Development of novel *in vitro* models to investigate the role of pathogens in the aetiology of feline chronic gingivostomatitis and to test therapeutic interventions

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Abstract

Feline chronic gingivostomatitis (FCGS) is a severe inflammatory disease of the oral cavity that causes extreme pain and distress in affected cats. Treatment options for FCGS are limited, and often unsatisfactory, and there is a poor understanding regarding its aetiology. Recent literature indicates that several putative bacterial pathogens, including *T. forsythia* and *P. circumdentaria*, could be involved in the development and progression of disease by stimulating an excessive host immune response. Understanding how the host responds to bacteria associated with FCGS is of importance to help identify novel targets for therapeutic strategies which are highly sought after. The aim of this study was to investigate the impact of putative pathogenic bacteria on the host inflammatory response and test the anti-microbial potential of several compounds on an FCGS-associated multispecies biofilm.

A panel of bacteria found to be prevalent in FCGS were selected as a basis for i*n vitro* research into the immune response from host cells following exposure to these bacteria. The QUANTI-Blue™ assay was used to measure toll-like receptor (TLR) activation in human THP1-XBlue™ pro-monocytes. IL-8 gene expression and protein release was measured from both human and feline cell lines by qPCR and ELISA, respectively. Moreover, the antimicrobial potential of novel agents including carbohydrate-derived fulvic acid (CHD-FA), xylitol, berberine, and ubiquinol were assessed against a multi-species biofilm consisting of bacteria prevalent in FCGS. The effect of these compounds on biofilm cell viability was determined using alamarBlue® and the impact on biofilm biomass was measured using the crystal violet assay.

The results from this study have generally shown that putative pathogens such as *T. forsythia*, *P. multocida* subsp. *multocida*, and *P. multocida* subsp. *septica* at a multiplicity of infection (MOI) of 200 produced the greatest increase in secreted embryonic alkaline phosphatase (SEAP) expression/TLR activation ($p<0.001$) in human cells and significant IL-8 release in human and feline cells. Commensal *B. zoohelcum* displayed an unexpected increase in IL-8 gene expression ($p<0.01$) and protein release ($p<0.001$) at an MOI of 200 in the squamous carcinoma cell feline cell line (SCCF1). Furthermore, the novel agent CHD-FA showed to significantly decrease biofilm cell viability ($p<0.01$) and biomass ($p<0.05$) at 0.8% compared to

the untreated control, while berberine disrupted only biofilm biomass at 100 µg/mL ($p<0.05$). Xylitol and ubiquinol had no significant impact on biofilm metabolic activity or biomass.

To conclude, this research has highlighted the inflammatory potential of bacteria associated to FCGS and how this may reflect the chronic inflammation presented by the host during disease. The development of a multi-species biofilm provided a platform to test novel compounds, highlighting its potential to be used as a tool in discovering appropriate therapeutic targets for FCGS and other biofilm infections. Such findings are valuable to enhance our understanding of the complex aetiology of FCGS and may help identify novel treatment interventions.

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Author's Declaration

I am aware of and understand the University's policy on plagiarism and I certify that this thesis is my own work, unless otherwise acknowledged or cited. This thesis has not been submitted for any other academic award.

Nicole Bonner, July 2020

Abbreviations

ANOVA	Analysis of variance
AP-1	Activating protein-1
AS	Artificial saliva
BHI	Brain heart infusion
BSA	Bovine serum albumin
CBA	Colombia blood agar
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming units
CHX	Chlorhexidine
CO_2	Carbon dioxide
COX	Cyclooxygenase
CV	Crystal violet
DANA	2-deoxy-2,3-dehydro-N-acetyleneuraminic acid
ddH_20	Double-distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPS	Extracellular protein matrix
FAA	Fastidious anaerobic agar
FBS	Fetal bovine serum
FCGS	Feline chronic gingivostomatitis
FCV	Feline calicivirus
FeLV	Feline leukaemia virus
FIV	Feline immunodeficiency virus
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2	Hydrogen
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
L1	Leucocyte antigen 1
MHC	Major histocompatibility complex

MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
MUNANA	4-methylumbelli-feryl N-acetyl-α-D-neuraminic acid
N_2	Dinitrogen
NAM	N-acetylmuramic acid
NF-κB	Nuclear factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
rFeIFN-ω	Recombinant feline interferon omega
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
RT-qPCR	Real-time quantitative polymerase chain reaction
SCCF1	Squamous cell carcinoma feline cell line
SD	Standard deviation
SEAP	Secreted embryonic alkaline phosphatase
Th	T helper
THB	Todd Hewitt broth
THP1-XBlue	Human pro-monocytic cell line
TLR	Toll-like receptor
TMB	3,3',5,5'-tetra-methylbenzide
TNF	Tumour necrosis factor
UK	United Kingdom

1 Introduction

1.1 General introduction

Feline chronic gingivostomatitis (FCGS) is a feline oral disease which is characterised by severe, widespread inflammation within the oral cavity. Cats affected with FCGS are often severely compromised, with inflammation typically lasting months to years, and may lead to the euthanisation of diseased cats in refractory cases. Various bacterial species involved in human periodontal disease have been associated with FCGS and it has been implicated that viruses may play an important role in disease pathogenesis (Lobprise and Dodd, 2018). However, the exact aetiology of FCGS remains elusive and the cause of chronic inflammation is considered to be multi-factorial involving interactions between oral bacteria, oral viruses, and the local immune system (Lyon, 2005; Lommer, 2013). Due to the complex aetiology of FCGS, there are currently no satisfactory treatment options available that show consistent positive outcomes. The idiopathic nature of FCGS contributes to the complexity of the disease and makes the aetiopathogenesis an important area of research to progress in the development of preventative measures and novel effective methods of treatment.

1.2 Feline Chronic Gingivostomatitis

1.2.1 Nomenclature

While feline chronic gingivostomatitis is the name commonly referred to in recent literature due to frequent occurrence in feline patients, various names have been used previously to refer to FCGS. These include lymphocytic plasmacytic gingivitis stomatitis, chronic ulcerative paradental stomatitis, and chronic mucositis (Baird, 2005; Lyon, 2005; Gengler, 2013). In these cases, the syndrome was named based either on the location of lesions within the oral cavity or on the inflammatory infiltrates present during disease (Diehl and Rosychuk, 1993; Healey et al., 2007).

1.2.2 Clinical presentation

FCGS is marked by chronic, ulcerative inflammation within the oral cavity of cats. Unlike gingivitis, which does not extend beyond the mucogingival junction, stomatitis is a unique syndrome that can cause proliferative and ulcerative inflammation of the whole oral cavity (White et al., 1992). It can affect all oral and pharyngeal soft tissues including the gingiva as well as the oral and pharyngeal mucosa. The inflammation is typically confined to specific tissues and locations within the mouth, and the disease name is often defined by the distribution of inflammation apparent during disease, as shown in Figure 1.1. In the most severe cases of FCGS, two main sites in the oral cavity are most commonly affected by painful ulcerous lesions. These include tissue lateral to the palatoglossal folds known as the fauces (caudal mucositis), and mucosa overlying the premolar/molars extending to the buccal mucosa (alveolar mucositis). In some cases, swelling of the lips and loss of papillae on the tongue is also apparent (Southerden, 2010; Hennet et al., 2011). The salient clinical signs of gingivostomatitis include extreme oral pain and discomfort in affected animals. Other common clinical symptoms include halitosis, ptyalism (excessive salivation) and dysphagia often followed by weight loss due to difficulty eating (Bellei et al., 2008). Some cats may also display a decrease in grooming behaviour. The high severity of symptoms associated with FCGS can additionally lead to changes in affected cats' demeanour including an increase in aggressiveness when handled as well as withdrawn behaviour (Lommer, 2013).

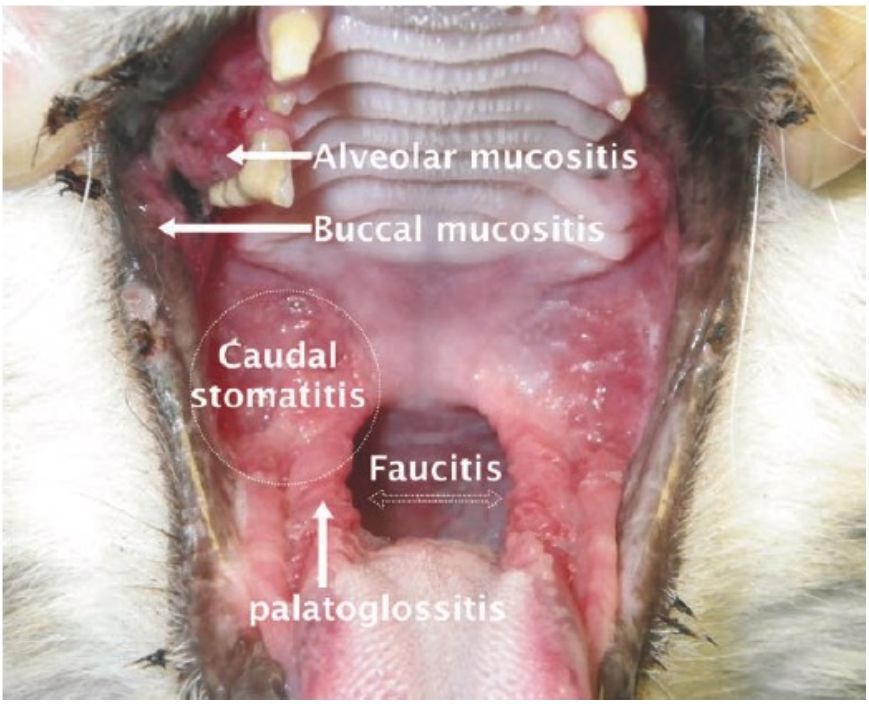

Figure 1.1: Terminology and localisation of inflammation in FCGS (Perry & Tutt, 2015).

1.2.3 Prevalence

Oral disease in cats is very common, with periodontal disease (affecting around 85% of cats) and feline tooth resorption lesions (affecting 25-75% of cats) seen most frequently in practice (Bonello, 2007). However, there is limited research investigating the prevalence of FCGS and so the commonness of the condition is uncertain. In one study investigating a total of 753 cats, 12% of cats were found to be affected with FCGS (Verhaert and Van Wetter, 2004). However, another larger study from the north-west of England showed an FCGS prevalence of 0.7% in a population of 4858 cats visiting veterinary practices (Healey et al., 2007). With approximately 10 million cats owned as pets in the United Kingdom (Murray et al., 2009), the estimated disease burden of FCGS translates to at least 70,000 cases (0.7%) and upwards of 1 million cases (12%) in the UK alone.

There are three distinct periods in a cat's life when significant oral inflammation and stomatitis may be present. The first is at 3-6 weeks during the time of kitten vaccinations. At this period, inflammation may be elicited in response to vaccinal elements or due to increased dental plaque that coincides with deciduous tooth eruption. The second period is marked by the eruption of permanent teeth at 4-

6 months old, where more severe oral inflammation can be present and enhanced oral hygiene may be required as a method of control. The largest group of cats found to be most commonly affected with oral inflammation and FCGS is at a mean age of seven years. Adult cases of FCGS present clinical signs of inflammation with varying severity and location, with some cats believed to be more susceptible to the aetiological stimuli (Johnston, 2012). The disease is seen in all breeds of domestic cats, however it has been suggested that pure-bred cats, including Maine Coon, are predisposed to the juvenile form of FCGS and can develop more severe lesions during disease (Wexler-Mitchell, 2018).

1.3 Aetiopathogenesis

The aetiopathogenesis of FCGS is not well understood, but it is believed that the chronic inflammation that is characteristic of FCGS is a result of an excessive immune response to oral antigenic stimulation. The disease is thought to be multifactorial, with various possible causes (Lyon, 2005). Oral antigens that are considered to be key factors in prompting the atypical immune response in FCGS include dental plaque accumulation, oral bacteria, and viral infections. It is also believed that the chronic nature of the syndrome could be partially due to an underlying immune abnormality of the host (Southerden, 2010). The multifactorial nature of FCGS is briefly summarised in Figure 1.2.

1.3.1 Periodontal disease and tooth resorption

Periodontal disease is often identified in cats with FCGS, due to inflammation spreading to the underlying bone and causing destruction of structures supporting the teeth (Hennet, 1997). As gingivitis increases in severity, the gingival tissue shows a loss of integrity and ulceration of the gingival sulcus occurs, which allows bacteria and their by-products to migrate to deeper gingival structures and this can have an exacerbating effect on FCGS. Moreover, type one tooth resorption, characterised by a normal periodontal ligament space and radiodensity of root structure, is also commonly present in cats with FCGS and is found in areas where periodontal inflammation is severe (Lobprise and Dodd, 2018). Tooth resorption poses a significant complication in many FCGS cases, increasing the difficulty of tooth extraction due to ankylosis and/or weakening of roots. A study investigating

101 cats with FCGS found that all cats with FCGS had periodontitis and 49% of FCGS cases displayed external inflammatory tooth resorption (Farcas et al., 2014).

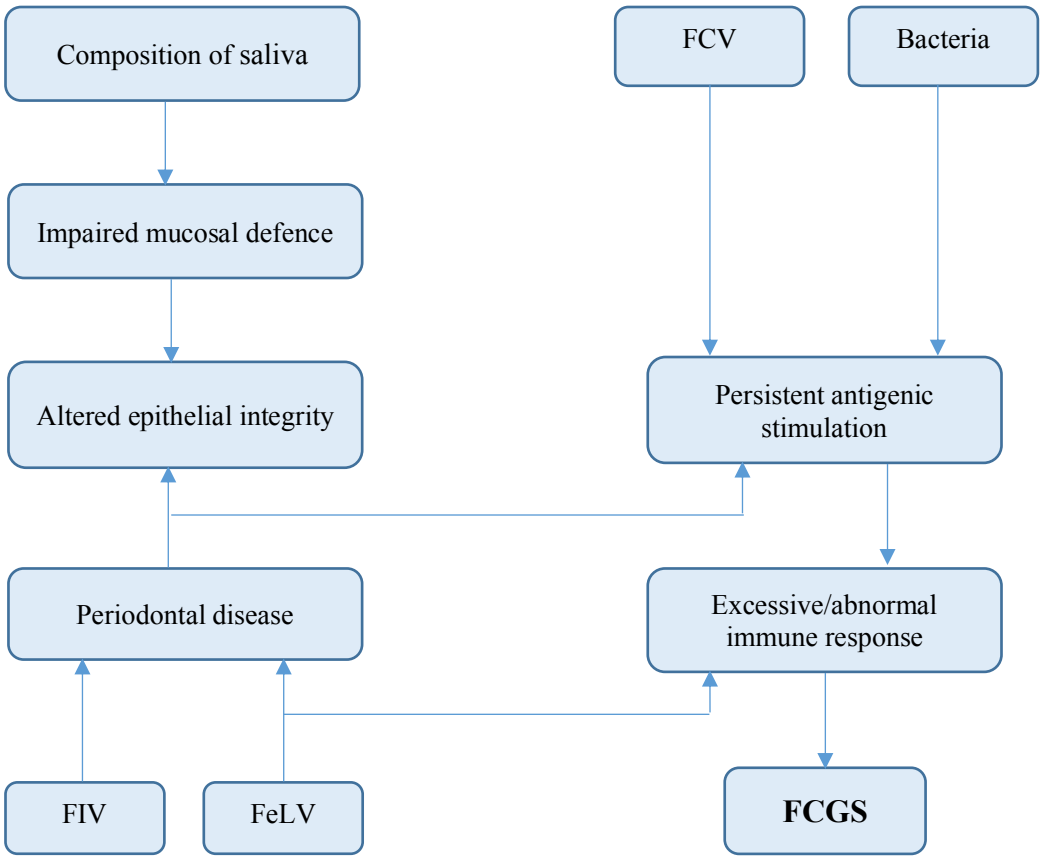

Figure 1.2: Algorithm of FCGS aetiology

FCV: Feline calicivirus, FIV: Feline immunodeficiency virus, FeLV: Feline leukaemia virus, FCGS: Feline chronic gingivostomatitis. Adapted from (Bonello, 2007).

1.3.2 Bacteriology

1.3.2.1 Dental plaque

Although research has indicated that FCGS is multifactorial in nature, it is generally accepted that plaque bacteria are a key contributing factor to the development of FCGS, with oral tissues overreacting to the presence of dental plaque. Bacteria are ubiquitous in the feline oral cavity, with a vast number of

bacterial species present in both healthy and diseased states in the form of a complex protective plaque biofilm (Perry and Tutt, 2015; Harris et al., 2015). Biofilms provide advantages to bacteria over planktonic states, including protection from environmental stresses by the production of an extracellular polymer matrix and increased resistance to antimicrobials (Sedlacek and Walker, 2007). The biofilm adheres to the tooth surface and accumulates readily in all species. It is rich in bacterial species, both commensal and pathogenic, as well as various glycoproteins and polysaccharides (Marsh & Bradshaw, 1995). In the feline oral cavity, initial plaque development occurs within hours of the eruption of teeth or following cleaning. An exposed tooth surface is covered by a layer of glycoprotein salivary pellicles, which act as a prime adhesive for bacteria as they provide a rich nutrient source (Zambori et al., 2012). The first bacteria (early colonisers) to attach are mostly Gram-positive aerobic organisms that possess adhesion structures such as fimbriae which allow them to colonise and grow on the surface of the tooth. Following growth of early colonisers, microcolonies begin to form which attracts more bacteria and triggers the development of the extracellular protein matrix (EPS). The EPS acts as a protective barrier against antimicrobial agents and environmental stresses. As dental plaque matures, the oxygen tension reduces and thus, the biofilm becomes more compliant to increased numbers of Gram-negative anaerobic species including bacilli and spirochaetes (Cate, 2006).

Plaque bacteria can reside harmlessly within the oral cavity if plaque is consistently removed through an upkeep of oral hygiene, however, failure to remove plaque allows for the accumulation of a thick layer of microbes and a subsequent enrichment of inflammatory by-products associated with active gingival inflammation. Consequently, plaque accumulation can lead to a variety of dental diseases in the host. Within feline hosts, where daily brushing is not a common practice, a build-up of plaque will proceed to calcify to form the brown malodorous material known as dental calculus. A positive correlation between feline age and calculus formation has been observed, as well as an increased frequency of calculus shown in cats fed wet, rather than dry, food (Gawor et al., 2006). Calculus has a rough surface and can promote further colonisation of bacteria (Perry and Tutt, 2015). Without intervention, plaque and calculus build-up can lead to the development of periodontal disease, which can progress from

gingivitis to irreversible periodontitis (Wolf and Hassell, 2006). While cats affected by FCGS will often have very little calculus in the early stages of disease, by the time of clinical presentation, cats are also often suffering from significant periodontal disease (Hennet, 1997). In cats with FCGS, plaque is considered as a major stimulant for the excessive oral inflammation, which is more severe than the typical progression of periodontal disease. Understanding the involvement of plaque deposition in FCGS is of importance for the clinical management of this disease.

1.3.2.2 The diseased feline oral microbiota

Extensive research on the bacteria of the human oral cavity has revealed that it harbours a widely diverse bacterial community which grow within complex biofilms. In human periodontal disease, as biofilms progress from supragingival to subgingival sites, there is a shift in the oral microbial community from predominantly commensal aerobes such as *Actinomyces* and *Streptococcus* species towards anaerobic, potentially pathogenic bacteria including *Porphyromonas gingivalis* and *Tannerella* species (Bascones and Figuero, 2005). This follows the ecological plaque hypothesis, by which the subgingival environment selects the oral bacterial composition, with a shift to pathogenic bacteria driving the transformation from health to disease (Bartold and Van Dyke, 2013). While the feline oral microbiome is less well studied, a shift in oral bacterial populations during the progression of periodontal disease has been implicated. Early research found black-pigmented *Bacteroides* and *Peptostreptococcus anaerobius* to be of higher prevalence in cats with increasingly severe periodontal disease (Mallonee et al., 1988). It has also been shown, through the use of next-generation sequencing, that there is a change in the proportion of phyla in feline subgingival plaque dependent on health state. Cats with periodontal disease were found to have double the number of Firmicutes than healthy cats, while Bacteroidetes and Proteobacteria decreased in comparison (Harris et al., 2015).

