The Putative Role of Pathogenic Oral Microorganisms in Chronic Kidney Disease

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The Putative Role of Pathogenic Oral Microorganisms in Chronic Kidney Disease

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LIST OF ABBREVIATIONS

Acronym	Definition
AGCF-P	Artificial gingival crevicular fluid - periodontal
APGN	Acute post-streptococcal glomerulonephritis
BE	Bacterial endocarditis
BHIB	Brain heart infusion broth
BMM	Basal media mucin
C13:10 iso	13-methyltetradecanoic acid
C4BP	Human complement inhibitor C4b-binding protein
CFU/mL	Colony forming units per mL
CKD	Chronic kidney disease
CP	Chronic periodontitis
Ct	Cycle threshold
DMEM	Dulbecco modified eagle medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFR	Estimated glomerular filtration rate
ELISA	Enzyme-linked immunosorbent assay
FGF	Fibroblast growth factor
GCF	Gingival crevicular fluid
GWAS	Genome wide association studies
HGF-1	Human gingival fibroblast 1
HK-2	Human kidney proximal tubule epithelial cells 2
IEGN	Infective endocarditis glomerulonephritis
IL-6	Interleukin 6
IL-8	Interleukin 8
Kgp	Lysine-specific gingipain
LCMS	Liquid chromatography mass spectroscopy
LPS	Lipopolysaccharide
MMCD	Madison metabolomics consortium database
MMPs	Matrix metalloproteases
NCBI	National centre for biotechnology information
NOD	Nucleotide-binding oligomerization domain containing
OD	Optical density
OMVs	Outer membrane vesicles
PA	Polyamines
PAI-1	Plasminogen activator inhibitor 1
PAR1	Protease activated receptor 1
PAR2	Protease activated receptor 2
PBS	Phosphate buffered saline
PCA	Principle component analysis
pCO2	Physiological carbon dioxide
PDGF	Platelet derived growth factor
PGE2	Prostaglandin E2

PGLYRP1	Peptidoglycan recognition protein 1
PLs	Polymorphonuclear leukocytes
PLS-DA	Partial least squares – discriminant analysis
PNL	Transfected plasmid with no insert
PSD	Polymicrobial synergy and dysbiosis
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RF	Random forest
RgpA	Arginine-specific gingipain A
RgpB	Arginine-specific gingipain B
SBEs	Sonicated bacterial extracts
SE	Standard error
TGF-β	Transforming growth factor beta
TIMPs	Tissue inhibitors of metalloproteases
TLRs	Toll like receptors
TSB	Tryptone soya broth
VEGF	Vascular endothelial growth factor
VIP	Variable Importance in Projection

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ABSTRACT

Chronic kidney disease and chronic periodontitis are common diseases that are found disproportionately comorbid with each other and have been reported to have a detrimental effect on the progression of each respective disease. They have an overlap in risk factors and both are underpinned by systemic inflammation along with a wide selection of immunological and non-specific effects that can affect the body over the lifespan of the conditions. The oral microorganisms involved in chronic periodontitis have demonstrated the ability to translocate and elicit distal effects in a variety of systemic diseases but currently there has been little research into the associations with chronic kidney disease. Previous studies have investigated the directionality of the relationship between these two diseases; however, there is a lack of evidence on how these diseases may be interacting at the local sites such as the oral cavity and systemic level, especially looking at periodontitis associated microbial products that could damage the kidneys.

The aim of this study was to investigate the microbial products produced by periodontitis associated microorganisms using a novel metabolomics approach and identify if any of these products are capable of eliciting *in vitro* a cytotoxic (cell viability testing), migratory (wound healing assay) and fibrotic response (qPCR) in renal cells.

A selection of microorganisms associated with periodontitis were cultivated in a novel media representative of the diseased environment, and the microbial products secreted in bacterial supernatants over their lifecycle were collected. These supernatants were prepared for testing by centrifugation and filtering at 0.2 µM in order to remove microbial cells; the prepared samples were stored at -80^{o}C. A novel metabolomics approach utilising liquid chromatography mass spectroscopy was developed and trialled as a fast screening method for identifying potentially toxic metabolites but it was not suitable for this study.

Testing of the collected microbial supernatants against human kidney cells identified that only *Porphyromonas gingivalis* supernatants were capable of inducing a cytotoxic and mild anti-migratory effect. Tests to identify the causative agent behind

these effects suggest this effect could be protein derived, as the addition of a protease inhibitor attenuated the cytotoxic effects of the supernatants, suggesting the role of microbe specific proteases known as Gingipains. Investigations in the fibrotic potential of the collected supernatants showed that only *Porphyromonas gingivalis* supernatants were capable of significantly inducing the production of a three pro-fibrotic markers: Collagen, type I, alpha 1, transforming growth factor β and tissue inhibitor of metalloproteinases 1.

In conclusion, this study showed that one periodontitis associated microorganism, *Porphyromonas gingivalis* is capable of eliciting cytotoxic and mild anti-migratory effects in renal cells, along with inducing the production of three pro-fibrotic markers. This study sheds light on how this key microorganism may pay a role in the mediation of chronic kidney disease.

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PREFACE

The work presented in this thesis was undertaken at Manchester Metropolitan University, UK.

The literature review undertaken for Chapter 1 was partly published in:

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Chapter 2 was partly published in:

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The author presented a 30-minute oral presentation at the International Conference on Oral Malodour – Role of oral microbiome in systemic diseases in Bristol, UK. In addition, the author was an assistant at the Biodeterioration and Biodegradation Society's Symposium 'IBBS 17' in Manchester, UK. Furthermore, the author has attended and presented posters at numerous international and national conferences such as the Healthcare Infection Society 2018 Conference in Liverpool, UK; MetaboMeeting 2018 Conference in Nottingham, UK and Eurobiofilms 2017 Conference, Amsterdam, Holland.

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I would like to dedicate this doctorate to my late father Seán Hickey who passed away during the first year of my PhD due to complications related to kidney disease. Sometimes it is hard to relate your research to a bigger picture and investigating a very small niche can feel far removed from the manifestations of the disease you work on. However, I like to believe that what little dent I have made into the unknown of systemic links to kidney disease may help in some small way to improve treatment and diagnosis, in turn allowing others to have some more time with their loved ones.

I would not have completed this without the support of my director of studies and friend Dr Rebecca Taylor, who really went above and beyond her supervisory role to help with my academic and personal issues throughout the last three years. My other four supervisors Prof Kathryn Whitehead, Dr Liliana Shalamanova, Dr Nina Dempsey-Hibbert and Prof Christopher van der Gast all provided a wide variety of experience and discussions to help with multiple areas that enabled my multidisciplinary project to succeed. A special thank you to Dr Jonathan Butler, who despite not being part of my supervisory team provided much support with experimental design, scientific discourse and laboratory assistance with this project and our many side projects.

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are exceptional scientists who epitomise what is to be inquisitive researchers; we have all pushed each other and I am proud to count them as friends.

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Seán Hickey
1964-2017

OVERVIEW OF THE THESIS STRUCTURE

The focus of this study was to investigate the putative link between two systemic diseases, chronic periodontitis and chronic kidney disease, and how they may interact with each other. This was investigated by identifying microorganisms implicated in chronic periodontitis and specifically observing at how the microbial products they produce may interact with chronic kidney disease. The first chapter (Chapter 1) provides an overview of this research topic and of how these two diseases may interact.

The first research chapter (Chapter 2) covers the selection of periodontitis associated microorganisms that have been implicated in systemic disease and/or related to chronic periodontitis and chronic kidney disease. These microorganisms were then cultivated in a novel developed growth medium that was designed to mimic the gingival crevicular fluid and the microbial supernatants were sampled over their life cycle in order to purify their microbial products for testing.

The second research chapter (Chapter 3) focuses on the design, development and proof of concept of a novel metabolomics approach from scratch to investigate microbial metabolites produced by periodontal pathogens. Liquid Chromatography - Mass Spectroscopy was utilised along with a specifically designed analysis pathway in order to identify compounds that disproportionately account for the difference between bacterial samples and the controls. While the development of a pipeline was successful, issues surrounding identification of compounds prevented this technique from being useful for screening purposes.

The third research chapter (Chapter 4) focuses on testing all microbial supernatants against human kidney cells and assaying a variety of parameters such as cytotoxicity, migration and fibrosis induction. The final research chapter (Chapter 5) focuses on the identification of the causative agent behind the kidney cell damage observed using a variety of different approaches. The second focus of chapter 5 was further investigating fibrosis induction using quantitative polymerase chain reaction.

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Chronic kidney disease (CKD) is one of the most common chronic diseases with the worldwide prevalence estimated to be approximately 13.4% and projected to continue to rise annually, especially in developing countries where renal healthcare is limited (Couser et al., 2011, Hamer and El Nahas, 2006, Coresh, 2017). Chronic Periodontitis (CP) is a chronic infection of the supporting structures of teeth that is predominately found in adults and the elderly. CP is ubiquitous, with worldwide prevalence estimated to be approximately 10% (Kassebaum et al., 2014, Frencken et al., 2017). As chronic diseases persist for long periods, there is a significant amount of comorbidity in patients, especially with other chronic diseases (Jones, 2010, Schellevis et al., 1993). Indeed, these comorbidities have been reported as a source of adverse outcomes (Tonelli et al., 2015) and highlight that complex interactions between chronic diseases could lead to effects that are detrimental for the health of the patient.

There have been reports highlighting a link between CKD and CP, which both have an overlap in terms of risk factors. While some studies have reported significant associations between both diseases, there is increasing evidence suggesting a directional link between CKD and CP that can affect their progression and cause adverse outcomes (Zhao et al., 2018). Both CKD and CP have well documented systemic effects on the body such as chronic inflammation and dysfunction of immune cells (Oberg et al., 2004, Vaziri et al., 2012, Akar et al., 2011, Loos, 2005); however, how their individual systemic effects may interact with each other is relatively unstudied. Furthermore, the combined effect of both chronic diseases on the localised site of periodontal disease (the oral cavity and periodontium) has not been fully elucidated and may explain why both diseases have been reported to have a detrimental effect on each other.

1.1.1 The Oral Microenvironment

The mouth is one of the most biologically complex regions in the mammalian body. It provides an entrance and transit point for both the digestive and respiratory systems, plays a role in rudimentary pre-processing of food, and is pivotal for oral

communication and sensory functions (Marsh et al., 2009). Within the oral cavity there are the hard tissues of the teeth and soft mucosal structures such as the tongue, soft and hard palate, cheeks, periodontium and the gingiva sulcus which provide an array of microhabitats for the large numbers of diverse microorganisms present (Figure 1.1) (German and Palmer, 2006, Marsh, 2000, Verran, 1991). In this environment the two key biofluids, saliva and gingival crevicular fluid (GCF), play an important physiological role. The saliva is the predominant oral fluid. It acts as a lubricant, assists in preliminary digestion through the action of enzymes such as lipase and amylase, and plays a role in the forming of a food bolus and taste (Edgar et al., 2004). In contrast, the GCF is normally present in small amounts bathing the gingiva. However, in pathological conditions (e.g. local inflammation), the secretion of GCF increases significantly as a defensive mechanism in the immune response (Taylor and Preshaw, 2016).

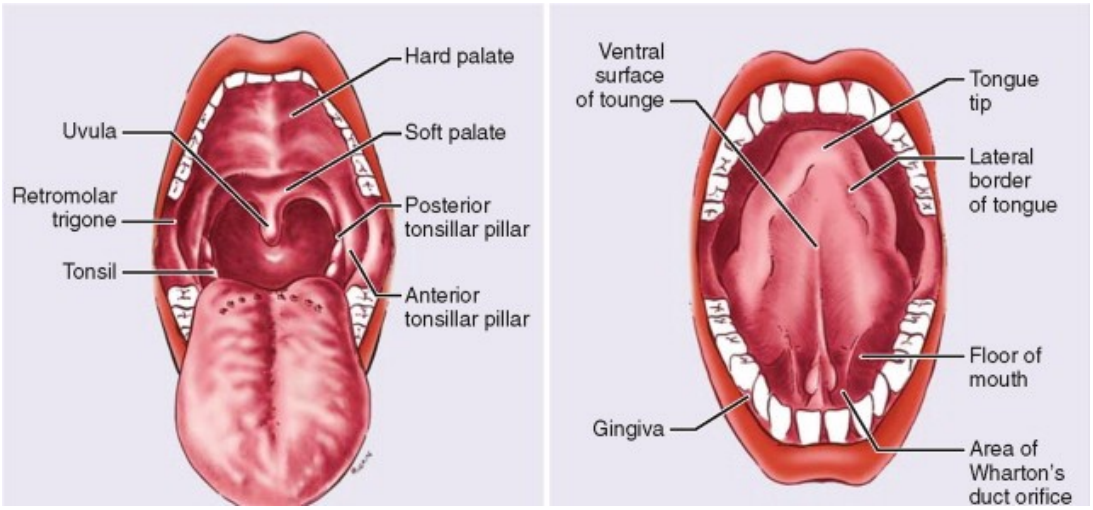

Figure 1.1 The selection of structures present in the oral cavity that can provide a differential habitat for oral microorganisms (adapted from (Goldenberg and Koch, 2009))

The microorganisms in the oral cavity change in response to a wide variety of stimuli such as dietary energy sources, age, host and bacterial metabolite production, poor oral hygiene and systemic diseases (Rasiah et al., 2005). Over 700 microbial species have been characterised in the human oral cavity alone, highlighting the high levels of diversity (Dewhirst et al., 2010). This resident microbiota does not have a singularly passive relationship with the host since it contributes to the host defences, can

synthesise vitamins which are beneficial to the host and plays a vital role in preventing colonisation of potential pathogens (Marsh, 2000, Kumar et al., 2013). However, as the oral cavity is a key barrier and entry point between the external environment and the body, it is open to opportunist pathogens that can infiltrate the blood, the lower respiratory tract and the digestive system (Tada and Hanada, 2010). Despite much of the microbiota living in symbiosis with the host, the oral cavity can further act as a reservoir for infection with many commensal microorganisms being the cause of opportunist infections in immunocompromised individuals (Gendron et al., 2000, Akpan and Morgan, 2002).

1.1.2 Chronic Periodontitis

Periodontitis is the inflammation and infection of the periodontium, which is due to the accumulation of sub-gingival dental plaque and is mediated by a wide spectrum of oral microorganisms (Hajishengallis, 2015). It presents as repetitive inflammation, recession of the gingiva, gingival bleeding and the formation of deep periodontal pockets between teeth which over time can leave to bone loss and loss of attachment (Figure 1.2) (Darveau, 2010, Schatzle et al., 2004). The disease can be classified into three different stages depending on the clinical connective tissue attachment loss: slight 1-2 mm of loss, moderate 3-4 mm of loss and severe >5 mm loss (Wiebe and Putnins, 2000).

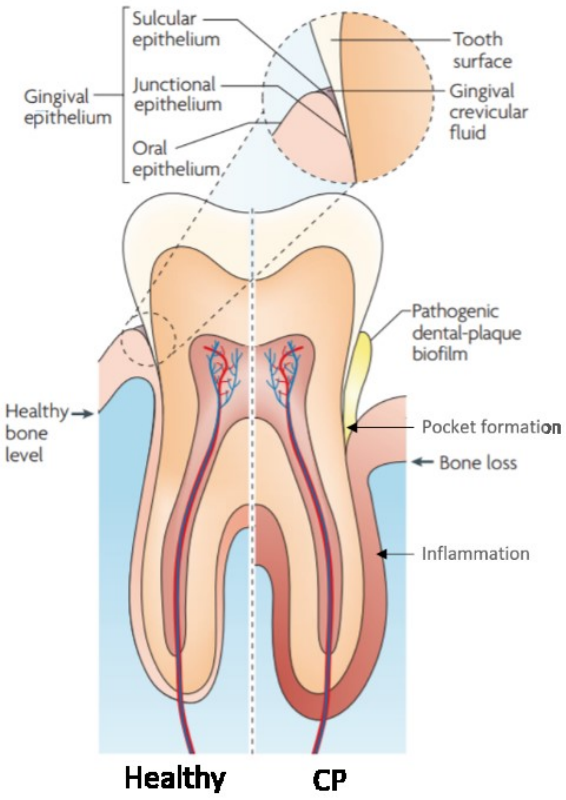

Figure 1.2 The presentation of chronic periodontitis (Right) in comparison to healthy individuals (Left). In healthy tissue, the teeth are supported by connective tissue, the gingival epithelium and alveolar bone. In chronic periodontitis, the accumulation of the pathogenic dental plaque biofilm leads to inflammation, formation of periodontal pockets and the destruction of the gingival epithelium along with alveolar bone loss (Adapted from (Darveau, 2010)).

There are seven identified subtypes of periodontitis with CP being by far the most common and severe of all forms in the adult population. CP is categorised by the formation of periodontal pockets of which increase in depth with the severity of the disease and provide a space for the aetiological microorganisms. It has been reported that 45% of adults in England have at least one periodontal pocket, with 8% of the population having severe periodontal pocketing (Heidari et al., 2015). The worldwide prevalence of severe CP was estimated to be 10%, making it the sixth most prevalent disease worldwide (Kassebaum et al., 2014, Frencken et al., 2017). CP is of particular interest as it is a global chronic disease and can be detrimental to the health of the patient and a risk factor for myriad other diseases such as Alzheimer's (Dominy et al., 2019), cardiovascular disease (Dietrich et al., 2008), diabetes mellitus (Engebretson et al., 2013), chronic obstructive pulmonary disease (Usher and Stockley, 2013) and pre-term birth (Marsh et al., 2009, Han et al., 2004).

The main aetiological agents driving the inflammatory processes in CP are the plaque associated oral bacteria which form biofilms and dysregulate the normal oral microbiota (Wahid et al., 2013). CP is caused by the inflammatory response to dental plaque accumulation and formation of calculus (hardened plaque) around the gingiva, which may result in an increased flow of GCF, and further favours the growth of anaerobic microorganisms (Marsh et al., 2009, Winning et al., 2015). These Gram-negative anaerobes include *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola,* which have been isolated from the majority of CP cases and are referred to as the 'red complex' (Wade, 2013). Indeed, the levels of *P. gingivalis* and *T. denticola* in subgingival plaque can predict the progression of CP, highlighting the key role of these organisms in the pathogenesis of CP (Byrne et al., 2009). Previously it was thought that these 'red complex' organisms affected the signalling pathways of the human host, which led to a dysbiosis of the oral microbiota thereby resulting in disease (Darveau, 2010). However, it has since become apparent that CP is more complex than a singular group of organisms, with a current predominating theory being the Polymicrobial Synergy and Dysbiosis (PSD) model (Hajishengallis and Lamont, 2012).

In the PSD model of CP, keystone pathogens such as those of the 'red complex' colonise the gingiva, interact with accessory organisms and increase the virulence of the community causing dysbiosis and disruption of homeostasis, which leads to destruction of tissue (Hajishengallis and Lamont, 2012, Payne et al., 2019). In this model, there are a wide range of microorganisms involved, with keystone pathogens such as the aforementioned 'red complex' and periodontitis accessory organisms (Table 1). These accessory organisms are predominately commensals commonly found in the healthy microbiota, which can interact with the keystone pathogens to assist in the pathogenesis of CP. However, due to the complicated nature of these interactions such as specific adhesion, metabolic symbiosis, complex biofilm formation and virulence modulation, many of the mechanisms are yet to be elucidated (Shaikh et al., 2018).

Table 1.1 List of key pathogens and accessory organisms with the reported roles in periodontitis

Keystone Pathogens		
Name	**Reported Roles**	**Reference**
Porphyromonas gingivalis	A key pathogen in CP because of complex host-pathogen and community interactions, which can cause dysbiosis and promote a pro-inflammatory phenotype. In addition, a multitude of virulence factors are produced which can cause damage and destruction of the periodontium.	(Hajishengallis, 2009)
Tannerella forsythia	A key pathogen associated with the clinical attachment loss seen in CP and has been shown to cause alveolar bone loss. It has a few identified virulence factors and many synergistic associations with accessory organisms.	(Kesavalu et al., 2007)
Treponema denticola	A key pathogen of CP of which the severity of the disease can be predicted by levels of *P. gingivalis* and *T. denticola*. Also has a multitude of virulence factors, which assist with adherence, instigating dysbiosis in the subgingival microbiota and damaging the gingival tissues.	(Byrne et al., 2009, Dashper et al., 2011)
Accessory Organisms		
Name	**Reported Roles**	**Reference**
Actinomyces israelii	Present in patients with CP more frequently than healthy patients, along with those with high scores on the plaque index. It may play a role in the maturation of sub and supra gingival plaque. It is commonly isolated alongside *A. actinomycetemcomitans* suggesting synergy in periodontal infection.	(Vielkind et al., 2015, Könönen and Wade, 2015)
Aggregatibacter actinomycetemcomitans	A facultative anaerobic organism, which is commonly found in CP and is considered a key pathogen. It has numerous reported virulence factors promoting periodontal disease with stimulation of inflammatory cytokines and the production of a leukotoxin, which is reported to be pro-apoptotic towards gingival epithelial cells.	(Henderson et al., 2010)
Campylobacter *(C. rectus, C. showae)*	*Campylobacter* organisms are not common commensals but present as transient members of the healthy microbiota. *C. rectus* is predominately found in initial periodontal lesions and shallower pockets, which leads to suggestions it may play a role in the initiation of periodontitis. *C. showae* is predominately found in deeper pockets and later stages of CP with its role in the pathogenesis relatively under studied.	(Macuch and Tanner, 2000)
Eubacterium nodatum	Commonly mistaken for *A. israelii* due to similar morphology. It is reported to be a key accessory organism in periodontitis along with having synergy with *T. denticola* in the induction of periodontitis. However, due to misidentification there is a lack of studies looking at the interplay of this organism in CP.	(Haffajee et al., 2006, Arora et al., 2014)
Fusobacterium nucleatum	Reported to play a role as a 'bridge' for early and late colonisers to co-aggregate. It has also been reported to have synergy with *T. forsythia* in inducing an immune response and causing alveolar bone loss.	(Settem et al., 2012)
Parvimonas micra	A commensal organism commonly found in the subgingival plaque is found in the top layer of periodontal plaque. It is not thought to form biofilm structures, but has a significant association with periodontal destruction. However, it is relatively understudied in CP so many the biochemical interactions are unknown.	(Zijnge et al., 2010, Al-hebshi et al., 2014)
Prevotella *(P. intermedia, P. melaninogenica, P. nigrescens)*	These are anaerobic commensal organisms within supragingival plaque, which increase over the course of periodontal disease. Biofilms produced by *Prevotella spp* can be used to subvert the innate immune system causing the periodontal plaques to chronically colonise the periodontal pockets. Furthermore, it has also been reported that *P. intermedia* can release pro-inflammatory cytokines and matrix metalloproteinases, which can stimulate periodontal destruction.	(Stingu et al., 2013, Tanaka et al., 2008, Fukui et al., 1999, Yamanaka et al., 2009, Guan et al., 2008)
Streptococcus anginosus group *(S. anginosus, S. constellatus, S. intermedia)*	This group are commensals that inhabit the subgingival periodontal pockets in those with periodontitis and are usually present even after treatment. It has been suggested that they provide an attachment substratum, which can allow for recolonization of periodontal pathogens after treatment as they have strong anti-phagocytic resistance to leukocytes.	(Rams et al., 2014)
Streptococcus mitis group *(S. gordonii, S. sanguinis, S. oralis)*	This group are thought to assist the key pathogens in adherence by providing an attachment substratum and facilitate signal transduction. Furthermore, synergy between *S. gordonii*, *P. gingivalis* and *A. actinomycetemcomitans* has been demonstrated causing increased pathogenicity when in co-culture.	(Whitmore and Lamont, 2011, Hendrickson et al., 2017)

Infection of the periodontium can provide a method of systemic entry into the body for microorganisms along with microbial products and potential antigens. These may induce inflammatory responses in distant tissues by dissemination using the circulatory system (Bastos et al., 2011), with evidence of this seen in infective endocarditis (Baddour et al., 2015) and infective glomerulonephritis (Brodsky and Nadasdy, 2017). In addition, CP associated microorganisms and products are released into the digestive system by the swallowing of the inflammatory exudate, periodontal bacteria and bacterial products (Olsen and Yamazaki, 2019).

1.1.3 Chronic Kidney Disease

The kidneys play a vital role in the body by filtering blood and metabolic waste, while also modify the content and composition of fluids in the body. They facilitate waste excretion, nutrient reabsorption, secrete hormones, regulate osmolarity, regulate blood pressure and maintain systemic acid base homeostasis (Jameson and Loscalzo, 2016). The anatomy of the kidney reflects its unique role in the body with the functional unit of the kidney being the nephrons, which filter the blood to form urine. Damage to the nephrons can lead to acute or chronic kidney diseases, which may predispose to end-stage renal failure which requires dialysis or transplantation (Hoy et al., 2005).

It is estimated that in England 2.6 million people aged 16 years and older have CKD stage 3-5 which equates to 6.1% of the general population (Aitken, 2014). CKD is defined as reduced kidney function that is present for over three months, and has implications for the health of the patient such as kidney failure and cardiovascular death (Inker et al.). The reduced kidney function is measured using the estimated glomerular filtration rate (eGFR), with the severity of CKD staged from 1-5 based on the decrease in eGFR (Hemmelgarn et al., 2010). CKD often goes undiagnosed and can present idiopathically along with co-morbidities such as diabetes, cardiovascular disease and chronic periodontitis (Levey et al., 2003, Dyck et al., 2012, Borawski et al., 2006). The progression of CKD is characterised by the loss of renal cells and the replacement of these with extracellular matrix, which leads to the reduction of renal function and related symptoms such as uremia, proteinuria and oedema (Nogueira et al., 2017).

The function of the kidney in acting as a filtering agent of waste products for the blood increases their risk of their damage by toxins (Finn, 1977). The blood flow to the kidney is greater per gram of tissue compared to any other organ (Finn, 1977) meaning that the total amount of waste/toxins delivered will be disproportionately high. Furthermore, as the kidneys are able to concentrate substances through filtration, reabsorption and secretion, this can cause various metabolites and toxins to build up to levels which can be damaging to the nephrons (Finn, 1977).

Many factors increase the risk of CKD such as age, gender, ethnicity, concurrent diseases such as hypertension and diabetes and socio-economic status (Kazancioglu, 2013). Indeed, a key strategy for management of CKD is identification of modifiable risk factors, which would allow for earlier detection of the disease. The identification of predisposing risk factors for CKD can also allow intervention-based strategies to facilitate early treatment of the disease. Current research has focused on investigating risk factors that may have a directional link with CKD independent of other risk factors such as Alzheimer's Disease (Shi et al., 2018b), obstructive sleep apnoea (Mirrakhimov, 2012) and CP (Wahid et al., 2013). Identification of these directional risk factors is of particular importance, as many of these can have a significant impact on progression of both diseases and if controlled could be a useful treatment to deal with both diseases.

1.2 Oral Bacteria and Distal Pathogenic Effects

Although the effect of pathogenic oral bacteria on the health and disease of the oral cavity has been acknowledged for many years, the interplay between these organisms and other systemic diseases has only recently re-emerged. The first proposal of distal pathogenic effects by microorganisms was in 1891 with W. Miller suggesting that oral microorganisms could access distal body parts, known as the focal infection theory (Miller, 1891). This was built upon and suggested to be the cause of a number of systemic diseases such as endocarditis, diabetes and nephritis (Billings, 1916). At the time these suggestions were not taken seriously, however the proposal re-emerged in the 1990's with the American Academy of Periodontology and the world workshop in periodontitis suggesting that oral infection is frequently

related to local and general disease (Scannapieco, 1998). This has led to modernisation of the focal infection theory to the periodontal medicine concept which highlights the relationship between chronic periodontitis and systemic disease, with oral status connected to systemic health (Pizzo et al., 2010).

Many of these putative links have been identified through epidemiological associations adjusted for known risk factors. However, the mechanisms behind many of these interactions still need to be elucidated (Seymour et al., 2007). There are a few known case studies of distal pathogen effects from oral microorganisms namely: bacterial endocarditis, acute post-streptococcal glomerulonephritis and infective endocarditis glomerulonephritis (Carapetis et al., 2005, Lusco et al., 2016, Majumdar et al., 2000).

1.2.1 Translocation of Oral Pathogens

In a healthy mouth, the mucous membrane along with host-produced antimicrobial molecules such as defensins provide a barrier to external pathogens, preventing the commensal and opportunist microbes from damaging the host. This barrier can be disrupted in multiple ways including physical disruption via trauma such as micro abrasions caused by tooth brushing (Addy and Hunter, 2003), by periodontal pathogens invading the gingival margin (Bosshardt, 2017) or if a host is immunocompromised through medication or disease where they are more at risk of infection.

There are three possible mechanisms of how oral microbes can facilitate further diseases: (1) infection due to metastatic transient bacteraemia; (2) metastatic immunological injury and; (3) metastatic toxic injury (Thoden van Velzen et al., 1984, Pizzo et al., 2010). Metastatic transient bacteraemia refers to the damage caused by translocation of microorganisms throughout the body. Metastatic immunological injury refers to the damage caused by the stimulation of an immune response such as the process of inflammatory mediation seen in chronic periodontitis by the microorganisms involved. Metastatic toxic injury is the damage caused by the release of toxic compounds which can be produced by microorganisms or by components of the cell ultrastructure such as Lipopolysaccharide (LPS) which can stimulate an

immune response through host toll like receptors (TLRs) (Gendron et al., 2000, Aderem and Ulevitch, 2000).

1.2.2 Bacteraemia

Bacteraemia is defined as the presence of viable bacteria in the bloodstream, with primary bacteraemia occurring through direct access from the outer environment such as through injection or venepuncture, and secondary bacteraemia being related to the disruption of a local barrier such as the mucosal membranes or wounds (Jawetz, 2007). Bacteraemia can be categorised based on the longevity of the microorganisms in the bloodstream: transient, intermittent or persistent (Poveda-Roda et al., 2008). Transient bacteraemia originating from the oral cavity is a relatively common event which can be caused by dental procedures including daily oral hygiene routines, such as tooth brushing and flossing, and while being asymptomatic it usually only lasts for up to fifteen minutes (Everett and Hirschmann, 1977, Roberts et al., 2006). In contrast, intermittent and persistent bacteraemia are not as common and are associated with increased severity of periodontal disease and accumulation of plaque within the gingival crevice and periodontal pocket.

Bacteraemia is particularly common in CP, with the bacteria and their products being able to enter the bloodstream through the inflamed gingiva and elicit a response in distal tissues (Fisher et al., 2010). For example the key CP pathogen *P. gingivalis* has been isolated from atherosclerotic plaques (Serra e Silva Filho et al., 2014), the placenta (Katz et al., 2009), the liver (Ishikawa et al., 2013) and is capable of invading arterial cells (Deshpande et al., 1998) and causing endothelial dysfunction (Tonetti et al., 2007). The accessory organism *A. actinomycetemcomitans* has also been isolated from periodontal pockets and atherosclerotic plaques (Padilla et al., 2006). While the microbiota of periodontal and atheromatous plaques has been reported to be highly similar, indicating translocation rather than sporadic infection (Serra e Silva Filho et al., 2014), there is currently no study to demonstrate the clonal nature between periodontal plaques and atherosclerotic plaques.

1.2.3 Bacterial Endocarditis

Bacterial endocarditis (BE) is an infection of the inner lining of the heart, most commonly the heart valves but it can also affect artificial implants such as pacemakers and artificial valves (Carmona et al., 2002). This infection is caused by a subset of circulating microorganisms that can bind to the endocardium, or an abrasion/micro-clot already present, forming a biofilm. This can lead to the activation of complement and formation of a clot with a notable absence of white blood cell recruitment (Cabell et al., 2003). It is a relatively uncommon disease with an incidence of 2.3 cases per 100,000 people in the UK, with a poor prognosis in lower income countries (Ambrosioni et al., 2017).

Oral microorganisms such as *Streptococcus mutans* and *Streptococcus sanguinis* were previously the most common microorganisms isolated as causative agents of bacterial endocarditis, most likely due to their strong thrombogenic potential which allows adherence to the surfaces of the heart (Coykendall, 1989). Bacterial adherence is related to intrinsic dextran production, which allows attachment to the heart valves along with fibrin and platelets leading to the formation of plaques (Scheld et al., 1978).

Renal glomerular lesions have been reported in cases of BE (Majumdar et al., 2000). The pathogenesis of endocarditis related glomerulonephritis is similar to acute post-streptococcal glomerulonephritis, as both are a form of infective glomerulonephritis. Infective Endocarditis Glomerulonephritis (IEGN) is be mainly caused by *Staphylococcus aureus* endocarditis with *Streptococcal* endocarditis being the second most common pathogen, they both can lead to tubular damage as identified by renal biopsies (Boils et al., 2015). Previous work investigating pathogenesis of the IEGN had identified the activation and deposition of C3 of the complement cascade and the involvement of IgG antibodies (Boils et al., 2015). However many of these studies used cadaver kidneys and more recent renal biopsy studies demonstrated that the activation of C3 is the primary cause of damage, with IgG and other antibodies being present in a much smaller amount (Bayer and Theofilopoulos, 1990). These studies demonstrate that IEGN is more complex than previously thought and the activation of the complement cascade may be stimulated by various virulence factors such as

Staphylococcal superantigens which may cause direct tissue damage (Salgado-Pabon et al., 2013) and various immunological complexes produced by *Streptococci* as further discussed in the related acute post-streptococcal glomerulonephritis condition.

1.2.4 Evidence for Kidney Damage Induced by Oral Microorganisms

Although several reports link oral microorganisms and kidney damage there are inconsistencies regarding the causative species, type of damage and putative mechanisms of damage (Nasr et al., 2008, Trivedi et al., 2017, Ramanathan et al., 2017, Nasr et al., 2011). Acute post-streptococcal glomerulonephritis (APGN) is a well-studied example of pathogenic oral microorganisms eliciting a distal response and damage to kidneys.

APGN represents an inflammation of the glomeruli in the kidneys and is widely attributed to group A streptococci (Ahn and Ingulli, 2008). AGPN is one of the most common nephric conditions, with over 490,000 cases estimated globally per year. The majority of the AGPN cases occur in developing countries and are easily treated with antibiotics (Carapetis et al., 2005). As group A streptococci can be present in the oral and skin microbiota, the source of infection mainly depends on the climate, with the colder climates associated with upper respiratory tract infections and warmer climates with skin infections (Earle, 1985). In one such case, swabs were taken from four neighbouring rural families who contracted acute glomerulonephritis (Almroth et al., 2005). Kidney biopsies of the patients showed acute diffuse proliferate glomerulonephritis, which could be a consequence of streptococcal nephritis, with *Streptococcus constellatus* and *Streptococcus pyogenes* being isolated from these biopsies. While the direct mechanism for this kidney damage is unknown, it has been suggested that the post-streptococcal glomerulonephritis could be caused by deposition of nephrogenic streptococcal proteins in the glomerulus and the production of an antibody-mediated response (Cronin et al., 1989) or deposition of bacterial toxins in the kidneys. A recent review on this topic suggests that this pathogenesis was probably immunological rather than toxin-related, as the level of observed kidney damage is not proportional to the infection as is the case in other toxin mediated diseases (Brodsky and Nadasdy, 2017). A number of streptococcal

proteins have been identified (e.g. nephritis strain-associated protein (Ohkuni et al., 1983), Endostreptosin (Yoshizawa et al., 1997) and streptococcal M protein (Vosti et al., 1971)) which induce detectable antibody titres in a large proportion of patients. However, the lack of specificity to acute post-streptococcal glomerulonephritis of these proteins is a major drawback in the identification of causative agent(s). This has led to other mechanisms such as autoimmunity (Zabriskie et al., 1970) and coagulation (Alkjaersig et al., 1976) to be proposed as playing a role in the disease.

1.3. THE SYSTEMIC EFFECTS OF CHRONIC DISEASE

Systemic disease refers to a disease affecting the whole body rather than a single body part such as the oral cavity (Li et al., 2000). With chronic diseases, the long-term exposure to various by-products of the disease and complications related to disruptions in homeostasis can lead to distal effects in a variety of body functions and sites. These distal effects could conceivably lead to predisposition to other chronic diseases, especially as many have overlapping risk factors, and could be a key reason as to why many chronic diseases are comorbid within chronic disease populations (Jones, 2010).

1.3.1 The Systemic Effects of CP

Although it has been suggested that the oral cavity might provide an entry point to multiple diseases, how this is accomplished and what systemic consequences it can produce has only been partly explained. Recent research has demonstrated that CP is linked with diseases outside of the oral cavity, such as atherosclerosis, diabetes, stroke and coronary heart disease, with the pathogenesis mechanisms being under investigation (Gualtero et al., 2018, Sen et al., 2018, Beck et al., 2018, Preshaw et al., 2012). Many of the systemic effects associated with CP are resultant of the dysbiosis of oral microbiota, and the dissemination of the microorganisms or their products to distal parts of the body. With this in mind, it may be hypothesized that a similar systemic oral link may be influencing the incidence of CKD (Figure 1.3).

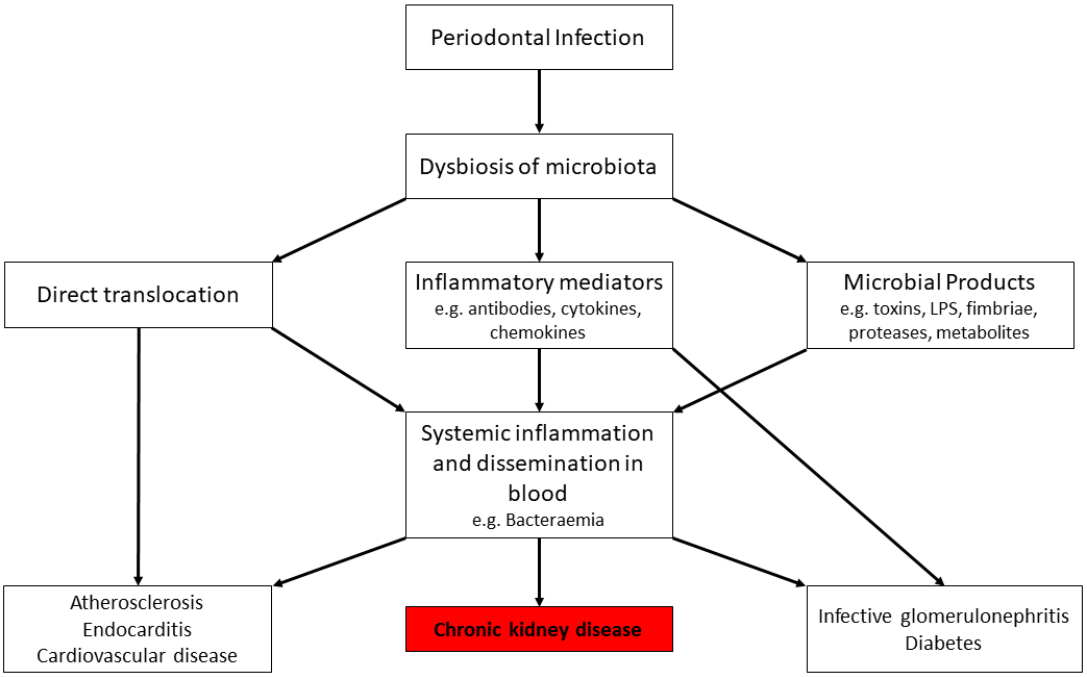

Figure 1.3 The systemic diseases linked with periodontal disease and putative mechanisms behind their relationship.

CP has an effect on the oral cavity over the course of the disease in numerous ways. It can modify the site of the infection, the periodontium, and the wider oral cavity. This is evidenced by disruption of the microvasculature (Zoellner et al., 2002), loss of attachment of teeth and bone damage (Flemmig, 1999), gingival bleeding (Albandar and Kingman, 1999) and the formation of periodontal pockets up to 1500-2000 mm^2 (Loos, 2006) with bacteria potentially in excess of 1 x 10^8 colony forming units in each pocket (Pihlstrom et al., 2005). The local production of inflammatory cytokines interleukin-6 and tumour necrosis factor-α in CP can increase the systemic levels of C-reactive protein and thus induce systemic inflammatory effects (Moutsopoulos and Madianos, 2006, Dye et al., 2005, Slade et al., 2003).

The localised damage to the oral cavity and periodontium along with the systemic inflammation can have a detrimental effect on the consumption of food and medication in CKD patients, affecting their quality of life and potentially leading to malnutrition and predisposition to other diseases (Anand et al., 2013, Akar et al., 2011, Chen et al., 2006). Indeed, the systemic inflammatory effects of CP could be

predicted to exacerbate the inflammation already present in CKD with potential interactions possibly acting as a further stressor on the already damaged kidneys.

1.3.2 The Systemic Effects of CKD

CKD causes a multitude of systemic effects on the human body, which are due to the kidneys progressively failing to remove waste products from the blood. Over time, this can lead to long-term exposures of the body to a variety of uremic toxins (e.g. amino acid metabolic products such as urea and creatinine) (Vanholder et al., 2003). This can lead to the development of uremic syndrome (uremia), which results in the accumulation of organic waste products which would normally be cleared by the kidneys (Inker and Levey, 2014, Meyer and Hostetter, 2007). Alongside this, in CKD changes are observed in processes linked to the normal function of the kidney such as the regulation of blood pressure and the homeostasis of electrolytes and acid-base balance (Dhondup and Qian, 2017), which lead to hypertension (Judd and Calhoun, 2015), hyperphosphatemia (Hruska et al., 2008), and hypocalcaemia (Kalantar-Zadeh et al., 2010). There are a multitude of other non-specific effects of CKD, such as a general inflammation, infection and protein wasting (Akar et al., 2011).

The oral cavity is a site where the systemic effects of CKD have been reported and is of particular interest as it encompasses gingival tissue, the site for CP pathogenesis. The effects are multifaceted and dependent on factors such as: the drugs used for the treatment of CKD, the stage of CKD, dialysis status, oral healthcare regime and confounding diseases such as diabetes. Increased plaque, gingival inflammation and calculus have been extensively reported (Craig, 2008). Although the cause is not clear, as other factors such as immune dysfunction, confounding diseases and poor oral hygiene may play a role. It has also been reported that oral lesions are much more common in CKD patients who display increased serum urea concentrations and elevated pH in the oral cavity (Honarmand et al., 2017, Oyetola et al., 2015). There are a large selection of other changes to the oral cavity reported to take place in CKD such as changes to teeth, periodontium, oral mucosa, bone and tongue (Akar et al., 2011, Craig, 2008). With multiple reports highlighting the increased incidence of periodontal disease in those with CKD it is possible that changes in the oral cavity may predispose CKD patients to developing CP (Ariyamuthu et al., 2013).

With the onset of CKD, increasing levels of urea in the oral cavity can lead to a range of changes that contribute to the development of CP. The hydrolysis of urea can increase salivary pH through the formation of alkaline ammonia compounds, which cause 'uremic fetor' (a fish like odour on the breath on patients with renal failure) (Simenhoff et al., 1977, Epstein et al., 1980). An alkaline oral environment favours periodontal pathogens and their colonisation, and promotes calculus deposition (Ismail et al., 2015, Bots et al., 2006). Xerostomia, a dryness of the mouth and mucosal surfaces, is also a side effect in CKD patients, which is thought to be caused by a spectrum of factors such the changes in oral mucosa, reduced salivary flow and drugs used for CKD management (de la Rosa Garcia et al., 2006). The reduced flow of saliva in xerostomia has been shown to predispose patients to infections, gingival inflammation and the development of chronic periodontitis (de la Rosa Garcia et al., 2006, Kho et al., 1999, Porter et al., 2004). Intriguingly, CKD patients have a decreased incidence of caries, which is due to the alkaline oral environment not being amenable for cariogenic organisms (Peterson et al., 1985).

The persistent long-term increase of urea and uremic toxins (uremic toxaemia) has an effect on the immune system and can lead to immune dysregulation and chronic inflammation (Cohen and Hörl, 2012). Uremic toxins can cause a dysfunction of polymorphonuclear leukocytes (PLs) such as basophils, monocytes and neutrophils and dysregulate the innate immune response (Haag-Weber and Horl, 1996, Vaziri et al., 2012). Interestingly, previous studies have also shown that the ammonia produced by the degradation of the increased urea concentrations can inhibit neutrophil function which could result in an additive effect to the already dysregulated innate immune system (Niederman et al., 1990). Such dysregulation in the innate immune system may lead to an increased risk of infection in the oral cavity, which can predispose CKD patients to CP.

The drugs utilised for immunosuppressive therapy after kidney transplantation (Calcinurin inhibitors) and calcium channel blockers, which are used to regulate the increased cytosolic calcium along with hypertension in those with renal disease, have also been reported to cause gingival hyperplasia (the overgrowth of the gingival tissue) (Somacarrera et al., 1994, Bharti and Bansal, 2013, Brown and Arany, 2015).

Interestingly, this gingival overgrowth can be enhanced by poor oral hygiene which is reported to be particularly endemic in CKD patients and it has been suggested that such gingival overgrowth can lead to gingival inflammation and subsequent increased risk of CP (Reali et al., 2009, Pihlstrom et al., 2005).

Evidence suggests a multifactorial effect of the systemic consequences of CKD on the oral cavity, the result of which is the predisposition to CP (Figure 1.4). However, it should be noted that these mechanisms and their interactions need further investigation and there may be more complex interactions not yet elucidated. Indeed, there may be redundant pathways that still predispose to CP. For example, while increased cytosolic calcium has been shown to cause dysfunction in immune cells, this can usually be treated by drugs such as calcium channel blockers. However, calcium channel blocker can induce gingival hyperplasia and inflammation, which can predispose to CP.

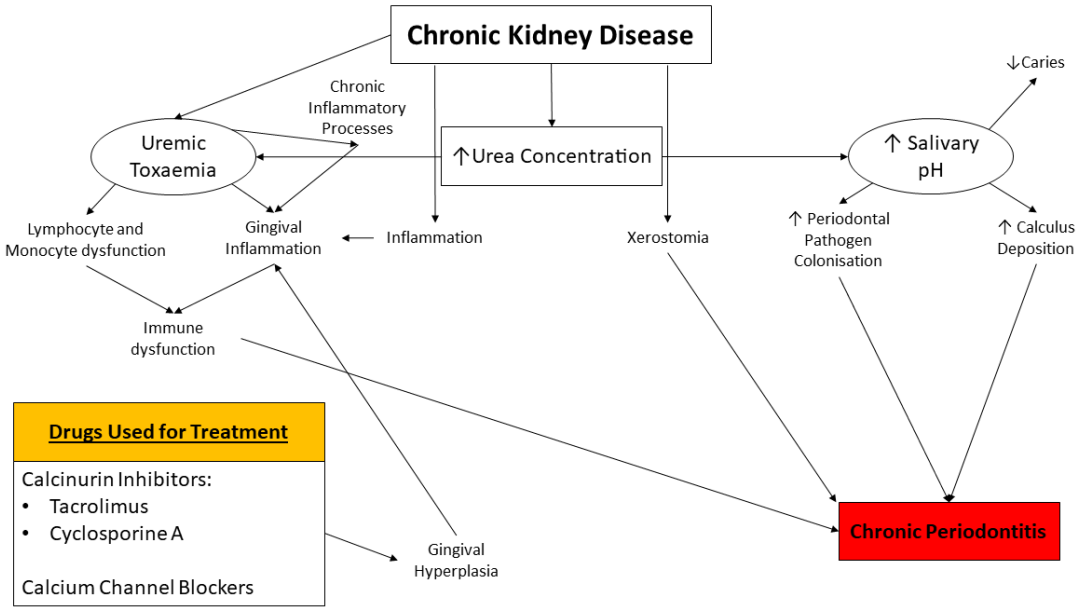

Figure 1.4 The systemic effects of chronic kidney disease in the oral cavity that can interact with chronic periodontitis. ↑=Increased; ↓=Decreased.

1.3.3 Interactions Between the Systemic Effects of CKD and CP

The systemic and localised effects of CKD and CP and their impact on each other is difficult to elucidate, partly due to the complexity of the diseases but also because this is an under studied area. However, with careful consideration of the known systemic and localised effects of each disease, it is possible to suggest ways in which they may interact with each other. The potential interaction of the effects of CP (Section 1.3.1) and CKD (Section 1.3.2) is summarised in Figure 1.5 to hypothesize how these two diseases may lead to a potential bidirectional relationship.

The pathogenesis of CKD induces systemic inflammation and a more amenable environment in the oral cavity for the growth of periodontal pathogens. Furthermore, a dysfunction of the polymorphonuclear leukocytes would make the environment more amenable for the colonisation and persistence of periodontal pathogens (Rijkschroeff et al., 2016). Considered alone, these changes would predispose CKD patients to an increased risk of CP, even without factoring in other known confounders such as age, smoking status and diseases such as diabetes. In addition, the establishment of CP has the potential to affect CKD due to the systemic inflammation produced along with the increased exposure to microorganisms and their products into the blood through persistent bacteraemia. With the kidneys

playing a vital role through the filtration of the blood, it may cause bacterial products to affect renal cells, potentially leading to glomerular and/or tubular damage and fibrosis.

In CKD, the damage to the oral cavity (e.g. loss of teeth and gingival tissue) may also cause difficulties in the intake of food and medicine, and may be one of the contributing factors to the significant association of CKD and CP. The loss of teeth and gingiva may be accelerated in CKD by poor oral hygiene, as it has been shown that CKD patients access dental care less frequently (Grubbs et al., 2012). Furthermore, uremic patients are reported to be more prone to bacterial infections due to malnutrition (Mitch and Maroni, 1999), which impedes the production of antibodies and immune cells (De Rossi and Glick, 1996).

At this current moment in time, much of the evidence for a directional relationship (onset of exposure takes places before the outcome) between CKD and CP, points in favour of CKD causing CP as there is less to support CP as a cause of CKD. However, current consensus suggest that it is more likely that there is a non-directional link (onset of the exposure can happen before, during or after the outcome) due to the lack of evidence currently available for directional associations. Furthermore, while the evidence for the association between CKD and CP is plausible due to interaction of systemic effects alone, it would be prudent to investigate the effects of the organisms involved in periodontal disease and how they may directly affect CKD and the kidneys. This is especially important as periodontal organisms and their products have been shown to be directly involved in other systemic diseases, and there is evidence for oral microorganisms damaging the kidneys already in other microbial diseases.

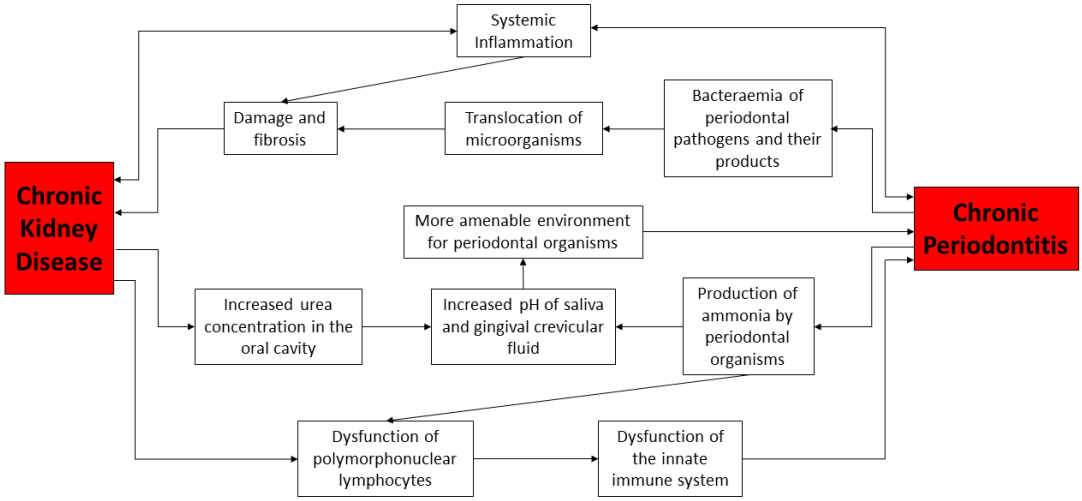

Figure 1.5 Possible interactions between the systemic effects of CKD and CP.

1.4. The Proposed Link Between CKD and CP

CKD and CP are both common diseases in the general populace and have common risk factors such as malnutrition and inflammation, along with their presence being more pronounced in developing countries (Van Dyke and Dave, 2005, Luyckx et al., 2017). As both diseases are highly prevalent independently of each other, it is to be expected that there would be some co-morbidity within patients. Indeed, it has been described that those patients who have CKD have an increased prevalence of periodontitis compared to a geographically matched non-diseased population (Jha et al., 2013), with a systematic review of the relationship confirming this in multiple populations with an odds ratio of 1.65 (Chambrone et al., 2013). Furthermore, until recently the effect of CP on mortality rates was relatively unstudied with only small sized cohort studies over short durations. This has made accurate determination of mortality effects difficult, as both diseases are multifactorial. Consequently, the evidence is contradictory with some studies reporting a detrimental effect (Linden et al., 2012, Chen et al., 2011) while other research suggests a more marginal effect (Avlund et al., 2009). However, a recent 10 year survival analysis of a large cohort of CKD patients found that the mortality rate increased from 32% to 41% when periodontitis existed (Sharma et al., 2016). This has also been confirmed by a meta-

analysis of cohort studies which showed that periodontal disease was associated with an increased risk of death in CKD (Zhang et al., 2017). It is therefore plausible to suggest that CP increases the severity of CKD and as both CKD and CP are inflammatory mediated, this provides an avenue of enquiry to investigate.

As discussed in Section 3.3, CKD can affect the oral microenvironment with pH changes and gingival hyperplasia, making the environment more amenable for certain periodontal pathogens that thrive in a more alkaline environment (Listgarten, 1986). CP also effects CKD by increasing the systemic inflammation, which is evident by increased C-reactive protein (Paraskevas et al., 2008). It is plausible that this could exacerbate systemic inflammation which is already present in CKD (Pihlstrom et al., 2005). Indeed, it is becoming more evident that the systemic inflammation induced by CP is a potential cause and/or non-traditional risk factor in the progression of CKD (Wahid et al., 2013). There have also been suggestions that there is a bidirectional relationship between CKD and CP, with gingival tissue being a source of chronic inflammation and treatment of CP having a positive effect of glomerular filtration rate (Bastos et al., 2011, Fisher et al., 2011).

While there is increasing evidence in support of a link between the two diseases, the mechanisms behind this relationship remain relatively unstudied. Much of the evidence reported for the mechanisms is purely observational through cohort studies and correlation, not resultant of a direct investigation. Furthermore, as the focal infection theory has been modernised to the periodontal medicine concept (Section 1.2.1), it is important to consider the potential ways in which these periodontal microorganisms might cause disease. It may not be as simple as a single causative mechanism; it is likely to be a summation of multiple mechanisms.

1.5 AIM AND OBJECTIVES

1.5.1 Aim

Despite the growing collection of evidence to support an association between CKD and CP, the relationship between how the diseases interact with each other is unclear. While there has been some large cohort studies investigating CKD and CP, it is particularly difficult to find a patient population without various other systemic diseases and mitigating factors that can be adjusted for confounding and derive a decisive conclusion of the putative interaction. Therefore, laboratory based investigations would be useful in order to investigate the effects of periodontal organisms at the biochemical level and determine whether the microorganisms implicated in CP have the ability to directly interact with CKD. As there is substantial evidence showing that oral microorganisms have multiple mechanisms to elicit a distal response in a multitude of diseases, it would be of interest to investigate whether these microorganisms and their products can have an effect on *in vitro* kidney based models.

Thus, the aim of this study was to investigate the microbial products produced by periodontitis associated microorganisms using a novel metabolomics approach and to identify if any of these products are capable of eliciting a cytotoxic and fibrotic response in kidney cell based models.

1.5.2 Objectives

- Identify and culture a selection of periodontitis keystone pathogens, accessory organisms and non-associated microorganisms in a growth medium representative of the diseased environment.

- Explore the use of metabolomics as a screening tool to investigate microbial products produced by selected microorganisms.

- Determine whether the microbial products produced by these microorganisms can induce cytotoxic and migratory effects in human kidney cells.

- Determine whether the microbial products produced by the selected microorganisms can induce fibrotic effects in human kidney cells.

- Identify which, if any, constituent/s of the microbial products have cytotoxic, migratory or fibrotic effects on human kidney cells.

CHAPTER 2: SELECTION AND GROWTH OF SELECTED PERIODONTAL PATHOGENS

2.1 INTRODUCTION

The oral bacteria associated with biofilm formation are the main aetiological agents behind the inflammatory processes seen in CP with dysregulation of the normal oral microbiota being observed (Wahid et al., 2013). CP is caused by the inflammatory response to the accumulation of dental plaque around the gingiva that results in an increased flow of GCF an inflammatory exudate which bathes the gingival crevice, and favours the growth of anaerobic microorganisms (Marsh et al., 2009, Winning et al., 2015).

Many of these periodontitis associated pathogens are difficult to culture *in vitro* which is partly compounded by the lack of available broad host range growth media. Indeed, many oral pathogens are also particularly fastidious anaerobes and in some notable examples, such as *Tannerella forsythia*, rely on nutrients secreted by other bacteria during synergism (Sharma, 2010). With the growth requirements of many of these organisms being specifically evolved to the unique environment of the gingival crevice, one of the best ways of cultivating a large collection of these microorganisms would be to use a growth medium, which can mimic the biofluid present at this environmental interface, which in this case is GCF. However, currently no such medium for the universal culture of periodontitis associated organisms is available.

As this project aims to investigate a selection of microorganisms involved in CP along with microbial products produced, it is of particular importance to culture all of these organisms in the same growth medium that is more representative of the diseased environment. Since the choice of the media that the bacteria are grown in influences the biochemical and metabolic state (Dalwai et al., 2006), the development of a single medium would allow for better comparisons between the bacteria and facilitate further *in vitro* investigations into the effects of microbial products.

2.1.1. Background behind Growth Medium Development

GCF is an inflammatory exudate, which is produced by the epithelial cells that line the gingival crevice in the oral cavity and bath the gingiva. The flow of GCF is relatively slow in healthy patients. However, in periodontal disease it can increase by up to 30 fold as part of the inflammatory response to the plaque accumulation (Marsh et al.,

2009). It is a useful biofluid that can be collected non-invasively and be used to monitor periodontal disease and is reported to have a multiple applications in health and disease (Mikkonen et al., 2016). The composition has been well characterised (Table 2.1) with the inorganic and organic ion content similar to that seen in plasma, which is because GCF is derived from this source (Ferguson, 2006). Small organic molecule content includes lactic acid, urea, hydroxyproline and Prostaglandin E2 (PGE_2) (Ferguson, 2006). Lactic acid and PGE_2 concentrations usually increase with severity of inflammation present while urea, which is present at concentrations several times higher than in plasma or saliva, appears to decrease with the severity of gingivitis, most likely because it is metabolised by periodontal organisms to ammonia (Ferguson, 2006). GCF also has a wide range of enzymes derived from bacterial leucocytes, and squamous cells with some inter-individual variability depending on the bacterial species present.

Table 2.1 Composition of human gingival crevicular fluid compared to saliva and plasma adapted from (Ferguson, 2006)

Component	GCF	Saliva	Plasma
Sodium (mmol/L)	91.6 ± 31.1	6-24	152
Potassium (mmol/L)	17.4 ± 11.7	13-40	4
Calcium (mmol/L)	5 ± 1.8	0.5-2.8	2.5
Magnesium (mmol/L)	0.4 ± 0.2	0.3-0.6	1.6
Phosphate (mmol/L)	1.3 ± 1	0.4-5	2.1
Protein (g/L)	70	1.2-1.7	70
Albumin (g/L)	35	0.025	35
Gamma globulins (g/L)	7.4	0.050	6-12

2.1.2 The Effect of CKD on the Oral Cavity

It has been well reported that CKD has a multitude of systemic effects on the human body, along with localised effects that can alter the balance of different environments such as the oral cavity in CP (Chapter 1.3.2). There are some suggestions that the increased incidence of periodontal disease in those with CKD is due to changes in the oral cavity such as increased urea concentration that may predispose CKD patients to increased plaque deposition and predisposition to developing CP (Ariyamuthu et al., 2013).

