014). However, evidence of the role of IL-17F in periodontitis progression is not conclusive, with a study demonstrating an IL-17F-associated protection from periodontitis in both humans and experimental models (Moretti *et al*., 2015). In addition, given the role of these cytokines in the regulation of neutrophils and the potential role of neutrophils in the periodontitis-diabetes relationship, their inclusion in the panel was crucial for the long-term aim of analysing the cytokine/chemokine profiles in diabetics with and without periodontitis.

The addition of MCP-1 to the cytokine/chemokine panel was based on the production of MCP-1 by a variety of cells in response to signals such as TNF-α, IL-1β and IFN-γ. Considering the increase in these cytokines associated with periodontitis and diabetes, there is a possibility that MCP-1 concentrations also increase in periodontal tissues during periodontitis. Indeed, MCP-1 has previously been shown to be increased at a gene and protein level in periodontitis (Hanazawa *et al*., 1993, Yu *et al*., 1993, Gupta *et al*., 2013). Furthermore, an animal study examining the concentration of MCP-1 in diabetic and periodontitis rat models showed a correlation between increased MCP-1 in both diabetic and periodontitis rats and increased gingival alveolar bone loss and inflammatory cell infiltration (Sakallioglu *et al*., 2008).

Similarly, the addition of MIP-1α was based on evidence demonstrating an increase in salivary levels in periodontitis with higher levels associated with bone loss (Fine *et al*., 2014). Furthermore, MIP1-α concentrations were shown to be higher in the GCF of

periodontitis patients with diabetes compared with those without diabetes regardless of site disease state (Duarte *et al*., 2014).

Neutrophil activation, in addition to release of cytokines/chemokines also involves the release of MMPs which aid in neutrophil migration and microbial clearance. However, if MMP release is dysregulated, an increase in MMP associated tissue destruction is observed (Silva *et al*., 2015). The role of MMPs in periodontitis, their impact on tissue destruction and their role in linking diabetes to periodontitis has been described in detail previously (section 1.2.3). As well as the above-mentioned analysis, this preliminary study aims to quantify the concentrations of MMP8 and MMP9 as well as their inhibitor (TIMP1) in GCF and saliva collected from the participants.

Finally, the intention of this preliminary study was to establish a microbial profile based on genetic information recovered directly from the saliva and GCF (metagenomics) (Thomas *et al*., 2012). The metagenomic NGS data were analysed for both taxonomy and microbial function. This could potentially provide invaluable information on the role of the microbiome in the pathogenesis of periodontitis. This is of particular interest when considering established differences in microbial profiles in periodontitis (including increases in red complex bacteria) and the advancement of culture independent methods for bacterial identification increasing our knowledge of the bacteria involved in periodontitis (Hiranmayi *et al*., 2017). As discussed in detail previously (section 1.4) information regarding microbiota profiles in diabetics with and without periodontitis are contradictory and more study is required.

6.2. Results

6.2.1. Neutrophil analysis

6.2.1.1. Migration

Leukocyte rich plasma was isolated from whole blood of five systemically healthy participants with periodontitis and five without and neutrophil migration was assessed using the Migratest assay (section 2.7.2). Although analysis was carried out on whole blood, and neutrophils were not isolated, an attempt was made to gate around neutrophils only (cell population with higher SSC due to greater intracellular complexity/higher granularity) during acquisition and analysis.

Data from all samples were acquired and analysed to quantify the number of migrated neutrophils, by counting the number of cells which had undergone migration as well as the changes in cell shape. The total number of cells that had migrated into the chamber was quantified, in addition their expression of L-selectin and cell size was analysed as indicators of activation by fMLP.

Neutrophils were identified and gated, followed by generation of FITC/L-selectin fluorescence histograms for this cell population (section 2.7.2). Thresholds were set on FITC histogram/L-selectin expression using non-migrated samples (samples remaining within the unstimulated cells inset). As show in Figure 6.1C, cells that failed to migrate and remained in the upper insert compartment had a higher FITC fluorescence as a consequence of higher L-selectin expression and, thus, more binding of the anti-L-selectin-FITC antibody. Activated cells (Figure 6.1B), on the other hand, have shed L-selectin and as such have a lower FITC fluorescence (Hafezi-Moghadam *et al*., 2001, Venturi *et al*., 2003). Unstimulated neutrophils had a proportion of cells with low L-selectin and a proportion of cells with higher L-selectin (Figure 6.1A). Furthermore, the total number of neutrophils migrating in unstimulated samples was lower than those migrating following stimulation (Figure 6.1).

A total count of migrated neutrophils was carried out (with counts normalised to counting beads as described previously) in addition to calculation of percentage of neutrophils with low L-selection (Figure 6.3). Changes in neutrophil size were also quantified. A decrease in FSC of stimulated neutrophils (Figure 6.2A and Figure 6.3C) compared with unstimulated neutrophils (Figure 6.2B and Figure 6.3C) was observed. This correlates to a decrease in cell size, a process neutrophils engage to allow more efficient movement through blood vessels and into tissues during infection.

Samples from all donors were analysed for total number of migrated neutrophils, percentage of activated cells (as indicated by reduction in L-selectin) and neutrophil size (Figure 6.3). Analysis of the data in this manner allowed determination of replicability of the methods. When analysing the number of cells that migrated through the membrane, there appeared to be varying degrees of replicability in technical repeats. Samples from certain participants (for example, participant 2 and participant 10) show large variations between technical repeats in both stimulated and unstimulated samples, while samples from other participants (e.g. participant 1 and participant 4) show limited differences within their technical repeats (Figure 6.3A). When analysing the percentage of cells with decreased L-selectin and changes in cell shape these variations were no longer apparent and little difference was observed between technical replicates (Figure 6.3B and Figure 6.3C).

Samples were also analysed in healthy and periodontitis groups using averages of technical repeats. Changes within participant groups were compared for significant differences (two-way ANOVA, Tukey, $p < 0.05$) followed by comparisons of healthy and periodontitis participant groups (two-way ANOVA, Bonferroni, $p < 0.05$).

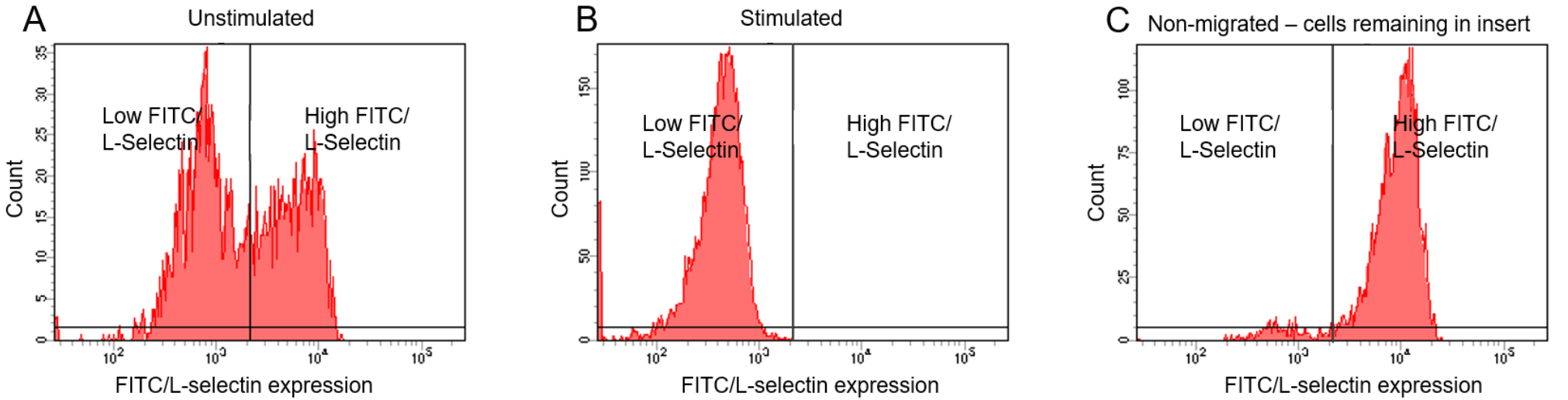


Figure 6.1: Representative flow cytometry data for neutrophil migration analysis. (A) Histogram showing L-selectin associated FITC against the number of neutrophils from unstimulated whole blood. (B) FITC histogram shows population shift to the lower L-selectin-FITC region upon neutrophil stimulation with fMLP. (C) Histogram for non-migrated cells remaining in the unstimulated insets (negative control).

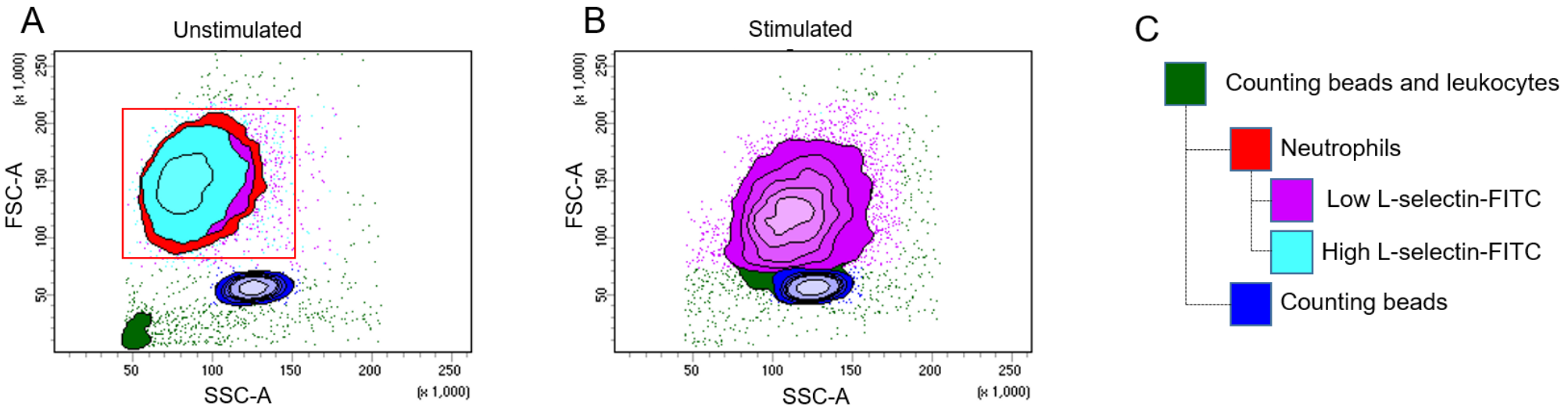


Figure 6.2: Representative flow cytometry data for neutrophil size changes following migration. (A) Contour plot showing FSC/SSC from unstimulated whole blood. (B) Contour plot following stimulation with fMLP shows a decrease in neutrophil cell size as indicated by a decrease in FSC (C) Population hierarchy.

In healthy participants there was no significant difference in the number of cells that had migrated from unstimulated samples compared with stimulated samples (Figure 6.4A), whereas significantly more neutrophils appeared to have migrated through the insert upon stimulation in samples from participants with periodontitis. Interestingly there was no significant difference in the number of migrated neutrophils when comparing healthy and periodontitis participants regardless of neutrophil stimulation (Figure 6.4A).

Although not significantly different, there appeared to be a lower number of migrated and non-migrated neutrophils in the unstimulated samples of periodontitis participants, perhaps indicating a lower total peripheral neutrophil number in periodontitis participants. Furthermore, the results suggest potentially more variation in the number of migrated neutrophils in patients with periodontitis compared with the healthy participants, particularly upon neutrophil stimulation. However, these observations could be a consequence of the small sample size and are therefore not conclusive.

When analysing the percentage of migrated neutrophils with decreased L-selectin, there was a significant difference between unstimulated, stimulated and non-migrated neutrophils in both periodontitis and healthy participants (Figure 6.4B). As expected, a higher percentage of migrated neutrophils had decreased L-selectin when stimulated compared with unstimulated and those that remained in the insert for both groups. Upon stimulation, 99.54% ± 0.14 (periodontitis) and 99.79% ± 0.083 (healthy) of neutrophils that passed through the membrane had decreased L-selectin, thus indicating stimulation encouraged migration which was not impacted by periodontitis. Although no difference was observed in stimulated migrated neutrophil L-selectin expression between health and periodontitis, fewer migrated neutrophils had low L-selectin (indicative of activation) in unstimulated samples. This suggests a higher proportion of neutrophils in healthy individuals were active despite a lack of stimulation.

During migration, in samples from healthy participants there was a significant decrease in cell size when neutrophils were stimulated (Figure 6.4C). Although no significant changes were observed in cell size of stimulated migrated neutrophils in participants with periodontitis compared with unstimulated neutrophils, stimulated neutrophils showed a decreased size compared with non-migrated neutrophils. In both healthy and periodontitis participants, both unstimulated and non-migrated neutrophils showed no significant differences in cell size.

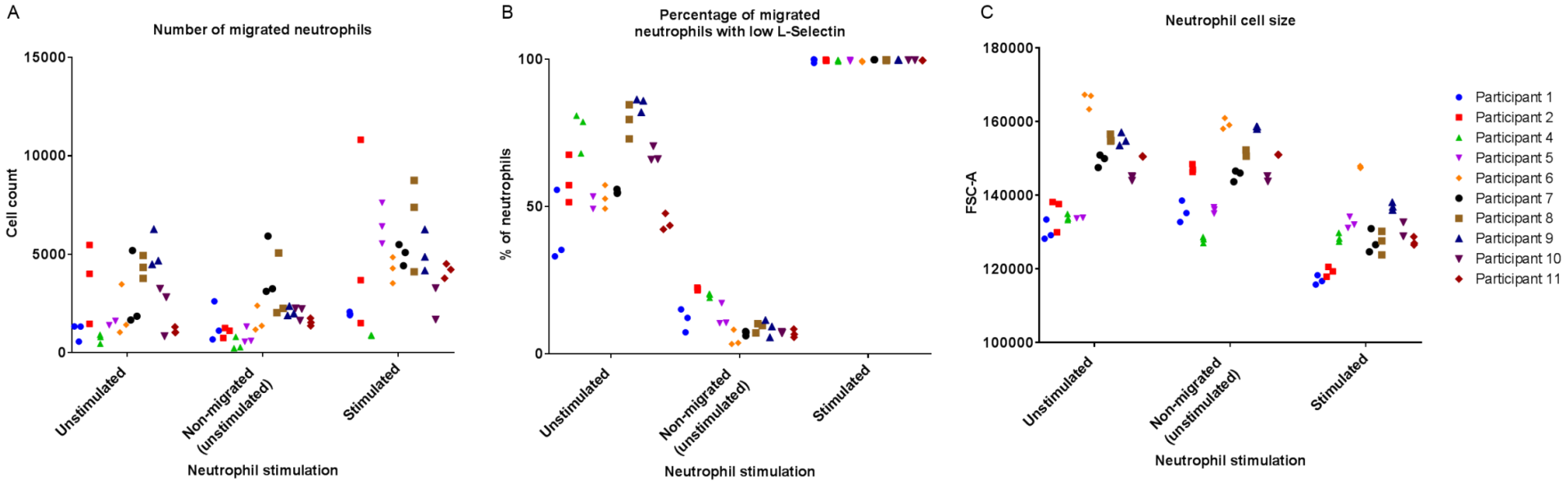


Figure 6.3: Analysis of neutrophils migration by participant. (A) Total number of neutrophils that migrated through the membrane, normalised to the counting beads. (B) Percentage of total migrated cells with low L-selectin-FITC (C) Changes in neutrophil FSC as an indication of changes in cell size.