There is strong evidence that bacterial composition may also influence the development of FCGS in felines, with specific species of bacteria shown to be more prevalent in diseased states, as is often seen in periodontal disease. One study suggested that cats affected with FCGS have a reduced diversity of oral

bacteria in comparison to healthy cats (Dolieslager et al., 2011). Early research indicated that FCGS could be caused by, or associated with, *Bartonella henselae*. It was discovered that cats co-infected with feline immunodeficiency virus (FIV) and *B. henselae* showed a higher prevalence of gingivitis, suggesting a possible connection in cats with clinical oral disease (Ueno et., al 1996). However, there has since been no credible link found between *Bartonella* and FCGS. It has since been discovered that felines with FCGS have an increased prevalence of *Pasteurella multocida* sub-species compared to healthy cats (Dolieslager et al., 2011). FCGS samples with a large overgrowth of *P. multocida* subsp. *multocida* have displayed a concurrent reduction of some bacteria found in high levels in healthy cats, such as *C. canimorsus*, possibly due to increased competition for nutrients. Furthermore, bacteria such as *Pseudomonas* species, *Tannerella forsythia* and *Porphyromonas circumdentaria* have been commonly detected in cats with FCGS, and may be significant periodontopathogens (Dolieslager et al., 2011). Diseased felines that harbour *T. forsythia* are thought to present FCGS with the highest clinical disease severity. Moreover, cats suffering from FCGS have shown to have statistically significantly increased levels of serum antibodies towards certain Gram-negative anaerobes such as *Bacteriodes* species (Sims et al., 1990). The increased prevalence of specific bacteria in FCGS cases suggests that they may be of aetiological significance in the development of disease.

1.3.2.3 Bacterial sialidases

Sialidases are a group of enzymes involved in the cleavage of terminal sialic acids from complex carbohydrates found on glycoproteins or glycolipids (Juge et al., 2016). Previous research has shown that the sialidase enzymes of some pathogenic bacteria, including *Vibrio cholerae* and *Streptococcus pneumoniae*, contribute to bacterial virulence within the host (Corfield, 1992). These enzymes are also thought to particularly increase the virulence of bacteria that are present on and/or invade mucosal surfaces, likely due to a greater abundance of sialic acid in these tissues. Sialidases have shown to act in tissue destruction, modulation of host innate immunity, as well as promoting biofilm formation (Soong et al., 2006). Research has found that organisms which have shown to be prevalent in the oral cavity during FCGS, including *P. multocida* species and *T. forsythia,* display medium-high sialidase activity (Scharmann et al., 1970). *T. forsythia* has also

been prevented from attaching to epithelial cells when its sialidase activity was inhibited (Honma et al., 2011). Investigating the sialidase activity of bacteria associated with FCGS could be valuable when developing targeted inhibitors to reduce bacterial attachment and invasion during disease.

1.3.3 Viral influence

Several viral agents have been linked to the occurrence of oral inflammation and FCGS in cats, including feline calicivirus, feline leukaemia virus, and feline immunodeficiency virus. This is primarily due to an increased prevalence of FCGS observed in cats with various viral diseases (Tenorio et al., 1991; Quimby et al., 2008; Belgard et al., 2010). However, the pathological significance of viruses in FCGS is unclear. It is thought that some viruses may not be a direct causative agent of disease, but instead exacerbate the symptoms of FCGS. One proposed mechanism of viral involvement in FCGS suggests that immune dysregulation associated with viral infection could lead to oral microbial dysbiosis and a subsequent increase in pathogen colonisation and infection (Taniwaki et al., 2013).

1.3.3.1 Feline calicivirus

Feline calicivirus (FCV) is a highly contagious ribonucleic acid (RNA) virus that is a major cause of respiratory infection in cats. Privately owned pet cats kept in small numbers generally have lower rates of infection (around 10%) by FCV, with a greater prevalence of up to 40% found in shelter cats or catteries (Radford et al., 2007). FCV replicates in the oral and respiratory tissues, and has been identified as a cause of ulceration in the oral cavity of infected cats. Several studies investigating felines chronically affected by FCGS have reported above 70% of these patients to also test positively for FCV following oropharyngeal swabbing (Knowles et al., 1989; Thompson et al., 1984; Harbour et al., 1991). In the study by Thompson et al. (1984), FCV was isolated from 80% of cats with FCGS, compared to 0% of the control group. However, more recent immunohistochemistry and PCR analysis of 26 FCGS-affected feline samples showed no detection of FCV in diseased cats (Rolim et al., 2017). Moreover, another study of specific pathogen-free cats infected with FCV were found to have associated acute oral

inflammation, but no cases developed into FCGS over a 10-month period (Knowles et al., 1991). While several studies have demonstrated a higher prevalence of FCV in cats with FCGS, a direct causal relationship between FCV and FCGS has not been established. Further research is required to determine the involvement of FCV in FCGS, as a possible cause or modifying agent in disease.

1.3.3.2 Feline leukaemia virus

Feline leukaemia virus (FeLV) is an RNA retrovirus that infects up to 3% of cats in the United States and up to 5% of healthy cats in European countries (Gleich et al., 2009). Infection with FeLV can have several outcomes including development into a persistent viraemia, development of an inactive latent form, or the immune system may eliminate the virus (Rezanka et al., 1992). A possible relationship between FeLV and FCGS has been suggested, since around 15% of cats infected with FeLV will show clinical signs of oral inflammation (Levy, 2005). Studies investigating a link between FeLV and FCGS have shown inconsistent results. In one study, 16.6% out of 36 cats with stomatitis tested positive for FeLV (White et., al 1992). However, a study of 23 cats with FCGS found that all cats were negative for FeLV (Hennet, 1997). Similarly, a study by Quimby et al. (2008) concluded that FeLV was not present in any of the cats with FCGS that were assessed. Therefore, the involvement of FeLV in FCGS is questionable.

1.3.3.3 Feline immunodeficiency virus

Feline immunodeficiency virus (FIV) is an RNA lentivirus that affects up to 4.4% of cats worldwide (Richards, 2005). FIV has been identified in cats with chronic oral lesions. The severity of oral lesions has also shown to increase in cats with co-infections of FIV with other viruses such as FCV (Dawson et al., 1991). Studies investigating the association of FIV and FCGS have found conflicting outcomes. A study that observed cats in the UK affected with FCGS found that 75% of cats were also positive for FIV compared to only 16% of the control group (Knowles et al., 1989). However, a more recent study was unable to identify a significant difference in antibodies for FIV in serum samples between cats with FCGS and healthy controls (Belgard et al., 2010). Some single case reports have also shown that felines affected with FCGS tested negative for FIV (Baird, 2005; Southerden

and Gorrel, 2007). It is possible that the presence of both FeLV and FIV in cats with FCGS is coincidental, as the prevalence of these viruses in affected cats has shown to not differ significantly from the general feline population (Dolieslager et al., 2011). Further research is required to fully understand the viral role in FCGS aetiology.

1.3.4 Immunology of FCGS

1.3.4.1 Mucosal cells

Various immune cells are present within the oral cavity including lymphocytes, dendritic cells, macrophages, and mast cells (Arzi et al., 2010). Inflammatory cell infiltrates are usually abundant in FCGS, with research suggesting that oral lesions present in affected cats represent a complex and destructive inflammatory process that frequently extends from the epithelium to submucosal tissues (Harley et al., 2011). While the inflammatory infiltrate present in the feline oral cavity during FCGS is not entirely understood, plasma cells and lymphocytes are believed to be predominant within FCGS oral lesions (Johnessee and Hurvitz, 1983). Histological imaging of moderate to severe FCGS has displayed an expansion of the mucosal lamina propria by sheets of mature plasma cells, including the presence of Mott cells (Murphy et al., 2019). Mott cells are atypical plasma cells which contain immunoglobulins within their cytoplasm (Bain, 2009). Moreover, increased numbers of CD3+ T lymphocytes, L1+ cells, and elevated levels of MHC class II expression have been associated with greater severity of inflammation in FCGS oral lesions (Harley et al., 2011). Small increases in the proportion of mast cells, dendritic cells and macrophages have also been noted (Harley et al., 2003a; Vapniarsky et al., 2020). There has also shown to be a shift in salivary immunoglobulins in cats with FCGS, from primarily IgA in healthy cats to primarily IgG and IgM in affected cats (Harley et al., 2003b). A decrease in IgA levels in saliva can weaken the mucosal defence against pathogens and therefore could contribute to the development of FCGS.

Furthermore, CD8+ T cells have been found in greater abundance than CD4+ T cells in mucosal samples of FCGS-affected cats (Harley et al., 2011). In general, CD4+ T cells primarily act by regulating other immune cells through the release of

cytokines or by direct cell contact. CD8+ T cells, however, are cytotoxic and typically kill target cells that have been virally infected or have undergone neoplastic transformation. When CD8+ cells encounter the target antigen, they differentiate into an effector phenotype which is marked by increased levels of pro-inflammatory cytokines and killing of target cells. Some of these cells also acquire a memory phenotype, which allows the cell to persist long-term in the absence of an antigen (Kalia and Sarkar, 2018). Memory cells with "effector memory" can attach and reside in sites of inflammation, such as within the oral mucosa (Sallusto et al., 2004). Elevated levels of CD8+ T cells in FCGS could therefore suggest an elicited cytotoxic cell-mediated immune response, supporting the possible involvement of viral agents in disease (Harley et al., 2011). In addition, one study has demonstrated that there is a notable increase in effector memory CD8+ lymphocytes and a corresponding decrease in central memory CD8+ lymphocytes in felines with FCGS in comparison to a healthy control group (Vapniarsky et al., 2020). This suggests that the persistent inflammation in FCGS could be due to CD8+ cells remaining in an activated state. The complex immune response found in FCGS most likely drives the chronic inflammation that presents during disease.

1.3.4.2 Toll-like receptors

Toll-like receptors (TLRs) are a class of proteins present on the surface of host leukocytes which can sense and respond to invading pathogens by initiating the production of cytokines and activating the innate and adaptive immune response (Janeway and Medzhitov, 2002). Signature molecules on the surface of microorganisms known as pathogen-associated molecular patterns (PAMPs) are recognised by the host with specific recognition receptors, including TLRs, which allows the host to determine the nature of a pathogen and induce an appropriate inflammatory response. Notable changes in host TLR expression can give insight into the possible pathogens involved in the aetiopathogenesis of a disease (Akira and Takeda, 2004). Using quantitative PCR, Dolieslager et al. (2013) demonstrated significantly elevated mRNA levels of TLR2 and TLR7 in mucosal tissue biopsies of cats affected with FCGS compared to a healthy control group. Additionally, increased levels of TLR mRNAs were displayed when putative pathogens *T. forsythia* (TLR2, TLR4, TLR7 and TLR9), *P. circumdentaria* (TLR2 and

TLR3) and FCV (TLR2) were present in affected cats. TLR2 and TLR9 both recognise many bacterial, fungal and viral substances, and mRNA expression of TLR2 has shown to increase when it is activated (Weiss et al., 2004). TLR4 activation can occur after binding of several Gram-negative bacteria and envelope proteins of viruses, while TLR7 responds to single-stranded RNA and anti-viral compounds (Akira and Takeda, 2004). The increased expression of these TLRs and heightened activity of the immune system is most likely the cause for the clinical manifestation of FCGS, possibly in response to the altered oral microbiota or viral presence that has been implicated in FCGS. Further research is required to evaluate the influence of FCGS-associated putative pathogens individually, and in combination, on TLRs and the immune response.

1.3.4.3 Cytokines and chemokines

Following TLR activation, there is an increase in the transcription of many genes including those for cytokines, which act as signalling molecules to mediate and regulate the inflammatory response. Studies have reported increased mRNA expression of a range of pro-inflammatory cytokines in felines with FCGS, including IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ, TNF-α and IL-1β (Harley et al., 1999; Dolieslager et al., 2013). Pro-inflammatory cytokines are involved in both innate and adaptive pathways, promoting the progression of the inflammatory response which may explain the severe inflammation characteristic to FCGS. Furthermore, there is a shift from predominantly T-helper lymphocyte type 1 (Th1) cells in healthy cats to a mixed environment of Th1 and Th2 cells in cats with FCGS (Harley et al., 1999). Th1 cells produce cytokines such as IFN- γ and IL-2 and play a role in the activation of macrophages and delayed hypersensitivity responses. Th2 cells produce several cytokines including IL-4, IL-6, IL-9, IL-10 and IL-13, and are involved in the stimulation of B cells (Cruse and Lewis, 2010). The chronic inflammation found in FCGS is likely a result of the amplified immune response in cats with FCGS.

Interleukin-8 (IL-8) is a pro-inflammatory chemokine that is a major chemotactic factor in acute inflammation, involved in the recruitment of neutrophils to sites of inflammation and tissue infiltration (Haas et al., 2016). IL-8 has been used in both *in vitro* and *in vivo* studies to measure inflammation in human periodontal

disease (Fukui et al., 2013; Dommisch et al., 2015). In humans, an increase in levels of IL-8 has been shown in gingival tissues, correlating to the severity of periodontal disease (Noh et al., 2013). While its specificity for oral disease in cats is not known, several studies have identified increased levels of IL-8 in other chronic felines inflammatory diseases (Habenicht et al., 2012; Gruen et al., 2017). Therefore, IL-8 may be useful as a marker in cats with an abnormal inflammatory response such as in FCGS.

1.4 Diagnosis

FCGS is a poorly defined condition where inflammation extends beyond the mucogingival line. As FCGS is likely to have multifactorial aetiology, there is no definitive diagnostic test as underlying causes may differ between cases. A veterinary surgeon will thoroughly examine a cat's oral cavity under anaesthesia before reaching an accurate diagnosis. This allows for other potential causes of inflammation in the mouth such as azotaemia, squamous cell carcinoma, and periodontal disease to be ruled out to avoid misdiagnosis. Furthermore, a full dental radiograph is strongly recommended to evaluate root remnants, as most cats with FCGS will also suffer from a form of periodontitis which will require treatment (Farcas et al., 2014). If there is still doubt over the diagnosis, biopsies should be taken which allows for the elimination of neoplasms (Milella, 2008). Biopsies can also be used to evaluate the symmetry of oral lesions which further helps to correctly diagnose the disease. Inflammatory lesions in FCGS are usually bilaterally symmetrical (Niemiec, 2008). PCR technology can be used to identify carriage of oral viruses.

1.5 Disease prevention

Establishing preventative measures for FCGS has proven difficult due to the elusive aetiology of the disease. However, a sensible method of maintaining the oral health of a cat as much as possible would be to upkeep good oral hygiene. This would focus on the removal of dental plaque and disruption of bacterial biofilms in the mouths of affected cats and in turn, reduce the risk of periodontal disease that can often develop in cats with FCGS. A variety of products are available to maintain dental hygiene in cats including toothpaste and mouthwash. A reduction

in dental calculus has been shown in cats that have their teeth brushed once or twice weekly (Richardson, 1965). A specific diet can also be implemented to improve dental care. The diets often include large kibbles that are designed to clean the teeth during eating by mechanically reducing plaque build-up from the surface of teeth. Dried food is generally believed to benefit oral health in comparison to moist food (Studer and Stapley, 1973). One recent study found that the probability of cats developing poor oral health is lower when fed a dry diet (Mata, 2015). It has also been suggested that the oral health of cats may improve when artificial ingredients are removed from food (Addie et al., 2003).

1.6 Treatment options

The treatment of FCGS is challenging as the response to treatment is unpredictable. In general, the treatment of FCGS can be approached with surgical and/or medical intervention. Improvement is often observed in around two-thirds of cases, while some cats will not recover (Jennings et al., 2015). Many cats will continue to show persistent or relapsing lesions and will require chronic management of disease. As the aetiology of FCGS is unknown, effective targeted treatment options remain limited. Therefore, most current treatments aim to reduce inflammation as well as minimise pain and discomfort that accompanies FCGS.

1.6.1 Dental extraction

Surgical management of FCGS typically involves the extraction of all premolars and molars with the hypothesis that this can remove stagnant plaque bacteria and decrease clinical symptoms (Bellei et al., 2008). This is currently the recommended method of treatment for FCGS as it has shown to provide the best long-term outcome. Studies have shown varying success rates of dental extraction in cats with FCGS, with 50-60% of cases requiring no further treatment, 20-40% of cases show signs of improvement and the remaining 10-20% of cases will not improve following the intervention (Hennet, 1997; Girard and Hennet, 2005; Bellei et al., 2008). In particularly severe cases, if there is no positive response to partial-mouth extraction, cats may require a full dental extraction (including canines and incisors) as a second stage of treatment (Jennings et al., 2015).

Post-operative plaque control often involves the use of a topical chlorhexidine (CHX) treatment. CHX paste or gel is believed to be one of the most important and effective treatment options available, both short- and long-term, to control FCGS and reduce the overall antigenic burden. CHX is widely accepted as the 'gold standard' in the management of human oral biofilm diseases, shown to have a broad-spectrum of activity against bacteria, fungi and viruses (Herrera, 2013; Lim and Kam, 2008). One problem with this treatment is finding a suitable product for affected felines, as some gels have a bitter taste and may cause side effects in some animals (Lobprise and Dodd, 2018). Identifying a potentially natural alternative compound that has the potency of CHX but with minimal adverse effects could be highly valuable in the search for therapeutic measures for FCGS.

1.6.2 Antibiotic therapy

Antibiotics are often advised as a first-line treatment in an attempt to alleviate excessive inflammation or allow oral tissue to heal following surgery. The antibiotics prescribed should preferably target both aerobic and anaerobic pathogenic bacteria in the oral cavity. Recommended antibiotics include clindamycin, amoxicillin, metronidazole, and doxycycline (Frost and Williams, 1986; Harvey, 1991; Lyon, 2005; Wiggs, 2007). However, the use of antibiotics in FCGS is thought to worsen the long-term prognosis of some cases, as they may become ineffective and lead to super-infections (Bonello, 2007). Moreover, *Pasteurella multocida* species, which have shown to be highly prevalent in cats with FCGS, appear to be clindamycin resistant and therefore this drug may not be effective in all cases (Dolieslager et al., 2011). Antibiotic therapy is more favourable as a short-term intervention for FCGS to reduce oral discomfort.

1.6.3 Anti-inflammatory and immunosuppressive drugs

Corticosteroids are drugs that mimic the effects of hormones that are normally produced in the adrenal glands. They have been used to treat FCGS due to having anti-inflammatory properties. Prednisolone is a short-acting corticosteroid that is often used to control inflammation in FCGS. In one study using several corticosteroids as a treatment method for FCGS, an improvement was observed in

up to 80% of cases (White et al., 1992). However, while these drugs may improve clinical symptoms of disease, they can also lead to a more progressive form of FCGS that will not respond to treatment and therefore, they are often used only as a "rescue treatment". Additionally, the use of corticosteroids is generally contraindicated because of an associated increased risk of obesity and diabetes mellitus (Niemiec, 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs) are another type of anti-inflammatory used to reduce the effect of the inflammatory response in FCGS. NSAIDs work by blocking the COX enzymes and reduce prostaglandins throughout the body. The best option of NSAID for FCGS appears to be meloxicam, which would often be prescribed in combination with an antibiotic (Hennet et al., 2011). However, there is limited research reporting the efficacy of NSAIDs for FCGS.