To investigate this, the presence of a urease gene was evaluated in microorganisms to indicate if the increased urea concentration and subsequent pH changes may act as a selection pressure. Using the National Centre for Biotechnology Information (NCBI) web platform, all urease like genes were searched for which were present in all bacterial species. This highlighted 38 bacteria of 17 different species which had this gene or homologous gene present. Of these 17 species identified, four were found to be related to periodontal disease and three of these species were from the same *Fusobacterium* genus (Table 2.2)

Table 2.2 List of microorganisms identified to have a urease gene and implicated in periodontal disease.

Species	Identifier	Identified Gene
Fusobacterium nucleatum subsp. animalis	N/A	Hypothetical protein FSDG_RS11485 and hypothetical protein FSDG_RS04160
Fusobacterium nucleatum subsp. nucleatum	ATCC 25586	Urease accessory protein UreG, hypothetical protein BKN39_RS06810 and hypothetical protein FN0683
Fusobacterium periodonticum		Hypothetical protein FSAG_RS08490 and hypothetical protein FSAG_RS07755
Fusobacterium necrophorum subsp. funduliforme	ATCC 51357	Hypothetical protein HMPREF1049_RS04085
Treponema denticola	ATCC 35405	hydrogenase nickel incorporation protein HypA and hypothetical protein TDE2660

The *Fusobacterium* genus is heavily linked to periodontal disease and is known to play an important role as an accessory organism due to its ability to co-aggregate with other organisms, in particular the keystone pathogens (Signat et al., 2011). Furthermore, due to its co-aggregation abilities it can act as an important bridge for early and late colonisers of the periodontal pocket and can form synergistic reactions with a variety of Gram negative and positive microorganisms, which can facilitate the development of CP (Bolstad et al., 1996). Indeed, *T. denticola* is one of the keystone pathogens highly associated with CP and can interact with *Fusobacterium* species to instigate the development of CP (Hajishengallis and Lamont, 2012).

The high prevalence of *Fusobacterium* species and *T. denticola* microorganisms with urease genes indicate that the CKD oral cavity may be a more suitable environment for these organisms. In particular, with *Fusobacterium* species playing a pivotal role in linking polymicrobial periodontal biofilms and early and late colonisers, the

establishment of these species in the CKD oral cavity could be potential factor behind the increased levels of periodontal diseases in those with CKD.

2.1.3 Changes in Oral Microbiota Related to CP and CKD

There has only been a few studies that have investigated changes in the microbiota associated with CKD and CP (Castillo et al., 2007, Torres et al., 2010, Takeuchi et al., 2007). There is also some confusion over whether there is a significant change in the microbiota between CKD patients with CP versus patients only with CP, which is compounded by differences between CKD patients on haemodialysis and those who are not. One such study observed haemodialysis patients compared with patients not receiving haemodialysis; this highlighted that the presence of periodontitis microorganisms were more prevalent in those without haemodialysis but there was no specific microbial signature and did not present as more severe (Castillo et al., 2007). A further study assessed the periodontal status and presence of red complex periodontal organisms in those with CP alone compared to haemodialysis and CP. This found haemodialysis patients had more red complex organisms even when shallower periodontal pockets were present compared to those without CKD (Torres et al., 2010). Indeed, the differences reported for periodontal status between haemodialysis CKD patients and those CKD patients not undergoing haemodialysis conceivably could be due to better control of the systemic disease by the haemodialysis treatment. This argument is supported by another study which showed that while CKD patients with and without haemodialysis have more detectable periodontal organisms than healthy controls, those undergoing haemodialysis having lower detection rates of these same organisms with the CKD subgroup (Takeuchi et al., 2007).

A pilot study which looked at the effect of end stage renal disease on the subgingival microbiome found little difference in the proportional makeup, except for a slight decrease in microorganism diversity in those undergoing long term dialysis (Araújo et al., 2015). However, a cross sectional study that determined the association of nine periodontal pathogens with CKD did not find a specific periodontal profile in CKD but did find that *T. denticola*, *T. forsythia* and *P. micros* were significantly associated with periodontitis within CKD patients (Ismail et al., 2015). Once a multivariate model had

been applied to the data, only age and *T. forsythia* was independently associated with periodontitis in CKD patients. The periodontal pathogens present in CP patients with and without CKD was also studied and a strong association was identified between more severe CP and an increased frequency of *C. albicans*, *P. gingivalis*, *T. forsythia*, and *T. denticola* in patients with CKD who were on renal replacement therapy and pre-dialysis (Bastos et al., 2011).

From the published works, it may be hypothesized that CP appears to be more severe in those with CKD. This could possibly be explained by the presence of two systemic diseases having detrimental and/or synergistic interactions with each other. As those on haemodialysis had higher detection of periodontal organisms compared to controls but less than CKD patients pre-dialysis, this evidence further supports this theory given those on haemodialysis would arguably have better control of their CKD. The association with a specific profile of periodontal organisms for CKD patients is yet to be proved as the small-scale studies that have identified periodontal pathogens associated with CKD do not all agree on specific organisms. Furthermore, those microorganisms identified as having increased frequency such as *P. gingivalis*, *T. forsythia* and *T. denticola* are the keystone pathogens of CP, so might be attributed to the development of CP as a whole rather than a specific signature for CKD patients alone.

2.1.4 Chosen Microorganisms

As there are a large number of periodontitis associated organisms, a selection of eight microorganisms were chosen for this study. Six were associated with periodontitis, some of which had associations with other systemic diseases. Two were not associated with periodontitis with one of these a common member of the oral microbiota and the other normally not associated with the oral cavity. The chosen microorganisms were divided into three groups: keystone pathogen, accessory organisms and non-associated organisms (Table 2.3).

Whilst there are three identified keystone pathogens in periodontal disease, *P. gingivalis* and *T. denticola* and *T. forsythia* (Hajishengallis and Lamont, 2012), only *P. gingivalis* was investigated due to the known difficulties in cultivation of the other

keystone pathogens. *P. gingivalis* is the easiest of the three keystone pathogens to cultivate and is consistently isolated in CP along with the bacterial products produced being implicated in various other systemic diseases such as Alzheimer's (Dominy et al., 2019).

For the accessory organism group five different organisms were chosen to reflect a selection of accessory organisms implicated as interacting within CP and also implicated in systemic diseases (Table 2.3).

Table 2.3 The microorganisms chosen to study for this study and the reasoning behind the choice.

Microorganism	Group	Reasoning	Reference
Porphyromonas gingivalis	Keystone Pathogen	A key pathogen in CP because of complex host-pathogen and community interactions, which can cause dysbiosis and promote a pro-inflammatory phenotype. In addition, a multitude of virulence factors are produced which can cause damage and destruction of the periodontium.	(Hajishengallis, 2009)
Actinomyces israelii	Accessory organism	Present in patients with CP more frequently than healthy patients, along with those with high scores on the plaque index. It may play a role in the maturation of sub and supra gingival plaque. It is commonly isolated alongside *A. actinomycetemcomitans* suggesting synergy in periodontal infection.	(Vielkind et al., 2015, Könönen and Wade, 2015)
Fusobacterium nucleatum	Accessory organism	Reported to play a role as a 'bridge' for early and late colonisers to co-aggregate. It has also been reported to have synergy with *T. forsythia* in inducing an immune response and causing alveolar bone loss. Has a putative urease gene.	(Settem et al., 2012)
Parvimonas micra	Accessory organism	A commensal organism commonly found in the subgingival plaque and in periodontal plaque forms in the top layer. It is not thought to form biofilm structures, but has a significant association with periodontal destruction. It has been associated with CP within the CKD subgroup.	(Zijnge et al., 2010, Al-hebshi et al., 2014)
Streptococcus constellatus	Accessory organism	A member of the '*Streptococcus anginosis*' group which are common commensals in the oral cavity and associated in periodontitis. Implicated in periodontitis as providing an attachment substratum allowing attachment and recolonization of other periodontitis associated organisms. Implicated in systemic disease with isolates identified from brain abscesses being linked to a periodontal pocket source.	(Colombo et al., 1998, Rams et al., 2014, da Silva et al., 2004)
Streptococcus sanguinis	Accessory organism	A member of the '*Streptococcus mitis*' group which are common commensals in the oral cavity and associated in periodontitis. *S. sanguinis* is suggested to play a role in binding of other periodontitis associated organisms. The group is also linked to systemic disease and are the most common cause of infectious endocarditis.	(Whitmore and Lamont, 2011, Hendrickson et al., 2017, Baker et al., 2019, Zhu et al., 2018)
Enterococcus faecalis	Non associated organism	A normal commensal microorganism commonly found in the gastro-intestinal tract and a opportunist pathogen in nosocomial infections. It is not part of the normal oral microbiota and is merely transient. While not linked with periodontitis it has been commonly found as a cause of root canal infections post treatment.	(Vidana et al., 2011, Zehnder and Guggenheim, 2009)
Streptococcus mutans	Non associated organism	A normal commensal microorganism found in the oral cavity. Not involved in periodontal disease but the most common cause of tooth decay (dental caries).	(Loesche, 1986, Nicolas and Lavoie, 2011)

2.2 AIM AND OBJECTIVES

2.2.1 Aim

To investigate a selection of periodontitis associated organisms, culture the organisms in a suitable medium and collect microbial supernatants over the microorganisms lifecycle.

2.2.2 Objectives

- Identify a selection of periodontitis associated microorganisms to investigate.
- Develop a new growth medium that is more representative of the gingival crevicular environment and can cultivate all chosen microorganisms.
- Cultivate the chosen microorganisms in this newly developed growth medium.
- Collect microbial supernatants from cultivated organisms and control media for further investigations.

2.3 METHODS

2.3.1 Development of Artificial Gingival Crevicular Fluid Periodontal (AGCF-P)

The development and reasoning behind the development of the AGCF-P growth medium is fully described in Hickey et al. (2019).

2.3.1.1 Composition of Base Medium

The base medium used to modify for the development of AGCF-P was basal medium mucin (BMM)(Table 2.4).

Table 2.4 Ingredients of Basal Medium Mucin

Ingredient	Amount	Supplier
Partially purified pig gastric mucin	2.5 g/L	Sigma Aldrich, UK
Protease peptone	10 g/L	Oxoid, UK
Trypticase peptone	5 g/L	Scientific Lab Supplies, UK
Yeast extract	5 g/L	Sigma Aldrich, UK
Potassium Chloride	2.5 g/L	Sigma Aldrich, UK
Urea	1 mmol/L	Sigma Aldrich, UK
Arginine	1 mmol/L	Sigma Aldrich, UK
Haemin	1 mg/L	Sigma Aldrich, UK
Menadione	1 mg/L	Sigma Aldrich, UK

2.3.1.2 Composition of Artificial Gingival Crevicular Growth Medium

The new growth medium, AGCF-P was composed of (BMM) (Wong and Sissions, 2001) with multiple modifications as described (Section 2.4.1). The full composition of AGCF-P is shown in (Table 2.5). All reagents were procured from Sigma Aldrich, UK with the exception of trypticase peptone (Scientific Laboratory Supplies, UK) and horse blood (TCS Biosciences, UK).

Table 2.5 Constituents of Artificial Gingival Crevicular Fluid – Periodontal (AGCF-P)

Ingredient	Amount (g/L)
Protease peptone	10
Trypticase peptone	5
Yeast extract	5
KCL	2.5
Pig Mucin	2.5
L-cysteine	0.5
L-arginine	0.17
Urea	0.18
Agar no 3 (If solid media required)	12
Potassium Phosphate monobasic anhydrous	1.68
Sodium Phosphate dibasic heptahydrate	41.31
Blood added	5% horse blood serum

2.3.2 Cell Culture

2.3.2.1 Human Gingival Fibroblast Cells

Human Gingival Fibroblast (HGF-1) primary cells (LGC standards, UK) were cultured in Dulbecco Modified Eagle Medium (DMEM) with Glutamine and Glucose (Lonza, UK), supplemented with 10% foetal calf serum (Lonza, UK), 50 µg/mL streptomycin (Lonza, UK) and 50 µg/mL penicillin (Lonza, UK). Cells were growth-arrested in serum free cell culture media, in the absence of foetal calf serum.

2.3.2.2 Human Kidney Proximal Tubule Cells

Immortalised Human Kidney Proximal Tubule cells (HK-2) (ATCC, UK) were cultured in 50:50 glucose free DMEM (Invitrogen, UK) and Hams F-12 medium (Lonza, UK) supplemented with 10% foetal calf serum (Lonza, UK), 50 µg/mL streptomycin (Lonza, UK), 50 µg/mL penicillin (Lonza, UK) and 2.5 mMol glutamine (Lonza, UK). Cells were growth-arrested in serum free cell culture media, in the absence of foetal calf serum.

2.3.2.3 Maintenance of Cells

All cells were maintained in a 37°C incubator with 5% CO_2, the respective cell culture media were changed every other day dependent on the confluency of the cells. When cell reached confluency trypsin-EDTA solution (Lonza, UK) was used to disassociate cells from the cell culture flasks allowing re-seeding of the cells and continued culturing. In order to seed cells, sterile filtered 0.4% trypan blue (Sigma Aldrich, UK) was used to check cell viability using a 1:1 ratio with cell suspension. Cell viability was

measured using either a haemocytometer or a TC10 automated cell counter (Bio-Rad, USA), the number of viable cells was used to work out number of cells seeded per well.

2.3.3 Cell Viability Testing

To assess the effect of AGCF-P bacterial growth medium on mammalian cell viability, the AGCF-P medium was incubated for 5 days in anaerobic conditions without bacterial inoculation. At 0 D, 1 D, 2 D, 3 D, 4 D and 5 D aliquots of the AGCF-P medium were plated out to check for bacterial contamination. The remaining media was centrifuged at 600 *x g* for 10 min, then filtered using a 0.2 µM Acrodisk filters (Scientific Lab Supplies, UK) and stored at -80°C until used.

The cell viability of HGF-1 and HK-2 cells were tested using the cell counting kit-8 (Tebu-bio, UK) (Tominaga et al., 1999) which is a more sensitive detector of viable cells in comparison to other tetrazolium salts. Cells were seeded into a 96 well plate at 5 x 10^3 cells per well and incubated at 37°C in 5% CO_2; a cell culture plate was seeded separately for 24 h and 48 h cell viability experiments to allow double dosing of the 48 h experiment. The cells were washed with phosphate buffered saline (PBS) (Lonza, UK) once 80% confluency was reached and the medium was changed to serum free medium. After 24 h in serum free medium, the medium was refreshed and bacterial supernatants were added at a 1:20 dilution.

For 24 h cell viability experiments the plates were observed and photographed before and after the experiment, at 24 h 10 µL of the CCK-8 dye was added to each well and absorbance readings were taken at 1 h, 2 h and 3 h at 450_{nm} and 600_{nm} using a microplate reader (Thermo-Scientific Multiskan 60, UK). For the 48 h incubations with supernatants, the serum free media with dilutions was refreshed after 24 h and re-incubated at 37°C in 5% CO_2. Then, at 48 h the CCK-8 dye was added and absorbance readings were taken at 1 h, 2 h and 3 h.

2.3.3.1 Cell Viability Data Analysis

All absorbance data was exported to excel and the absorbance at 600_{nm} was taken away from the absorbance at 450_{nm}, then the dilutions and dye without cells absorbance was taken away in order to produce accurate absorbance values.

$$Cell\ Viability\ = \frac{((450\ ABS\ -\ 600\ ABS)\ -\ Blank\ Media\ with\ Dye)}{Cell\ viability\ of\ cell\ culture\ control} \times 100$$

Individual percentage standard error was calculated and graphs were plotted on Prism GraphPad 8.1.1

$$\%\ Standard\ error = \frac{(Standard\ error\ of\ samples)}{Cell\ viability\ of\ cell\ culture\ control} \times 100$$

2.3.4 Bacterial Strains and Culture Technique

2.3.4.1 Microorganisms for testing AGCF-P media

The microorganisms used to test AGCF-P media were *Actinomyces israelii* NCTC 12972, *Candida albicans* ATCC 2091, *Capnocytophaga ochracea* NCTC 12371, *Prevotella intermedia* NCTC 13070, *Rothia dentocariosa* NCTC 10917, *Streptococcus oralis* NCTC 11427, *Streptococcus mitis* NCTC 12261 and *Streptococcus salivarius* NCTC 8618.

C. albicans, *R. dentocariosa*, *S. oralis*, *S. mitis* and *S. salivarius* were grown on AGCF-P agar or broth at 37^{o}C for 48 h before pictures were taken. *A. israelii*, was grown on AGCF-P agar or broth at 37^{o}C in anaerobic conditions (80% N_2 - 10% H_2 - 10% CO_2) in a Baker-Ruskin Concept Plus anaerobic incubator (Ruskin, UK) for 48 h before pictures were taken.

2.3.4.2 Chosen Microorganisms to study

The microorganisms used in this study were *Actinomyces israelii* NCTC 12972, *Enterococcus faecalis* NCTC 775, *Fusobacterium nucleatum sub fusiforme* NCTC 11326, *Parvimonas micra* NCTC 11808*, Porphyromonas gingivalis* NCTC 11834, *Streptococcus constellatus* NCTC 11325*, Streptococcus mutans* NCTC 10449 and *Streptococcus sanguinis* NCTC 7863.

A. israelii, *F. nucleatum*, *P. micra* and *P. gingivalis* were grown on AGCF-P agar or broth at 37 ^{o}C in anaerobic conditions. *E. faecalis, S. constellatus*, *S. mutans* and *S. sanguinis* were grown on AGCF-P agar or broth at 37^{o}C in carbon rich conditions (5% CO_2) in a CO_2 incubator (LEEC, UK).

2.3.5 Preparation of Overnight Cultures

Overnight cultures were prepared by inoculating a single colony of a chosen organism into 5 mL of AGCF-P liquid media and incubated for 24 h in anaerobic conditions or in 5% CO_2 for *E. faecalis, S. constellatus*, *S. mutans* and *S. sanguinis*. Due to the slow growth of *P. gingivalis* overnight cultures, they were cultured for 48 h to allow sufficient growth. Once grown, the cultures were centrifuged at 600 *x g* for 10 min taking care to use Suba Seals (Sigma, UK) for strict anaerobic cultures when centrifuging outside the anaerobic environment. Once centrifuged, the supernatants were removed and the cells were re-suspended in PBS (Sigma Aldrich, UK). The bacteria were adjusted to an optical density (OD) of 1.0 ± 0.05 using a spectrophotometer (Jenway 6305, UK) which was calibrated with PBS.

2.3.6 Growth Curves

Three overnight cultures were grown of the chosen microorganism and once an OD of 1.0 ± 0.05 was obtained, 1 mL per 100 mL of overall culture was inoculated into three sterile culture flasks with liquid AGCF-P growth media (*n=3*). In order to account for the degradation of growth media over time, three non-inoculated culture flasks with liquid AGCF-P growth media were also sampled alongside the chosen microorganisms and for the same time points. Alternatively, large vented cell culture flasks (Fisher Scientific, UK) were used for long-term cultures in the anaerobic cabinet to reduce contamination.

For the chosen keystone pathogen, *P. gingivalis*, six time points were chosen (0 D, 1 D, 2 D, 3 D, 4 D, 5 D) to reflect the different stages of bacterial growth. For all these time points, CFU/mL of culture (Section 2.2.5) were recorded to quantify the numbers of viable microorganisms over time and bacterial supernatants (Section 2.2.6) were sampled at (0 D, 1 D, 2 D, 3 D, 4 D, 5 D) for further analysis.

For all chosen accessory organisms and non-associated organisms, six time points were chosen (0 h, 2 h, 4 h, 6 h, 24 h and 48 h) to reflect the different stages of bacterial growth taking into account the manufacturer's recommendations. CFU/mL of culture were recorded at all time points to quantify the numbers of viable microorganisms over time. Bacterial supernatants were also sampled at four time

points (0 h, 6 h, 24 h, 48 h), due to the first four time points being close and limitations regarding long-term storage space for the supernatants.

2.3.7 Colony Forming Unit per mL Quantification

At each time point CFU/mL were quantified using the Miles-Misra droplet method (Miles et al., 1938). Serial dilutions were completed from 10^{-1} to 10^{-8} of the triplicate bacterial cultures and three technical replicate droplets of 20 µL for each serial dilution was plated out on AGCF-P agar plates divided into thirds. These agar plates were incubated for a 24-48 h period in the chosen growth conditions dependent on the microorganism. Once grown the plates were stored at 4°C in a refrigerator until ready to be counted. For each time point the droplets with the lowest dilution and a countable number of colonies (between 3 and 30) was chosen. The average number of colonies for the three technical replicates was recorded for each biological and exported into an Excel spreadsheet. The CFU/mL was calculated using this formula:

$$Colony\ Forming\ Units\ per\ mL\ (CFU/mL) = \frac{Number\ of\ colonies \times\ dilution\ factor}{Volume\ of\ culture\ plate}$$

Standard error was calculated of the biological replicates using this formula:

$$Standard\ Error\ (SE)\ = \frac{Standard\ deviation\ of\ biological\ replicates}{\sqrt{\text{number of replicates}}}$$

2.3.8 Preparation of Bacterial Supernatants

Bacterial supernatants were prepared for further analysis by sampling the control and inoculated AGCF-P broth. At each chosen time point, 5 mL of inoculated broth and respective controls were sampled into separate 10 mL plastic universals and centrifuged at 3018 *x g* for 10 min. Ensuring the pellet was not disturbed; the supernatants were filtered into two sterile glass bijoux per sample using 5 mL disposable syringes and 0.2 µM Acrodisk filters (Scientific Lab Supplies, UK). These samples were labelled and stored at -80°C until ready for use.

2.3.9 Statistical Analysis

A two-way ANOVA with multiple comparisons was used to determine significant differences between the cell viability control and different time points of AGCF-P. All statistical analysis was conducted using Prism GraphPad 8.1.1 software.

2.4 RESULTS

2.4.1 Development of a Suitable Growth Medium for this Study

After a literature search for a suitable media to use as the base to develop a new medium, it was determined that the best candidate was an artificial saliva based media called Basal Medium Mucin (BMM) (Table 2.4) (Wong and Sissions, 2001).

2.4.1.1 Modifications to Create the New Growth Medium AGCF-P

While BMM was an excellent base to design a medium that represents the GCF, it needed to be modified to be more representative of the GCF, as BMM was designed to mimic saliva along with having supplements for the growth of *Treponema spp* and haemin and menadione supplemented in the absence of blood. From the base BMM medium, haemin and menadione were removed.

As the inorganic and organic constituents of GCF are most similar to serum (Table 2.1) the addition of 5% horse blood was added to the medium after autoclaving. In order to more accurately mimic the GCF in periodontal disease the pH was modified to physiological level.

The full composition of AGCF-P is shown in (Table 2.5).

2.4.1.2 Testing Suitability for Liquid and Solid Culture

A selection of microorganisms present in the laboratory and related to chronic periodontitis or oral health (Section 2.3.4.1) were tested for their ability to grow in both liquid and solid AGCF-P medium. The microorganisms were cultured in liquid culture (Images not shown as the media is viscous) and streaked onto solid AGCF-P agar plates, with (Figure 2.1) showing a selection of these organisms grown on agar.

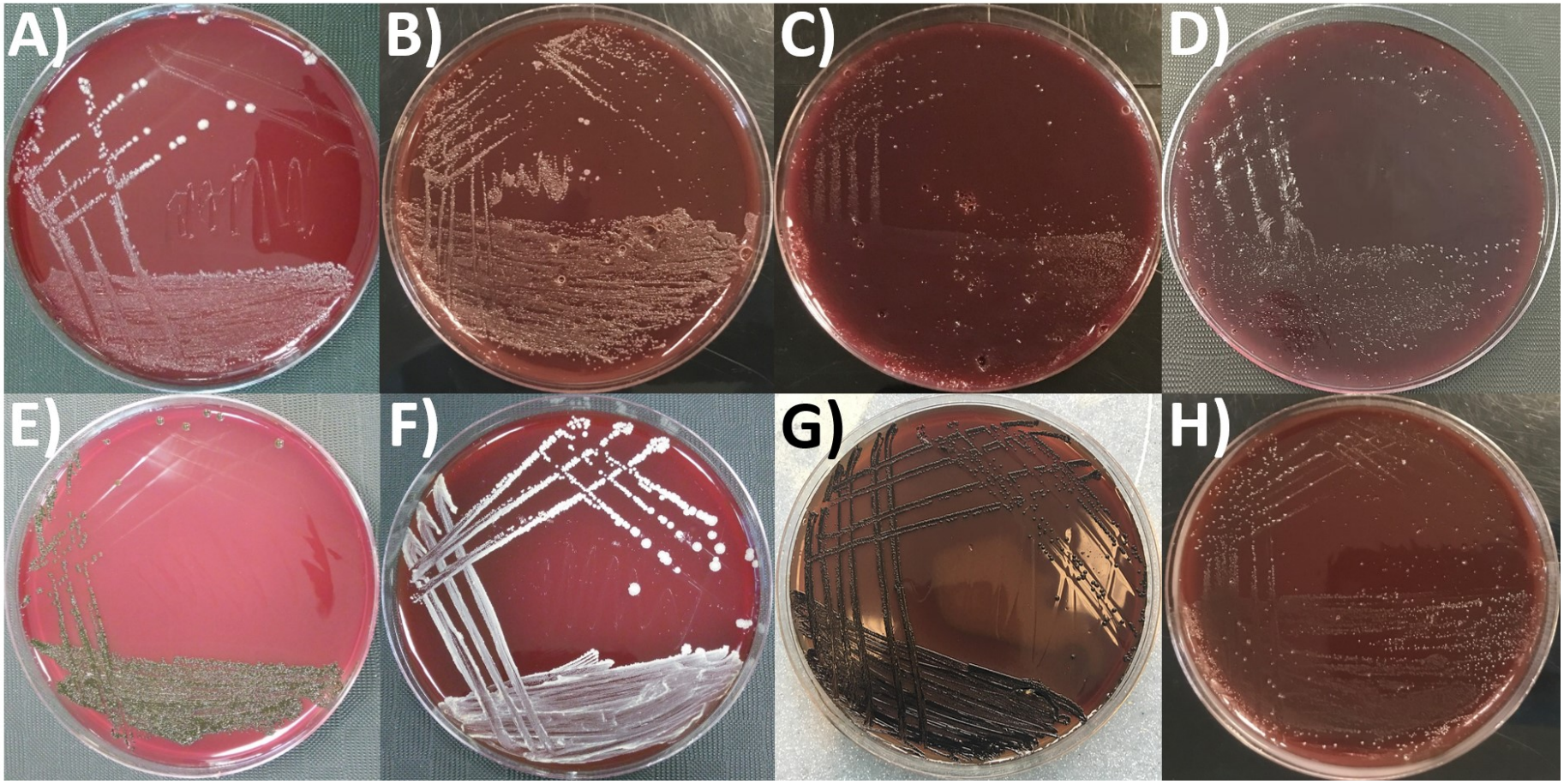

Figure 2.1 A selection of images of oral associated microorganisms grown on AGCF-P agar. A) *A. israelii* B) *C. albicans* C) *C. ochracea* D) *F. nucleatum* E) *P. intermedia* F) *R. dentocariosa* G) *P. gingivalis* H) *S. salivarius*

2.4.1.3 Testing Suitability for Cell Culture Applications

The effect of the AGCF-P medium on the cell viability of HGF-1 (Figure 2.2), and HK-2 (Figure 2.3) cells was assessed by exposure to 1:20 dilutions of AGCF-P supernatants incubated over a 5-day period in anaerobic conditions. Although the cell viability of HGF-1 was slightly increased (Figure 2.3), there was no significant effect of AGCF-P supernatants on the viability of these cells or HK-2 cells after 24 h (Figure 2.2A and 2.3A) or 48 h (Figure 2.2B and 2.3B) of incubation, as compared to control cells exposed to serum-free cell culture medium only.

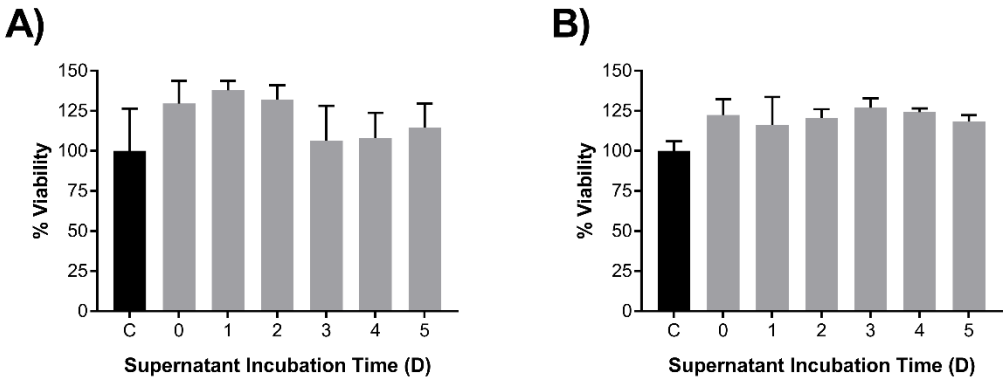

Figure 2.2 Cell viability of HGF-1 cells after 24 h (A) or 48 h (B) of incubation with 1:20 dilutions of AGCF-P supernatants collected over 5 days. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM (*n=3*).

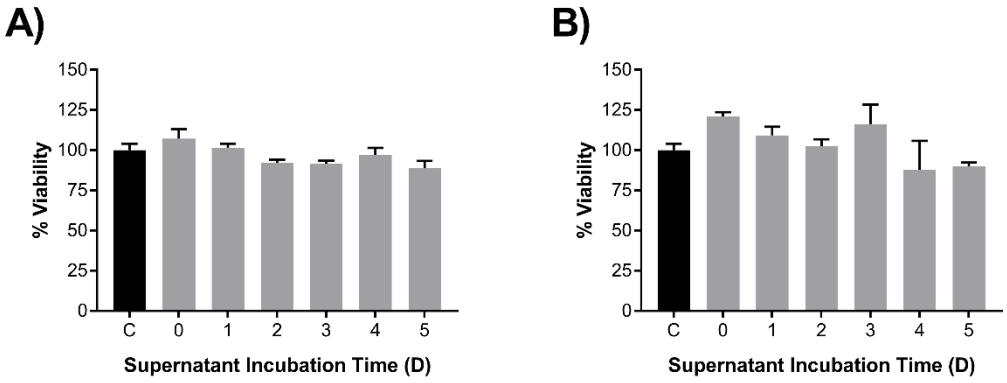

Figure 2.3 Cell viability of HK-2 cells after 24 h (A) or 48 h (B) of incubation with 1:20 dilutions of AGCF-P supernatants collected over 5 days. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and the error bars represent SEM (*n=3*).

2.4.2 Cultivation of Keystone Pathogens

2.4.2.1 Porphyromonas gingivalis

Growth curves of *P. gingivalis* were conducted in AGCF-P broth over 5 days in triplicate. CFU/mL were recorded of all biologicals and standard error was calculated

and plotted on a graph (Figure 2.4). *P. gingivalis* reached a good level of growth after 1 D, averaging at 10^8 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-1 D, stationary phase from 1-4 D and death phase from 4-5 D (Figure 2.4).

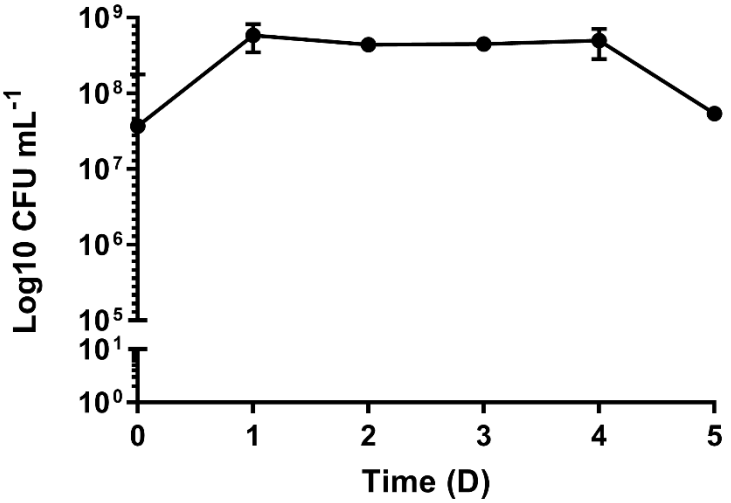

Figure 2.4 Colony forming units per mL of *Porphyromonas gingivalis* cultured in AGCF-P and anaerobic conditions for 5 D (*n=3*).

2.4.3 Cultivation of Accessory Organisms

2.4.3.1 Actinomyces israelii

Growth curves of *A. israelii* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.5). *A. israelii* reached a good level of growth after 1 D, averaging at 10^7 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-8 h, stationary phase from 8-48 h with death phase beginning upon 48 h (Figure 2.5).

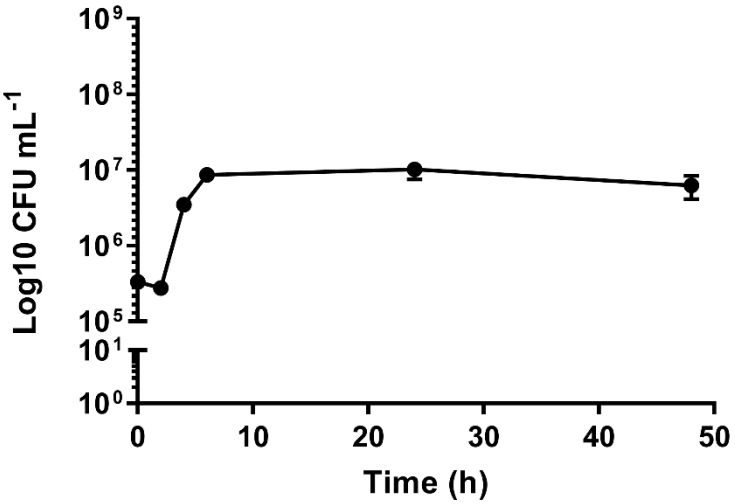

Figure 2.5 Colony forming units per mL of *Actinomyces israelii* cultured in AGCF-P and anaerobic conditions for 48 h (*n=3*).

2.4.3.2 Fusobacterium nucleatum

Growth curves of *F. nucleatum* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.6). *A. israelii* reached a good level of growth after 1 D, averaging at 10^7 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-8 h, stationary phase from 8-48 h with death phase beginning upon 48 h (Figure 2.6).

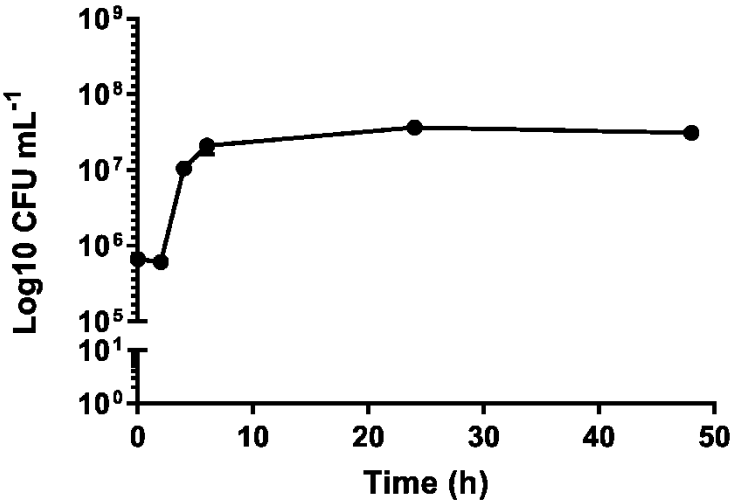

Figure 2.6 Colony forming units per mL of *Fusobacterium nucleatum* cultured in AGCF-P and anaerobic conditions for 48 h (*n=3*).

2.4.3.3 Parvimonas micra

Growth curves of *P. micra* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.7). *P. micra* reached a good level of growth after 1 D, averaging at 10^8 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-20 hours, stationary phase from 20-40 h with death phase beginning upon 40 h (Figure 2.7).

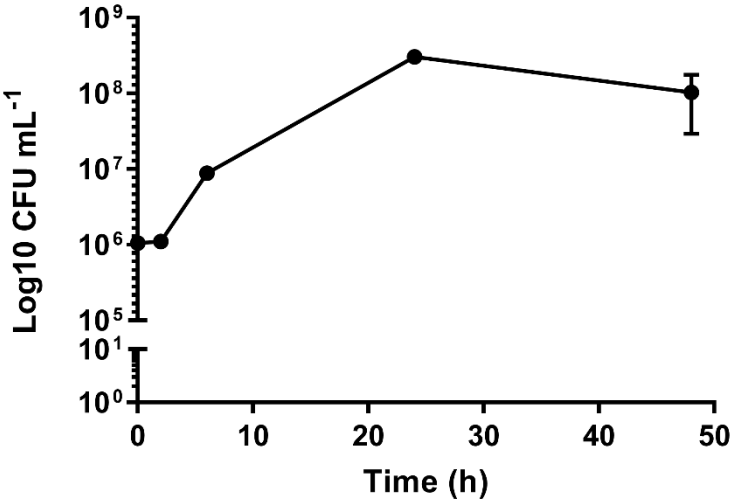

Figure 2.7 Colony forming units per mL of *Parvimonas micra* cultured in AGCF-P and anaerobic conditions for 48 h (*n=3*).

2.4.3.4 Streptococcus constellatus

Growth curves of *S. constellatus* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.8). *S. constellatus* reached a good level of growth after 1 D, averaging at 10^7 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-20 h, stationary phase from 20-40 h with death phase beginning upon 40 h (Figure 2.8).

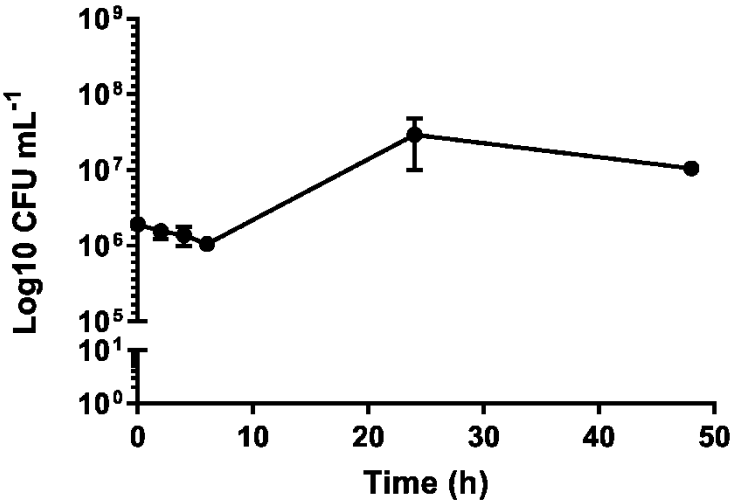

Figure 2.8 Colony forming units per mL of *Streptococcus constellatus* cultured in AGCF-P and carbon rich conditions (5% CO_2) for 48 h (*n=3*).

2.4.3.5 Streptococcus sanguinis

Growth curves of *S. sanguinis* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.9). *S. sanguinis* reached a good level of growth after 1 D, averaging at 10^8 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-25 h, stationary phase from 25-48 h with death phase beginning upon 48 h (Figure 2.9).

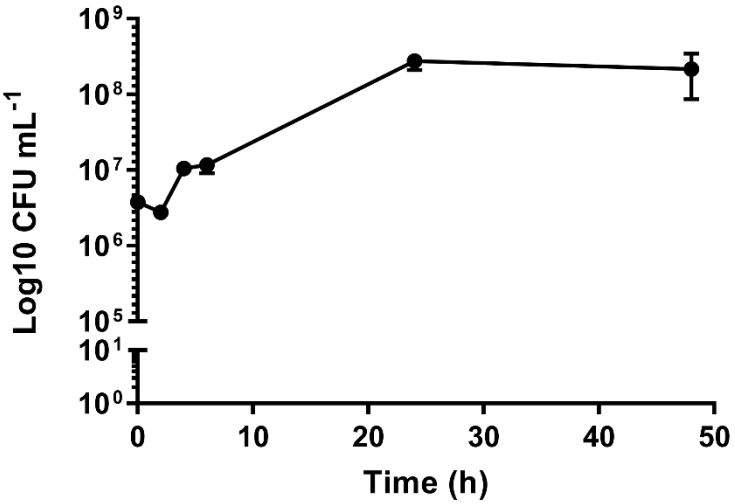

Figure 2.9 Colony forming units per mL of *Streptococcus sanguinis* cultured in AGCF-P and carbon rich conditions (5% CO_2) for 48 h (*n=3*).

2.4.4 Other Organisms Related to Oral Disease

2.4.4.1 Enterococcus faecalis

Growth curves of *E. faecalis* were conducted in AGCF-P broth over 48 h in triplicate, with CFU/mL recorded (Figure 2.10). *E. faecalis* reached a good level of growth after 1 D, averaging at 10^8 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-8 h, stationary phase from 8-48 h with death phase beginning upon 48 hours (Figure 2.10).

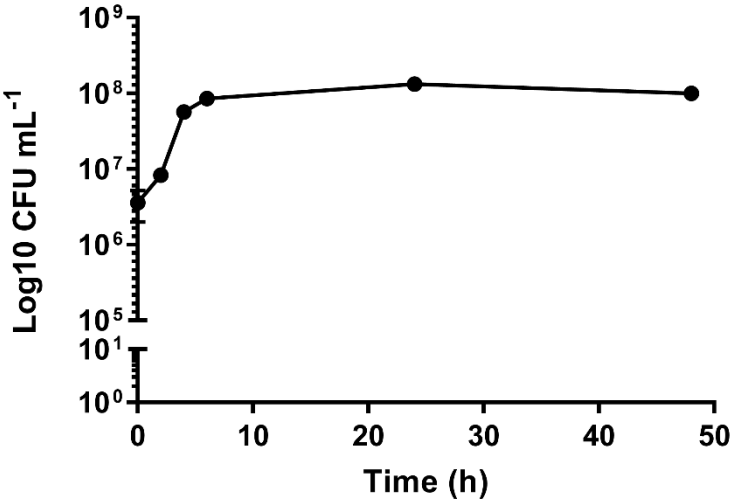

Figure 2.10 Colony forming units per mL of *E. faecalis* cultured in AGCF-P carbon rich conditions (5% CO_2) for 48 h (*n=3*).

2.4.4.2 Streptococcus mutans

Growth curves of *S. mutans* were conducted in AGCF-P broth over 48 h in triplicate with CFU/mL recorded (Figure 2.11). *S. mutans* reached a good level of growth after 1 D, averaging at 10^7 CFU/mL indicating that the developed AGCF-P media was sufficiently nutritious to support the growth. The exponential phase of bacterial growth approximately takes place from 0-20 h, stationary phase from 20-30 h with death phase beginning upon 48 h (Figure 2.11).

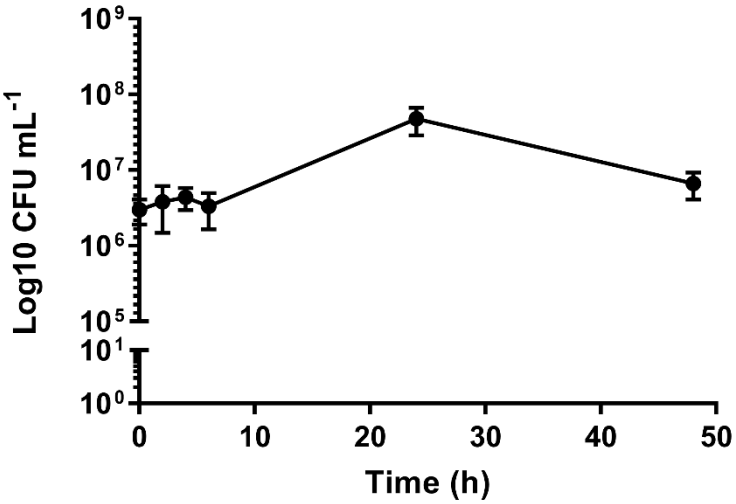

Figure 2.11 Colony forming units per mL of *Streptococcus mutans* cultured in AGCF-P and carbon rich conditions (5% CO_2) for 48 h (*n=3*).

2.5 DISCUSSION

2.5.1 The Development of a Representative Growth Medium

BMM was chosen as base medium for modification as it has been successfully used to emulate saliva and grow dental plaque bacteria in an 'artificial mouth' system. Furthermore, it is useful as it has been reported GCF has a similar composition to saliva while having similar organic and inorganic components to those found in blood plasma (Table 2.1) (Ferguson, 2006).

2.5.1.1 Reasoning Behind Modifications

The pH of the growth medium was modified as the pH of GCF is often stated to be between 7.5 and up to 8.0, it is also has been shown that the pH of GCF increases with severity of GCF. One thing to consider is many readings of pH take place in absence of physiological CO_2 (pCO_2). However, when the PCO_2 of 4.7 kPa (35 mmHg) and physiological temperature (37^oC) was taken into account, the measured pH was 7.96 ± 0.10 (Wong and Sissions, 2001). Furthermore, at pCO_2 the total buffering power becomes high above pH 8.0 because of the high bicarbonate concentration, therefore the pH of GCF cannot surpass 8.0 regardless of the severity of inflammation. In order to attain the desired pH of 7.9 ± 0.1 for AGCF-P the phosphate buffer system was utilised which uses potassium phosphate monobasic anhydrous (KH_2PO_4) and sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$). In order to achieve the desired pH of 7.9 ± 0.1, even after the addition of blood it was calculated that the addition 1.68 g/L potassium phosphate monobasic anhydrous and 49.89 g/L sodium phosphate dibasic heptahydrate was required.

Urea composition was modified as it is several times higher in GCF compared to saliva (Table 2.4). The actual urea concentration is reported to be similar to the amount in serum at between 1-10 mM and it is also reported that urea levels decrease with severe gingivitis (Golub et al., 1971). This may be related to the pH as it has been suggested that the rise in pH seen in periodontitis is related to urea being hydrolysed by periodontal pathogens to ammonia potentially explaining the inverse relationship between urea concentrations and pH of the GCF in severe periodontitis. Urea concentration in the saliva or controls was reported at around 18 mg/dL ± 2 (Peterson

et al., 1985) while that is the saliva concentration we can relate this to GCF as urea levels are similar between the two biofluids. The chosen urea concentration to reflect the amount found CP GCF was 3 mmol/L (0.18 g/L). Cysteine was also added as a reducing agent to further mimic the reducing capacity and support the growth of anaerobic organisms. Indeed, the addition of horse blood rather than horse serum takes into account the red blood cell infiltration that is reported in GCF (Barros et al., 2016).

2.5.1.2 Summary

Currently, the development of media optimally designed for the growth of periodontal organisms has predominately focused on selective growth media for specific organisms such as for *Aggregatibacter actinomycetemcomitans* and their clinical isolation (Rurenga et al., 2012, Tsuzukibashi et al., 2008). Mimicking the chemical components of the physiological environment is a strategy that has been used to cultivate particularly fastidious or uncultivatable organisms and can influence the bacterial products present (Kaeberlein et al., 2002, Vartoukian et al., 2010). Emulating some of the elements of GCF should allow for more representative growth of disease-related organisms. As alternative nutrient sources available may stimulate regulatory changes in gene expression profiles leading to the production of environment specific bacterial products, such as metabolites and secreted proteins (Shimizu, 2014, Balleza et al., 2009).

One of the objectives of this chapter was to develop a general-purpose growth medium that was representative of the gingival crevicular environment and could cultivate a large selection of periodontitis associated organisms. The developed AGCF-P medium was successful at cultivating a selection of periodontitis associated pathogens and should further facilitate research into their role in the pathogenesis of CP as it produces more biologically relevant conditions. Furthermore, cell viability testing of incubated AGCF-P media against human cells also indicated that the developed media had no adverse effects on either cell type after 24 h or 48 h of exposure. Indeed, the mild positive effect of AGCF-P on the viability of primary gingival fibroblasts can be attributed to an increased cell proliferation due to nutrients provided by AGCF-P. The lack of negative effects of AGCF-P medium on

human cell viability can further facilitate the development of *in vitro* and *ex vivo* models of CP.

2.5.2 The Growth of the Chosen Microorganisms

The microorganisms selected to investigate were representative of the two groups of microorganisms reported to be involved in CP, the keystone pathogens and accessory organisms, along with two non-associated organisms as controls. All of these microorganisms grew successfully in the developed growth medium AGCF-P, indicating its usefulness as a growth media for investigating CP.

The starting CFU/mL of all microorganisms was not necessarily consistent; this is because the AGCF-P medium could not be used to adjust absorbance to the desired OD due to opacity so PBS had to be used. This may also be the reason behind a wider difference in starting CFU/mL between all microorganisms especially in the different growth conditions of anaerobic and carbon rich. All the microorganisms reached exponential and stationary stages of growth with the majority reaching death phase. This is of particular importance as microbial products can change dependent on stage of growth and these microbial products are the subject of this study. From each microorganism, bacterial supernatants were successfully collected and stored for further experiments (Chapters 3, 4 and 5).

2.6 CONCLUSIONS

The developed AGCF-P was a robust medium for cultivating a selection of periodontitis associated pathogens that will further facilitate research into their role in the pathogenesis of CP by producing more biologically relevant conditions. This singular medium can allow for more comprehensive and robust comparisons between organisms in order to further identify key microbial products produced and effects on the human host. The lack of negative effects of AGCF-P medium on human cell viability can further facilitate the development of *in vitro* and *ex vivo* models of CP infection.

The cultivation of a selection of microorganisms implicated in CP along with control organisms and the collection of bacterial supernatants can facilitate further investigations into the microbial products produced. The collected supernatants can be investigated for microbial products that may play a role in pathogenesis of CP and play a role in interacting with other systemic diseases such as CKD. Initially, it would be of interest to utilise a method such as untargeted metabolomics to mass-screen these supernatants for any microbial products of interest; this forms the content of the next chapter (Chapter 3).

CHAPTER 3: METABOLOMICS BASED INVESTIGATIONS

3.1 Introduction

3.1.1 Metabolomics

The study of metabolites, metabolomics, is an emerging 'omics' field which focuses on the study of the chemical pathways and products which are present within an organism. Unlike genes and proteins which are subject to various post translational or epigenetic changes, metabolites are the direct biochemical signature of the reactions and biochemical pathways being undertaken and are a therefore a more realistic comparison to the phenotype (Patti et al., 2012). Metabolomics is increasingly being used to study plant (Allwood et al., 2008), microbial (Bradley et al., 2009) and mammalian (Kenny et al., 2010) systems in order to elucidate which metabolites are of biological importance in relation to genes, environmental interactions or diseased states. With advances in mass spectroscopy it is now possible to use untargeted metabolomics to rapidly measure thousands of metabolites from small samples and interpret them, while overlaying genetic and proteomic data to provide a greater overview of interactions (Pirhaji et al., 2016).

Bacterial metabolites can be considered the language of the microbiota as they change directly based on host-organism interactions, organism-organism interactions, diet and disease state. Microorganisms can also be directly responsible for changes seen in the diseased microenvironment; with the metabolism of urea to ammonium ions thought to be responsible for the rise in pH seen in the gingival crevice in CP (Ferguson, 2006). Microorganisms are known to generate primary and secondary metabolites and some of these have been shown to interact with the human body through immune regulation (Brestoff and Artis, 2013), protection against pathogens (Clarke et al., 2010) and metabolism of synthetic substances (Kuhn et al., 2014). With CP providing a point of entry to systemic circulation through its blood supply and bacteraemia, it is plausible that oral microorganisms and their metabolites can freely circulate around the body, with the potential of deleterious effects on the mammalian host especially in those organs such as the liver and kidneys that are responsible for filtration and detoxification.

3.1.2 Evidence for the Role of Metabolites in CKD

While the field of metabolomics is a relatively new phenomenon, identification of individual metabolites that are dysregulated within diseases has been an area of interest for a long period of time in CKD, particularly for clinical diagnoses. The most well-known example of this is urea which is a nitrogenous degradation product of protein in mammals and was identified as dysregulated in CKD in the 1850s and useful for clinical diagnosis of the disease (Thudichum, 1857). In addition, other endogenous filtration factors representative of glomerular filtration rate such as Creatinine and Cytostatin C have thereafter been identified and successfully employed for the clinical diagnosis of CKD in gold standard blood tests for kidney function (Sandilands et al., 2013). While these metabolites have been identified as useful for the diagnosis of kidney disease, it is predominately due to the increased presence of these metabolites being indicative of the kidney not functioning properly rather than being involved in the pathogenesis of the disease. However, more recent investigations into identification of 'dietary metabolites' which are indicative of CKD and the progression of the disease has highlighted advanced glycated end products and indoxyl sulphate as being closely linked to progression as well as being uremic toxins that can directly affect the disease (Hasegawa et al., 2017).

Advances in technology have allowed a host of other metabolites to be identified from various methods such as Genome Wide Association Studies (GWAS) and Liquid Chromatography Mass Spectroscopy (LCMS) analysis of urine and serum metabolites and their association with kidney decline. A GWAS investigating the excretion of serum and urine metabolites in relation to CKD highlighted genetic influences on metabolite concentrations that can effect CKD. Some of the main genetic influences detected were in polyamines such as putrescine, medium-chain acylcarnitines and various amino acid ratios such as lysine/glutamine ratio that may be associated with CKD (Li et al., 2018). The role of polyamines such as putrescine, spermidine and spermine along with acrolein which is the by-product of polyamine degradation, has previously been highlighted in CKD patients with acrolein levels found to be fivefold higher compared to controls in serum and may act as a uremic toxin (Igarashi et al., 2006). Indeed, LCMS based investigations further highlighted the polyamine spermidine along with two other metabolite ratios, the phosphatidylcholine diacyl

C42:5-to-phosphatidylcholine acyl-alkyl C36:0 ratio and the kynurenine-to-tryptophan ratio to higher incidences of CKD (Goek et al., 2013).

3.1.3 Evidence for the Role of Metabolites in CP

In CP, investigations into the role various metabolites may play in progression or for the clinical diagnosis of the disease is much less studied. This is possibly due to the success of oral periodontal pocket examination for accurate staging of the progression of disease. However, the diversity of the microbiota and the different organisms existent in disease states such as CP, present a unique opportunity for metabolic profiling to take place. Preliminary metabolomic analysis of saliva samples has shown that healthy patients can be separated from those who have various forms of periodontitis by analysing the metabolites present, with CP having a distinct metabolic signature (Aimetti et al., 2012). Indeed, metabolomic analysis of saliva samples before and after removal of supragingival plaque has highlighted two metabolites, cadavarine and hydrocinnamate, which are associated with periodontal inflammation (Sakanaka et al., 2017). Other pathways such as polyamine metabolism, arginine and proline metabolism, butyric acid metabolism, and lysine degradation have also been associated with periodontal inflammation (Sakanaka et al., 2017, Shah and Swiatlo, 2008). Some progress has been made into the development of prediction models for CP based on metabolite concentrations, with the combination of cadavarine, 5-oxoproline, and histidine biomarkers being shown to be the most accurate predictor of periodontitis out of the metabolites identified so far (Kuboniwa et al., 2016).

3.1.4 Metabolites Reported to be Dysregulated between Both Groups

Despite various metabolites having been reported to be dysregulated in both CP and CKD, the only common metabolites/metabolite class identified is polyamines and their degradation by-products. Polyamines (PA) such as Spermine, Putrescine and Cadavarine are a fundamental class of small polycations that are relatively genetically conserved within nature and are reported to have a wide range of roles in mammalian cells such as transcription, stress response, translation, growth and apoptosis (Pegg, 2009). Indeed, in microorganisms their role is not fully elucidated either with suggestions of being involved in stress response along with stabilisation

of DNA and RNA and gene deletion studies predominately highlighting cell proliferation and survival (Gevrekci, 2017). PAs are predominantly produced from the amino acids ornithine and methionine (Kuksa et al., 2000), which corresponds with the theme of amino acid metabolic by-products which have been identified as potentially dysregulated metabolites in CP and CKD. There has been some toxicity studies on PAs with high levels of spermine being reported to be potentially nephrotoxic and most likely capable of renal damage (Tabor and Rosenthal, 1956, Pegg, 2009). Indeed, dysregulation of PAs and associated metabolites has been reported to potentially lead to conditions such as cancer, diabetes, renal failure, stroke and inflammation (Pegg, 2013, Park and Igarashi, 2013)

However, despite being essential across all kingdoms of life and tightly regulated there is no unifying biochemical role of PAs identified as of yet (Miller-Fleming et al., 2015). With a large number of essential cellular processes being linked to PAs it is understandable that the wide number of associated diseases has led to PAs being heavily identified as dysregulated in many metabolomics studies (Miller-Fleming et al., 2015). The relative non-specificity of PAs and associated metabolites along with the ubiquitous nature of the molecules would be a large hindrance for associations with diseases, especially as multiple sources such as number of specific microorganisms, inflammation, other diseases and genetics may affect the concentration of PAs.

3.1.5 Approach to Investigating Potential Metabolites Dysregulated in CP and CKD

With little research having been carried out on this topic area and only one metabolite class of interest, it would not be beneficial in conducting a targeted metabolic investigation. Instead, by utilising an untargeted screening approach it should allow the identification of a wide selection of molecules/classes of molecules that change with the addition of microorganisms of interest. With an untargeted approach the only metabolite group linked as dysregulated in both diseases, PAs, can be looked for alongside a wide selection of other known molecules as the PA mass spectrum has already been well reported (Magnes et al., 2014).

We hypothesize that development of an LCMS based untargeted metabolomics pipeline can facilitate rapid screening of microorganisms associated with CP and potentially identify metabolites of interest that may elicit an effect on renal cells.

In order to trial an untargeted metabolomics approach to identification of dysregulated metabolites one microorganism, *Streptococcus constellatus*, was chosen to be provisionally investigated due to its role as a normal member of the microbiota associated with periodontitis (Colombo et al., 1998) and systemic diseases such as bacteraemia and abscesses (Balleza et al., 2009, da Silva et al., 2004). *S. constellatus* was cultured over 48 hours in two separate growth media, brain heart infusion broth (BHIB) and tryptone soya broth (TSB) to investigate discernible microbial products following growth in different broths. The newly developed medium AGCF-P (Chapter 2) was not used for this provisional test as it was being developed concurrently to this metabolomics pipeline. The *S. constellatus* samples cultured in BHIB and TSB were analysed through an untargeted metabolomics workflow developed in house and the results analysed to evaluate the suitability of this workflow for this study.

3.2 AIM AND OBJECTIVES

3.2.1 Aim

To develop a microbial metabolomics workflow in house utilising LCMS, that is capable of analysing the supernatant of microbial samples for dysregulation of metabolites.

3.2.2 Objectives

- Develop a workflow for the metabolomic analysis of bacterial products.
- Test the developed workflow with one oral microorganism.
- Evaluate the developed workflow and its suitability for identification of dysregulated metabolites.
- Evaluate the workflow for the potential to mass screen a selection of periodontitis associated microorganisms.

3.3 METHODS

3.3.1 Bacterial Culture and Statistical Analysis

The *S. constellatus* type strain NCTC 11325 was grown on Brain Heart Infusion Agar (Oxoid, UK) and Tryptone Soya Agar (Oxoid, UK) in a 5% CO_2 incubator for 48 h. A single colony was used to inoculate 5 mL of BHIB (Oxoid, UK) and 5 mL of TSB (Oxoid, UK) for static overnight cultures. Three biological replicates of each organism were grown in 250 mL culture flasks for 48 h for each growth media with a starting bacterial absorbance of 1.0 ± 0.1 at an optical density (OD) of 540_{nm} and control broths were also prepared and sampled alongside. OD and CFU/mL monitored growth over a 48 h period at 0 h, 2 h, 4 h, 6 h, 24 h and 48 h hours. A t-test with Welch's correction was applied to the OD and CFU/mL results using Prism GraphPad 8.1.1 software to determine any significant difference between samples. Cell supernatants were taken at 0 h, 6 h, 24 h and 48 h to be prepared for LCMS analysis.

3.3.2 Preparation of Microbial and Control Supernatants for LCMS Analysis

Cold phase methanol extraction was used to prepare microbial and growth media supernatants for LCMS analysis. At chosen time points, 5 mL samples were taken in triplicate from bacterial cultures and untreated media and centrifuged at 3018 *x g* for 10 min. The resultant supernatant was transferred into a sterile universal and the pellet discarded. From each sample, 1000 µL was transferred into a 1.5 mL microcentrifuge tube and 300 µL of cold methanol with 0.1 M formic acid (Sigma Aldrich, UK) was added, followed by centrifugation at 13400 *x g* for 20 min. From each sample 400 µL was transferred into a sterile Bijoux and diluted with 800 µL of LCMS grade water, then 200 µL was taken and diluted with 800 µL of methanol (Sigma Aldrich, UK). The resultant solution was filtered using a 0.2 µM Acrodisk filter (Scientific Lab Supplies, UK) into 2 mL LCMS vials (Agilent, USA) and labelled. The calculated dilution factor was 0.256.