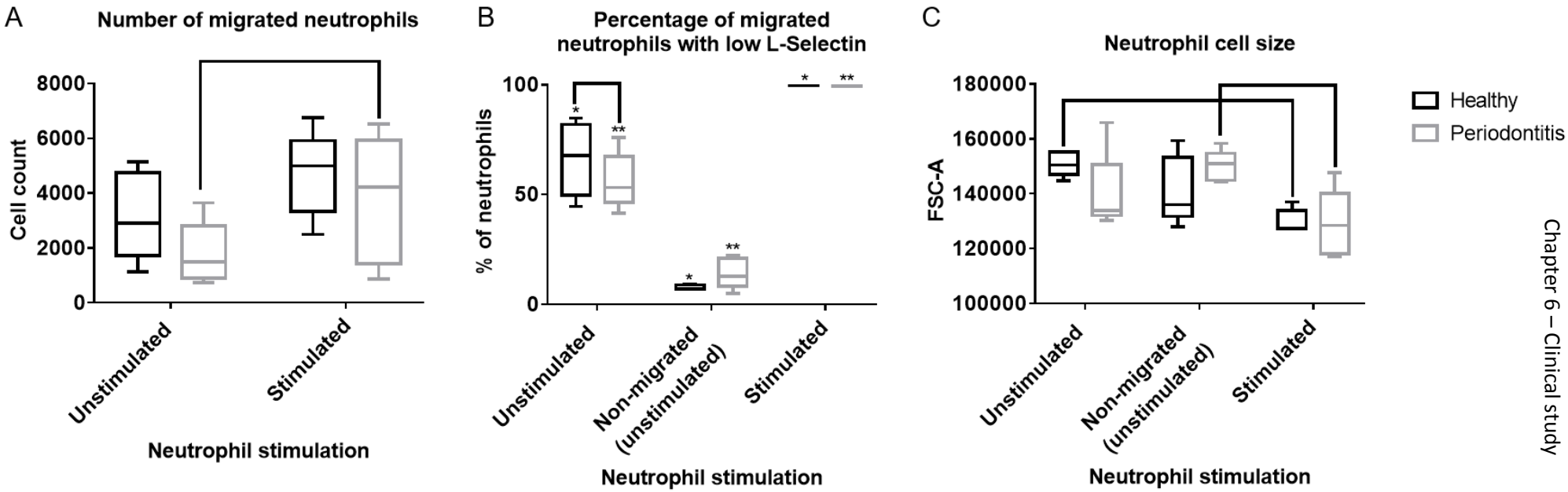


Figure 6.4: Migration of leukocyte in samples from healthy and periodontitis participants. (A) Total number of leukocytes that migrated through the insert membrane when unstimulated and stimulated with fMLP. All samples were normalised to a set of 2000 counting beads. (B) Percentage of migrated cells with decreased L-selectin expression. Symbols (*, **) indicate significant differences between groups with matching symbols. (C) Changes in leukocyte cell size as detected by change in FSC. Statistical significance was determined by comparing samples within groups and between groups using two-way ANOVA followed by a Tukey/Bonferroni, with significance $p < 0.05$.

In addition to no differences being observed in neutrophil L-selectin expression and number of migrated cells between healthy and periodontitis patients, there also appeared to be no significant differences in cell size changes when comparing the two groups, regardless of neutrophil stimulation. Taking both of these indicators of neutrophil migration/activation together, we can conclude that in this cohort, the assay provided no evidence of changes in neutrophil migration in periodontitis.

6.2.1.2. Analysis of neutrophil phagocytosis

Phagocytosis by the peripheral neutrophils from the periodontitis and healthy participants was also analysed. Whole blood was processed to generate LRP, and data acquired as previously described (section 2.7.3). As with migration analysis, neutrophils were not isolated specifically prior to assay but were gated and specified during analysis.

Determination of neutrophil phagocytosis followed stimulation of neutrophils with various FITC labelled *E. coli* concentrations, so that measurement of FITC correlated with the amount of labelled *E. coli* phagocytosed by neutrophils (described in section 2.7.3). Thresholds for FITC were set using unstimulated samples (Figure 6.5A), such that any FITC fluorescence seen above the fluorescence of the unstimulated samples (background fluorescence), was an indication of neutrophil phagocytosis. Upon stimulation, a dose dependent uptake of *E. coli* was observed (Figure 6.5).

As with the analysis of neutrophil migration, technical repeats of neutrophil phagocytosis were analysed for validation of methodology (Figure 6.6). There was limited variation between technical repeats when analysing the percentage of neutrophils phagocytosing (Figure 6.6A). This was particularly evident when cells were stimulated with 2×10^7 *E. coli*, perhaps due to the stimulation of maximum phagocytosis. Similarly, when measuring the mean fluorescent intensity (MFI) of phagocytosed *E. coli* (Figure 6.6B) as an indication of phagocytic activity, limited variation was observed between technical repeats, thus, indicating the replicability of this method.

Participant data were then analysed according to periodontal health. The results indicated a significant increase in the percentage of neutrophils phagocytically active upon stimulation, regardless of stimulant concentration (Figure 6.7A), in both healthy and periodontitis participant groups. Stimulation with 2×10^7 *E. coli* induced maximum proportions of neutrophils to be phagocytically active; 97.4% ± 1.1 (healthy) and 97.71% ± 0.83 (periodontitis), with a significant dose dependent decrease in the percentage of neutrophils performing phagocytosis (Figure 6.7B).

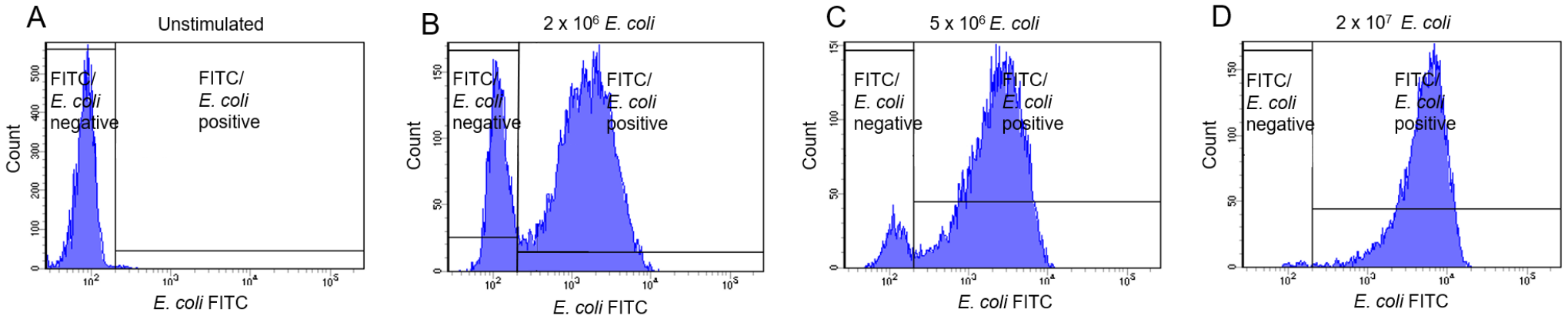


Figure 6.5: Representative flow cytometry data for phagocytosis analysis. (A) Histogram of *E. coli* FITC fluorescence of unstimulated neutrophils. Fluorescence observed within the FITC negative region is considered background fluorescence due to lack of *E. coli* in samples. (B) – (D) histograms demonstrating FITC fluorescence associated with stimulation with 2 x 10^6 *E. coli* (B), 5 x 10^6 *E. coli* (C) and 2 x 10^7 *E. coli*

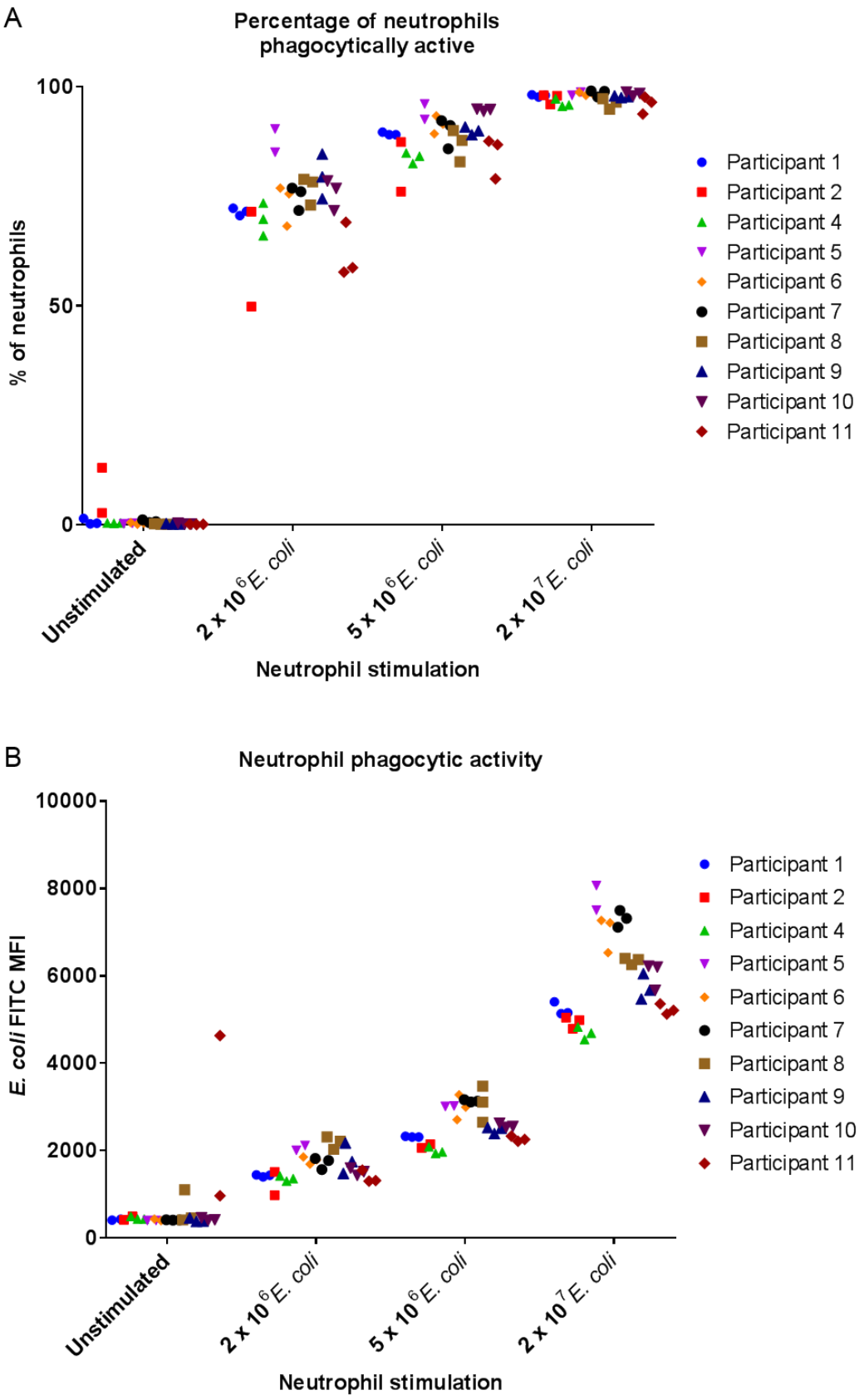


Figure 6.6: Analysis of neutrophil phagocytosis by participants. (A) Percentage of neutrophils phagocytically active as determined by the percentage of neutrophils which have phagocytosed FITC labelled *E. coli*. (B) Mean fluorescence intensity (MFI) of ingested FITC labelled *E. coli* as an indication of neutrophil phagocytic activity.

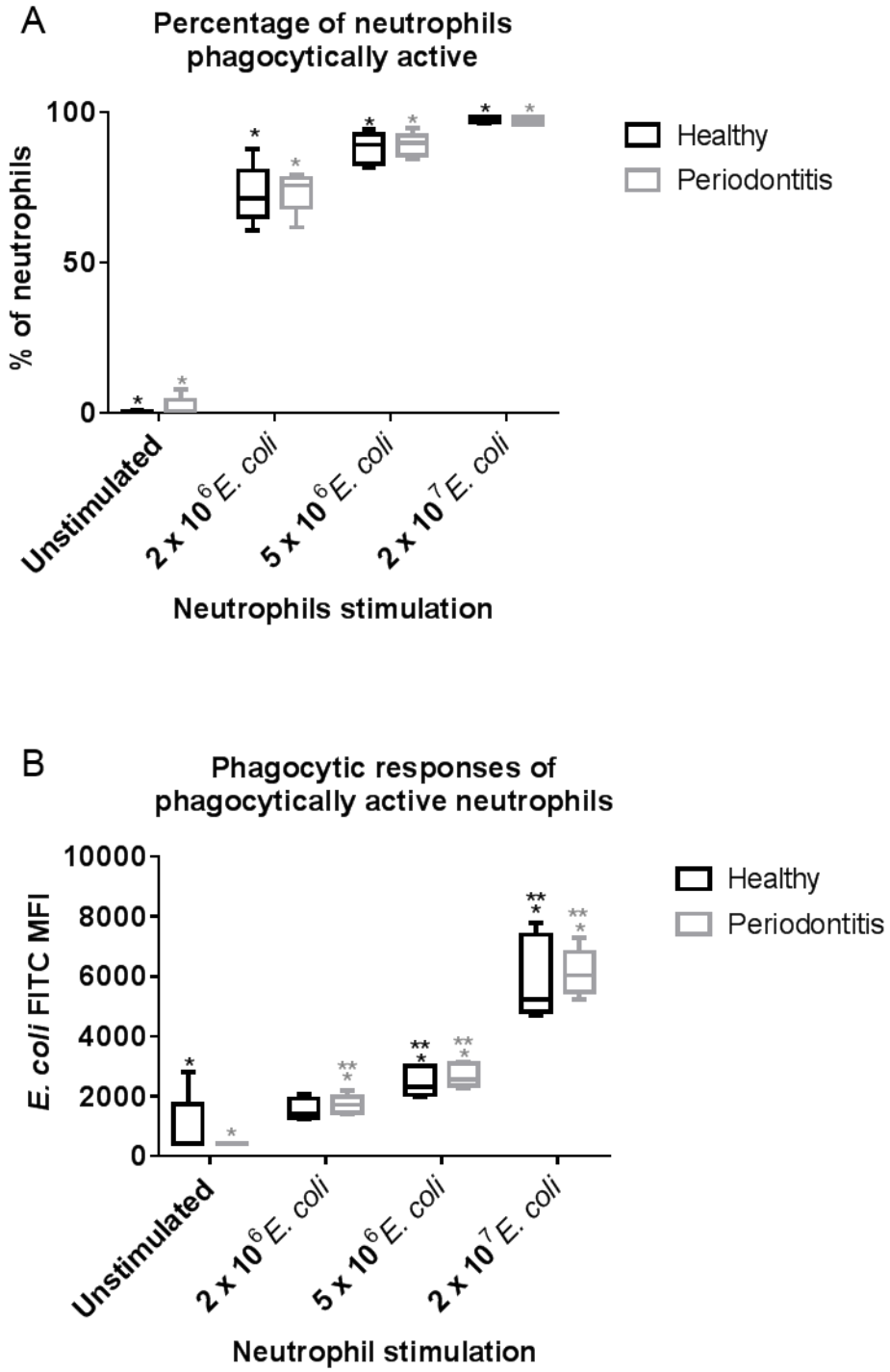


Figure 6.7: Phagocytosis by neutrophils in samples from healthy and periodontitis patients. (A) Percentage of neutrophils which had engulfed FITC labelled *E. coli* as an indication of phagocytically active neutrophils. (B) MFI of FITC labelled *E. coli* engulfed by neutrophils as an indication of neutrophil phagocytic activity. Statistical significance was determined by comparing samples within groups (two-way ANOVA, Tukey) and between healthy and periodontitis (two-way ANOVA, Bonferroni) with significance $p < 0.05$. Symbols (*, **) indicate significant differences between stimulation groups with matching symbols (black * indicate differences in healthy participants, grey * indicate differences in periodontitis participants).

In healthy participants, stimulation with at least 5 x 10^6 *E. coli* was required to induce phagocytosis sufficient to significantly increase the fluorescence of the neutrophils. In periodontitis participants, stimulation with 2 x 10^6 *E. coli* was sufficient to induce phagocytosis to significantly increase the fluorescence of the neutrophils. In addition, a significant dose dependent decrease in phagocytic activity was observed between the stimulated neutrophils.

Along with analysis within groups, percentage of neutrophils undergoing phagocytosis and the phagocytic activity of these neutrophils was compared between healthy and periodontitis groups (two-way ANOVA, Bonferroni, $p < 0.05$) and in both cases no significant differences were observed. Similar to migration analysis, we can conclude that in this cohort, the assay provided no evidence of changes in neutrophil phagocytosis in periodontitis.

6.2.1.3. Analysis of neutrophil respiratory burst

Finally, neutrophil respiratory burst from whole blood of these participants was analysed. In a similar manner to phagocytosis analysis; LRP was generated, respiratory burst was activated using various concentrations of *E. coli* and fluorescence correlating to ROS dependent oxidation of DHR 123 to the fluorogenic R123 was detected at wavelength 488 nm (section 2.7.4). As with previous assays, neutrophils were identified during analysis.

An initial analysis gate was set using unstimulated samples to distinguish between R123 negative (background fluorescence – Figure 6.8A) and R123 positive (neutrophils undergoing respiratory burst). An *E. coli* stimulation dose dependent increase was observed in R 123 fluorescence (Figure 6.8B-D).

Methodology for respiratory burst was validated by ensuring replicability between technical repeats. Limited variation between samples from the same participant was observed for analysis of both the percentage of neutrophils undergoing respiratory burst (Figure 6.9A) and the amount of ROS generated by these neutrophils (Figure 6.9B). In addition to stimulation of respiratory burst by *E. coli*, neutrophils were also stimulated by PMA (which directly activates protein kinase C for downstream ROS release) and fMLP (which binds to its cell surface receptors to activate signalling cascades that result in ROS release) (Hu *et al*., 2011) to activate the respiratory burst independently of phagocytosis.