1.6.4 Interferon treatment

Interferons (IFNs) are a group of proteins used in cell communication throughout the immune system that can interfere with viral replication (Hennet et al., 2011). Recombinant feline interferon omega (rFeIFN-ω) is an immunomodulatory drug which has been described as effective for the treatment of feline viruses including FCV and feline herpesvirus-1 (Truyen et al., 2002). One study investigated the use of rFeIFN-ω compared to prednisolone in FCGS-affected cats that were refractory to extraction therapy. There was found to be no statistically significant difference in clinical signs when using rFeIFN-ω compared to prednisolone, however the use of rFeIFN-ω did show to significantly reduce the pain, and increase the activity, of affected cats throughout the study (Hennet et al., 2011). Furthermore, Leal et al. (2013) conducted a case study with 2 cats and showed that treatment of oral lesions with rFeIFN-ω resulted in a significant decrease in inflammation. Research suggests that long-term prognosis with the use rFeIFN-ω exceeds the potential of other methods of treatment for this condition (Hennet et al., 2011).

1.6.5 Mesenchymal stem cell therapy

The efficacy of mesenchymal stem cells (MSCs) administered intravenously to cats with refractory FCGS has been reported with promising results. MSCs are multipotent stem cells that can modulate innate and adaptive immunity through inhibition of T-cell proliferation, downregulation of MHC II, and inhibition of dendritic cell maturation (Clark et al., 2017; Quimby and Borjesson, 2018). Autologous MSCs have shown to be nonimmunogenic and safe in both humans and animals, with few reports of adverse reactions following long-term use (Quimby et al., 2013; Kol et al., 2015). One study showed a positive response rate of over 70% when cats with refractory FCGS were treated with autologous adipose-derived MSCs, with over 40% showing complete resolution of disease (Arzi et al., 2016). Further investigations to determine the potential of MSCs as a treatment option for FCGS are ongoing, including clinical trials to gain a greater understand of the mechanism of action of MSCs in disease (Quimby and Borjesson, 2018).

1.7 *In vitro* biofilm models

Many studies have highlighted the diverse nature of dental plaque biofilms and that biofilm composition may influence a healthy or disease state in both human and feline oral disease. Various plaque biofilm models have been developed in the study of human oral diseases to investigate biofilm formation and antimicrobial susceptibility, and to further understand the impact of specific bacteria on disease (Periasamy and Kolenbrander, 2009; Park et al., 2014; Guggenheim et al., 2001). Biofilm models of human supra- and sub- gingival plaque have previously been developed using defined bacterial species or from undefined plaque samples. While using an undefined bacterial model more closely reflects the species diversity present in the oral cavity, defined biofilm models have shown to be favourable in the study of biofilms due to their reproducibility. It also allows the study of specific bacterial biofilms of interest, where exact mechanisms of biofilm formation and species-specific interactions can be identified.

While plaque biofilm models are extensively used in the study of human periodontal disease, there is no evidence of the development of a feline oral

plaque biofilm. A five species human caries biofilm model with *S. oralis*, *S. mitis*, *A. naeslundii*, *S. downei* and *S. sanguinis* was created to study the biofilm formation when treated with bovine milk osteopontin, with an overall reduction in the biofilm biomass shown (Schlafer et al., 2012). Furthermore, a complex eleven species oral biofilm has been developed to assess the viability of individual species following treatment with oral cleansing regimes (Sherry et al., 2016). The use of *in vitro* biofilm models could be beneficial in the study of feline oral disease.

1.8 Summary

FCGS is a poorly defined disease, marked by localised chronic inflammation of soft oral tissues including the gingiva and oral mucosa. The aetiology is believed to be a manifestation of an overreactive immune response to antigenic stimulation, with bacteria and viruses believed to be important contributing factors to the development and progression of disease. While there are a several treatments available for FCGS, no method of intervention is completely satisfactory to cure the disease in all cases. Until the complex multifactorial nature of FCGS can be understood, it will remain difficult to find an appropriate method of intervention. This makes the aetiopathogenesis of FCGS an important area of research to ultimately allow for the development of effective preventative measures and novel methods of treatment to tackle this debilitating disease.

1.9 Aims of study

1. To exploit multiple cell lines to investigate, *in vitro*, the initial innate immune responses (via cytokine release and mRNA expression) to putative pathogenic bacteria associated with FCGS. The cell models include THP1-XBlue™ human pro-monocytes and SCCF1 feline oral squamous carcinoma cells.
2. To develop a multi-species biofilm with FCGS putative pathogens and commensal microorganisms and test with biologically active agents.

2 Host cell inflammatory response following exposure to bacteria associated with FCGS

2.1 Introduction and Aims

FCGS is a complex syndrome with a myriad of factors that may contribute to disease aetiology. It is commonly believed that cats with FCGS suffer from an over-reactive immune response to low levels of oral antigens, including dental plaque. Specific bacteria have been identified as highly prevalent in diseased cats showing abnormal levels of inflammation, with *Tannerella* species and *Porphyromonas* species implicated to be key pathogens involved in the progression of disease. Moreover, *Pasteurella multocida* species have shown to be highly represented in FCGS, suggesting an association with disease pathogenesis (Dolieslager et al., 2013).

The colonisation and accumulation of dental plaque bacteria in the feline oral cavity, like in periodontal disease, may stimulate a variety of host inflammatory responses. Phagocytes are a first line of host defence and are involved in clearing pathogens by phagocytosis, degranulation, and through the recruitment of other immune cells to the site of infection (Paltrinieri, 2008). Some phagocyte cells have toll-like receptors (TLRs) on their surface which allow for the initial detection of pathogens (Janeway and Medzhitov, 2002). TLRs activate downstream signalling pathways, such as the nuclear factor NF-κB pathway, that lead to the induction of innate immune responses by releasing pro-inflammatory chemokines and cytokines like interleukin-8 (IL-8) and tumour necrosis factor alpha (TNFα) (Kawasaki and Kawai, 2014). The pro-inflammatory chemokine IL-8 has been implicated in chronic inflammation (Harada et al., 1996). These processes are not only involved in eliciting immediate host responses such as inflammation, but also orchestrate antigen-specific adaptive immune responses (Janeway and Medzhitov, 2002).

The ability of the host to mount an appropriate inflammatory response is critical for maintaining oral health and host-microbial symbiosis. Due to lack of oral hygiene in felines, plaque bacteria may accumulate at the gingival margin and lead to the over-reactive immune response and consequent chronic inflammation that is characteristic during FCGS. Investigating the interactions between host cells and putative pathogens of FCGS is vital to provide a greater understanding of the abnormal inflammation observed in affected cats.

The aim of this chapter is to investigate how FCGS-associated bacteria may modulate innate immune inflammatory responses at a gene and protein level, which may contribute to the chronic inflammation observed in FCGS.

2.2 Materials and Methods

2.2.1 Bacterial culture and standardisation

A selection of laboratory strains of commensal, known pathogenic and potentially pathogenic bacteria associated with FCGS were used in this study. These included *Pasteurella multocida* subsp. *multocida* DSM 16031 and *Pasteurella multocida* subsp. *septica* DSM 23071 which were grown on Colombia blood agar (CBA) [Oxoid, Basingstoke, UK] supplemented with 5% sheep blood and maintained for 24 h at 37°C in 5% CO_2. *Bergeyella zoohelcum* DSM 16783 was grown on CBA supplemented with 5% horse blood and maintained for 48 h at 37°C in 5% CO_2. *Porphyromonas circumdentaria* DSM 103022 and *Porphyromonas gingivalis* ATCC 33277 were maintained at 37°C on fastidious anaerobic agar (FAA) [Lab M, Lancashire, UK] supplemented with 5% horse blood under anaerobic conditions (85% N_2, 10% CO_2 and 5% H_2 [Don Whitley Scientific Limited, Shipley, UK]) for 4 days. *Tannerella forsythia* ATCC 43037 was maintained at 37°C on FAA supplemented with 5% horse blood containing 10 µg/mL N-acetylmuramic acid (NAM) for 4 days under anaerobic conditions. All isolates were stored in Microbank® vials [Pro-Lab Diagnostics, Wirral, UK] at -80°C until required.

A loopful of each isolate was inoculated into 10 mL of the appropriate growth medium for bacterial quantification. *P. multocida* sub-species and *B. zoohelcum* were propagated in 10 mL brain heart infusion broth (BHI) [Sigma-Aldrich, Gillingham, UK] for 24 h at 37°C in 5% CO_2. *T. forsythia* was grown in 10 mL BHI containing 5% fetal bovine serum (FBS) [Sigma-Aldrich] and 0.001% NAM for 4 days under anaerobic conditions. *P. circumdentaria* and *P. gingivalis* were grown in 10 mL Schaedler's anaerobic broth [Oxoid] for approximately 4 days under anaerobic conditions. Cultures were washed twice by centrifugation at 3000 rpm for 10 minutes and re-suspended in 10 mL phosphatase buffer saline (PBS) [Sigma-Aldrich]. All cultures were standardised to a final stock concentration of 1 x 10^9 cells/mL in PBS by measuring the optical density at 550 nm. The bacterial suspensions were subjected to heat at 55°C for 30 minutes to kill the bacteria and then stored at -20°C for further studies.

2.2.2 THP1-XBlue™ human cell culture

THP-1 cells are a human pro-monocytic cell line, derived from the peripheral blood of an acute monocytic leukaemia patient (Tsuchiya et al., 1980). The THP1-XBlue™ cell line [InvivoGen, UK] is a derivative of THP-1, specifically engineered to monitor the NF-κB signal transduction pathway. THP1-XBlue™ cells feature an NF-κB and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene. SEAP is secreted by THP1-XBlue™ cells in response to TLR stimulation and can be measured using QUANTI-Blue™, a SEAP detection reagent that turns purple/blue in the presence of SEAP.

THP1-XBlue™ cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum (FBS) [Sigma-Aldrich], 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL Zeocin™ and 100 µg/mL Normocin™. Cells were seeded at 7 x 10^5 cells/mL in a 75 cm^2 flask [Corning Life Sciences, NY] and maintained at 37°C in 5% CO_2. Cells were allowed to reach approximately 80% confluency before passage. To passage cells, cells were washed twice in PBS by centrifugation for 5 minutes at 1000 rpm and then re-suspended in 5 mL of RPMI-1640 [Sigma-Aldrich]. The cells (10 µL) were stained with 0.4% trypan blue solution (10 µL) and live cells were counted on a haemocytometer under a light microscope. Cells were re-seeded at 1 x 10^6 cells/flask in RMPI-1640. THP1-XBlue™ cells were used as a comparative human control in this study.

Frozen stocks of THP1-XBlue™ were prepared for long-term storage in liquid nitrogen. Cell suspensions were standardised to 1 x 10^6 cells/mL in RPMI-1640 supplemented with 20% fetal bovine serum (FBS). Equal volumes of 20% dimethyl sulfoxide (DMSO) and THP1-XBlue™ cells were transferred to a cryovial to obtain a final volume of 1 mL. The vials were stored overnight at -80°C to ensure the cells were cooled slowly before final storage in liquid nitrogen. Cells were revived from frozen stocks by thawing at 37°C before transferring into a cell culture flask containing warm RPMI-1640. For experiments, cells were seeded at 2 x 10^5 cells/mL in RPMI-1640.

2.2.3 SCCF1 feline cell culture

The squamous cell carcinoma feline (SCCF1) cell line was developed from a laryngeal squamous cell carcinoma of a cat (Tannehill-Gregg et al., 2001). SCCF1 cells were acquired from the original developers of the cell line at The Ohio State University (USA). Briefly, SCCF1 cells were propagated in William's E medium [Sigma-Aldrich] supplemented with 2 mM L-glutamine, 0.05 mg/mL gentamicin, 10 ng/mL epidermal growth factor and 10% FBS. Cells were maintained at 37°C in 5% CO_2 until 90% confluence. To passage cells, the medium was removed and cells were washed twice with Dulbecco's phosphate-buffered saline [ThermoFisher, Epsom, Surrey, UK] followed by the addition of 3 mL of 0.25% trypsin-0.53 mM EDTA [ThermoFisher] to disaggregate the cells from the surface. Once detached, 10 mL of media was added to the cells to terminate trypsinisation. A cell count was performed by staining cells with 0.4% trypan blue and counting viable cells on a haemocytometer under a light microscope. Cells were re-seeded at 1 x 10^6 cells/flask in supplemented William's E medium.

Frozen stocks of SCCF1 cells were prepared as described for THP1-XBlue™ previously (section 2.2.2). For experiments, SCCF1 cells were seeded at 2 x 10^5 cells/mL in William's E medium.

2.2.4 Bacterial stimulation of cell lines

Each cell line (THP1-XBlue™ and SCCF1) was stimulated with a panel of bacteria thought to be involved in the aetiology and chronic inflammatory response found in FCGS. Cells were seeded in a 96-well plate at a concentration of 2 x 10^5 cells/mL in RPMI-1640 and incubated overnight at 37°C in 5% CO_2. The bacterial stocks of 1 x 10^9 cells/mL were subject to a series of double serial dilutions using PBS to prepare a range of multiplicities of infection (MOI) of bacteria – 200, 100, 50, 25 and 12.5 – with bacterial concentrations from 4 x 10^7 bacteria/mL (MOI 200) to 1.25 x 10^6 bacteria/mL (MOI 12.5). Following incubation, 20 µL of each bacterial dilution was added to the cell suspension. RPMI-1640 served as a negative control. The plate was then incubated at 37°C in 5% CO_2 for 24 h. At 24 h, the plate was centrifuged at 1000 rpm for 1 minute before extracting 100 µL of supernatant which was stored at -20°C. The remaining cell/bacterial suspension was stored at -80°C. Supernatants and cell lysates were retained to assess the

release of pro-inflammatory mediator and changes in mRNA expression following stimulation. RNA was extracted as described in section 2.2.6 and ELISA techniques were performed as described in section 2.2.11.

2.2.5 QUANTI-Blue™ assay

Toll-like receptor (TLR) activation was measured using the THP1-XBlue™ cell line which produces SEAP in response to TLR activation, and induction of the NF-κB transcription factor. To quantify the levels of SEAP from the exposed THP1-XBlue™ cells, a QUANTI-Blue™ colorimetric enzyme assay [InvivoGen] was used to assess all supernatants harvested from the bacterial stimulation experiments. QUANTI-Blue™ turns purple/blue in the presence of SEAP. Each sample was diluted 1:10 with warmed QUANTI-Blue™ and incubated at 37°C for 4 h. Following the incubation period, the optical density (OD) was recorded at 630 nm using a plate reader.

2.2.6 RNA extraction

Following stimulation of cell lines with various FCGS-associated bacteria, cell supernatants were removed and RNA was extracted from host cells using the RNeasy Mini kit [Qiagen Ltd, Crawley, UK] in accordance with the manufacturer's instructions. Host cell samples were lysed with the addition of 350 µL buffer RLT. The resultant lysate was transferred to an RNase-free microfuge tube and mixed thoroughly with 350 µL of 70% ethanol. Each 700 µL sample was then transferred to an RNeasy spin column placed within a 2 mL collection tube and centrifuged at 13000 rpm for 15 seconds, with the flow-through discarded. The RNA on the column membrane was washed with 350 µL of buffer RW1 by centrifugation at 13000 rpm for 15 seconds and flow-through discarded. A solution containing 10 µL of DNase 1 stock solution and 70 µL of buffer RDD was added onto the RNeasy column membrane and incubated at room temperature for 15 minutes. Next, buffer RW1 was again added to the column and centrifuged at 13000 rpm for 15 seconds with flow-through discarded. The RNA on the membrane was precipitated by the addition of 500 µL of buffer RPE and centrifugation at 13000 rpm for 15 seconds, followed by an additional centrifugation at 13000 rpm for 1 minute to dry the membrane. Finally, 30 µL of RNase-free water was added directly to the membrane before centrifugation at 13000 rpm for 1 minute to elute the RNA. RNA

was collected and quantified using a spectrophotometer or stored at -80°C until required.

2.2.7 RNA quantification

The RNA extracted from host cells was quantified using the NanoDrop 1000 spectrophotometer [Thermo Scientific, Wilmington, USA] to assess the quality and concentration of RNA obtained. The NanoDrop pedestal was loaded with 1.5 µL of RNA from each sample. To measure the purity of the RNA, the ratio of absorbance was measured at 260 and 280 nm, where RNA with a 260/280 ratio greater than 1.8 was deemed to be of high quality. Samples were stored at -20°C until required for cDNA synthesis.

2.2.8 cDNA synthesis

Complementary DNA (cDNA) synthesis was achieved using the High-Capacity RNA-to-cDNA™ reverse transcription (RT) kit [Thermofisher]. Two hundred nanograms of RNA was added to 4 µL of RT master mix, with a final volume of 20 µL made up using RNase-free water. Controls which did not contain the reverse transcriptase enzyme were also included. Samples were centrifuged at 1000 rpm for 2 minutes to remove air bubbles. Samples were then loaded on to the thermal cycler [Bio-Rad, Watford, UK]. The cDNA was synthesised using the following thermal cycling conditions: 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C and a final hold stage at 4°C. Samples were then stored at -20°C for use in PCR.

2.2.9 Primer design

Primers used for PCR were either found in previously published literature or designed based upon their sequence, which was obtained from the National Centre for Biotechnology Information (NCBI, Bethesda, USA). To design primer sets in-house, the web-based Primer-Blast software (NCBI) was used (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed to yield a PCR product size of 70-120 base pairs. The National Institute of Health's Basic Local Alignment Search Tool (NIH-BLAST) was used to check primer specificity (http://www.nlm.nih.gov/BLAST). Oligonucleotides matching the

resulting primer sequences were synthesised [Invitrogen, Paisley, UK] for the target genes of interest (Table 2.1).

2.2.10 Real-time quantitative PCR

Cytokine gene expression was analysed using SYBR® Green [Invitrogen, Paisley, UK] based real-time quantitative PCR (RT-qPCR), using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene (Barber et al., 2005). The primers used are listed in Table 2.1. Each well contained 12.5 µL of SYBR® green master mix [Invitrogen], 0.5 µL of forward and 0.5 µL of reverse primers, 2 µL of cDNA and 9.5 µL RNase-free water [Qiagen, UK] to make a final volume of 25 µL. The thermal cycle was as follows: 2 minutes at 55°C, 10 minutes at 95°C followed by 40 cycles of 30 seconds at 95°C, and 60 seconds at 60°C. Each parameter was analysed in duplicate on three independent occasions using the MxProP Quantitative PCR machine and MxProP 3000 software [Stratgene, Amsterdam, Netherlands]. The expression of genes of interest was normalised to the housekeeping gene *GAPDH* using the $2^{-\Delta Ct}$ method, and then the relative expression of gene transcripts to the media control was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 2.1: Primer sequences used in real-time quantitative PCR

Target	Primer sequence (5'-3')	Reference
Feline *IL-8*	F - TCCAAGCTGGTTGTTGCTCT R - TGCACTGGCATCGAAGTTCT	In-house
Human *IL-8*	F - CAGAGACAGCAGAGCACACAA R - TTAGCACTCCTTGGCAAAAC	(Ramage et al., 2012)
GAPDH	F - GAGCTGAATGGGAAGCTCAC R - CGTATTTGGCAGCTTTCTCC	(Dolieslager et al., 2013)

F, forward; R, reverse.

2.2.11 Enzyme-linked immunosorbent assay (ELISA)

Supernatants from each cell line (THP1-XBlue™ and SCCF1) challenged with a panel of FCGS-associated bacteria for 24 h were retained to assess the release of

pro-inflammatory protein by ELISA. ELISA kits for human [Peprotech, London, UK] and feline [R&D Systems, Abingdon, UK] interleukin-8 (IL-8) were used according to manufacturer's instructions. All incubations were carried out at room temperature. A standard curve was constructed by plotting the mean absorbance for each standard against the IL-8 concentration and R-squared was calculated. Results were determined using a 4-parameter curve fit to determine the concentration of protein IL-8 released in samples tested. All reactions were performed in duplicate on three separate occasions.