3.3.3 Preparation of Wash Samples and Blanks

Wash samples were created to clean out the column after quantification of analytical standards to enable better sensitivity; 1 mL of methanol with 0.1 M formic acid was filtered through a 0.2 µM Acrodisk filter into 2 mL LCMS vials. Blanks were created to

account for any residual contaminants in the LCMS column; 1 mL of HPLC grade water (Sigma Aldrich, UK) was filtered through 0.20 µM filter into 2 mL LCMS vials. All samples were frozen at -80^{o}C until ready for analysis.

3.3.4 Instrument Parameters

LCMS analysis was performed using an Agilent 6540 UHD Q-TOF LCMS system (Agilent, USA) equipped with an ACE UltraCore 2.5 µm SuperC18™ 100 mm x 2.1 mm column (Advanced Chromatography Technologies, Scotland). The analysis was conducted in positive ion mode at 45°C with a MS range of 100-3000 and calibrated using four reference masses. The mobile phase used was LCMS grade acetonitrile with 0.1% formic acid (Sigma Aldrich, UK) and LCMS grade H_2O with 0.1% formic acid (Sigma Aldrich, UK) was the polar solvent. A flow rate of 0.3 mL/min was used with each sample run for 30 min and 10 µL injections taken from each sample.

3.3.5 Data Processing

3.3.5.1 Conversion of Files

All .D output files from the LCMS were converted to the open source .mzML format to facilitate further analysis using the open source converter ProteoWizard MSConvert (Chambers et al., 2012). The output format selected was .mzML and the peak-picking filter was chosen as this uses centroidisation, which facilitates the use of the centWave for feature detection.

3.3.5.2 Deconvolution of Data

Deconvolution of the data was completed through the open source LCMS data processor XCMS online (Gowda et al., 2014). A single job was conducted of all samples using a customised parameter created based on HPLC/UHD Q-TOF template, which is the closest to our LCMS analyser Agilent LCMS 6540 UHD Q-TOF and a feature detection limit of <50% and relative standard deviation of <20 was chosen. This customised parameter was saved as 'MMU LCMS Parameter' and used for analysis of both datasets. The results folder was then downloaded and the annotated excel results file was used for further analysis.

3.3.5.3 Quality Assurance

In order to reduce the number of false positives and negatives, quality assurance of raw data files was undertaken prior to further analysis. In the excel spreadsheet the average intensity for each peak of all the samples was calculated along with the average intensity of the blanks. The percentage contribution of the blanks to the sample intensity of each peak was calculated using this formula:

$$\%\ Blank\ Contribution = \left(\frac{Average\ intensity\ of\ blanks}{Average\ intensity\ of\ samples}\right) \times 100$$

The results were then filtered with peaks removed with a percentage blank contribution of >5%.

3.3.5.4 Analysis of Data

Once the data had undergone quality assurance, the data matrix was changed to facilitate analysis using the open source LCMS analysis and interpretation software MetaboAnalyst (Xia and Wishart, 2016). When processing the MS peak list into MetaboAnalyst a mass tolerance of 0.025 (m/z) and a retention time tolerance of 30.0 was employed.

3.3.5.5 Missing Values

Missing value correction using MetaboAnalyst was undertaken in order to improve the quality of the data. Features with >50% of missing values were removed and those with less that that were replaced using the K-nearest neighbour methodology.

3.3.5.6 Normalisation and Scaling

Data normalisation, transformation and scaling was undertaken using MetaboAnalyst. Samples were normalised by quantile normalisation and Pareto scaling was employed which is mean-centered and divided by the square root of standard deviation of each variable.

3.3.5.7 Univariate and Multivariate Analysis

Using MetaboAnalyst, a univariate volcano plot was plotted of all bacterial samples against the blank media samples with a fold change threshold of 2.0 and a *p-value* of 0.05 used as significance cut-offs. The volcano plot image was saved and the data table was exported as a .csv file. A multivariate principle component analysis was plotted to ascertain whether the variance within the data could be used to sort the

data into the bacterial and control classes without referring to them, the 2D scores plot was saved as a .png file. Next, multivariate partial least squares - discriminant analysis was conducted to work out which variables have the class separating information. The 2D scores plot was saved as a .png file and the Variable Importance in Projection (VIP) score graph which shows the top 15 features showing a larger contribution to class discrimination, was plotted and saved as a .png file.

3.3.5.8 Supervised Data Analysis

Using MetaboAnalyst a supervised learning algorithm Random Forest (RF) was used to analyse the dataset, which uses random feature selection and classification trees to predict class based on majority vote. RF can identify features which are highly associated with a class and when removed decrease the classification accuracy of the model. The top 15 features which are associated with classification accuracy were plotted in graph and exported as a .png file.

3.3.5.9 Metabolite Identification

All significant features identified from univariate, multivariate and supervised data analysis were collated into a single excel spreadsheet and prepared for metabolite identification using MetaboSearch (Zhou et al., 2012). The five database plugins used for comparison were the Human Metabolome Database (Wishart et al., 2012), the Metlin mass spectral database (Smith et al., 2005), the Madison metabolomics consortium database (MMCD) (Cui et al., 2008), LIPID MAPS database (Fahy et al., 2009) and MetaCyc database (Caspi et al., 2013). The metabolite search was run in positive ion mode with a molecular weight tolerance of five parts per million and exported to Excel when completed. The results were then manually curated, removing matches to synthetic drugs and filling in the putative role of the metabolite match, the results were then formulated into a table.

3.4 RESULTS

3.4.1 Growth Curves

Concurrent growth curves were undertaken of *S. constellatus* in both BHIB and TSB to see if there were any discernible difference in metabolic products and to test the suitability of the designed untargeted metabolomics pathway. The growth, measured as absorbance at 540 nm (Figure 3.1), of *S. constellatus* over 48h for both BHIB and TSB indicated log phase between 6 and 24 h and stationary phase between 24 and 48 h, with slightly slower initiation of log phase in TSB. However, the difference between the absorbance at 540 nm over 48 hours of BHIB and TSB was found to be non-significant (p=0.8377). The growth, measured as CFU/mL (Figure 3.2), of *S. constellatus* over 48 h for both BHIB and TSB indicated log phase between 6 and 24 h, stationary from 24h and the potential beginning of death phase at 48h for both media. TSB appeared to initiate log phase slower than BHIB. However, the difference between the CFU/mL over 48 hours of BHIB and TSB was found to be non-significant (p=0.6341).

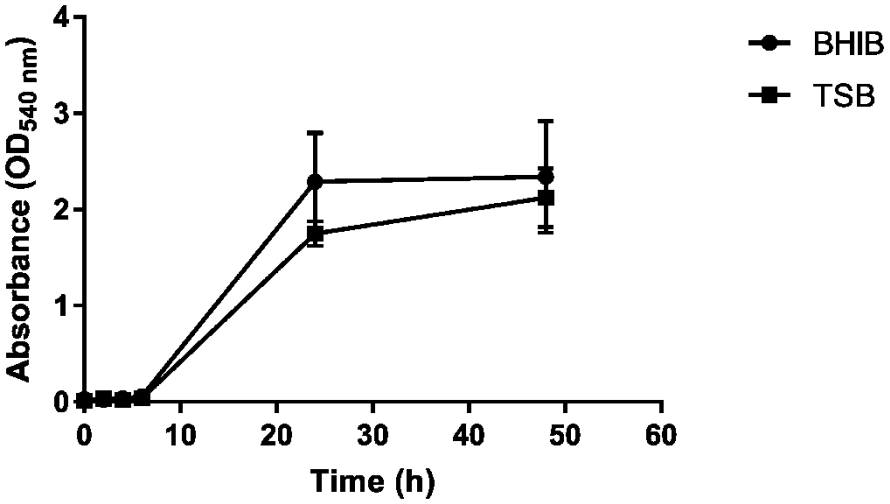

Figure 3.1 Absorbance at 540_{nm} of *Streptococcus constellatus* cultivated in Brain Heart Infusion Broth (BHIB) and Tryptone Soya Broth (TSB) over 48 h and in carbon rich conditions (5% CO_2). Error bars presented as standard error (n=3).

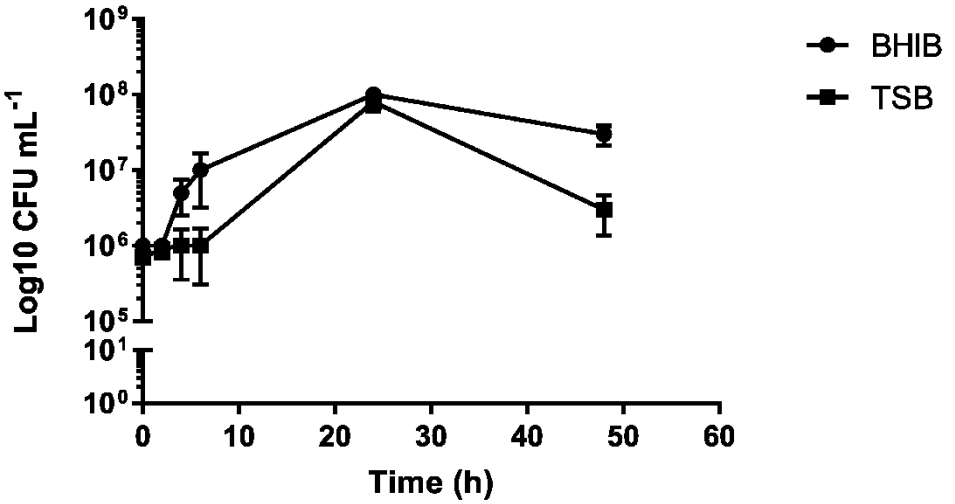

Figure 3.2 Colony forming units per mL of culture of *Streptococcus constellatus* cultivated in Brain Heart Infusion Broth (BHIB) and Tryptone Soya Broth (TSB) over 48 h and in carbon rich conditions (5% CO_2). Error bars presented as standard error (*n=3*).

3.4.2 LCMS Analysis of *S. constellatus* Cultured in BHIB

From the growth curve of *S. constellatus* in BHIB described in Section 3.2.1, sampling in triplicate was undertaken at 0 h, 6 h, 24 h and 48 h and these samples were prepared for untargeted LCMS analysis. A media control of BHIB kept in the same conditions (5% CO_2 and 37°C) was also sampled in triplicate at 0, 6, 24 and 48 h and prepared for untargeted LCMS analysis alongside the bacterial cultures in order to account for abiotic degradation.

After LCMS analysis and deconvolution using XCMS online, quality assurance of the raw data files reduced the number of total features from all samples from 8003 to 5428. Peak alignment, missing value imputation and data normalisation was then conducted through MetaboAnalyst with (Figure 3.3) showing the normalisation step which allows for better comparison of features.

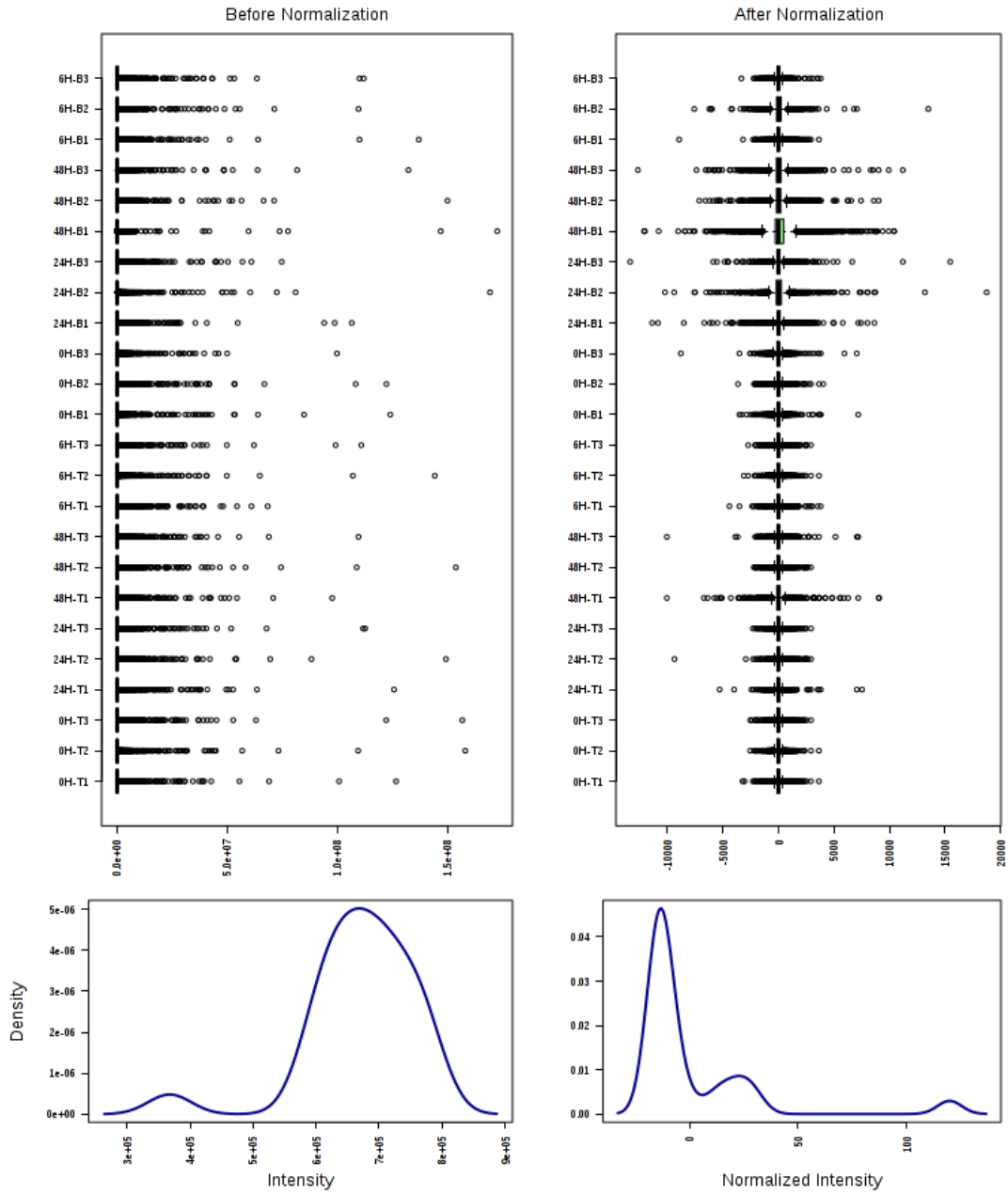

Figure 3.3 Box plots and kernel density plots before and after normalization of LCMS data of *S. constellatus* cultured in BHIB (Indicated as B) and control BHIB samples (Indicated as T) at 0, 6, 24 and 48h. The normalisation using quantile normalization and Pareto scaling. (*n=3*).

3.4.2.1 Univariate Analysis

Univariate analysis was conducted of all features to ascertain which features were significantly upregulated or downregulated (p=<0.05 and fold change of 2 ≥ or -2 ≤) from the BHIB control to *S. constellatus* cultured in BHIB. From the 5428 features initially identified 124 were identified to be significantly changed with 43 upregulated and 81 downregulated (Figure 3.4).

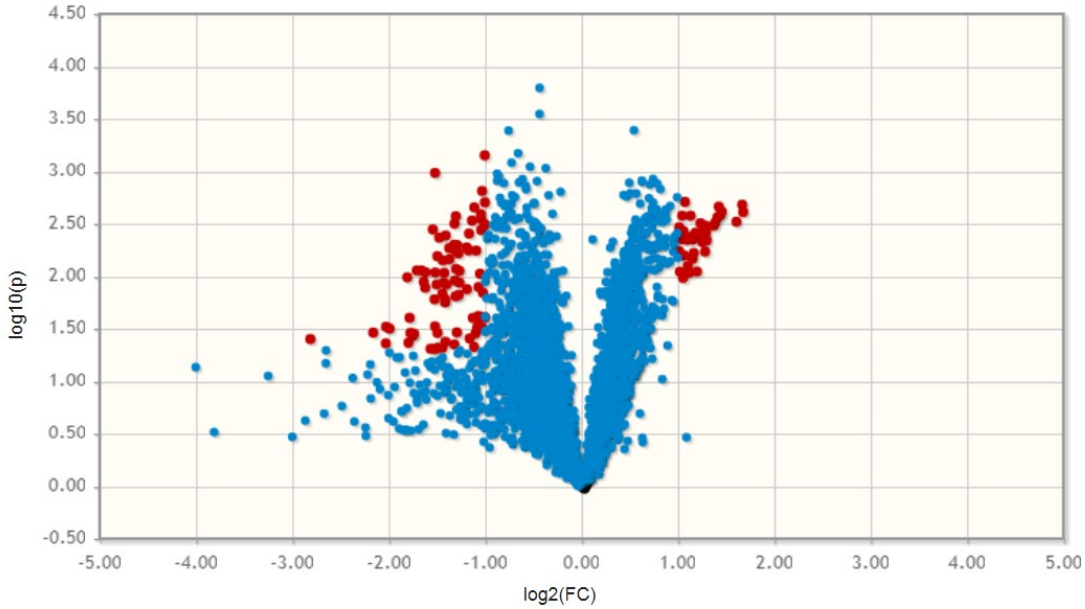

Figure 3.4 Important features from BHIB LCMS analysis selected by univariate volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.05. The red circles represent features above the threshold and all values are log transformed. The further its position away from the (0, 0) the more significant the feature.

3.4.2.2 Principle Component Analysis

Principle component analysis (PCA) is an unsupervised method that attempts to find directions that can explain the variance in data irrespective of class. The scores plot of all BHIB bacterial and control samples (Figure 3.5) shows that the BHIB control is much more tightly clustered (Red) than BHIB inoculated with *S. constellatus* (Green). All 0 h and 6 h samples are tightly clustered together indicating that not much biotic degradation has taken place. The 24 h and 48 h bacterial samples with the exception of 24H-B1 are very different from a large selection of the samples highlighting a larger difference in the components present. Another notable outlier is a control 24H-1 which is clustered with the bacterial samples rather than all the other controls.

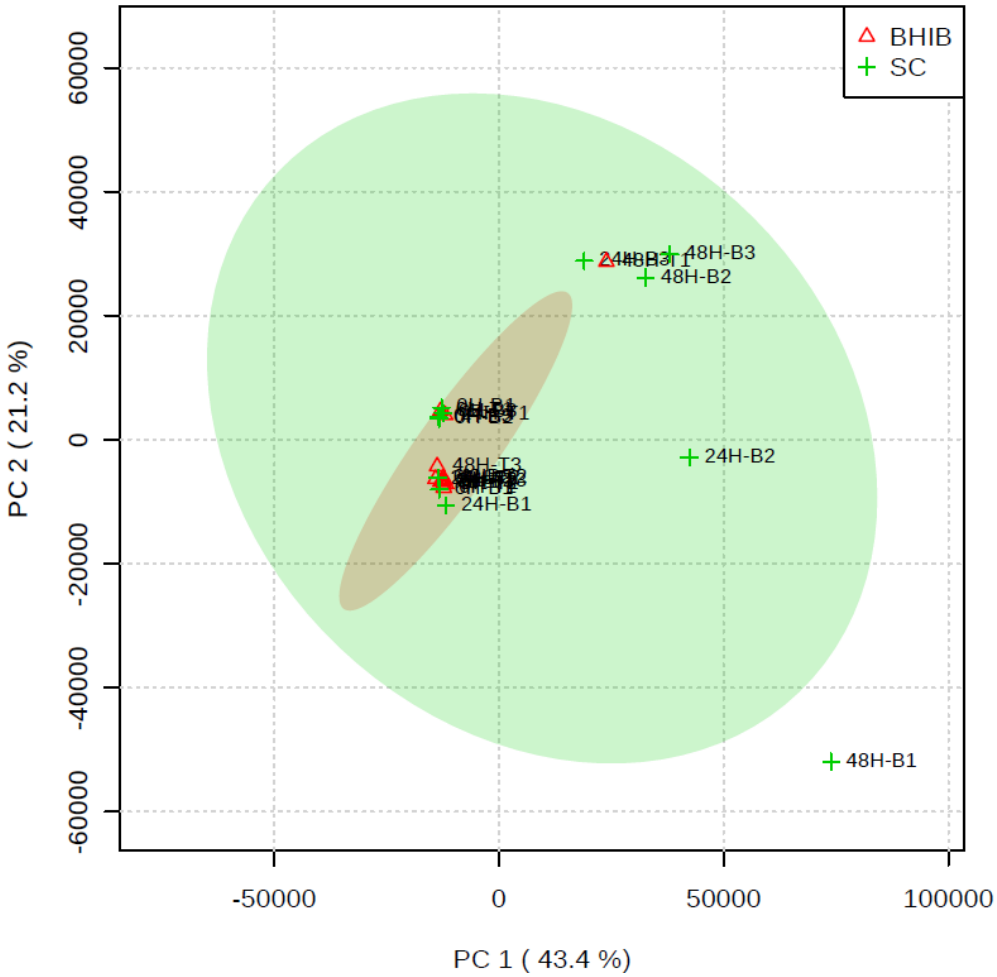

Figure 3.5 A 2D scores plot of a PCA between all BHIB bacterial (SC) and control (BHIB) classes. The explained variances are shown in brackets. B after a class denotes Bacterial and T denotes controls with the number denoting a biological replicate.

3.4.2.3 Partial Least Squares – Discriminant Analysis

Partial least squares – discriminant analysis (PLS-DA) is a supervised method of multivariate regression which is similar to PCA, however it takes into account the classes of samples and attempt to work out which variables have the class separating information. The 2D scores plot of all BHIB bacterial and control samples (Figure 3.6) shows that the BHIB control is much more tightly clustered (Red) than BHIB inoculated with *S. constellatus* (Green), this correlates with the PCA (Figure 3.5). PLS-DA usually has sharper separation between classes than PCA, which is shown with the difference between (Figure 3.5 and Figure 3.6). All 0H and 6H samples from each class are tightly clustered together indicating that not much biotic degradation has taken place, with the exception of 6H-B2. The 48 h bacterial samples have the largest variance between them, which is most likely due to the metabolic by-products. A

notable outlier is a 48-T1 that is distinctly variable from the rest of the BHIB control samples, however the reason for this is unknown. A Variable Importance in Projection (VIP) score graph was plotted (Figure 3.7) which calculates weighted sum of squares of the PLS loadings taking into account the amount of explained Y-variation in each dimension for each component. A higher VIP indicates that the feature in question has a larger contribution to class discrimination in the PLS-DA model. The top 15 VIP features identified were selected for metabolite identification.

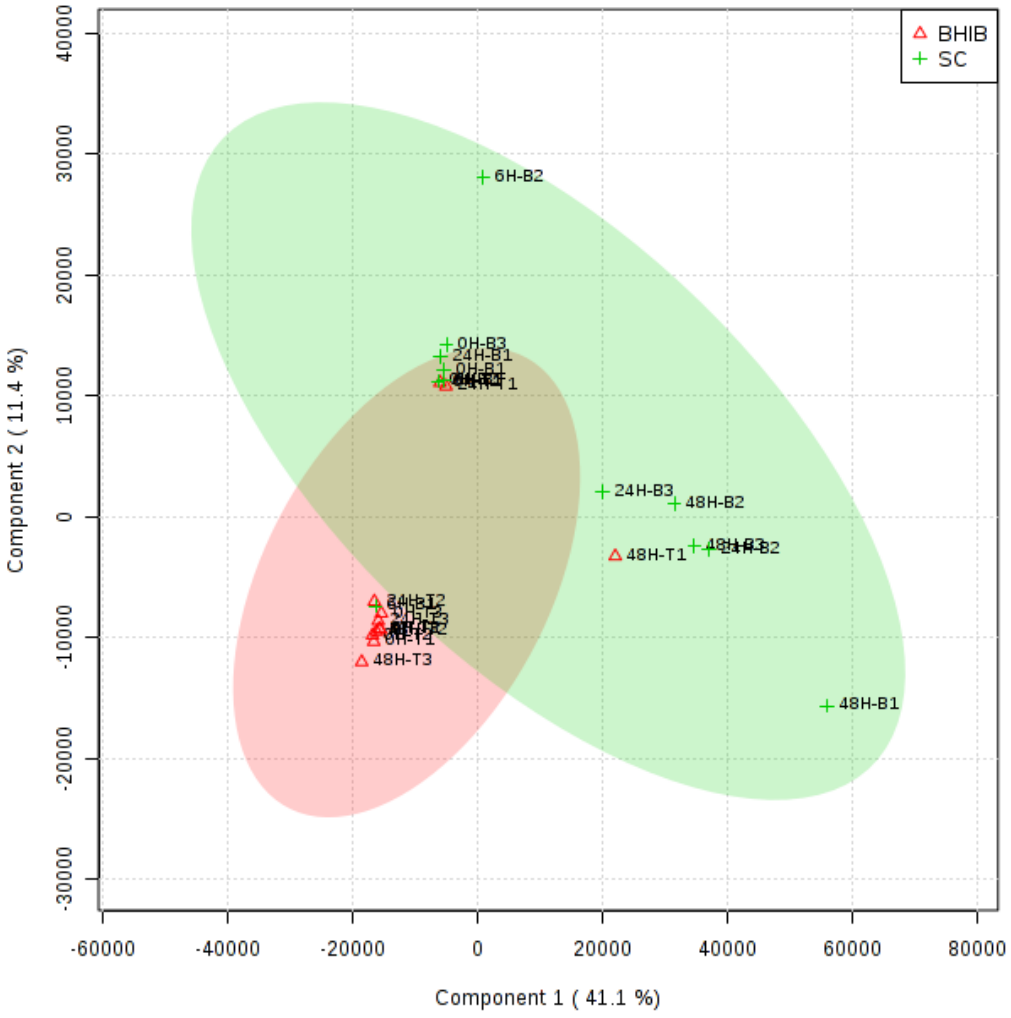

Figure 3.6 A 2D scores plot of a PLS-DA between all BHIB bacterial (SC) and control (BHIB) classes. The explained variances are shown in brackets. B after a class denotes Bacterial and T denotes controls with the number denoting a biological replicate.

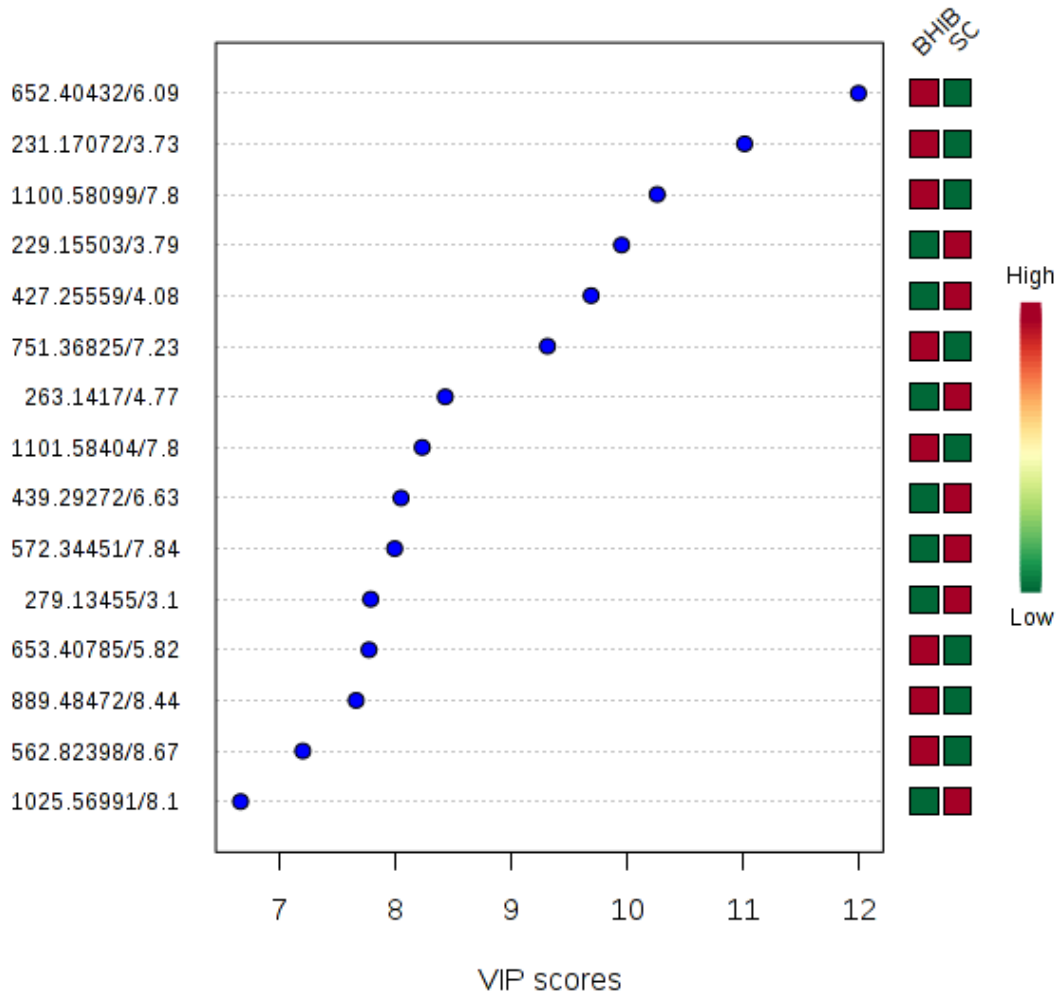

Figure 3.7 Important features identified by a PLS-DA of BHIB bacterial and control samples. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. The x axis denotes VIP scores and y axis the feature m/z and retention time separated by /.

3.4.2.4 Significant feature selection using Random Forest algorithm

RF is a supervised algorithm for data analysis which uses random feature selection and classification trees to predict class based on majority vote. It is useful for feature selection as it can identify important features by measuring the increase error in class identification when a feature is omitted. The BHIB control and bacterial samples were analysed using RF (Figure 3.8) in order to identify features that are important for class segregation. The top 15 significant features identified were selected for metabolite identification.

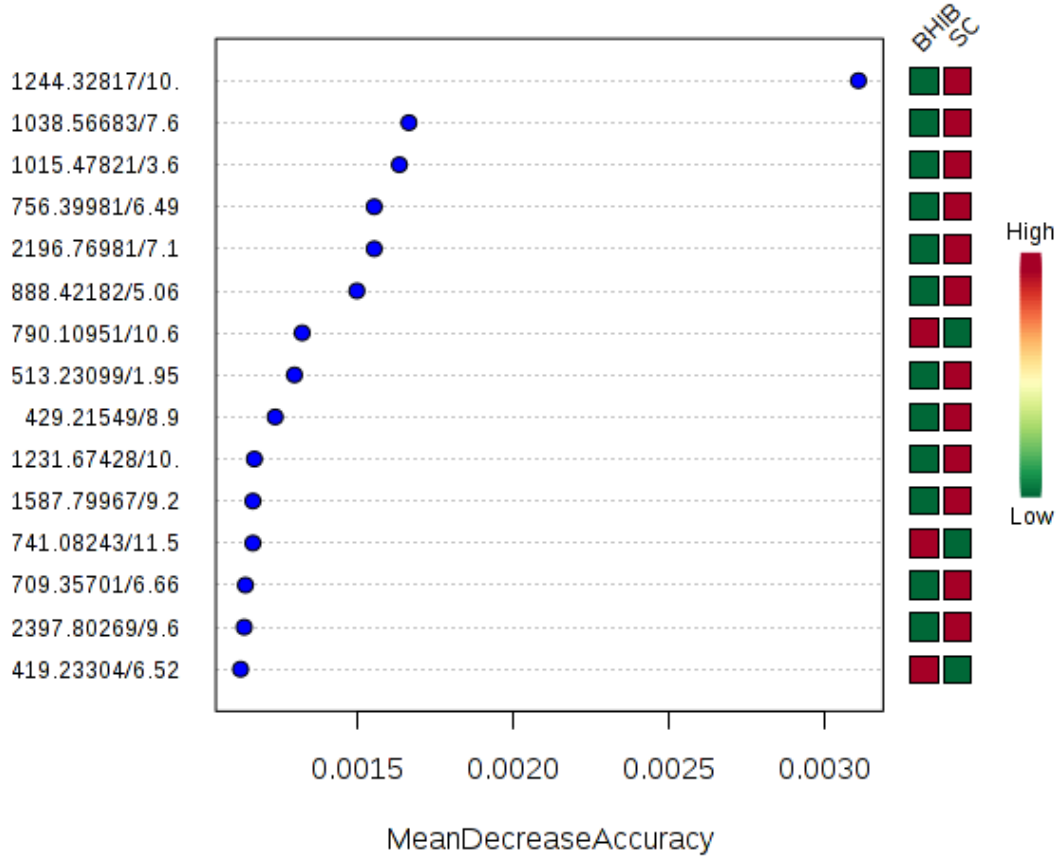

Figure 3.8 Significant features from BHIB (control) and SC inoculated of BHIB identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. The x axis denotes mean decrease in accuracy and y axis the feature m/z and retention time separated by /.

3.4.2.5 Metabolite identification

All significantly upregulated and downregulated features identified by volcano plots, Variable Importance in Projection features identified by PLS-Da and important features in classification identified by RF were extracted into a Microsoft Excel spreadsheet for identification of metabolites. The program MetaboSearch was used to compare the m/z and retention times of the metabolites of interest with a selection of mass spectral databases for putative identification and the results extracted. For all significant features, there was a 7.1% success rate in identification. There are no clear metabolite identification trends associated with the control and the bacterial groups due to the low metabolite identification rate. The only exception

being amino acids and their various degradation products being associated with the BHIB control group most likely due to the enriched nature of the medium.

Table 3.1 Putative metabolite identification of significantly up and downregulated metabolites identified by volcano plots of BHIB bacterial and control samples.

Upregulation								
m/z	RT	FC	*p* value	Name	Formula	Mass of Match	ppm deviation	Putative Role
2256.227	10.58	3.2003	0.002432	-	-	-	-	-
2255.226	10.56	3.1777	0.002067	-	C104H183N5O47	2254.2084	4.332174693	Unknown LipidMaps match
2257.23	10.57	3.0488	0.003	-	C104H185N5O47	2256.224	0.541038062	Unknown LipidMaps match
1128.62	10.58	2.7427	0.002408	-	-	-	-	-
1128.118	10.58	2.7131	0.002601	-	-	-	-	-
2279.211	10.58	2.6852	0.002157	-	-	-	-	-
2278.209	10.57	2.6592	0.002719	-	-	-	-	-
2277.206	10.56	2.6171	0.003046	-	-	-	-	-
1194.642	11.11	2.5857	0.003271	-	-	-	-	-
1129.622	10.58	2.4603	0.004522	-	-	-	-	-
1129.122	10.31	2.4456	0.003755	-	-	-	-	-
2157.169	10.23	2.4334	0.005785	-	-	-	-	-
1130.123	10.58	2.4331	0.003263	-	-	-	-	-
2280.214	10.58	2.4238	0.004028	-	-	-	-	-
1195.646	11.11	2.4156	0.00353	-	-	-	-	-
1078.588	10.23	2.3877	0.004693	-	-	-	-	-
1216.625	11.11	2.385	0.003773	-	-	-	-	-
1217.628	11.11	2.3528	0.003103	-	-	-	-	-
2156.167	10.22	2.2947	0.008951	-	-	-	-	-
752.7498	10.58	2.2739	0.003819	-	-	-	-	-
719.7299	10.23	2.2641	0.004179	-	-	-	-	-
1079.092	9.76	2.2622	0.003944	-	-	-	-	-
2179.152	10.22	2.2438	0.005953	-	-	-	-	-
753.084	10.58	2.2404	0.004108	-	-	-	-	-
598.8284	11.11	2.2238	0.00443	-	-	-	-	-
2178.148	10.23	2.2219	0.006794	-	-	-	-	-
1218.631	11.1	2.1905	0.002629	-	-	-	-	-
1149.618	8.07	2.1708	0.009203	-	-	-	-	-
720.0641	10.23	2.1597	0.004207	-	-	-	-	-
1150.621	8.07	2.1471	0.007951	-	-	-	-	-
752.4154	10.58	2.1347	0.004444	-	-	-	-	-
1196.649	11.11	2.1052	0.001943	-	-	-	-	-
1079.591	10.23	2.0962	0.006308	-	-	-	-	-
1139.108	10.58	2.0919	0.004198	-	-	-	-	-
1140.111	10.58	2.0815	0.00368	-	-	-	-	-
2294.184	10.58	2.077	0.010277	-	-	-	-	-
1139.61	10.58	2.0637	0.004416	-	-	-	-	-
597.8198	9.59	2.0634	0.002626	-	-	-	-	-
1689.93	10.12	2.0302	0.008977	-	-	-	-	-
1130.623	10.58	2.0205	0.003392	-	-	-	-	-
1155.079	10.58	2.0187	0.005722	-	-	-	-	-
1688.925	10.11	2.016	0.006074	-	-	-	-	-

m/z	RT	FC	*p* value	Name	Formula	Mass of Match	ppm deviation	Putative Role
1136.527	7.36	0.49921	0.001961	-	-	-	-	-
204.6169	3.83	0.49875	0.024434	-	-	-	-	-
448.2438	11.98	0.49873	0.003225	-	-	-	-	-
666.8456	6.92	0.49852	0.000699	-	-	-	-	-
622.3146	8.3	0.49721	0.003161	-	-	-	-	-
585.2516	1.7	0.49205	0.014277	-	-	-	-	-
738.8582	4.11	0.49182	0.03096	-	-	-	-	-
1026.307	2.8	0.48892	0.001528	-	-	-	-	-
1010.421	7.72	0.48569	0.003604	-	-	-	-	-
960.9337	7.99	0.4849	0.00254	-	-	-	-	-
1009.919	7.71	0.48425	0.00281	-	-	-	-	-
1122.955	6.84	0.48192	0.009388	-	-	-	-	-
287.1172	1.16	0.47889	0.029239	Methionyl-Histidine	C11H18N4O3S	286.1099612	0.106663811	AA degradation product
263.6354	2.87	0.47707	0.012558	-	-	-	-	-
1027.306	2.78	0.47709	0.023711	-	-	-	-	-
1236.012	9.9	0.47209	0.030586	-	-	-	-	-
858.2423	2.64	0.46912	0.005648	-	-	-	-	-
691.2196	0.96	0.46639	0.034861	-	-	-	-	-
255.1321	2.87	0.4625	0.002188	-	-	-	-	-
599.307	6.95	0.46174	0.046807	-	-	-	-	-
623.3427	9.16	0.45758	0.024729	-	-	-	-	-
254.6301	2.87	0.45481	0.002925	-	-	-	-	-
911.4864	7.07	0.44715	0.038863	-	-	-	-	-
264.1368	2.87	0.44537	0.00389	-	-	-	-	-
2117.807	7.2	0.4437	0.005626	-	-	-	-	-
2019.83	7.52	0.4388	0.013175	-	-	-	-	-
2116.804	7.19	0.43785	0.005281	-	-	-	-	-
2018.828	7.52	0.41565	0.01141	-	-	-	-	-
1060.411	7.19	0.41546	0.008823	-	-	-	-	-
526.2414	5.16	0.41366	0.014891	-	-	-	-	-
1058.907	7.18	0.41237	0.006014	-	-	-	-	-
489.2571	5.56	0.40846	0.005003	-	-	-	-	-
865.7988	5.9	0.4081	0.034019	-	-	-	-	-
1059.91	7.18	0.40552	0.008451	-	-	-	-	-
1137.528	7.38	0.40496	0.002661	-	-	-	-	-
902.4643	7.32	0.40406	0.01542	-	-	-	-	-
1059.409	7.18	0.40405	0.005674	-	-	-	-	-
497.3256	5.06	0.4021	0.010901	PG(P-18:0/0:0)	C24H49O8P	496.3165	3.627800239	Glycerophospholipid, no known role
1000.914	8.02	0.40099	0.003134	-	-	-	-	-
430.2006	9.46	0.40067	0.044284	-	-	-	-	-
1001.416	8.02	0.40042	0.00497	-	-	-	-	-
1050.903	7.77	0.38686	0.006815	-	-	-	-	-
2197.772	7.14	0.38609	0.00535	-	-	-	-	-
815.2732	5.25	0.37905	0.011886	-	-	-	-	-
1049.902	7.76	0.37633	0.004057	-	-	-	-	-
512.2882	7.43	0.37591	0.041827	-	-	-	-	-
550.3341	4.97	0.37513	0.01648	-	-	-	-	-
1060.912	7.19	0.3745	0.017569	-	-	-	-	-
445.1952	5.12	0.37258	0.009205	-	-	-	-	-
317.1358	5.63	0.37065	0.011438	-	-	-	-	-
418.1469	3.57	0.36895	0.007001	N6-ISOPENTENYL-ADENOSINE-5'-MONOPHOSPHATE	C15H24N5O7P	417.1413347	4.09689538	Unknown MMCD match
999.8795	7.19	0.3676	0.014563	-	-	-	-	-
372.2499	8.04	0.36704	0.047759	-	-	-	-	-
706.6092	7.18	0.35931	0.004251	-	-	-	-	-
575.285	5.43	0.35615	0.047476	-	-	-	-	-
1001.916	8.03	0.35476	0.034334	-	-	-	-	-
732.9282	7.1	0.35471	0.006386	-	-	-	-	-
999.3794	7.19	0.35293	0.011896	-	-	-	-	-
1100.394	7.1	0.34935	0.009142	-	-	-	-	-
1294.515	6.95	0.34861	0.029785	-	-	-	-	-
875.9217	8.53	0.34853	0.001028	-	-	-	-	-
938.3601	6.15	0.34813	0.049058	-	-	-	-	-
1100.895	7.1	0.34754	0.016444	-	-	-	-	-
2196.77	7.14	0.34318	0.003553	-	-	-	-	-
749.3458	7.54	0.33753	0.048659	-	-	-	-	-
2198.775	7.11	0.32591	0.009097	-	-	-	-	-
903.467	7.31	0.32483	0.012736	-	-	-	-	-
1039.362	7.1	0.32137	0.01123	-	-	-	-	-
1039.863	7.1	0.31609	0.00874	-	-	-	-	-
733.2653	7.11	0.30588	0.008742	-	-	-	-	-
937.3592	6.13	0.29974	0.034927	-	-	-	-	-
113.0219	1.23	0.29656	0.036827	-	-	-	-	-
1185.462	6.96	0.2921	0.034409	-	-	-	-	-
1040.365	7.08	0.29038	0.024667	-	-	-	-	-
1295.519	6.96	0.28804	0.042714	-	-	-	-	-
733.5986	7.1	0.2855	0.010161	-	-	-	-	-
982.2709	4.97	0.2514	0.031258	-	-	-	-	-
446.1993	5.12	0.24519	0.030043	-	-	-	-	-
1262.354	6.66	0.24461	0.043261	-	-	-	-	-
577.2204	8.43	0.2235	0.034154	-	-	-	-	-
427.2556	4.08	0.14218	0.039381	-	-	-	-	-

Table 3.2 Putative metabolite identification of Variable Importance in Projection features identified by PLS-Da of BHIB bacterial and control samples. Grey = Control BHIB, Blue = BHIB inoculated with *S. constellatus*

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	ppm deviation	Putative Role
1	652.4043	6.09	C	-	-	-	-	-
2	231.1707	3.73	C	Valyl-Leucine	C11H22N2O3	230.1630426	1.723684721	Incomplete AA degradation product
3	1100.581	7.8	C	-	-	-	-	-
4	229.155	3.79	B	Leucyl-Proline	C11H20N2O3	228.1473925	1.538269332	Incomplete AA degradation product
5	427.2556	4.08	B	-	-	-	-	-
6	751.3683	7.23	C	OKOOA-PI	C35H59O15P	750.3592	0.00177002	Oxidised glycerophospholipid
7	263.1417	4.77	B	Leucyl-Methionine	C11H22N2O3S	262.1351133	2.561224023	
8	1101.584	7.8	C	-	-	-	-	-
9	439.2927	6.63	B	-	-	-	-	-
10	572.3445	7.84	B	-	-	-	-	-
11	279.1346	3.1	B	Tyrosyl-Proline	C14H18N2O4	278.1266571	2.304234942	Incomplete AA degradation product
12	653.4079	5.82	C	-	-	-	-	-
13	889.4847	8.44	C	PIP(16:1(9Z)/18:0)	C41H78O16P2	888.4765095	1.099142744	Phosphatidylinositol, cell wall component
14	562.824	8.67	C	-	-	-	-	-
15	1025.57	8.1	B	-	-	-	-	-

Table 3.3 Putative metabolite identification of important features in classification identified by RF analysis of BHIB bacterial and control samples. Grey = Control BHIB, Blue = BHIB inoculated with *S. constellatus*

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	ppm deviation	Putative Role
1	1244.328	10	B	-	-	-	-	-
2	1038.567	7.6	B	-	-	-	-	-
3	1015.478	3.6	B	-	-	-	-	-
4	756.3998	6.49	B	-	-	-	-	-
5	2196.77	7.1	B	-	-	-	-	-
6	888.4218	5.06	B	-	-	-	-	-
7	790.1095	10.6	C	-	-	-	-	-
8	513.231	1.95	B	-	-	-	-	-
9	429.2155	8.9	B	-	-	-	-	-
10	1231.674	10	B	-	-	-	-	-
11	1587.8	9.2	B	-	-	-	-	-
12	741.0824	11.5	C	-	-	-	-	-
13	709.357	6.66	B	-	-	-	-	-
14	2397.803	9.6	B	-	-	-	-	-
15	419.233	6.52	C	-	-	-	-	-

3.4.3 LCMS analysis of *S. constellatus* cultured in TSB

From the growth curve of *S. constellatus* in TSB described in Section 3.2.1, sampling in triplicate was undertaken at 0 h, 6 h, 24 h and 48 h and these samples were prepared for untargeted LCMS analysis. A growth medium control of TSB kept in the same conditions (5% CO_2 and 37°C) was also sampled in triplicate at 0 h, 6 h, 24 h and 48 h and prepared for untargeted LCMS analysis alongside the bacterial cultures in order to account for abiotic degradation.

After LCMS analysis and deconvolution using XCMS online, quality assurance of the raw data files in Microsoft Excel reduced the number of total features from all samples from 5568 to 5460. Peak alignment, missing value imputation and data normalisation was then conducted through MetaboAnalyst with Figure 3.9 showing the normalisation step that allows for better comparison of features.

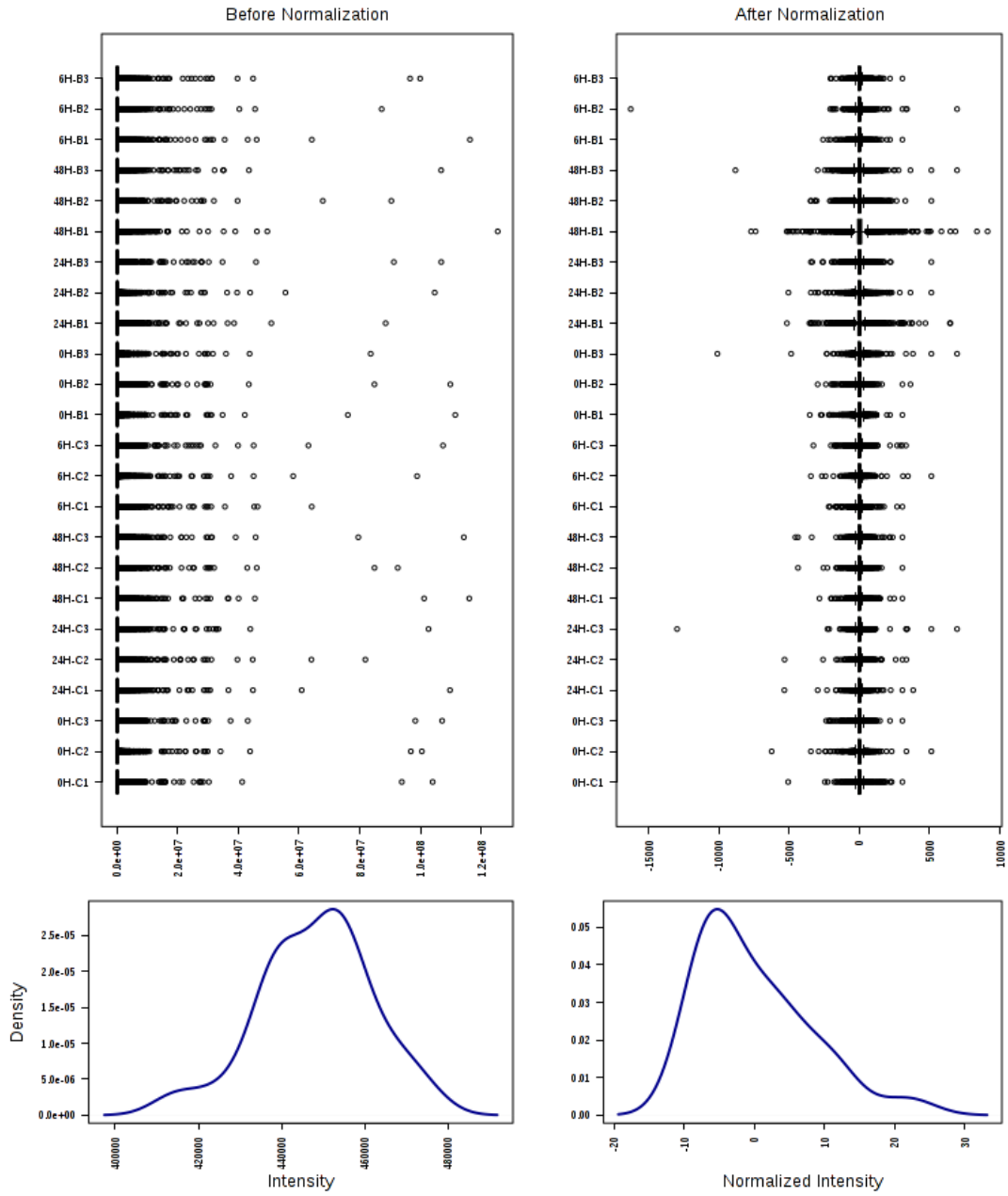

Figure 3.9 Box plots and kernel density plots before and after normalization of LCMS data of *S. constellatus* cultured in TSB (Indicated as B) and control TSB samples (Indicated as T) at 0,6,24 and 48h. The normalisation using quantile normalization and Pareto scaling. (*n=3*).

3.4.3.3 Univariate Analysis

Univariate analysis was conducted of all features to ascertain which features were significantly upregulated or downregulated (p=<0.05 and fold change of 2≥ or -2≤) from the BHIB control to *S. constellatus* cultured in TSB. From the 5460 features initially identified, 13 were identified to be significantly changed with 1 upregulated and 12 downregulated (Figure 3.10).

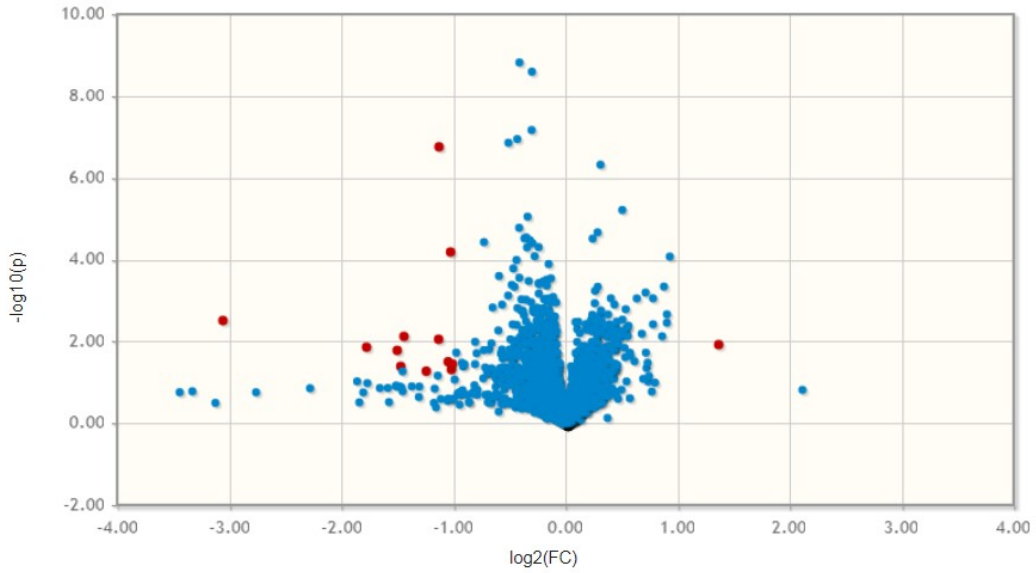

Figure 3.10 Important features from TSB LCMS analysis selected by univariate volcano plot with fold change threshold (x) 2 and t-tests threshold (y) 0.05. The red circles represent features above the threshold and all values are log transformed. The further its position away from the (0, 0) the more significant the feature is.

3.4.3.2 Principle Component Analysis

The 2D scores plot of all TSB bacterial and control samples (Figure 3.11) showed that the TSB control is much more tightly clustered (Red) than BHIB inoculated with *S. constellatus* (Green). All 0 h and 6 h samples are tightly clustered together indicating that not much biotic degradation has taken place along with the control media being tightly clustered even after 48 h, indicating that little has changed chemically. The 24 h and 48 h bacterial samples are different from a large selection of the samples highlighting a larger difference in the components present, most likely due to microbial metabolism.

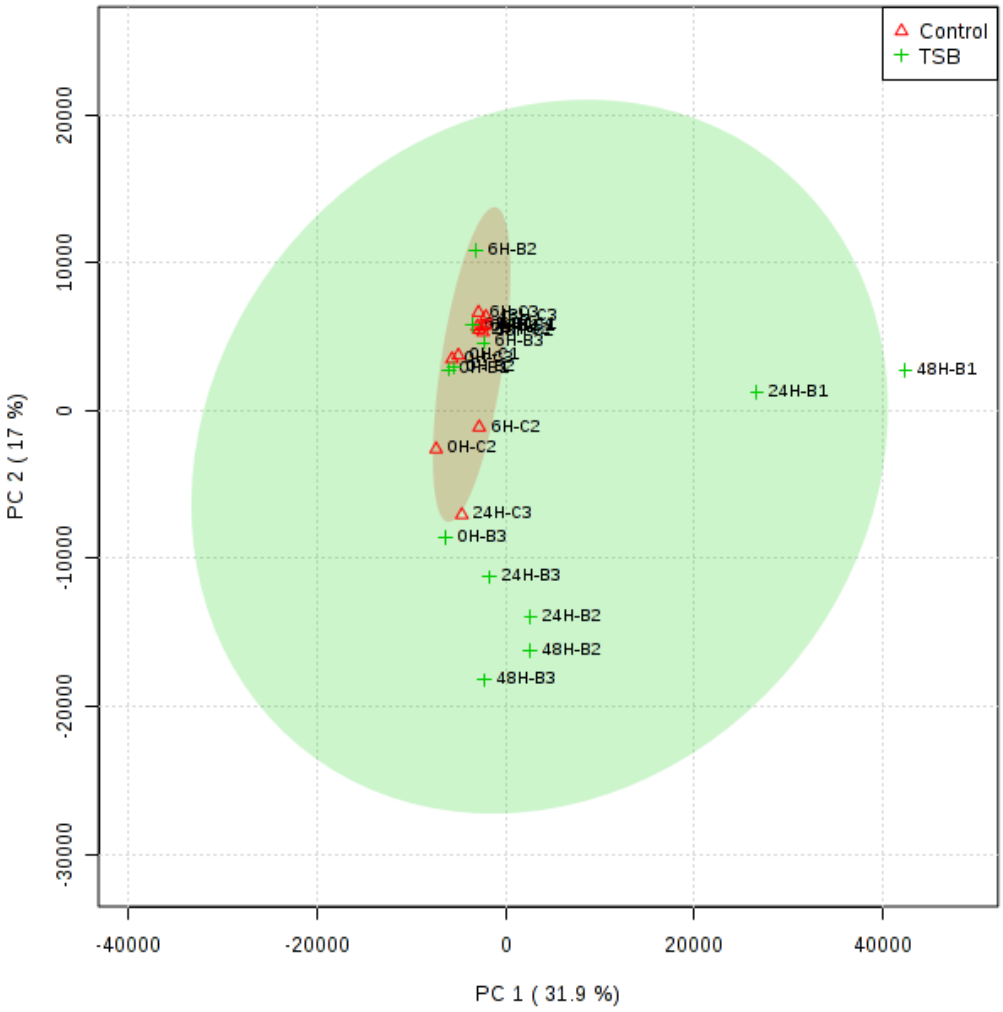

Figure 3.11 A 2D scores plot of a PCA between all TSB bacterial (TSB) and control (Control) classes. The explained variances are shown in brackets. B after a class denotes Bacterial and T denotes controls with the number denoting a biological replicate.

3.4.3.3 Partial Least Squares – Discriminant Analysis

The 2D scores plot of all TSB bacterial and control samples (Figure 3.12) shows that the TSB control is much more tightly clustered (Red) than TSB inoculated with *S. constellatus* (Green), this correlates with the PCA (Figure 3.11). PLS-DA usually has sharper separation between classes than PCA, which is shown with the difference between (Figure 3.11 and Figure 3.12). All 0 h and 6 h samples from each class are tightly clustered together indicating that not much biotic degradation has taken place. A Variable Importance in Projection (VIP) score graph was plotted (Figure 3.13) which calculates weighted sum of squares of the PLS loadings taking into account the amount of explained Y-variation in each dimension for each component. A higher VIP

indicates that the feature in question has a larger contribution to class discrimination in the PLS-DA model. The top 15 VIP features identified were selected for metabolite identification.

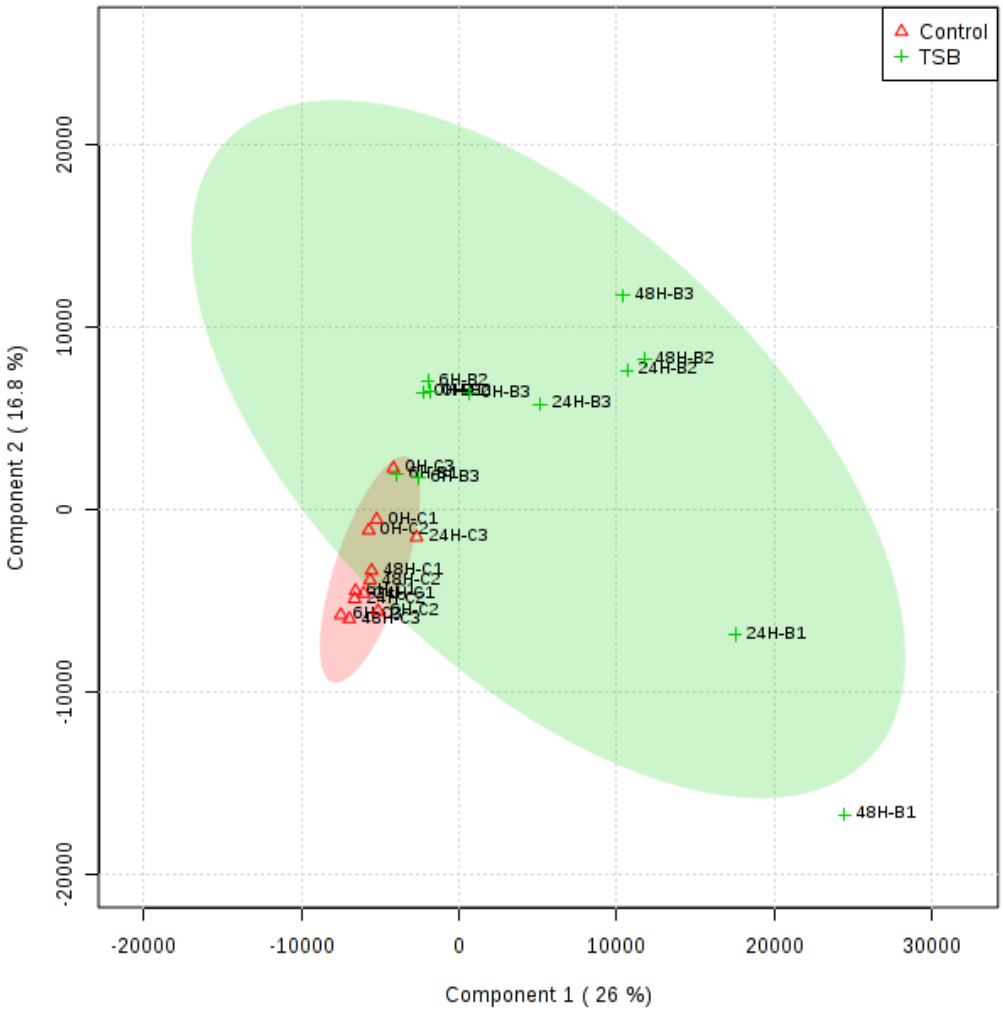

Figure 3.12 A 2D scores plot of a PLS-DA between all TSB bacterial (TSB) and control (Control) classes. The explained variances are shown in brackets. B after a class denotes Bacterial and T denotes controls with the number denoting a biological replicate.