A significant increase (two-way ANOVA, Tukey, $p < 0.05$) was observed in the percentage of neutrophils undergoing respiratory burst upon stimulation with *E. coli* (regardless of *E. coli* concentration) and PMA compared with unstimulated samples in

both healthy and periodontitis participant groups (Figure 6.10). Furthermore, a significant dose dependent increase in the percentage of neutrophils undergoing respiratory burst was observed (Figure 6.10A) but no differences were detected in the amount of ROS released by these neutrophils, except upon stimulation with PMA (Figure 6.10B). In both cases no significant differences between healthy and periodontitis groups was seen.

In addition, a dose dependent decrease was observed in the percentage of neutrophils undergoing respiratory burst upon stimulation with *E. coli* in both groups. This was significant comparing stimulation with 2×10^7 *E. coli* and 2×10^6 *E. coli*, but not when comparing stimulation with 2×10^7 *E. coli* and 5×10^6 *E. coli* or 5×10^6 *E. coli* and 2×10^6 *E. coli*. This perhaps indicates that the percentage of neutrophils undergoing respiratory burst was at its maximum when stimulated by both 2×10^7 *E. coli* and 2×10^6 *E. coli* but a 10 fold reduction in the stimulant was required to significantly decrease the percentage of neutrophils undergoing respiratory burst. Although stimulation by PMA led to 93.98%±3.5 (healthy) and 96.8%±1.76 (periodontitis) of neutrophils undergoing respiratory burst, stimulation by fMLP showed no significant difference compared with unstimulated samples. Furthermore, there appeared to be no significant difference when comparing responses from healthy participants with those with periodontitis.

As with previous neutrophil analysis, we can conclude that in this cohort, the assay provided no evidence of changes in neutrophil respiratory burst in periodontitis.

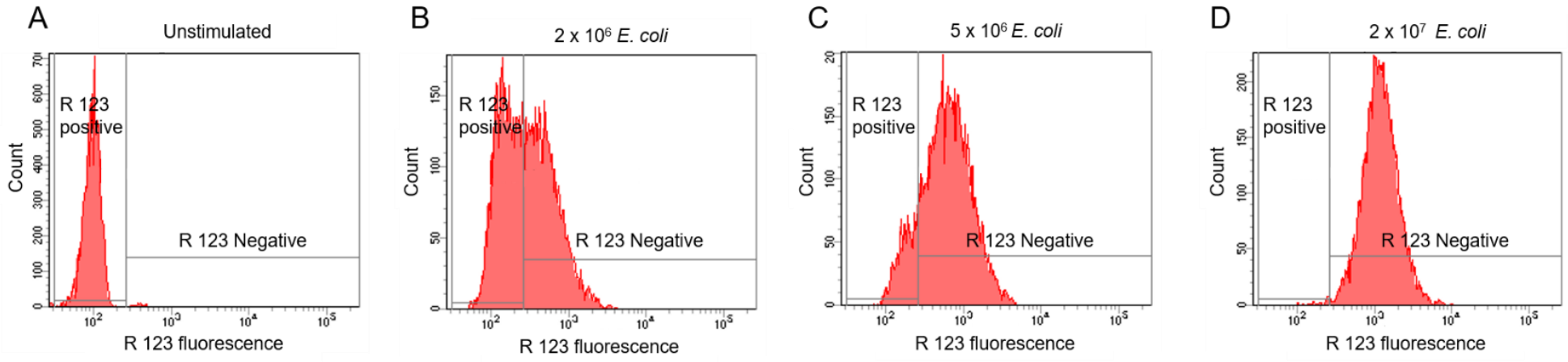


Figure 6.8: Representative flow cytometry analysis of neutrophil respiratory burst. (A) Histogram of background neutrophil R123 fluorescence in unstimulated samples. (B) – (D) Histograms of R123 fluorescence in samples stimulated with 2 x 10^6 *E. coli* (B), 5 x 10^6 *E. coli* (C) or 2 x 10^7 *E. coli* (E).

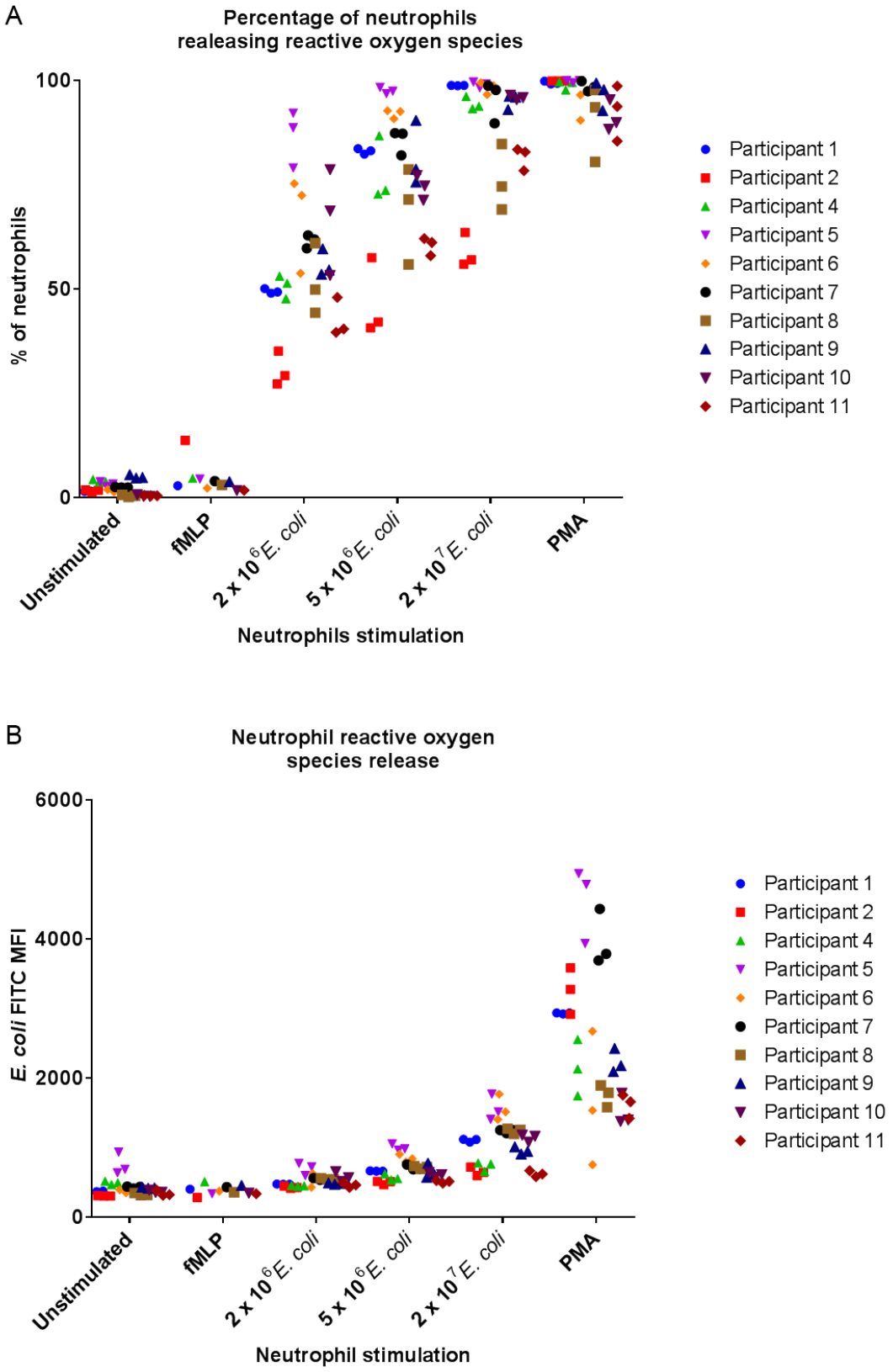


Figure 6.9: Participant neutrophil respiratory burst analysis. (A) Percentage of neutrophils undergoing respiratory burst as determined by the percentage of R123 positive neutrophils. (B) MFI R123 fluorescence in the R123 positive neutrophils as an indication of the amount of ROS released.

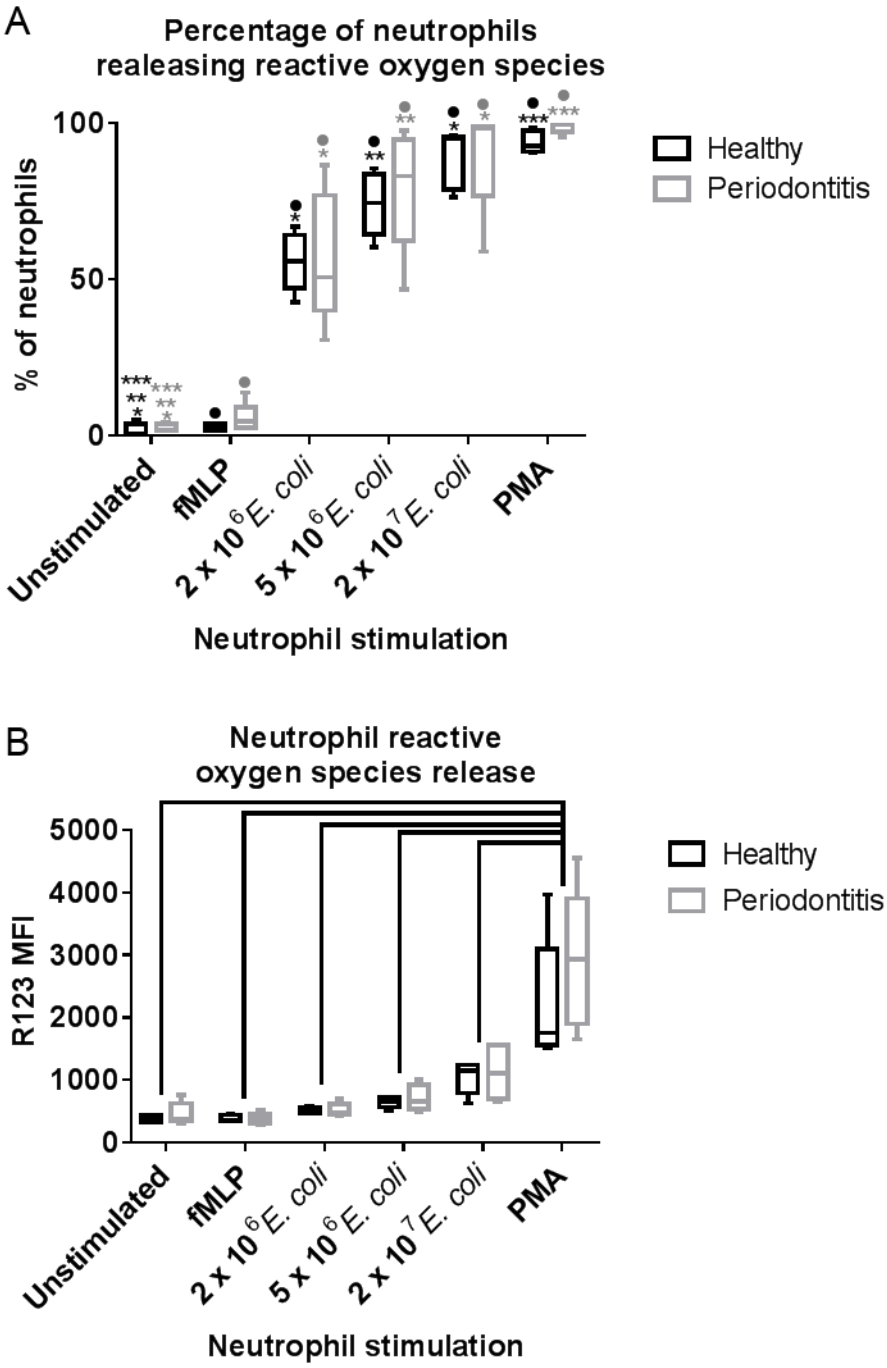


Figure 6.10: Neutrophil respiratory burst in PMNs of healthy participants and those with periodontitis. (A) Percentage of neutrophils undergoing respiratory burst as determined by the percentage of PMNs falling into the FITC positive region (Figure 6.8B – histogram, boxed region). (B) MFI of FITC fluorescence in the FITC positive region as an indication of the amount of PMN respiratory burst. Statistical significance was determined by comparing samples within groups (two-way ANOVA, Tukey) and between healthy and periodontitis (two-way ANOVA, Bonferroni) with significance $p < 0.05$. Symbols (*, **, ***, •) indicate significant differences between stimulation groups with matching symbols and colours (black symbols indicate differences in healthy participants, grey symbols indicate differences in periodontitis participants).

6.2.2. Cytokine/chemokine profiles of saliva, plasma and GCF

Saliva, plasma and GCF were collected and processed from five systemically healthy participants with periodontitis and five without as described previously (section 2.7.1). The samples were analysed to determine the presence and concentration of a panel of cytokines/chemokines using a multiplex bead array method (section 2.7.5).

The multitude of proteins contributing to the complex matrix of saliva has the potential to interfere with the detection of the cytokine/chemokines. Taking this into account cytokines/chemokines in saliva were quantitated both undiluted and diluted to determine the efficiency of the multiplex bead array method with saliva. Dilution methods outlined by the manufacturer for plasma analysis was utilised for saliva for a 1:1 dilution with the provided diluent (section 2.7.5). Once data were collected, standard curves were generated, and cytokine/chemokine concentrations determined using the multiplex bead array manufacturer's analysis software (LegendPlex), with corrections for background fluorescence (Figure 6.11).

There were limited differences between undiluted and diluted samples. However, IL-1β and IL-23 were detected in more samples following dilution while detection of IL-17A and IL-17F appeared to be lost in some samples following dilution (Figure 6.11). In all cases, differences in cytokine/chemokine detection were observed when concentrations were low and potentially below the reliable detection limit of the assay. Considering this and the potential for interference from salivary proteins, saliva analysis was carried out on diluted samples.

Concentrations of cytokine/chemokines from participant samples were determined using the LegendPlex, the results from healthy and periodontitis participants grouped and compared for statistically significant differences using two-way ANOVA followed by a post-hoc Bonferroni ($p < 0.05$). The results indicated the presence of eight out of nine analysed cytokines/chemokines in saliva to varying degrees in both healthy and periodontitis participants (Figure 6.12 and Table 6.1). Of the cytokines/chemokines, IL-8, IL-1β and MCP-1 were the most prevalent in saliva of both healthy and periodontitis groups. In periodontitis IL-1β had the highest concentrations, averaging 12311.63 ± 1318.28 pg/mL followed by IL-8 (8940.2 ± 7550.71 pg/mL) and MCP-1 (792.02 ± 978.84 pg/mL), while IL-17 was not detected and IL-17A had the lowest average concentration (1.62 ± 3.62 pg/mL). Contrastingly, in healthy participants IL-8 was detected at the highest concentrations (1606.75 ± 1331.43 pg/mL) followed by MCP-1 (188.604 ± 80.33 pg/mL) and IL-1β (122.07 ± 184.94 pg/mL). Both groups, however, exhibited large variations in cytokine/chemokine concentrations between participants (Figure 6.12). This was particularly evident within the periodontitis group for IL-8, IL-1β and MCP-1

concentrations (Figure 6.12 and Table 6.1). It is perhaps for this reason that comparisons between healthy and periodontitis groups resulted in no significant differences in any of the analysed cytokines/chemokines in saliva.

In plasma, all nine of the analysed cytokines/chemokines were detected at varying concentrations (Figure 6.12 and Table 6.1). In healthy participants MCP-1 had the highest average concentration (287.604 ± 27.56 pg/mL) followed by MIP-1α (84.948 ± 59.37 pg/mL) and IL-23 (72.264 ± 46.4 pg/mL). In plasma of the periodontitis group, MCP-1 also had the highest concentration (212.1 ± 53.93 pg/mL) followed by IL-8 (104.688 ± 139.71 pg/mL) and MIP-1α (74.502 ± 44.71 pg/mL).

In contrast to saliva, lower concentrations of IL-8, IL-1β and MCP-1 were observed in plasma whereas higher concentrations of IL-6, MIP-1α, TNF-α, IL-17A, IL-17F and IL-23 were seen. However, as with saliva, a large amount of variation was observed in the IL-8 concentration in periodontitis participants (Figure 6.12A, Figure 6.12B and Table 6.1). Although cytokine/chemokine expression appeared to differ comparing the plasma of healthy and periodontitis participants these differences were not significant.

For cytokine/chemokine profiling of GCF, samples from four participants with periodontitis and five healthy participants were analysed. GCF strips from one participant were contaminated with blood and so data from this sample were not included in the final analysis of cytokines/chemokines. Results are presented as concentrations of cytokines absorbed at a site onto Perio Paper strips in 30 seconds with samples from five sites pooled (section 2.7.1.3 and 2.7.5).