2.2.11.1 Human IL-8 ELISA

A human IL-8 ELISA kit was used to detect IL-8 release from human THP1-XBlue™ cells following bacterial stimulation. Capture antibody (0.5 µg/mL) was prepared in PBS and 100 µL added to wells of Corning® 96-well high binding microplates [Sigma-Aldrich]. Plates were sealed and incubated overnight. Contents were then discarded and plates washed with 300 µL of PBS containing 500 µL Tween20 [Sigma-Aldrich] per litre. Plates were blocked with 300 µL of block buffer containing 1% bovine serum albumin (BSA) in PBS for 1 h to block non-specific binding. Plates were washed and 100 µL of each sample was loaded in triplicate. In addition, standards of known concentrations of IL-8 (15.6 to 1000 pg/mL) were included on each plate in duplicate and incubated for 2 h. Contents were discarded and 100 µL of detection antibody (0.5 µg/mL) was added to each well containing sample or standard and incubated for a further 2 h. Next, plates were washed and 100 µL of a 1:2000 dilution of avidin-HRP conjugate in assay diluent (0.05% Tween20, 0.1% BSA in PBS) was added to wells and incubated for 30 minutes. Finally, contents were discarded and 100 µL of 3,3',5,5'-tetra-methylbenzide (TMB) [Sigma-Aldrich] was added to each well and incubated in the dark for 20 minutes before the addition of 100 µL of 1 mM HCl to stop the reaction. The absorbance was read using the FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) at 405 nm with a 650 nm wavelength correction.

2.2.11.2 Feline IL-8 ELISA

A feline IL-8 ELISA kit was used to assess the release of IL-8 from feline SCCF1 cells following bacterial stimulation. Briefly, 100 µL of capture antibody (4 µg/mL)

prepared in PBS was added to Corning® 96-well high binding microplates and incubated overnight. Contents were discarded and plates washed with 400 µL of wash buffer (0.05% Tween20 in PBS). Plates were then blocked with reagent diluent (1% BSA in PBS) for 1 h to prevent non-specific protein interactions. Plates were washed and 100 µL of each sample was loaded in duplicate as well as standards of known concentrations of IL-8 ranging from 62.5 to 4000 pg/mL and incubated for 2 h. Contents were again discarded and 100 µL of detection antibody (500 ng/mL) prepared in reagent diluent was added to wells with samples and standards and incubated for 2 h. Plates were washed and 100 µL of streptavidin-HRP (diluted 1:200 with reagent diluent) was added to each well and incubated for 20 minutes. Finally, plates were washed and 100 µL of substrate solution TMB was added to wells and incubated in the dark for 20 minutes, and then 50 µL of 1mM HCl was added to stop the reaction. The absorbance was measured at 450 nm with a 570 nm wavelength correction.

2.2.12 Statistical analysis

Graph production and statistical analysis were performed using GraphPad Prism (version 5; La Jolla, USA). Data which followed a normal distribution was analysed by using a one-way analysis of variance (ANOVA) to investigate significant differences between the mean of two or more independent groups. A two-way ANOVA was used to compare the mean of two or more independent groups at multiple time points. A Bonferroni correction (two-way ANOVA) was used to determine statistically significant differences between groups. A Dunnett's post-test (one-way ANOVA) was used to measure significant differences between independent groups and the control group. Statistical significance was achieved at $p<0.05$ for all analyses.

2.3 Results

2.3.1 THP1-XBlue™ TLR activation is influenced by concentration-dependent stimulation with FCGS putative pathogens

An *in vitro* model system was developed involving the stimulation of cell lines with bacteria involved in FCGS to measure the ability of these bacteria to induce an inflammatory response within the host. A human cell line containing an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct was exposed to varying concentrations of *T. forsythia* and *P. circumdentaria* (believed to be key pathogens in FCGS), *Pasteurella multocida* species (putative pathogens in FCGS), *B. zoohelcum* (a representative feline commensal bacterium) and *P. gingivalis* (a major human periodontal pathogen). This allowed for the ability of FCGS-associated bacteria to activate toll-like receptors (TLRs), and consequently promote host inflammation, to be assessed. THP1-XBlue™ cells incubated without bacteria were used as a cells only control (MOI of 0).

Following 4 hour stimulations, there were significant increases in the level of SEAP production in cells exposed to *T. forsythia* and *P. gingivalis*, with the cell only control (MOI of 0) absorbance of 0.04 and 0.05 increasing to 1.13 ($p<0.001$) and 0.34 ($p<0.001$), respectively, at an MOI of 200 (Figure 2.1). Following 24 hour stimulations, there were significant increases in the level of SEAP release in cells exposed to *T. forsythia* (MOI 0, 0.06; MOI 200, 2.06; $p<0.001$), *P. multocida* subsp. *multocida* (MOI 0, 0.25; MOI 200, 1.31; $p<0.001$), *P. multocida* subsp. *septica* (MOI 0, 0.41; MOI 200, 1.29; $p<0.001$), and *P. gingivalis* (MOI 0, 0.06; MOI 200, 1.30; $p<0.001$) at an MOI of 200 compared to an MOI of 0. There was also a notable increase in SEAP production in cells following exposure to an MOI of 200 for 24 hours compared to 4 hours in *T. forsythia* (4 h, 1.13; 24 h, 2.06; $p<0.001$) , *P. multocida* subsp. *multocida* (4 h, 0.40; 24 h, 1.31; $p<0.001$), *P. multocida* subsp. *septica* (4 h, 0.40; 24 h, 1.29; $p<0.001$) and *P. gingivalis* (4 h, 0.34; 24 h, 1.30; $p<0.001$). Although there appeared to be an increase in SEAP production in the 24 h supernatant with *P. circumdentaria*, no significant differences in were shown in cells following exposure to *P. circumdentaria* or *B. zoohelcum*.

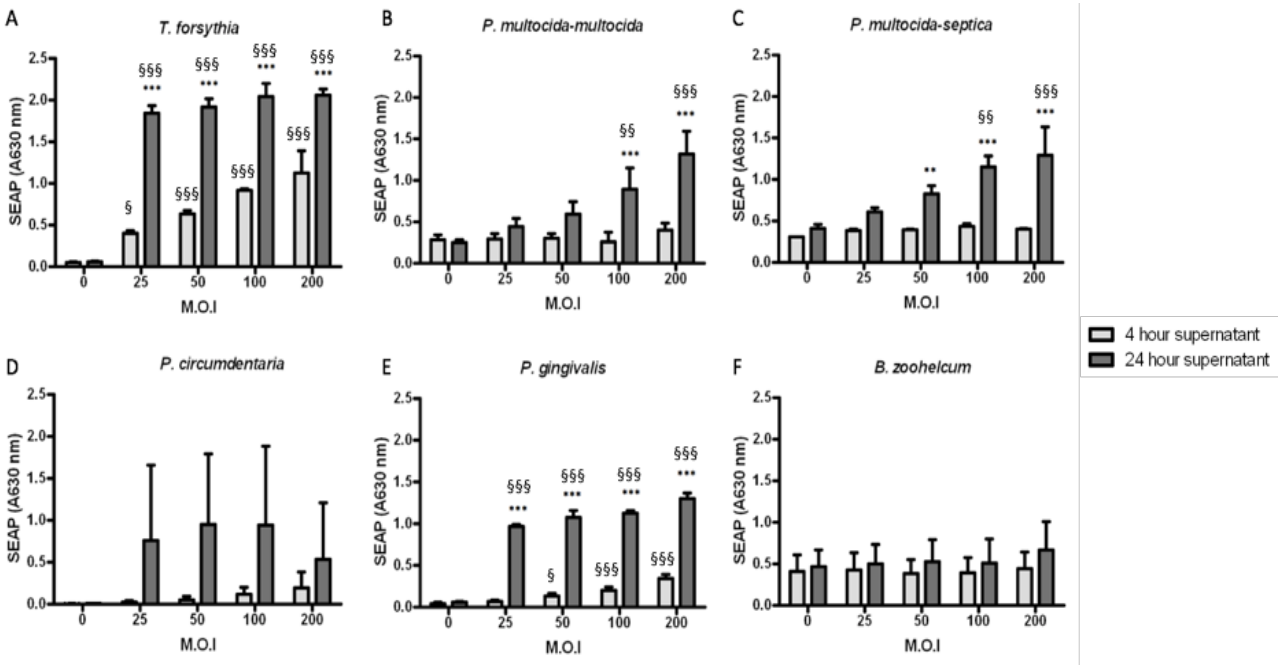

Figure 2.1: SEAP expression of THP1-XBlue™ cells following exposure to bacterial panel of interest

THP1-XBlue™ cells were seeded at the concentration of $2x10^5$ cells/mL in a 96-well plate for SEAP studies. Cells were exposed to heat-killed bacteria ([A] *Tannerella forsythia* [B] *Pasteurella multocida* subsp. *multocida* [C] *Pasteurella multocida* subsp. *septica* [D] *Porphyromonas circumdentaria* [E] *Porphyromonas gingivalis* [F] *Bergeyella zoohelcum*) at various multiplicities of infection (M.O.I) (0-200) for a period of 4 hours and 24 hours. QUANTI-Blue™ assay was then used to measure SEAP with colour development measured at an absorbance wavelength of 630 nm. All groups were assayed in triplicate on three separate occasions. Statistical analysis was performed using two-way ANOVA and Bonferroni post-hoc test to compare all groups. Data represents mean ± SD (Comparison to cells only (0) § $p<0.05$, §§ $p<0.01$, §§§ $p<0.001$) (4 hours vs. 24 hours **$p<0.01$, ***$p<0.001$).

2.3.2 THP1-XBlue™ cell IL-8 mRNA expression is influenced by concentration-dependent stimulation with FCGS putative pathogens

To further investigate the response of the THP1-XBlue™ cells to FCGS-associated bacteria, the impact of varying MOIs of bacteria of interest on the regulation of pro-inflammatory mediator IL-8 was assessed. IL-8 is a key mediator of periodontal inflammation and so was an appropriate indicator for chronic inflammation in this study. First, THP1-XBlue™ cell IL-8 mRNA expression, normalised to the housekeeping gene *GAPDH*, was measured (Figure 2.2).

Following 24 h exposure, THP1-XBlue™ *IL-8* expression was significantly up-regulated in cells following exposure to *T. forsythia* (168.78-fold increase; $p<0.001$), *P. circumdentaria* (3.07-fold increase; $p<0.05$), and *P. gingivalis* (199.57-fold increase; $p<0.001$) at an MOI of 200 compared to the cell only control (MOI 0). Cells also showed an increase in *IL-8* mRNA expression when stimulated with *T. forsythia* at a lower MOI of 50 (56.88-fold increase; $p<0.05$) and 100 (101.44-fold increase; $p<0.01$). No statistically significant differences were observed in gene expression following exposure to *Pasteurella* species and *B. zoohelcum* species at any MOI; however, there was a notable trend of a concentration-dependent increase in IL-8 expression from all bacteria at an MOI of 200 compared to 0.

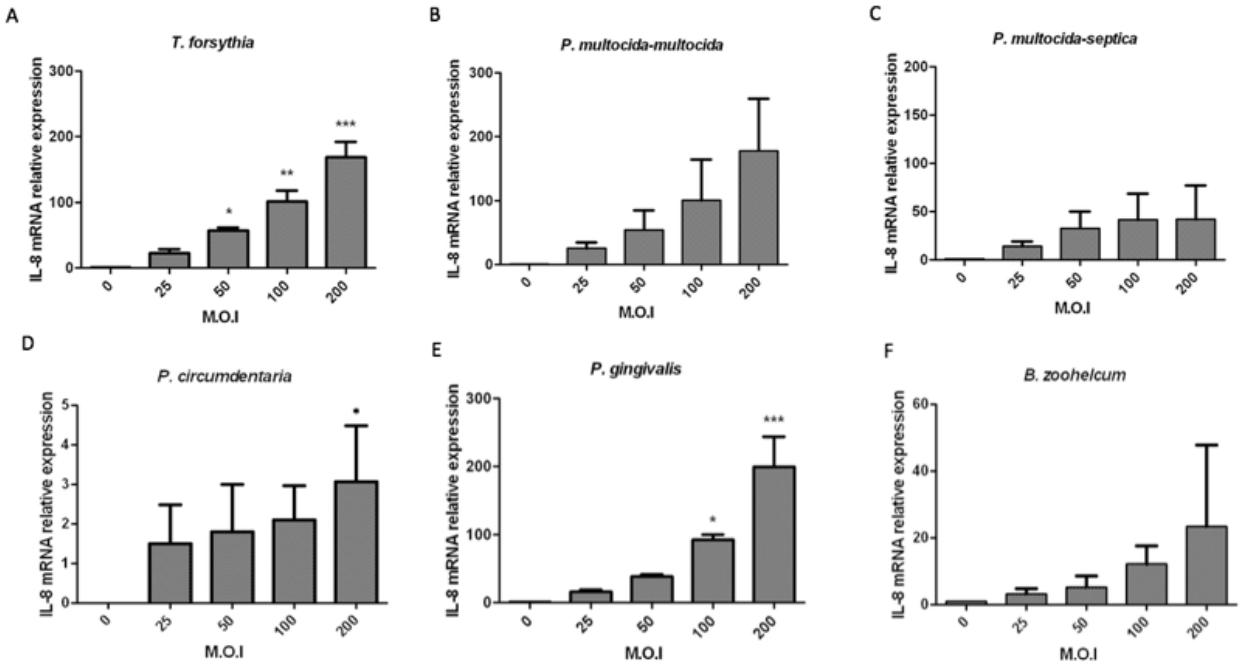

Figure 2.2: THP1-XBlue™ cell IL-8 mRNA expression following exposure to bacterial panel of interest

THP1-XBlue™ cells were seeded at the concentration of $2x10^5$ cells/mL in a 96-well plate. Cells were exposed to heat-killed bacteria ([A] *Tannerella forsythia* [B] *Pasteurella multocida* subsp. *multocida* [C] *Pasteurella multocida* subsp. *septica* [D] *Porphyromonas circumdentaria* [E] *Porphyromonas gingivalis* [F] *Bergeyella zoohelcum*) at various multiplicities of infection (0-200) for 24 h. RT-qPCR was performed using SYBR® green to determine IL-8 relative expression, normalised to *GAPDH*. Samples were assayed in duplicate on three independent occasions. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test. Data represents mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

2.3.3 THP1-XBlue™ cell IL-8 protein release is influenced by concentration-dependent stimulation with FCGS putative pathogens

Due to the previous data (section 2.3.2) showing an altered IL-8 mRNA expression in THP1-XBlue™ cells in response to FCGS-associated bacteria, protein release was then examined. Levels of IL-8 protein in cell culture supernatants following 24 h stimulation with the bacterial panel of interest were assessed (Figure 2.3).

THP1-XBlue™ cells incubated without bacteria were used as a cells only control (MOI 0). A significant increase in IL-8 protein release was shown in cells stimulated with *T. forsythia* at an MOI of 100 (835.99 pg/mL; $p<0.01$) and 200 (2542.61 pg/mL; $p<0.001$) compared to the cells only control of 10.20 pg/mL. There was also a significant increase in cell IL-8 following exposure to *P. multocida* subsp. *multocida* at 50 (262.49 pg/mL; $p<0.01$), 100 (1132.19 pg/mL; $p<0.0001$), and 200 (3441.62 pg/mL; $p<0.001$) MOI compared to the control of 15.44 pg/mL. Exposure to *P. gingivalis* showed a large significant increase in IL-8 release at all MOIs, with 3652.62 pg/mL present at an MOI of 200 ($p<0.001$). A significant increase in IL-8 production was shown in cells following exposure to *P. multocida* subsp. *septica* (1421.41 pg/mL; $p<0.01$) and *B. zoohelcum* (104.64 pg/mL; $p<0.001$) at an MOI of 200 only. No significant increase in protein release was noted following exposure to *P. circumdentaria*.

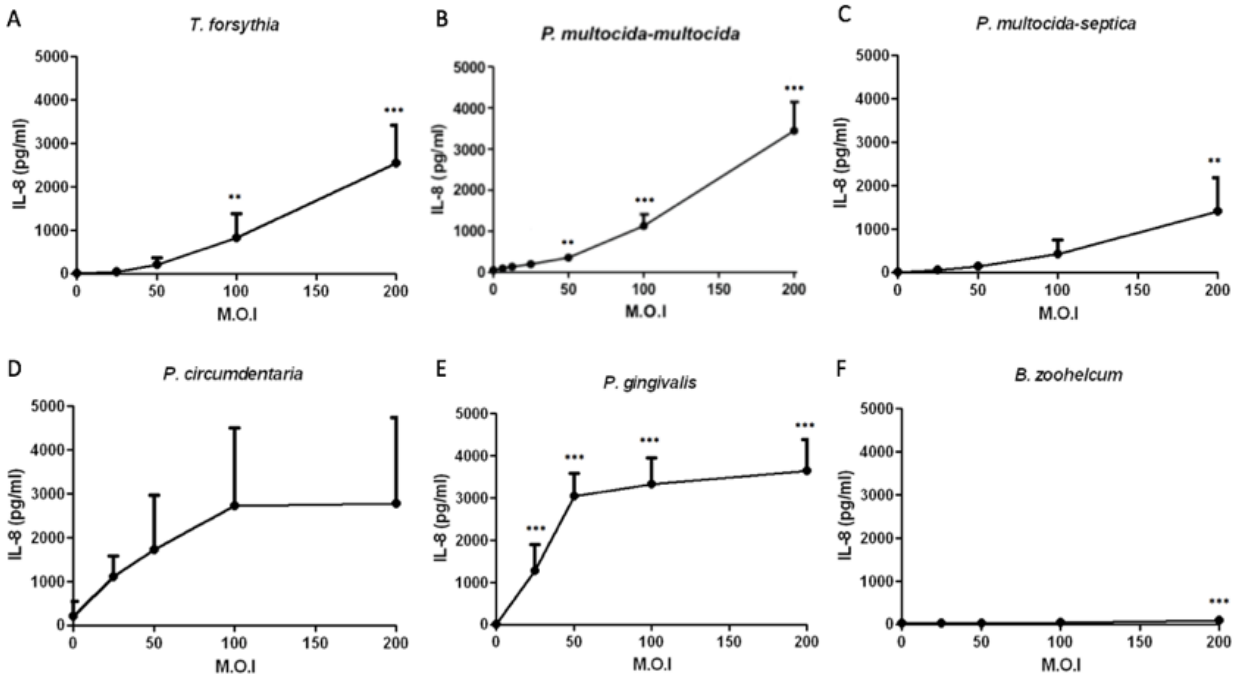

Figure 2.3: THP1-XBlue™ cell IL-8 protein response to bacterial panel of interest

THP1-XBlue™ cells were seeded at the concentration of $2x10^5$ cells/mL in a 96-well plate. Cells were exposed to heat-killed bacteria ([A] *Tannerella forsythia* [B] *Pasteurella multocida* subsp. *multocida* [C] *Pasteurella multocida* subsp. *septica* [D] *Porphyromonas circumdentaria* [E] *Porphyromonas gingivalis* [F] *Bergeyella zoohelcum*) at various multiplicities of infection (0-200) for 24 h. IL-8 protein release in culture supernatants was measured by ELISA. Samples were assayed in duplicate on three independent occasions. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test. Data represents mean ± SD (**p<0.01, ***p<0.001).

2.3.4 SCCF1 cell IL-8 mRNA expression is influenced by concentration-dependent stimulation with FCGS-associated bacteria

Next, it was investigated whether the FCGS-associated bacteria evoked a similar biological response in a feline oral squamous cell carcinoma cell line, SCCF1, by measuring changes in pro-inflammatory mediator IL-8.