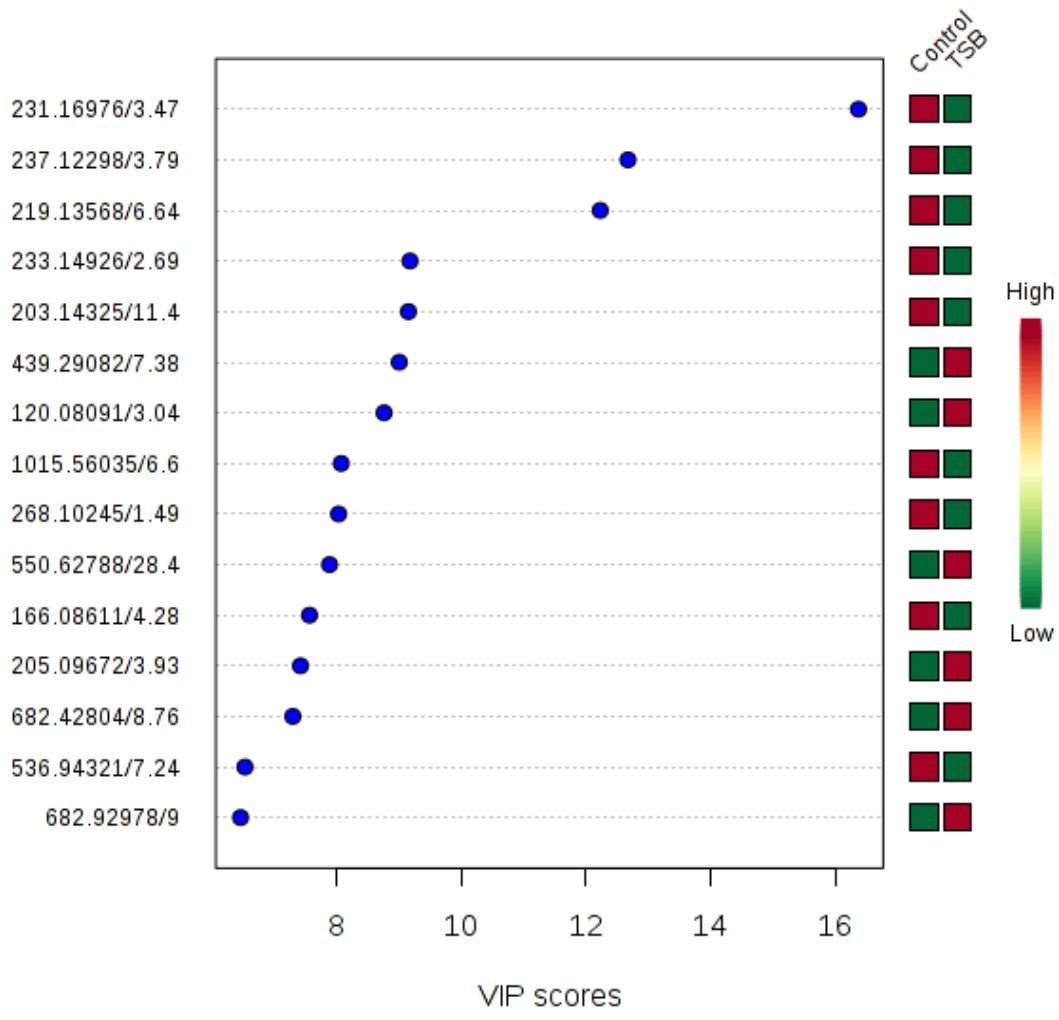

Figure 3.13 Important features identified by a PLS-DA of TSB bacterial and control samples. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. The x axis denotes VIP scores and y axis the feature m/z and retention time separated by /.

3.4.3.4 Significant feature selection using Random Forest algorithm

The TSB control and bacterial samples were analysed using RF (Figure 3.14) in order to identify features that are important for class segregation. The top 15 significant features identified were selected for metabolite identification.

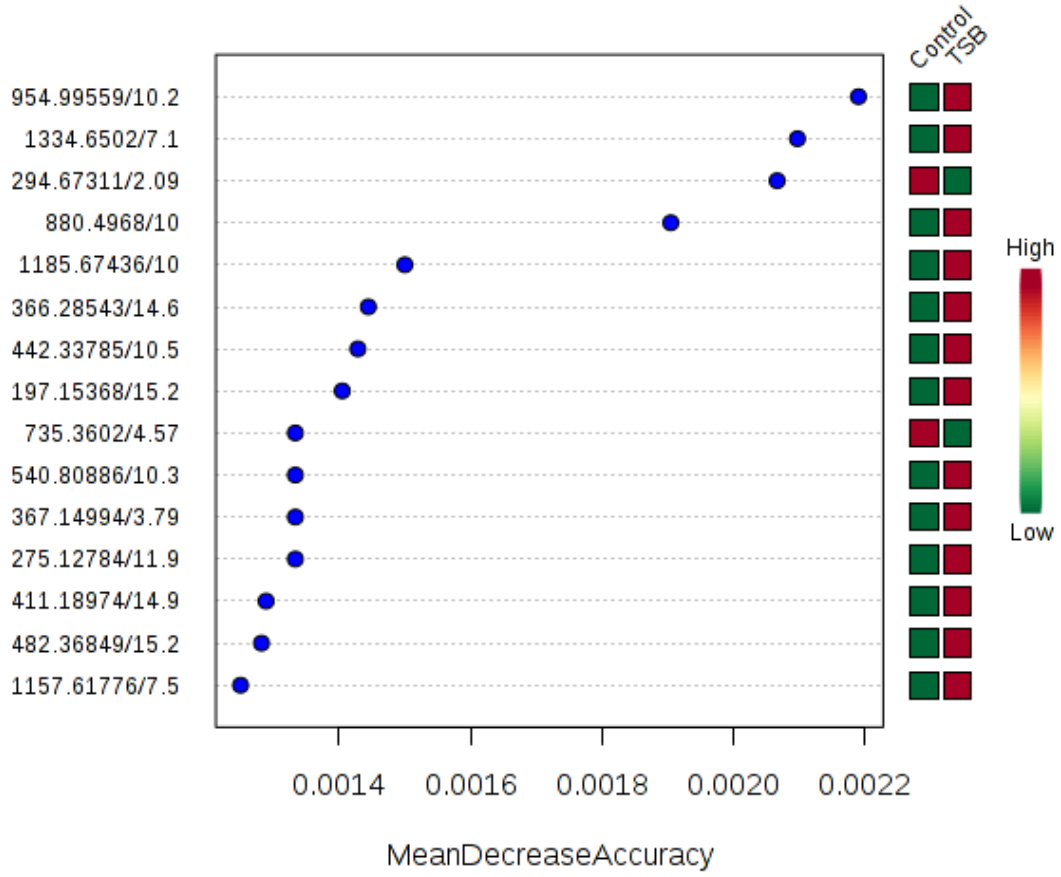

Figure 3.14 Significant features from TSB (control) and SC inoculated of TSB identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. The x axis denotes mean decrease in accuracy and y axis the feature m/z and retention time separated by /.

3.4.3.5 Metabolite identification

All significantly upregulated and downregulated feature identified by volcano plots, Variable Importance in Projection features identified by PLS-Da and important features in classification identified by RF were extracted into an excel spreadsheet for identification of metabolites. The program MetaboSearch was used to compare the m/z and retention times of the metabolites of interest with a selection of mass spectral databases for putative identification and the results extracted. For all significant features, there was a 30% success rate in identification. Of the metabolites identified with the TSB control, the majority were amino acids and their derivative products, which is expected due to the nature of TSB as an enriched growth medium. Subsequently, the majority of identified metabolites associated with *S. constellatus*

cultured in TSB are degradation products of amino acids indicating microbial metabolism of TSB.

Table 3.4 Putative metabolite identification of significantly up and downregulated metabolites identified by volcano plots of TSB bacterial and control samples.

Upregulation								
m/z	RT	FC	*p* value	Name	Formula	Mass of Match	ppm deviation	Putative Role
268.10245	1.49	2.579	0.011201	Neuraminic acid	C9H17NO8	267.0954165	0.91405767	Common sialic acid, bacterial by-product
Downregulation								
m/z	RT	FC	*p* value	Name	Formula	Mass of Match	ppm deviation	Putative Role
1102.59141	10.37	0.49762	0.033504	-	-	-	-	-
1081.61181	10.37	0.49418	0.045468	-	-	-	-	-
291.2165	11.29	0.49158	6.12E-05	-	-	-	-	-
1101.5889	10.37	0.48451	0.029317	-	-	-	-	-
290.18624	11.71	0.45793	1.65E-07	-	-	-	-	-
212.10094	1.54	0.45664	0.008294	-	-	-	-	-
540.80886	10.37	0.42304	0.049912	-	-	-	-	-
558.32232	4.44	0.36865	0.007062	-	-	-	-	-
474.58769	4.3	0.36135	0.038094	-	-	-	-	-
752.37342	7.2	0.35351	0.015483	-	-	-	-	-
751.3674	7.2	0.29249	0.012941	OKOOA-PI	C35H59O15P	750.3592	1.220118783	Oxidised glycerophospholipid
132.06547	3.23	0.12041	0.002889	Glutaramic acid	C5H9NO3	131.0582	0	Non-essential amino acid

Table 3.5 Putative metabolite identification of Variable Importance in Projection features identified by PLS-Da of TSB bacterial and control samples. Grey = Control TSB, Blue = TSB inoculated with *S. constellatus.*

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	ppm deviation	Putative Role
1	231.2	3.47	C	Valyl-Leucine	C11H22N2O3	230.1630426	2.452935949	Incomplete AA degradation product
2	237.1	3.79	C	Phenylalanyl-Alanine	C12H16N2O3	236.1160924	1.615602428	Incomplete AA degradation product
3	219.1	6.64	C	-	-	-	-	-
4	233.1	2.69	C	Threoninyl-Leucine	C10H20N2O4	232.1423071	1.380336826	Incomplete AA degradation product
5	203.1	11.4	C	2-Pentyl-3-phenyl-2-propenal	C14H18O	202.1357652	0.981341675	Aromatic cinnamaldehyde compounds
6	439.3	7.38	B	-	-	-	-	-
7	120.1	3.04	B	-	-	-	-	-
8	1016	6.6	C	-	-	-	-	-
9	268.1	1.49	C	Neuraminic acid	C9H17NO8	267.0954165	0.91405767	Common sialic acid, bacterial by-product
10	550.6	28.4	B	-	-	-	-	-
11	166.1	4.28	C	Multiple phenylalanine degradation products	-	-	-	By-products of phenylalanine metabolism
12	205.1	3.93	B	Multiple tryptophan degradation Products	-	-	-	By-products of tryptophan metabolism
13	682.4	8.76	B	-	-	-	-	-
14	536.9	7.24	C	-	-	-	-	-
15	682.9	9	B	-	-	-	-	-

Table 3.6 Putative metabolite identification of important features in classification identified by RF analysis of TSB bacterial and control samples. Grey = Control TSB, Blue = TSB inoculated with *S. constellatus.*

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	ppm deviation	Putative Role
1	955	10.2	B	-	-	-	-	-
2	1335	7.1	B	-	-	-	-	-
3	294.7	2.09	C	-	-	-	-	-
4	880.5	10	B	-	-	-	-	-
5	1186	10	B	-	-	-	-	-
6	366.3	14.6	B	-	-	-	-	-
7	442.3	10.5	B	-	-	-	-	-
8	197.2	15.2	B	Multiple uncatorgorised fatty acid esters	-	-	-	-
9	735.4	4.57	C	-	-	-	-	-
10	540.8	10.3	B	-	-	-	-	-
11	367.1	3.79	B	-	-	-	-	-
12	275.1	11.9	B	4,4'-dihydroxy-3,5-dimethoxydihydrostilbene	C16H18O4	274.1205	0.222658113	Cinnamylphenols, no known role
13	411.2	14.9	B	-	-	-	-	-
14	482.4	15.2	B	-	-	-	-	-
15	1158	7.5	B	PIM2(18:1(9Z)/16:2(9Z,12Z))	C55H97O23P	1156.6158	4.643800943	Glycerophospholipids, no known role

3.5 DISCUSSION

3.5.1 Differences in Growth between BHIB and TSB

Both BHIB and TSB successfully cultivated *S. constellatus* as both media are nutrient rich and contain components such as dextrose, which can have a substantial impact on bacterial growth as well as supporting high cell densities (Jaradat and Bhunia, 2002). Despite statistically there being no significant difference between the growth in both media, there was an observed disparity in growth, with TSB slightly slower at initiating growth and a shorter stationary stage. Studies have previously shown a potential cause for this reported trend, with BHIB being reported to stimulate proteinase production leading to favourable conditions for some streptococcal species (Jack and Tagg, 1992). One of the reasons behind this proteinase production could be because of the addition of the component protease peptone within BHIB. Protease peptone is one of the main differences in formula between BHIB and TSB, with TSB containing no amount of protease peptone. Another previous study grew *Streptococci* in a variety of media and showed that growth of several strains was best supported in a protease peptone broth even in comparison with TSB (Ferretti and Ward, 1976). Protease peptone contains elements of nitrogen as well as providing an effective buffering range for bacteria, which is potentially a favourable environment for *S. constellatus* and could be responsible for the slight disparity in growth between BHIB and TSB. With a notable disparity between two relatively similar media having a large effect on the metabolites, this further solidifies the reasoning for the development and utilisation of a more representative periodontal growth medium (Chapter 2) for comparisons between microorganisms and collection of microbial products.

3.5.2 Trends From Metabolites Identified

The developed untargeted metabolomics pathway was successful in being able to translate raw LCMS results from a large number of peaks to a selection of ~100 metabolite features for each dataset, which are putatively responsible for differences between control media and microbial inoculation. It is also clear from the PCA and PLS-DA analyses that there is a statistically significant difference between the control

and bacterial classes, confirming that microbial degradation of both BHIB and TSB took place and the classes can be separated based on the products present.

The use of a variety of methods such as univariate analysis, multivariate analysis and machine learning for identification of features that significantly contribute to class separation between control and bacteria was successful in highlighting features of interest. However, the identification of these features was poor with a 7.1% success rate in identification for BHIB and 30% for TSB. Of these features identified, none were a match for the known mass values for PAs, which does not necessarily indicate they are not there, rather that they were not able to be detected. The majority of the features that were identified were nonspecific degradation products related to amino acid metabolism along with non-specific glycophospholipids known to be related to microbial metabolism (Shaw, 1975).

Not being able to identify the large majority of the features of interest limits the experiment as it restricts translation of potential metabolites to look at their biological role. Indeed, of the metabolites successfully identified the majority were very non-specific such as amino acids and amino acid degradation products, which are not useful candidate molecules for potential biomarkers involved in disease as they are ubiquitous in all forms of life.

3.5.3 The Problems Facing the Emergent Metabolomics Field

Currently there are multiple issues facing untargeted metabolomics, which hinder the translation of LCMS analysis to results of biological significance. With the data generated for *S. constellatus,* even after three different methods of metabolite identification and four different databases utilised, only a small selection of metabolites were identified. This can be down to a variety of reasons, they may indeed be truly novel, they may be false positives or they may be fragments of known metabolites.

Unambiguous metabolite identification is a bottleneck, which is limiting the success of metabolomics (Dunn et al., 2013), as multiple metabolites can match one mass peak it makes identification of that peak harder to undertake. Even with algorithms such as the ‘Seven Golden Rules’ (Kind and Fiehn, 2007) which utilises filtering by

chemical rules such as hydrogen carbon ratios, there is a substantial problem in untargeted metabolomics of actually deciphering what peak corresponds to which metabolite.

To compound the problem of identification, there is also a limited number of metabolites, which have actually been characterised. Of the four major metabolite databases, there is approximately ~40,000 metabolites with structures and associated spectra and MS/MS. If this is compared to the proteomic databases such as UniProt, which has ~550,000 manually annotated and ~89,000,000 automatically annotated proteins, it is clear that there is a lack of characterised data for comparison, which is needed to decipher what m/z value corresponds to a metabolite.

Furthermore, if you consider that each organism will have a different genome, proteome and transcriptome leading to wholly unique metabolites, it means that identification of these metabolites is at present difficult unless working with model organisms. The suitability for untargeted metabolomics for identification of novel compounds and/or mediators of disease is hindered by a combination of all these factors.

3.6 CONCLUSIONS

The development of an untargeted metabolomics pathway for analysis was successful in principle, as raw LCMS data was successfully translated from thousands of features to a smaller selection of significant features putatively responsible for difference between the control and bacterial inoculation. However, the suitability of untargeted metabolomics for screening of multiple periodontal associated organisms is questionable as while some metabolite identification took place the majority of significant features could not be identified and of the metabolites identified the majority were relatively non-specific degradation products. In order to find 'novel' compounds that may be involved in disease a microbial library for each organism would need to be made which would be an expensive and time-consuming process that is not feasible for this pilot study.

Rather than screening a wide selection of periodontitis associated organisms through this untargeted pathway the decision was taken that it would be more practical, provide less ambiguous results and be less time consuming to consider an alternative approach. The alternative approach was cell culture based experiments (Chapter 4), that would allow testing of periodontitis microbial supernatants against kidney cells in order to test a variety of parameters. This could serve as a provisional screening step to cut down the number of organisms of interest and pave the way for future more targeted LCMS based investigations and/or reanalysis of untargeted LCMS data to look for identified molecules.

CHAPTER 4: KIDNEY CELL BASED INVESTIGATIONS

4.1 INTRODUCTION

In healthy humans, host-microbiome interactions are critical to maintaining homeostasis and play a key role in a wide selection of processes such as production of key metabolites, providing a barrier to pathogenic attack and priming the immune system (Cho and Blaser, 2012, Engevik and Versalovic, 2017). In diseases states, such as CP, the oral microbiota undergoes dysbiosis and a move to a more Gram negative dominated environment with distinct differences between healthy and diseased patients seen in the saliva and plaque (Shi et al., 2018a, Chen et al., 2018). This more pathogenic periodontal microbiota has been reported to have a wide variety of potentially deleterious effects such as tissue invasion, production of toxic compounds and evasion of the immune system (Guthmiller and Novak, 2002). The production of secreted toxic compounds are of particular interest when investigating CP and CKD as they could potentially interact with the kidneys through translocation from the oral cavity via the bloodstream to the kidneys (Chapter 1.2.1). Using the supernatants collected from the chosen periodontal pathogens (Chapter 2.3.8), cell-based investigations can be used to investigate whether the microbial products can elicit a deleterious effect on kidney cells.

4.1.1 Evidence for periodontal supernatants eliciting cellular effects

Numerous studies have investigated the cellular effects of periodontitis-associated microbial extracts, but this research has predominately focused around the effect of sonicated bacterial extracts (SBEs) on cells of the oral cavity such as gingival fibroblasts. With these studies there is evidence to show that SBEs of *Aggregatibacter actinomycetemcomitans* (Stevens et al., 1983, Stevens and Hammond, 1988), *Porphyromonas endodontalis* (Yamasaki et al., 1998), *Porphyromonas gingivalis* (Yamasaki et al., 1998, Fotos et al., 1990, Stevens and Hammond, 1988, Morioka et al., 1993) and *Fusobacterium nucleatum* (Yamasaki et al., 1998, Stevens and Hammond, 1988) elicit cytotoxic effects on gingival fibroblasts. However, it is important to understand the difference between the two types of microbial preparations; microbial supernatants are more representative of the potential exposure human cells would receive compared to the SBEs (Figure 4.1). This is because microbial supernatants do not contain the lysis and intracellular products

of the microorganisms that become released upon microbial cell death may be more cytotoxic than secreted products in supernatants. In some cases the SBEs may be toxic to cells, while the microorganisms alone are not (Shah et al., 1992). Furthermore, SBEs can contain greater concentrations of cell wall components like LPS and fimbriae that are known to be cytotoxic (Nishio et al., 2013, Rossol et al., 2011) and can be released by the sonication step as this disrupts the cell wall.

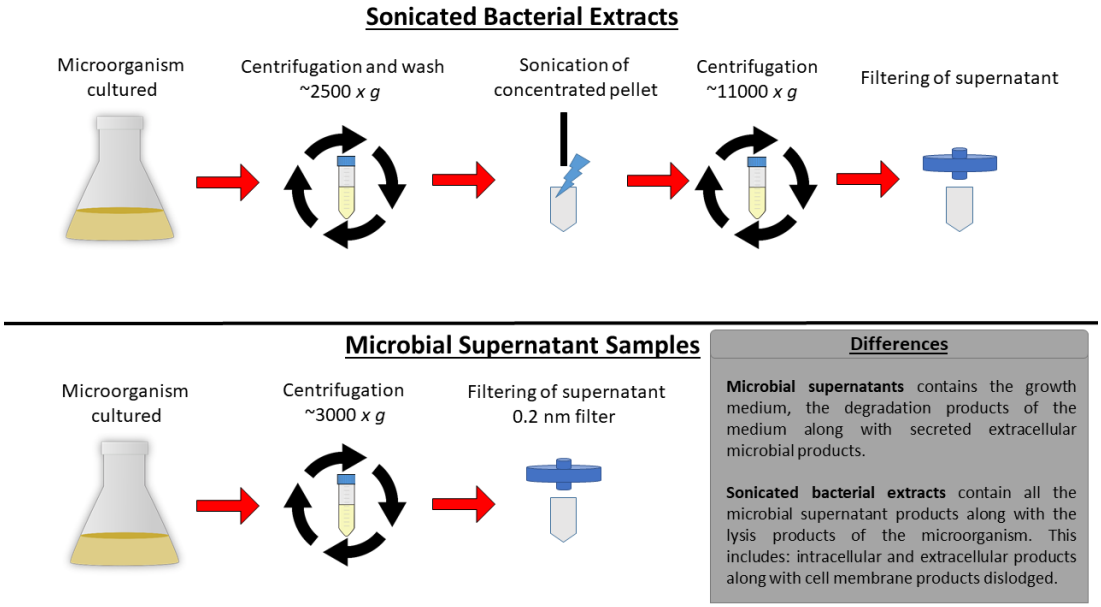

Figure 4.1 The differences between sample preparation of sonicated bacterial extracts and microbial supernatant samples. Sample preparation aggregated from a selection of publications (Yamasaki et al., 1998, Stevens and Hammond, 1988, Morioka et al., 1993).

However, evidence for the effects of periodontal microbial supernatants on various human cell types is sparse. Although, some experiments have investigated the effect of supernatants on gingival fibroblasts, with *A. actinomycetemcomitans* (Letzelter et al., 1998), *F. nucleatum* (Johansson et al., 1994) and *P. gingivalis* (Letzelter et al., 1998, Johansson et al., 1996) being reported to be cytotoxic and *Capnocytophaga ochracea* and *Peptostreptoccocus micros* reported to produce toxins that can inhibit fibroblast proliferation (Letzelter et al., 1998). It has been suggested that the cytotoxicity of microbial products, be it supernatant or SBE, can vary depending on the different cell types exposed, which may be due to different target sites and capacity for neutralisation of toxicity (Johansson et al., 1994, Gibbons, 1989). With this in mind, testing the effect of a selection of CP associated microbial supernatants

against kidney cell lines may provide an insight into how secreted periodontal microbial products could potentially be interacting with the kidneys and how this may be different to interactions with cells of the oral cavity.

4.1.2 Renal Fibrosis

Fibrosis is a non-specific wound healing process in which injured organs and tissues are repaired by recruitment of fibroblasts and local and/or circulating immune cells (Wynn, 2007). Renal fibrosis, which presents as glomerular or tubulointerstitial fibrosis, is the excessive accumulation and deposition of excess extracellular matrix (ECM) within the kidney parenchyma, which over time can lead to loss of function (Efstratiadis et al., 2009). Renal fibrosis occurs in the vast majority of chronic kidney diseases and the progressive excessive accumulation of ECM can ultimately lead to end-stage renal failure (Liu, 2006, Hewitson, 2012).

The process of renal fibrosis is depicted in Figure 4.2. Initially, renal fibrosis starts as a protective response to an insult to the epithelial or endothelial cells of the kidney. A number of factors can damage these cells, for example infections, metabolic factors (e.g. hyperglycaemia in diabetes), toxins and hypertension (Hewitson, 2012). Upon initial injury of the cells and/or activation of endothelial cells, tissue factor is exposed to the blood thereby promoting platelet activation, fibrin formation and feedback inhibition (Madhusudhan et al., 2016). The damage along with tissue factor stimulates the complement cascade and the development of a fibrin clot with the release of pro-fibrotic growth factors such as transforming growth factor beta (TGF-β) (López-Hernández and López-Novoa, 2012) and platelet-derived growth factor (PDGF) (Floege et al., 2008) along with various chemokines. These chemokines and growth factors drive the recruitment of leukocytes from the circulatory system which in-turn initiate inflammation and tissue remodelling (Wynn, 2007). These immune cells secrete a selection of components which drive these processes such as matrix metalloproteases (MMPs) (Zhao et al., 2013), tissue inhibitors of metalloproteases (TIMPs) (Bieniaś and Sikora, 2018), interleukins 6 and 13 (Su et al., 2017), TGF-β and Epidermal growth factor (EGF) (Rayego-Mateos et al., 2018).

After the initial inflammation and remodelling caused by the components secreted by the leukocytes, the fibroblasts are activated by the pro-fibrotic fibroblast growth factor (FGF). These differentiated fibroblasts, known as myofibroblasts, proliferate heavily and are a feature of all forms of kidney disease (Meran and Steadman, 2011). Accumulation of this specific cell type is heavily associated with CKD disease progression due to the production of ECM components such as collagen that are integral to the scarification seen in CKD (Hewitson, 2012). In the normal process of inflammation, the growth factor vascular endothelial growth factor (VEGF) is key in the stimulation of angiogenesis, which takes place alongside the activation of myofibroblasts and normally would lead to wound contraction and eventual regeneration of tissues (Doi et al., 2010, Wynn, 2007). However in CKD this normal healing process is disrupted with failed angiogenesis and a more pro-fibrotic response characterised by persistent myofibroblast activation, inflammation, imbalance in MMPs and TMPS and excessive ECM deposition (Ballermann and Obeidat, 2014).

The cause of the difference between healthy wound healing and the fibrotic response seen in CKD is hard to decipher despite it being clear that the chronic persistence of myofibroblasts plays a key role (Meran and Steadman, 2011). One key distinction is the duration of the injury, which provides a sustained pro-fibrotic signal that can lead to accumulation of interstitial matrix and unchecked fibrosis (Liu, 2006).

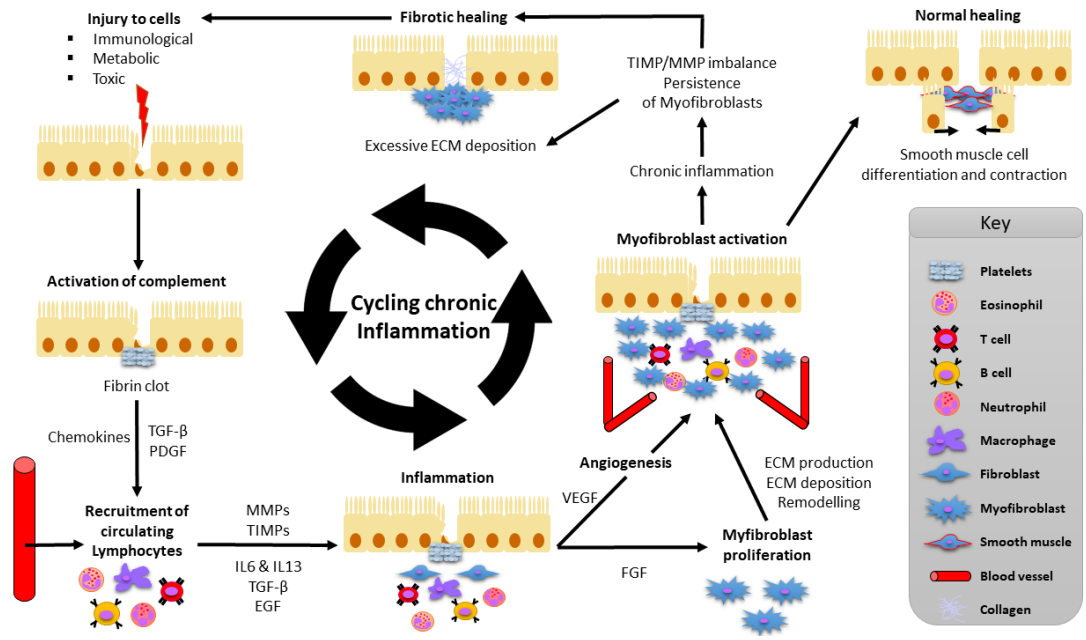

Figure 4.2 The fibrotic pathway in chronic kidney disease. Adapted from (Wynn, 2007)

It is clear that there are myriad factors involved in the excessive fibrotic phenotype seen in CKD (Figure 4.2) with no singular cell type or secreted component responsible for this phenotype. Because of this, choosing a singular marker for use as a fast test for fibrosis induction in cells is a difficult conundrum. A potential marker which is specific enough for fibrosis and sensitive enough to react to changes in the environment and damage such as reactive oxidative species and stress is plasminogen activator inhibitor 1 (PAI-1). PAI-1 is an inhibitor of tissue plasminogen activator and urokinase plasminogen activator, which convert plasminogen to plasmin and are key in the formation of clots (Figure 4.3) (Malgorzewicz et al., 2013). In addition to its role in clot formation there is a large body of evidence to support PAI-1 as a powerful fibrosis promoting molecule which can effect cellular migration, recruiting fibroblasts and inflammatory cells along with inhibiting ECM degradation, the activation of MMPs and being directly related to TGF-β expression (Eddy and Fogo, 2006). Due to this, there has been some success in transfecting cells with PAI-1 luminescence reporters in order to monitor TGF-β expression and general fibrosis in cell based luciferase reporter assays (Abe et al., 1994); this will be utilised in this chapter to provisionally investigate fibrotic potential of the selected microorganism supernatants.

4.2 AIM AND OBJECTIVES

4.2.1 Aim

To test the effect of microbial supernatants on cytotoxicity, migration, and induction of a fibrosis in human proximal tubule epithelial (HK-2) cells.

4.2.2 Objectives

- Evaluate whether selected microbial supernatants have cytotoxic effects on human kidney cells.
- Evaluate whether the selected microbial supernatants have migratory effects on human kidney cells.
- Evaluate whether the selected microbial supernatants induce fibrosis in transfected human kidney cells.

4.3 METHODS

4.3.1 Preparation of Bacterial Supernatants for Cell Based Investigations

The bacterial supernatants used were prepared as described earlier in Chapter 2.3.8.

4.3.2 Cell Culture

HK-2 cell culture and maintenance was conducted as described earlier in Chapter 2.3.2.

4.3.3 Cell Viability Testing

Cell viability testing of HK-2 cell was conducted as described in Chapter 2.3.3. The only modification was incubation of the bacterial supernatant alongside the AGCF-P media collected for 1 D, 2 D, 3 D, 4 D and 5 D or 24 h and 48 h depending on the microorganism tested. The HK-2 cell viability tests were conducted for 24 and 48 h and the medium was refreshed daily. The data analysis of cell viability results was undertaken as described in Chapter 2.3.3.1.

4.3.4 Scratch Test – Cell Migration Assay

The *in vitro* scratch assay was adapted from (Liang et al., 2007) and used to study the effect of periodontal microorganism supernatants on cell migration of HK-2 cells. HK-2 cells were seeded into 24-well plates and cultured in growth media with serum until cell confluence of 70-80% was reached. The growth medium was removed, the cells were rinsed twice with PBS and serum free medium was added. After 24 h, a scratch was made with a sterile p200 pipette tip straight across the monolayer of each well. The debris and detached cells were removed by washing twice with serum free media. A 1:20 dilution of prepared bacterial supernatant to serum-free medium was used for all tests (25 µL : 475 µL). Images were taken at 0 h, 6 h, 24 h, 48 h and 72 h, and the same frame of reference for each well was kept approximately by marking the bottom of each well with a pen. Images were captured using GXCapture-T camera attached to a light microscope (Gtvision, UK) and saved for further analysis.

In order to calculate the migration of cells over time the images taken were analysed using the open source image processor Image J (Schneider et al., 2012). A macro, MRI Wound Healing Tool, was used to estimate the area of each image and this value was

exported to a Microsoft Excel document. The percentage wound closure over time was then calculated and plotted in a graph.

4.3.5 PAI-1 Luciferase Reporting Assay

A PAI-1 luciferase-reporting assay was developed using the pNL2.3 luciferase reporter vector (Promega, UK) which produces soluble luciferase and the human Plasminogen activator inhibitor-1 (PAI-1) 955 bp promoter region (upstream of start codon -853bp; downstream +82bp) was cloned into the vector. The pNL2.3 vector with no insert was used as a negative control. The control and pNL2.3 PAI-1 reporter vector were transfected into the HK-2 kidney cell line to create HK-2 pNL and HK-2 PAI-1 cell lines. The cloning and transfection experiments were completed in the Renal group of Dr Liliana Shalamanova in Manchester Metropolitan University prior to the start of this project.

HK-2 cell line clones containing either the native pNL2.3 vector or the PAI-1 reporter vector were cultured in the same culture media as non-transfected HK-2 cells. Cells were seeded into 24-well tissue culture plates and cultured until a confluence of 80-90% was reached. The cells were then washed twice with PBS and the medium was changed to serum free medium. After 24 h, the medium was replaced with bacterial supernatants 25 µL, diluted with serum free medium 475 µL (1:20).

After 24 h, 20 µL aliquots were taken from all samples after aspirating to avoid concentration gradients; these were then centrifuged at 12470 *x g* for 5 min to deposit any protein fragments. These aliquots were mixed with 40 µL of distilled water and 50 µL of prepared NanoLuc Luciferase substrate (Promega, UK) in a 96 well black cell culture plate (Sigma-Aldrich, UK). The samples were left to stand for 5 min and then luminescence readings were taken using a Synergy HTX Multi-mode plate reader (Agilent, UK) thrice with 15 min in-between. The results were normalised to total cellular protein content.

4.3.6 Normalisation of PAI-1 Luciferase Reporter Assay Results

Normalisation of the PAI-1 luciferase reporter assay results was completed using a Bicinchoninic acid (BCA) kit (Merck, UK). The 24 well culture plates used in the PAI-1 Reporter Assay were aspirated and washed twice with PBS after the assay was

completed. All liquid was then removed and the plates were stored at -20^{o}C prior further analysis.

The total cellular protein in the 24 wells was harvested in 80 µL of 0.1 NaOH, transferred into sterile 1.5 ml tubes and hydrolysed with 0.1M NaOH for 2 hours at 37^{o}C.

The samples were then diluted at 1:5 with deionised water in preparation for BCA assay. A set of protein standards (0, 100, 200, 250, 400 µg/mL) was created using bovine serum albumin from the BCA kit. A further control consisting of 0.1 M NaOH diluted 1:5 into deionised water was also used. From each sample 20 µL were added to a 96 well plate in duplicate and 200 µL of BCA working solution, consisting of 50:1 reagents A (Bicinchoninic acid) and B (Copper II Sulphate Pentahydrate), were added to each well.

After a 30 min incubation at 37^{o}C the absorbance was measured at 562 nm using a microplate reader (Thermo-Scientific Multiskan 60, UK). A standard curve of absorbance against standards of known BSA concentration was generated and used to determine the concentration of total protein in cell lysates via interpolation after factoring in the dilution factor of 1:5.

The calculated total protein content of each well was converted to mg and the PAI-1 fluorescence-reporter assay results were divided by the total protein content, with the finalised results plotted as Luminescence Arbitrary Unit per mg of protein.

4.3.7 Statistical Testing

All statistical testing was undertaken using GraphPad Prism 8.1.1 software. A two-way ANOVA with multiple comparisons was used to determine significant differences between the cell viability control and the different dilutions of AGCF-P and for the scratch assay comparisons and fibrosis reporting comparisons between control, AGCF-P media and bacterial supernatants.

4.4 RESULTS

4.4.1 Cell Viability Testing

Cell viability testing was undertaken of all chosen microorganisms against HK-2 cells in order to assess the effect bacterial products excreted in the supernatants may have on the viability and proliferation of human kidney cells over 24 h and 48 h with the media refreshed daily.

4.4.1.1 Keystone Pathogen

Porphyromonas gingivalis

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *P. gingivalis* bacterial supernatants collected over 5 days (Figure 4.3).

After 24 hours (Figure 4.3a), the viability of cells treated with all *P. gingivalis* supernatants was significantly lower than the control, with the exception of the 3 D *P. gingivalis* supernatant. There was no significant differences between the viability of all the AGCF-P media samples in comparison to the control.

Similar effects were observed after 48 hours (Figure 4.3b); with the viability of cells treated with all *P. gingivalis* supernatants significantly lower than the control. There was also no significant differences between the viability of all the AGCF-P media samples in comparison to the control.

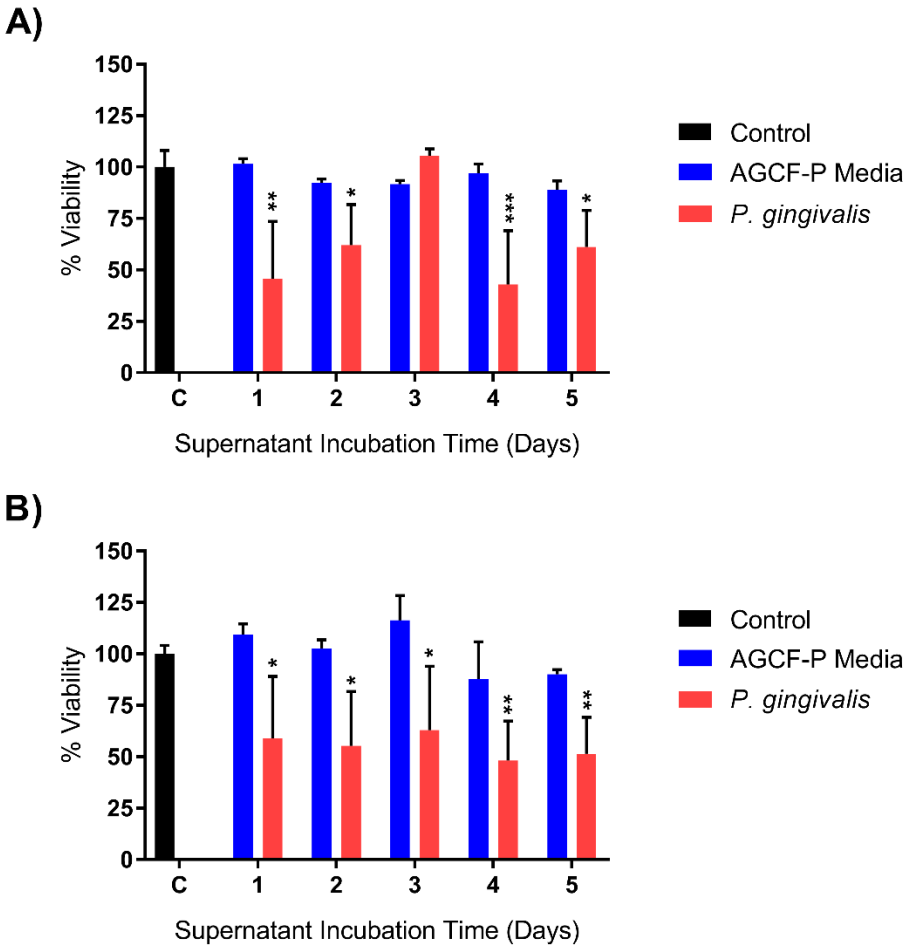

Figure 4.3 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *P. gingivalis* supernatants collected over 5 days. Viability is presented as percentage change from the control (C: HK-2 cells exposed to serum-free cell culture medium). The error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; ($n=3$).

The detrimental viability effects exhibited by the *P. gingivalis* supernatants (Figure 4.3) correlate with images taken of the same wells prior to sampling (Figure 4.4). Cell damage manifests by cell detachment (red arrows), as visible in images from cells exposed to *P. gingivalis* supernatants (Figure 4.4c) and (Figure 4.4e) compared to (Figure 4.4a) serum free media control and (Figure 4.4b) and (Figure 4.4d) which are AGCF-P media controls.

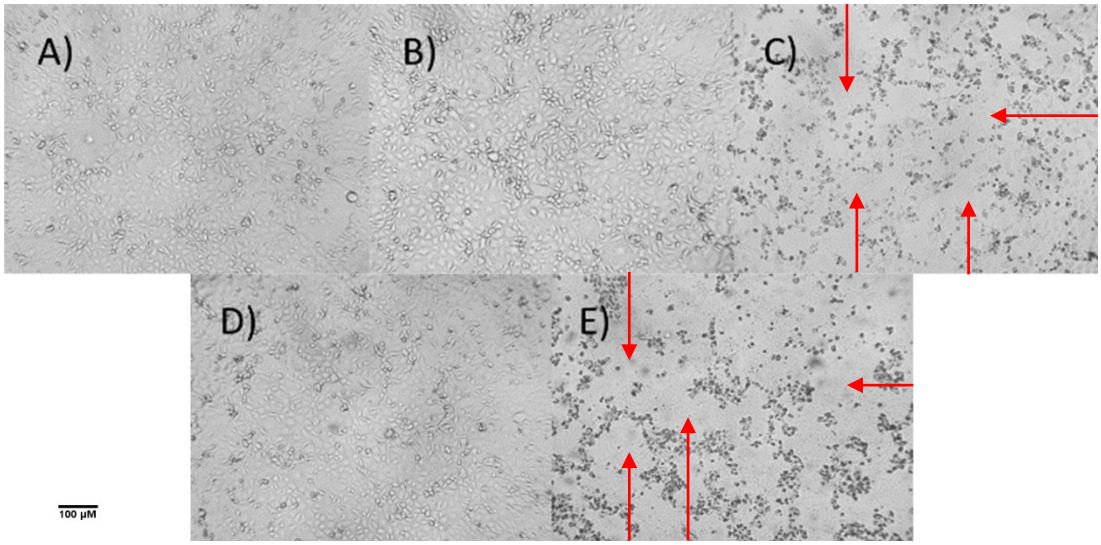

Figure 4.4 Images of HK-2 cells taken during cell viability testing of AGCF-P supernatants and *P. gingivalis* supernatants collected over 5 days exposed to HK-2 cells for 24 h and 48 h. A) Control (Serum-free cell culture medium) B) 5 day AGCF-P after 24 h exposure of HK-2 cells C) 5 day *P. gingivalis* after 24 h exposure of HK-2 cells D) 5 day AGCF-P after 48 h exposure of HK-2 cells E) 5 day *P. gingivalis* after 48 h exposure of HK-2 cells. Scale length = 100µM. Red arrows = cell detachment indicated by lack of cells in areas pointed to.

4.4.1.2 Accessory Organisms

Actinomyces israelii

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *A. israelii* bacterial supernatants collected over 48 hours (Figure 4.5).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

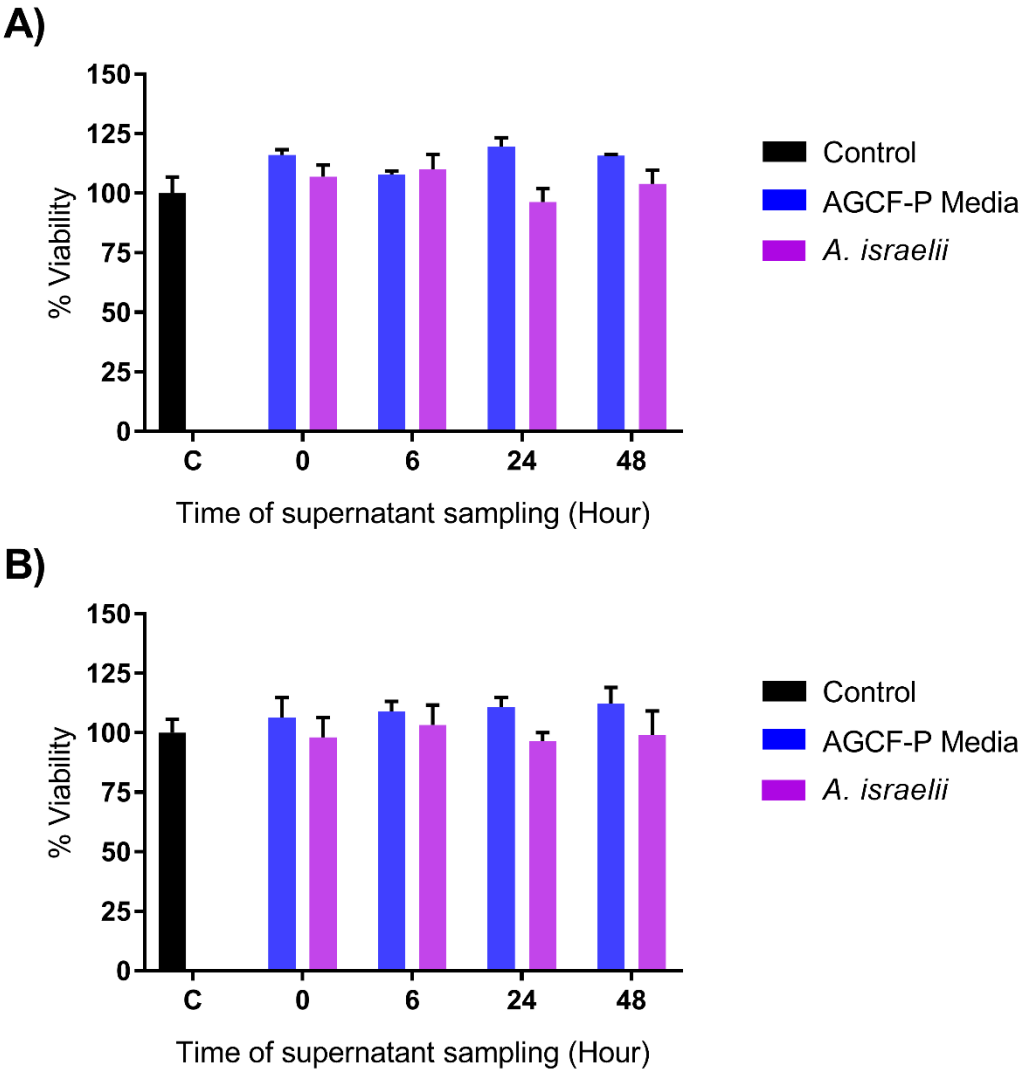

Figure 4.5 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *A. israelii* supernatants collected over 48h. Viability is presented as percentage change from the control (C: HK-2 cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

Fusobacterium nucleatum

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *F. nucleatum* bacterial supernatants collected over 48 hours (Figure 4.6).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

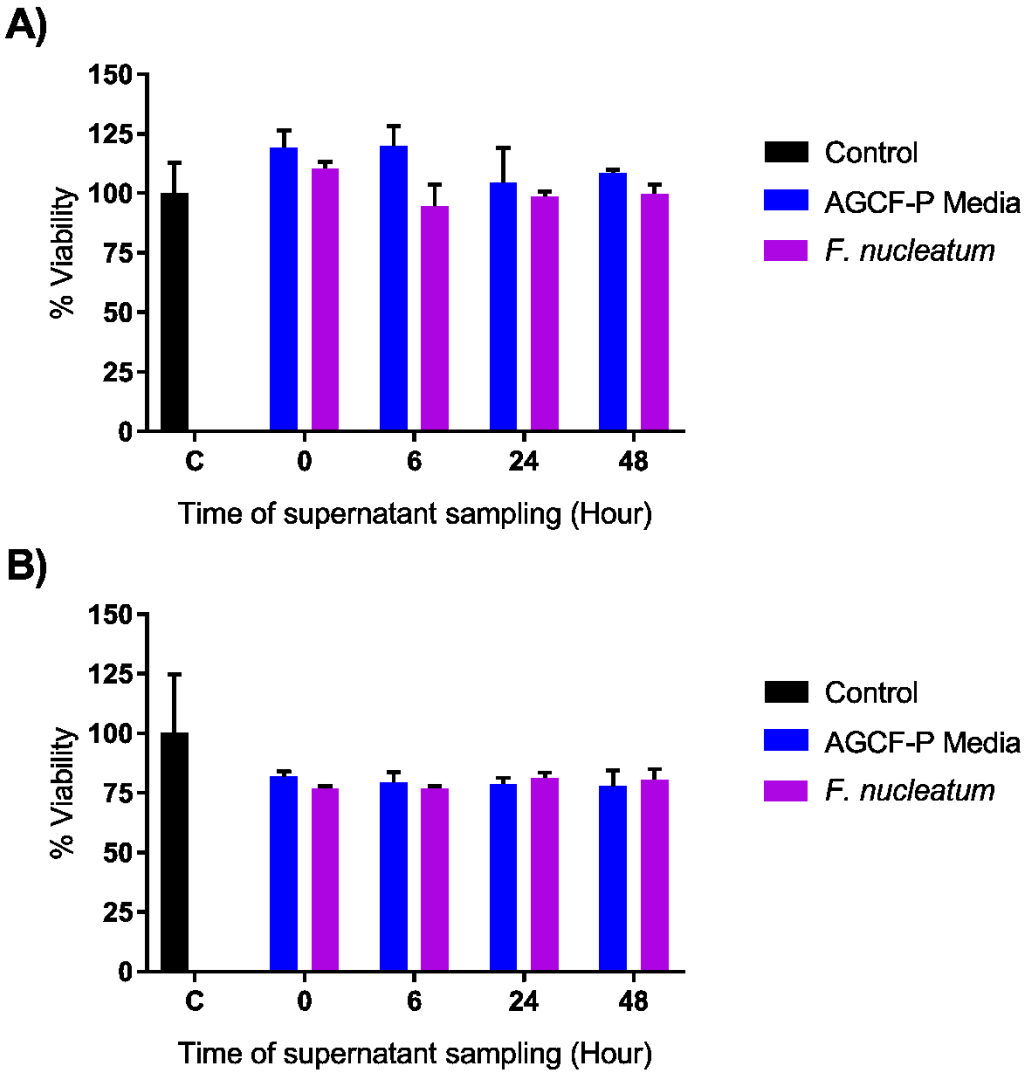

Figure 4.6 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *F. nucleatum* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

Parvimonas micra

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *P. micra* bacterial supernatants collected over 48 hours (Figure 4.7).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

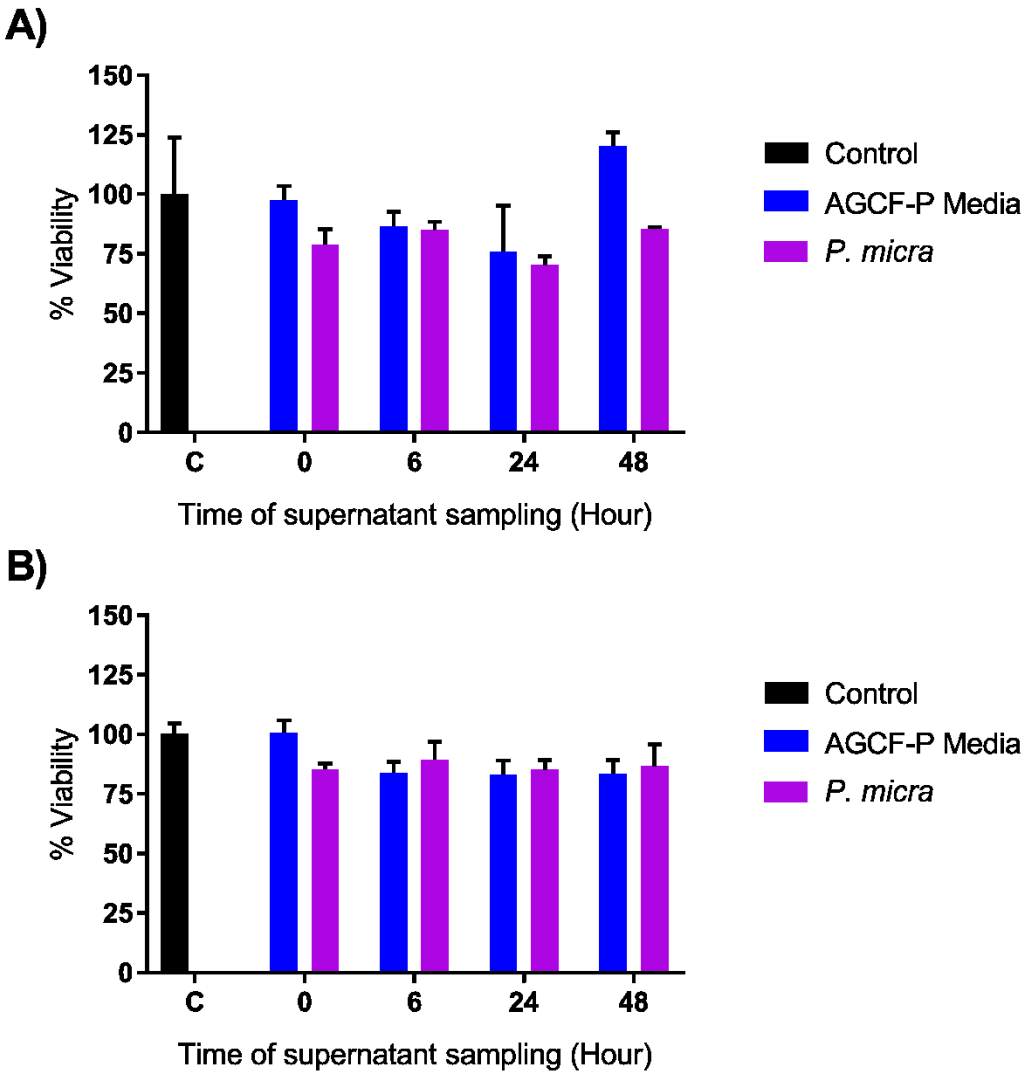

Figure 4.7 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *P. micra* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

Streptococcus constellatus

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *S. constellatus* bacterial supernatants collected over 48 hours (Figure 4.7).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

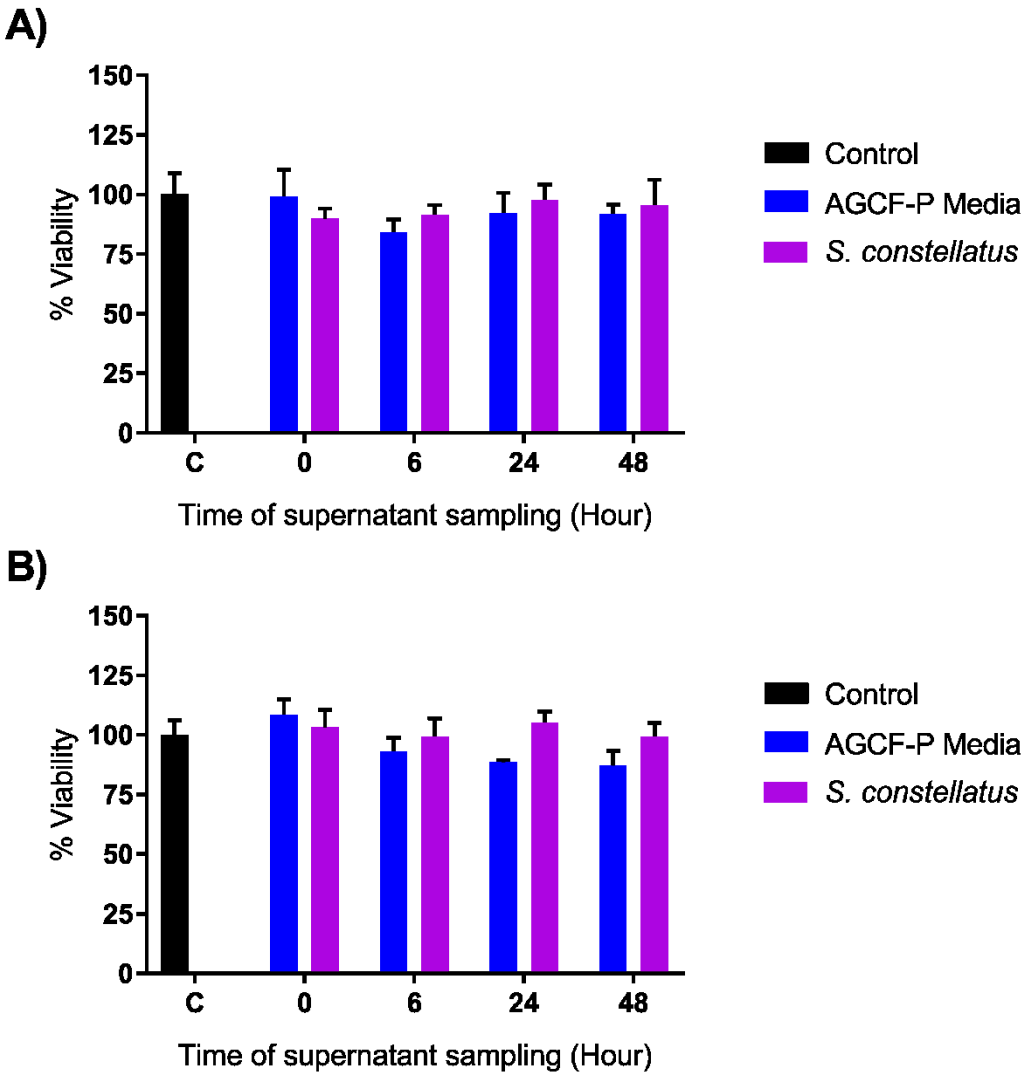

Figure 4.8 Cell viability of HK-2 cells after A) 24 h or B) 48 incubation with AGCF-P supernatants and *S. constellatus* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

Streptococcus sanguinis

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *S. sanguinis* bacterial supernatants collected over 48 hours (Figure 4.9).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

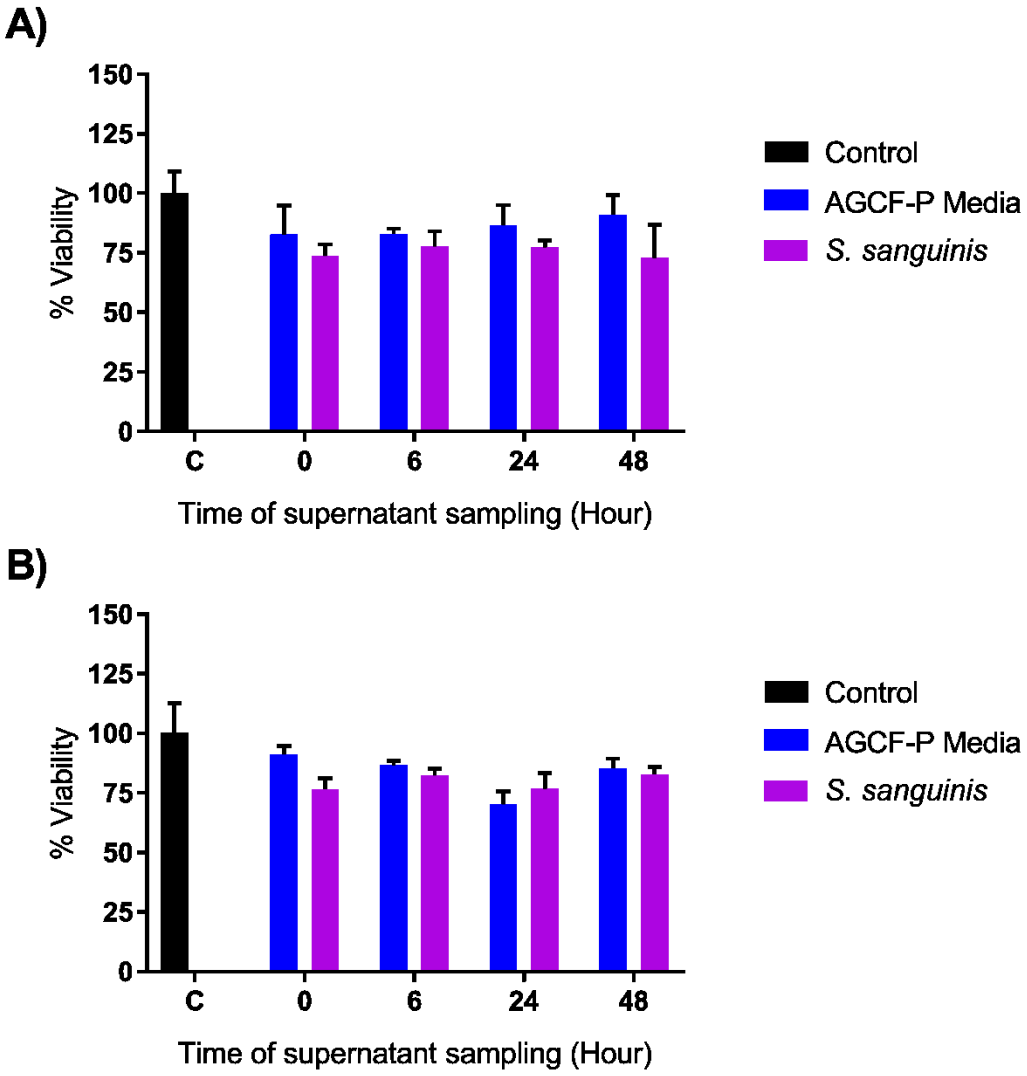

Figure 4.9 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *S. sanguinis* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

4.4.1.3 Other Organisms related to Oral disease

Enterococcus faecalis

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *E. faecalis* bacterial supernatants collected over 48 hours (Figure 4.10).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

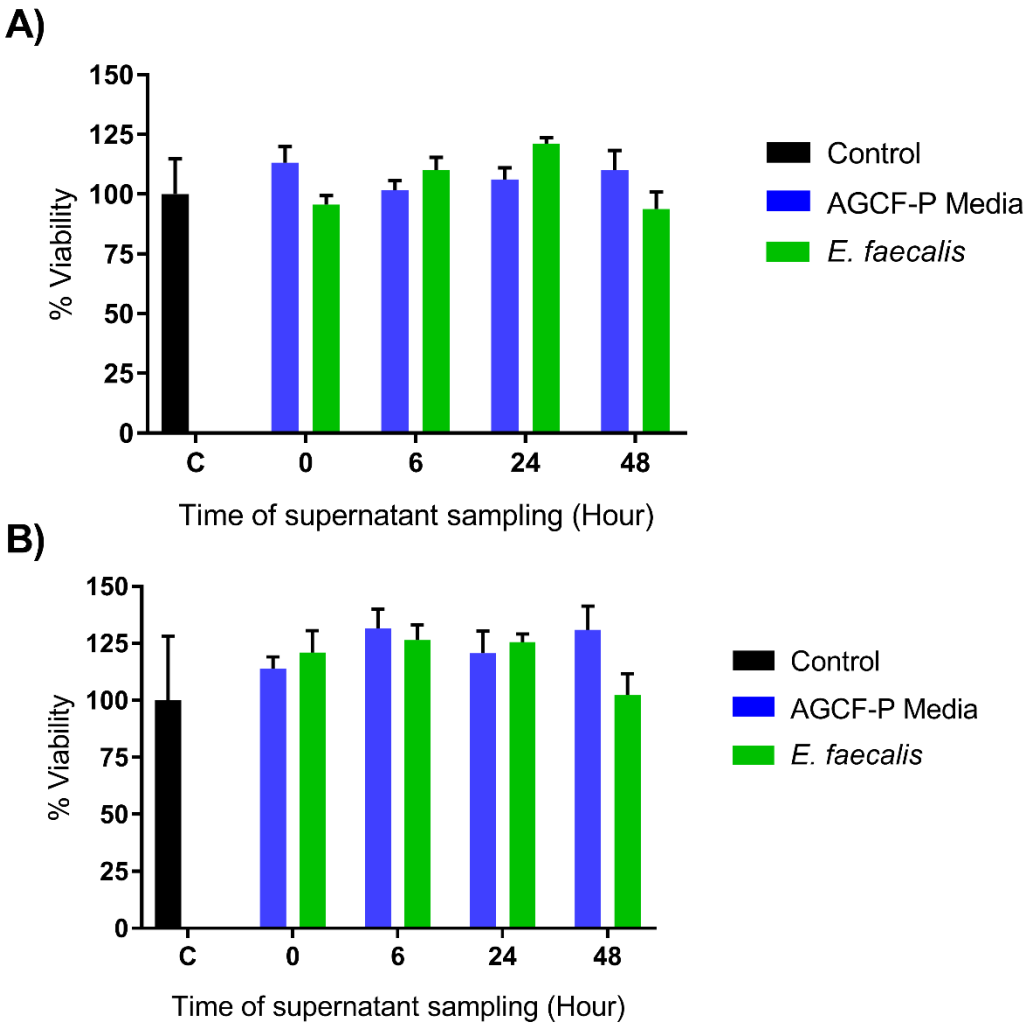

Figure 4.10 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *E. faecalis* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

Streptococcus mutans

Cell viability testing was undertaken following exposure of HK-2 cells for 24 h or 48 h to *S. mutans* bacterial supernatants collected over 48 hours (Figure 4.11).