In GCF, only IL-8 and IL-1β were detectable in both healthy and periodontitis participant groups (Figure 6.12C and Table 6.1). This contrasted to saliva and plasma, where 8 out of 9 cytokines/chemokines or all cytokines/chemokines respectively were found to be present to some degree. In addition, MCP-1 was also seen in CGF of participants with periodontitis. In a similar manner to the trend observed in saliva and plasma, a larger variation in concentrations of IL-8 was observed in periodontitis participants compared with healthy participants. In the case of GCF, significantly higher concentrations of IL-8 were observed in periodontitis participants compared with healthy participants. However, no difference between health and disease was observed in concentrations of IL-1β and MCP-1. Despite the lack of significant difference, on average the concentration of IL-1β was higher in periodontitis (10941.52 ± 17932.9 pg/mL) compared with health (687.840 ± 1416.37 pg/mL).

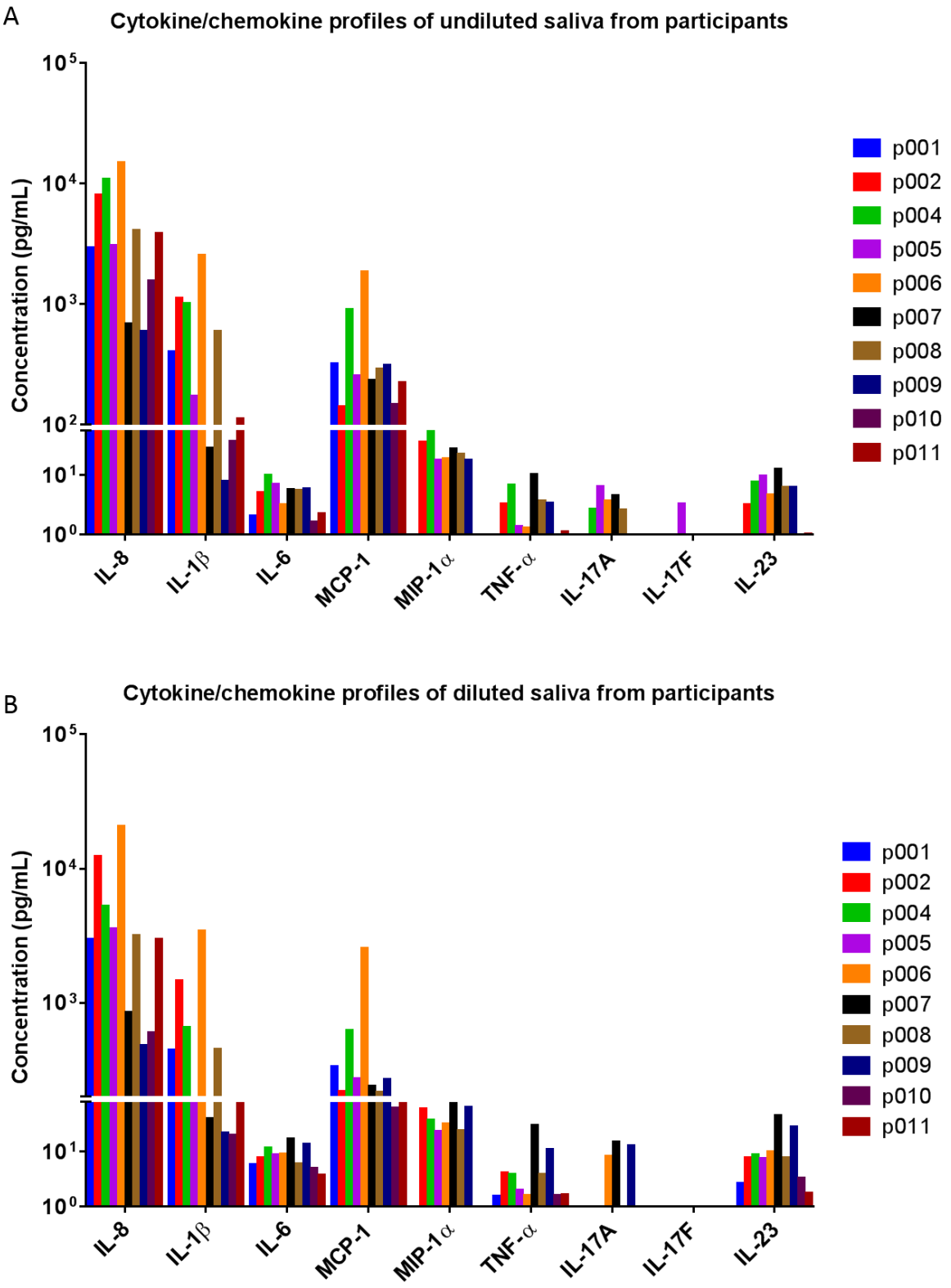


Figure 6.11: Cytokine/chemokine profiles of saliva from healthy and periodontitis participants. (A) Cytokine/chemokine concentrations in undiluted saliva of participants detected using a multiplex bead array. (B) Cytokine/chemokine concentrations (with dilution factors accounted for) in saliva diluted 1:1 prior to detection using a multiplex bead array.

Table 6.1: cytokine/chemokine concentrations in saliva, plasma and GCF of healthy and periodontitis participants. Results are mean ± SD.

		Concentration (pg/mL) in samples from:	
Sample matrix	Cytokine/chemokine	Periodontitis participants	Healthy participants
Saliva	Interleukin-8	8940.20 ± 7550.71	1606.75 ± 1331.43
	Interleukin-1β	12311.63 ± 1318.28	122.07 ± 184.94
	Interleukin-6	8.55 ± 2.06	9.02 ± 5.90
	Monocyte chemoattractant protein 1	792.02 ± 978.84	188.60 ± 80.33
	Macrophage inflammatory protein 1α	30.42 ± 2.79	33.26 ± 36.27
	Tumour necrosis factor-α	2.60 ± 1.25	9.54 ± 11.96
	Interleukin-17A	1.62 ± 3.62	5.48 ± 7.54
	Interleukin-17F	0	0
	Interleukin-23	7.26 ± 2.76	17.31 ± 19.06
Plasma	Interleukin-8	104.69 ± 139.71	28.84 ± 18.06
	Interleukin-1β	30.81 ± 20.76	18.97 ± 13.80
	Interleukin-6	30.54 ± 20.07	30.88 ± 13.60
	Monocyte chemoattractant protein 1	212.10 ± 53.93	287.60 ± 27.56
	Macrophage inflammatory protein 1α	74.50 ± 44.71	84.95 ± 59.37
	Tumour necrosis factor-α	39.82 ± 23.57	19.51 ± 10.90
	Interleukin-17A	35.84 ± 27.46	23.08 ± 9.96
	Interleukin-17F	6.81 ± 9.61	11.25 ± 14.61
	Interleukin-23	65.77 ± 45.09	72.26 ± 46.40
Gingival crevicular fluid (GCF)	Interleukin-8	75997.68 ± 115189.00	151767.08 ± 15613.00
	Interleukin-1β	10941.52 ± 17932.90	687.84 ± 1416.37
	Interleukin-6	0	0
	Monocyte chemoattractant protein 1	75.12 ± 167.97	0
	Macrophage inflammatory protein 1α	0	0
	Tumour necrosis factor-α	0	0
	Interleukin-17A	0	0
	Interleukin-17F	0	0
	Interleukin-23	0	0

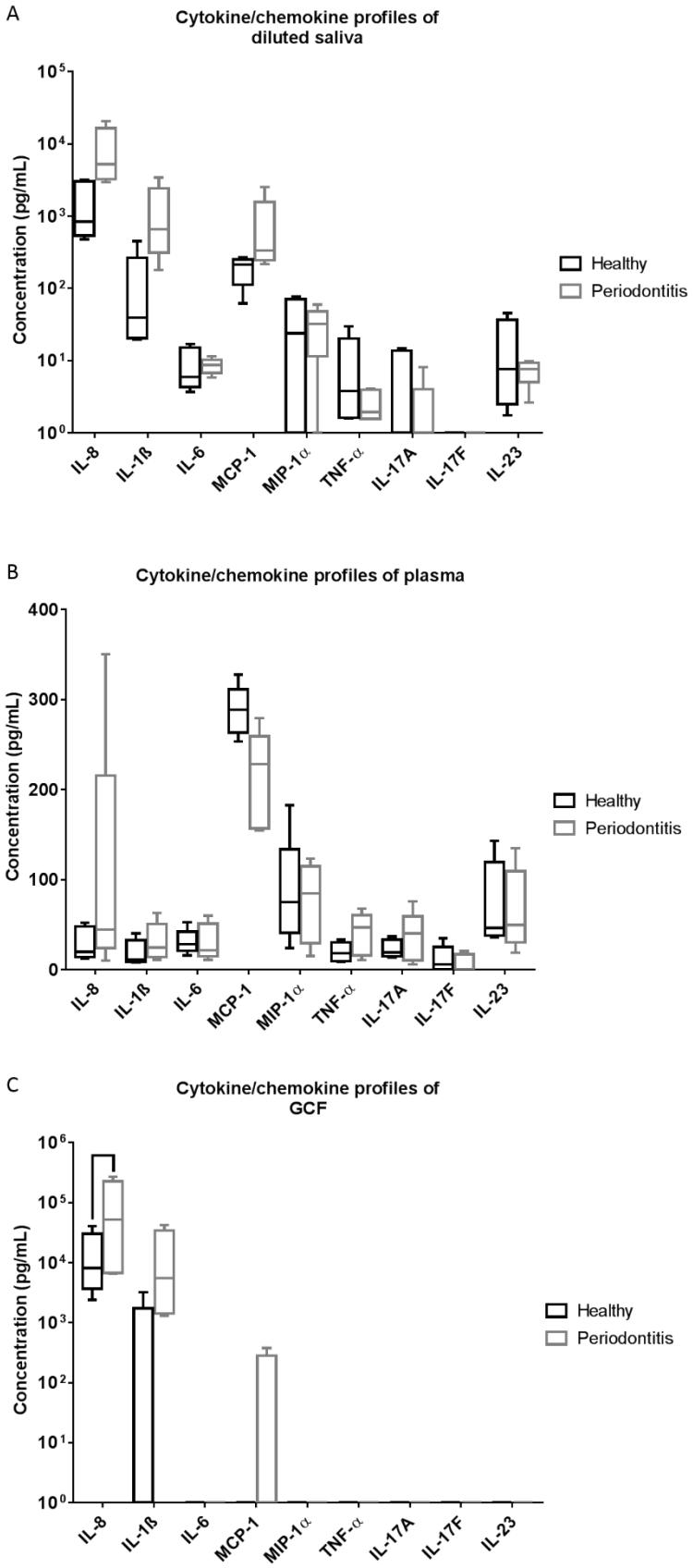


Figure 6.12: Cytokine/chemokine profiles in samples from healthy and periodontitis participants. Saliva (A), plasma (B) and gingival crevicular fluid (C) samples were assayed using a multiplex bead array. Significant differences were calculated using two-way ANOVA followed by a post-hoc Bonferroni ($p < 0.05$).

6.2.3. MMP8, MMP9 and TIMP1 protein concentrations in saliva and GCF

Saliva and GCF from healthy and periodontitis participants were processed and analysed using ELISAs to determine concentrations of MMP8, MMP9 and TIMP1 as described previously (section 2.7.6).

An increase in the concentration of MMP8 and MMP9 in both saliva and GCF from periodontitis participants was observed, however statistical analysis showed no significant difference when comparing periodontitis and healthy participants (T-test, Mann-Whitney, $p > 0.05$).

In both saliva and GCF the MMP8 concentrations in periodontitis participants appeared to have a larger range; 445.1 – 1420.3 ng/mL in GCF and 121.53 – 603.42 ng/mL in saliva, compared with healthy participants; 118.03 – 1006.46 ng/mL in GCF and 13.382 – 181.1 ng/mL in saliva (Figure 6.13A and Figure 6.13D). Although not significantly different, on average the MMP8 concentration in periodontitis participant saliva (326 ± 237.9 ng/mL) and GCF (923.6 ± 438.5 ng/mL) appeared to be higher compared with health (55.93 ± 70.48 ng/mL in saliva and 519.6 ± 325.8 ng/mL in GCF).

Results for MMP9, indicated a similar pattern to MMP8 in saliva and GCF. In saliva samples from participants with periodontitis, the data indicated a larger variation in MMP9 concentration (192 – 1381 ng/mL) compared with healthy (0 – 586.7 ng/mL) (Figure 6.13B). This pattern was reflected in GCF but with lower overall concentrations compared with saliva (211.9 – 550.2 ng/mL in periodontitis and 41.96 – 404.3 ng/mL) (Figure 6.13D). Although, no significant differences were observed in MMP9 concentrations in saliva and GCF of participants with and without periodontitis, a higher average concentration in periodontitis (544.9 ± 480.2 ng/mL in saliva and 433 ± 159 ng/mL in GCF) was observed compared with health (184.3 ± 237.5 ng/mL in saliva and 270 ± 152 ng/mL in GCF) (Figure 6.13B and Figure 6.13D)

In both healthy and periodontitis saliva samples, MMP inhibitor TIMP1, had similar mean concentrations (30.02 ± 14.18 ng/mL and 26.02 ± 16 ng/mL respectively) and similar concentration ranges (11.84 – 53.21 ng/mL in periodontitis and 14.48 – 41.71 ng/mL in health) (Figure 6.13C) with no significant differences between the two groups. In GCF, a higher average concentration of TIMP1 was observed in periodontitis (19.1 ± 16.96 ng/mL) compared with health (2.02 ± 4.52 ng/mL) although the difference did not reach significance (Figure 6.13F).

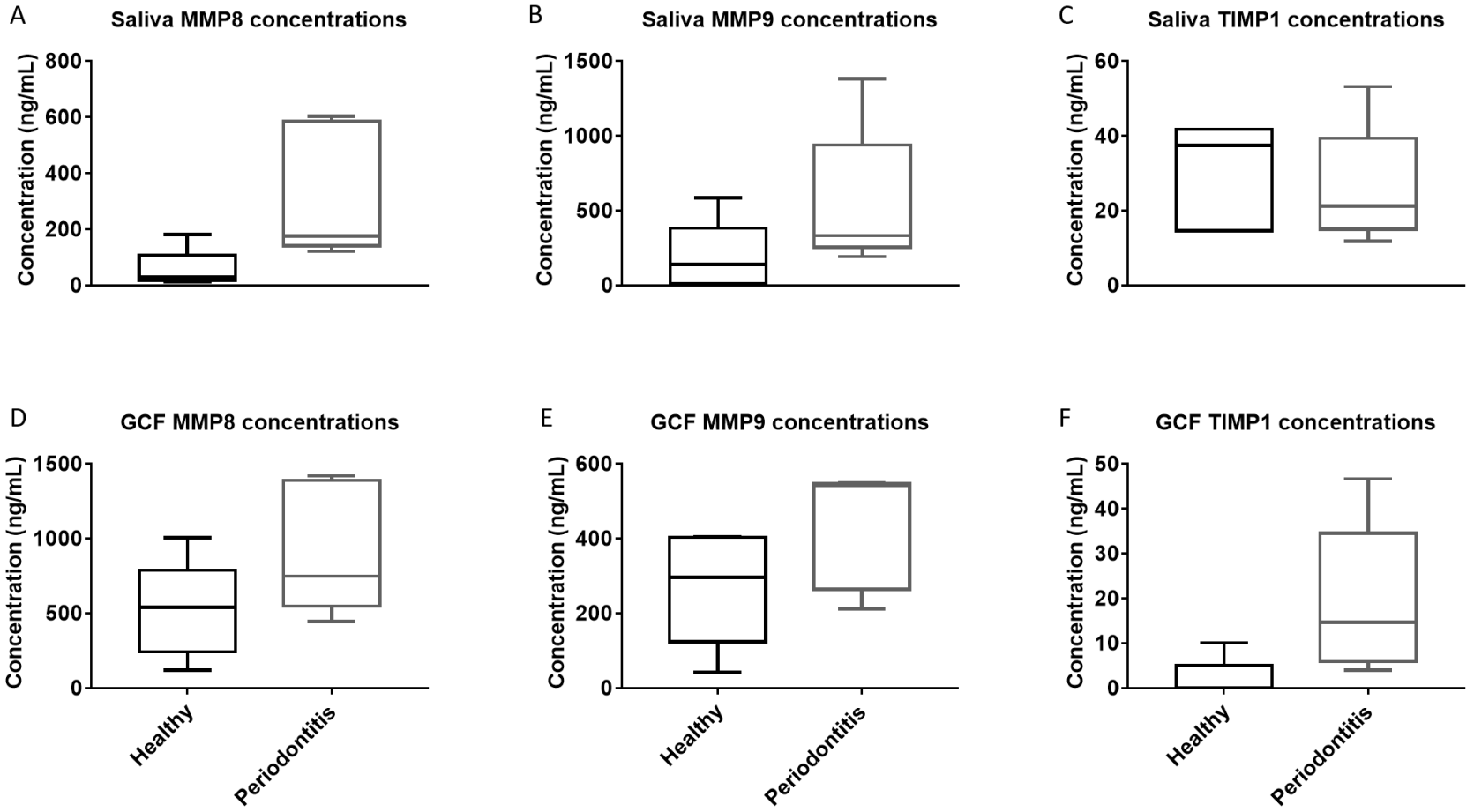


Figure 6.13: Matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinase 1 (TIMP1) concentrations in healthy and periodontitis participants. (A) – (C) Concentrations of MMP8 (A), MMP9 (B) and TIMP1 (C) in saliva determined via ELISAs. (D) – (F) Concentrations of MMP8 (D), MMP9 (E) and TIMP1 (F) in gingival crevicular fluid (GCF) determined via ELISAs.