The *IL-8* gene expression from SCCF1 cells following exposure to the bacterial panel of interest was firstly measured, as shown in Figure 2.4. All bacteria showed to significantly increase *IL-8* expression in cells at an MOI of 200 compared to the

cell only control, with an 8.52-fold increase from *T. forsythia* ($p<0.001$); 13.53-fold increase from *P. multocida* subsp. *multocida* ($p<0.001$); 4.81-fold increase from *P. multocida* subsp. *septica* ($p<0.05$); 38.89-fold increase from *P. circumdentaria* ($p<0.001$); 2.77-fold increase from *P. gingivalis* ($p<0.001$); and 47.46-fold increase from *B. zoohelcum* ($p<0.01$). *IL-8* expression was significantly up-regulated following exposure to *P. circumdentaria* at the lowest MOI (25) of all the bacteria ($p<0.01$).

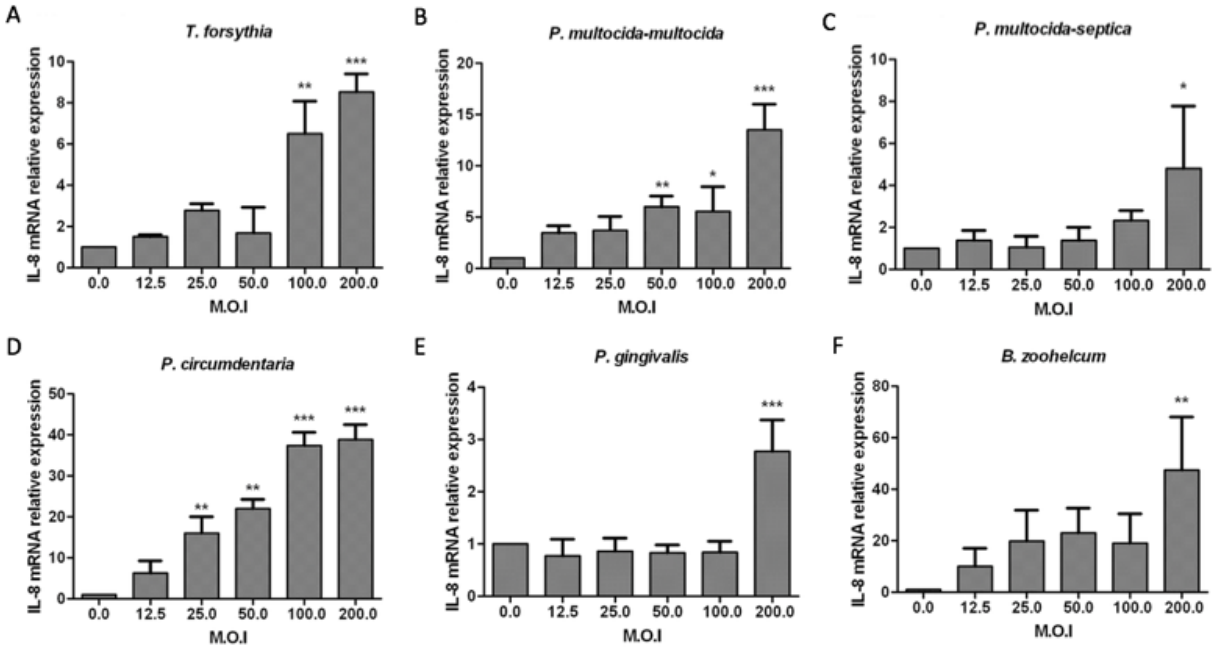

Figure 2.4: SCCF1 cell IL-8 mRNA expression following exposure to bacterial panel of interest

SCCF1 cells were seeded at the concentration of $2x10^5$ cells/mL in a 96-well plate. Cells were exposed to heat-killed bacteria ([A] *Tannerella forsythia* [B] *Pasteurella multocida* subsp. *multocida* [C] *Pasteurella multocida* subsp. *septica* [D] *Porphyromonas circumdentaria* [E] *Porphyromonas gingivalis* [F] *Bergeyella zoohelcum*) at various multiplicities of infection (0-200) for 24 h. RT-qPCR was performed using SYBR® green to determine IL-8 relative expression, normalised to *GAPDH*. Samples were assayed in duplicate on three independent occasions. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test. Data represents mean ± SD (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

2.3.5 SCCF1 cell IL-8 protein release is influenced by concentration-dependent stimulation with FCGS-associated bacteria

As *IL-8* mRNA expression was shown to be generally up-regulated in SCCF1 cells following exposure to FCGS-associated bacteria (Figure 2.4), the production of this chemokine by host cells was further investigated by ELISA.

IL-8 release from SCCF1 cells in response to bacterial stimulation was synonymous to IL-8 release from the human THP1-XBlue™ cell line, with an increasing IL-8 concentration found in supernatants as MOI increased. A significant increase in IL-8 protein was measured following stimulation with *T. forsythia* (4382.34 pg/mL; $p<0.001$), *P. multocida* subsp. *multocida* (3049.44 pg/mL; $p<0.001$), *P. multocida* subsp. *septica* (4888.57 pg/mL; $p<0.001$), *P. circumdentaria* (3988.95 pg/mL; $p<0.05$), *P. gingivalis* (6175.58 pg/mL; $p<0.05$), and *B. zoohelcum* (4855.11 pg/mL; $p<0.001$) at an MOI of 200 compared to the cells only control (MOI 0) (Figure 2.5). IL-8 protein release in cells was also increased after exposure with an MOI of 100 of *T. forsythia* ($p<0.05$), *P. multocida* species ($p<0.05$) and *B. zoohelcum* ($p<0.001$).

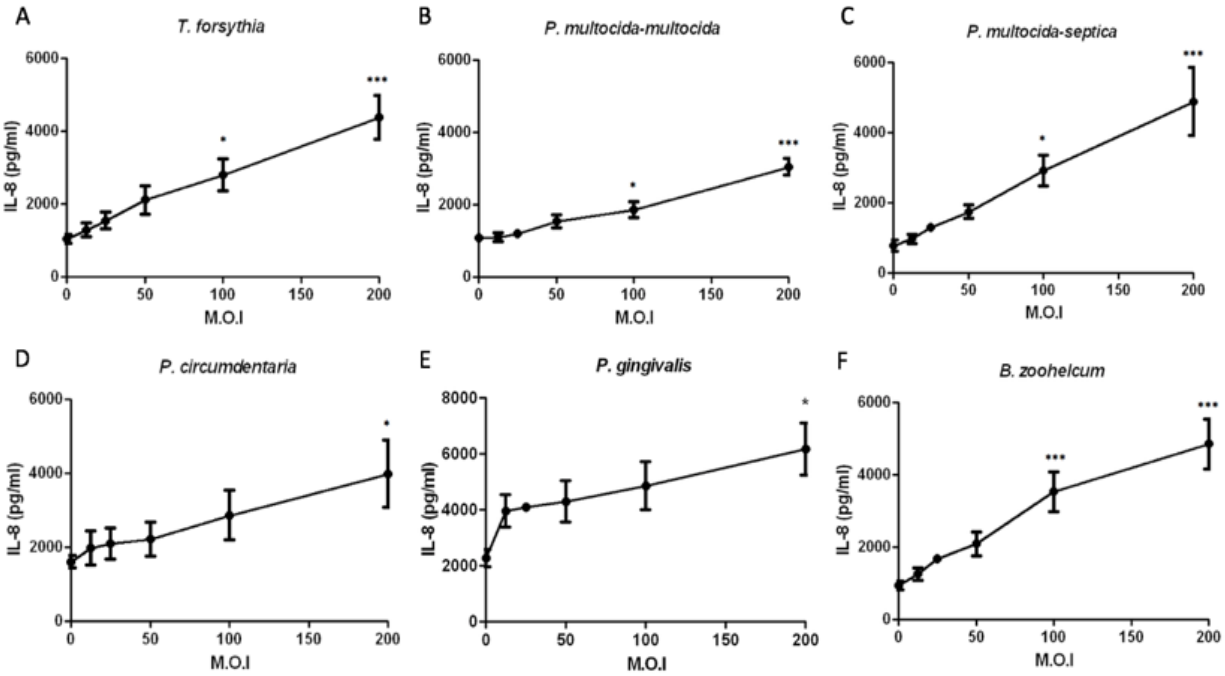

Figure 2.5: SCCF1 cell IL-8 secretion following exposure to bacterial panel of interest

SCCF1 cells were seeded at the concentration of $2x10^5$ cells/mL in a 96-well plate and incubated at 37°C in 5% CO_2 for 24 h. Cells were exposed to heat-killed bacteria ([A] *Tannerella forsythia* [B] *Pasteurella multocida* subsp. *multocida* [C] *Pasteurella multocida* subsp. *septica* [D] *Porphyromonas circumdentaria* [E] *Porphyromonas gingivalis* [F] *Bergeyella zoohelcum*) at various multiplicities of infection (0-200) for 24 h. IL-8 protein release in culture supernatants was measured by ELISA. Samples were assayed in duplicate on three separate occasions. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test. Data represents mean ± SD (*$p<0.05$, ***$p<0.001$).

2.4 Discussion

While the aetiology of FCGS is believed to be multifactorial, it is thought that the dysbiosis of interactions between oral plaque bacteria and the host immune system is a key contributor to the chronic inflammation present during FCGS. Specific bacteria, as seen in periodontal disease, have been identified as more predominant within oral communities in diseased cats with abnormal levels of inflammation, including *Pasteurella* species (Dolieslager et al., 2011). In this chapter, the interactions between FCGS-associated bacteria and host cells have been investigated in order to establish a greater understanding of the cell response to bacteria of interest, and how the presence of specific bacteria may modulate the immune response during disease.

The key finding was that both human and feline host cell lines demonstrated a distinct IL-8 gene and protein response to varying concentrations of heat-killed FCGS-associated bacteria, particularly in response to bacteria thought to be putative pathogens in disease. This result showed the immune function consequences of feline bacterial species of differing pathogenicity and their potential to contribute to the chronic inflammation that is evident in FCGS.

The data, firstly, showed the changes in TLR activation when THP1-XBlue™ cells were exposed to FCGS-associated bacteria. THP1-XBlue™ cells could be used in this study as a comparative positive control as it is already known that *P. gingivalis* is a major periodontal pathogen in human oral disease (Hajishengallis et al., 2012). Pathogen-associated molecules are recognised by several sensors of the innate immunity, including TLRs (Akira and Takeda, 2004). TLRs can mediate the inflammatory response when activated by specific pathogens. Thus, various enzymatic bio-assays have been developed which co-express a TLR-inducible reporter gene encoding SEAP, which allows TLR stimulation to be conveniently monitored by using a phosphatase detection assay. In this study, TLR activation, represented by levels of SEAP production, was significantly increased in cells exposed to feline putative pathogens *T. forsythia* and *P. multocida* species as well as human pathogen *P. gingivalis*. A study by Dolieslager et al. (2013) found that cats harbouring *T. forsythia* had significant increases in TLR2, TLR4, TLR7 and TLR9 compared to cats in which this bacterial species was absent. As *T. forsythia*

showed the greatest influence on TLR activation in this study, followed by *P. multocida* species which have shown to be highly prevalent during disease, this suggests that these bacteria may be of importance in stimulating a host immune response to FCGS.

Moreover, THP1-XBlue™ cells showed significantly increased IL-8 gene expression in response to *T. forsythia*, *P. circumdentaria* and *P. gingivalis* and significantly increased levels of IL-8 protein were present in response to *T. forsythia*, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. gingvalis* and *B. zoohelcum*. IL-8 acts by recruiting immune cells such as neutrophils and T lymphocytes to the site of infection and stimulates phagocytosis, and is therefore widely acknowledged as a causative agent of inflammation in both humans and felines (Shahzad et al., 2010; Alavi-Moghaddam et al., 2011). As *T. forsythia* and *P. gingivalis* are known pathogens in human oral disease, the THP1-XBlue™ immune response observed was as expected in this study. A previous study has shown *T. forsythia* contains a PrtH protein that is able to stimulate inflammation by inducing the production of IL-8 (Ksiazek et al., 2015). The notable increase in TLR activation by *T. forsythia* and *P. multocida* species and concurrent increase in IL-8 protein release in this study suggests these bacteria may be key in eliciting an innate immune response during disease and could be significant in the aetiopathogenesis of FCGS.

Furthermore, the inflammatory response to FCGS-associated bacteria was investigated in a feline squamous carcinoma cell line, SCCF1. All bacteria tested (key pathogens, putative pathogens and commensal) elicited an increase in IL-8 mRNA expression at an MOI of 200, as well as an increase in IL-8 protein expression which increased with an increasing concentration of bacteria applied. Previous research has discovered that *P. multocida* species are highly prevalent in the feline oral cavity during FCGS, comprising 51.8% of the bacterial population and therefore considered significant in disease (Dolieslager et al., 2011). These results suggest that *P. multocida* species can stimulate pro-inflammatory IL-8 gene expression and protein production in a feline cell line and therefore may play a key role in stimulating the chronic inflammation found during disease. Moreover, Dolieslager et al. (2011) found *B. zoohelcum* to be highly prevalent in the oral cavity of healthy cats compared to those with FCGS; however, the data in the

current study suggests that *B. zoohelcum* is capable of stimulating an inflammatory response during disease.

While this model proved to demonstrate the initial innate cell responses to putative bacteria in this study, it is important to consider that heat-killed bacteria were used to stimulate cells due to the ability of many live bacteria to modulate pro-inflammatory cytokines and chemokines. It has been shown that *P. gingivalis* can subvert the host proinflammatory response, through lysine gingipain, by direct degradation of chemokines such as IL-8 (Stathopoulou et al., 2009). One study has also observed that differing levels of pro-inflammatory cytokines were induced in blood cultures by live *Streptococcus suis* compared to the heat-killed bacterium (Segura et al., 2006). Therefore, the host response to heat-killed bacteria in this study may not reflect the inflammatory response induced by live bacteria.

In summary, focusing on how specific bacteria may differentially modulate the host immune response is crucial to understanding the abnormal inflammatory levels evident in FCGS. It would be interesting to use this model further to test FCGS-associated bacteria in combination, or within a biofilm to measure the differences in immune response.

3 Development of an *in vitro* FCGS biofilm model for therapeutic testing

3.1 Introduction and Aims

The human oral microbiome is known to play a significant role in human health and disease. While the feline oral microbiome is less well studied, it is thought that over 400 bacterial species associated with the feline oral cavity contribute to feline oral health (Adler et al., 2016).

Bacterial communities that reside in the oral cavity form complex biofilms (plaque) on teeth and soft gingival tissues (Zijnge et al., 2010). Within the biofilm, bacteria are more resistant to antibiotics and antimicrobials, and are highly capable of evading host defences (Bjarnsholt, 2013). As microbial communities can readily alter the local environment, biofilm composition can shift to an increasing proportion of disease-associated bacteria which ultimately leads to disease such as periodontitis (Berezow and Darveau, 2011). It is thought this microbial dysbiosis may also occur in the feline oral cavity during the development of FCGS. Due to the large diversity of bacteria found in dental plaque, studying the roles and impact of bacteria within oral biofilms has proved difficult. Therefore, a variety of *in vitro* biofilm models have previously been created to replicate a diseased microbial environment within the oral cavity, allowing the study of bacterial interactions within biofilms and the evaluation of orally relevant antimicrobial compounds.

Due to the complex nature of FCGS, an adequate treatment is not yet available. A first-line treatment for all affected cats involves the improvement of basic oral hygiene in attempt to reduce the oral antigen burden as well as antibiotics in some cases to control excessive inflammation. However, in almost all cases, first-line treatment is a short-term resolution, and diseased teeth will then be extracted to reduce the chronic inflammation. Chlorhexidine (CHX) is also used as an effective post-op plaque control, however, reported prolonged use of CHX in studies of human oral disease has shown adverse reactions such as pain of the oral mucosa and anaphylaxis (Frank et al., 2001, Dyer et al., 2013). Furthermore, CHX does not always prove to be entirely effective or suitable for all cats (Lobprise and Dodd, 2018).

Discovering alternative compounds which have the same, or greater, antimicrobial potency of CHX but low toxicity is of importance in the search for appropriate

treatments to reduce the bacterial burden during FCGS. Antimicrobials such as CHD-FA and xylitol have both previously shown to be an effective treatment option used on multi-species biofilms devised to mimic human oral disease (Badet et al., 2008; Sherry et al., 2013). Berberine, an isoquinoline alkaloid, has also shown to have anti-microbial properties on single-species biofilms of *Pseudomonas* and *Staphylococcus* species (Aswathanarayan and Vittal, 2018; Wang et al., 2009). These studies highlight the potential of these novel compounds to inhibit biofilm activity, and therefore testing these compounds on an FCGS biofilm was worth investigation in this study. Moreover, ubiquinol is an active ingredient found in the anti-microbial and anti-inflammatory product, Oralmat®, so was of interest to discover the antimicrobial potential of this compound.

Therefore, the aim of this study was to create an *in vitro* multi-species biofilms model to mimic disease-associated plaque, containing bacteria which are orally relevant to FCGS, which could be used to test the effect of various novel anti-microbial compounds.

3.2 Materials and Methods

3.2.1 Bacterial culture and standardisation

A multispecies biofilm model consisting of 10 bacterial species associated with FCGS was developed for antimicrobial testing (Dolieslager et al., 2011). *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *B. zoohelcum*, *P. circumdentaria* and *T. forsythia* were cultured and standardised as previously described in 2.2.1. Additionally, *Streptococcus mitis* NCTC 12261, *Streptococcus intermedius* ATCC 27335, and *Streptococcus oralis* ATCC 35037 were grown and maintained at 37°C on CBA in 5% CO_2. *Actinomyces naeslundii* DSMZ 17233 and *Fusobacterium nucleatum* DSMZ 10953 were cultured at 37°C on FAA under anaerobic conditions.

S. mitis, *S. intermedius*, and *S. oralis* were propagated in 10 mL tryptic soy broth [Sigma-Aldrich]. *A. naeslundii* and *F. nucleatum* were grown in 10 mL of Schaedler's anaerobic broth under anaerobic conditions. Cultures were grown for 24-48 h at 37°C as necessary and washed as previously described in 2.2.1. All bacteria were then standardised and adjusted to a final working concentration of 1 x 10^8 cells/mL for downstream biofilm development and sessile susceptibility testing.

3.2.2 Biofilm growth medium

All biofilm cultures were initially grown using artificial saliva (AS) as previously described (Pratten et al., 1998). This was comprised of porcine stomach mucins (0.25% w/v) [Sigma-Aldrich], sodium chloride (0.35 w/v) [VWR, Leuven, Belgium] potassium chloride (0.02 w/v) [VWR], calcium chloride dihydrate (0.02 w/v) [VWR], yeast extract (0.2 w/v) [Formedium, Hunstanton, UK], lab lemco powder (0.1 w/v) [Oxoid] and proteose peptone (0.5 w/v) [Sigma-Aldrich] in ddH2O [Thermo Scientific]. Urea [Sigma-Aldrich] was diluted in ddH2O (40% w/v) and added to a final concentration of 0.05% (v/v) in AS.

A second medium was also used to grow biofilms during this study. This consisted of Todd Hewitt broth (THB) [Sigma-Aldrich] supplemented with 10 µg/mL hemin [Sigma-Aldrich] and 2 µg/mL menadione [Sigma-Aldrich]. To make a working

broth, the supplemented THB was added to an equal volume of RPMI-1640. Bacterial growth and biofilm formation were not affected when cultured in the THB: RPMI medium.