After 24 and 48 h, statistical tests showed no significant differences between the effects of the bacterial supernatants and AGCF-P media on cellular viability in comparison with the control.

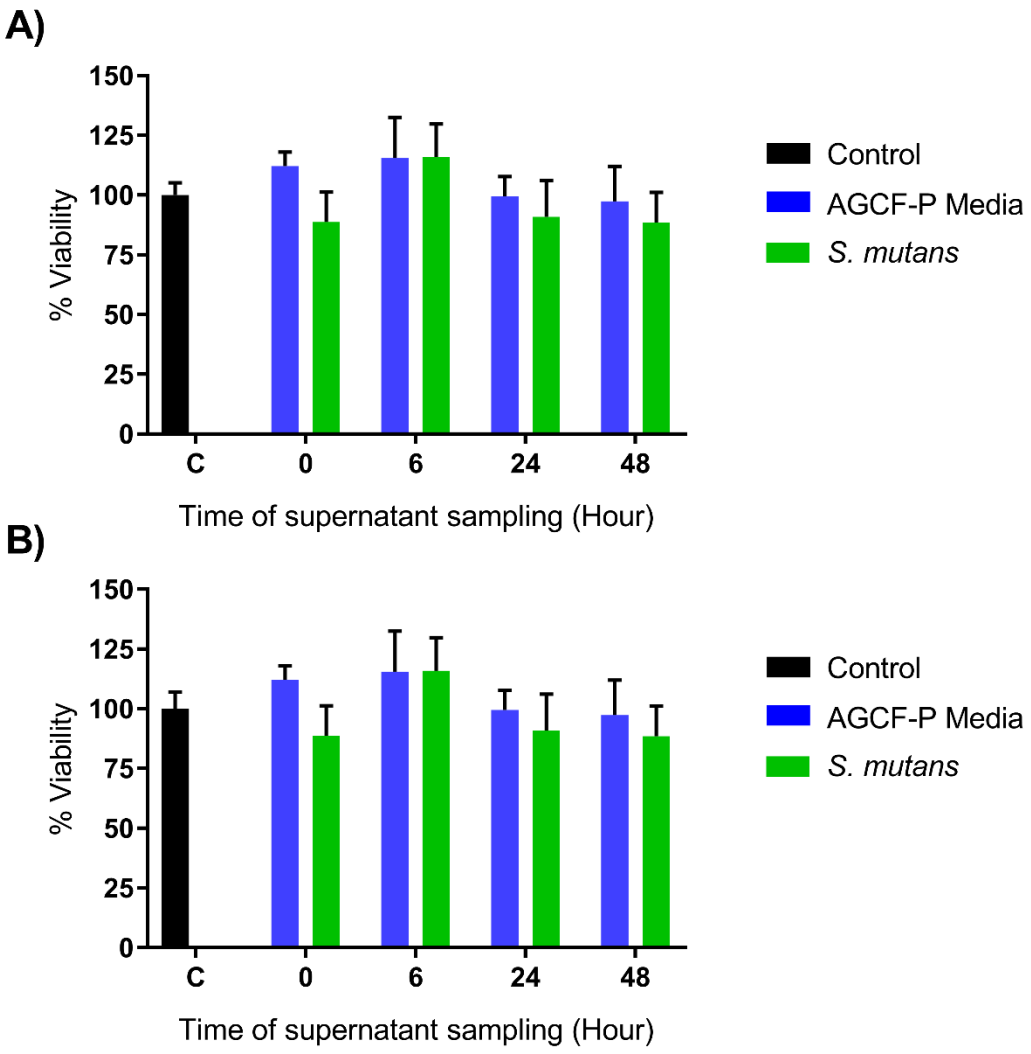

Figure 4.11 Cell viability of HK-2 cells after A) 24 h or B) 48 h incubation with AGCF-P supernatants and *S. mutans* supernatants collected over 48h. Viability is presented as percentage change from the control (C: cells exposed to serum-free cell culture medium) and error bars represent SEM; (*n=3*).

4.4.2 Cell Migration and Wound Healing Assay

The scratch wound healing assay was undertaken with all chosen microorganisms supernatants, in order to investigate the effect of the bacterial products on the migration and wound closure of human kidney cells.

4.4.2.1 Keystone Pathogen

Porphyromonas gingivalis

A cell migration assay was undertaken testing the effect of *P. gingivalis* and AGCF-P supernatants sampled for 5 D and the ability of these supernatants to effect cell migration and wound closure of HK-2 cells (Figure 4.12). Each graph represents a different day of bacterial supernatant and non-inoculated AGCF-P media supernatant sampled from 1 D to 5 D.

Statistical analysis of all scratch assays was undertaken comparing the means of the control (serum-free media), the AGCF-P media and *P. gingivalis* percentage wound closure with each other at each time point using a two-way ANOVA with multiple comparisons. All wounds achieved closure after 72 h incubation in the respective media. With the exception of cells exposed to AGCF-P medium collected at 1 D, all AGCF-P supernatant control media had a statistically significant positive effect on wound closure after 24 and 48 h when compared with cells exposed to the control serum-free medium.

For all 1 D results (Figure 4.12a) the only significant result was the 48 h AGCF-P Media vs *P. gingivalis*. For 2 D (Figure 4.12c) there were three significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs. *P. gingivalis* and 48 h Control vs. AGCF-P Media. However, there was no significant difference between the 2D 24 h *P. gingivalis* and 24 h AGCF-P Media. For 3 D (Figure 4.12d) there were three significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs. *P. gingivalis* and 48 h Control vs. AGCF-P Media. However, there was no significant difference between the 3D 24 h *P. gingivalis* and 24 h AGCF-P Media. For 4D (Figure 4.12e) there were two significant results, 24 h Control vs. AGCF-P Media and 48 h Control vs. AGCF-P Media. For 5 D (Figure 4.12f) there was two significant results, 24 h Control vs. *P. gingivalis* and 48 h

Control vs. AGCF-P Media. However, there was no significant difference between the 24 h *P. gingivalis* and 24 h AGCF-P Media.

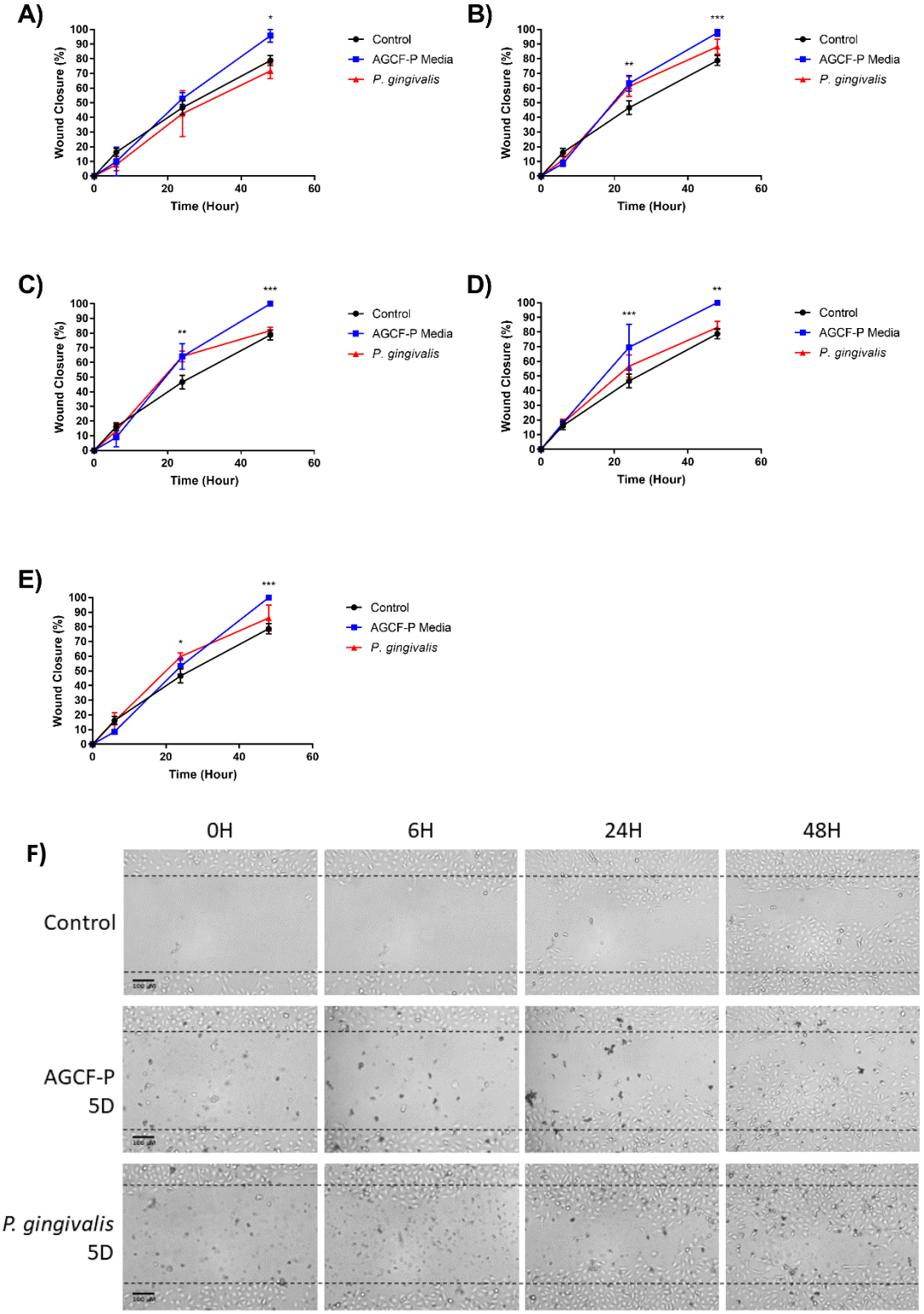

Figure 4.12 Percentage wound closure over 48 h of HK-2 cells exposed to serum-free medium alone (Black), AGCF-P media supernatants in serum-free medium (1:20) (Blue), or *P. gingivalis* supernatants in serum free medium (1:20) (Red), all concurrently collected over 5 days. A) 1 d B) 2 d C) 3 d D) 4 d E) 5 d F) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure of individual wells and error bars represent SEM. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Scale length = 200µM (*n=3*).

The observed variance from the control that was reported as statistically significant was for faster wound closure and this can be attributed to the AGCF-P media. Indeed, in most instances where there was significance reported for *P. gingivalis* supernatants, there was no statistical significance from the matched AGCF-P non-inoculated media for that time point. In two instances 2D 48 h and 5D 48 h the observed variance was statistically significant between the AGCF-P media and *P. gingivalis,* with *P. gingivalis* supernatant delaying the wound closure over time, as compared to the AGCF-P medium alone.

4.4.2.2 Accessory Organisms

Actinomyces israelii

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *A. israelii* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.13A) and 48h (Figure 4.13B) of exposure. However, the effect of the *A. israelii* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.13a) there were four significant results, 24 h Control vs *A. israelii,* 24 h Control vs AGCF-P media, 48 h Control vs *A. israelii* and 48 h Control vs. AGCF-P Media. For the 48 h supernatants (Figure 4.13b) there were four significant results, 24 h Control vs *F. nucleatum*, 24 h Control vs AGCF-P, 48 h Control vs *A. israelii* and 48 h Control vs. AGCF-P Media.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

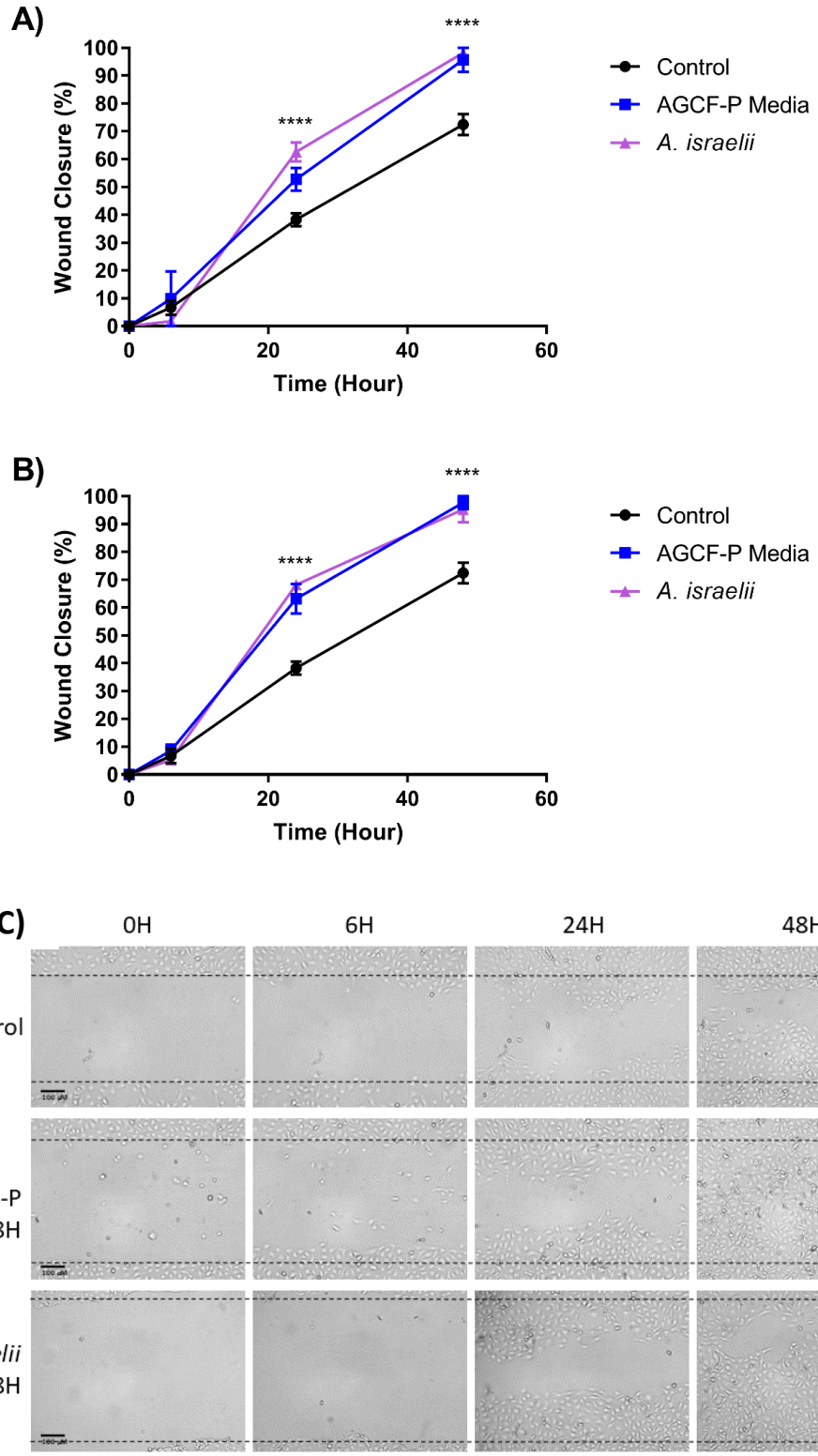

Figure 4.13 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *A. israelii* supernatants (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale length = 200µM (*n=3*).

Fusobacterium nucleatum

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *F. nucleatum* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.14A) and 48h (Figure 4.14B) of exposure. However, the effect of the *F. nucleatum* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.14a) there were two significant results, 24 h Control vs *F. nucleatum* and 48 h Control vs. AGCF-P Media. For the 48 h supernatants (Figure 4.14b) there were two significant results, 48 h Control vs *F. nucleatum* and 48 h Control vs AGCF-P Media.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

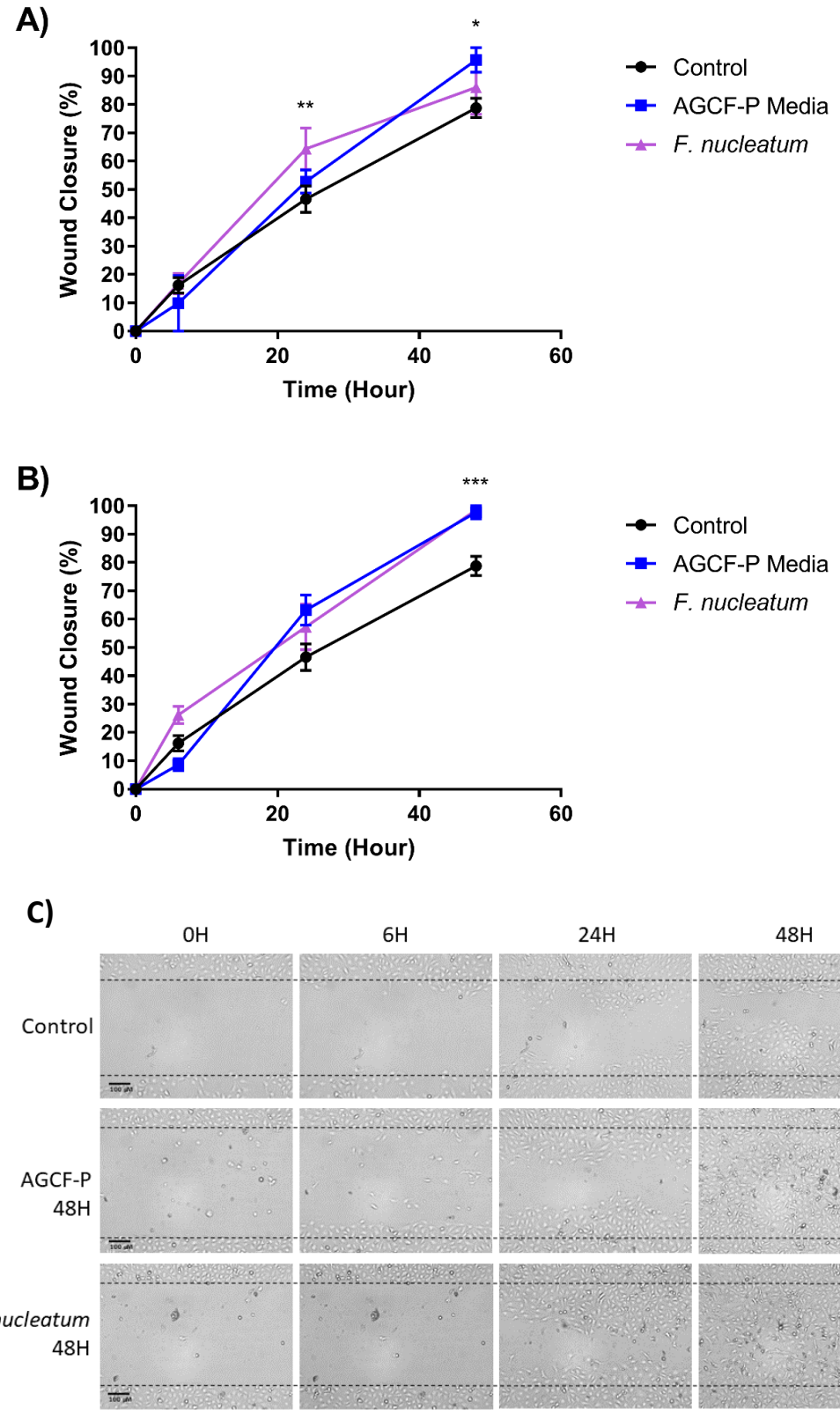

Figure 4.14 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *F. nucleatum* (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale length = 200µM (*n=3*).

Parvimonas micra

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *P. micra* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.15A) and 48h (Figure 4.15B) of exposure. However, the effect of the *P. micra* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.15a) there was one significant result, 48 h Control vs. AGCF-P Media. For the 48 h supernatants (Figure 4.15b) there were three significant results, 24 h Control vs. AGCF-P Media, 48 h Control vs. AGCF-P Media and Control vs. *P. micra*.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

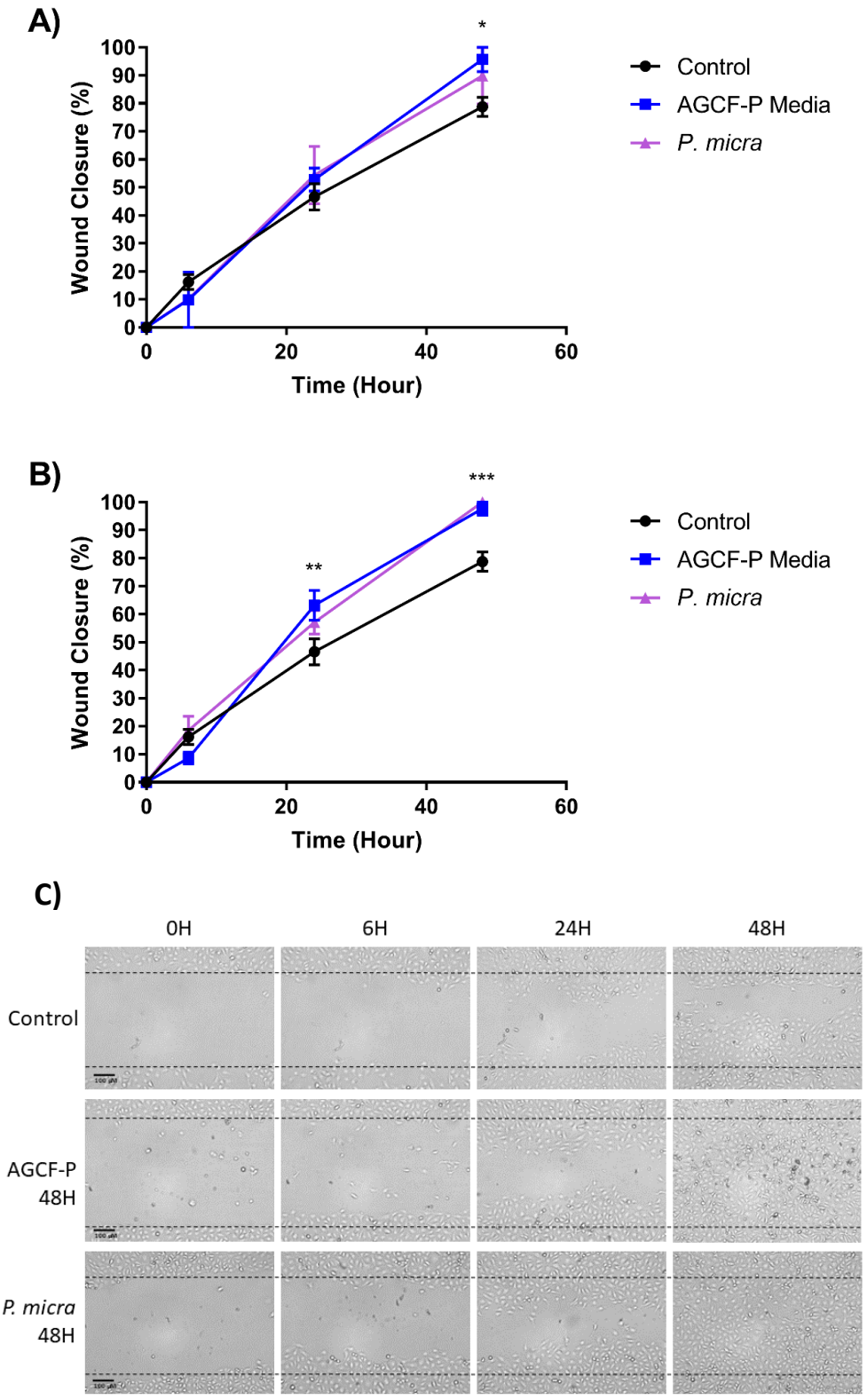

Figure 4.15 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *P. micra* (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale length = 200µM (*n=3*).

Streptococcus constellatus

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *S. constellatus* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.16A) and 48h (Figure 4.16B) of exposure. However, the effect of the *S. constellatus* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.16a) there were five significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs *S. sanguinis*, 24 h AGCF-P Media vs. *S. constellatus*, 48 h Control vs. AGCF-P Media and 48 h Control vs *S. sanguinis*. For the 48 h supernatants (Figure 4.16b) there were four significant results 24 h Control vs. AGCF-P Media, 24 h Control vs *S. sanguinis*, 48 h Control vs. AGCF-P Media and 48 h Control vs *S. sanguinis*.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media. The only exception to this is 24 h AGCF-P Media vs. *S. constellatus*.

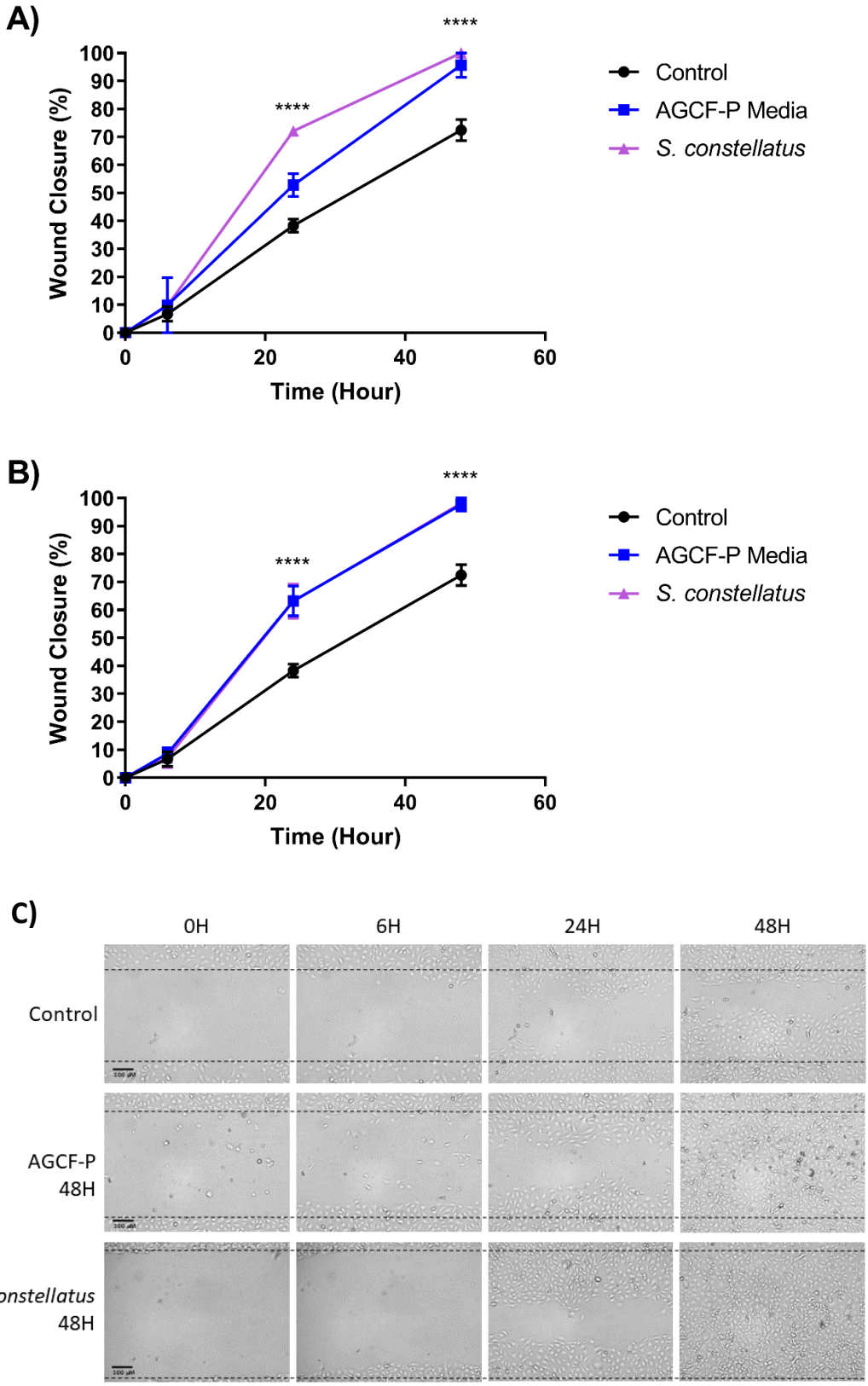

Figure 4.16 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *S. constellatus* (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale length = 200µM (*n=3*).

Streptococcus sanguinis

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *S. sanguinis* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.17A) and 48h (Figure 4.17B) of exposure. However, the effect of the *S. sanguinis* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.17a) there were two significant results, 48 h Control vs. AGCF-P Media and 48 h Control vs *S. sanguinis*. For the 48 h supernatants (Figure 4.17b) there were three significant results, 24 h Control vs. AGCF-P Media, 48 h Control vs. AGCF-P Media and 48 h Control vs *S. sanguinis*.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

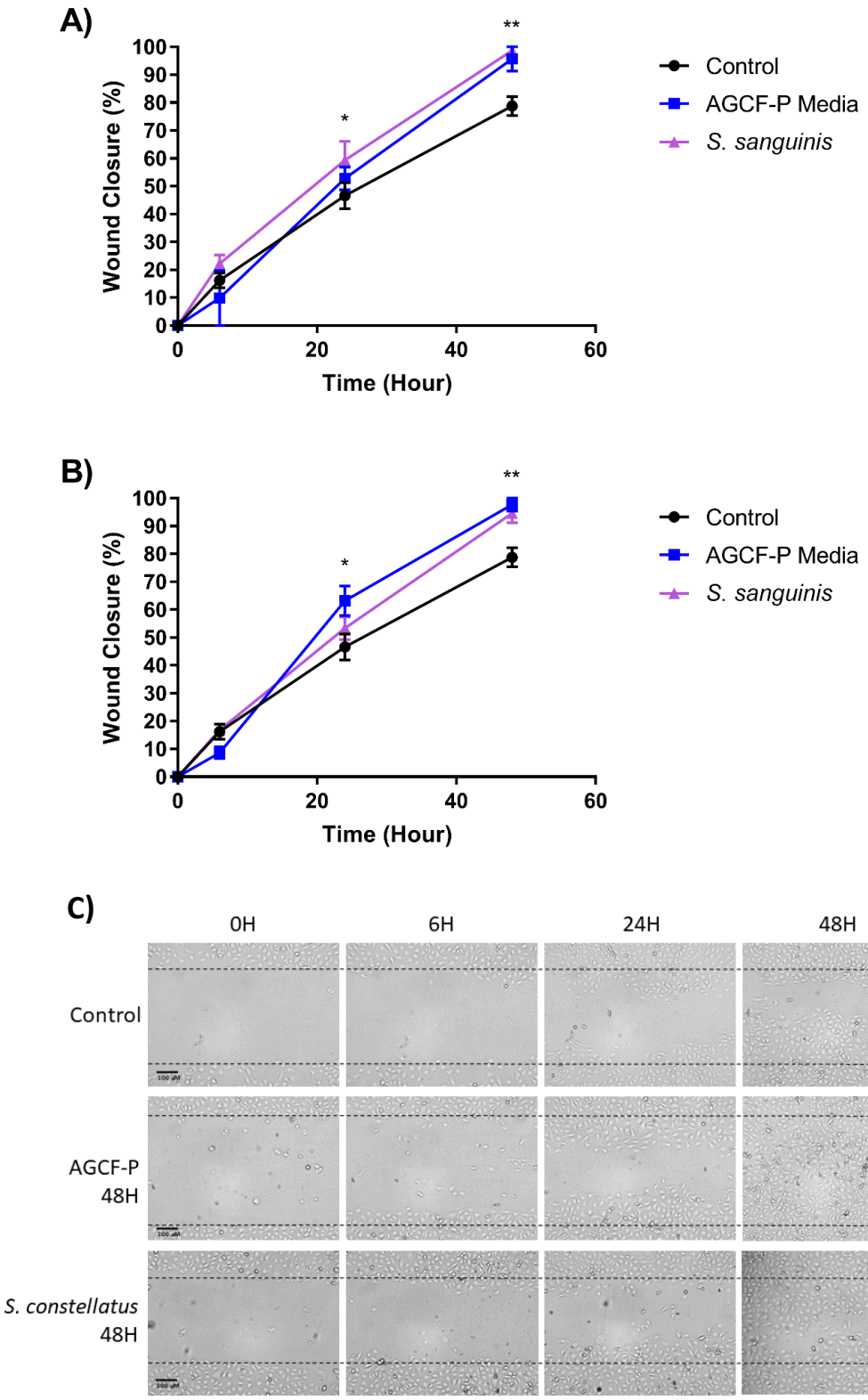

Figure 4.17 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *S. sanguinis* (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$. Scale length = 200µM (*n=3*).

4.4.2.3 Other Organisms related to oral disease

Enterococcus faecalis

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *E. faecalis* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.18A) and 48h (Figure 4.18B) of exposure. However, the effect of the *E. faecalis* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.18a) there were two significant results, 48 h Control vs. AGCF-P Media and 48 h Control vs *E. faecalis*. For the 48 h supernatants (Figure 4.18b) there were four significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs *E. faecalis*, 48 h Control vs. AGCF-P Media and 48 h Control vs *E. faecalis*.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

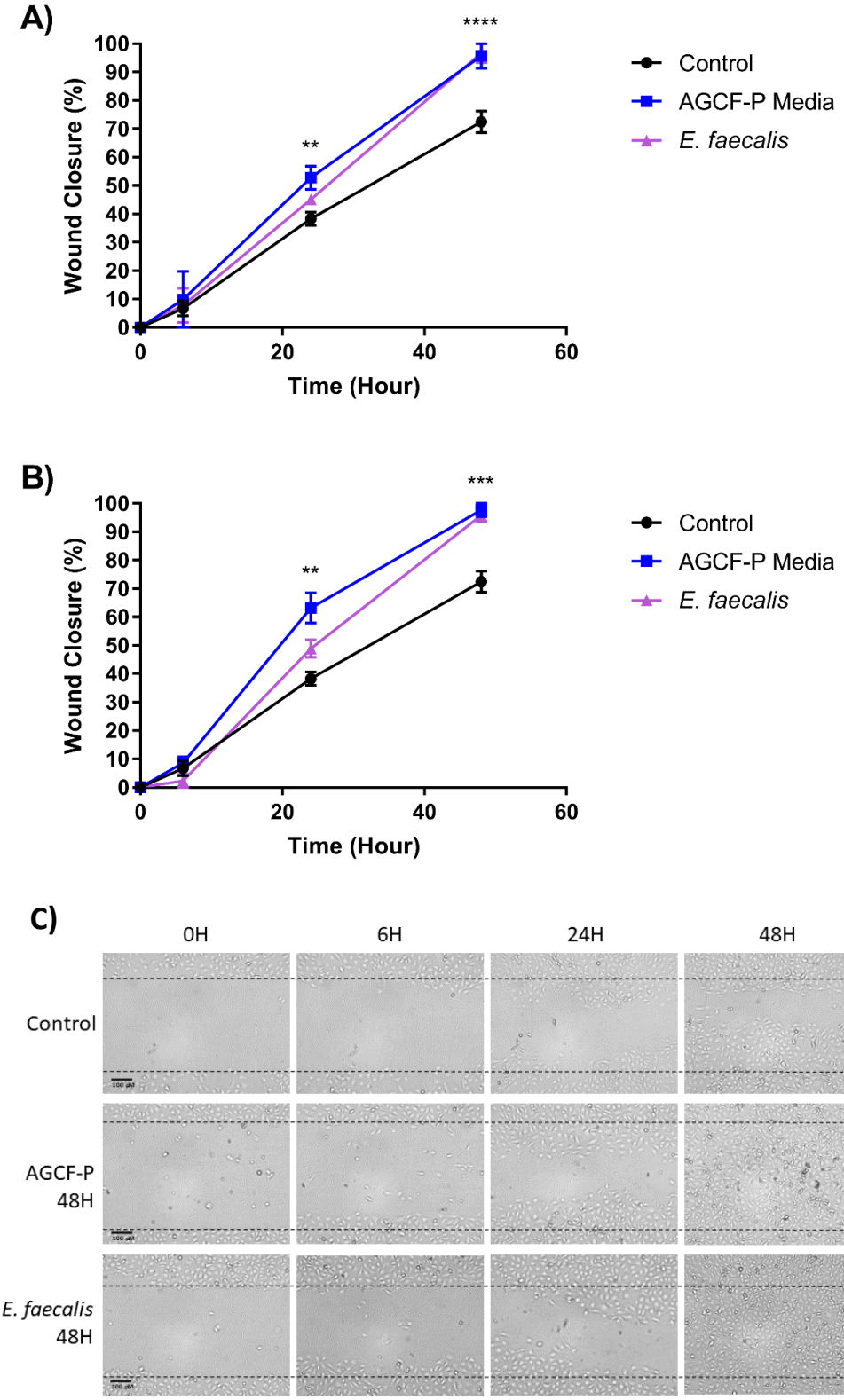

Figure 4.18 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media supernatants (Blue) and treatment with diluted *E. faecalis* (Purple). A) 24 h B) 48 h C) Representative images captured of assay, dashed line indicates starting wound. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale length = 200µM (*n=3*).

Streptococcus mutans

A cell migration assay demonstrated that in comparison to the control serum free medium, supernatants from *S. mutans* sampled at 24 h to 48 h significantly accelerated wound closure of HK-2 cells after 24h (Figure 4.19A) and 48h (Figure 4.19B) of exposure. However, the effect of the *S. mutans* supernatants were not significantly different from the wound closure effect of the non-inoculated AGCF-P media.

Statistical analysis of all scratch assay results were compared to the control (no addition). For the 24 h supernatants (Figure 4.19a) there were four significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs *S. mutans*, 48 h Control vs. AGCF-P Media and 48 h Control vs. *S. mutans*. For the 48 h supernatants (Figure 4.19b) there were four significant results, 24 h Control vs. AGCF-P Media, 24 h Control vs *S. mutans*, 48 h Control vs. AGCF-P Media and 48 h Control vs *S. mutans*.

However, in all instances where there was significance for bacterial supernatants there was no significant difference between the bacterial supernatant and AGCF-P media.

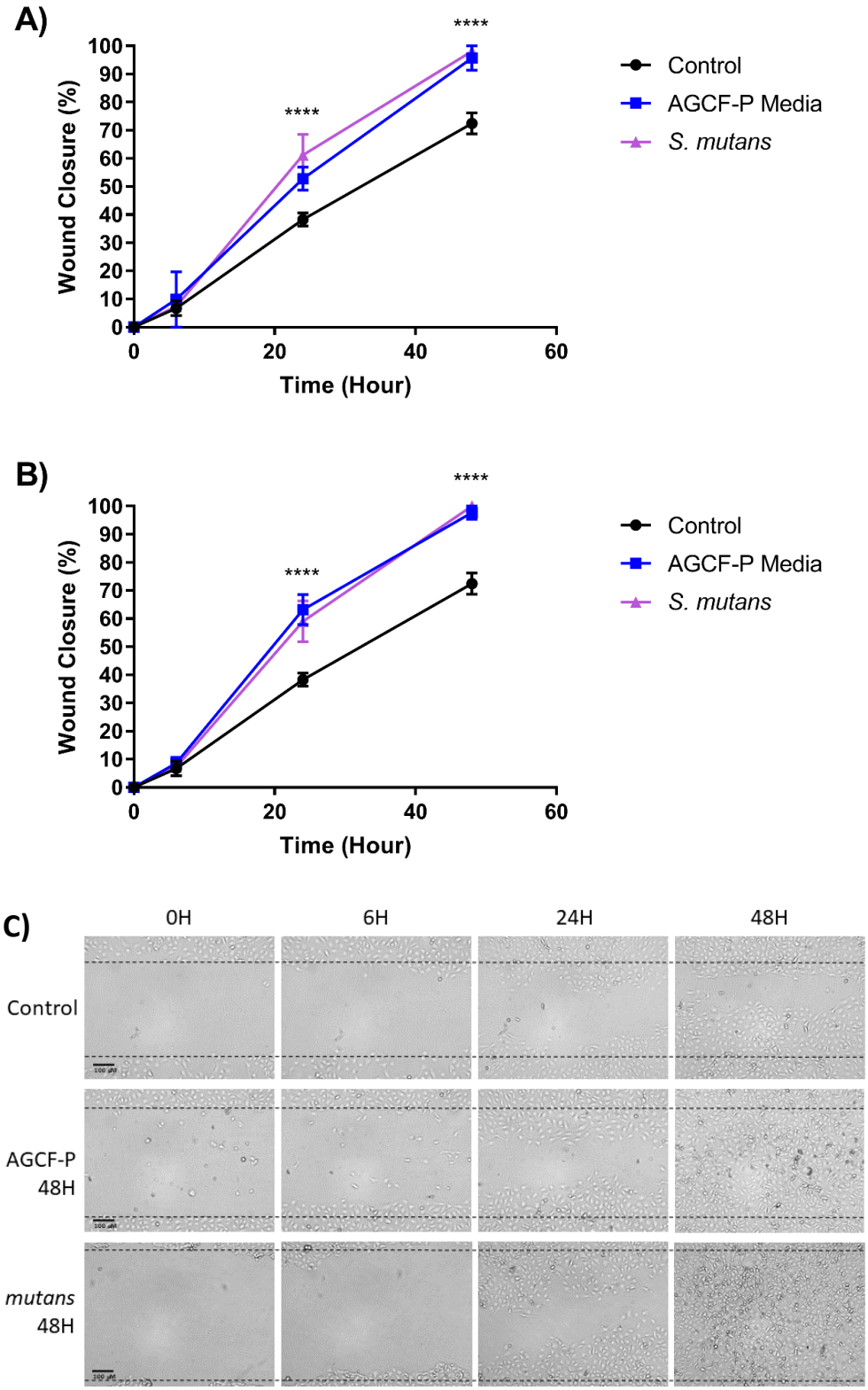

Figure 4.19 Percentage wound closure over 48 h of HK-2 cells incubated with serum-free medium (Black), treatment with diluted AGCF-P media (Blue) and treatment with diluted *S. mutans* supernatants (Purple). A) 24 h B) 48 h. Wound closure is presented as percentage closure in individual wells and error bars represent SEM. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Scale length = 200µM (*n=3*).

4.4.3 PAI-1 Luciferase Reporter Assay

A PAI-1 luciferase reporter assay was undertaken to examine the effect of bacterial products on the expression of the marker for fibrosis (PAI-1) in human kidney cells over 24 h. Diluted supernatants of *P. gingivalis*, *F. nucleatum* and *S. mutans* were incubated with HK-2 cells transfected with either the native reporter vector pNL2.3 or with the PAI-1-pNL2.3 reporter construct. Only three microorganisms were chosen due to time constraints and the cost of the components used. The three microorganisms represent each of the three categories of microorganisms investigated so far in this study: keystone pathogens, accessory organisms and other organisms related to disease.

4.4.3.1 Testing the Transfected Cell Lines

To evaluate the effectiveness of the PAI-1-pNL2.3 construct in reporting on the transcription regulation of PAI-1, soluble luciferase activity was measured in supernatants of transfected HK-2 cells exposed either to serum free medium or to AGCF-P supernatants. The AGCF-P supernatants had been collected over 5 days and were incubated for 24 h with HK-2 cells transfected either with PAI-1-pNL2.3 or with the native pNL2.3 vector. Luminescence was quantified in the HK-2 cell supernatants and it was then normalised per mg of total cellular protein (Figure 4.20). The results demonstrate that no detectable luciferase was secreted by cells transfected with the native pNL2.3 (PNL) vector neither in response to serum free medium, nor AGCF-P supernatants. In contrast, HK-2 cells transfected with the PAI-1 reporter construct demonstrated significant fluorescence in response to serum free and AGCF-P media. Although there was no significant difference of PAI-1 transcription regulation between the different AGCF-P media collection dates, all AGCF-P media significantly upregulated the expression of PAI-1, as compared to serum-free medium treatment.

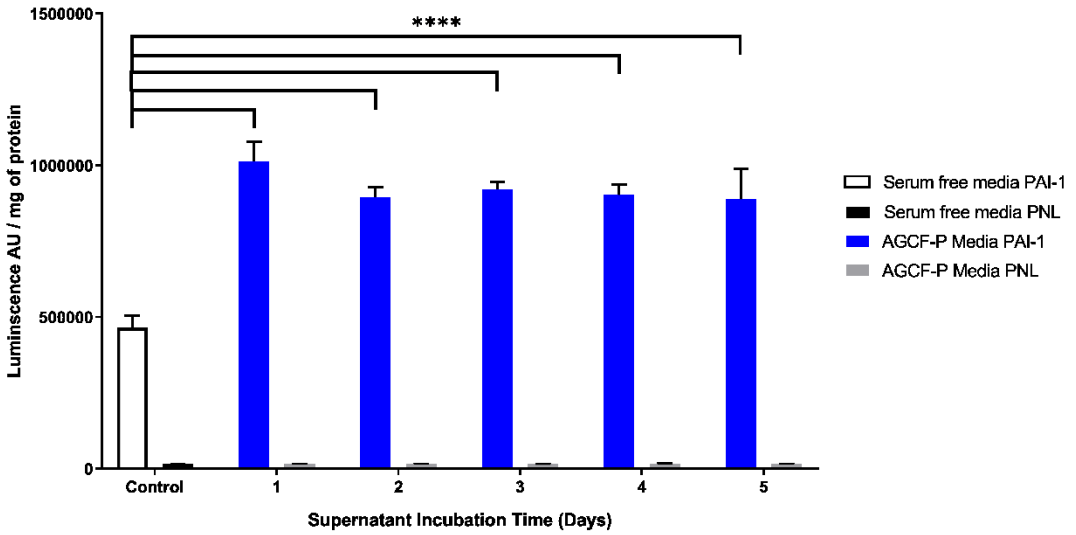

Figure 4.20 Effect of AGCF-P supernatants on the transcription regulation of PAI-1 expression. HK-2 cells transfected either with the PAI-1-pNL2.3 reporter construct (PAI-1) or with native pNL2.3 vector (PNL) were exposed for 24 h to AGCF-P supernatants collected over 5 days. The soluble luciferase activity (presented as Luminescence Arbitrary Units normalised to mg of total cellular protein) was measured in HK-2 supernatants. Serum Free Media PAI-1 (Black), PAI-1 transfected HK-2 (Blue) and PNL transfected HK-2 (Grey). Error bars represent SEM; (*n=3*).

4.3.3.2 Keystone Pathogen

The transcription regulation of PAI-1 was measured for *P. gingivalis* bacterial supernatants and AGCF-P control supernatants collected over 5 days against HK-2 cells transfected with the PAI-1-pNL2.3 construct (Figure 4.21). The results demonstrate that after 24 h, all AGCF-P media and *P. gingivalis* supernatants have statistically significant differences compared with serum free media indicating increased PAI-1 expression. However, there is no significant difference between all matched AGCF-P media and *P. gingivalis* supernatants indicating that the AGCF-P media was responsible for the increased PAI-1 expression.

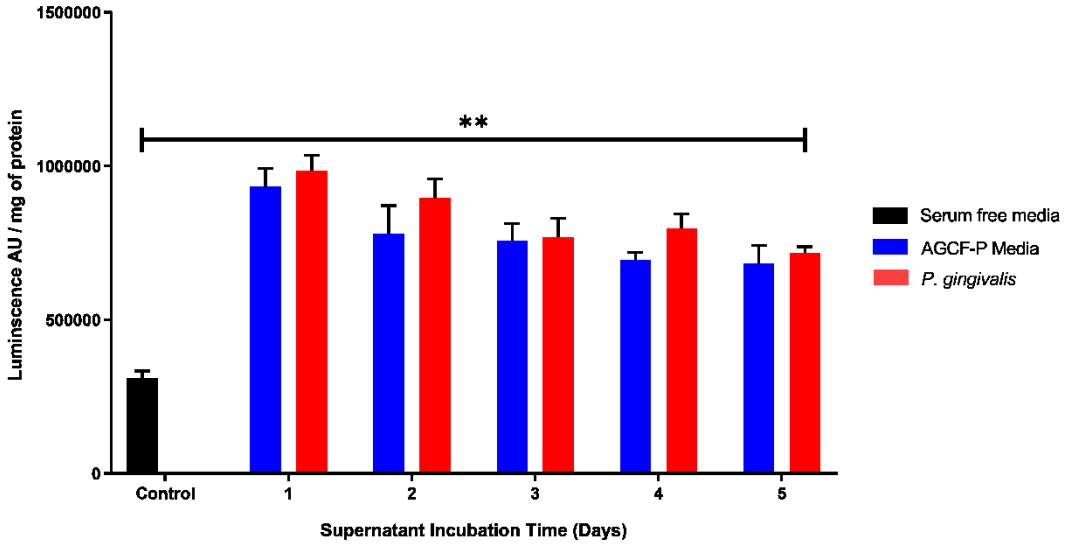

Figure 4.21 Effect of AGCF-P and *P. gingivalis* supernatants on the transcription regulation of PAI-1 expression. HK-2 cells transfected with the PAI-1-pNL2.3 reporter construct (PAI-1) were exposed for 24 h to AGCF-P and *P. gingivalis* supernatants collected over 5 days. The soluble luciferase activity (presented as Luminescence Arbitrary Units normalised to mg of total cellular protein) was measured in HK-2 supernatants. Serum Free Media (Black), AGCF-P supernatants (Blue) and *P. gingivalis* supernatants (Red). |-| indicates significance between the first bar and all others. Error bars represent SEM ** $p \leq 0.01$; *(n=3)*.

4.3.3.3 Other Microorganisms

The transcription regulation of PAI-1 was measured for *F. nucleatum* and *S. mutans* bacterial supernatants along with AGCF-P control supernatants collected over 48 hours against HK-2 cells transfected with the PAI-1-pNL2.3 construct (Figure 4.22). The results demonstrate that after 24 h, all AGCF-P media along with for *F. nucleatum* and *S. mutans* supernatants have statistically significant differences compared with serum free media indicating increased PAI-1 expression. However, there is no significant difference between all matched AGCF-P media and bacterial supernatants indicating that the AGCF-P media was responsible for the increased PAI-1 expression.

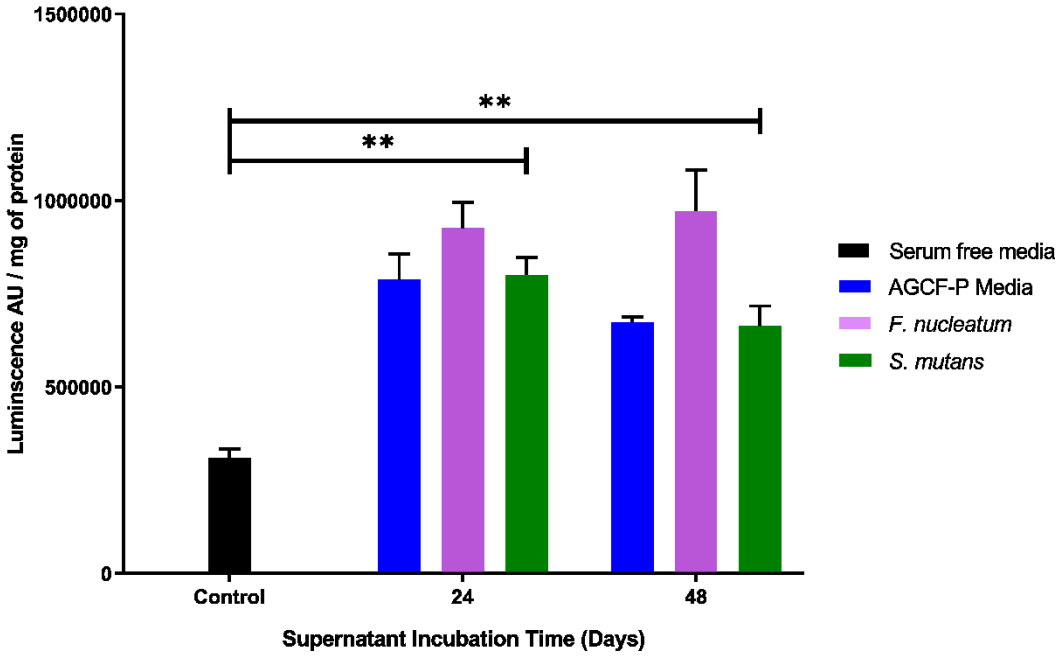

Figure 4.22 Effect of AGCF-P, *F. nucleatum* and *S. mutans* supernatants on the transcription regulation of PAI-1 expression. HK-2 cells transfected with the PAI-1-pNL2.3 reporter construct (PAI-1) were exposed for 24 h to AGCF-P and *P. gingivalis* supernatants collected over 5 days. The soluble luciferase activity (presented as Luminescence Arbitrary Units normalised to mg of total cellular protein) was measured in HK-2 supernatants. Serum Free Media (Black), AGCF-P supernatants (Blue), *F. nucleatum* supernatants (Pink) and *S. mutans* supernatants (Green). |-| indicates significance between the first bar and all others. Error bars represent SEM. ** $p \leq 0.01$; *(n=3)*.

4.5 DISCUSSION

4.5.1 Cell Viability

The results of all the cell viability assays indicated that only the *P. gingivalis* supernatants had a statistically significant effect on the viability of the kidney cells. This corresponds with evidence of the cytotoxicity of *P. gingivalis* supernatants to various human cell lines such as human gingival fibroblasts (Desta and Graves, 2007, Morioka et al., 1993), human aortic endothelial cells (Roth et al., 2007) and various human urothelial cells (Shah et al., 1992). One of these studies investigated various components of *P. gingivalis* on three different urothelial cell lines and identified a few different virulence factors that could have cytotoxic effects on cells such as LPS, and Gingipain preparations with heterogeneity between cell type with epithelial cells less effected (Shah et al., 1992, Johansson et al., 1996). There has also been reports of heterogeneity between *P. gingivalis* subtypes, with *P. gingivalis* 33277 (NCTC 11834 - used in this study) being more cytotoxic than *P. gingivalis* 381 and *P. gingivalis* W50 which has been suggested is due to the different immunogenicity of the serotypes (Johansson et al., 1996, Ebersole and Steffen, 1995).

The majority of these reported studies have identified the main cause of this *P. gingivalis* cytotoxicity to be protein based (Chen et al., 2001, Desta and Graves, 2007, Morioka et al., 1993), with a cytotoxic effect that could be dose-dependently neutralised by heat inactivation (Johansson et al., 1996) or the addition of anti-sera (Chen et al., 2001) . Furthermore, several studies have identified *P. gingivalis* specific Gingipains as one of the major causes of this protein mediated cytotoxicity (Desta and Graves, 2007, Morioka et al., 1993, Chen et al., 2001). It is reported that these Gingipains are secreted in outer membrane vesicles and are capable of degradation of the intracellular matrix (Johansson et al., 1996, Chen et al., 2001) and induction of apoptosis (Desta and Graves, 2007) in human cells.