6.2.4. Next generation sequencing analysis of clinical samples

6.2.4.1. Taxonomical analysis of plaque and saliva samples from healthy and periodontitis participants

A Shotgun metagenomic approach was used to process plaque and saliva samples from healthy and periodontitis participants. Results were pre-processed (section 2.6) prior to separation of reads into rDNA and DNA. rDNA reads were analysed following the QIIME pipeline (section 2.6.1) while short DNA reads were *de novo*-assembled to form DNA contigs and analysed used MEGAN (section 2.6.2). All analysis was limited to bacterial reads only.

Taxonomic composition of the microbiota in saliva and plaque was analysed using the operational taxonomic unit (OTU) counts based on alignments against the human oral microbiome database (HOMD) for rDNA reads and NCBI non-redundant protein databases for DNA contigs (section 2.6).

Alpha diversity plots were used to determine species richness (number of taxa/OTUs) within each sample. Multiple calculations of alpha diversity were carried out and analysis based on rDNA OTU counts in this cohort demonstrated saliva samples were richer in species compared with plaque samples, regardless of calculation method (Figure 6.14A). Contrastingly, using DNA contig OTU counts for alpha diversity calculations in these samples resulted in limited differences in plaque and saliva when using the Chao1, ACE and Fisher methods and higher alpha diversity in saliva using the Simpson, inverse Simpson and Shannon methods (Figure 6.14B). In both rDNA and DNA contig analysis methods and regardless of calculation method, no significant difference was observed (unpaired T-test, Mann-Whitney, $p > 0.05$).

In alpha diversity measurements of both rDNA and DNA contig counts, the plaque samples appeared to have more variation in the alpha diversity measure, indicating more variation in richness compared with the saliva samples. Interestingly, in rDNA samples no differences were apparent when comparing healthy to periodontitis regardless of calculation method. However, when using DNA counts Shannon, Simpson and InvSimpson alpha diversity measures were significantly lower in periodontitis than in health (unpaired T-test, Mann-Whitney, $p > 0.05$) (Figure 6.14B).

Principle coordinate analysis (PCoA) with Bray-Curtis dissimilarity plots indicated saliva samples clustered separately from plaque samples in counts from both rDNA and DNA contig, with less sample relatedness in plaque samples (Figure 6.15). Additionally, saliva and plaque samples from periodontitis and healthy clustered together. There was, however, more crossover between health and periodontitis in both saliva and plaque when using rDNA counts (Figure 6.15A). Together these data suggest that communities

from plaque are relatively different from those found in saliva and communities found in health are different compared with those found in periodontitis.

Following analysis of sample relatedness, taxonomic composition at a phylum level using OTU counts from both rDNA and DNA contigs were analysed. Results are presented as percentage relative abundances (abundance of each OTU relative to all OTUs in each sample) to allow for a more accurate comparison between sample types and sample sites (Figure 6.16).

When looking at the percentage abundance of OTUs based on rDNA data in these oral samples it is clear that a difference exists not only between the healthy and periodontitis samples, but also between sample sites (Figure 6.16A). In plaque, healthy samples had a higher abundance of *Proteobacteria* compared with periodontitis samples, where abundances of *Proteobacteria* were very low, while abundances of *Spirochaetes* had the opposite trend. In saliva, however, the percentage of *Proteobacteria* remained unchanged and *Spirochaetes* were undetectable. Furthermore, in both saliva and plaque more *Firmicutes* were observed in health, with abundances higher in saliva compared with plaque. Less *Bacteroidetes* were present in healthy plaque and a similar trend was found in saliva. *Synergistetes*, interestingly, were only detectable in plaque from periodontitis participants. Finally, *Actinobateria* appeared to be more prevalent in health and this difference was more evident in plaque than in saliva.

Analysis of OTUs based on DNA contigs demonstrated similar patterns of prevalence in *Bacteroidetes*, *Firmicutes, Fusobacteria* and *Synergistetes* to that of rDNA (Figure 6.16B). Compared with rDNA based analysis, *Fusobacteria* appeared to be more abundant in plaque and saliva DNA contig OTUs but in both cases little difference was observed between health and periodontitis. *Proteobacteria* percentages based on DNA contigs also differed with higher abundances in periodontitis plaque and lower abundance in saliva and healthy plaque compared with rDNA based OTU percentages. Despite this, *Proteobacteria* appeared to follow the same trend as described for rDNA based abundances. Furthermore, *Actinobacteria* percentages in DNA contig based OTU counts were increased in saliva, unchanged in periodontitis plaque and decreased in healthy plaque compared with rDNA but with a similar trend in regard to differences between health and periodontitis.

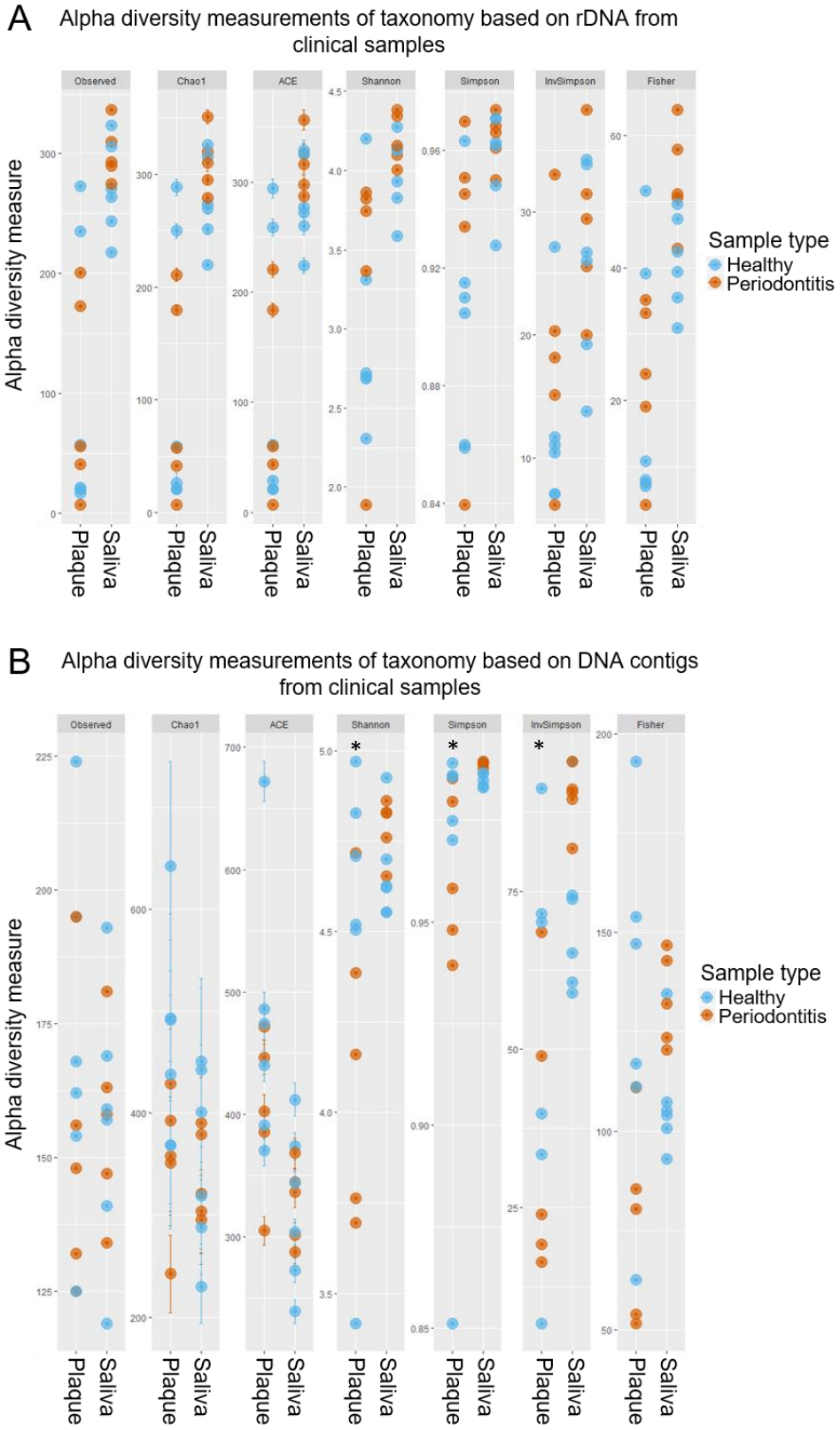


Figure 6.14: Alpha diversity measurements of clinical samples. (A) Alpha diversity measures calculated using a variety of methods for OTU counts based on rDNA alignments. (B) Alpha diversity measures calculated using a variety of methods for OTU counts based on DNA contig alignments. Significant differences between health and periodontitis at each site was calculated using an unpaired t-test (*, $p < 0.05$).

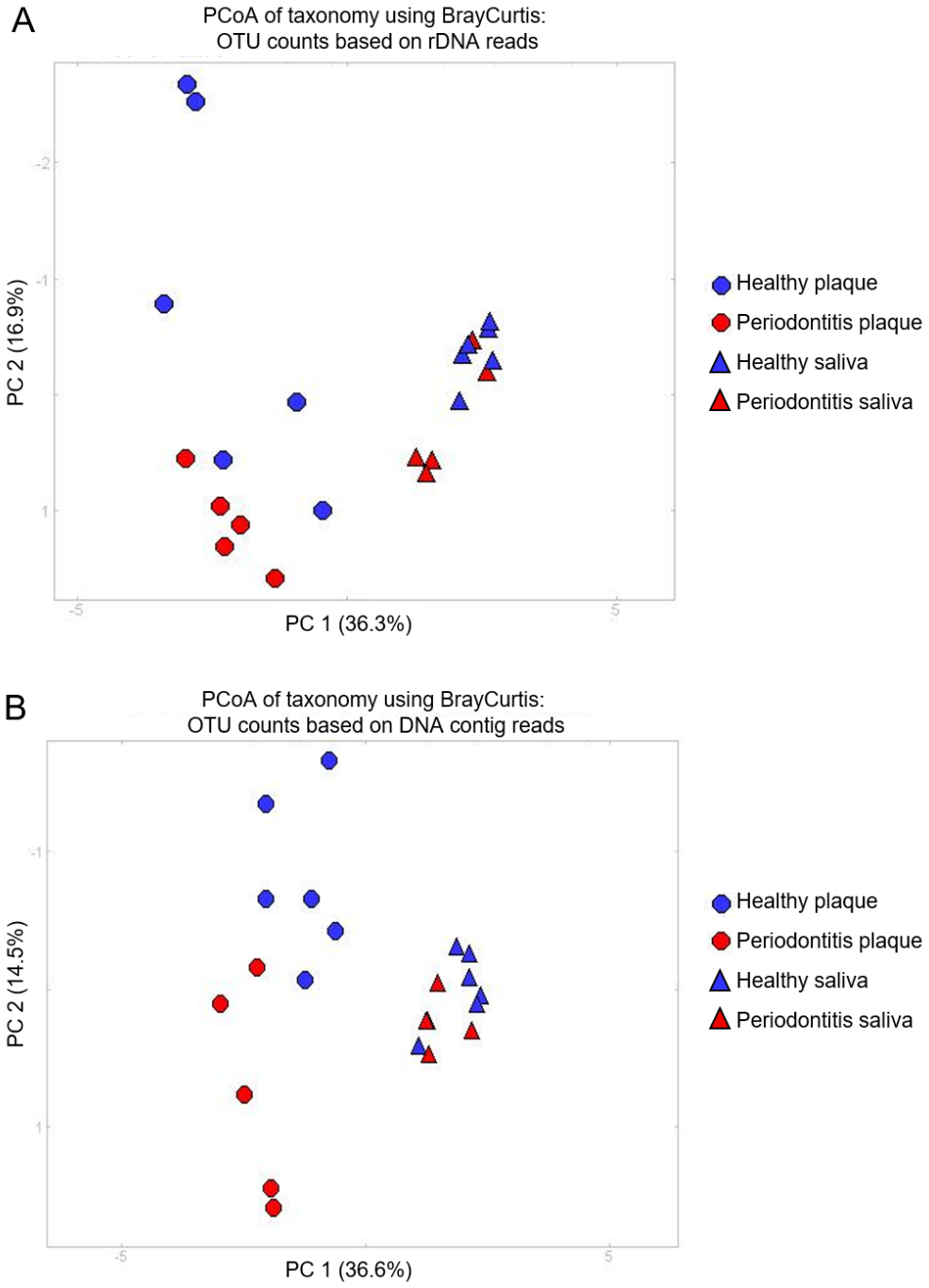


Figure 6.15: Principal coordinate analysis (PCoA) with Bray-Curtis dissimilarity based on taxonomic profiles of samples. (A) Plot for taxonomic clustering of plaque and saliva samples at a species level based on rDNA reads. (B) Plot for taxonomic clustering of plaque and saliva samples at a species level based on DNA contigs.

When using DNA contigs for taxonomy analysis, *Spirochaetes* abundances were higher in plaque than those seen when using rDNA with the increase in periodontitis compared with health more prominent (Figure 6.16B). Interestingly, in DNA contig analysis *Spirochaetes* were also detected in periodontitis saliva while in rDNA analysis *Spirochaetes* were not detected. In addition to these changes in the most abundant phyla, DNA contig based analysis also allowed the detection of less prevalent phyla such as *Acidobacteria*, *Chloroflexi*, *Chlamydiae*, *Cynobacteria*, *Tenericutes* and others.

DNA contigs were then used for a more in-depth analysis of the taxonomy because comparisons between health and periodontitis remained unchanged between rDNA and DNA contig based taxonomical analysis, more phyla were detected using the DNA contig method, and there was increased accuracy using DNA based alignment methods (rather than rDNA/16S alignment methods) for metagenomics.

Analysis of taxonomy at a genus level was limited to the 50 most abundant genera with removal of unclassified bacteria to increase clarity (Figure 6.17). Salivary bacterial profiles at this genus level were similar in both healthy and periodontitis participants. However, of the 50 most abundant genera, higher percentages were observed in *Porphyromonas*, *Tannerella*, *Treponema* and *Prevotella* in saliva samples of periodontitis participants. On the other hand, in periodontitis saliva lower abundances of *Veillonella*, *Streptococcus*, *Haemophilus* and *Actinomyces* were seen.

In plaque, there were higher percentages of *Fretibacterium*, *Treponema*, *Prevotella*, *Tannerella*, *Porphyromonas*, *Fusobacterium, Bacteroides* and *Anaerolineaceae bacterium* oral taxon 439 in periodontitis compared with healthy. Contrastingly, lower abundances of *Streptococcus, Paenibacillus, Mycobacterium, Haemophilus, Rothia, Escherichia, Salmonella, Corynebacterium, Chlamydia, Lautropia, Bacillus* and *Actinomyces* were observed in periodontitis compared with health. Although the results showed changes in the bacterial profiles of both saliva and plaque from periodontitis, given the lack of technical replicates and small cohort size it could not be determined whether these differences were significant.

As described above, differences in bacterial composition appeared to be more evident in plaque than saliva when comparing health and periodontitis. There were also clear differences in the prevalence of certain bacteria between the two sample types. For example, *Bradyrhizobium* was undetectable in saliva but was found at an approximately 5% abundance in healthy and periodontitis plaque. Similarly, *Fretibacterium*¸ *Bacteroides* and *Anaerolineaceae bacterium* oral taxon 439 were observed predominantly in periodontitis plaque although detected in small amounts in saliva and healthy plaque. On the other hand, *Rothia* and *Bacillus* were observed predominantly in

healthy plaque with smaller abundances in periodontitis plaque and saliva (periodontitis and healthy). There was also an increase in the percentage of *Actinomyces, Porphyromonas* and *Treponema* in plaque compared with saliva for both healthy and periodontitis samples. Conversely, *Campylobacter* were almost undetectable in plaque but was seen in both healthy and periodontitis saliva at similar levels. Similarly, *Streptococcus, Veillonella,* and *Haemophilus* were found at higher percentages in saliva compared with plaque.