3.2.3 Multi-species biofilm culture (10 species)

An *in vitro* multi-species biofilm was developed to represent and recapitulate the diseased microbial environment present in the oral cavity during FCGS. The 10 species model consisted of *S. mitis*, *S. intermedius*, *S. oralis*, *B. zoohelcum*, *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *A. naeslundii*, *F. nucleatum*, *T. forsythia* and *P. circumdentaria*. Biofilms were prepared in 24-well plates [Corning, NY, USA] containing Thermanox™ coverslips (13 mm diameter) [Fisher Scientific, Loughborough, UK]. For the addition of each bacterial species to the biofilm, bacterial suspensions were standardized to 1 x 10^7 CFU/mL in 500 µL of THB: RPMI medium. Firstly, *S. mitis*, *S. intermedius*, and *S. oralis* were added together and incubated at 37°C in 5% CO_2 for 24 hours. Next, the supernatant was removed and *B. zoohelcum*, *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* standardised in THB: RPMI were added to the biofilms and grown for 24 hours at 37°C in 5% CO_2. On the third day, the supernatant was again removed and *A. naeslundii*, *F. nucleatum*, *T. forsythia* and *P. circumdentaria* standardised in THB: RPMI were added before the biofilms were incubated anaerobically at 37°C for a further 4 days. Each day, the supernatants were removed and fresh THB: RPMI added.

Biofilms were either used for testing directly after culture, or the supernatant was removed and biofilms were washed with PBS before storage at -80°C until required. Frozen biofilms were revived by the addition of 500 µL of THB: RPMI and incubation for 24 h in the anaerobic cabinet before experimental use.

3.2.4 Investigating sialidase inhibitors as a novel treatment option

3.2.4.1 Bacterial sialidase activity

The sialidase activity of FCGS putative pathogens (*P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. circumdentaria* and *T. forsythia*) was determined.

Firstly, 1 mL of 1 x 10^6 CFU/mL of each bacterium was added to a 0.1 mM 4-methylumbelli-feryl N-acetyl-α-D-neuraminic acid (MUNANA) sodium salt [Carbosynth Limited, Compton, UK] solution to create a reaction mix. The samples were incubated for 24 h in aerobic (*P. multocida* species) or anaeorobic (*P. circumdentaria* and *T. forsythia*) conditions, with the transfer of 50 µl of each reaction mix to separate wells of a clear-bottomed black 96-well plate following incubation period. The reaction was stopped by the addition of 75 µL of 100 mM sodium carbonate buffer [Sigma-Aldrich] to each well and then the fluorescence measured at 355 nm and 460 nm on a microplate reader.

3.2.4.2 Bacterial sialidase inhibition

Two potential sialidase inhibitors were investigated in this study: a novel plant alkaloid, berberine, which has previously shown to inhibit sialidase activity in various strains of influenza virus (Enkhtaivan et al., 2017), and sialic acid analogue, 2-deoxy-2,3-dehydro-N-acetyleneuraminic acid (DANA), which has shown to inhibit viral, bacterial and mammalian sialidases (Meindl and Tuppy, 1969; Burmeister et al., 1993). A 40 mM stock solution of inhibitor was serially diluted to 10 µm in PBS. Each bacterial species was standardized to 1 x 10^6 CFU/mL and 450 µL was added each to 50 µL of inhibitor and incubated for 1 h in appropriate conditions. Following incubation, 225 µL of 0.1 mM MUNANA was added to create the reaction mix. Samples were then incubated for up to 24 h, with transfer of sample to separate wells of a clear-bottomed black 96-well plate at time intervals of 0, 1, 2, 3, 4, 22 and 24 h during incubation. The reactions were quenched at each time point by the addition of 75 µL of 100 mM sodium carbonate buffer, and fluorescence measured at 355 nm and 460 nm on a microplate reader.

3.2.5 Antibacterial susceptibility testing of multi-species biofilm

During this study, four active compounds were tested to determine the antibacterial potential on FCGS biofilm cells. The compounds included xylitol [Sigma-Aldrich] which has shown to inhibit human oral multi-species biofilm growth (Badet et al., 2008); carbohydrate-derived fulvic acid (CHD-FA) [Fulhold Ltd, Cape Town, South Africa] which has shown to be effective against a multi-

species periodontal biofilm (Sherry et al., 2013); berberine [Sigma-Aldrich] which is a potential sialidase inhibitor and has shown anti-microbial activity (Aswathanarayan and Vittal, 2018); and ubiquinol [Sigma-Aldrich] which is an active agent in the natural anti-inflammatory Oralmat® product. Chlorhexidine (CHX) [Sigma-Aldrich] at 0.2% was also used as a positive control.

For testing on multi-species biofilms, biofilms were grown as previously described in section 3.2.3. Compounds were prepared at a range of concentrations for anti-microbial testing, according to inhibitory concentrations found in literature. Xylitol was prepared at 0.25, 0.5, 1, and 5% (w/v). CHD-FA was prepared at 0.25, 0.5, and 0.8% (v/v). Berberine and ubiquinol were prepared at 50, 100, and 200 µg/mL. Mature biofilms were treated for 5 minutes (CHD-FA, xylitol, ubiquinol) or 24 h (berberine) with each concentration of compound before the addition of neutralising buffer to stop the agents from acting on the biofilms for longer than desired. Biofilms were then washed with PBS before further downstream testing to measure for disruption of biofilms. The experiment was carried out in triplicate on three independent occasions.

3.2.6 alamarBlue® cell viability assay

The resazurin-based solution alamarBlue® [Invitrogen] was used to measure multi-species biofilm cell viability following treatments with each compound. Following treatment, multi-species biofilms were carefully washed with PBS before the addition of alamarBlue® (at a 1:10 dilution in THB: RPMI medium) to each well. The alamarblue® is a colorimetric assay whereby a colour change (blue to pink) occurs as a result of an oxidation/reduction reaction based upon cellular metabolic activity. The alamarBlue® was incubated for 1-2 hours under anaerobic conditions. Following incubation, the fluorescence was read at 544 nm with a reference wavelength at 590 nm. The mean fluorescence of each treatment group was used to calculate the percentage of cell viability compared to the untreated control as follows: percentage of cell viability = $(A_{treatment} - A_{blank}) / (A_{control} - A_{blank})$ x 100% (where, A = fluorescence). Biofilms were washed with PBS and left to dry on the bench overnight for crystal violet assay, as described in section 3.2.8.

3.2.7 Biofilm quantification: Miles and Misra

Live bacterial cells in the treated multi-species biofilms were quantified using the Miles and Misra method (Miles et al., 1938). Briefly, treated biofilms (not used in cell viability or biomass testing) were removed from Thermanox™ coverslips in a sonic bath at 35 kHz for 10 minutes in 1 mL of PBS. Each inoculum was then serially ten-fold diluted from neat supernatant to 10^{-8} in PBS. For each dilution, 10 µL was drop-plated in triplicate on both CBA and FAA plates, left to dry on the bench for 30 minutes, and then cultured in the appropriate conditions for 24 and 48 h, respectively. Following incubation, colonies were then counted at each dilution where the number of colonies ranged between 30 – 300 and the colony-forming units (CFU) were calculated as follows: CFU = no. of colonies / volume plated (mL) x dilution factor.

3.2.8 Biofilm biomass quantification by crystal violet assay

To quantify biofilm biomass following antimicrobial treatment, crystal violet (CV) assays were performed. Five hundred microlitres of 0.05% (w/v) CV solution was added to each biofilm and incubated for 20 minutes at room temperature, allowing uptake of the dye. The CV solution was then discarded and biofilms were carefully washed with water to remove any excess dye. Next, 500 µL of 100% ethanol was added to biofilms and mixed thoroughly to release the CV dye before transferring 75 µL from each well to a fresh 96-well flat-bottomed plate. Biofilm biomass was then measured by reading absorbance at 570 nm in a microtitre plate reader. A negative control, containing media only, was included to allow for blank correction of all absorbance values.

3.2.9 Statistical analysis

Graph production and statistical analysis was carried out using GraphPad Prism (version 5; La Jolla, USA). Independent sample data was analysed using a one-way ANOVA. A Dunnett's post-test and Bonferroni correction for multiple comparisons was applied to the data where appropriate. A p value of less than 0.05 was considered significant.

3.3 Results

3.3.1 FCGS putative pathogens display sialidase activity

Since bacterial sialidases are believed to play a role during bacterial colonisation and pathogenesis of mammalian mucosal surfaces, it is possible that bacterial sialidases are used in the oral cavity to enhance biofilm formation and bacterial survival. If present, the bacterial enzyme sialidase cleaves MUNANA into N-acetylneuraminic acid and 4-methylumbelliferone, which is a fluorescent molecule. The sialidase activity of putative pathogens of FCGS was investigated based on the measurement of fluorescence (Figure 3.1).

A reaction mix control was included containing no bacteria. A significant difference in sialidase activity was observed after 24 h between the control, with a fluorescence of 2432, and each bacterial species: *P. multocida* subsp. *septica* (22-fold increase; $p<0.001$), *P. mutocida* supsp. *multocida* (26-fold increase; $p<0.001$), *T. forsythia* (25-fold increase; $p<0.001$), and *P. circumdentaria* (4-fold increase; $p<0.001$). Hence, *P. circumdentaria* displayed the lowest sialidase activity.

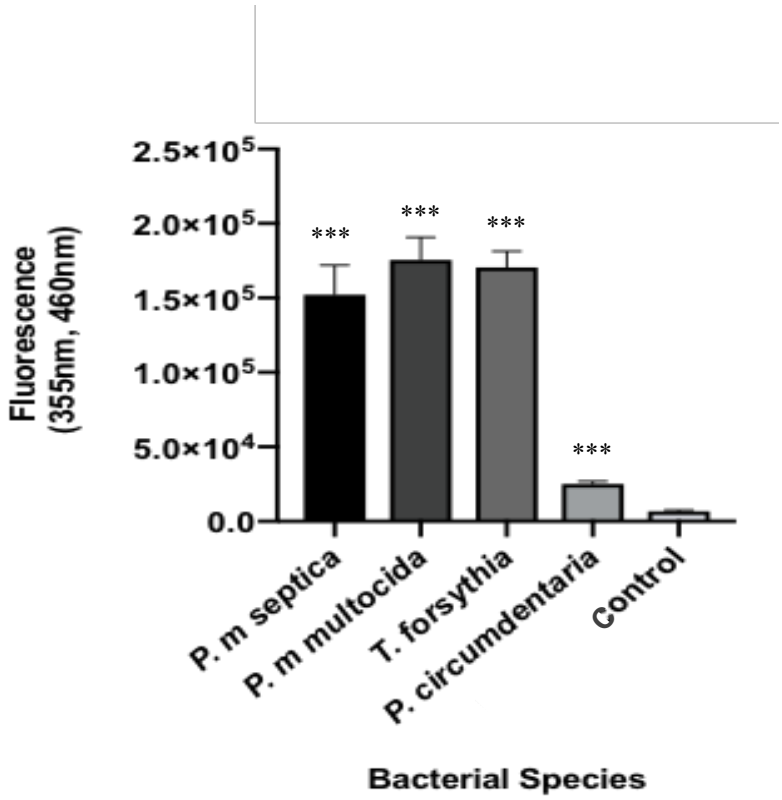

Figure 3.1: Sialidase activity of FCGS putative pathogens

Bacteria were standardized to 1 x 10^6 CFU/mL before incubation with MUNANA for 24 h. Following 24 h, each reaction was quenched using sodium carbonate buffer and sialidase activity was determined by measuring fluorescence at 355 nm and 460 nm. A control containing no bacterial cells was included. Statistical analysis was performed using one-way ANOVA and Dunnett's post-hoc test to compare the means of each species to the control group mean. Data represents mean ± SD (***$p<0.001$) of three independent experiments each performed in triplicate.

3.3.2 Berberine treatment inhibits FCGS putative pathogen sialidase activity

As the previous data (section 3.3.1) demonstrated that the putative pathogens of FCGS displayed sialidase activity, all species were then used to investigate the efficacy of a potentially natural sialidase inhibitor, berberine (Figure 3.2). Bacteria were incubated over 24 h with inhibitor and fluorescence was measured at specific time intervals. A bacteria only control was included (0 μM berberine).

At 24 h, following treatment with 5 μM berberine, there was a significant difference in the fluorescence of *T. forsythia* (41% decrease; $p<0.05$) compared to the bacteria only control (0 μM) which contained no inhibitor. No significant

differences were shown between *P. multocida* subsp. *septica*, *P. multocida* subsp. *multocida*, or *P. circumdentaria* following treatment of 5 µM berberine compared to the bacteria only control. At 24 h, following treatment with 20 mM berberine, there was a significant difference in the fluorescence of *P. multocida* subsp. *septica* (89% decrease; $p<0.001$), *P. multocida* subsp. *multocida* (87% decrease; $p<0.001$), *P. circumdentaria* (79% decrease; $p<0.001$) and *T. forsythia* (71% decrease; $p<0.001$) compared to the bacteria only control (0 µM). There was a notable concentration-dependent effect on the sialidase activity of all bacteria following treatment with berberine for 24 hours.

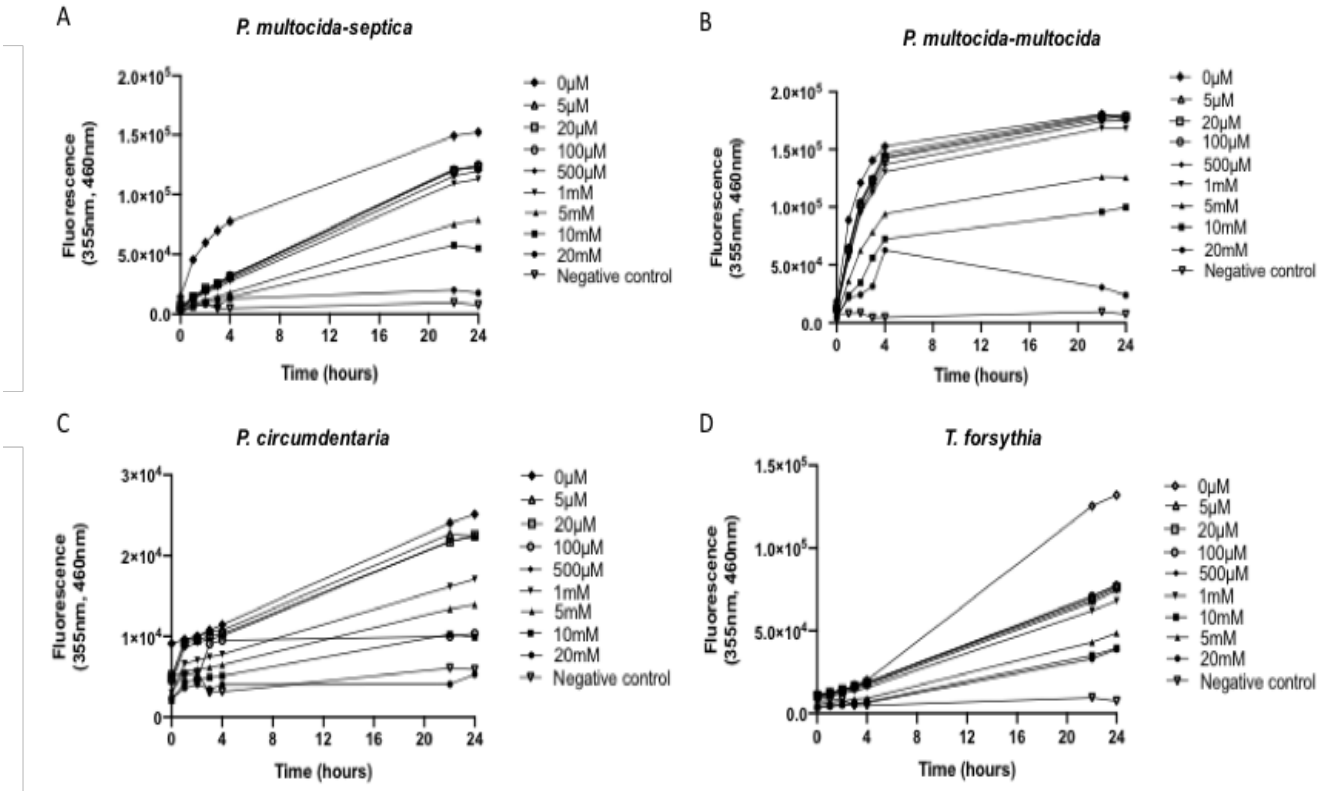

Figure 3.2: Sialidase activity of putative pathogens in response to berberine

Bacteria ([A] *Pasteurella multocida* subsp. *septica* [B] *Pasteurella multocida* subsp. *multocida* [C] *Porphyromonas circumdentaria* [D] *Tannerella forsythia*) were standardized to 1 x 10^6 CFU/mL before incubation with MUNANA and a range of concentrations of berberine for 24 h. At time intervals of 0, 1, 2, 3, 4, 22 and 24 h, each reaction was quenched using sodium carbonate buffer and sialidase activity was determined by measuring fluorescence at 355/460 nm. A negative control containing no bacterial cells was included. Samples were assayed in triplicate on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test. Data represents mean ± SD (*$p<0.05$).

3.3.3 DANA treatment inhibits FCGS putative pathogen sialidase activity

Another known synthetic sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetyleneuraminic acid (DANA), was investigated for its potential to inhibit the sialidase activity of putative pathogens in FCGS (Figure 3.3). The bacteria were incubated with inhibitor over 24 h with fluorescence measured at set time intervals. A bacteria only control, containing no inhibitor, was included (0 μM DANA).

Following 24 h, there showed to be a statistically significant decrease in the fluorescence of *T. forsythia* compared to the bacteria only control (0 μM) after incubation with the lowest concentration of DANA used (5 μM) (51% decrease; $p<0.01$). No significant differences in fluorescence were observed for the *P. mutocida* species or *P. circumdentaria* at 24 h incubation with 5 μM DANA. At 24 h, following treatment with berberine at a concentration of 20 mM, there was a significant decrease in the fluorescence of *P. multocida* subsp. *septica* (80% decrease; $p<0.0001$), *P. multocida* subsp. *multocida* (60% decrease; $p<0.01$), and *T. forsythia* (74% decrease; $p<0.001$) compared to the bacteria only control. DANA showed to have no effect on the sialidase activity of *P. circumdentaria*, with no statistically significant differences in fluorescence displayed at any concentration. DANA generally showed to inhibit sialidase activity in a concentration-dependent manner.