Indeed, alongside visible cell death, morphology changes were also observed in cells treated with *P. gingivalis* supernatants compared to those treated with AGCF-P media and serum media alone. These results are in agreement with evidence in the literature that *P. gingivalis* supernatants can induce morphological changes in

gingival fibroblasts to become rounded (Yamasaki et al., 1998). It is suggested that the cause of these effects could be related to degradation of E-cadherin and the intracellular matrix (Chen et al., 2001) and this may account for the increased HK-2 cell detachment observed in this study as a result of *P. gingivalis* supernatant treatments. However, it has also been suggested that the mechanism of cellular morphology changes is different from that of cell death (Morioka et al., 1993) or bacteria induced apoptosis (Desta and Graves, 2007).

4.5.2 Cell Migration

The results of the wound healing assays demonstrated that the AGCF-P media alone increased the migration compared to serum free media. This is most likely due to the AGCF-P medium containing more nutrients and potential growth factors from the addition of blood, in comparison to serum free media, potentially inducing cellular proliferation and migration (Brunner et al., 2010). In contrast to the other bacterial supernatants that mirrored the AGCF-P media, two *P. gingivalis* supernatants and the 24 h *S. constellatus* supernatant had a significant effect on wound closure. The 24 h *S. constellatus* supernatant at 24 h had a statistically significant positive effect on the migration and proliferation of HK-2 cells and this could potentially be due to the production of fermented sugars which could enhance growth (Vos et al., 2011). There were also two significant reductions in migration of cells treated with *P. gingivalis* supernatants (Figure 4.12), compared with the AGCF-P media control. These instances may be the result of the cytotoxic activity of the *P. gingivalis* supernatants (Section 4.4.1) counteracting the positive effect of the AGCF-P media, especially as the viability of cells was not known.

4.5.3 PAI-1 Luciferase Reporter Assay

The successful transfection of HK-2 cells with the PAI-1 reporter vector allowed for the establishment of a fast assay indicative for fibrosis. The test can be completed in a relatively short amount of time and is specifically useful for screening compounds for pro-fibrotic effects along with being successful in prior publications (Abe et al., 1994). Of the three microorganisms tested, none of them appeared to be inducing the increased production of PAI-1 in comparison with the AGCF-P media control. However, this does not necessarily mean PAI-1 is not being induced as this test is an

indicator of gene transcription, with protein translation being regulated by miRNA which could be interfered with by the complexity of the microbial growth medium used. Indeed, as PAI-1 is one of many markers associated with fibrosis there is also a possibility that while PAI-1 is not induced, other pro-fibrotic markers could be induced by a variety of microbial secreted products so it still warrants further testing.

After normalisation of the data, it is clear that the AGCF-P medium is inducing the production of PAI-1 and this highlights that this assay may not be useful for the analysis of complex microbial samples. The induction of PAI-1 by the AGCF-P media does not necessarily mean the AGCF-P media is causing fibrosis; it may be that the chosen indicator of fibrosis is too non-specific and the test is too sensitive, especially as PAI-1 can be induced by a variety of different non-fibrosis related stimuli. This may be because of AGCF-P is a complex medium which most likely has a selection of non-specific stimuli such as growth factors and metabolic factors such as glucose present from the addition of blood and this could be interfering with the production of PAI-1 (Eddy and Fogo, 2006, Ghosh and Vaughan, 2012) (Figure 4.23).

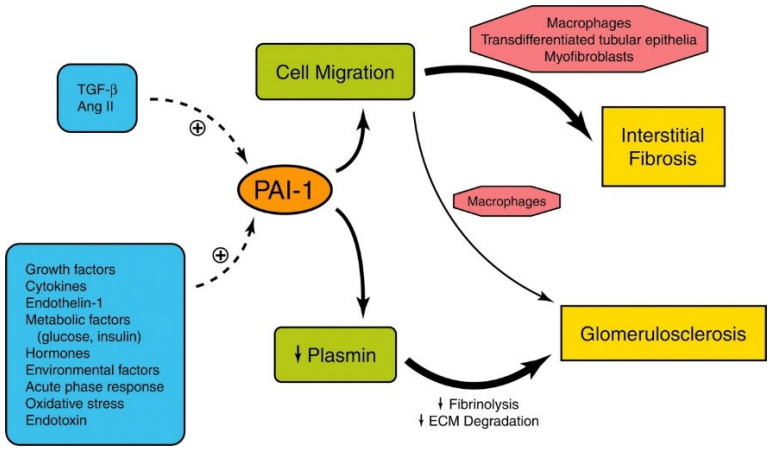

Figure 4.23 Summary of potential ways PAI-1 production can be induced and its interactions in the pathogenesis of renal fibrosis. Taken from (Eddy and Fogo, 2006).

Despite some drawbacks, this luciferase reporter assay was utilised in order to provide an early indicator of potential fibrotic effects and while the assay appeared to work the consistency of AGCF-P medium may be interfering. In order to provide a real indicator of the induction/moderation of pro-fibrotic effects a wide selection of protein and genetic markers would need to be quantitatively investigated, including PAI-1.

4.6 CONCLUSIONS

The utilisation of cell-based assays for evaluation of the viability, proliferation and migration effects of all chosen microbial supernatants against HK-2 cells were successful in screening these organisms for harmful effects. From these tests it has been possible to narrow down the number of organisms that had secreted products capable of eliciting a damaging effect to kidney cells to only *P. gingivalis*, with significant cell death observed. This leads onto investigating the causative agent/agents behind this injury with particular interest in Gingipains, investigated in the final research chapter (Chapter 5).

However, the developed reporter assay for the fibrosis associated PAI-1 gene did not allow elucidation of potential fibrotic activity of the selection of microbial supernatants due to high background reading with the utilised microbial growth medium AGCF-P. This problem is most likely due to the relatively broad nature of how PAI-1 can be activated which has led to the AGCF-P media being responsible for a 'pro-fibrotic effect' and potentially masking any pro-fibrotic effects from the microbial supernatants. In order to investigate whether these microbial supernatants can elicit any pro-fibrotic effects it would be of interest to use a more specific test for a wider selection of pro-fibrotic markers such as quantitative reverse transcriptase polymerase chain reaction (qPCR); this forms the content of the final research chapter (Chapter 5).

CHAPTER 5: ELUCIDATION OF THE CAUSE OF EFFECT

5.1 INTRODUCTION

It is clear that oral microorganisms in periodontal disease are the main aetiological agents of disease and that they have the potential to damage the host in CP itself and in a multitude of other associated diseases (Chapter 1). However, of the chosen microorganisms investigated in this study, *P. gingivalis* supernatants were the only ones cytotoxic to kidney cells (Chapter 4). In order to understand what component/s of *P. gingivalis* is causing damage it is important to consider all potential virulence factors of this microorganism and identify those that could be isolated in the microbial supernatants. As *P. gingivalis* has been identified as one of the keystone pathogens in CP and a key member of the 'red complex', many of its virulence mechanisms such are well reported (Hajishengallis and Lamont, 2012). Figure 5.1 depicts the wide variety of reported virulence mechanisms that *P. gingivalis* possesses such as direct translocation, LPS, fimbriae, outer membrane vesicles and peptidoglycan and highlights how these may interact with the human host. The capacity for the involvement of *P. gingivalis* with CKD via these virulence factors are discussed in depth in this chapter.

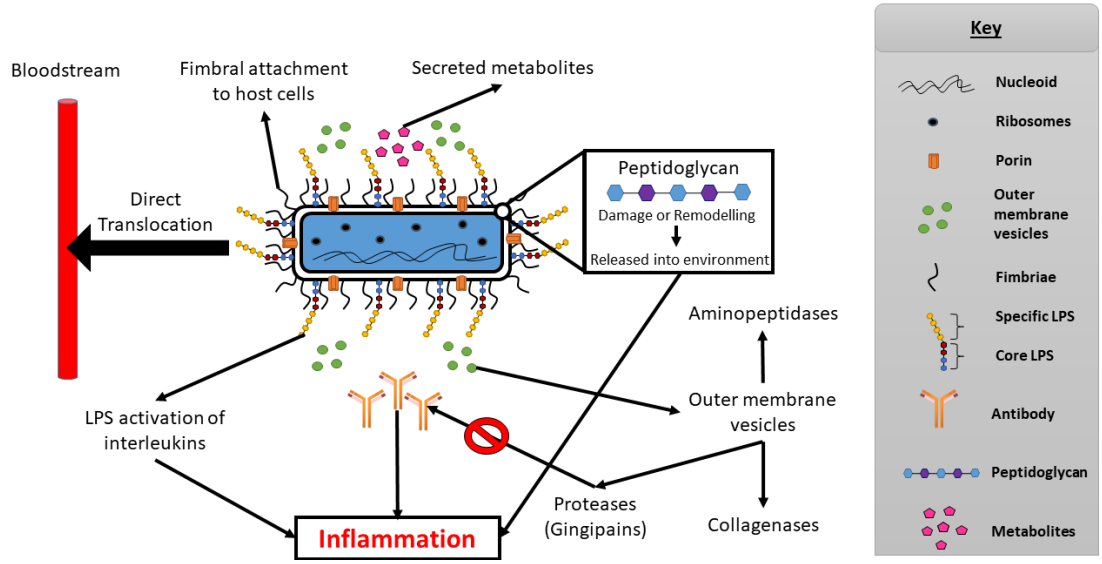

Figure 5.1 The virulence mechanisms of *Porphyromonas gingivalis* and how these may interact with the human host.

5.1.1 Evidence for Direct Translocation and Invasion

Currently there is no evidence showing the direct translocation of oral microorganisms to the kidneys. However, there is evidence for the translocation and invasion of periodontitis associated microorganisms in a variety of human cell types not present in the oral cavity. *P. gingivalis* has been reported to be able to invade the following human cell lines: coronary artery (Dorn et al., 1999), osteoblasts (Zhang et al., 2010), oral epidermoid epithelial (Dorn et al., 2000, Duncan et al., 1993), umbilical vein endothelial (Dorn et al., 2000) and gingival fibroblasts (Amornchat et al., 2003). Indeed, a causal link between CP and atherosclerosis has been suggested as the genomic DNA of *P. gingivalis* has been found in atherosclerotic plaques (Velsko et al., 2014), along with viable bacteria of invasive *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* (Kozarov et al., 2005).

It is clear that oral bacteraemia is a common event in diseases such as CP (Chapter 1.2.2) and with the mechanistic nature of the cardiovascular system; it is highly plausible that periodontal and oral microorganisms that enter the blood could encounter a part of the kidney such as the glomerulus or tubules. For example, in individuals who already have kidney disease the glomeruli can be damaged (López-Novoa et al., 2011) which could allow translocated organisms to penetrate further into the kidney to the interstitial, tubular and vascular compartments. With *P. gingivalis* able to adhere and invade a variety of oral and non-oral human tissues, it is possible that this organism has the capacity to adhere and/or invade kidney epithelial cells, which has similarities with oral epithelial cells. There is also evidence that *P. gingivalis* can degrade the cell junctional complexes of human gingival epithelial cells allowing access to deeper structures (Katz et al., 2002b); these are the same junctional proteins that exist in kidney epithelial cells (Katz et al., 2000).

5.1.2 Evidence of Acquired Immune Response to Periodontal Pathogens in Relation to CKD

Evidence from cross-sectional studies of serum antibody titres to periodontal pathogens in cohorts with CP and CKD, highlights a link between CP and CKD. In one such study of elderly individuals in Japan (mean age of aged 79 yrs), individuals with elevated serum antibody levels to *P. gingivalis* were 2.6 times more likely to have

CKD and decreased kidney function when compared with other quartiles (Iwasaki et al., 2012). High serum IgG levels to *P. gingivalis*, *T. denticola* and *A. actinomycetemcomitans* are significantly associated with impaired kidney function when adjusted for confounders in a similar elderly community living population (Kshirsagar et al., 2007). However, a larger cross-sectional study of 4,053 American adults all aged 40 or older (mean age of 57.4 yrs) showed that 9% of the population had CKD and 9% had CP, while 24% had high *A. actinomycetemcomitans* titres and 22% had high *P. gingivalis* titres (Fisher et al., 2008). Of these results, a low antibody titre of *A. actinomycetemcomitans* was found to be statistically significant as a risk factor for CKD.

While most of these studies appeared to show alternate correlations between individual periodontal organisms and CKD it is important to consider their population demographics. They are not directly comparable especially as CP is usually observed in those over 40 and increases in prevalence with advancing age. Another interesting point to consider is it has been reported that periodontal pathogens such as *P. gingivalis* produce proteases that can degrade various immunoglobulins such as IgA1, IgA2 and IgG (Guthmiller and Novak, 2002, Kilian, 1981). They can even hydrolyse antibodies that have bound to the bacterial cell surface allowing the key pathogen to evade host defences (Grenier et al., 1989).

5.1.3 Lipopolysaccharides

LPS, commonly referred to as endotoxin, is integral to the structure of the cell wall in most Gram-negative bacteria such as the black pigmented keystone pathogens. LPS is considered one of the main proponents of the systemic inflammatory burden in periodontal disease and is known to enhance production of the inflammatory cytokines such as the interleukins IL-1, IL-6, IL-8 and Tumour Necrosis Factor α (Wang and Ohura, 2002). Normally LPS can bind to CD14 in cell membranes from which it can be transferred to toll-like receptor 4 (TLR-4) initiating cell activation and innate host defence mediator production (Bainbridge and Darveau, 2001). However it has been reported that periodontal LPS such a *P. gingivalis* LPS can bind to TLR-2 along with being an antagonist for TLR-4; this may allow inhibition of immune responses in the fibroblasts in human inflamed gingiva and the chronic inflammation seen

(Bainbridge and Darveau, 2001). Consequently, a recent study highlighted that *P. gingivalis* LPS can inhibit a secondary immune response from gingival fibroblasts, preventing cytokine and chemokine release, thus the LPS enables the sustained inflammation characteristic of periodontitis (Fitzsimmons et al., 2018). Furthermore, high serum LPS levels have been associated with development of kidney disease in those with diabetes (Nymark et al., 2009), which highlights a potential link to kidney damage. However, this may be predominant in those patients with comorbidities such as diabetes. For LPS to be implicated in kidney disease, it would need to be stable within the blood and cause a distal response at the site of the kidney. Although, there is evidence that LPS is rapidly eliminated by the liver in healthy patients, this may be different in those with chronic diseases (Yao et al., 2016). If the LPS could remain viable in the blood it could potentially be concentrated within the kidney and stimulate an immune response in the kidney. This is evidenced with the association of LPS in sepsis with acute kidney injury; 40-50% of septic patients develop an acute kidney injury with suggestions this is mediated by lipopolysaccharide binding protein (Gómez and Kellum, 2016, Stasi et al., 2017). The source of LPS does not necessarily need to be from Gram-negative periodontitis associated pathogens; there is some evidence that dysbiosis of the gut microbiota can lead to LPS entering the bloodstream and this is associated with inflammation in CKD patients (Salguero et al., 2019).

5.1.4 Peptidoglycan

Peptidoglycan is an exoskeleton-like mesh made up of sugars and amino acids, which is a structural component of Gram-negative and predominantly Gram-positive bacterial cell walls. Peptidoglycan is a common target for beta-lactam drugs and can be an endotoxin which can induce an inflammatory response, such as the production of numerous cytokines (Okugawa et al., 2010). Peptidoglycan is constantly degraded and remodelled in the bacterial cell wall through growth and can be released into the environment where it can be a ligand for detection by the innate immune system (Johnson et al., 2013). In particular, the Gram-negative periodontitis associated organisms such *A. actinomycetemcomitans*, *P. gingivalis* and *F. nucleatum* can activate Nucleotide-binding oligomerization domain containing 1 (NOD1) and NOD2

receptors on macrophages which can cause inflammation in periodontitis (Chaves de Souza et al., 2016). In patients with CKD, it has also been found that peptidoglycan recognition protein 1 (PGLYRP1) is upregulated in those with poor oral health and was correlated with the number of periodontal pockets (Nylund et al., 2017); however the putative effect of peptidoglycan on the pathogenesis of CKD outside of inflammation is unknown.

5.1.5 Fimbriae

Fimbriae are present as numerous appendages on some Gram-negative and Gram-positive microorganisms; they form a key part in the adhesion of bacterial cells to surfaces, other microorganisms and nutrients (Marsh et al., 2009). It has been proposed that *P. gingivalis* fimbriae act as pathogen-associated molecular patterns (Enersen et al., 2013). These can activate toll-like receptors and their signalling pathways, resulting in the release of inflammatory cytokines and chemokines such as IL-1β, IL-6, and TNF-α and IL-8 in monocytes (Hajishengallis et al., 2004) along with separately producing IgA2 anti-fimbriae antibodies (Ogawa et al., 1991). There is also evidence showing that in *P. gingivalis*, adhesion and invasion of epithelial cells is promoted by the major fimbriae fimA (Umeda et al., 2006). Fimbriae would most likely play an important role in attachment of periodontal pathogens to kidney tissues if invasion and/or damage took place.

5.1.6 Outer Membrane Vesicles

P. gingivalis is not known to produce any exotoxins but it does produce a variety of enzymes and metabolites which are secreted into the local environment and have been reported to cause a variety of deleterious effects (Lamont and Jenkinson, 1998). Many Gram negative bacteria secrete spherical buds of periplasmic material called outer membrane vesicles (OMVs), which can be bound to the microbial surface or secreted directly into the environment (Schwechheimer and Kuehn, 2015). *P. gingivalis* is known to secrete OMVs of 50-250 nm in size which can act as vehicles for virulence factors such as LPS, adhesins and proteolytic enzymes (Xie, 2015). It has been reported that *P. gingivalis* specific proteases, Gingipains, are disproportionately packed into these OMVs (Veith et al., 2014, Haurat et al., 2011). Furthermore, treatment of human epithelial cells (Nakao et al., 2014) and human gingival

fibroblasts (Mantri et al., 2015) with *P. gingivalis* OMVs of >3 µg ml^{-1} has been shown to detach cells from confluent monolayers with the effect diminished upon heat activation.

5.1.7 Proteases

A protease is an enzyme that can break down peptides and proteins. It has been known for some time that proteases are produced by periodontal pathogens and are important virulence factors, which can cause tissue destruction and inflammation (Nakayama, 2003). In *P. gingivalis* there are a selection of proteases which are secreted extracellularly or predominately in OMVs, with the major group being cysteine proteinases called Gingipains which have been reported to account for 85% of the proteolytic activity and 99% of the trypsin-like activity of OMVs (Potempa et al., 2003).

Three types of Gingipains are produced by *P. gingivalis*: Lysine-specific gingipain (Kgp), arginine-specific gingipain A (RgpA), and arginine-specific gingipain B (RgpB) (Li and Collyer, 2011). These Gingipains are known to activate Proteinase Activated Receptors (PAR) in the periodontium and circulating immune cells which can lead to an inflammatory response (Rovai and Holzhausen, 2017). As a predominant virulence factor, the Gingipains can have a variety of interactions with the host immune system leading to destructive loss of attachment and a pro-inflammatory response with disease sustained inflammation which facilitates invasion and tissue damage (Figure 5.2) (Sandholm, 1986).

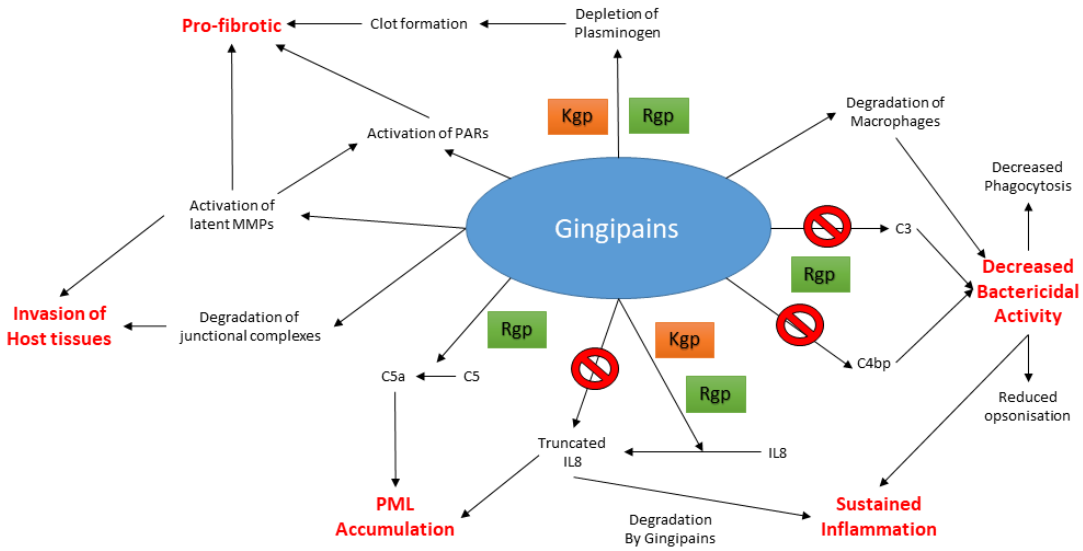

Figure 5.2 The interactions Gingipains produced by *P. gingivalis* has with the immune system. Lysine-specific gingipain (Kgp), arginine-specific gingipain A (RgpA), and arginine-specific gingipain B (RgpB), Complement components (C3, C4bp, C5, C5a), protease activated receptors (PARs), polymorphonuclear lymphocytes (PML).

P. gingivalis has been shown to be highly resistant to the bactericidal activity of human complement and this has been reported to be due to Rgp Gingipains binding to human complement inhibitor C4b-binding protein (C4BP) along with degradation of complement component C3 (Potempa et al., 2008, Wingrove et al., 1992). Moreover, the degradation of the complement component C5 to C5a can lead to the release of this potent chemoattractant which can recruit PMLs to the gingival lesion, thus promoting sustained inflammation (Wingrove et al., 1992). Gingipains have also been shown to degrade CD14 macrophages preventing LPS activating leukocytes, facilitating the continual colonisation of the gingival crevice (Imamura, 2003).

An interesting discovery was that secreted Gingipains could stimulate the accumulation of PML by the conversion of interleukin 8 (IL-8) to a more potent truncated version, while Gingipains present in OMV can degrade the truncated IL-8 locally which may be a reason behind the chronic inflammation combined with persistence of *P. gingivalis* seen in periodontitis (Mikolajczyk-Pawlinska et al., 1998). It is also reported that these proteases stimulate the activation of dormant matrix metalloproteinases such as MMP-9 that degrade the extracellular matrix, which combined with depletion of plasminogen and the destruction of cell junctional

complexes can promote fibrosis and invasion (Kuula et al., 2009, Fleetwood et al., 2015, Katz et al., 2002b, Katz et al., 2000).

5.1.8 Metabolites

Metabolites are low molecular weight compounds, which represent the precursors, intermediates and reactants of biochemical reactions which take place intracellularly and extracellularly (Dunn et al., 2011). It has previously been highlighted that certain microorganisms can generate primary and secondary metabolites and some of these have been shown to interact with the human body through immune regulation (Brestoff and Artis, 2013), protection against pathogens (Clarke et al., 2010) and metabolism of synthetic substances (Kuhn et al., 2014).

Secreted metabolites in *P. gingivalis* are relatively unstudied, however the anaerobic nature of *P. gingivalis* growth can lead to the production of major fermentation products such as butyric acid and indole (Parte et al., 2011). A selection of other metabolic end products have also been reported such as: Acetic acid, Isobutryric, Isovaleric, Phenylacetic acid, Propionic and Succinic with the major fatty products being 13-methyltetradecanoic acid (C13:10 iso) (Parte et al., 2011). Of the metabolites known to be produced, little is known about their potential effects on the host, however their abundance can be measured using a metabolomics-based approach discussed in Chapter 3.

5.1.9 Components that would be present in microbial supernatants

As discussed in more depth in Chapter 4.1.1 and Figure 4.1, the use of microbial supernatants for this study narrows down the potential causes of the damage observed to the HK-2 cells. These bacterial supernatants do not contain any microbes and do not contain the lysis and intracellular products of the microorganisms. There would also be lower amounts of LPS, peptidoglycan and fimbriae due to the lack of sonication. The supernatants would contain degradation products of the growth medium (AGCF-P) along with outer membrane vesicles, secreted metabolites and small amounts of LPS and peptidoglycan. This chapter focuses on identifying the factors in these microbial supernatants that cause the deleterious effect observed

against HK-2 cells along with using further tests to assess whether these supernatants can induce fibrosis in HK-2 cells.

5.2 AIM AND OBJECTIVES

5.2.1 Aim

To identify the cause of the cytotoxic effects seen in HK-2 cells treated with *P. gingivalis* supernatants and to further investigate the fibrotic potential of microbial supernatants.

5.2.2 Objective

- Determine whether LPS can have cytotoxic effects on human kidney cells.
- Determine whether secreted microbial proteins can have cytotoxic effects on human kidney cells.
- Utilise the metabolomics pipeline developed in Chapter 3 to investigate *P. gingivalis* for any potentially cytotoxic compounds.
- Determine whether *P. gingivalis* supernatants can induce a selection of fibrosis markers using qPCR.

5.3 METHODS

5.3.1 Endotoxin Quantification

The Pierce LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific, UK) was utilised to create a standard curve of *Escherichia coli* lipopolysaccharide to compare against microbial supernatant samples. In order to quantify the amount of lipopolysaccharide present in the microbial supernatants, these supernatants were diluted at 1:10 with endotoxin free water (ThermoFisher Scientific, UK) because of the high innate colour of the microbial samples. The standard protocol was used and in order to account for the slight colour of the microbial samples, the 96 well plate was read before and after the addition of the chromogenic substrate at 405_{nm} on a Multiskan FC Microplate Photometer (ThermoFisher Scientific, UK). All results were exported to Microsoft Excel, the blank endotoxin free water values were averaged and subtracted from all samples and a standard curve of high and low concentrations of LPS was produced with a linear regression. Using the linear equation, the LPS concentrations of the microbial samples were derived by averaging the replicates for each sample and inputting the results into the equation of the standard curves.

5.3.2 LPS Testing Against Kidney and Gingival Cell Lines

HGF-1 and HK-2 cells were maintained and viability testing was conducted as discussed in Chapter 2.3.3-2.3.4.

P. gingivalis LPS (Sigma Aldrich, UK) and *Escherichia coli* O111:B4 LPS (Sigma Aldrich, UK) were reconstituted in HGF-1 and HK-2 cell medium prior to use and inoculated into the cell culture medium at a range of concentrations to be investigated for effects on cell viability.

5.3.3 Protein Inactivation and testing of *P. gingivalis* and AGCF-P supernatants

An aliquot (100 µL) of *P. gingivalis* supernatants (1D-5D) which elicited viability effects and AGCF-P supernatants (1D-5D) were heat inactivated by heating at 90^{o}C for 30 minutes in a dry heat block. Another aliquot (100 µL) of the *P. gingivalis* and AGCF-P supernatants were inactivated by the addition of 10 µL of filter sterilised Trypsin-EDTA (Lonza, UK). Cell viability testing of these inactivated supernatants was

repeated as in Chapter 4.3.1.1 using the cell viability method described in Chapter 2.3.3-2.3.4.

5.3.4 Testing of Protease Inhibitors

The cell viability testing of *P. gingivalis* and AGCF-P media was repeated as completed in Chapter 4.3.1.1 alongside the same bacterial and control supernatants, with the addition of 1 µL of cell culture safe protease inhibitor cocktail mixture (Sigma Aldrich, UK) using the cell viability method described in Chapter 2.3.3-2.3.4.

5.3.5 LCMS Preparation and Analysis

P. gingivalis supernatant samples and matched AGCF-P media controls were prepared to untargeted LCMS analysis as described in Chapter 3.3.2-3.3.5.

Further additions were made to these reported LCMS methods, with preparation of pooled quality controls taking place after section 3.3.3 and quality control and assurance after section 3.3.5.3.

5.3.5.1 Preparation of Pooled Quality Control (QC)

Pooled quality control samples were created by taking 150 µL from every sample, methanol washes and blanks and mixing in a universal. The number of quality control samples required was then worked out and 750 µL of the pooled QC was aliquoted into the number of 2 mL LCMS vials required. To work out the number of quality controls needed per run the equation used was:

$$Number\ of\ QC\ samples\ required = 3 + (Number\ of\ samples\ /\ 6\ )$$

5.3.5.2 Quality Control and Quality Assurance

In order to reduce the number of false positives and negatives, quality control and assurance of raw data files was undertaken in Microsoft Excel prior to further analysis. The quality control and assurance methods used are described below:

Step 1 – Work out % control contribution

- Work out average intensity of controls and average intensity of QCs =average
- % Control contribution formula =(Average of controls/Average of QC)*100

Step 2 – Work out Relative Standard Deviation of Quality Control and Samples

- Work out Standard Deviation of QC and Samples =STDEV

- Work out mean of QC and Samples =AVERAGE
- Work out RSD of QC and samples =SUM(SD/mean)*100

Step 3 – Work out % count of QC and Samples

- Work out count of QC and samples which contain more than 0 (make sure they are in chronological order i.e. QC1, QC 2, QC 3 etc) =COUNTIF(FirstSample:LastSample,">0")
- Work out % count =SUM(COUNT/Total number of QC or Sample)*100

Step 4 – Work out Relative Standard Deviation ratio of Samples / Quality Control

- =SUM(RSDsamples/RSDQC)*100

Step 5 – Filter data

- Delete all rows with % QC Count of <60
- Delete all rows with QC RSD >20%
- Delete all rows with blanks contribution >5%

5.3.6 Quantitative Polymerase Chain Reaction (qPCR)

5.3.6.1 Cell Culture and Experimental Plan

HK-2 cells were maintained as discussed in Chapter 4.2.2. Six well tissue culture plates were seeded with HK-2 cells (4 x 10^4 cells) and grown to 70% confluency in growth medium. The cells were exposed to 15 conditions (Serum free media, AGCF-P media supernatants x 5, *P. gingivalis* supernatants x 5, *S. mutans* supernatants x 2 and *F. nucleatum* supernatants x 2), with each one in triplicate.

5.3.6.2 RNA Extraction

After 24 hours of exposure to the respective treatment, the media were aspirated and the cells were washed three times with ice cold Phosphate Buffered Saline (Lonza, UK). Total RNA was extracted on ice using an RNeasy kit (Qiagen, UK) as per manufacturer's protocol. A Thermo Scientific™ NanoDrop™ One Spectrophotometer (Fisher Scientific, UK) was used to measure the total RNA concentration and its purity, and the RNA samples were diluted with DNase/RNase-free water to 0.6 µg of RNA per sample to ensure comparability. All samples were stored at -80^{o}C until needed.

5.3.6.3 cDNA Synthesis

A Bioline Tetro™ cDNA Synthesis Kit (Sigma Aldrich, UK) was utilised to convert mRNA to cDNA to allow quantification of gene expression by qPCR. The standard protocol was followed with Oligo(dT) primers rather than random hexamer primers as this

qPCR experiment aims to look at mature messenger RNA. Once converted all cDNA samples were kept at -20^{o}C for long-term storage.

5.3.6.4 Primers

The primers used for the qPCR experiments and the respective sequences are shown in Table 5.1. All primers were diluted to stock solutions of 70 µM with PCR grade water to allow for 1:10 dilutions for the qPCR master mix.

Table 5.1 All primer genes and sequences for qPCR experiments.

Primer		Sequence	Fragment Size Expected (Base Pairs)
β-actin (Housekeeping)	Forward	CACTCTTCCAGCCTTCCTTC	90
	Reverse	GGATGTCCACGTCACACTTC	
Protease Activated Receptor 1 (PAR1)	Forward	AGAAGTCAGGAGAGAGGGTGAA	177
	Reverse	CTTTTGATTCTGGCCTGCGG	
Protease Activated Receptor 2 (PAR2)	Forward	GCTCTGAGTTTCGAATCGGC	152
	Reverse	TCTACTGGTTCCTTGGATGGT	
Transforming growth factor beta 1 (TGF-βR1)	Forward	CCTCGAGATAGGCCGTTTGT	121
	Reverse	GCAATGGTAAACCAGTAGTTGGA	
Vascular Endothelial Growth Factor (VEGF)	Forward	GCCTTGCTGCTCTACCTCCAC	75
	Reverse	ATGATTCTGCCCTCCTCCTTCT	
Tissue Inhibitor of Metalloproteinases 1 (TIMP1)	Forward	CGCAGCGAGGAGTTTCTCAT	188
	Reverse	CTCTGCAGTTTGCAGGGGATG	
Plasminogen Activator Inhibitor-1 (PAI-1)	Forward	CGAGGTGAACGAGAGTGGCA	170
	Reverse	CCGAGGGTCAGGGTTCCATC	
Collagen type 1 Alpha 1 (COL1A1)	Forward	GTCGAGGGCCAAGACGAAGA	114
	Reverse	GTTGTCGCAGACGCAGATCC	

5.3.7.5 qPCR

The QuantiNova SYBR Green PCR kit (Qiagen, UK) was used for all qPCR experiments. The reagent mixture used (Table 5.1) was based off the standard protocol which came with the kit and the reaction volume was 20 µL. The ROX reference dye was not used as the qPCR instrument used (BioRad CFX96) did not require ROX.

Table 5.2 Reaction mixture for all qPCR experiments

Component	Amount (µL)	Final Concentration
2 x SYBR Green QPCR master mix	10	1x
FW Primer	2	0.7 µM
RV Primer	2	0.7 µM
Template DNA	1	<100 ng/reaction
Nuclease free PCR grade water	5 (Adjust to make 20 µL final)	
Total	20	20 µL

The PCR cycling program (Table 5.3) was based upon the standard protocol from the kit.

Table 5.3 PCR cycling program for all qPCR experiments

Cycles	Duration of Cycle	Temperature (°C)	Ramp Rate
PCR initial heat activation	2 min	95	Maximal/fast
Denaturing	5 seconds	95	Maximal/fast
Combined annealing/extension	10 seconds	60	Maximal/fast
Number of cycles = 39 Use melting curve analysis			

5.3.7.6 Agarose Gel Electrophoresis

In order to ensure the correct fragment size and a lack of multiple products present in the qPCR reactions, qPCR products were separated by 2% agarose gel electrophoresis. To make this gel, 1.5 g of molecular grade agarose powder (Bioline, UK) was added to 50 mL of 1 x Tris base, boric acid and EDTA (TBE) buffer and heated until dissolved. Next, 2 µL of midori green dye (Bioline, UK) was mixed into the agarose solution and the gel was poured and allowed to set. Subsequently, 450 mL of 1 x TBE buffer was use to fill an electrophoresis tank and 4 µL of all samples was mixed with 1 µL of 5 x loading buffer (Bioline, UK). These mixed samples were then added to the wells along with 5 µL of a 1kb hyperladder (Bioline, UK) loaded on each gel to facilitate the determination of the size of the PCR products. The gel electrophoresis was carried out for 45 minutes at 80 mV and the PCR products were then visualised with UV light.

5.3.7.7 Data Analysis

All qPCR data was checked prior to exporting cycle threshold (Ct) values to Microsoft Excel. The baseline setting was set to baseline subtracted curve fit for all plates and

the threshold value was manually set as the average of plate 1 and plate 2 for each gene to ensure consistency. Fold gene expression was calculated in Microsoft Excel using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with β-actin used as the housekeeping gene. The $2^{-\Delta\Delta Ct}$ values were used to depict fold change and statistical analysis was completed on Log^2 of the $2^{-\Delta\Delta Ct}$ values to reduce error.

5.3.7.8 Graph Plotting and Statistical Analysis

Prism 8.1.1 was used for all graph plotting and statistical analysis of qPCR data. A two way ANOVA with multiple comparisons (Tukey) was used to compare all Log^2 fold changes between all samples at each time point.

5.3 RESULTS

5.3.1 Investigating the Role of LPS in Perceived Cell Damage

Quantification of LPS amounts in microbial and control supernatant samples was undertaken to evaluate whether *P. gingivalis* LPS could be responsible for the cytotoxic effects observed in Chapter 4 caused by *P. gingivalis* supernatants.

5.3.1.1 Quantification of LPS in Cell Damaging Samples

The Limulus Amebocyte Lysate (LAL) assay was used to quantify LPS in all *P. gingivalis* samples and 1 D and 5 D AGCF-P media supernatant samples (Figure 5.3). Not all AGCF-P media samples were investigated due to the prohibitive cost of the kit and the lack of microbial inoculation.

All *P. gingivalis* samples averaged around 0.3 EU/mL which equates to 0.3 ng/mL while AGCF-P media supernatants averaged around 0.1 EU/mL which equates to 0.1 ng/mL. There was no significant difference between LPS concentration of 1D *P. gingivalis* supernatants compared with 1D AGCF-P supernatants along with 5D *P. gingivalis* supernatants compared with 5D AGCF-P supernatants.

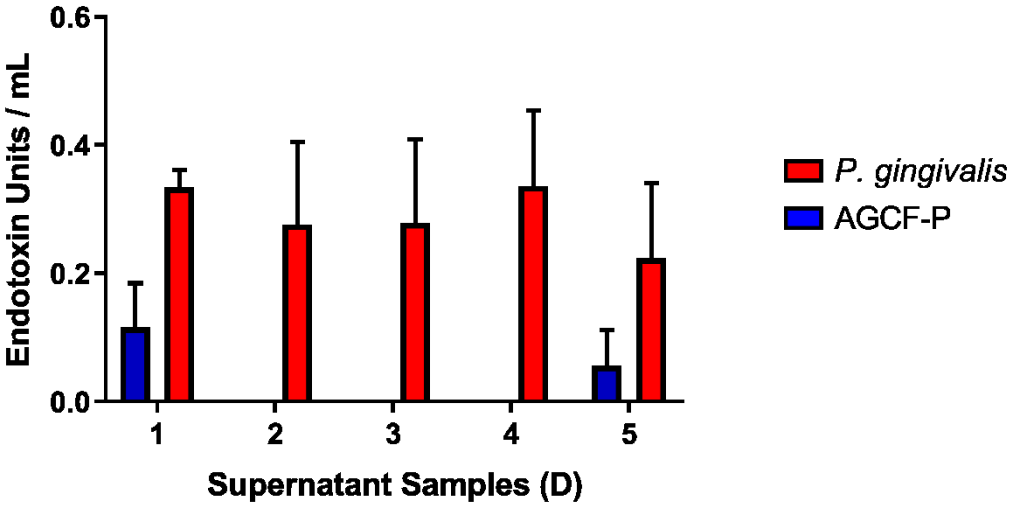

Figure 5.3 Endotoxin amount in 1-5 Day (D) *Porphyromonas gingivalis* (PG) and 1 and 5D AGCF-P (C) supernatant samples. Error bars represent SEM (*n=3*).

5.3.1.2 Creating a Standard Curve of Cell Damage with E. coli and P. gingivalis LPS

In order to determine whether the EU/mL concentration present in the supernatant samples could elicit a cytotoxic effect on kidney cells, titration experiments were carried out using of *P. gingivalis* LPS (Figure 5.4) and *E. coli* O111:B4 LPS (Figure 5.5). Only up to 10 µg/mL of *P. gingivalis* LPS could be tested due to the starting concentration of *P. gingivalis* LPS not being sufficiently high enough for appropriate dilution into cell culture media.

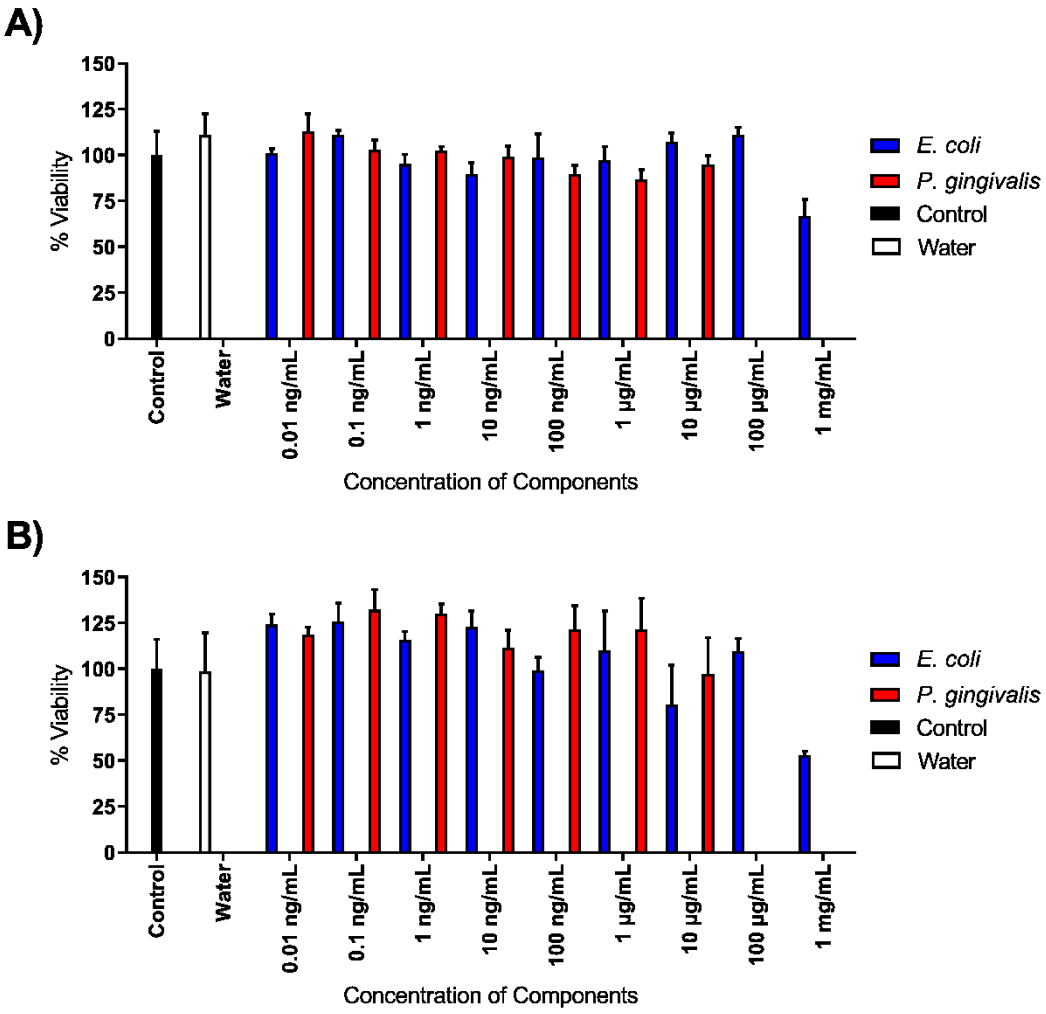

Figure 5.4 Cell viability of HK-2 cells incubated with serum free media (Control), 1:20 dilution of endotoxin free water and a dilution series of *P. gingivalis* and *E. coli* LPS for A) 24 h and B) 48 h. Error bars represent SEM (*n=3*).

After 24 and 48 hours incubations of LPS with HK-2 cells, neither *E. coli* nor *P. gingivalis* LPS had a statistically significant effect on the cellular viability (Figure 5.4). The same effect was also observed after 24 and 48 hours exposure of HGF-1 cells to *E. coli* or *P. gingivalis* (Figure 5.5).

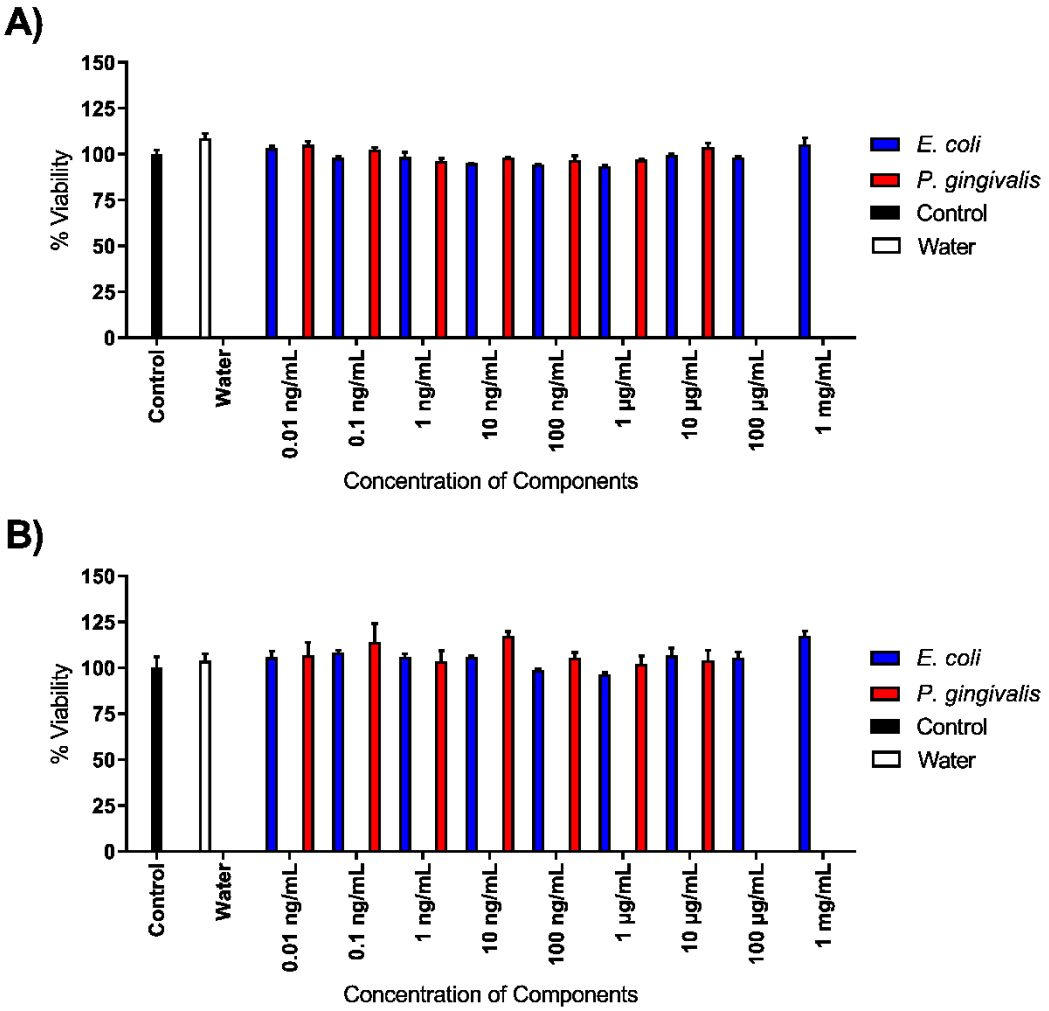

Figure 5.5 Cell viability of HGF-1 cells incubated with serum free media (Control), 1:20 dilution of endotoxin free water and a dilution series of *P. gingivalis* and *E. coli* LPS for A) 24 h and B) 48 h. Error bars represent SEM (*n=3*).

5.3.2 Investigating the Role of Secreted Proteins in Perceived Cell Damage

In order to determine whether the bacterial proteins secreted in the supernatant samples could elicit a cytotoxic effect on kidney cells, denaturing and inhibition of potential target proteins (Gingipains) was undertaken.

5.3.2.1 Inactivation of Proteins Present in P. gingivalis and AGCF-P samples

Heat inactivation of an aliquot of all *P. gingivalis* samples and all AGCF-P media supernatant samples was undertaken and the cell viability experiment of *P. gingivalis* supernatants incubated with HK-2 cells (Chapter 4.3.1.1) was repeated with or without inactivated supernatants. However, the viscosity caused by protein inactivation interfered colorimetrically with the viability assay dye, providing inaccurate results (Data not shown). The same issue was found when trypsin was used to inactivate the *P. gingivalis* and AGCF-P supernatants and the cell viability experiments repeated (Data not shown).

5.3.2.2 Incubation with Protease Inhibitors

In order to determine whether *P. gingivalis* specific proteases, Gingipains, may be responsible for the cytotoxic effects observed in Chapter 4.3.1.1 a protease inhibitor cocktail mix was used to repeat the cell viability experiments with and without the addition to the bacterial supernatants (Figure 5.6).

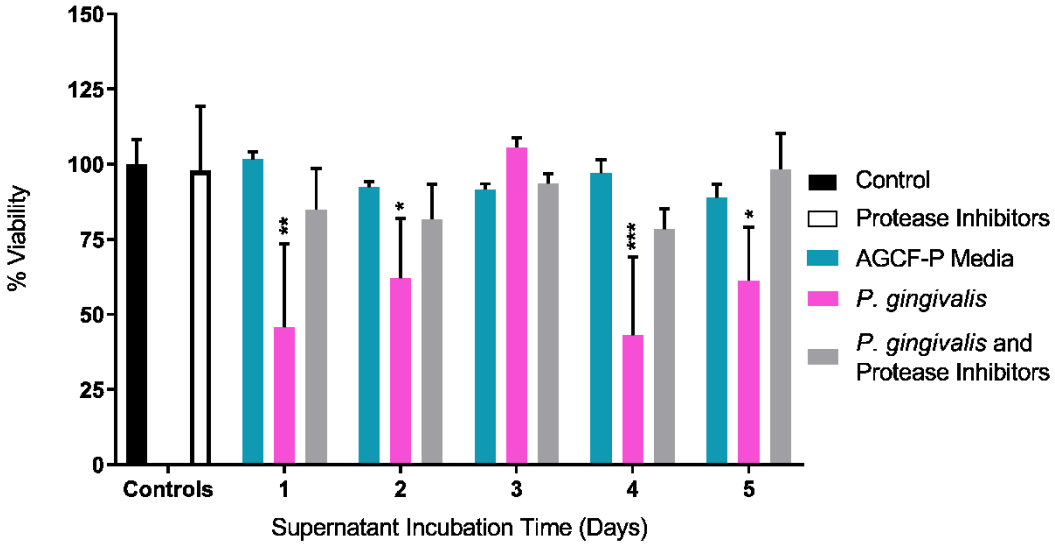

Figure 5.6 Cell viability of HK-2 cells after 24 h incubation with AGCF-P supernatants and *P. gingivalis* supernatants collected over 5 days with and without protease inhibitors. Viability is presented as percentage change from the control (Control: cells exposed to serum-free cell culture medium). Protease inhibitors (White) is serum free media with the addition of protease inhibitors alone. Error bars represent SEM. * = *p*-value ≤ 0.05, ** = *p*-value ≤ 0.01, *** = *p*-value ≤ 0.0001 (*n=3*).

After 24 hours, with the exception of the 3 D *P. gingivalis* supernatant, a significant decrease in cell viability was observed in response to all *P. gingivalis* supernatants, as compared to the control. In contrast, there was no significant difference in viability

of cells exposed to AGCF-P media and the control (serum free) medium. The protease inhibitor cocktail added to serum free media had no significant effect on cellular viability compared to the control. However, the addition of the protease inhibitor cocktail preventing the loss of viability of cells exposed to *P. gingivalis* supernatants.

5.3.2.3 qPCR of Protease Activated Receptors

It has been demonstrated the protease activated receptors 1 and 2 (PAR1 and PAR2) are activated by Gingipains (Chapter 5.1.7). In order to establish their involvement in the cytotoxic effects of Gingipains, the mRNA levels of PAR1 and PAR2 were evaluated in HK-2 cells exposed to serum free media (control), *P. gingivalis* supernatants and AGCF-P supernatants using qPCR (Figure 5.7a) and (Fig 5.7b) respectively.

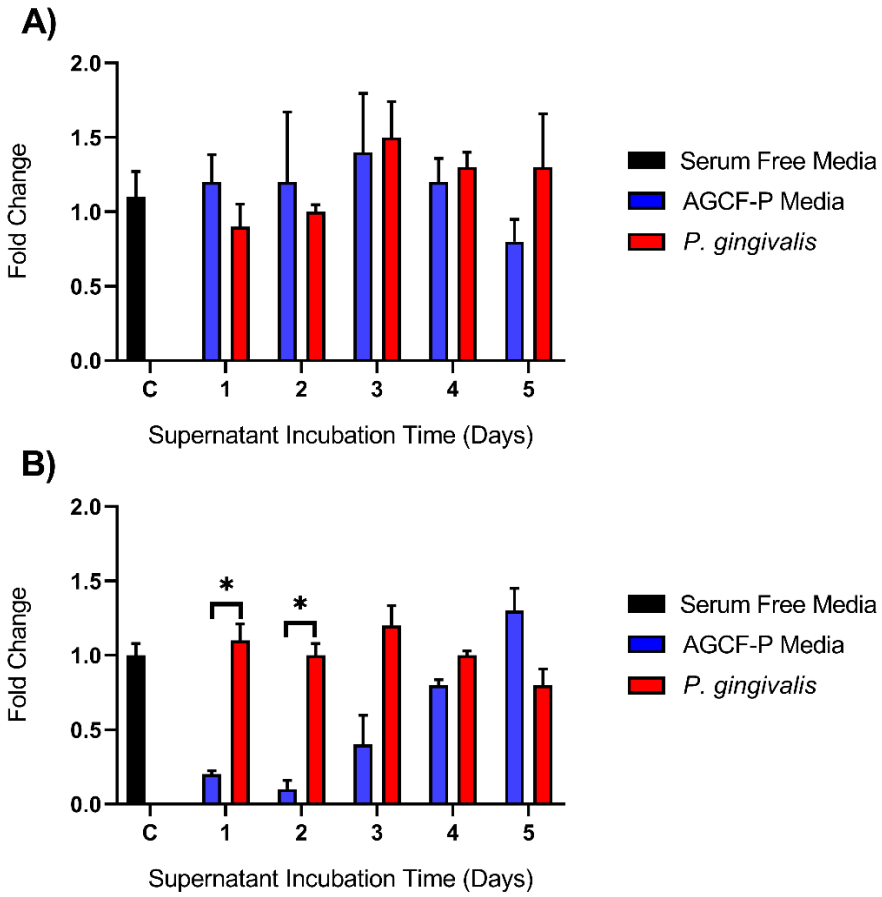

Figure 5.7 Relative gene expression in comparison to β actin of A) PAR1 and B) PAR2 mRNA in HK-2 cells after exposure to *P. gingivalis* and AGCF-P supernatants for 24 hours. C = serum free medium. Error bars represent SEM. * = *p*-value ≤ 0.05; (*n=3*).

After 24 hours of treatment with *P. gingivalis* and AGCF-P supernatants, no significant differences were observed between the PAR1 mRNA levels in cells exposed to *P. gingivalis* supernatants and AGCF-P supernatants in comparison with the serum free media control. There was also no significant difference between PAR1 mRNA levels of *P. gingivalis* supernatants in comparison with the matched AGCF-P supernatants for each day.

For the PAR2 receptor, there was no significant difference between PAR2 receptor mRNA in cells exposed to *P. gingivalis* supernatants and AGCF-P supernatants in comparison with the serum free media control. However, a significant reduction of PAR2 receptor mRNA was observed between 1 and 2 D AGCF-P supernatants and the respective 1 and 2 D *P. gingivalis* supernatants.

5.3.3 LCMS of *P. gingivalis* Supernatants

The *P. gingivalis* and AGCF-P supernatants collected over 5 days in triplicate (Chapter 2.4.2.1) were prepared for untargeted metabolomics with the AGCF-P media used to account for abiotic degradation and quality control samples. After LCMS analysis and deconvolution using XCMS online, quality assurance of the raw data files reduced the number of total features from all samples from 5576 to 4047 by blank contribution and 4047 to 2273 by quality assurance with QC samples. Peak alignment, missing value imputation and data normalisation was then conducted through MetaboAnalyst, (Figure 5.8) showing the normalisation step which allows for better comparison of features.

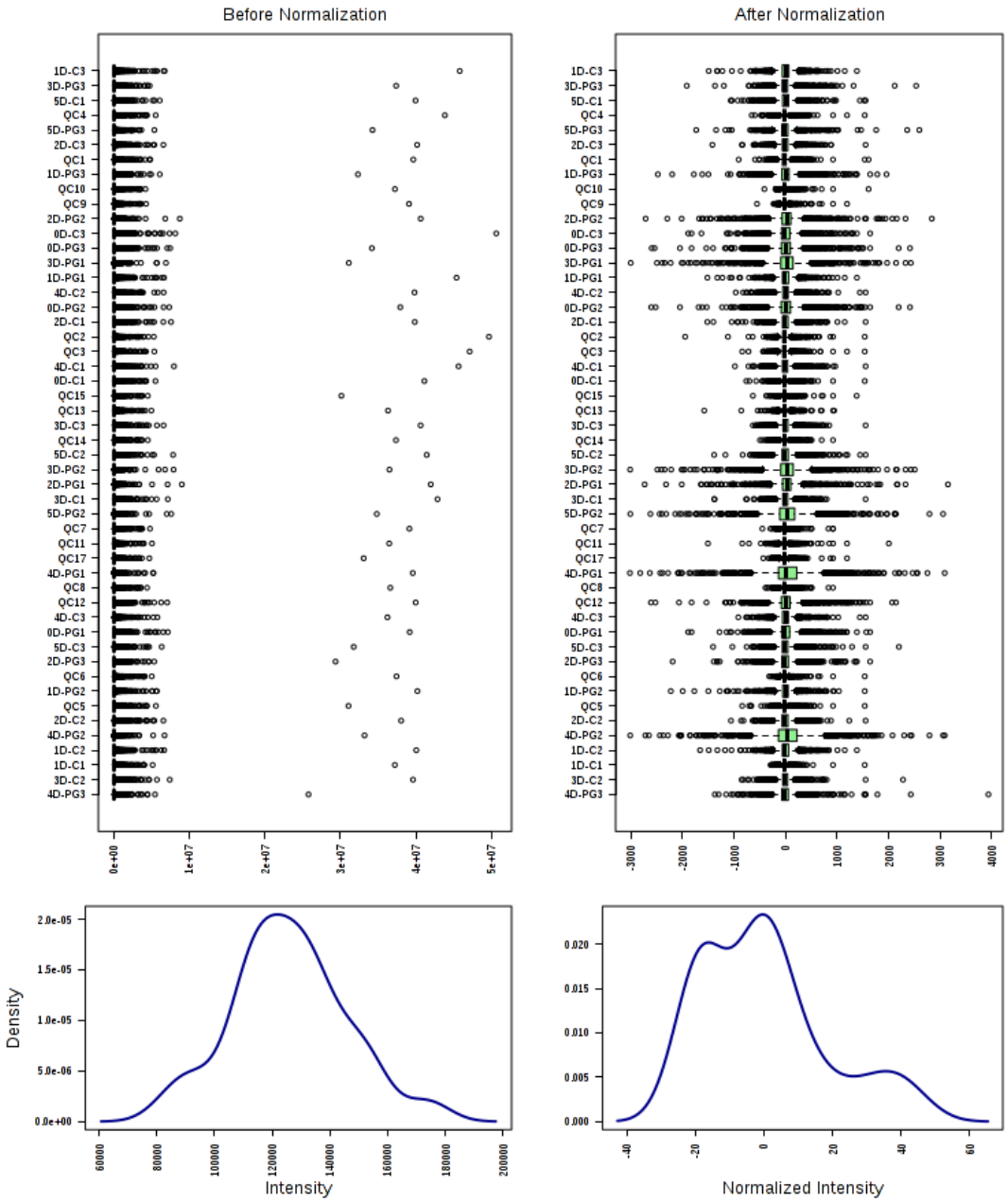

Figure 5.8 Box plots and kernel density plots before and after normalization of LCMS data of *P. gingivalis* cultured in AGCF-P (PG), control AGCF-P samples (C) at 0, 1, 2, 3, 4 and 5 D and quality control samples (QC). Quantile normalization and Pareto scaling were used. (*n=3*).

5.3.3.1 Univariate Analysis

Univariate analysis was conducted on all features to ascertain which features were significantly upregulated or downregulated (p=<0.05 and fold change of 2≥ or -2≤) from the AGCF-P control to *P. gingivalis* cultured in AGCF-P. From the 2273 features initially identified, 406 were identified to be significantly changed with 117 upregulated and 289 downregulated (Figure 5.9).