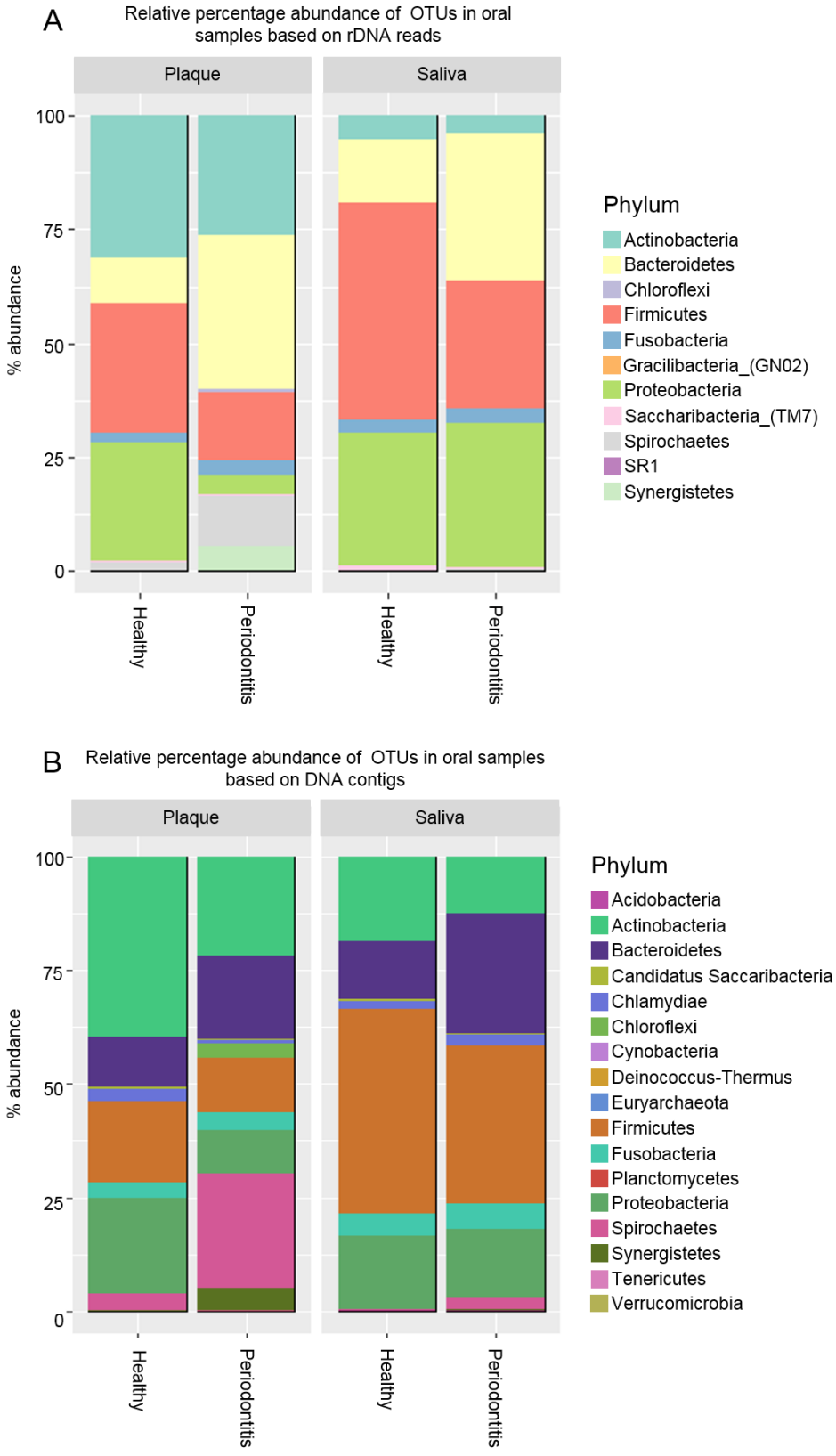


Figure 6.16: Relative percentage abundances of phyla in clinical samples. (A) Taxonomic percentage abundances based on rDNA reads. (B) Taxonomic percentage abundances based on DNA contigs.

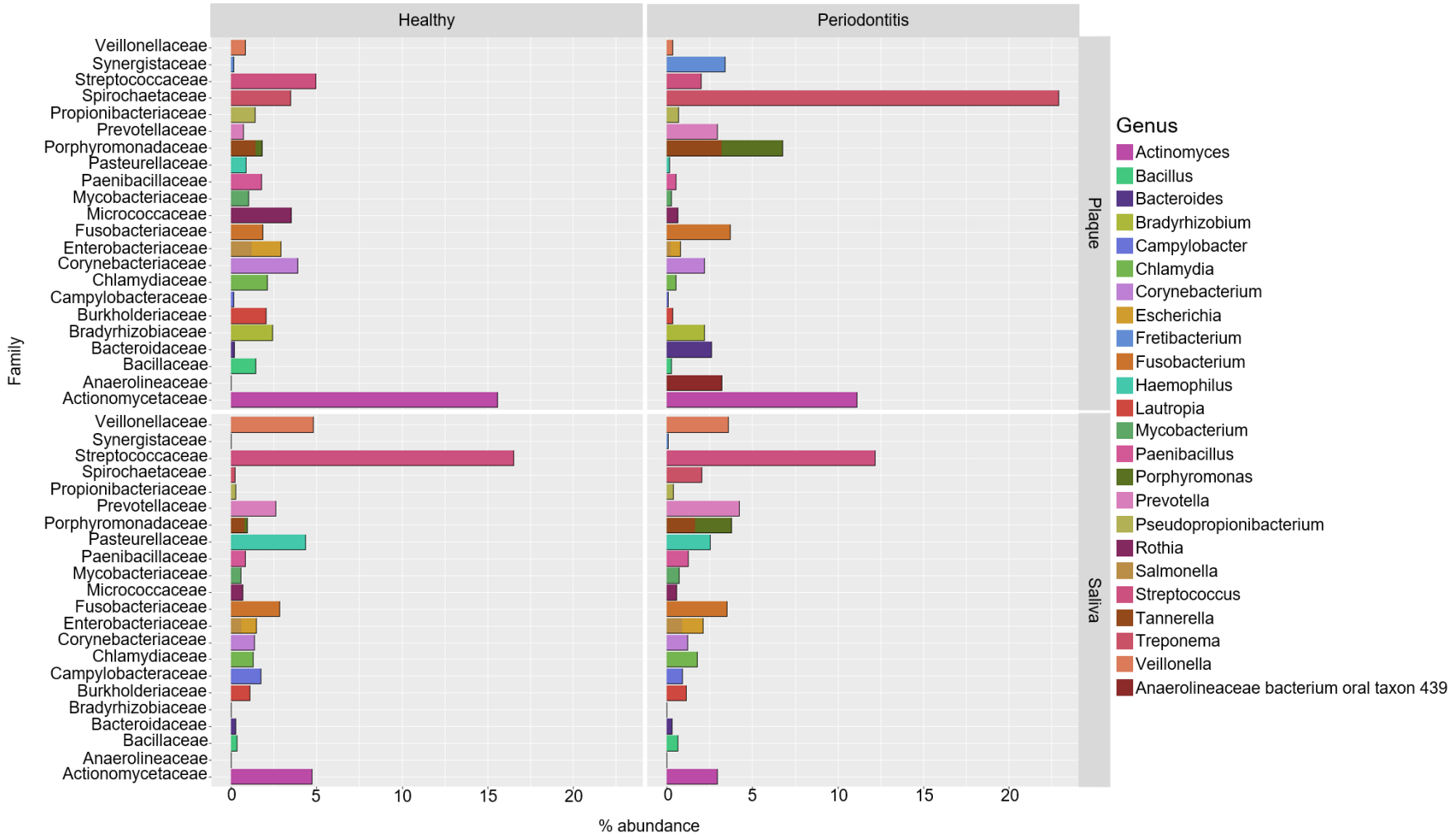


Figure 6.17: Relative percentage abundance of genera in clinical samples. Analyses at a genera level were limited to the top 50 most abundant genera.

6.2.4.2. Metagenomics functional analysis of healthy and periodontitis participants' saliva and plaque samples

In addition to the above described taxonomic analysis of plaque and saliva samples from participants with and without periodontitis, analysis was also carried out to determine overall functional profiles of the microbiota in these environments. Short DNA reads were assembled into contigs prior to being aligned against the NCBI non-redundant protein database. The resulting protein assignments were then aligned using SEED and EggNOG for annotation (section 2.6).

As with taxonomical analysis, a PCoA using the Bray-Curtis distribution was carried out for counts based on both SEED and EGGNOG functions. The analysis showed a cluster of the majority of the samples with outliers of six or seven samples. No obvious patterns were seen in the clustering of samples in both analyses (Figure 6.18).

The resulting data demonstrated little variation in the SEED functional profiles of saliva samples regardless of periodontal health status (Figure 6.19). Indeed, these profiles appeared similar to healthy plaque. Differences were observed in periodontitis plaque samples, however, with evidently higher counts in genes related to virulence, stress response, RNA metabolism, protein metabolism, DNA metabolism, carbohydrates and cell wall and capsule proteins. Interestingly, periodontitis plaque also had higher counts for unclassified proteins.

Using EGGNOG databases for annotations resulted in a similar trend with healthy plaque, healthy saliva and periodontitis saliva having no discernible differences in counts (Figure 6.20). As with the SEED profiles, periodontitis plaque appeared to have more counts for certain protein groups. These included carbohydrate transport and metabolism, amino acid transport and metabolism, energy production and conversion, defence mechanisms, post translational modification, protein turnover and chaperones, cell wall/membrane/envelope biogenesis, cell motility and replication, recombination and repair. On the other hand, lower counts for defence mechanisms, cell cycle control, cell division, chromosome partitioning and translation, ribosomal structure and biogenesis were observed in healthy plaque compared not only to periodontitis plaque but also healthy and periodontitis saliva.

Although some differences were observed, as with taxonomical analysis, significance could not be determined due to lack of technical replicates and limited cohort size in this pilot study.

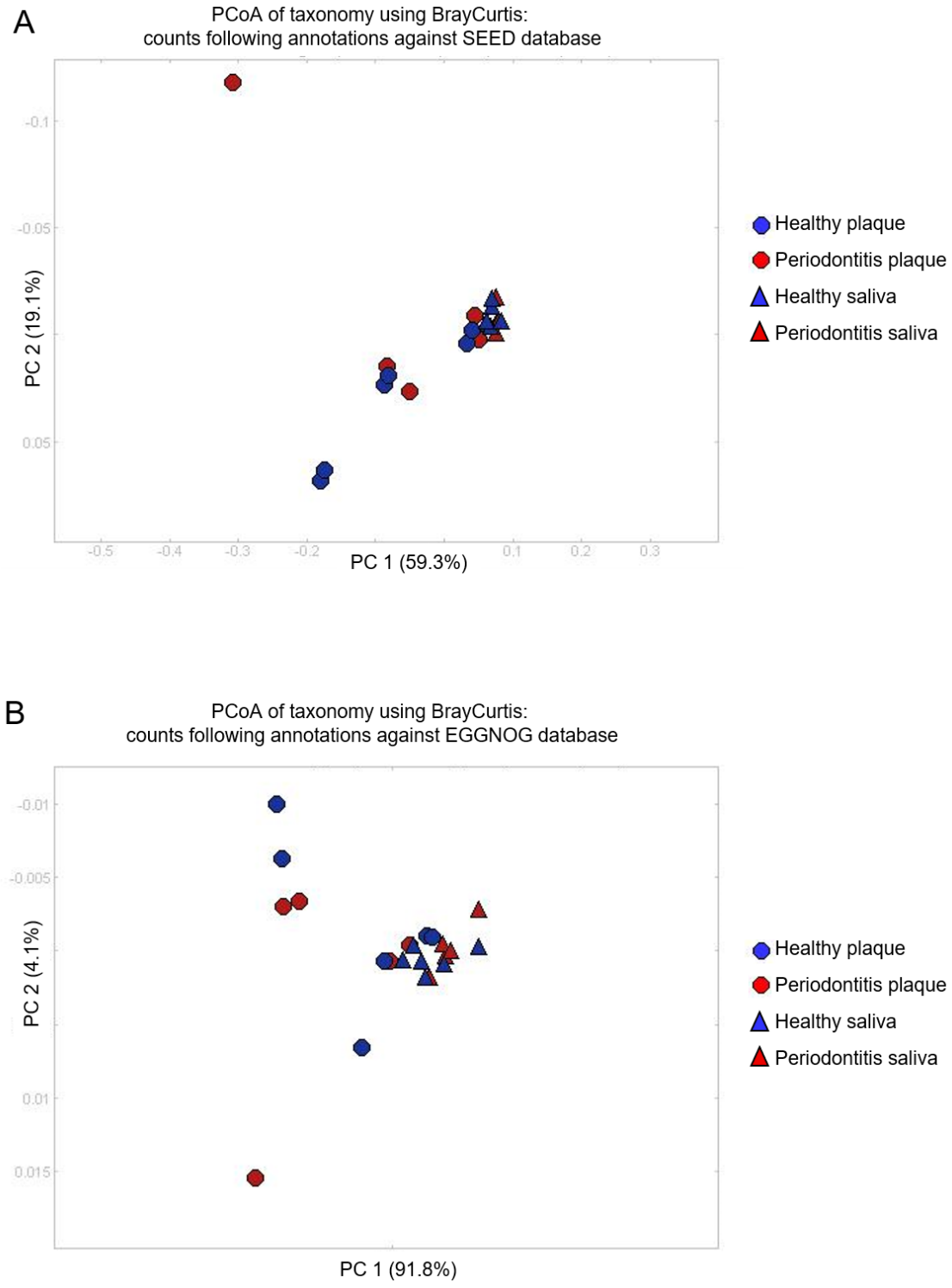


Figure 6.18: Principal co-ordinate analysis (PCoA) with Bray-Curtis dissimilarity based on functional profiles of samples. (A) Plot for functional clustering of plaque and saliva samples based on SEED assignments. (B) Plot for functional clustering of plaque and saliva samples at based on EGGNOG assignments.

Figure 6.19: Functional analysis of clinical sample microbiota using SEED database for annotations

Figure 6.20: Functional analysis of clinical sample microbiota using EGGNOG database for annotations

6.3. Discussion

A disruption in the balance of immune responses and ineffective clearance of pathogenic or excessive bacteria in the oral cavity can be detrimental to the periodontal tissues but the exact mechanisms involved in maintaining health are not fully understood (Moutsopoulos and Konkel, 2017). In this preliminary study we aimed to establish a methodology to examine immune responses in periodontitis participants by determining changes in systemic neutrophils, systemic and local cytokines/chemokines and local MMPs with the aim to implement these methods in a study including diabetics with and without periodontitis. In addition, this preliminary study also analysed the microbiota profiles of these participants with the long term aim to correlate microbiota changes to changes in immune responses.

6.3.1. Peripheral blood neutrophils in periodontitis and health

Although neutrophil analysis (migration, phagocytosis and respiratory burst) in systemically healthy participants with and without periodontitis showed that there was no significant difference between the two groups, the feasibility of analysing the blood samples from participants for all three assays within the appropriate time was confirmed. In addition, for both test groups, responses to stimulus in all neutrophil assays were as expected, i.e. an increase in neutrophil migration towards the chemoattractant fMLP, a dose dependent increase in phagocytosis of increasing *E. coli* concentrations and a dose dependent increase in ROS release when stimulated with increasing concentrations of *E. coli*.

The study indicated limited variation between participant technical replicates for analysis of phagocytosis and respiratory burst regardless of treatment group, indicating that in future samples can be analysed without the need for replicates as these flow cytometry bases methods analyse the response of 10000 neutrophils per sample. This could decrease analysis time and volume of clinical sample used.

However, during analysis of migration, it was evident that variation in migrated cell counts existed between technical replicates, perhaps leading to skewing of final results. This indicates that the migration assay requires optimising. Perhaps, calculating migrated neutrophils as a percentage of total neutrophils could compensate for any differences caused by the number of total cells loaded onto the insert membrane. To accomplish this, unmigrated cells would need to be quantified in addition to migrated cells and the number of migrated cells calculated as a percentage of the total. Despite this variation, it was evident that the neutrophils were migrating in response to the chemoattractant fMLP.