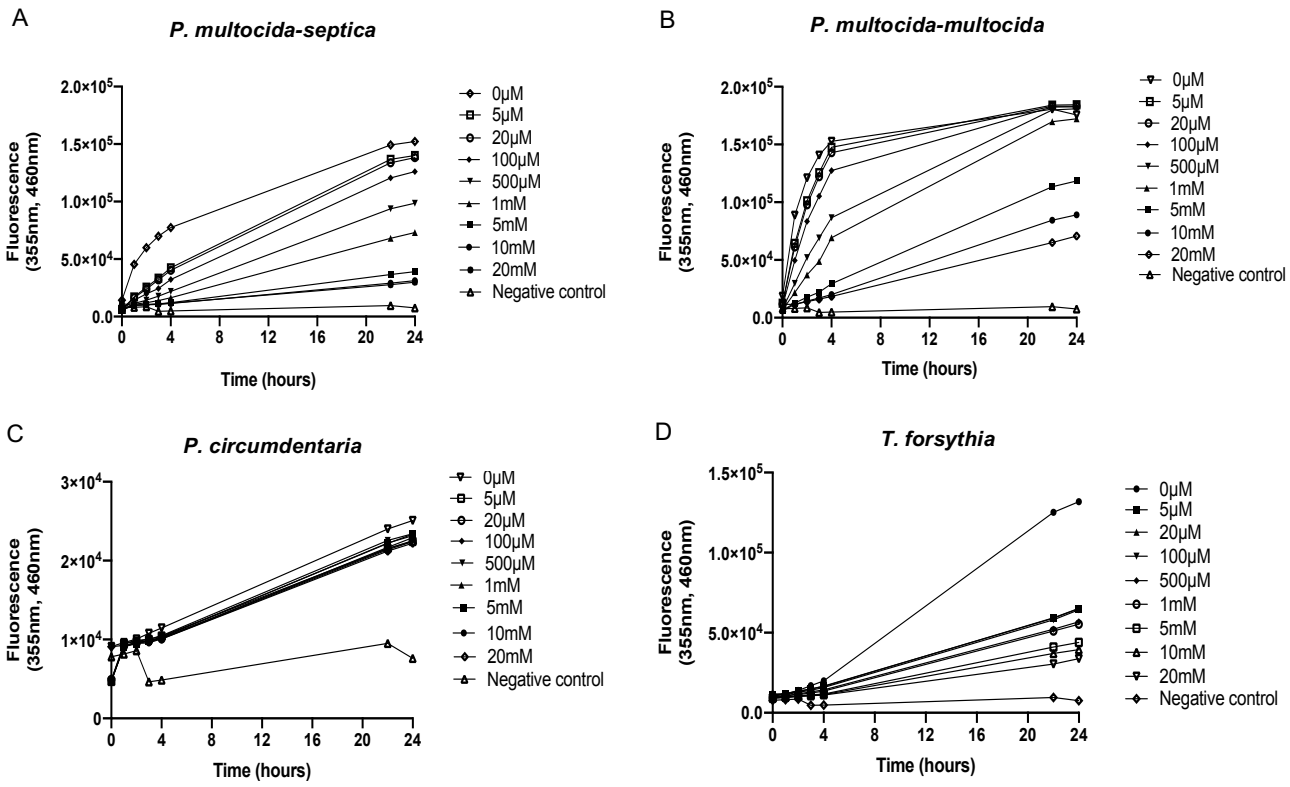

Figure 3.3: Sialidase activity of putative pathogens in response to DANA

Bacteria ([A] *Pasteurella multocida* subsp. *septica* [B] *Pasteurella multocida* subsp. *multocida* [C] *Porphyromonas circumdentaria* [D] *Tannerella forsythia*) were standardized to 1 x 10^6 CFU/mL before incubation with MUNANA and a range of concentrations of DANA for 24 h. At time intervals of 0, 1, 2, 3, 4, 22 and 24 h, each reaction was quenched using sodium carbonate buffer and sialidase activity was determined by measuring fluorescence at 355/460 nm. A negative control containing no bacterial cells was included. Samples were assayed in triplicate on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test. Data represents mean ± SD (*$p<0.05$).

3.3.4 Berberine treatment affects biofilm biomass

To measure the antimicrobial potential of the actives, multi-species biofilms were developed containing the FCGS bacteria which may reside in a diseased feline oral cavity and contribute to pathogenicity. Biofilms were treated with berberine for 24 h before cell viability was assessed using alamarBlue® metabolic dye. Biofilm biomass following treatment was measured using crystal violet dye, and the number of viable cells were counted using the Miles and Misra method (Figure 3.4).

As berberine was previously found to inhibit sialidase activity in FCGS-associated bacteria, the effect of berberine on the FCGS multi-species biofilm was then investigated. Untreated and positive (0.2% CHX) controls were included for comparison. Cell viability was presented as a percentage of the untreated control. The viability of biofilms treated with various concentrations of berberine was not comparable to the killing effect of CHX, and no significant differences were found between concentrations despite a notable decrease in viability in the biofilm treated with 200 µM compared to 50 µM. The biofilm biomass of the untreated control was 1.83, which was significantly decreased by 50 µM and 100 µM to 1.35 ($p<0.05$) and 1.40 ($p<0.01$), respectively. No significant differences in the number of CFUs were found following treatment with berberine at each concentration, however, there was a notable decrease in the number of live aerobic and anaerobic cells in biofilms treated with 200 µM berberine compared to the untreated control.

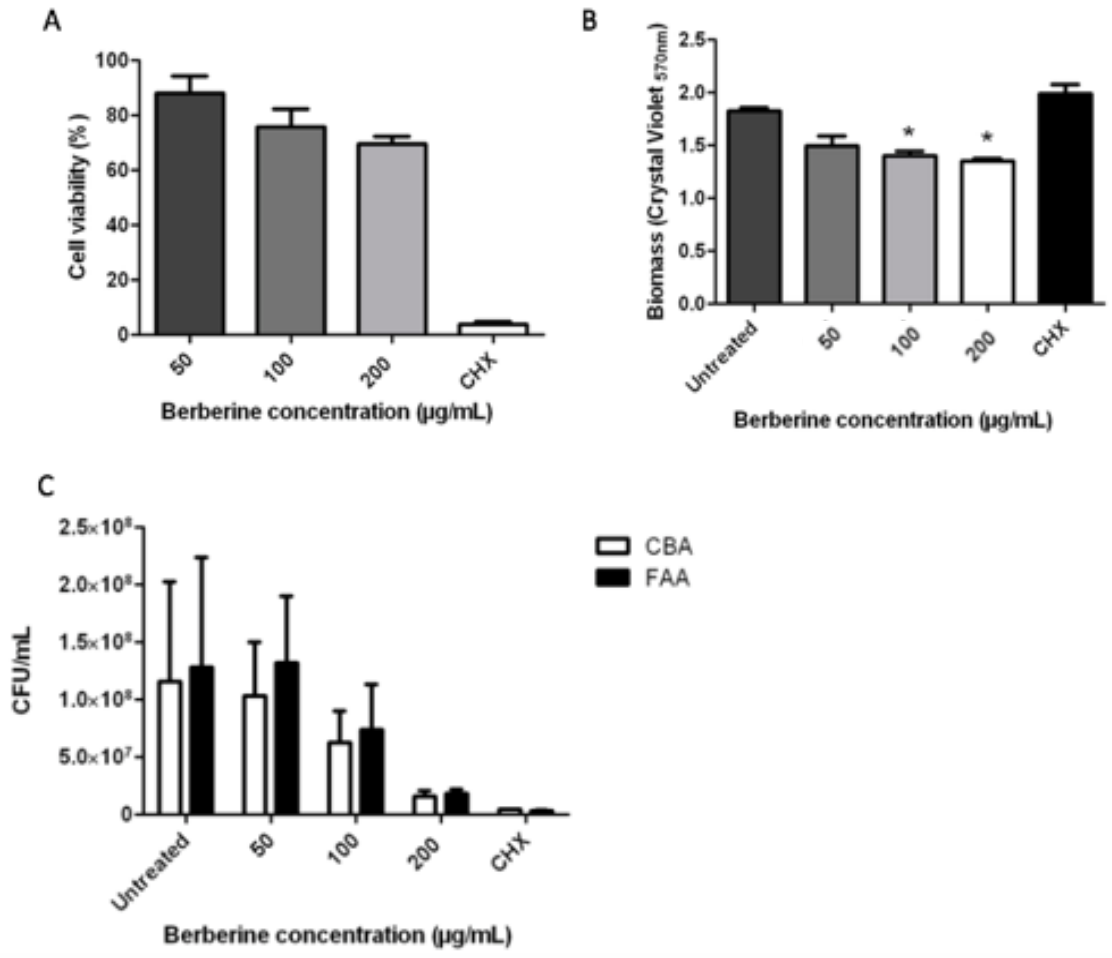

Figure 3.4: Biofilm viability and biomass following berberine treatment

Multi-species biofilms containing FCGS-associated bacteria were grown on Thermanox™ coverslips and treated with 50, 100 and 200 µg/mL berberine for 24 hours. Biofilms were also treated with 0.2% (v/v) CHX used as a positive control. Metabolic activity was measured using alamarBlue® and data presented as a percentage of the untreated control [A]. Biofilms were retained following treatment and biofilm biomass determined by staining with 0.05% (w/v) crystal violet solution and quantified spectrophotometrically by reading at 570 nm [B]. Bacteria were sonicated in PBS for 10 min and viable bacteria enumerated using Miles and Misra plate counting method on CBA and FAA plates supplemented with 5% horse blood [C]. All samples were assayed in triplicate, on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test. Data represents mean ± SD (*$p<0.05$).

3.3.5 CHD-FA treatment affects biofilm viability and biomass

Due to its effectiveness on previous multispecies biofilm models, the anti-microbial activity of CHD-FA was measured by testing on FCGS multi-species biofilms as described previously (section 3.3.4). Mature biofilms were treated for 5 minutes with CHD-FA before the addition of a neutralising buffer to stop the reaction (Figure 3.5).

Following a 5-minute treatment, CHD-FA at 0.8% was shown to significantly reduce cell viability to less than 5.94% of the untreated control, comparable to the CHX treatment which reduced cell viability to 2.96%. Biofilms treated with a concentration of 0.8% CHD-FA also displayed significantly decreased cell viability compared to 0.5% and 0.25%, with observed viability of 40.82% and 73.64%, respectively. Compared to the untreated control (1.00), biofilm biomass was also shown to significantly decrease to 0.66 ($p<0.05$) and 0.71 ($p<0.05$) following treatment with 0.8% and 0.5% CHD-FA, respectively. Moreover, treatment of biofilms with CHD-FA showed a concentration-dependent decrease in the number of viable aerobic and anaerobic CFUs, however this was not deemed statistically significant compared to the untreated control.

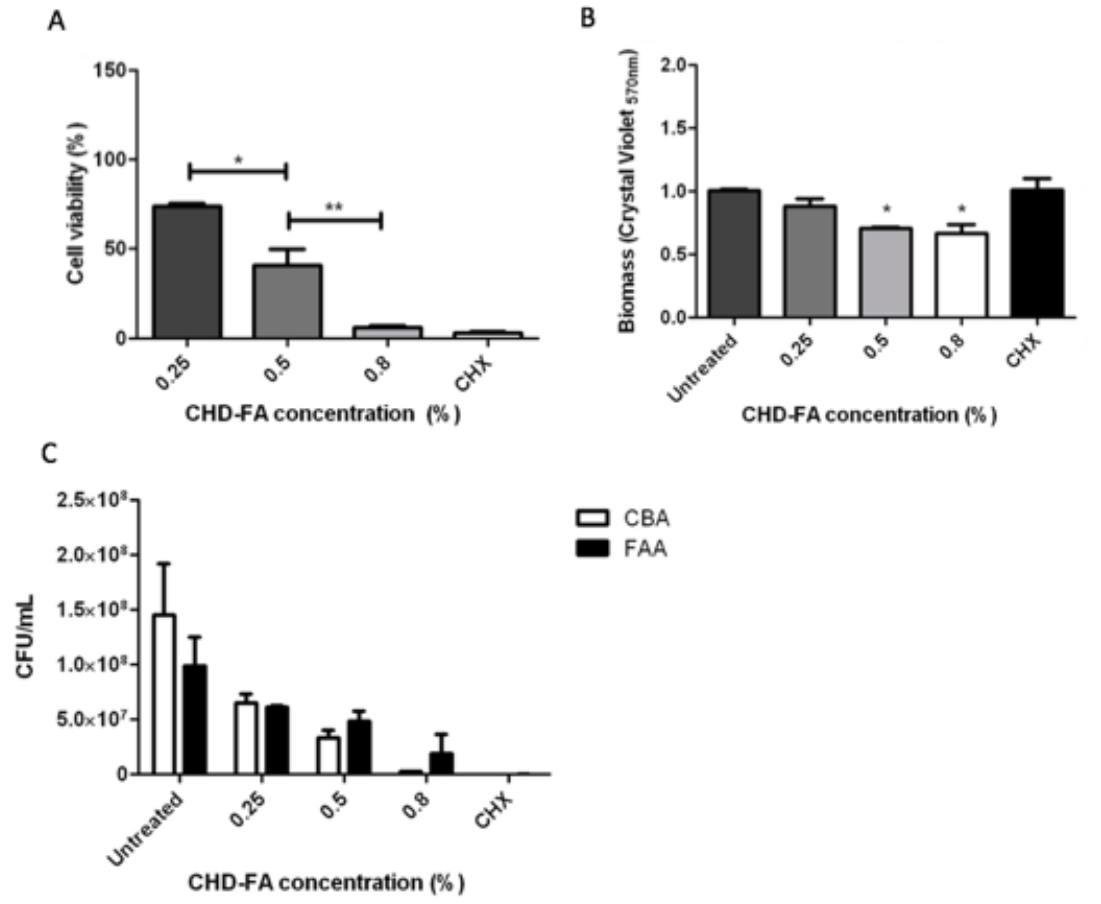

Figure 3.5: Biofilm viability and biomass following CHD-FA treatment

Multi-species biofilms containing FCGS-associated bacteria were grown on Thermanox™ coverslips and treated with 0.25% (v/v), 0.5% (v/v) and 0.8% (v/v) CHD-FA for 5 min. Biofilms were also treated with 0.2% (v/v) CHX used as a positive control. Metabolic activity was measured using alamarBlue® and data presented as a percentage of the untreated control [A]. Biofilms were retained following treatment and biofilm biomass determined by staining with 0.05% (w/v) crystal violet solution and quantified spectrophotometrically by reading at 570 nm [B]. Bacteria were sonicated in PBS for 10 min and viable bacteria enumerated using Miles and Misra plate counting method on CBA and FAA plates supplemented with 5% horse blood [C]. All samples were assayed in triplicate, on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test. Data represents mean ± SD (*$p<0.05$, **$p<0.001$).

3.3.6 Xylitol treatment does not affect FCGS biofilm viability or biomass

Furthermore, using the multi-species biofilm model, the activity of xylitol was investigated and biofilm viability, biomass and CFU counts were measured following a 5-minute treatment (Figure 3.6).

No significant difference in biofilm cell viability was discovered between biofilms treated with xylitol at various concentrations (0.25-5%), with viability found to be over 90% of the untreated control at all concentrations tested. Similarly, biofilm biomass did not significantly change following treatment with a range of xylitol concentrations compared to the untreated control. The number of CFUs counted were shown to be decreased when plated on CBA compared to FAA, indicating that xylitol may have slightly higher toxicity to aerobic bacteria in the biofilm, however this was not statistically significant.

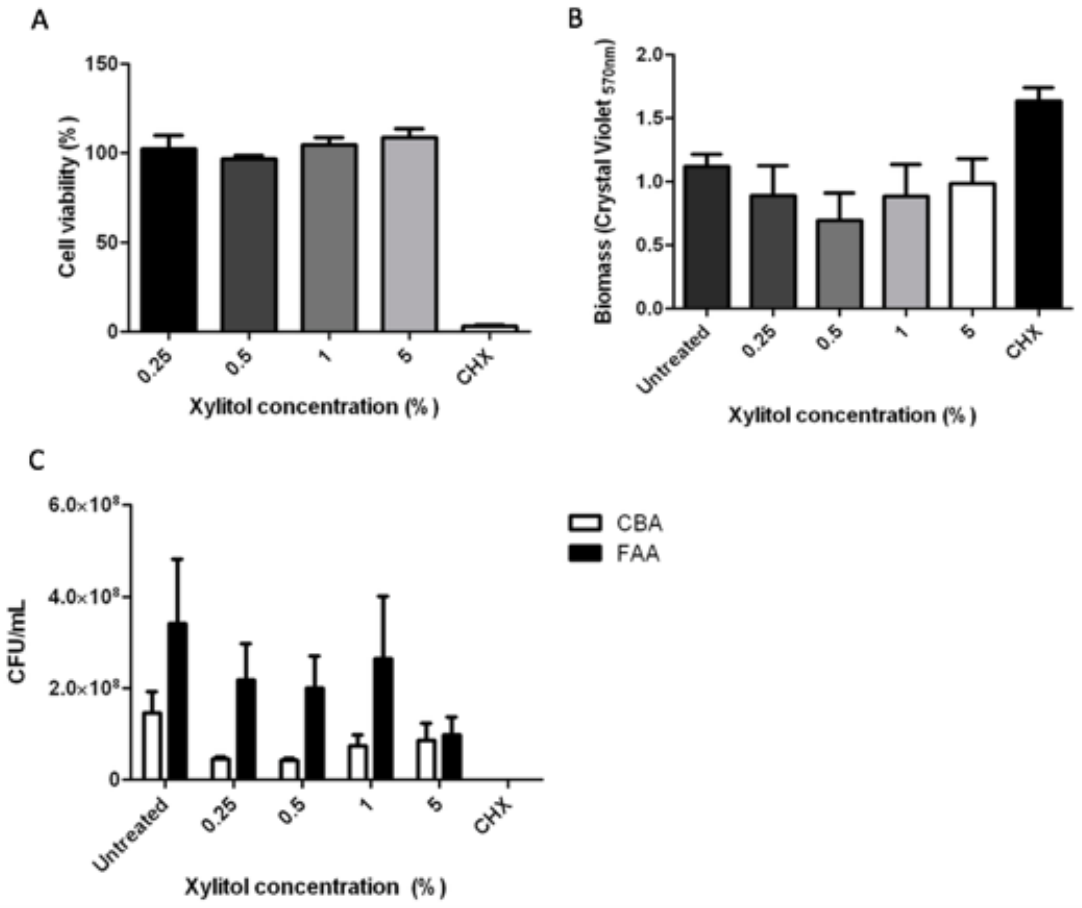

Figure 3.6: Biofilm viability and biomass following xylitol treatment

Multi-species biofilms containing FCGS-associated bacteria were grown on Thermanox™ coverslips and treated with 0.25% (w/v), 0.5% (w/v), 1% (w/v) and 5% (w/v) xylitol for 5 min. Biofilms were also treated with 0.2% (v/v) CHX used as a positive control. Metabolic activity was measured using alamarBlue® and data presented as a percentage of the untreated control [A]. Biofilms were retained following treatment and biofilm biomass determined by staining with 0.05% (w/v) crystal violet solution and quantified spectrophotometrically by reading at 570 nm [B]. Bacteria were sonicated in PBS for 10 min and viable bacteria enumerated using Miles and Misra plate counting method on CBA and FAA plates supplemented with 5% horse blood [C]. All samples were assayed in triplicate, on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test.

3.3.7 Ubiquinol treatment does not affect FCGS biofilm viability or biomass

The anti-microbial potential of ubiquinol, an active component of Oralmat® anti-inflammatory remedy, was investigated on the FCGS multi-species biofilm (Figure 3.7). Following a 5-minute treatment with ubiquinol, biofilms showed no significant differences in cell viability or between various concentrations tested. There was also no significant difference in the level of biofilm biomass of biofilms treated at each concentration of ubiquinol compared to the untreated control. There was a notable decrease in the number of live aerobic cells within biofilms treated with 50 and 200 µg/mL of ubiquinol compared to the untreated control, however this decrease was not found to be significant.