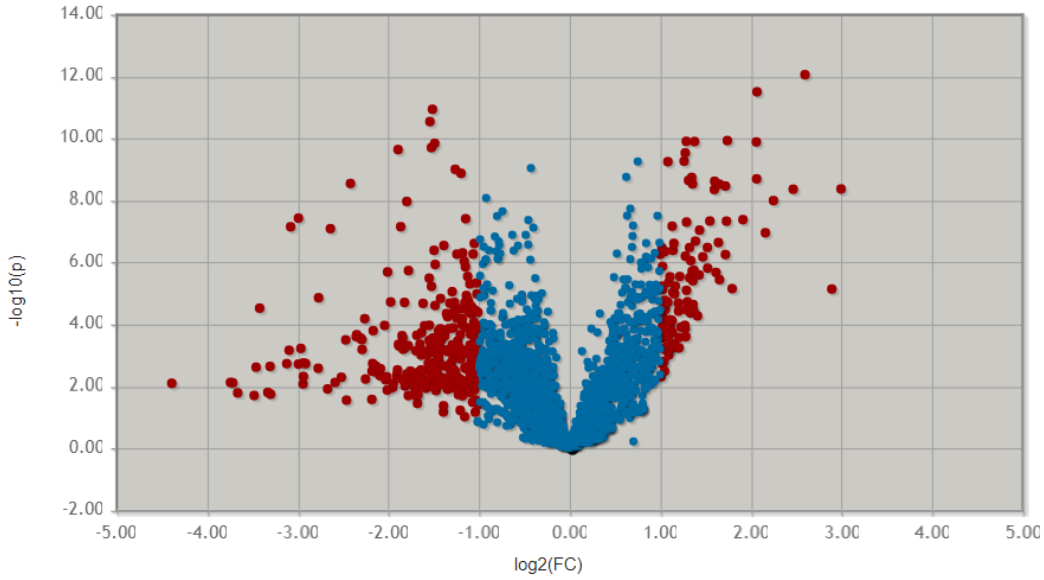

Figure 5.9 Important features identified from *P. gingivalis* supernatants in comparison to AGCF-P control supernatants, selected by a univariate volcano plot with a fold change threshold (x) 2 and t-tests threshold (y) 0.05. The red circles represent features above the threshold, the further the position away from the (0, 0) the more significant the feature. All values are log transformed.

5.3.3.2 Principle Component Analysis

PCA is an unsupervised method that attempts to find directions that can explain the variance in data irrespective of class. The scores plot of all QC, *P. gingivalis* and AGCF-P control samples (Figure 5.10) shows that the quality control samples (QC) are tightly clustered, indicating good experimental design. Furthermore, the AGCF-P media (AGCF-P) samples are more tightly clustered than the *P. gingivalis* (P.G) samples highlighting microbial degradation of the growth media is taking place.

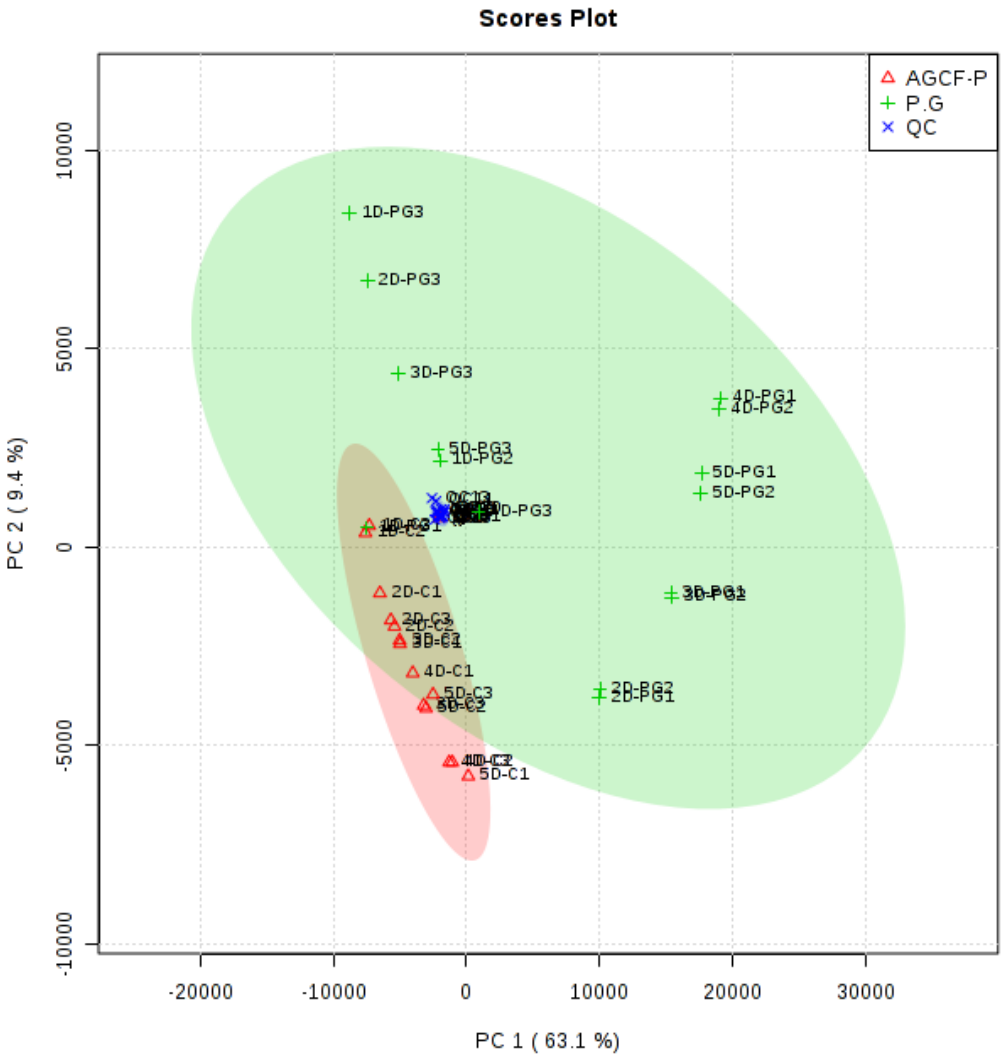

Figure 5.10 A 2D scores plot of a PCA between all QC, *P. gingivalis* and AGCF-P control classes. The explained variances are shown in brackets.

5.3.3.3 Partial Least Squares – Discriminant Analysis

PLS-DA is a supervised method of multivariate regression that is similar to PCA, however it takes into account the classes of samples and attempts to work out which variables have the class separating information. The 2D scores plot of all QC, *P. gingivalis* and AGCF-P control samples (Figure 5.11) shows that the AGCF-P control is much more tightly clustered (Red) than AGCF-P inoculated with *P. gingivalis* (Green) along with a tight QC cluster, this correlates with the PCA (Figure 5.10). PLS-DA usually has sharper separation between classes than PCA, which is shown with the difference between Figure 5.10 and Figure 5.11.

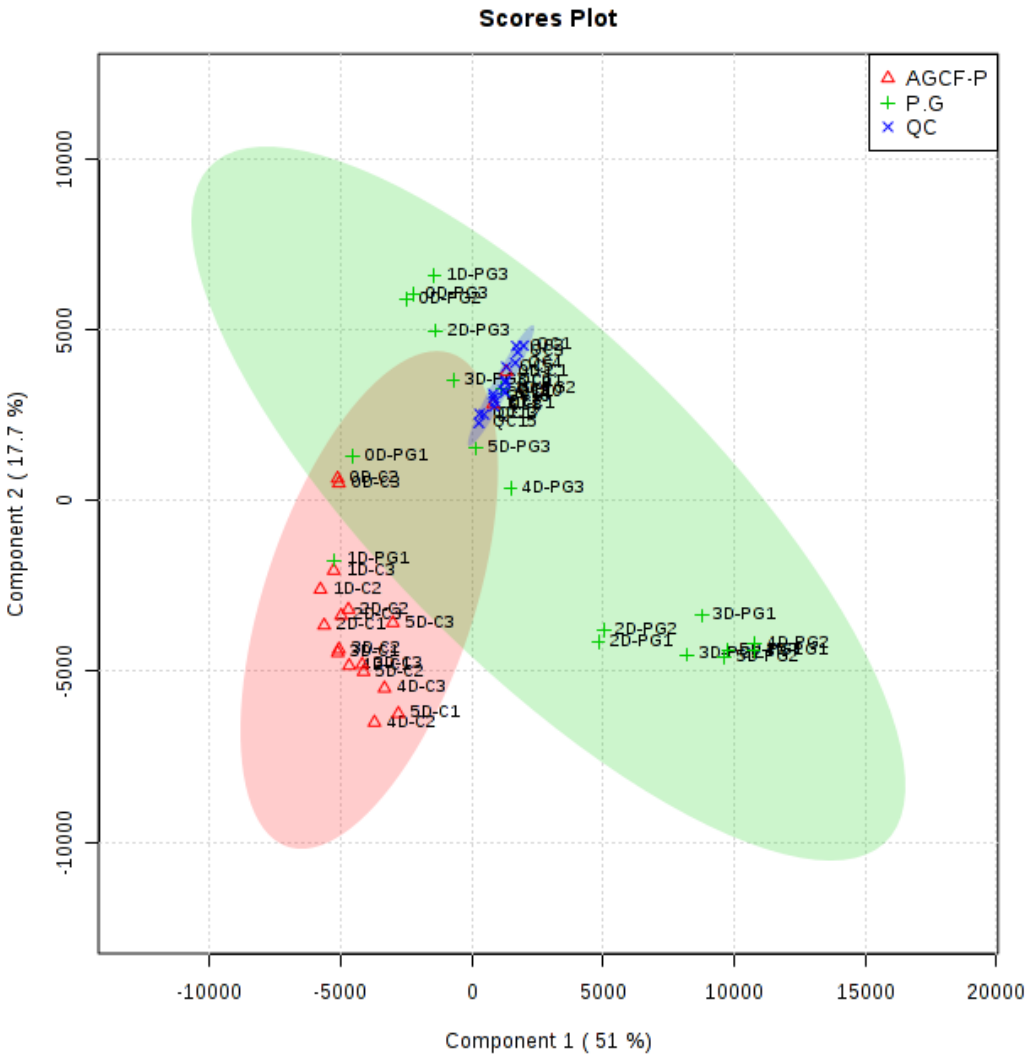

Figure 5.11 A 2D scores plot of a PLS-DA between all AGCF-P bacterial and control classes. The explained variances are shown in brackets. B after a class denotes Bacterial and T denotes controls with the number denoting a biological replicate.

A VIP score graph was plotted with the QC class removed (Figure 5.12), which calculates weighted sum of squares of the PLS loadings taking into account the amount of explained Y-variation in each dimension for each component. A higher VIP indicates that the feature in question has a larger contribution to class discrimination in the PLS-DA model. The top 15 VIP features identified were selected for metabolite identification.

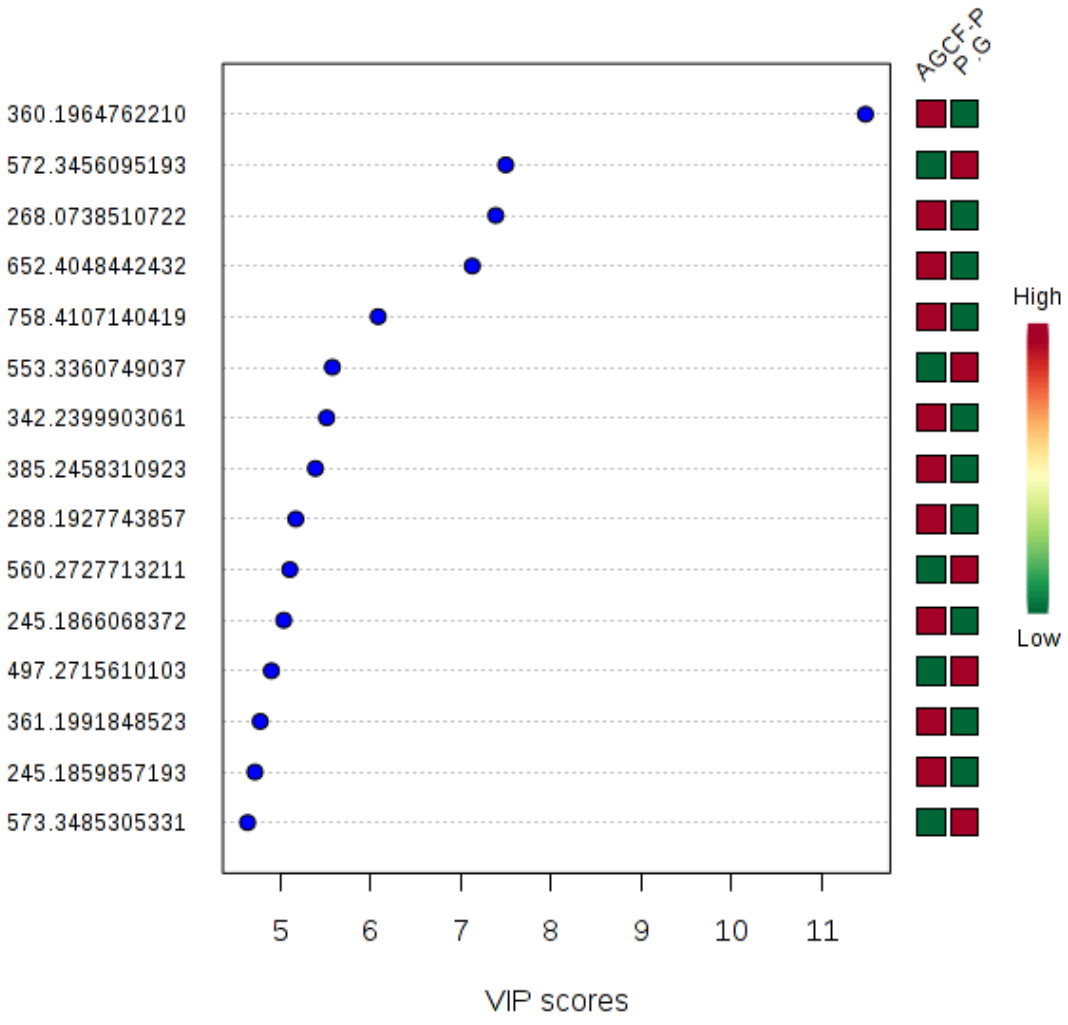

Figure 5.12 Important features identified by a PLS-DA of *P. gingivalis* and AGCF-P bacterial control samples. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study. The x-axis denotes VIP scores and y-axis the feature m/z value.

5.3.3.4 Significant Feature Selection Using Random Forest Algorithm

RF is a supervised algorithm for data analysis, which uses random feature selection and classification trees to predict class based on majority vote. It is useful for feature selection as it can identify important features by measuring the increased error in class identification when a feature is omitted. The *P. gingivalis* and AGCF-P control samples were analysed using RF (Figure 5.13) in order to identify features that are important for class segregation. The top 15 significant features identified were selected for metabolite identification.

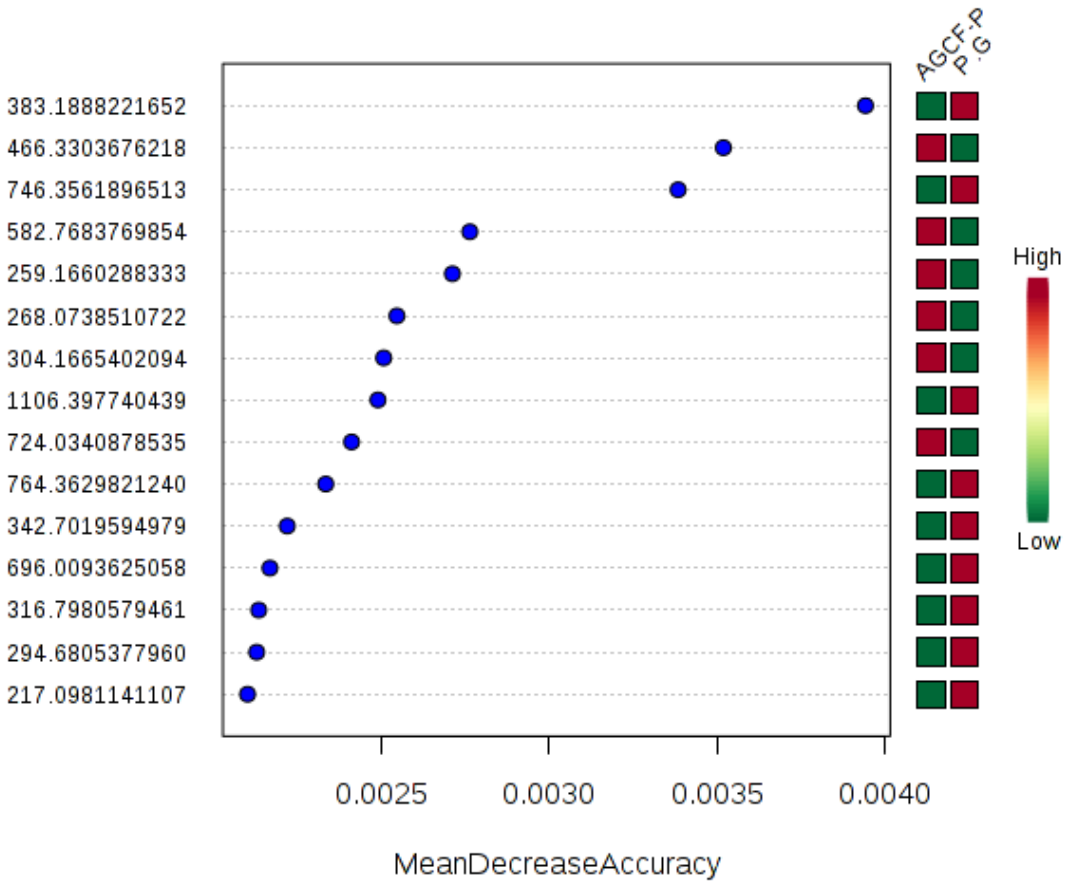

Figure 5.13 Significant features from AGCF-P (control) and *P. gingivalis* inoculated into AGCF-P identified by Random Forest. The features are ranked by the mean decrease in classification accuracy when they are permuted. The x axis denotes mean decrease in accuracy and y axis the feature m/z and retention time separated by /.

5.3.3.5 Metabolite Identification

All significantly upregulated and downregulated features identified by volcano plots, Variable Importance in Projection features identified by PLS-DA and important features in classification identified by RF were extracted into an Microsoft Excel spreadsheet for identification of metabolites. The program MetaboSearch was used to compare the m/z and retention times of the metabolites of interest with a selection of mass spectral databases for putative identification and the results extracted. For all significant features, there was a 6.6% success rate in identification. There were no clear metabolite identification trends associated with the classes due to the low metabolite identification rate. The only exception being amino acids and their various degradation products being increased in the *P. gingivalis* samples.

Table 5.4 Metabolite identification of significantly up and downregulated metabolites identified by volcano plots of AGCF-P bacterial and control samples.

Upregulation								
m/z	**Retention Time**	**Fold Change**	***p value***	**Name**	**Formula**	**Mass of Match**	**PPM Deviation**	**Putative Role**
197.102	0.79	7.97	0.00000	-	-	-	-	-
292.201	0.83	7.43	0.00001	-	-	-	-	-
268.074	1.24	6.05	0.00000	-	-	-	-	-
287.073	0.78	5.53	0.00000	-	-	-	-	-
355.061	0.77	4.75	0.00000	-	-	-	-	-
245.187	5.62	4.47	0.00000	Isoleucyl-Leucine/Leucyl-Isoleucine	C12H24N2O3	244.179	2.562	Amino acid degradation product
269.077	1.23	4.19	0.00000	Portulacaxanthin II	C11H12N2O6	268.070	1.594	Amino acid related compounds
245.186	5.15	4.18	0.00000	Isoleucyl-Leucine/Leucyl-Isoleucine	C12H24N2O3	244.179	0.062	
246.189	5.15	4.17	0.00000	-	-	-	-	-
265.155	5.47	3.76	0.00000	Phenylalanyl-Valine	C14H20N2O3	264.147	1.733	Amino acid degradation product
371.035	0.78	3.47	0.00001	-	-	-	-	-
336.168	7.02	3.34	0.00000	-	-	-	-	-
247.192	5.63	3.32	0.00000	-	-	-	-	-
440.315	12.98	3.29	0.00000	LysoPE(16:0/0:0)	C21H46NO6P	439.306	2.292	Phospholipid degredation product
423.048	0.77	3.29	0.00000	-	-	-	-	-
356.255	7.82	3.15	0.00000	-	-	-	-	-
259.166	7.05	3.14	0.00000	-	-	-	-	-
304.167	5.85	3.12	0.00000	Tryptophyl-Valine	C16H21N3O3	303.158	3.221	Amino acid degradation product
233.123	1.21	3.06	0.00000	-	-	-	-	-
266.159	5.47	3.03	0.00000	-	-	-	-	-
466.330	13.30	3.02	0.00000	PE(P-18:0/0:0)	C23H48NO6P	465.322	2.558	Phospholipid degredation product
295.166	5.40	2.92	0.00000	Tyrosyl-Leucine	C15H22N2O4	294.158	2.905	Amino acid degradation product
491.036	0.77	2.87	0.00000	-	-	-	-	-
260.167	1.19	2.87	0.00000	-	-	-	-	-
260.131	1.19	2.77	0.00000	-	-	-	-	-
318.183	6.35	2.70	0.00000	-	-	-	-	-
288.170	1.19	2.69	0.00000	-	-	-	-	-
491.233	6.95	2.66	0.00005	-	-	-	-	-
370.235	8.14	2.62	0.00000	-	-	-	-	-
290.053	1.24	2.60	0.00000	-	-	-	-	-
482.362	13.08	2.59	0.00000	PC(O-16:0/0:0)	C24H52NO6P	481.353	2.536	Phospholipid degredation product
993.637	6.29	2.58	0.00004	-	-	-	-	-
404.134	5.74	2.57	0.00003	-	-	-	-	-
492.236	6.95	2.57	0.00002	-	-	-	-	-
377.227	6.84	2.57	0.00000	-	-	-	-	-
362.196	6.11	2.56	0.00000	-	-	-	-	-
791.418	7.52	2.55	0.00000	-	-	-	-	-
468.346	14.38	2.54	0.00000	PE(O-18:0/0:0)	C23H50NO6P	467.338	3.069	Phospholipid degredation product
395.712	7.50	2.53	0.00000	-	-	-	-	-
219.109	1.19	2.52	0.00004	-	-	-	-	-
790.416	7.52	2.51	0.00000	-	-	-	-	-
396.213	7.52	2.50	0.00000	-	-	-	-	-
360.196	6.10	2.48	0.00000	-	-	-	-	-
376.224	6.84	2.48	0.00003	-	-	-	-	-
1049.009	7.07	2.46	0.00002	-	-	-	-	-
905.478	6.12	2.44	0.00000	-	-	-	-	-
405.137	5.74	2.44	0.00001	-	-	-	-	-
270.079	1.24	2.44	0.00000	-	-	-	-	-
1165.667	7.19	2.44	0.00003	-	-	-	-	-
522.357	13.06	2.43	0.00025	PC(P-16:0/2:0)	C26H52NO7P	521.348	2.459	Phospholipid degredation product
792.421	7.52	2.42	0.00000	-	-	-	-	-
361.199	6.10	2.42	0.00000	Tryptophyl-Arginine	C17H24N6O3	360.191	2.627	Amino acid degradation product
523.360	13.06	2.42	0.00014	-	-	-	-	-
1164.665	7.19	2.41	0.00011	-	-	-	-	-
363.198	6.11	2.40	0.00000	-	-	-	-	-
994.640	6.29	2.35	0.00012	-	-	-	-	-
289.173	1.19	2.32	0.00000	-	-	-	-	-
281.152	2.26	2.31	0.00027	-	-	-	-	-
289.196	5.55	2.31	0.00058	-	-	-	-	-
582.837	7.19	2.30	0.00004	-	-	-	-	-
546.293	6.10	2.30	0.00002	-	-	-	-	-
343.243	6.27	2.24	0.00001	-	-	-	-	-
497.344	12.75	2.24	0.00047	-	-	-	-	-
342.240	6.27	2.23	0.00001	-	-	-	-	-
186.161	0.76	2.22	0.00000	-	-	-	-	-
683.472	6.27	2.22	0.00052	-	-	-	-	-
496.341	12.75	2.21	0.00056	PE(19:0/0:0)	C24H50NO7P	495.333	2.772	Phospholipid degredation product
380.713	7.15	2.21	0.00001	-	-	-	-	-
670.341	6.95	2.20	0.00000	-	-	-	-	-
306.145	5.24	2.20	0.00018	Threoninyl-Tryptophan	C15H19N3O4	305.138	1.300	Amino acid degradation product
700.010	7.04	2.19	0.00000	-	-	-	-	-
213.160	6.29	2.19	0.00008	-	-	-	-	-
524.345	6.75	2.18	0.00042	-	-	-	-	-
680.400	7.64	2.17	0.00050	-	-	-	-	-
491.287	6.53	2.17	0.00014	-	-	-	-	-
1166.670	7.19	2.16	0.00007	-	-	-	-	-
344.246	6.27	2.16	0.00000	-	-	-	-	-
759.413	7.15	2.15	0.00009	-	-	-	-	-
493.235	6.95	2.15	0.00008	-	-	-	-	-
527.287	8.75	2.14	0.00069	-	-	-	-	-
525.377	14.10	2.13	0.00099	PC(O-18:1(9Z)/0:0)	C26H54NO6P	507.369	1.925	Phospholipid degredation product
508.377	13.38	2.13	0.00000	-	-	-	-	-
327.209	6.29	2.13	0.00073	-	-	-	-	-
681.402	7.64	2.12	0.00025	-	-	-	-	-
786.405	8.26	2.12	0.00010	-	-	-	-	-
447.312	13.73	2.12	0.00000	-	-	-	-	-
583.338	7.19	2.12	0.00002	-	-	-	-	-
327.710	6.29	2.11	0.00020	-	-	-	-	-
290.198	5.55	2.11	0.00017	-	-	-	-	-

525.348	6.75	2.11	0.00022	-	-	-	-	-
330.677	6.51	2.11	0.00002	-	-	-	-	-
428.296	6.40	2.09	0.00056	-	-	-	-	-
313.156	6.62	2.08	0.00065	-	-	-	-	-
330.176	6.50	2.08	0.00003	-	-	-	-	-
380.211	7.15	2.08	0.00014	-	-	-	-	-
724.034	7.02	2.07	0.00000	-	-	-	-	-
887.468	7.60	2.07	0.00002	PIP(16:1(9Z)/16:1(9Z))	C41H76O16P2	886.461	0.000	Phospholipid degredation product
379.710	7.15	2.07	0.00021	-	-	-	-	-
340.704	7.64	2.07	0.00018	-	-	-	-	-
288.193	5.55	2.07	0.00327	-	-	-	-	-
604.347	7.24	2.06	0.00024	-	-	-	-	-
241.088	1.19	2.06	0.00016	-	-	-	-	-
653.408	6.29	2.06	0.00159	-	-	-	-	-
760.416	7.15	2.05	0.00018	-	-	-	-	-
660.344	6.50	2.04	0.00009	-	-	-	-	-
498.347	12.76	2.04	0.00028	-	-	-	-	-
766.922	7.84	2.04	0.00006	-	-	-	-	-
691.679	6.71	2.04	0.00001	-	-	-	-	-
724.701	7.04	2.03	0.00000	-	-	-	-	-
326.707	6.29	2.03	0.00193	-	-	-	-	-
316.188	7.05	2.03	0.00001	-	-	-	-	-
888.471	7.60	2.03	0.00003	-	-	-	-	-
605.350	7.24	2.03	0.00013	-	-	-	-	-
282.111	1.19	2.03	0.00000	-	-	-	-	-
492.291	6.53	2.02	0.00025	-	-	-	-	-
524.373	14.09	2.01	0.00496	PC(O-16:0/2:0)	C26H54NO7P	523.364	4.082	Phospholipid degredation product
1049.510	7.05	2.00	0.00000	PIP2(18:0/20:3(5Z,8Z,11Z))	C47H87O19P3	1048.505	2.561	Phospholipid degredation product

Downregulation								
m/z	**Retention Time**	**Fold Change**	***p value***	**Name**	**Formula**	**Mass of Match**	**PPM Deviation**	**Putative Role**
774.883	5.76	0.50	0.00011	-	-	-	-	-
268.660	5.95	0.50	0.00177	-	-	-	-	-
506.704	5.86	0.50	0.00878	-	-	-	-	-
1011.582	7.02	0.50	0.00681	-	-	-	-	-
277.674	5.95	0.50	0.00173	-	-	-	-	-
561.296	5.65	0.50	0.00212	-	-	-	-	-
398.534	5.15	0.50	0.00015	-	-	-	-	-
278.176	5.95	0.50	0.00227	-	-	-	-	-
402.728	5.28	0.50	0.03961	-	-	-	-	-
590.355	6.55	0.50	0.00004	-	-	-	-	-
718.300	6.42	0.49	0.00073	-	-	-	-	-
417.215	6.34	0.49	0.00362	-	-	-	-	-
714.366	7.52	0.49	0.00068	-	-	-	-	-
1206.020	6.39	0.49	0.00012	-	-	-	-	-
411.016	0.78	0.49	0.00042	-	-	-	-	-
768.416	6.56	0.49	0.00001	-	-	-	-	-
662.163	6.45	0.49	0.00037	-	-	-	-	-
1247.361	10.78	0.49	0.03234	-	-	-	-	-
190.914	0.79	0.49	0.00000	-	-	-	-	-
630.480	13.87	0.49	0.00015	-	-	-	-	-
427.220	5.77	0.49	0.00133	-	-	-	-	-
863.432	7.59	0.49	0.00038	-	-	-	-	-
666.319	5.69	0.49	0.00052	-	-	-	-	-
1250.361	10.78	0.49	0.02783	-	-	-	-	-
402.228	5.28	0.49	0.06934	-	-	-	-	-
989.356	6.25	0.49	0.00278	-	-	-	-	-
763.782	6.40	0.49	0.00351	-	-	-	-	-
477.238	5.24	0.48	0.00004	-	-	-	-	-
960.382	5.97	0.48	0.00200	-	-	-	-	-
665.316	5.69	0.48	0.01320	-	-	-	-	-
642.379	5.23	0.48	0.00000	-	-	-	-	-
275.041	0.78	0.48	0.00004	-	-	-	-	-
433.549	5.31	0.48	0.00009	-	-	-	-	-
489.869	1.23	0.48	0.00024	-	-	-	-	-
515.782	7.02	0.48	0.00512	-	-	-	-	-
124.923	0.80	0.48	0.00000	-	-	-	-	-
1000.526	6.05	0.48	0.00685	-	-	-	-	-
584.330	8.05	0.48	0.03291	-	-	-	-	-
709.408	7.15	0.47	0.00221	-	-	-	-	-
709.909	7.15	0.47	0.00318	-	-	-	-	-
498.402	14.03	0.47	0.00003	-	-	-	-	-
1418.810	7.17	0.47	0.00141	-	-	-	-	-
313.140	1.23	0.47	0.00160	-	-	-	-	-
586.454	13.92	0.47	0.00005	-	-	-	-	-
266.657	7.24	0.47	0.00014	-	-	-	-	-
534.712	6.09	0.47	0.00098	-	-	-	-	-
931.406	5.75	0.47	0.00245	-	-	-	-	-
864.435	7.59	0.47	0.00021	-	-	-	-	-
331.198	6.79	0.47	0.00097	-	-	-	-	-
940.376	5.61	0.47	0.00442	-	-	-	-	-
456.317	5.08	0.47	0.01593	-	-	-	-	-
493.170	5.74	0.47	0.00047	-	-	-	-	-
445.315	13.30	0.47	0.00003	-	-	-	-	-
296.260	15.23	0.47	0.00001	-	-	-	-	-
636.343	6.64	0.46	0.00651	-	-	-	-	-
542.428	13.97	0.46	0.00004	-	-	-	-	-
542.286	5.95	0.46	0.00228	-	-	-	-	-
451.221	5.54	0.46	0.00002	-	-	-	-	-
1096.552	6.18	0.46	0.00002	-	-	-	-	-
909.392	6.05	0.46	0.00083	-	-	-	-	-
649.818	5.31	0.46	0.00000	-	-	-	-	-
905.479	7.72	0.45	0.00998	-	-	-	-	-
217.098	5.44	0.45	0.00001	-	-	-	-	-
990.359	6.25	0.45	0.00274	-	-	-	-	-
345.699	5.37	0.45	0.00070	-	-	-	-	-
746.356	5.99	0.45	0.00000	-	-	-	-	-
369.194	14.16	0.45	0.00013	-	-	-	-	-
650.821	5.31	0.45	0.00000	-	-	-	-	-
759.376	5.65	0.45	0.00699	-	-	-	-	-
196.880	0.83	0.45	0.00002	-	-	-	-	-
439.255	7.27	0.45	0.00254	-	-	-	-	-
802.444	5.28	0.45	0.09672	-	-	-	-	-

451.720	5.54	0.45	0.00000	-	-	-	-	-
307.161	5.74	0.45	0.00077	-	-	-	-	-
340.188	6.45	0.44	0.00873	-	-	-	-	-
593.330	7.91	0.44	0.00000	-	-	-	-	-
1846.520	10.78	0.44	0.02030	-	-	-	-	-
998.511	6.37	0.44	0.01017	-	-	-	-	-
447.962	1.31	0.44	0.00007	-	-	-	-	-
616.301	7.98	0.44	0.01241	-	-	-	-	-
617.468	13.13	0.44	0.00010	-	-	-	-	-
636.272	6.35	0.44	0.00000	-	-	-	-	-
390.834	0.83	0.44	0.00003	-	-	-	-	-
1252.362	10.78	0.44	0.01306	-	-	-	-	-
629.366	7.50	0.44	0.00132	PI(20:0/0:0)	C29H57O12P	628.359	0.486	Monoacylglycerophosphoinositol
359.683	5.32	0.43	0.00601	-	-	-	-	-
308.223	8.02	0.43	0.00022	-	-	-	-	-
803.447	5.28	0.43	0.05996	-	-	-	-	-
710.911	7.17	0.43	0.00070	-	-	-	-	-
507.213	5.83	0.43	0.00053	-	-	-	-	-
797.433	7.59	0.43	0.01391	-	-	-	-	-
775.258	5.55	0.43	0.01453	-	-	-	-	-
1847.520	10.78	0.43	0.01871	-	-	-	-	-
338.173	2.19	0.42	0.00034	-	-	-	-	-
521.722	6.33	0.42	0.00082	-	-	-	-	-
635.341	6.65	0.42	0.00495	-	-	-	-	-
529.415	13.21	0.42	0.00006	-	-	-	-	-
652.330	6.64	0.42	0.00000	-	-	-	-	-
612.802	5.79	0.42	0.00671	-	-	-	-	-
628.374	5.80	0.42	0.00081	-	-	-	-	-
343.846	5.09	0.42	0.00000	-	-	-	-	-
469.233	6.25	0.42	0.00333	-	-	-	-	-
440.411	12.13	0.41	0.00002	Pentacosanoylglycine	C27H53NO3	439.403	2.292	Indicator of fatty acid oxidation
627.372	5.80	0.41	0.00060	-	-	-	-	-
489.340	13.26	0.41	0.00004	-	-	-	-	-
514.289	5.90	0.41	0.00432	-	-	-	-	-
1062.478	6.66	0.41	0.00296	-	-	-	-	-
567.907	1.31	0.41	0.00013	-	-	-	-	-
337.186	0.78	0.41	0.00130	-	-	-	-	-
495.183	6.25	0.41	0.00116	-	-	-	-	-
1024.493	7.17	0.41	0.00482	-	-	-	-	-
1248.365	10.78	0.41	0.01119	-	-	-	-	-
587.457	13.92	0.41	0.00005	-	-	-	-	-
499.278	5.38	0.41	0.00047	-	-	-	-	-
1031.557	7.02	0.41	0.00277	-	-	-	-	-
560.333	6.99	0.41	0.00048	-	-	-	-	-
1097.557	6.19	0.41	0.00001	-	-	-	-	-
795.426	7.59	0.40	0.00866	-	-	-	-	-
991.359	6.25	0.40	0.00175	-	-	-	-	-
629.375	5.80	0.40	0.00070	-	-	-	-	-
515.291	5.89	0.40	0.00334	-	-	-	-	-
562.280	6.15	0.40	0.00065	-	-	-	-	-
455.379	14.08	0.40	0.00002	-	-	-	-	-
220.012	1.23	0.40	0.00014	-	-	-	-	-
796.429	7.59	0.40	0.00970	-	-	-	-	-
1273.617	7.52	0.39	0.00076	-	-	-	-	-
764.857	5.35	0.39	0.00006	-	-	-	-	-
1848.524	10.78	0.39	0.01258	-	-	-	-	-
398.218	7.59	0.39	0.00835	-	-	-	-	-
630.369	7.50	0.39	0.00223	-	-	-	-	-
915.412	5.32	0.39	0.00609	-	-	-	-	-
511.277	8.37	0.39	0.00005	-	-	-	-	-
170.079	5.84	0.38	0.00024	-	-	-	-	-
1272.613	7.52	0.38	0.00068	-	-	-	-	-
627.971	6.59	0.38	0.00000	-	-	-	-	-
450.211	5.19	0.38	0.00901	-	-	-	-	-
449.207	5.19	0.38	0.04292	-	-	-	-	-
780.499	5.90	0.38	0.06795	-	-	-	-	-
843.443	6.19	0.38	0.00532	-	-	-	-	-
339.204	6.44	0.38	0.00839	-	-	-	-	-
526.323	6.35	0.38	0.00221	-	-	-	-	-
666.419	7.17	0.37	0.00034	-	-	-	-	-
817.409	7.59	0.37	0.00664	-	-	-	-	-
302.118	6.75	0.37	0.00001	-	-	-	-	-
559.742	6.05	0.37	0.00014	-	-	-	-	-
238.108	7.20	0.37	0.00022	-	-	-	-	-
637.313	7.52	0.37	0.00054	-	-	-	-	-
314.189	5.80	0.37	0.00026	-	-	-	-	-
637.814	7.52	0.37	0.00101	-	-	-	-	-
495.683	6.25	0.37	0.00148	-	-	-	-	-
427.391	13.65	0.37	0.00016	-	-	-	-	-
383.230	5.87	0.37	0.00123	-	-	-	-	-
624.274	5.75	0.36	0.00031	-	-	-	-	-
914.409	5.32	0.36	0.00386	-	-	-	-	-
636.811	7.52	0.36	0.00074	-	-	-	-	-
553.336	6.45	0.36	0.00963	-	-	-	-	-
1274.619	7.52	0.36	0.00057	-	-	-	-	-
268.167	6.45	0.36	0.01384	-	-	-	-	-
640.366	5.85	0.36	0.00035	-	-	-	-	-
533.367	13.21	0.36	0.00000	-	-	-	-	-
218.101	5.44	0.36	0.00002	-	-	-	-	-
744.404	6.11	0.36	0.00470	-	-	-	-	-
776.262	5.55	0.36	0.01155	-	-	-	-	-
383.189	5.34	0.36	0.00000	-	-	-	-	-
623.270	5.74	0.36	0.00083	-	-	-	-	-
384.233	5.87	0.36	0.00247	-	-	-	-	-
652.663	6.64	0.35	0.00000	-	-	-	-	-
740.459	7.34	0.35	0.00174	-	-	-	-	-
654.382	6.07	0.35	0.00028	-	-	-	-	-
382.687	5.35	0.35	0.00000	-	-	-	-	-
614.357	6.35	0.35	0.00319	-	-	-	-	-
758.374	5.65	0.35	0.00511	-	-	-	-	-
999.517	5.99	0.35	0.00590	-	-	-	-	-
387.273	12.53	0.35	0.00001	Glycerol trihexanoate	C21H38O6	386.267	3.950	Triacylglycerol - Fatty Acid
764.363	5.35	0.35	0.00000	-	-	-	-	-
578.287	5.49	0.35	0.00658	-	-	-	-	-
498.274	5.37	0.34	0.00056	-	-	-	-	-
325.716	5.87	0.34	0.00778	-	-	-	-	-
485.316	6.30	0.34	0.00726	-	-	-	-	-
383.689	5.34	0.34	0.00000	-	-	-	-	-
612.302	5.79	0.34	0.00264	-	-	-	-	-

443.184	7.52	0.34	0.00068	-	-	-	-	-
525.303	5.86	0.34	0.00011	-	-	-	-	-
561.276	6.15	0.34	0.00033	-	-	-	-	-
515.399	12.47	0.34	0.00000	-	-	-	-	-
909.504	6.75	0.34	0.00186	-	-	-	-	-
456.219	6.19	0.34	0.00097	-	-	-	-	-
769.444	6.40	0.33	0.00936	-	-	-	-	-
554.339	6.45	0.33	0.01169	-	-	-	-	-
708.430	6.40	0.33	0.00320	-	-	-	-	-
502.291	8.02	0.33	0.00011	PE(20:4(5Z,8Z,11Z,14Z)/0:0);LysoPE(20:4(5Z,8Z,11Z,14Z)/0	C25H44NO7P	501.286	4.018	Glycerophospholipid - In blood
998.515	5.99	0.33	0.00666	-	-	-	-	-
468.442	12.95	0.33	0.00002	-	-	-	-	-
997.512	5.99	0.32	0.00597	-	-	-	-	-
560.273	6.15	0.32	0.00019	-	-	-	-	-
455.215	6.19	0.32	0.00069	-	-	-	-	-
475.284	7.12	0.32	0.00100	-	-	-	-	-
516.791	7.37	0.32	0.00299	-	-	-	-	-
743.402	6.11	0.32	0.00436	-	-	-	-	-
280.641	6.15	0.32	0.00044	-	-	-	-	-
371.705	6.10	0.32	0.00422	-	-	-	-	-
316.168	5.74	0.31	0.01854	-	-	-	-	-
391.255	5.90	0.31	0.03590	-	-	-	-	-
855.498	7.57	0.31	0.00368	PI(16:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))	C45H75O13P	854.495	4.357	Glycerophosphoinositol
499.261	5.99	0.31	0.01117	-	-	-	-	-
497.272	5.37	0.31	0.00023	-	-	-	-	-
329.151	5.91	0.31	0.01691	Phenylalanyl-Tyrosine	C18H20N2O4	328.142	3.348	Amino acid degradation product
781.502	5.90	0.31	0.02047	PG(15:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C43H73O10P	780.494	1.017	Glycerophosphoglycerol
1023.488	7.15	0.31	0.00544	-	-	-	-	-
519.255	5.37	0.31	0.00072	-	-	-	-	-
317.169	5.74	0.31	0.01200	-	-	-	-	-
613.357	6.35	0.31	0.00402	-	-	-	-	-
829.431	6.00	0.30	0.00580	-	-	-	-	-
379.194	5.64	0.30	0.00652	-	-	-	-	-
757.373	5.65	0.30	0.00326	-	-	-	-	-
1159.592	7.37	0.29	0.00783	-	-	-	-	-
471.373	12.50	0.29	0.00000	-	-	-	-	-
316.669	5.74	0.29	0.02009	-	-	-	-	-
594.327	7.47	0.29	0.00049	-	-	-	-	-
499.763	5.99	0.29	0.00805	-	-	-	-	-
492.221	5.12	0.29	0.00000	-	-	-	-	-
742.400	6.10	0.29	0.00408	-	-	-	-	-
637.285	6.15	0.29	0.00051	-	-	-	-	-
1158.591	7.37	0.29	0.00827	-	-	-	-	-
254.169	6.45	0.28	0.00983	-	-	-	-	-
308.104	6.35	0.28	0.00002	-	-	-	-	-
647.801	7.51	0.28	0.00064	-	-	-	-	-
281.142	6.15	0.28	0.00028	-	-	-	-	-
1157.588	7.35	0.28	0.00609	-	-	-	-	-
563.225	6.10	0.28	0.00024	-	-	-	-	-
316.798	0.89	0.27	0.00000	-	-	-	-	-
638.289	6.15	0.27	0.00050	-	-	-	-	-
945.946	6.19	0.27	0.00045	-	-	-	-	-
491.217	5.12	0.27	0.00000	-	-	-	-	-
856.500	7.57	0.27	0.00298	-	-	-	-	-
568.298	7.17	0.27	0.00444	-	-	-	-	-
579.799	7.37	0.26	0.00800	-	-	-	-	-
560.293	5.65	0.26	0.01113	-	-	-	-	-
359.003	0.78	0.25	0.00002	-	-	-	-	-
406.809	0.81	0.25	0.00000	-	-	-	-	-
440.249	5.34	0.25	0.01330	-	-	-	-	-
540.339	6.47	0.25	0.00496	-	-	-	-	-
579.297	7.37	0.24	0.00622	-	-	-	-	-
231.114	5.71	0.24	0.00011	-	-	-	-	-
893.537	7.15	0.24	0.00273	-	-	-	-	-
408.537	5.79	0.23	0.00461	-	-	-	-	-
569.299	7.15	0.23	0.00222	-	-	-	-	-
474.280	7.10	0.23	0.00335	-	-	-	-	-
473.277	7.10	0.22	0.00335	-	-	-	-	-
572.346	7.49	0.22	0.00016	-	-	-	-	-
566.331	7.12	0.22	0.00185	-	-	-	-	-
511.746	7.14	0.22	0.00327	-	-	-	-	-
563.295	6.47	0.22	0.02675	PKOOA-PA	C27H47O10P	562.291	4.776	Oxidized glycerophosphoglycerol
1022.483	7.14	0.21	0.00588	-	-	-	-	-
575.353	7.49	0.21	0.00007	-	-	-	-	-
1105.663	5.95	0.20	0.00066	-	-	-	-	-
287.179	7.49	0.20	0.00031	-	-	-	-	-
574.351	7.49	0.20	0.00028	-	-	-	-	-
573.349	7.49	0.20	0.00022	-	-	-	-	-
286.677	7.47	0.20	0.00025	-	-	-	-	-
1106.398	5.57	0.19	0.00000	-	-	-	-	-
562.292	6.47	0.18	0.02824	-	-	-	-	-
137.030	1.19	0.18	0.00032	-	-	-	-	-
433.753	6.59	0.17	0.00516	-	-	-	-	-
866.497	6.59	0.17	0.00758	-	-	-	-	-
177.062	0.78	0.16	0.00000	-	-	-	-	-
595.325	5.79	0.16	0.01228	OOB-PG	C28H51O11P	594.317	2.054	Oxidized glycerophosphate
504.922	6.24	0.15	0.00001	-	-	-	-	-
421.211	5.85	0.15	0.00264	-	-	-	-	-
892.534	7.15	0.13	0.00186	-	-	-	-	-
326.207	5.31	0.13	0.00484	-	-	-	-	-
446.269	7.14	0.13	0.00177	-	-	-	-	-
594.323	5.79	0.13	0.00852	-	-	-	-	-
691.859	6.12	0.13	0.00060	-	-	-	-	-
133.098	0.78	0.13	0.00000	-	-	-	-	-
891.531	7.15	0.13	0.00194	-	-	-	-	-
155.081	0.79	0.12	0.00000	-	-	-	-	-
461.576	6.14	0.12	0.00069	-	-	-	-	-
446.771	7.14	0.11	0.00187	-	-	-	-	-
685.396	6.53	0.10	0.01802	-	-	-	-	-
517.286	6.21	0.10	0.00230	-	-	-	-	-
342.702	6.53	0.10	0.01585	-	-	-	-	-
867.535	8.24	0.09	0.00003	PI(P-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C47H79O12P	866.531	4.015	Fatty acid
516.283	6.21	0.09	0.00244	-	-	-	-	-
684.394	6.53	0.09	0.01991	-	-	-	-	-
480.281	6.19	0.08	0.01644	-	-	-	-	-
669.402	6.37	0.08	0.00774	-	-	-	-	-
334.703	6.37	0.07	0.00755	-	-	-	-	-
668.398	6.37	0.05	0.00804	-	-	-	-	-

Table 5.5 Putative metabolite identification of Variable Importance in Projection features identified by PLS-DA of AGCF-P bacterial and control samples. Grey = AGCF-P Control, Blue = *P. gingivalis*.

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	PPM Deviation	Putative Role
1	360.196	6.10	C	-	-	-	-	-
2	572.346	7.49	PG	-	-	-	-	-
3	268.074	1.24	C	-	-	-	-	-
4	652.405	6.29	C	-	-	-	-	-
5	758.411	7.15	C	-	-	-	-	-
6	553.336	5.95	PG	-	-	-	-	-
7	342.240	6.27	C	-	-	-	-	-
8	385.246	5.92	C	-	-	-	-	-
9	288.193	5.55	C	-	-	-	-	-
10	560.273	6.15	PG	-	-	-	-	-
11	245.187	5.62	C	Leucyl-leucine	C12H24N2O3	244.179	2.562	Amino acid degradation product
12	497.272	5.37	PG	-	-	-	-	-
13	361.199	6.10	C	Arginyl-Tryptophan	C17H24N6O3	360.191	2.627	Amino acid degradation product
14	245.186	5.15	C	Leucyl-Isoleucine	C12H24N2O3	244.179	0.062	Amino acid degradation product
15	573.349	7.49	PG	-	-	-	-	-

Table 5.6 Putative metabolite identification of important features in classification identified by RF analysis of AGCF-P bacterial and control samples. Grey = AGCF-P Control, Blue = *P. gingivalis*.

VIP Rank	m/z	RT	Class	Name	Formula	Mass of Match	PPM Deviation	Putative Role
1	383.189	5.34	PG	-	-	-	-	-
2	466.330	13.30	C	PC(P-15:0/0:0)	C23H48NO6P	465.3219	2.557768178	Breakdown of phosphatidylcholine
3	746.356	5.99	PG	-	-	-	-	-
4	582.768	6.64	C	-	-	-	-	-
5	259.166	7.05	C	-	-	-	-	-
6	268.074	1.24	C	-	-	-	-	-
7	304.167	5.85	C	Tryptophyl-Valine	C16H21N3O3	303.1582916	3.221295664	Amino Acid degradation product
8	1106.398	5.57	PG	-	-	-	-	-
9	724.034	7.02	C	-	-	-	-	-
10	764.363	5.35	PG	-	-	-	-	-
11	342.702	6.53	PG	-	-	-	-	-
12	696.009	6.69	PG	-	-	-	-	-
13	316.798	0.89	PG	-	-	-	-	-
14	294.681	6.55	PG	-	-	-	-	-
15	217.098	5.44	PG	-	-	-	-	-

5.3.4 qPCR of Fibrosis Associated Genes

In order to further investigate the potential fibrosis inducing properties of bacterial supernatants on kidneys, a more sensitive assay was utilised. Multiple qPCR experiments were conducted on mRNA extracted from kidney cells treated with *P. gingivalis, F. nucleatum, S. mutans* supernatants or with AGCF-P media controls. A selection of markers that are indicative of fibrosis were used as discussed in Chapter 4.1.2: Collagen Type 1 Alpha 1 (ColA1), Plasminogen Activator Inhibitor 1 (PAI-1), Tissue Inhibitor of Metalloproteases 1 (TIMP1), Transforming growth factor beta 1 (TGF-β) and Vascular Endothelial Growth Factor (VEGF).

5.3.4.1 Collagen Type 1 Alpha 1 mRNA Expression

After 24 hours of treatment with serum free media along with *P. gingivalis* and AGCF-P supernatants collected over 5 days, mRNA levels of the ColA1 were measured by qPCR (Figure 5.14). ColA1 mRNA levels were significantly reduced in HK-2 cells exposed to *P. gingivalis* or AGCF-P supernatants in comparison to cells exposed to serum free medium. Although a time-dependent decrease of ColA1 mRNA levels was observed in HK-2 cells exposed to *P. gingivalis* supernatants, the ColA1 mRNA levels were significantly higher for 1, 2 and 4 D supernatants, as compared to AGCF-P media. In contrast, at 5 D ColA1 mRNA was significantly higher in cells exposed to AGCF-P media, as compared to *P. gingivalis* supernatants.

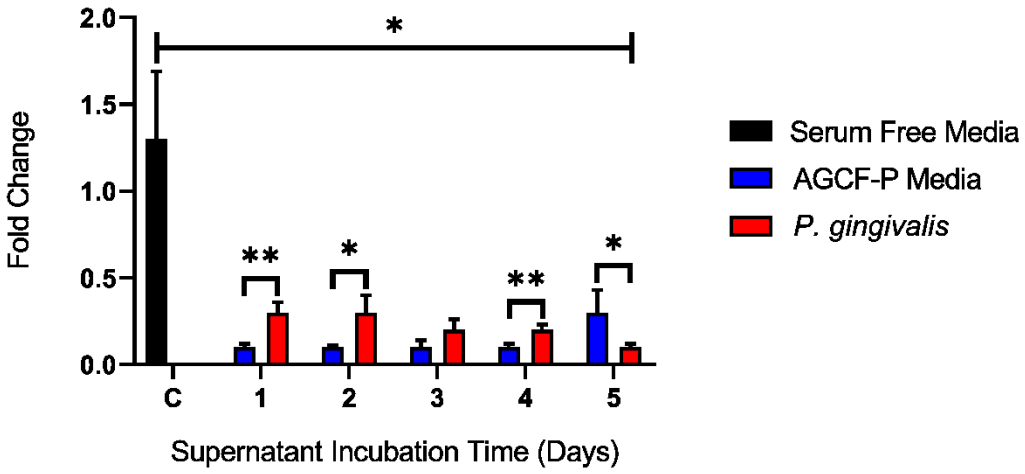

Figure 5.14 Relative gene expression in comparison to β actin of Collagen Type 1 Alpha 1 mRNA in HK-2 cells after exposure to exposure to serum free media along with *P. gingivalis* or AGCF-P supernatants for 24 hours. |-| indicates significance between the first bar and all others. Error bars represent SEM. * = *p*-value ≤ 0.05, **= *p*-value ≤ 0.01 (*n=3*).

Similar results were obtained when HK-2 cells were exposed to *F. nucleatum* and *S. mutans* supernatants (Fig 5.15). The ColA1 mRNA levels were significantly reduced HK-2 cells exposed to 1 D *F. nucleatum, S. mutans* or AGCF-P supernatants and 2 D AGCF-P supernatants in comparison to cells exposed to serum free medium. There were also significant differences between both respective 2 D bacterial supernatants in comparison to the 2 D AGCF-P media control.

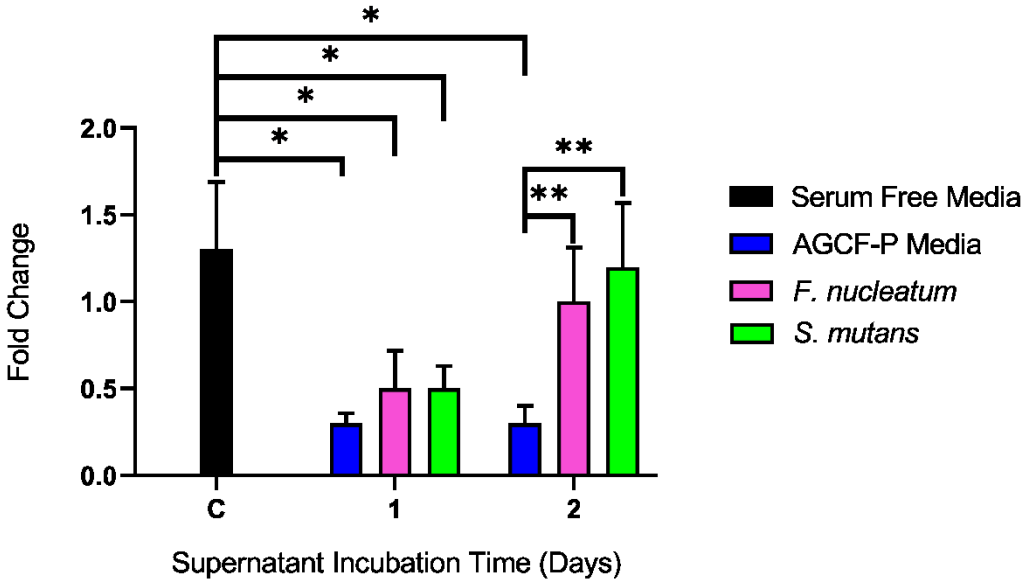

Figure 5.15 Relative gene expression in comparison to β actin of Collagen Type 1 Alpha 1 mRNA in HK-2 cells after exposure to serum free media along with *Fusobacterium nucleatum, Streptococcus mutans* or AGCF-P supernatants for 24 hours. Error bars represent SEM. * = *p*-value ≤ 0.05, ** = *p*-value ≤ 0.01, *** = *p*-value ≤ 0.001 (*n=3*).

5.3.4.2 Plasminogen Activator Inhibitor 1 mRNA Expression

After 24 hours of treatment with serum free media along with *P. gingivalis* and AGCF-P supernatants collected over 5 days, mRNA levels of the PAI-1 gene were significantly downregulated by *P. gingivalis* or AGCF-P supernatants in comparison to the serum free media control (Fig 5.16). There was also a significant increase between 3 D *P. gingivalis* supernatants and 3 D AGCF-P media.

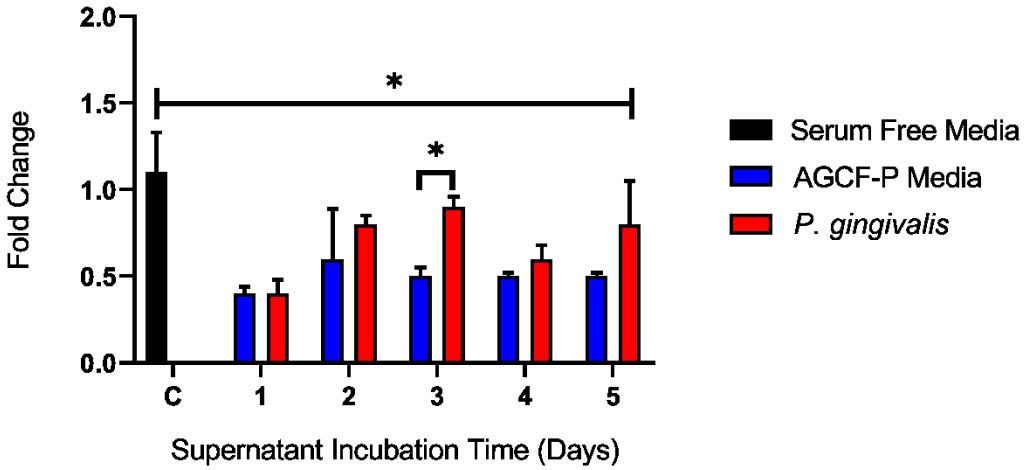

Figure 5.16 Relative gene expression in comparison to β actin of Plasminogen Activator Inhibitor 1 mRNA in HK-2 cells after exposure to serum free media along with *P. gingivalis* or AGCF-P supernatants for 24 hours. |-| indicates significance between the first bar and all others. Error bars represent SEM. * = *p*-value ≤ 0.05 (*n=3*).

In agreement with the experiment above, the PAI-1 mRNA levels were significantly downregulated in HK-2 cells exposed to 1 and 2D AGCF-P media as compared to the serum free media control (Fig 5.17). However, 1 and 2 D *F. nucleatum* and *S. mutans* supernatants, had no significant difference in comparison to the serum free media control. There was also a significant increase between both respective 2 D bacterial supernatants in comparison to the 2 D AGCF-P media control.

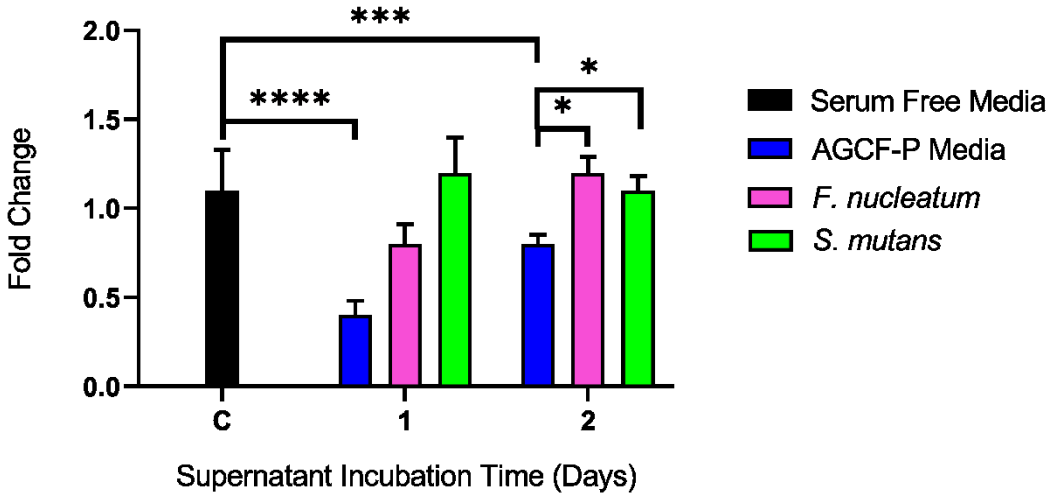

Figure 5.17 Relative gene expression in comparison to β actin of Plasminogen Activator Inhibitor 1 mRNA in HK-2 cells after exposure to serum free media along with *Fusobacterium nucleatum, Streptococcus mutans* or AGCF-P supernatants for 24 hours. Error bars represent SEM. * = *p*-value ≤ 0.05, ** = *p*-value ≤ 0.01, *** = *p*-value ≤ 0.001, **** = *p*-value ≤ 0.0001 (*n=3*).