Although no significant differences in responses were observed between periodontitis and healthy participants, previous studies have shown that neutrophil dysfunction plays an imperative role in periodontitis. Studies examining the impact of conditions affecting neutrophil homeostasis and functions in periodontal health exemplify this. Chediak-Higashi syndrome, for example, is a condition which is characterised by the fusion of cytoplasmic granules leading to a decrease in the number of myeloid precursors. Any surviving neutrophils contain giant granules which impacts the process of diapedesis and so neutrophil chemotaxis. This links the rapid development of periodontitis and the associated bone loss in patients with Chediak-Higashi syndrome to defective chemotaxis of neutrophils (Clawson *et al*., 1978, Kaplan *et al*., 2008, Hajishengallis and Hajishengallis, 2014, Cortes-Vieyra *et al*., 2016). Furthermore, patients with Chediak-Higashi syndrome have also been shown to have delayed intracellular killing of phagocytosed bacteria due to a reduced content of hydrolytic enzymes in their granules. In cases of Chediak-Higashi syndrome, patients often present with periodontitis, thus, suggesting the progression of periodontitis is linked to neutrophil function (Delcourt-Debruyne *et al*., 2000, Nualart Grollmus *et al*., 2007). Another example is Papillon-Lefevre syndrome, which is characterised by defective neutrophil chemotaxis and reduced bacterial killing and also show increased susceptibility to periodontitis (Hajishengallis and Hajishengallis, 2014, Cortes-Vieyra *et al*., 2016).

The disorders discussed above highlight some functions important in oral health maintenance, but there is also evidence highlighting changes in neutrophils in systemically healthy patients with periodontitis. As discussed in detail previously (section 1.2.1) neutrophils are the most prevalent leukocytes recruited to the oral cavity and are the first line of defence against microorganisms (Dutzan *et al*., 2016, Nicu and Loos, 2016). A study examining neutrophils isolated from peripheral blood demonstrated decreased speed, velocity and chemotactic accuracy of neutrophils from periodontitis patients compared with healthy following stimulation by fMLP and IL-8. Non-surgical periodontitis treatment only recovered velocity and chemotactic activity in response to IL-8 while no changes were observed in response to fMLP (Roberts *et al*., 2015). Furthermore, comparison of peripheral neutrophils isolated from both chronic and aggressive periodontitis indicated a significant decrease in chemotactic activity in both forms of periodontitis but no difference between the two (Kumar and Prakash, 2012).

Despite evidence of decreased chemotaxis of neutrophils in periodontitis patients, an increase in neutrophil number has also been demonstrated in periodontitis at both a local and systemic level (Loos *et al*., 2000, Landzberg *et al*., 2015). Increases in oral neutrophil counts have also been shown to be reduced following periodontal treatment (Bender *et al*., 2006). Furthermore, Del-1 (developmental endothelial locus-1) deficient mice have

also been shown to spontaneously recruit excessive neutrophils to the gingiva, causing a destructive inflammatory response as well as alveolar bone loss. Del-1 has been shown to be antagonistic to β2 integrin dependent neutrophil adhesion and, as such, loss of Del-1 could disrupt neutrophil homeostasis (Eskan *et al*., 2012, Hajishengallis and Hajishengallis, 2014). Localised aggressive periodontitis (LAP) further exemplifies the potential of hyperactive neutrophil migration. Although LAP was previously thought to be a consequence of hypofunctional neutrophils, evidence now suggests that in combination with dysregulated chemotaxis and phagocytosis, LAP patients also have increased secretion of inflammatory mediators and increased oxidative stress (Kantarci *et al*., 2003, Ryder, 2010).

Similarly, hyper-activity or hyper-reactivity is also observed in isolated peripheral neutrophils from patients with periodontitis. For example, a study examining the release of ROS in peripheral neutrophils from both chronic and aggressive periodontitis indicated higher ROS release in unstimulated neutrophils in periodontitis compared with health and showed elevated ROS release in unstimulated neutrophils isolated from periodontitis patients (Guentsch *et al*., 2009). Recruitment of these hyper-active neutrophils to the periodontal tissue at the site could contribute to the oxidative tissue damage associated with periodontitis in the absence of excessive activation. However, following stimulation of these neutrophils with opsonised *P. gingivalis* and *A. actinomycetemcomitans*, ROS release was increased in chronic periodontitis but decreased in aggressive periodontitis compared with health. The same study demonstrated increased phagocytosis of *P. gingivalis* in periodontitis compared with health (Guentsch *et al*., 2009). Ling *et al*. (2016), also showed increased ROS release in both stimulated (by non-opsonised *P. gingivalis* and *F. nucleatum*) and unstimulated peripheral neutrophils in periodontitis compared with health, with a reduction following periodontitis treatment. Mariano *et al*. (2012) demonstrated elevated LL-37 in neutrophils from periodontitis patients following stimulation with *P. gingivalis, A. actinomycetemcomitans* and *E. coli* LPS compare with health. However, the same study also showed a decrease in NO in neutrophils from periodontitis compared with health (Mariano *et al*., 2012). Although not examined here, NET degradation has also been shown to be decreased in periodontitis patients with correlating decreased DNase-1 but with no changes in NET production (White *et al*., 2016a, White *et al*., 2016b). Given the importance of NETs as a mechanism employed by neutrophils for bacterial trapping and killing and the ability of certain periodontal pathogens to degrade NETs, this perhaps indicates a need to examine NETs in addition to other functions of neutrophils (Palmer *et al*., 2012). Together previous studies suggest that some neutrophil antimicrobial responses are elevated while others are depressed in periodontitis with differences between chronic and aggressive periodontitis in some

functions and these patterns of hyper- and hypo- reactivity could contribute to periodontitis progression.

While the results presented above show no significant differences in chemotaxis, phagocytosis and ROS release in peripheral neutrophils isolated from periodontitis compared with health, previous studies (as described above and previously in section 1.2.1) generally demonstrate decreased chemotaxis and either increased or decreased phagocytosis and increased ROS release. The sample size of the clinical study described here could potentially be the reason behind the contradictory results compared with previous studies. This small sample size indicates that, although the feasibility of the study was confirmed, no concrete conclusions can be made with regards to differences observed, or their lack of, comparing healthy participants and those with periodontitis.

Given that both insufficient and excessive numbers of neutrophils, in addition to changes in functionality, can contribute to the progression of periodontitis, an assay to analyse the total number of neutrophils within the local environment of the oral cavity should also be included. This change in total number of cells could potentially go towards explaining, at least partially, the progression of periodontitis associated with neutrophils without any obvious dysregulation of chemotaxis, phagocytosis or respiratory burst. Moreover, considering that the majority of cells recruited to the gingival crevice are neutrophils, analysis of local and systemic neutrophils (as studied here) could be beneficial in examining their role in periodontitis (Schiott and Loe, 1970, Loos *et al*., 2000). Indeed, apoptosis resistant neutrophils in GCF of periodontitis patients have previously been demonstrated (Gamonal *et al*., 2003). These neutrophils could contribute to increased numbers of neutrophils in the oral cavity in periodontitis as well as contribute to the tissue degradation associated with excessive neutrophil activation and/or presence.

A recent study has shown the role of *P. gingivalis* in altering neutrophil anti-microbial responses, whereby *P. gingivalis* co-activates C5a receptor-1 and TLR2 in human neutrophils causing a decrease in myeloid differentiation primary response protein-88 (MyD88), via ubiquitylation dependent proteasomal destruction, which leads to suppression of antimicrobial responses. In addition, this pathway activates phosphoinositide 3-kinase leading to the inhibition of RhoA GTPase and actin polymerisation, thus, blocking phagocytosis (Hajishengallis and Korostoff, 2017). Not only does this highlight the necessity for analysis of local neutrophils, it also suggests a potential need to alter the stimulus used for the study carried out above as exposure to periodontitis associated bacteria may alter neutrophil responses in a manner not seen with *E. coli.* It is also important to note that local tissues have regulatory roles with regard

to neutrophils (as described previously) through the release of cytokine/chemokines, thus providing more reason to analyse local neutrophils in addition to systemic neutrophils.

6.3.2. Local and systemic cytokine/chemokine profiles

In addition to observing no significant differences in the analysed neutrophil functions between healthy and periodontitis participant groups, the results indicated no significant difference in the concentrations of the panel of cytokines/chemokines at both a local and systemic level with the exception of increased IL-8 in GCF of periodontitis participants (Figure 6.12). Previous studies are often contradictory with regards to GCF concentrations of IL-8, with most studies indicating an increase (Gamonal *et al*., 2001, Giannopoulou *et al*., 2003, Holzhausen *et al*., 2010, Konopka *et al*., 2012, Ertugrul *et al*., 2013, Khalaf *et al*., 2014, Lutfioglu *et al*., 2016) and a few showing decreases (Jin *et al*., 2000, Luo *et al*., 2011) in periodontitis compared with health.

In general, there also appeared to be increased salivary and plasma IL-8 in periodontitis compared with healthy participants, although this did not reach significance in saliva and plasma (Figure 6.12). In contrast to GCF, this correlates with previously described salivary concentrations of IL-8 in periodontitis (Cesar-Neto *et al*., 2007, Michiels *et al*., 2009, Venza *et al*., 2010, Lisa Cheng *et al*., 2014, Khalaf *et al*., 2014, Souto *et al*., 2014). The lack of significance in the differences, much like with the neutrophil study, could be due to the small cohort of participants used during the study and the participant to participant variation observed. It is unsurprising that IL-8 concentrations appeared to be higher in participants with periodontitis at a local level considering its role as a chemoattractant and consequently its role in recruiting neutrophils (as discussed previously).

In addition to IL-8, the only other cytokine detected in both healthy and periodontitis participant GCF was IL-1β which was observed at higher concentrations in periodontitis, in agreement with previous studies (Giannopoulou *et al*., 2003, Holzhausen *et al*., 2010, Tymkiw *et al*., 2011, Konopka *et al*., 2012, Ertugrul *et al*., 2013, Luo *et al*., 2014). Similarly, salivary IL-1β concentrations were higher in periodontitis, correlating with previous studies, but did not reach significance (Tobon-Arroyave *et al*., 2008, Gursoy *et al*., 2009, Mirrielees *et al*., 2010, Kaushik *et al*., 2011, Ebersole *et al*., 2013, Rathnayake *et al*., 2013).

In GCF, MCP-1 was detected in periodontitis but not in health. An increase in GCF concentration of MCP-1 in periodontitis patients has been shown in previous studies (Anil *et al*., 2013, Gupta *et al*., 2013). Similarly, MCP-1 has also demonstrated to be increased

in saliva reflecting the trend observed during this study and highlighting the need for an increased sample size for results to reach significance (Gupta *et al*., 2013).

Although IL-6, TNF-α, IL-17A, IL-23 and MIP-1α were all detected in saliva, there was no significant difference between healthy and periodontitis and average concentrations were similar in both groups. As with other cytokines, previous experiments have had conflicting results with regards to IL-6 concentrations in saliva and GCF with some indicating an increase in periodontitis (Cesar-Neto *et al*., 2007, Costa *et al*., 2010, Holzhausen *et al*., 2010, Venza *et al*., 2010, Tymkiw *et al*., 2011, Prakasam and Srinivasan, 2014) and others demonstrating no significant differences (Gursoy *et al*., 2009, Rathnayake *et al*., 2013). Similarly, local TNF-α concentrations have been reported to be signficantly higher (Frodge *et al*., 2008, Singh *et al*., 2014) in periodontitis as well as having no significant difference (Gumus *et al*., 2014). On the other hand, salivary concentrations of IL-17, IL-23 and MIP-1α, are generally demonstrated to be elevated in periodontitis (Fu *et al*., 2013, Awang *et al*., 2014, Fine *et al*., 2014, Luo *et al*., 2014, Ebersole *et al*., 2015, Mitani *et al*., 2015, Liukkonen *et al*., 2016). Interestingly, all nine cytokines/chemokines were detected in plasma although the profiles of periodontitis participants and healthy participants showed no significant difference and similar averages. Considering the detection of all cytokines/chemokines in plasma and the detection of these in other studies in GCF and saliva but the lack of their detection in the samples analysed in this study, there is a possibility that the method utilised (i.e. bead array) is inappropriate for analysis of GCF and saliva. Both saliva and GCF are protein rich samples, and so there is a potential of masking of cytokines/chemokines especially when taking into account the assay was designed for analysis of tissue culture and plasma samples. Alternative methods (e.g. ELISAs) are required to ensure results are not false negatives. In addition, the general low concentrations of many of the cytokines/chemokines in saliva and undetected cytokines/chemokines in GCF could be due to the complex nature of the sample matrix.

The analysis of MMP8, MMP9 and TIMP1 in saliva and GCF, again showed no significant differences. As with the analysis of cytokines/chemokines and neutrophils, there did appear to be higher concentrations of both MMPs in saliva and GCF of periodontitis participants which if significant would draw parallels with previous reports (Marcaccini *et al*., 2010, Leppilahti *et al*., 2014, Salminen *et al*., 2014, Ebersole *et al*., 2015, Gupta *et al*., 2015). For TIMP1, however, there appeared to be no difference in saliva but a higher concentration (although not significant) in GCF in periodontitis participants. This could perhaps reflect an attempt to control the excessive MMP-associated tissue breakdown characteristic of periodontitis. As discussed previously, this increase correlates with studies that demonstrate elevated TIMP1 concentrations in GCF of periodontitis patients

(Popat *et al*., 2014, Ghodpage *et al*., 2014). Interestingly, a previous study has demonstrated decreased TIMP-1 concentrations in saliva of periodontits patients compared with healthy (Nizam *et al*., 2014).

In the analysis of all the immune responses discussed above, it is apparent that variation between participants in this study occurs more in participants with periodontitis compared with those without. This could perhaps explain the lack of consensus in previous studies with regards to changes seen in periodontitis associated immune responses. In addition, it is now accepted that periodontitis is a disease with stages of immune activity followed by inactivity which could further explain the variations observed. This emphasises the necessity to increase the cohort size and potentially include samples taken over a period of time in all participants.

6.3.3. Shotgun metagenomics of saliva and plaque from healthy and periodontitis participants

The analysis of NGS data from periodontitis and healthy plaque and saliva samples provides a taxonomic and functional profile of bacteria present. A general decrease in microbial alpha diversity in plaque from participants with periodontitis was observed with significant differences following analysis of DNA contig counts. Recent studies using metagenomic or metatranscriptomic data correlates with these results (Jorth *et al*., 2014, Ai *et al*., 2017). On the other hand, there is also evidence suggesting a higher alpha diversity in periodontitis compared with health (Griffen *et al*., 2012, Abusleme *et al*., 2013, Dabdoub *et al*., 2016). As seen previously, no significant differences were seen in saliva between health and periodontitis and no trends observed regardless of read counts used for the calculations (Belstrom *et al*., 2017).

At a phylum level, the decrease in *Actinobacteria* and *Proteobacteria* and increases in *Bacteroides*, *Synergistetes* and *Spirocheates* in plaque from periodontitis patients described in this study has previously been reported (Griffen *et al*., 2012, Wang *et al*., 2013). Enrichment of the genera *Prevotella*, *Fretibacterium*, *Porphyromonas, Treponema* and lower abundances of *Streptococcus*, *Corynebacterium* and *Actinomyces* in periodontitis plaque have also been reported previously which is reflected in the results presented here (Liu *et al*., 2012, Wang *et al*., 2013, Dabdoub *et al*., 2016).

Metagenomics analysis of saliva from periodontitis subjects is limited, with fewer studies reported compared with analysis of plaque. Belstrom *et al*. (2017) compared healthy and periodontitis saliva using both metagenomics and metatranscriptomics and demonstrated an increased prevalence of pathogens traditionally associated with periodontitis such as *P. gingivalis*, *T. forsythia* and *Parvimonas*. This is reflected in the

present study where *Porphyromonas* and *Tannerella* were both enriched in saliva from periodontitis participants. Furthermore, a previous study also demonstrated distinct taxonomic clustering of samples suggesting the plaque microbiota is different to that seen in saliva (Yamanaka *et al*., 2012).

As with analysis of saliva taxonomy, limited studies have evaluated differences in healthy and periodontitis salivary microbiota at a functional level using metagenomics. Evidence suggests a downregulation of carbohydrate metabolism in periodontitis compared with health using metatranscriptomic data (Belstrom *et al*., 2017). The metagenomic data from this study, however showed similar functional profiles for saliva regardless of health status. This could be a consequence of using metagenomic analysis, which evaluates the functional capability of the microbiota from each environment but does not indicate gene pathways in operation. The metatranscriptomic data, however, allowed the evaluation of active gene pathways for growth and survival on microbiota from healthy and periodontitis participants.

A slight downregulation in carbohydrate and amino acid related genes was observed but whether these changes are significant requires further analysis. In contrast, evident differences were seen in periodontitis plaque compared with healthy plaque with similar changes seen in previous NGS studies (Liu *et al*., 2012, Wang *et al*., 2013, Jorth *et al*., 2014, Dabdoub *et al*., 2016). Of interest was the upregulation of virulence/disease/defence mechanisms, stress, cell motility and chemotaxis, cell wall/membrane/envelope biogenesis, transcription and DNA/RNA/carbohydrate/protein metabolism. Together this suggests that the microbiota of periodontitis plaque samples were more virulent but also suggests a higher bacterial load as indicated by increases in various metabolism and transport systems.