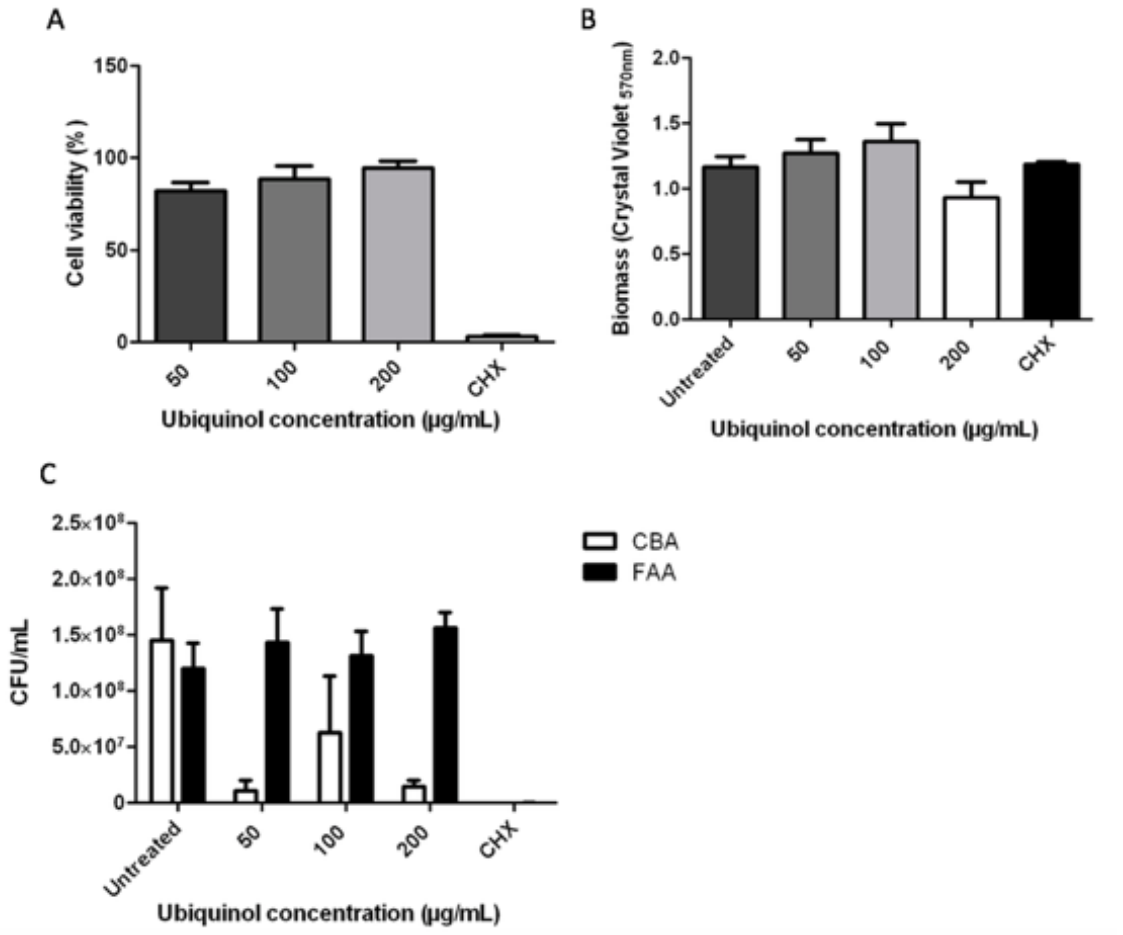

Figure 3.7: Biofilm viability and biomass following ubiquinol treatment

Multi-species biofilms containing FCGS-associated bacteria were grown on Thermanox™ coverslips and treated with 50, 100 and 200 µg/mL ubiquinol for 5 min. Biofilms were also treated with 0.2% (v/v) CHX used as a positive control. Metabolic activity was measured using alamarBlue® and data presented as a percentage of the untreated control [A]. Biofilms were retained following treatment and biofilm biomass determined by staining with 0.05% (w/v) crystal violet solution and quantified spectrophotometrically by reading at 570 nm [B]. Bacteria were sonicated in PBS for 10 min and viable bacteria enumerated using Miles and Misra plate counting method on CBA and FAA plates supplemented with 5% horse blood [C]. All samples were assayed in triplicate, on three separate occasions. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post-test.

3.4 Discussion

Within the oral cavity, bacteria are known to reside within multi-species biofilms, where changes in the composition of biofilm bacterial communities may disrupt host-microbial symbiosis and lead to the association of specific biofilms with health or disease. It is possible that cats affected with FCGS have an intolerance to even small quantities of bacterial plaque on the tooth or tissue surface, and while some cats respond well to improved oral hygiene alone, others will respond poorly to all treatments. Extraction of teeth is the standard treatment option for FCGS, of which 87% of cats show signs of improvement while 13% do not respond and may be considered refractory cases (Hennet, 1997; Girard and Hennet, 2005). Many drug-therapies, including anti-inflammatories, have been advocated for FCGS with limited efficacy, highlighting the urgency for novel treatment options. Previous research into human oral disease has led to the development of several multi-species biofilm models such as for gingivitis and periodontitis, which has allowed the study of specific bacterial interactions which mimic a disease-associated environment (Millhouse et al., 2014; Park et al., 2014). However, as feline oral disease is less-well studied, there is not currently a comparative model to study bacterial roles in feline oral disease. In this chapter, a multi-species biofilm containing bacteria associated with disease during FCGS was developed to evaluate the ability of novel actives to disrupt biofilms.

All bacterial species considered as putative pathogens of FCGS (*P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *T. forsythia*, and *P. circumdentaria*) (Dolieslager et al., 2011) were shown to display sialidase activity in this study. This was consistent with previous studies which have shown *P. multocida* species to produce medium-strong sialidase activity, and shown *T. forsythia* to display sialidase activity allowing it to utilise sialoglycoproteins for biofilm growth (Müller and Mannheim, 1995; Roy et al., 2011). *P. circumdentaria* has also shown to display levels of sialidase activity (Assis et al., 2013). Moreover, previous studies have shown a novel plant-derived compound, berberine, to cause inhibitory effects on both viral and bacterial sialidases making this a compound of interest in this study (Wu et al., 2011; Kim et al., 2014). When treated with berberine, sialidase activity of all bacteria was shown to significantly decrease when increasing concentrations of up to 20 mM were used, concurrent with previous research. The compound DANA has previously shown to be an effective

viral sialidase inhibitor, with less research reporting its potential as a bacterial sialidase inhibitor. In this study, an 80% decrease in the fluorescence of *P. multocida* subsp. *septica* was shown following treatment with DANA, as well as concentration-dependent reductions in fluorescence for *P. multocida* subsp. *multocida* and *T. forsythia*. This suggests that DANA may be a suitable sialidase inhibitor for some FCGS-associated bacteria.

A comparative assessment of anti-microbial compounds: berberine, CHD-FA, xylitol and ubiquinol against a 10-species biofilm containing FCGS-associated bacteria was performed. Berberine was found to reduce the biomass of the biofilm in a concentration-dependent manner up to 200 µg/mL, correlating to a previous study which found berberine to have anti-biofilm potential against *Pseudomonas* and *Salmonella* species (Aswathanarayan and Vittal, 2018). The reduction in biofilm biomass with berberine treatment may support its potential to inhibit bacterial sialidase activity, as shown in the current study. Furthermore, one study by Sherry et al. (2013) observed a killing and disruptive effect of CHD-FA on multi-species periodontal biofilms when treated with 0.5% (v/v) for 24 h. In this study, results corroborated with previous research, with the greatest reduction in biofilm cell viability shown when biofilms were treated with CHD-FA at 0.8% (v/v); an antimicrobial effect comparative to that caused by the positive CHX control. Biofilm biomass was also decreased by CHD-FA in a concentration-dependent manner. Xylitol did not significantly affect cell viability or biomass when treated at each concentration. A previous study has shown xylitol to inhibit the formation of an oral multi-species biofilm containing species such as *Streptococcus* and *Porphyromonas* (Badet et al., 2008). However, the research by Badet et al. (2008) did not investigate the ability of xylitol to decrease the viability and biomass of a mature biofilm, as carried out in this study, which may explain the differences observed. Similarly, treatment with ubiquinol did not cause any significant changes in the viability or biomass of the biofilm at any concentration. Thus far, there is limited research on the effect of ubiquinol on bacterial biofilms *in vitro*, however an *in vivo* study by Sugawara (2011) found that patients with periodontal disease treated with ubiquinol for 2 months showed a significant reduction in bacterial plaque adhesion. Biofilms treated with xylitol and ubiquinol, however, did show to have a notable increase in the recovery of anaerobic bacteria (FAA) compared to aerobic bacteria (CBA). This may suggest

that aerobic bacteria were more susceptible to these treatments or may have been outcompeted by anaerobes for nutrients. Quantifying the changes in the proportion of each bacterial species within the biofilm following treatment would be highly advantageous with further use of this model to allow susceptible and resistant species to be identified.

In summary, the FCGS multi-species model presented in this study has displayed ability to test anti-microbial compounds and, with further quantification, could be valuable in more widespread testing of potential active compounds.

4 General Discussion

4.1 Introduction

FCGS is a complex disease, with excessive oral inflammation believed to be driven by a multitude of interactions between various external stimuli and host immune cells in the oral cavity. The series of observations herein addresses how the presence of specific bacteria within the oral cavity may influence the host response. This was demonstrated by the use of an *in vitro* model system to measure host cell inflammatory changes upon challenge with bacteria associated with FCGS, and this system successfully displayed clear differences in the host response to each bacterial species. Furthermore, a biofilm model that represents FCGS-associated plaque was developed to investigate novel treatment options for this. This model system has demonstrated the efficacy of biologically active molecules on FCGS-associated biofilms, and the utility of this model could allow for future investigations on bacterial-host interactions including use in a co-culture model with appropriate host cells.

4.2 Conclusions and future work

The major aims of this study were to investigate the inflammatory effects of bacteria associated with FCGS on host cells and examine the ability of novel compounds to disrupt FCGS-associated bacteria within a multi-species biofilm model. Through the development of an *in vitro* assay, the TLR activation and pro-inflammatory IL-8 gene and protein expression of host cells could be measured following exposure to varying multiplicities of infection of FCGS bacteria. IL-8 was appropriate in this study as it is used as a biomarker for inflammation in many clinical conditions (Shahzad et al., 2010). With suspected pathogens of FCGS showing to elicit the activation of TLRs in this study, it was suggested that these bacteria are capable of triggering the metabolic pathologies of the immune response. Furthermore, it was shown that human THP1-XBlue™ cells had the greatest increase in both IL-8 gene and protein expression when stimulated by *T. forsythia* and *P. gingivalis* at an MOI of 200, which could be expected as these bacteria are routinely identified in subgingival plaque in humans with chronic oral disease and contribute to disease pathogenesis (Socransky et al., 1998). The release of IL-8 in cell culture supernatants further suggests that these bacteria may be involved in the initiation of the NF-κB pathway leading to a cascade of

chemokines and cytokines, and subsequent inflammation (Bennett et al., 2012). However, further research is required to prove whether the initiation of an IL-8 response in cells is the direct result of TLR stimulation. Using human cells provided an effective comparator to the feline SCCF1 cell line as bacterial involvement in human oral disease is more well understood. SCCF1 cells were used in this study to make the results more relevant to FCGS.

The feline SCCF1 cells showed a significantly increased IL-8 mRNA and protein expression following exposure to the bacteria suspected to be involved in FCGS (*T. forsythia*, *P. circumdentaria* and *P. multocida* species), which supports the idea that these bacteria have some involvement in the induction of a chronic inflammatory response during FCGS. However, SCCF1 cells also presented a similar pattern of gene expression and protein release in the presence of *B. zoohelcum*. As *B. zoohelcum* is regarded as a commensal organism in the feline oral cavity, an elevation of IL-8 levels was not expected and so the results using this cell line do not support *B. zoohelcum* as a commensal. Previous research has also suggested that *B. zoohelcum* could cause harm to cats when it was isolated in pure culture from lung tissues of cats with respiratory disease, indicating at least partial involvement in disease (Decostere et al., 2002). However, it must be considered that commensals work in unison with other species to form the healthy oral microbiota *in vivo*, and this behaviour may not be evident when studying this bacterium in isolation. Therefore, *B. zoohelcum* may play a greater role in the progression of FCGS than initially expected but future work would be necessary to determine the inflammatory potential of *B. zoohelcum* and investigate its role in oral disease in the presence of FCGS recognised pathogens. This *in vitro* assay could be used in further investigations to measure and compare the influence of specific FCGS-associated bacteria on other signature pro-inflammatory cytokines including tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) which have shown to have elevated mRNA expression in tissue biopsies of cats with FCGS compared to a healthy group (Dolieslager et al., 2013). It would also be of interest to determine the impact on the host immune response if host cells were stimulated with these bacteria in combination, or within a biofilm. Furthermore, additional experiments using the *in vitro* model system to look at innate immune responses from epithelial cells isolated from feline gingival tissue would be useful

to investigate the aetiology and pathogenesis of FCGS, as these cells would most closely model the response of host oral cells *in vivo*.

Many treatments have been advocated for FCGS, with most aiming to reduce the abnormal inflammatory response affecting the oral cavity. While the pathological mechanism of FCGS remains unclear, it is thought that increased plaque accumulation within the oral cavity may be a key predisposing factor of FCGS. Elective tooth extraction, which removes plaque retentive surfaces in the mouth, has become the accepted standard method of managing FCGS in cats and has proven efficacy with up to 80% of cats showing substantial or complete improvement (Hennet, 1997; Girard and Hennet, 2005). Another recommended method of management for FCGS is to implement the improvement of feline oral hygiene throughout all stages of treatment. This involves daily teeth brushing with the use of chlorhexidine toothpaste and oral rinses with the aim to reduce the level of plaque build-up and help minimise opportunistic infection of inflamed oral tissue. However, the majority of cats will not fully respond to this cleaning or extraction alone and extended or continuous medical treatments are required (Jennings et al., 2015). It is apparent that the elimination of bacterial plaques is of importance in the management of FCGS and investigating new treatment options to target these external stimuli could be vital to reduce refractory cases of FCGS.

It has been proposed that sialidase inhibitors could be of pharmacological relevance in the treatment of FCGS. Previous studies have implied the importance of sialidase enzymes of some pathogenic bacteria for biofilm formation and colonisation of mucosal surfaces (Oggioni et al., 2006; Parker et al., 2009). Sialidases likely contribute to virulence through cleavage of sialic acids in mucosal tissues, which bacteria utilise to obtain food and energy during the formation of a dental biofilm. Several pathogens have shown to use sialidases in virulence such as *Streptococcus pneumoniae* and *T. forsythia* (Corfield, 1992). *T. forsythia* has previously shown to use the sialidase enzyme NanH in initial biofilm formation (Roy et al., 2011). The use of sialidase inhibitors for the treatment of FCGS has been suggested due to the ability of these inhibitors to target a common binding site (α-2,6-linked sialic acid) in putative pathogenic bacteria, which could prevent plaque biofilm formation and invasion of bacteria in disease. In this study, it was

shown that all putative pathogens of FCGS (*T. forsythia*, *P. circumdentaria*, *P. multocida* supsp. *multocida*, and *P. multocida* subsp. *septica*) displayed a medium to high level of sialidase activity, suggesting that the sialidase enzyme could contribute to the virulence of these bacteria during disease. Furthermore, with bacteria showing a 70-90% decrease in sialidase activity following berberine treatment and a 60-80% decrease in sialidase activity following DANA treatment, this work provided evidence that these compounds could be considered as alternative drug therapies for the reduction of dental plaque biofilms in FCGS. It would be necessary to investigate the toxicity of the inhibitors at effective concentrations as well as consider associated costs.

A variety of research has shown the development of modelled oral biofilms that recapitulate the microbial environment in oral disease in attempt to understand bacterial interactions during disease and allow development of new chemotherapeutic agents (Periasamy and Kolenbrander, 2009; Sherry et al., 2016). Given that most bacteria exist as multi-species consortia in the oral cavity, creating a multi-species model for testing is advantageous as it allows a controlled and reproducible environment with a more realistic measure of bacterial susceptibility *in vivo* than studies of an individual component species (Tan et al., 2017). Moreover, the importance of biofilm models for the study of antimicrobials has been highlighted when the minimum inhibitory concentration (MIC) of oral bacterial species such as *F. nucleatum* and *P. gingivalis*, following treatment with CHX, was found to be 10,000 times lower for planktonic cells than single species biofilms (Park et al., 2014). In this study, a complex biofilm model comprising bacteria associated to a diseased microbial environment in the feline oral cavity during FCGS was created as a platform for testing the efficacy of novel compounds.

The feline oral cavity has a diverse and unique microbiota, comprising over 400 bacterial species (Adler et al., 2016). A defined group of bacteria were chosen to use in the FCGS biofilm model with properties that increased the chances of a mature biofilm to successfully develop. This included early colonisers (*Streptococcus* species) which initially attach to the tooth surface in early biofilm growth, and *F. nucleatum* which is a known bridging organism that other organisms will bind to promoting maturation of the dental plaque biofilm (Settem et al.,

2012). *F. nucleatum* has also shown to facilitate the survival of obligate anaerobes within a biofilm in aerated environments; a property that would be advantageous for this biofilm model due to the addition of strictly anaerobic FCGS-associated pathogens (*T. forsythia* and *P. circumdentaria*). The bacteria were added in a sequential manner to Thermanox™ coverslips in a biologically relevant media to allow the formation of the biofilm model to mimic plaque development *in vivo*. In this study, biological agents of interest were applied directly to the mature multi-species biofilm and the efficacy determined by measuring biofilm viability and biomass.

Our group has previously shown a natural compound (CHD-FA) to be highly effective against a multi-species periodontal biofilm at a concentration of 0.5% (Sherry et al., 2013). In this study, CHD-FA was the most effective compound tested against the FCGS multispecies biofilm, where 0.8% CHD-FA was able to disrupt cell viability to the same level as CHX, which is currently used as a treatment for FCGS. CHD-FA could potentially be used as an alternative natural agent to CHX to eliminate plaque biofilms in FCGS as it has shown no sign of toxicity in rats and humans and has shown to have anti-inflammatory properties (Gandy et al., 2012; Gandy et al., 2011). Furthermore, in this study, significant disruption of the multispecies biofilm biomass was observed when treated with 0.5% CHD-FA which corroborates with research by Sherry et al. (2013). That study found, through SEM imaging, disaggregation of bacterial biofilms following CHD-FA treatment, indicating possible action against the bacterial cell membrane. Collectively, these properties make CHD-FA a desirable option for the development of a topical agent such as a toothpaste or mouthwash to control microbial dysbiosis in FCGS. However, further studies to assess the effect of CHD-FA on the host and determine its efficacy *in vivo* would be required.

Previous work has found berberine, a naturally occurring chemical compound, to have anti-biofilm properties (Aswathanarayan and Vittal, 2018). In this study, it was also shown that the biomass of the multispecies biofilm was significantly disrupted following treatment with berberine. Compounds with the ability to disrupt biofilm structure are valuable in oral disease to increase the vulnerability of plaque bacteria to antimicrobial agents. Berberine has been used as a therapeutic agent in clinical applications due to its inherent low cytotoxicity

(Chung et al., 1999). Although the mode of action of berberine is not fully understood, this investigation indicated the potential for berberine to block bacterial sialidase activity as a mechanism to disrupt bacterial attachment and biofilm formation. Further investigations to understand the mode of action, and assessment of the cytotoxicity of berberine on feline host cell lines, would be of importance to evaluate the safety of this compound for clinical use.

Despite previous work showing the five-carbon polyol, xylitol, to have anti-biofilm properties and reduce plaque accumulation, it had no significant effect on the multispecies biofilm at the concentrations tested in this study (Badet et al., 2008; Milgrom et al., 2012). Moreover, treatment with ubiquinol did not have any effect on the biofilm viability or biomass. It is possible that the mature multispecies biofilm was too robust for the concentrations of these compounds tested. It could be of value to perform additional experiments to determine the MIC of these compounds on planktonic cells or single species biofilms of the bacteria of interest, which are more susceptible than the multi-species biofilm, to identify if these compounds have any antibacterial potential on FCGS-associated bacteria. It would also benefit to measure the effect of compounds on the biofilm at various timepoints, to determine if there is a time-dependent impact. One limitation of this study was the inability to quantify the live/dead bacteria in the multispecies biofilm before and after treatment using molecular techniques due to time restrictions, which is of importance to provide a reproducible model. With further work to validate this FCGS model, it could be used in future applications with local tissue and immune cells to study how biofilm composition modulates pro-inflammatory cytokine and chemokine gene expression, as well as protein release.

To conclude, the work in this thesis provides valuable information regarding the role of bacterial composition in the modulation of host-pathogen interactions and evaluated the antimicrobial activity of novel compounds for potential use in FCGS treatment. Together, these allow for a greater understanding of the aetiopathogenesis of FCGS and provide a basis for the development of alternative therapies to tackle this complex feline disease.

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