5.3.4.3 Transforming Growth Factor Beta 1 mRNA Expression

After 24 hours of treatment with serum free media along with *P. gingivalis* and AGCF-P supernatants collected over 5 days, mRNA concentrations of the TGF-β gene were measured (Figure 5.18). In comparison to the serum free medium control, 1 and 2 D *P. gingivalis* supernatants were significantly upregulated whereas 3 D AGCF-P supernatants were significantly downregulated. There rest of the microbial and AGCF-P supernatants had no significant difference in comparison to the control. There was also a significant increase in mRNA concentrations of the TGF-β gene between 1, 2 and 4 D *P. gingivalis* supernatants in comparison to 1, 2 and 4 D AGCF-P media.

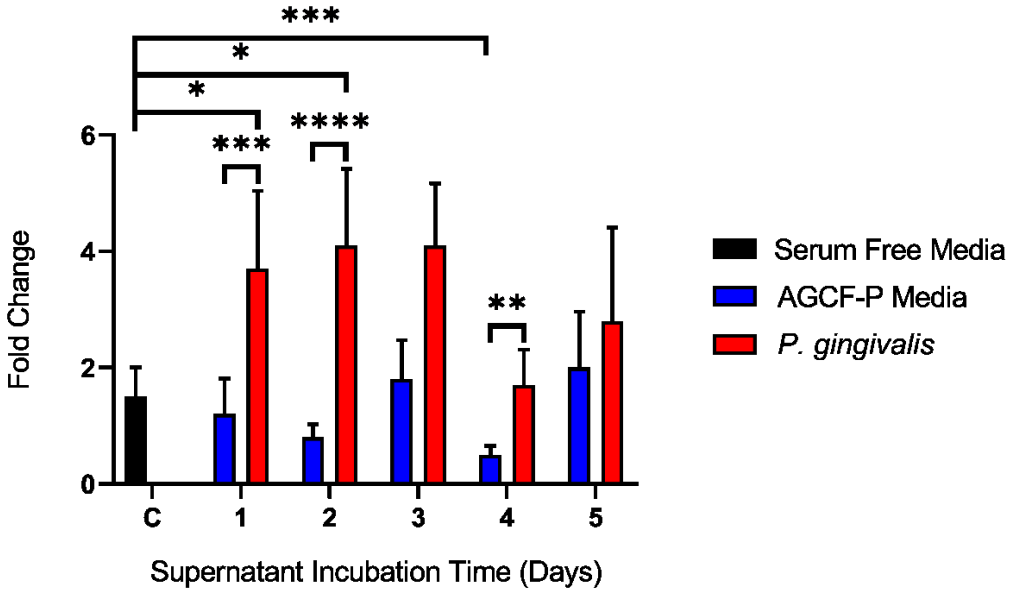

Figure 5.18 Relative gene expression in comparison to β actin of Transforming Growth Factor beta 1 mRNA in HK-2 cells after exposure to serum free media along with *P. gingivalis* and AGCF-P supernatants for 24 hours. Error bars represent SEM. * = *p*-value ≤ 0.05, ** = *p*-value ≤ 0.01, *** = *p*-value ≤ 0.001, **** = *p*-value ≤ 0.0001 (*n=3*).

For the *F. nucleatum* and *S. mutans* supernatants (Figure 5.19), statistical tests showed that both 1 D *F. nucleatum* and 2D AGCF-P supernatants were significantly downregulated in comparison to the serum free media control. There was also a significant increase between both respective 2 D bacterial supernatants in comparison to the 2 D AGCF-P media control.

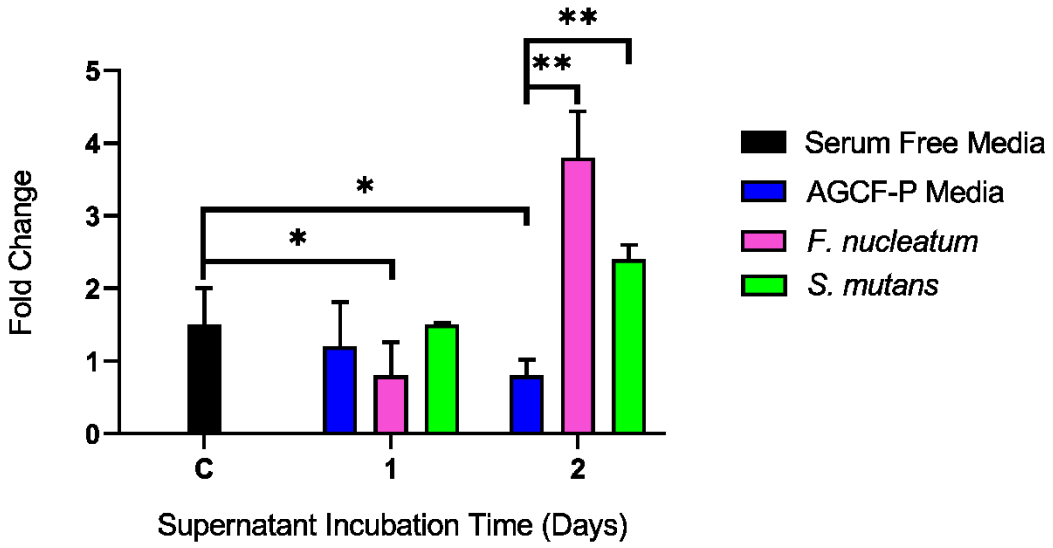

Figure 5.19 Relative gene expression in comparison to β actin of Transforming Growth Factor beta 1 mRNA in HK-2 cells after exposure to serum free media along with *Fusobacterium nucleatum, Streptococcus mutans* or AGCF-P supernatants for 24 hours. Error bars represent SEM. * = *p*-value ≤ 0.05, ** = *p*-value ≤ 0.01 (*n=3*).

5.3.4.4 Tissue Inhibitor of Metalloproteases 1 mRNA Expression

After 24 hours of treatment with serum free media along with *P. gingivalis* and AGCF-P supernatants collected over 5 days, mRNA levels of the TIMP1 were measured by qPCR (Figure 5.20). There was no significant change between all *P. gingivalis* and AGCF-P supernatants in comparison to cells exposed to serum free medium control. However, there was a significant increase between 1 and 4 D *P. gingivalis* supernatants and 1 and 4 D AGCF-P media.

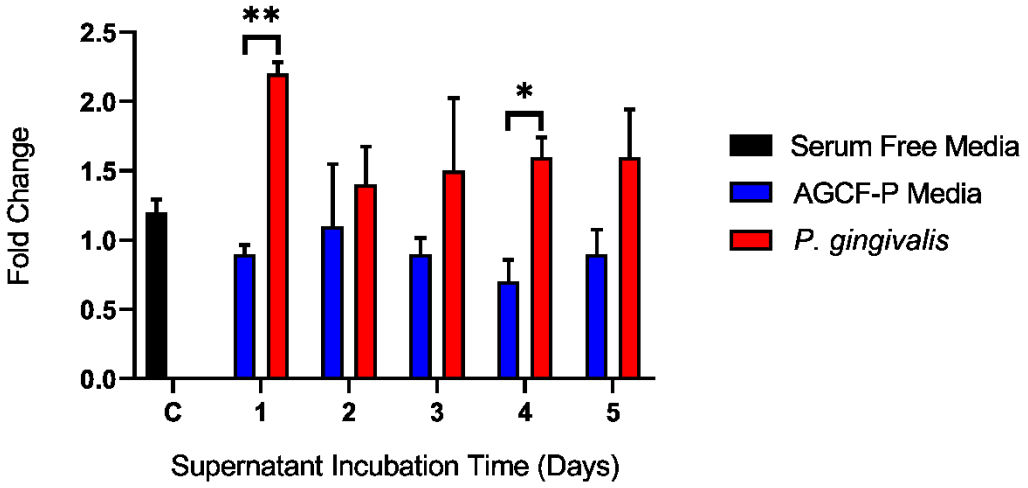

Figure 5.20 Relative gene expression in comparison to β actin of Tissue Inhibitor of Metalloproteases 1 mRNA in HK-2 cells after exposure to serum free media along with *P. gingivalis* or AGCF-P supernatants for 24 hours. Error bars represent SEM. * = *p*-value ≤ 0.05 (*n=3*).

Similar results were obtained when HK-2 cells were exposed to *F. nucleatum* or *S. mutans* supernatants (Fig 5.21). There was no significant change between all *P. gingivalis* and AGCF-P supernatants in comparison to cells exposed to serum free medium control. There was also no significant differences between the bacterial supernatants in comparison to the AGCF-P media control.

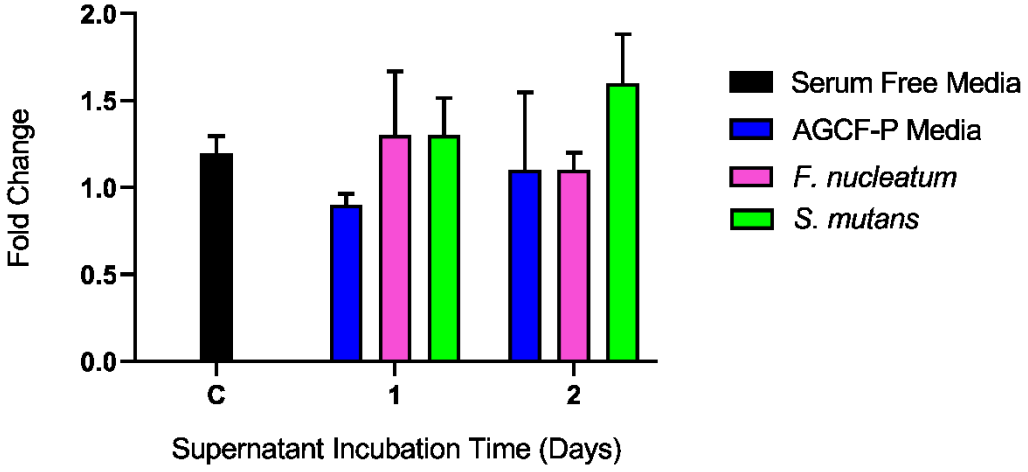

Figure 5.21 Relative gene expression in comparison to β actin of Tissue Inhibitor of Metalloproteases 1 mRNA in HK-2 cells after exposure to serum free media along with *Fusobacterium nucleatum, Streptococcus mutans* and AGCF-P supernatants for 24 hours. Error bars represent SEM (*n=3*).

5.3.4.5 Vascular Endothelial Growth Factor mRNA Expression

After 24 hours of treatment with serum free media along with *P. gingivalis* or AGCF-P supernatants collected over 5 days, mRNA levels of the VEGF were measured by qPCR (Figure 5.22). There was no significant change between all *P. gingivalis* and AGCF-P supernatants in comparison to cells exposed to serum free medium control. In addition, there were no significant differences between all *P. gingivalis* and AGCF-P media supernatants at each respective time point.

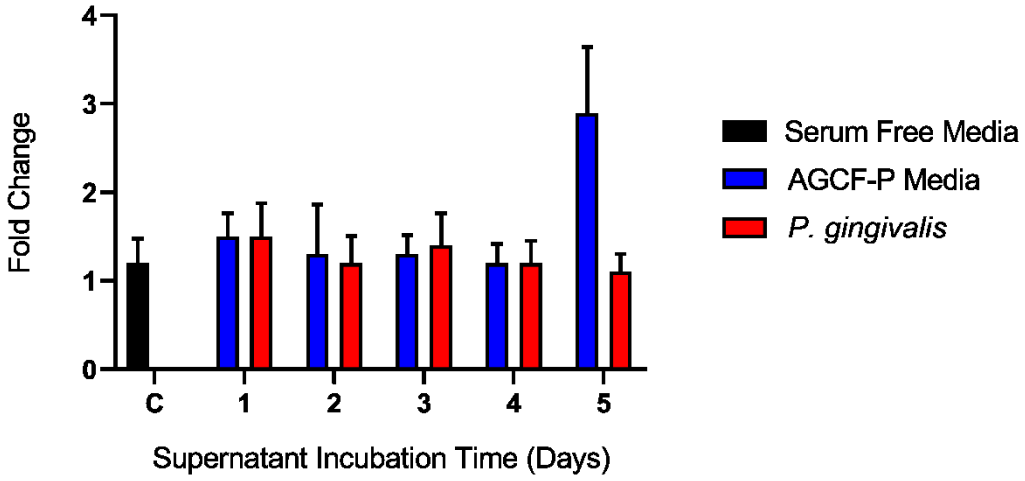

Figure 5.22 Relative gene expression in comparison to β actin of Vascular Endothelial Growth Factor mRNA in HK-2 cells after exposure to serum free media along with *P. gingivalis* or AGCF-P supernatants for 24 hours. Error bars represent SEM (*n=3*).

Similar results were obtained when HK-2 cells were exposed to *F. nucleatum* or *S. mutans* supernatants (Fig 5.23). There was no significant change between all *P. gingivalis* and AGCF-P supernatants in comparison to cells exposed to serum free medium control. There was also no significant differences between the bacterial supernatants in comparison to the respective AGCF-P media control.

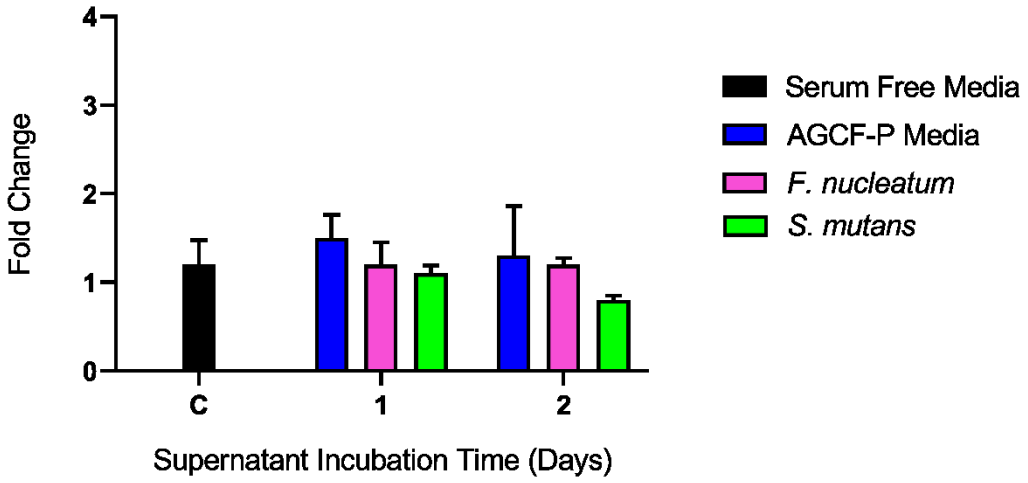

Figure 5.23 Relative gene expression in comparison to β actin of Vascular Endothelial Growth Factor mRNA in HK-2 cells after exposure to serum free media along with *Fusobacterium nucleatum, Streptococcus mutans* or AGCF-P supernatants for 24 hours. Error bars represent SEM (*n=3*).

5.4 DISCUSSION

5.4.1 LPS Quantification and Testing

The results of the LPS testing of supernatant samples shows that there is a very low intrinsic amount of LPS within the *P. gingivalis* (~0.3 EU/mL) and AGCF-P (~0.1 EU/mL) samples that is relatively constant. The low amount of LPS in the *P. gingivalis* samples is mostly likely due to the lack of an LPS extraction protocol utilised during the preparation of microbial supernatants, without this the LPS would stay relatively intact and attached to the cell wall even after death. In order for LPS to be extracted, more aggressive methods need to be employed to break the bacterial cell wall, for example the phenol water method used to purify *P. gingivalis* LPS (Asai et al., 2005), this was not used as this study did plan on studying the effect of LPS on cells specifically. The presence of more LPS within the *P. gingivalis* compared to the AGCF-P control is most likely an indicator of release of small amounts of LPS over the growth cycle of *P. gingivalis*. The presence of small amounts of LPS within the non-inoculated media is most likely due to the long-term storage vessels of these supernatants being autoclaved glass bijoux, with it being well reported that endotoxin can survive filtering and autoclaving (Wang and Doyle, 1998, Holland et al., 2000). However, it is important to note that the EU/mL levels reported are comparatively low and within the Federal Drug Administration guidelines for sterile water (0.25-0.5 EU/mL) depending on intended use (2014).

There are suggestions that *P. gingivalis* LPS elicits a weaker immune response compared to pathogenic *E. coli*, with a heterogeneous population of lipid A molecules allowing for a weak agonistic and antagonistic response in order to disrupt oral homeostasis and ensure the chronic nature of CP (Jain and Darveau, 2010). However, the results of this study have demonstrated that pathological levels of these specific strains of *E. coli* or *P. gingivalis* derived LPS have no effect on the viability of HK-2 and HGF-1 cells *in vitro*. The lack of effect by both LPS preparations is most likely due to this model using a singular cell line and may be different in other cell types; there are also known strain variations between different clinical subtypes and this could have a different effect. The pathogenic effects of LPS are known to be related to the

induction of an inflammatory response by activation of toll like receptors and immune cell recruitment (Underhill and Ozinsky, 2002, Biswas and Lopez-Collazo, 2009). In this case, it is clear that the *P. gingivalis* LPS released and present in the supernatant samples are not responsible for the cytotoxic cell viability effects reported in Chapter 4.

5.4.2 Secreted Protein Testing

The heat and trypsin inactivation of protein complexes in the microbial and control supernatants was unsuccessful due to interference with the colorimetric cell viability assays. However, by restoring the viability of HK-2 cells, the more targeted protease inhibitor cocktail mix was successful in providing evidence for the putative cause of the cytotoxic effect of the *P. gingivalis* supernatants. This is consistent with evidence in the literature of the proteinase-dependent cytotoxicity towards various cell lines (Johansson and Kalfas, 1998, Sheets et al., 2005, Sheets et al., 2008, Frisch and Francis, 1994) which has been suggested to be caused by a special type of apoptosis called 'anoikis' caused by the disruption of cell-matrix interactions (Frisch and Francis, 1994). There is also evidence that Gingipains have been specifically implicated in Alzheimer's disease, with evidence of the presence in the cerebral spinal fluid and brains of patients and correlations with the levels of tau and ubiquitin pathology (Dominy et al., 2019). Gingipains are also reported to stimulate the secretion of pro-inflammatory cytokines through protease-activated receptors and is suggested to be one of the ways *P. gingivalis* can induce inflammation in CP (Uehara et al., 2008).

In this research chapter, qPCR was used to look at mRNA levels of two coupled G-protein receptors, PAR1 and PAR2, known to be associated with serine proteases such as Gingipains (Olczak et al., 2001). The PAR1 receptor is reported to be activated by serine proteases such as thrombin, while the PAR2 receptor is activated by serine proteases like trypsin and both are thought to be a detector system for tissue injury and play a role in initiating inflammation (Coughlin and Camerer, 2003). In the literature there is some evidence to suggest PAR2 receptors play a role in periodontitis associated inflammation (Vergnolle, 2004), with *P. gingivalis* Gingipains being implicated in activating PAR2 receptors and stimulating the release of

interleukin-6 (Holzhausen et al., 2006, Lourbakos et al., 2001). Currently there is evidence from enzyme based assays that *P. gingivalis* derived proteases can activate PAR2 receptors in neutrophils (Lourbakos et al., 1998) and oral epithelial cells (Lourbakos et al., 2001) but there are no studies that have investigated the effect on PAR2 activation in kidney epithelial cells or utilising qPCR, highlighting the novelty of the work completed. The qPCR evidence (Figure 5.7) shows no significant change with mRNA levels of the PAR1 receptor (Figure 5.7a) with the addition of *P. gingivalis* and AGCF-P supernatants in comparison with the serum free media control, which is consistent with the literature (Dommisch et al., 2007). The qPCR determining mRNA levels of the PAR2 receptor showed only a significant difference between 1 and 2 D *P. gingivalis* and AGCF-P supernatant samples but no difference in comparison with the serum free media, however activation of a receptor does not necessarily affect receptor mRNA levels. Evidence in the literature suggests a high affinity of Gingipains for activation of PAR2 receptors, which can recruit inflammatory cells, and as a major virulence factor of CP (Holzhausen et al., 2006, Lourbakos et al., 2001, Lourbakos et al., 1998). The lack of a statically significant mRNA upregulation in comparison to serum free media could be due to a variety of reasons such as: mRNA levels of the receptors not being significantly increased on activation of the PAR receptors or even the presence of siRNA in the AGCF-P medium preventing the increased expression (Dommisch et al., 2007). With this in mind, it would be of interest in the future to measure the calcium signalling response of the receptors from activation and cleavage rather than mRNA levels of the genes encoding PARs, as this has been successfully used to quantify PAR activation in various other studies (Holzhausen et al., 2006, Lourbakos et al., 2001).

5.4.3 LCMS Testing of *P. gingivalis* and AGCF-P Supernatants

As similarly discussed in Chapter 3 (Section 3.4-3.5) the untargeted metabolomics pathway was successful in being able to translate raw LCMS results to a selection of significant metabolite features associated with microbial degradation of AGCF-P media. This is confirmed from the PCA and PLS-DA analyses that there is a statistically significant difference between the QC, AGCF-P control and bacterial classes, confirming that microbial degradation of AGCF-P took place and the classes can be

separated based on the products present. The modifications to the methods from Chapter 3 (Section 3.3.5) such as extra quality assurance steps with the addition of the QC samples allows the data to be much more robust and can allow for re-analysis in the future.

However, as also discussed in depth in Chapter 3 (Section 3.4-3.5), metabolite identification of features reported to be 'significant' was poor with a 6.6% success rate in identification. Of the metabolites that were identified the majority were relatively non-specific being amino acid degradation products upregulated and nutrient sources downgraded which are not specific enough biomarkers. Not being able to identify the large majority of the features of interest limits the experiment as it restricts translation of potential metabolites to look at their biological role. While currently many of these significant features cannot be identified, there is a high chance that they will be elucidated in the future. Currently there are only a few microorganisms who have had their metabolome studied thoroughly such as *E. coli* (Guo et al., 2012) and *Pseudomonas aeruginosa* (Huang et al., 2017) after following on from the examples of the curated yeast metabolome database (Jewison et al., 2011) and the human metabolome database (Wishart et al., 2017). With more experiments being undertaken on the role of the collective metabolome of the oral cavity in periodontitis (Gawron et al., 2019, Velsko et al., 2017) and growth in the use of metabolomics in studies (Kell and Oliver, 2016), a project to create a CP metabolome database looking at key microorganisms could be achievable in the near future and will allow the data generated here to be re-analysed.

5.4.4 mRNA levels of markers of fibrosis in response to bacterial supernatants

5.4.4.1 ColA1 mRNA Levels

The ColA1 mRNA was a significantly downregulated by all *P. gingivalis* supernatants, 1 D *F. nucleatum* and *S. mutans* supernatants and AGCF-P media in comparison with the serum free medium. However, there were no significant differences between 2 D *F. nucleatum* and *S. mutans* supernatants compared with serum free medium and they were significantly upregulated from the matched 2 D AGCF-P media.

The reason behind the significant downregulation could be due to the pro-proliferative and pro-migratory nature of the AGCF-P medium (Section 4.3.2 and 4.4.2). As the collagen family of proteins play an important part of the ECM, they are more likely to be upregulated if there is more space between cells and need for ECM production (Brunner et al., 2010). With the AGCF-P medium inducing proliferation and migration of HK-2 cells, there will be less space between the cells compared to the serum free medium, so the expression of collagen mRNA may be decreased. The significant upregulation of collagen mRNA by 1, 2 and 4 D *P. gingivalis,* 2 D *F. nucleatum* and *S. mutans* supernatants compared to the matched AGCF-P control medium suggests that these bacteria release pro-fibrotic factors in their supernatants. This may be due to a feedback loop of regulation due to the collagen proteolytic activity of *P. gingivalis* secreted Gingipains (Tokuda et al., 1998). The upregulation of 2 D *F. nucleatum* and *S. mutans* supernatants compared to the matched 2 D AGCF-P control could potentially be related to the reported abilities of both organisms to bind to collagen type 1 (Xie et al., 1991, Chow et al., 2019). The downregulation of collagen mRNA expression by 5 D *P. gingivalis* supernatants may be related to the growth cycle of the microorganisms being in stationary/death phase, with less pro-fibrotic mediators being secreted.

5.4.4.2 PAI-1 mRNA Levels

The PAI-1 mRNA levels were significantly downregulated by all *P. gingivalis* and AGCF-P supernatants in comparison with the serum free medium, whereas there was no significant differences with *F. nucleatum* and *S. mutans* supernatants. Indeed, only 3 D *P. gingivalis* supernatants significantly upregulated PAI-1 mRNA expression compared to matched 3 D AGCF-P medium, while 2 D *F. nucleatum* and *S. mutans* supernatants were significantly upregulated compared to the matched 2 D AGCF-P medium.

There is some evidence to suggest that *P. gingivalis* Gingipains can degrade PAI-1 with Gingipain defective strains having a negligible effect on degradation (Klarström Engström et al., 2015). However, this degradation would not have an impact on the mRNA levels of PAI-1 so would not be apparent via PCR. Conversely, there is also some evidence *P. gingivalis* can increase PAI-1 and suppress t-PA in aortic cells

(HAEC), which induces a pro-coagulant response (Roth et al., 2006). Currently there is nothing in the literature to describe potential interactions with both *F. nucleatum* and *S. mutans* and PAI-1.

5.4.4.3 TGF-β mRNA Levels

The TGF-β mRNA levels were significantly upregulated by only 1 and 2 D *P. gingivalis* supernatants while and 3 D AGCF-P supernatants were downregulated in comparison with the serum free medium, whereas there was no significant differences with *F. nucleatum* and *S. mutans* supernatants. In addition 1 and 2 D *P. gingivalis* supernatants had significantly upregulated TGF-β mRNA expression compared to matched 1 and 2 D AGCF-P medium, while 2 D *F. nucleatum* and *S. mutans* supernatants were significantly upregulated compared to the matched 2 D AGCF-P medium.

There is evidence in the literature that treatment of human fibroblasts (Palm et al., 2015), human aortic smooth muscles (Zhang et al., 2013) and human monocytes (Gemmell and Seymour, 1993) with *P. gingivalis* induced expression of TGF-β. This effect is attenuated in a Gingipain deficient strain (Palm et al., 2015) and appears to be mediated by the notch signalling pathway (Zhang et al., 2013). However, despite the upregulation reported in these results, there is evidence that TGF-β may have tissue specific activation, suggesting comparisons of expression with other cell lines may not be useful (Annes et al., 2003). There is some evidence for upregulation of TGF-β by *F. nucleatum* (Zhang et al., 2013) but none for *S. mutans*.

5.4.4.4 TIMP1 mRNA Levels

The TIMP1 mRNA levels did not significantly change for all microbial and AGCF-P media supernatants in comparison to the mRNA levels of the serum free media control. However, there was significant upregulation between TIMP1 mRNA levels of 1 and 4 D *P. gingivalis* supernatants in comparison to matched 1 and 4 D AGCF-P medium supernatants.

This outcome could potentially be due to TIMP1 gene expression activation by *P. gingivalis* LPS, as demonstrated in gingival fibroblasts (Herath et al., 2013). Conversely, there is also evidence that *P. gingivalis* strains with Gingipains (Type strain) and deficient in Gingipains (KDP128) do not significantly affect mRNA levels of

TIMP1 but affect the expression of TIMP2 in an engineered oral mucosa model of fibroblasts and epithelial cells (Andrian et al., 2007). Therefore, the regulation of TIMP-1 gene expression may vary between tissue types and it remains to be examined in HK-2 cells.

5.4.4.5 VEGF mRNA Levels

The TIMP1 mRNA levels did not significantly change for all microbial and AGCF-P medium supernatants in comparison to the mRNA levels of the serum free media control. There was also no significance between any microbial supernatant and matched AGCF-P medium supernatant sample, indicating a lack of effect for all preparations on the mRNA regulation of VEGF. This does appear to be contrary to evidence of VEGF gene expression being upregulated in gingival fibroblasts by *P. gingivalis* (Domon et al., 2014). However, this was after incubation with a more virulent *P. gingivalis* strain (W83) which may mean this upregulation is due to a multiple of other virulence factors or due to the fact this was direct live incubation rather than supernatant samples such as like we were testing.

5.4.4.6 Overall trend

There does not appear to be any significant trend associated with the five different fibrosis associated mRNA markers. However, there does seem to be a trend related to downregulation of the mRNA of both ColA1 and PAI-1 after treatment with the AGCF-P medium and all microbial samples compared to serum free medium, indicating that it may be due to the AGCF-P medium. As seen in the cellular migration studies (Section 4.3.2 and 4.4.2) the AGCF-P media appears to significantly increase the migration and proliferation of HK-2 cells and this is most likely due to the increased nutrients and potential growth factors from the addition of horse blood compared to the serum free media control. As serum free media is known to be less proliferative and lacking in soluble factors to stimulate growth (Brunner et al., 2010), it may be that the increased ColA1 and PAI-1 levels in the serum free medium could be due to a compensatory mechanisms to ensure integrity of the cell monolayer of the proximal tubule. Therefore, the decreased proliferation of cells cultured in serum free media could lead to compensatory changes leading to the upregulation of ColA1

and PAI-1 among other mRNA products, which induce the production of extracellular matrix and ensure the tubules maintain integrity in the monolayer.

Only *P. gingivalis* supernatants collected on day 1 and 2 had a significant upregulation compared to serum free media and the AGCF-P media control for TGF-β mRNA and this appears to be consistent with the literature for *P. gingivalis* upregulating TGF-β. For *F. nucleatum* and *S. mutans* there were no instances of significance when comparing to both serum free medium and AGCF-P medium.

If the results of the mRNA amounts in HK-2 cells after treatment with all bacterial supernatants are compared to the matched AGCF-P medium alone, it does appear that there is a potential pro-fibrotic effect with three different markers (TGF-β, ColA1 and TIMP1). There are much higher TGF-β mRNA levels when treated with 1 and 2 D *P. gingivalis* supernatants, increased ColA1 levels when treated with 1, 2 and 4 D *P. gingivalis* supernatants and increased TIMP1 mRNA levels when treated with 2 and 4 D *P. gingivalis* supernatants. In addition, if the PAI-1 results are compared with the PAI-1 Luciferase Reporter Assay (Chapter 4.5.3) and the qPCR tests in this chapter, there does appear to be a corroboration between levels for *P. gingivalis* and AGCF-P supernatants, with the exception of 3 D *P. gingivalis* supernatants that have increased levels in the qPCR.

5.5 Conclusions

This chapter has identified potential factors behind the cellular damage elicited by *P. gingivalis* supernatants. This work has demonstrated that in contrast to secreted proteases such as Gingipains, LPS does not play a role in HK-2 cytotoxicity and the role of secreted proteins such as Gingipains appearing as the lead candidate in both these experiments and in the literature. Indeed, further experiments need to be conducted in order to confirm this association.

The investigations into the fibrotic potential of periodontitis associated pathogens still remains unclear, with no distinct up-regulatory trends across the five markers. The only mRNA marker with significant upregulation compared to serum free and the AGCF-P growth medium control was TGF-β for 1 and 2 D *P. gingivalis* supernatants. However, if fold change in comparison to the AGCF-P medium is only considered there does appear to be a potential pro-fibrotic effect with increased expression of three different markers: TGF-β, ColA1 and TIMP1. Further studies investigating this upregulation are also warranted in order to confirm this.

CHAPTER 6: DISCUSSION AND FUTURE WORK

6.1 DISCUSSION

CKD and CP are both common diseases, which are frequently comorbid with each other and have an overlap in risk factors (Jones, 2010, Schellevis et al., 1993, Stringer et al., 2013). They are also both risk factors for each other and there is evidence that the concurrent presence of both diseases has a detrimental effect on the progression of each respective disease (Sharma et al., 2016, Zhang et al., 2017). CKD is reported to have a variety of effects on the body such as systemic inflammation and retention of uremic toxins, along with localised effects at the site of the oral cavity such as changes in the increased pH of oral fluids and inflammation of oral tissue (Akar et al., 2011, Oyetola et al., 2015). In addition, CP is also reported to have a variety of effects on the body with evidence of systemic inflammation along with bacteraemia of microbes and their products into the bloodstream (Moutsopoulos and Madianos, 2006, Fisher et al., 2010). These effects have led to reports of its involvement in a variety of other diseases such as Alzheimer's and cardiovascular disease (Dominy et al., 2019, Dietrich et al., 2008). With the known association between CKD and CP, understanding how they may influence each over is paramount as the identification of novel interventions/modifiable risk factors to slow disease progression is an area of urgency particularly in CKD (James et al., 2010). Previous studies have investigated the directionality of the relationship between these two diseases (Zhao et al., 2018, Fisher et al., 2011, Wahid et al., 2013); however there is a lack of literature that has examined how these diseases may be interacting at systemic and microbial level to cause this association.

This research has delivered an in depth review of the potential interactions between CKD and CP (Chapter 1) and developed a more representative growth medium for cultivation of a selection of periodontitis associated pathogens which better enabled the studying of their prospective effects (Chapter 2). Furthermore, a novel metabolomics based method using LCMS was developed in order to trial the use of metabolomics as a way of rapidly screening microbes for their ability to produce uremic toxins (Chapter 3). Additionally, the ability of bacterial products from selected microorganisms to elicit a detrimental effect on kidney cells was investigated (Chapter 4), with a specific focus on investigation of the potential to initiate fibrosis

(Chapters 4 and 5). Only one chosen microorganism elicited a detrimental effect on kidney cells, *Porphyromonas gingivalis*, and the causative component behind this effect was also explored (Chapter 5).

One of the objectives of this study was to identify a selection of microorganisms implicated in CP, along with developing a growth medium representative of the diseased environment. The selection of periodontitis organisms to study is complex due to a large number of associated organisms reported as being involved in periodontal disease (Table 1.1) (Wade, 2013) and it was not feasible to investigate all of them individually along with the various permutations of multispecies biofilms. With that being the case, the organisms chosen to be investigated (Chapter 2.1.4) represent a small subsection of periodontitis associated pathogens that have been implicated either in association with CP and CKD or just CP and systemic disease, along with two non-associated microorganisms to act as controls. The development of a new growth medium (Hickey et al., 2019) whilst not an original intention of the research, became apparent from the literature the difficulties of culturing periodontal pathogens and the lack of one commercially available growth medium that would support the growth of the selected organisms. One reasoning behind the development of this new growth medium was that cultivation of particularly fastidious organisms is reported to be achievable by mimicking components of the physiological environment where certain microbes are found (Kaeberlein et al., 2002, Vartoukian et al., 2010). Furthermore, there is evidence that different nutrient sources could potentially induce regulatory changes in gene expression and downstream products (Shimizu, 2014, Balleza et al., 2009), which may induce the chosen microorganisms to produce potential uremic compounds. The initial metabolomic proof of concept tests between two growth media BHIB and TSB partially confirmed the variance based on nutrient source, with a wide variety of potential metabolites produced by the same microorganism grown in two different media (Chapter 3). Overall, the use of a singular medium for all microorganisms allowed for better comparisons between the potential effects or lack of between the chosen microorganisms and a common control and facilitated further *in vitro* investigations in later chapters. However, these *in vitro* investigations did highlight a

disadvantage, with the complex formulation of this medium appearing to have some effects when in contact with human kidney cells which caused issues when investigating specific fibrosis markers.

The next objective was to develop an untargeted LCMS based metabolomics pipeline as a screening tool of microbial samples for potential toxic products. This was successfully completed with a microbial metabolomics pipeline developed detailing the purification of microbial supernatants from microbial culture, preparation for LCMS, analysis using LCMS and data processing and interpretation (Chapter 3.3 and optimised in Chapter 5.3.5). This developed pathway was primarily tested using *S. constellatus* cultivated in BHIB and TSB and was successful in translating thousands of different mass peaks to a selection of ~100 metabolite features, which are putatively responsible for differences between control media and microbial inoculation (Chapter 3). However, there was poor success in identification of these statistically significant metabolic features with a 7.1% success rate in identification for BHIB and 30 % for TSB. Of the features identified, the majority were very non-specific products such as amino acids and amino acid degradation products, which are not useful candidate molecules for potential biomarkers. This partial metabolite identification is a well-known bottleneck of metabolomics (Dunn et al., 2013) and is compounded by the lack of microorganism specific metabolite libraries to enable successful metabolite identification by cross reference. Due to the large amount of time needed for the employment of metabolomics and the ambiguous metabolite identification, its use for fast screening of microbial supernatants was deemed limited for this project.

Consequently, the collected microbial supernatants were tested against a kidney cell line to investigate their cytotoxic and pro-fibrotic effects (Chapter 4 and 5). The aim of this research was to identify pathogenic supernatants to conduct metabolomics experiments on them. With *P. gingivalis* supernatants inducing a cytotoxic effect on kidney cells (Chapter 5), an optimised version of the untargeted metabolomics pipeline was completed. However, the same inherent issues highlighted with *S. constellatus* supernatants arose with the *P. gingivalis* supernatants. While thousands of features were narrowed down to ~100 significant features, only 6.6% were

identified and those identified were non-specific products. While untargeted metabolomics was not successful for this project, the meticulous nature of the pipeline with multiple QA and QC steps should allow reanalysis in the future when mass spectral databases are more developed for microorganisms. Additionally, the developed metabolomics pipeline was successfully employed for a targeted study, with pyocyanin being quantified and used to determine the highest producing *Pseudomonas aeruginosa* strains for use in optimising microbial fuel cells (Slate, 2019). This highlights this pipeline has the potential to be applied to various other projects outside of this body of work.

The third objective was to determine whether the microbial products produced by the chosen microorganisms can induce cytotoxic and migratory effects in human kidney cells. This was completed by testing all the microbial supernatants against human kidney cells and measuring their effect on cellular viability (via assessment of biochemical activity) after 24 and 48 hours, along with separately testing the migratory effects by testing wound closure over time (Chapter 4). Of the supernatants tested, only one organism (*P. gingivalis*) significantly reduced cellular viability and migration. This research presents novel data that shows *P. gingivalis* derived microbial products elicit cytotoxic effects against the human kidney cells along with a weak anti-migratory effect. The results support the findings of *P. gingivalis* mediated cytotoxicity in other cell lines with direct incubation cytotoxicity in human gingival fibroblasts (Desta and Graves, 2007, Morioka et al., 1993), human aortic endothelial cells (Roth et al., 2007) and various human urothelial cells (Shah et al., 1992). However, there is evidence of variable cytotoxicity against different human cell types treated with the same periodontal microorganisms supernatants (Johansson et al., 1994), which may be the reason behind associations of CP with cardiovascular disease, endocarditis and kidney disease. There is also reported heterogeneity between different subtypes of *P. gingivalis* and cytotoxicity, which is suggested to be related to the different immunogenicity of the serotypes (Johansson et al., 1996, Ebersole and Steffen, 1995). Furthermore, the results support evidence that growth medium supernatants of *P. gingivalis* are cytotoxic to human gingival fibroblasts (Johansson et al., 1996) and human oral epithelial cells (Chen et al., 2001).

The mild anti-migratory effect we report of *P. gingivalis* supernatants after 24 h may just be due to the cytotoxic effects slowing cell migration, although there have been reports of anti-migratory activity due to the degradation of adhesion molecules such paxillin and focal adhesion kinase, resulting in cellular impairment (Furuta et al., 2009). This is also corroborated by evidence of hydrolysis of kidney epithelial junctional proteins such as E-cadherin by Gingipains, which may also have an anti-migratory effect as well as potentially promoting invasion of kidney tissue to underlying connective tissues in CKD (Katz et al., 2002a).

The fourth objective was to determine whether the microbial products secreted by the selected microorganisms could induce fibrotic effects in human kidney cells. This was completed initially by the use of transfected luminescent HK-2 cells with a fibrosis associated reporter gene (PAI-1) (Chapter 4). However, this was not successful due to the complexity of the AGCF-P medium control masking any potential effect. This was probably due to non-specific stimuli such as growth factors and metabolic factors (e.g. glucose) most likely present in the AGCF-P growth media from the addition of blood which could be interfering with the production of PAI-1 (Eddy and Fogo, 2006, Ghosh and Vaughan, 2012). Consequently, a more sensitive qPCR based approach was used to quantify the change in the mRNA of a selection of fibrosis associated genes (ColA1, PAI-1, TGF-β, TIMP1 and VEGF) in kidney cells treated with *F. nucleatum, P. gingivalis* and *S. mutans* microbial products. There was a lack of overall trends observed with these results. However, there was downregulation of the mRNA of both ColA1 and PAI-1 after treatment with the AGCF-P media and all microbial samples, indicating that it may be due to the AGCF-P media or due to the serum free media causing the cells to be more stressed due to a lack of growth factors present in the blood added to AGCF-P media. There was a significant upregulation of TGF-β after treatment with *P. gingivalis* supernatants in comparison to both the serum free medium and AGCF-P supernatants, but only for day 1 and 2 of the growth. This supports evidence in the literature that Gingipains (specifically lysine Gingipains) can induce the production of TGF-β alongside the suppression of CXCL8 and IL-6 (Bengtsson et al., 2015, Khalaf et al., 2014b). This effect was also shown to be attenuated in a Gingipain deficient strain indicating a potential role of

Gingipains (Palm et al., 2015) and there are also suggestions this induction by *P. gingivalis* may be mediated by the notch signalling pathway (Zhang et al., 2013). While notch signalling is not normally detectable in mature human kidneys (Hu and Phan, 2016), there is evidence that notch pathway proteins correlate with albuminuria, glomerulosclerosis, and renal function (Murea et al., 2010) and epithelial notch signalling can regulate interstitial fibrosis development (Bielesz et al., 2010). This suggests a possible mechanism of *P. gingivalis* microbial product interaction with CKD, mediated by Gingipains. However, if the upregulation/downregulation of the mRNA of the selection of fibrosis-associated genes is only considered in regards to differences between the microbial supernatants and matched AGCF-P control medium supernatants, there does appear to be a potential trend. For the *P. gingivalis* supernatants, there is upregulation of three different pro-fibrotic markers TGF-β, ColA1 and TIMP1 at various days of supernatant sampling with the upregulation of TGF-β being the largest fold change. This data does appear promising and highlights a potential future avenue to further unravel the putative role of CP associated microorganisms in CKD.

The final objective was to identify any microbial products capable of producing a cytotoxic, migratory or fibrotic effect on human kidney cells. As *P. gingivalis* supernatants were the only microbial supernatant to elicit a cytotoxic and anti-migratory effect. It was therefore the only supernatant fully investigated. Firstly, a summary of the different mechanisms by which *P. gingivalis* could conceivably cause cellular damage and the different components and virulence factors that would be present in microbial supernatants was discussed (Chapter 5.1). Next, LPS was investigated, and evidence was presented showing that LPS concentrations found in the microbial and control supernatant samples were insufficient to cause the reported cytotoxic effects and that even at high concentrations (100 µg/mL) of *P. gingivalis* and *E. coli* LPS, no statistically significant cytotoxicity was reported against human kidney and human fibroblast cell lines. The low quantity of LPS was most likely due to the preparation of the microbial supernatants as there was no sonication step. Despite *P. gingivalis* and *E. coli* LPS not initiating cytotoxic cell effects in these two cells lines, there is known variation in clinical and lab strains of microbial LPS along

with LPS having different effects on different cell lines. The pathogenic effects of LPS are known to be mediated by the induction of an inflammatory response by activation of toll like receptors and immune cell recruitment (Underhill and Ozinsky, 2002, Biswas and Lopez-Collazo, 2009). This research also presents evidence that the addition of a cell culture specific protease inhibitor cocktail mix attenuated the cytotoxicity of *P. gingivalis* supernatants against human kidney cells. This supports the literature regarding *P. gingivalis* specific proteases (Gingipains) as the cause of the observed cytotoxic effects against various cell lines (Johansson and Kalfas, 1998, Sheets et al., 2005, Sheets et al., 2008, Frisch and Francis, 1994) and the attenuation of this effect reported with heat treatment or proteinase inhibitors (Johansson and Kalfas, 1998) along with the addition of anti-sera (Chen et al., 2001). However, the literature lacks agreement regarding how Gingipains cause cytotoxicity, with some studies suggesting this occurs through the caspase dependent apoptosis (Sheets et al., 2005, Wang et al., 1999, Roth et al., 2007) whilst others have suggested cell death was caspase independent (Desta and Graves, 2007). More recent studies have proposed caspase independent apoptosis mediated by an apoptosis-inducing factor and involving the disruption of epithelial-cell adhesion by integrin β1 degradation (Li et al., 2019, Qiu et al., 2018). Indeed, this is consistent with a special type of apoptosis called 'anoikis' which is induced by the disruption of cell-matrix interactions (Frisch and Francis, 1994). To further investigate whether this effect was caused by Gingipains, multiple qPCR tests were conducted on RNA extracted from HK-2 cells, treated with and without *P. gingivalis* supernatants. The expression of protease-activated receptors 1 and 2 was investigated but there was no significant difference between the control and *P. gingivalis* over 48 h. In the literature there is evidence for the activation of PAR2 receptors by Gingipains in neutrophils (Lourbakos et al., 1998) and oral epithelial cells (Lourbakos et al., 2001), with activation of PAR2 stimulating release of interleukin-6 (Holzhausen et al., 2006, Lourbakos et al., 2001). The reasoning behind this lack of effect may be due to previous literature utilising calcium signalling to investigate PAR2 activation whereas this study utilised qPCR to investigate mRNA upregulation of the receptors, which may not take place upon activation (Holzhausen et al., 2006, Lourbakos et al., 2001, Lourbakos et al., 1998). More recent research has shown the activation and cleavage of PAR2 receptors can

stimulate a calcium signalling response and trigger platelet activation (Klarström Engström et al., 2015).

Overall, the main finding of this study is that microbial product/s derived from key CP pathogen, *P. gingivalis*, can elicit a cytotoxic and slight anti-migratory effect on human kidney cells. A secondary finding is that a microbial product/s derived from key CP pathogen *P. gingivalis* can induce the production of TGF-β mRNA in comparison to the AGCF-P medium and serum free medium controls. In both cases, it seems probable that this microbial product is *P. gingivalis* derived proteases, Gingipains, with evidence of Gingipains inducing cytotoxicity and the production of TGF- β (Khalaf et al., 2014a), although further work is warranted to confirm this. Another secondary finding is that in comparison to the ACGF-P control medium supernatant, microbial product/s derived from *P. gingivalis* supernatants can induce upregulation of three different pro-fibrotic markers TGF-β, ColA1 and TIMP1 over the 5 D sampling of *P. gingivalis* growth. These findings suggest evidence for a potential role of the keystone CP pathogen *P. gingivalis* in the mediation of inflammation and fibrosis, which is associated with CKD, and highlight potential avenues for future work. It is clear that chronic bacteraemia and translocation of periodontitis associated pathogens and their microbial products in CP takes place over the lifespan of the disease, which is evidenced by the multiple associations and isolations with systemic disease (Otomo-Corgel et al., 2012, Segura-Egea et al., 2015). This combined with the evidence of cellular damage to kidney cells, the reported hydrolysis of kidney epithelial junctions to allow invasion, along with the capability to trigger platelet activation through PAR2 receptors and pro-fibrotic effects through TGF-β and the notch-signalling pathway suggest a potential role for this key periodontal pathogen in mediating CKD.

6.2 Limitations and Future work

- As this study was conducted on singular, predominantly planktonic microorganisms (Chapter 2) this represents a limitation as it is well established that periodontitis consists of oral biofilms that are formed of polymicrobial communities that undertake a microbial shift with the progression of the disease

(Berezow and Darveau, 2011, Hajishengallis, 2015). These oral biofilms can have structural and functional heterogeneity along with a microcosm of environmental conditions and interactions between multiple oral microorganisms through quorum sensing which can modulate the environment (Kolenbrander et al., 2002, Dalwai et al., 2006). With the stark differences between singular planktonic and polymicrobial biofilms, the secreted microbial products could differ especially as there was limited biofilm characterisation, so confirmation of the effects seen in this study would be useful. Repeating some of these experiments using fractions collected from reproducible subgingival biofilm models such as described by Fernandez y Mostajo et al. (2017) along with a dental plaque biofilm models described by Davrandi et al. (2017) would be beneficial as batch culture is artificial and continuous culture or steady-state biofilms are more representative of the disease. This will enable confirmation of an effect on kidney cells and further warrant investigations into the potential interactions between CKD and CP.

- In this study, experiments (Chapter 2) were only conducted on one keystone pathogen of the red complex (*P. gingivalis,* not *T. forsythia* or *T. denticola*) (Hajishengallis and Lamont, 2012), predominately due to difficulties in cultivation (Wardle, 1997). In order to build a better picture of whether CP associated microorganisms can secrete products that have a potential effect on kidney cells it would be particularly useful to investigate these remaining two keystone pathogens. Progress has been made in cultivating previously uncultivatable members of the *Tannerella* sp. (Vartoukian et al., 2016) and it is known that *T. forsythia* requires supplementation of a exogenous source of N-acetyl muramic acid (Sharma, 2010). *T. denticola*, being a spirochete, is known to be particularly hard to grow but this is possible with modifications to the media to ensure a low redox potential along with sodium bicarbonate (Fenno, 2006). Future modifications to the developed growth medium AGCF-P (Hickey et al., 2019) would be warranted to allow investigations into these microbes of interest.
- In Chapter 3 and Chapter 5 a limitation highlighted of metabolomics and LCMS for mass screening of microbial supernatants is the lack of an appropriate mass

spectroscopy database for confirmation and identification of microbial products. Future work to look at the creation of a periodontitis metabolome database would rectify this issue and facilitate further investigations into the complex interactions of CP. In order to go about doing this it would be prudent to follow the example of the *Pseudomonas aeruginosa* metabolome database (PAMDB) (Huang et al., 2017).

- In this study, the only cell line used to investigate the cytotoxic and migratory effects of the microbial products was HK-2 cells (Chapter 4). This is a potential limitation because it has been reported that cytotoxicity can vary depending on the cell type due to cell specific toxins produced by the different periodontal pathogens (Johansson et al., 1994). While human gingival fibroblast cells (HGF-1) were used for testing the AGCF-P media in Chapter 2, they were found not to be appropriate for cytotoxicity testing due to difficulties regarding prolonged cultivation, as they are primary cells. Repeating the cytotoxicity and migratory tests in a wider variety of cell lines, more representative of the tissues the microbial products would come in contact with would be beneficial to further elucidate potential effects. Some cell lines that would be useful to investigate would be: human endothelial cells which are indicative of the contact with the circulatory systems, human oral epithelial cells and human gingival fibroblast cells which are indicative of the oral cavity and human macrophages which are indicative of the immune cells. Another avenue to consider would be co-culture of the kidney proximal tubules with other immune cells such as macrophages, which are present in the local tubule environment.

- Another limitation of the cytotoxicity investigations in this study (Chapter 4) is the microorganisms used; type strains were used to make it more repeatable however they may not be as representative of the clinical disease as clinical strains. This is shown by certain strains of *P. gingivalis,* specifically the type strain (NCTC 11834) used in this study, being more cytotoxic to oral cell lines than clinical 381 and W 50 strains which has been suggested to be due to different immunogenicity of the serotypes (Ebersole and Steffen, 1995). Repeating the

cytotoxicity, migration and fibrosis tests with different clinical serotypes of *P. gingivalis* would be of use to investigate whether the cytotoxicity and induction of fibrosis markers reported against kidney cells is reproducible in clinical based samples.

- The use of the colorimetric tetrazolium based CCK-8 dye for cell viability assays is a limitation due to the potential of the purified supernatants to potentially interact with the readings. Tetrazolium-based methods for assessing cellular viability looks at biochemical activity; the ability of cells to reduce the tetrazolium to formazan in the presence of electron carriers (Riss et al., 2016). This method gives an indirect measure of cellular viability directly proportional to absorbance at two wavelengths ($450/600_{nm}$). There is the potential of chemical interference from components of the supernatants with this reaction along with the issue of damaged cells still being viable and thus a false positive. In order to provide a more specific measure of cell death, assays utilising flow cytometry such as Annexin V/Propidium Iodide could be utilised which can provide an direct indication of necrosis and apoptosis (Rieger et al., 2011).

- The investigations into the pathogenic involvement of proteases such as Gingipains in Chapter 5 used degradation of the *P. gingivalis* supernatants by trypsin and heat inactivation along with the use of protease inhibitors. However, a limitation of these tests is that the cytotoxic effects were not completely proven to be due to the activity of Gingipains. Future work to definitively confirm this could be to utilise a Gingipains deficient strain of *P. gingivalis* such as (KDP128) (Brochu et al., 2001) and repeat the cytotoxicity and migration assays. An alternative option could be to purify Gingipains from the outer membrane vesicles using acetone precipitation followed by gel filtration (Potempa and Nguyen, 2007) or by the generation of a poly histidine tagged recombinant Gingipain that can be recovered by affinity chromatography (Veillard et al., 2015).

- The investigations into Gingipains in Chapter 5 utilised qPCR to assess upregulation of mRNA of the PAR1 and PAR2 receptors, in order to determine if

Gingipains are activating the receptors and stimulating upregulation. In the literature there was evidence for activation of PAR2 receptors in neutrophils and oral epithelial cells (Lourbakos et al., 1998, Lourbakos et al., 2001) and this activation stimulating the release of interleukin-6 (Holzhausen et al., 2006, Lourbakos et al., 2001), however there was no evidence of upregulation mRNA levels. The reasoning behind the non-significant results may be due to the activation of the PAR2 receptor not upregulating the amount of receptor produced and/or that mRNA levels do not correlate to activation; this highlights the assay chosen as a potential limitation. In order to better investigate activation of PAR2 receptors it would beneficial to study the calcium signalling response to activation of PAR2 receptors, as has been completed in other studies investigating Gingipain based activation (Holzhausen et al., 2006, Lourbakos et al., 2001, Lourbakos et al., 1998). Another avenue to consider would be investigating the secretion of interleukin-6 using an enzyme-linked immunosorbent assay (ELISA) or cytometric bead array to see if secretion is comparable from kidney cells to neutrophils and oral epithelial cells.

- A limitation of the qPCR conducted in Chapter 5 was that it was conducted on cDNA created from RNA extracted from HK-2 cells after a 24 h incubation with the microbial or control supernatants; this incubation may not be long enough to induce measurable changes in gene expression over time. In order to overcome this limitation it would be prudent to repeat the qPCR results on cDNA created from RNA extracted from HK-2 cells after a 48 h incubation with the microbial or control supernatants and compare these results to the 24 h results. This cDNA was generated alongside the 24 h cDNA but was not tested because of time constraints.

- The qPCR experiments in Chapter 5 investigating a selection of fibrosis related markers highlighted upregulation of only TGF-β by *P. gingivalis* supernatants compared to both the control media and serum free media. Future work to elucidate this would be warranted as there is already evidence of *P. gingivalis* upregulation in a variety of other cell lines such as fibroblasts and monocytes

(Palm et al., 2015, Gemmell and Seymour, 1993) along with suggestions this may be mediated by the notch signalling pathway (Zhang et al., 2013). Investigating the notch signalling pathway could be useful to provide further understanding about how this may affect cell fate as there is reported crosstalk between TGF-B and notch signalling (Kluppel and Wrana, 2005). Two possible ways of studying notch signalling in cell culture is qPCR for the specific gene products or developing a luciferase reporter cell line as developed in Chapter 4 for the PAI-1 gene (Zacharioudaki and Bray, 2014).

- As this study was an *in vitro* investigation, the results are hard to translate to the complexities of chronic disease interactions. Designing a cohort study looking at the subgingival microbiome of CKD patients in relation to their disease status and periodontal health, as has been ran for rheumatoid arthritis (Beyer et al., 2018), would be particularly useful. This would enable further identification of community specific populations within CKD using multiplex qPCR (Ciric et al., 2010) along with being able to study the effects of the different staging of CKD on CP in parallel with concurrent diseases, which is impossible to do in *in vitro* investigations and particularly hard in non-specific cohorts.

- Another avenue to investigate for future work is the generation of a vaccine based on outer membrane vesicles (OMVs) against *P. gingivalis*. As Gingipains have been identified as one of the primary virulence factors for *P. gingivalis* and suggested as the key causative agent behind its interactions with chronic disease, such as Alzheimer's (Dominy et al., 2019), targeting Gingipains could be highly effective at attenuating toxic effects. Furthermore, there is evidence that OMVs from *P. gingivalis* can cause platelet aggregation, which could link to fibrosis along with evidence that vesicles can travel to distal sites and play a role in the secondary pathogenesis related to links with systemic disease (Sharma et al., 2000). The development of an OMV vaccine is a possibility in the future as a vaccine against OMVs has already been successfully employed against *Neisseria meningitides* (van der Ley and van den Dobbelsteen, 2011). OMVs make excellent choices to target vaccines against, as they are heat stable, non-replicative and act

as storage contains for a variety of enzymes implicated in disease for various microorganisms (Cai et al., 2018).

- In money was no barrier, it would be of great benefit to this new area of research to conduct a longitudinal matched cohort study of CKD patients. In this study, a large cohort of patients with various stages of chronic kidney disease and a matched control population would be tracked over a 5-10 year period. During this timeframe blood samples, subgingival plaque samples and urine samples would collected and stored alongside clinical data on various co-morbidities and other known risk factors for CKD progression. Within this study, a dentist would record dental status and periodontitis status in all patients and controls, to enable effective comparisons. With this large dataset and the availability of clinical samples, a large number of area specific parameters could be investigated. Metagenomics could be employed to look at the difference between the subgingival microbial community between patients and controls along with between different stages of CKD in relation to periodontal disease, both at a cross-sectional level and temporally over the 5-10 year timeframe. The blood and urine samples could be investigated to see if bacteraemia of periodontal microorganisms can be cultured and/or sequenced and if there is any correlations between any culture/sequence and severity of CKD and CP. Furthermore, LC-MS could be utilised to look at the blood, serum and urine metabolites between control and CKD patients to look for dysregulated metabolites or toxins of interest. An extension to this study could be an interventional study in which CKD patients with CP are treated for periodontitis and followed up with matched non-treated patients, over a 5-10 year timeframe. This would provide direct evidence on whether CP is a modifiable risk factor in CKD progression. All these experiments would provide further evidence on the interactions between CKD and CP and may provide vital insights between CP and other systemic diseases.

6.3 Conclusion

In conclusion, this thesis describes in detail how two common chronic diseases, CKD and CP, can potentially interact with each other to the detriment of patients, addressing a gap in the knowledge. It also delivers a body of experimental evidence demonstrating that a key periodontitis associated pathogen has the ability to secrete products that can cause deleterious effects to kidney cells and induce the production of a selection of key pro-fibrotic factors: TGF-β, ColA1 and TIMP1. This evidence suggests that secreted microbial products could potentially be part of the mechanism of how these chronic diseases putatively interact. Furthermore, a novel microbial metabolomics pipeline was developed from scratch and optimised to successfully translate thousands of results to a select number of significant features responsible for differences between samples. While it was not able to be applied successfully to this project, it was successfully employed in a separate microbial study and has numerous potential uses in future studies.

Based on content in this thesis, further investigations into the ways CKD and CP may interact are warranted especially as there is growing evidence both diseases can have a detrimental effect on the progression of each other. With the evidence presented in this thesis a short term proposal would be investigations into the fibrotic potential of periodontitis associated microorganisms along with utilising patient samples to look for evidence of periodontal pathogen infiltration into the blood and potentially urine of CKD patients. A long-term proposal would be utilising vigilance and treatment of CP within the CKD patient population as a novel treatment for slowing progression of the disease.

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