While the data presented above validates the methods and indicates differences in composition and function of the microbiota when comparing health to periodontitis, the lack of technical sequencing replicates hinders the ability to analyse significance and as such limits understanding. It is also important to acknowledge the differences in sequencing methods (such as 454/Roche and Illumina/Solexa) and analysis pipelines currently used for metagenomic studies and the limitations this imposes on comparing between studies. These variations in sequencing and analysis methods, in addition to experimental factors (such as sampling sites and participant exclusion criteria) could explain conflicting results in previous studies. However, with constantly improving bioinformatics tools and increasing genomic data in databases, analysis of NGS data is becoming faster, easier and more accurate. Metagenomics provides valuable information with regards to functional potential of a microbiome but addition of

metatranscriptomic analysis could enhance this and provide a deeper understanding of active microbiota gene expression and function.

6.3.4. Implications for the diabetes-periodontitis link

Due to time limitations, the scope of this study could not include participants with diabetes. The inclusion of this group is imperative to elucidating mechanistic links between periodontitis and diabetes as patients with diabetes have previously been shown to have impairment in neutrophils, changes in concentrations of chemokines/cytokines and increased levels of MMPs (Preshaw *et al*., 2012). To date, studies investigating the relationship between diabetes and neutrophil function have been contradictory, with some studies showing up-regulation in total neutrophil counts, respiratory burst, migration and apoptosis while other studies have shown a decrease in neutrophil counts, adhesion, microbial activities, respiratory burst, migration and phagocytosis as discussed in detail in section 1.4.2 (Manouchehr-Pour *et al*., 1981, Salvi *et al*., 1997a, Salvi *et al*., 1997b, Alba-Loureiro *et al*., 2006, Graves *et al*., 2006, Alba-Loureiro *et al*., 2007, Preshaw *et al*., 2012). In a similar manner, studies on the local and systemic cytokine concentrations in diabetic patients with and without periodontitis have shown contradictory results (section 1.4.1). With regards to MMPs, studies have mainly shown increases in MMP concentrations associated with increased neutrophil activity (hyperactivity or increased number) (Kumar *et al*., 2006, Correa *et al*., 2008, Costa *et al*., 2010, Hardy *et al*., 2012). Differences in microbial composition in the oral cavity of diabetics have also been implicated in the increased prevalence of periodontitis. Indeed, previous studies have shown shifts in bacterial composition although reports are somewhat contradictory with regards to specific genera/species involved (Thorstensson et al., 1995, Lalla et al., 2006, Hintao et al., 2007, Ebersole et al., 2008, Casarin et al., 2013, Xiao et al., 2017). It is proposed that the methodologies validated in this feasibility study for the evaluation of the discussed immune responses alongside bacterial profiles, could reasonably be implemented in diabetics with and without periodontitis.

7. Conclusions and future directions

The studies presented in this thesis examine potential links between diabetes and periodontitis. The two diseases have complex aetiologies, and while it has long been demonstrated that the two are linked (Preshaw *et al*, 2012), the exact mechanisms are unclear. The most promising of these mechanisms has been discussed in detail previously (section 1.4). This thesis aimed to evaluate if increased AGEs (associated with hyperglycaemia) effected inflammatory responses of gingival keratinocytes, bacterial composition of a five species periodontitis biofilm model, and composition and functional potential of a complex inoculum biofilm model. In addition, the thesis outlines a methodology to evaluate how biofilms grown with concentration of AGEs that are commensurate with diabetes and health impact inflammatory responses when co-cultured with gingival keratinocytes.

As described previously (section 1.4.3), hyperglycaemia has been linked to the accumulation of AGEs and subsequent chronic inflammatory responses. Given the central role of inflammatory responses to both diabetes and periodontitis, increased periodontitis severity has been correlated to hyperglycaemia (Shlossman *et al*., 1990, Emrich *et al*., 1991, Taylor *et al*., 1998, Tsai *et al*., 2002, Hiroshima *et al*., 2018).

Although previous studies have shown that the increase in serum levels of AGEs from diabetic patients correlated with an increase in periodontitis-linked attachment loss and increased immunoreactivity of AGEs in gingival tissues (Takeda *et al*., 2006, Zizzi *et al*., 2013, Hiroshima *et al*., 2018), no studies to date have linked increased AGEs to changes in oral microbiota. The experiments presented above, demonstrate the potential use of a five species biofilm model, cultured to represent a simplistic periodontitis associated biofilm, as a way to analyse the effect of increased AGEs on biofilm composition. The results from this study demonstrated a change in the composition of the biofilm whereby *A. naeslundii* was the predominant species following introduction of AGE to the growth media (Figure 4.2). This was surprising when considering *Actinomyces* species (alongside *Streptococcus*) are the predominant early colonisers of tooth surfaces (Li *et al*., 2004) and have been long associated with health (Socransky and Haffajee, 2002). This was associated with a decrease in pH, potentially as a consequence of AGE metabolism. Given the inability of *P. gingivalis*¸ *P. intermedia* and *F. nucleatum* to survive in acidic environments, this could go towards explaining the biofilm composition following AGE addition (Takahashi *et al*., 1997).

While a series of studies have demonstrated AGEs produced by *E. coli* K12 are metabolised intracellularly by metalloproteases and secreted as low-molecular weight AGE peptides into the growth media (Katz *et al*., 2010, Cohen-Or *et al*., 2011, Cohen-Or

et al., 2013), these studies are limited to intracellular bacterial AGEs and did not investigate bacterial uptake of extracellular AGEs and subsequent metabolism. However, Hellwig *et al*. (2015), showed the ability of human colonic microbiota to metabolise AGEs which were incubated with faecal suspensions, through analysis of the resulting metabolites by HPLC. A similar approach could be taken to determine the metabolism of AGEs in the five species biofilm model. To fully determine AGE metabolism by the five species biofilms future work should also include quantifying the AGE levels in the growth media following the incubation period and analysing the metabolic pathways activated through transcriptomics.

The changes observed in the five species biofilm model confirmed the possibility of AGEs altering biofilm composition. However, given the complex nature of the oral microbiota, the implications of this are limited. As such, the effect of AGEs on a biofilm generated with a physiological inoculum to create a more complex biofilm, was evaluated. Using NGS, it was concluded that results from the complex biofilm model reflected those observed in the five species model. In both models, the addition of AGE encouraged the growth of health associated bacteria (section 5.2). In the complex biofilm model, it is likely the pre-conditioning of the HA pegs with high HSA-AGE concentrations selects for the bacteria that are present in the mature biofilms, as early (day 2) biofilms also favour health associated bacteria. Further analysis is required to elucidate the reason the health associated bacteria are selected for growth under high HSA-AGE concentrations. It is possible the complex biofilm reflect the results seen in the five species model so that metabolism of the HSA-AGE alters pH of the media causing the periodontitis associated bacteria growth to be halted and as such future studies should include pH monitoring. There is also the possibility that changes associated with the biofilms grown with low HSA-AGE concentrations are a consequence of the addition of HSA-C (which was added to account for protein differences) so that comparisons between low and high HSA-AGE are taking into account changes associated with the combination of HSA-AGE and HSA-C. To account for this biofilms without any supplementation of the media and supplementation of the media with low HSA-AGE concentrations alone are needed.

While these experiments highlight the potential of using HA pegs to generate complex biofilms, the study presented above has its limitations. In particular, the cultured biofilms were used to model the periodontitis microbiota and the effect of hyperglycaemia on these but the inocula used was from healthy volunteers. This is reflected in the composition of the inocula which is consistent with HMP data from seven healthy oral sites, where the predominant phyla were shown to be *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria* (Human Microbiome Project, 2012, Zhou *et al*., 2013b, Moon and Lee, 2016). Although the media used for culturing the

biofilms was designed to encourage the growth of periodontitis associated bacteria (Naginyté, 2017, unpublished), in future studies it would be beneficial to produce inocula from periodontitis patients, in an attempt to model periodontitis more closely. This is of particular importance when considering the observed shift in composition where genera typically associated with periodontitis, such as *Fusobacterium*, *Campylobacter, Treponema* and *Haemophilus*, were less prevalent while genera generally associated with periodontal health (including *Actinomyces*, *Streptococcus* and *Veillonella*) were more prevalent in early (day 2) biofilms compared to the inocula (section 5.2). This could perhaps suggest that health associated bacteria are outcompeting the less abundant pathogens or opportunistic pathogens found in healthy participants, for colonisation of the HA pegs.

It is also possible that expansion of the AGE concentrations is required. The modelling was based on previous evidence from a single study which demonstrated increased HSA-AGE concentrations in the GCF of periodontitis patients with diabetes (Kajiura *et al*., 2014). While this study provided a range of HSA-AGE concentrations to use, it is likely that analysis of a larger cross section of GCF (and perhaps saliva) of periodontitis patients, periodontitis patients with diabetes and healthy volunteers is required for better representation of AGE GCF concentrations. In addition, to truly evaluate the validity of this method to model the bacterial composition of biofilms in the periodontal pockets of diabetics with periodontitis, a direct comparison (using NGS/transcriptomics) of model biofilms and patient biofilms is required. As discussed in section 6.3.3 a comparison of clinical samples with model biofilms using the same analysis methods is imperative to eliminate any method bias.

The interactions between oral microbiota and the host cells also plays a crucial role in the progression of periodontitis and may be exacerbated by the immune dysregulation associated with diabetes (Preshaw *et al*¸ 2012). The methods presented here, attempt to model the microbiota-host cell interactions using a co-culture method. Close attention was paid to the inflammatory responses of the TIGK cells in response to the biofilms with or without AGE in the co-culture media. Biofilms were cultured under varying conditions (to reflect health and hyperglycaemia associated AGE concentrations) and introduced to TIGK cells, cultured for 8 hours and inflammatory responses analysed.

Prior to analysis of AGE associated changes in host inflammatory responses in TIGK cells, the expression of RAGE was first confirmed in the gingival keratinocytes cell line utilised in this study (section 3.2). The results demonstrated RAGE was expressed as both the cell surface bound and secreted versions. However, the overall expression of RAGE on these cells was not altered as a consequence of increased AGE

concentrations. To ensure the lack of difference is not a consequence of the methodologies utilised, further analysis is required. In particular, analysing the ratio of cell surface bound RAGE receptor and secreted RAGE receptor at both a protein and mRNA level is required. To accomplish this at a protein level an ELISA can be optimised to quantitate RAGE concentrations in both WCL and culture supernatant. For the analysis of RAGE mRNA, primers specific to the various isoforms of RAGE can be designed for use with qRT-PCR methods. Establishing these ratios is important when considering the sequestering of AGE by secreted RAGE prevents the activation of signalling cascades associated with AGE-RAGE interactions and could prevent the feedback required for the upregulation of RAGE expression (Yonekura *et al*., 2003, Hudson *et al*., 2008, Kalea *et al*., 2009, Ott *et al*., 2014, Wautier *et al*., 2017, Leung *et al*., 2016). In addition to RAGE ratio analysis, quantifying the levels of ADAM10 and MMP9 (which have been associated with RAGE cleavage to generate secreted RAGE) may prove prudent to determine if changes in these proteolytic proteins are responsible for the changes in secreted RAGE (Yonekura *et al*., 2003, Hudson *et al*., 2008, Kalea *et al*., 2009).

While the data presented in this thesis, demonstrated a lack of difference in cytokine/chemokine release following AGE addition to culture media (section 3.2), it is possible this was due to a lack of interactions between AGE and RAGE. It is possible that AGE interacts with various other receptors (including AGE-R1/OST-48, AGE-R2/80K-H, AGE-R3/galectin-3) which may not result in tested cytokine/chemokine changes (Araki *et al*., 1995, Vlassara *et al*., 1995, Li *et al*., 1996, Ohgami *et al*., 2001a, Ohgami *et al*., 2001b, Ohgami *et al*., 2001c, Jono *et al*., 2002, Tamura *et al*., 2003). To ensure AGE interaction with RAGE, investigation into the NFκB, MAPK and other signalling pathways would be beneficial, particularly when considering these have been linked to downstream signalling cascades for AGE-RAGE interaction (Figure 3.1) (Schmidt *et al*., 2000, Ishihara *et al*., 2003, Kokkola *et al*., 2005, Mallidis *et al*., 2007).

Although the presence of RAGE on TIGK cells was confirmed, the addition of AGE appeared not to alter cytokine/chemokine levels. However, co-stimulation of the TIGK cells with biofilms generated under various conditions appeared to dampen IL-6 and IL-8 responses (section 5.2.3). This could potentially be a consequence of bacterial degradation of cytokines/chemokines. This would correlate with other studies which have demonstrated *P. gingivalis* modulation of IL-8 (Darveau *et al*., 1998, Huang *et al*., 2001, Bainbridge *et al*., 2010) and IL-6 (Lourbakos *et al*., 2001, Uehara *et al*., 2008, O'Brien-Simpson *et al*., 2009). Interestingly, concentrations were higher with diabetes and periodontitis biofilms compared with periodontitis biofilms alone with changes more evident with direct biofilm-cell contact. This suggests the biofilm composition or biofilm

functionality is altered to modulate cellular inflammatory responses and perhaps highlights the possibility that hyperglycaemia of diabetics activates pathways that lead to an increase in inflammation (Preshaw *et al*., 2012).

However, as this was a feasibility study and thus limited to one replicate, the results are inconclusive and repeats are required for validation. The data do however, indicate a potential change in the cytokine/chemokine profile of TIGK cells following co-culture with biofilms and as such highlights the necessity for further analysis. Furthermore, this thesis demonstrated the feasibility of co-culture and may be applied in the future to analyse the effect of oral biofilms on immune cells. This would be of interest, given the high predominance of immune cells in the oral cavity and their crucial role in the progression of periodontitis and their increase in number during periodontitis (Dutzan *et al*., 2016). Furthermore, high expression of RAGE was shown in gingival epithelial cells and circulating leukocytes which contrasted with healthy gingiva, where RAGE expression was limited (Abbass *et al*., 2012). Co-culture of leukocytes under hyperglycaemic AGE conditions with complex biofilm, may therefore result in a more exacerbated inflammatory response.

In both TIGK responses to AGE and co-culture experiments, the analysis of MMPs would aid in understanding the cytokines/chemokines response. Particularly when considering they are targets for MMP cleavage (Franco *et al*., 2017). Indeed, in previous studies, MMP13 has been implicated in activation of other MMPs through a proteolytic cascade in gingival cells from periodontitis patients (Hernandez Rios *et al*., 2009, Franco *et al*., 2017). An increase in MMPs in co-culture experiments could account for the decreases in cytokines/chemokines observed.

In addition to the *in vitro* studies discussed above, the thesis presents a preliminary clinical study analysing the immune and inflammatory response of periodontitis and healthy participants at both a local and systemic level (section 6.2). Given the limited cohort size, no differences in neutrophil phagocytosis, respiratory burst and migration were detected and as such an increase in participant number is required. In addition to the functions of neutrophils described in this thesis, neutrophil extracellular traps (NETs) have recently been indicated as important in neutrophil killing (Brinkmann *et al*., 2004). In chronic periodontitis NETs have been observed in periodontal pockets and GCF (Vitkov *et al*., 2009). Furthermore, *P. gingivalis*, *T. forsythia*, *F. nucleatum* and *Prevotella intermedia*, have been shown to have DNAse activity and can degrade NETs under certain growth conditions, thus, avoiding NET-associated killing (Palmer *et al*., 2012). Together these studies suggest the addition of NET analysis would be beneficial to elucidating the links between diabetes and periodontitis. As described previously, an

increase in local and systemic neutrophil number has been observed in periodontitis and *P. gingivalis* has been shown to increase migration and retention of neutrophils into tissues (Wingrove *et al*., 1992, Meyle and Chapple, 2015, Dutzan *et al*., 2016), perhaps suggesting a need to evaluate the number of systemic and local neutrophils in periodontitis patients with and without diabetes.

Similar to neutrophil data, the panel of cytokines/chemokines analysed indicated limited significant differences between healthy and periodontitis patients at both a systemic and local level. However, IL-8 (GCF, saliva and plasma), IL-1β (GCF, saliva), MCP-1 (GCF, saliva) all appeared to have increased concentrations in periodontitis. Likewise, the MMPs analysed appeared to be present at increased concentrations in the GCF and saliva of periodontitis participants. Although the differences didn't always reach significance, the data suggest that periodontitis participants had an increased inflammatory response. To confirm these results an increase in cohort size is required. The clinical study also indicates differences in composition and function of the microbiota when comparing health to periodontitis. It is proposed that the methodologies validated in this clinical feasibility study for the evaluation of cytokine/chemokine concentrations, neutrophil functions and bacteral profiles, can be implemented in diabetics with and without periodontitis.

The data described in this thesis presents a framework for future investigation. Alongside expanding the clinical study to include diabetic patients and increasing the cohort size, future work includes optimizing the complex biofilm model to better represent long term consequence of hyperglycaemia on periodontitis biofilms and co-culture experiments to analyse the effects of biofilms on oral cells.

v. References

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