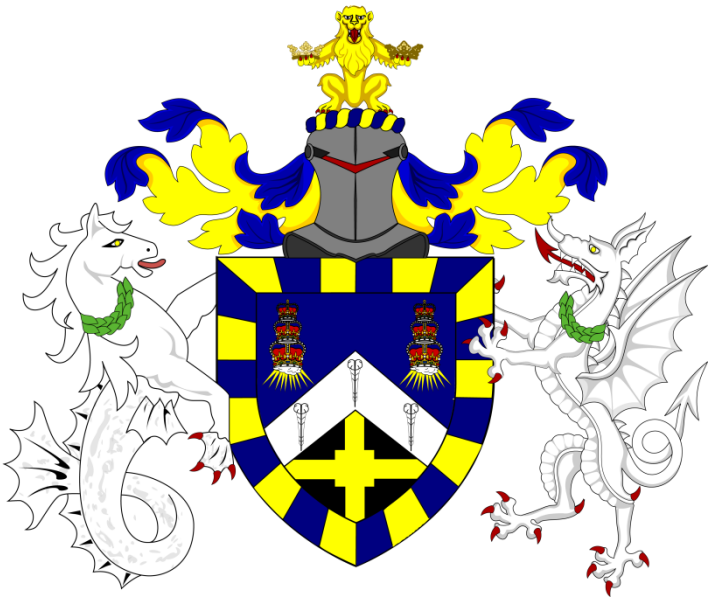
The Relationship between Oral Malodour and Chronic Periodontitis: Role of Volatile Sulfur Compounds



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This thesis is submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

May 2016

We are in much the same position as an observer trying to gain an idea of the life of a household by careful scrutiny of the persons and material arriving or leaving the house; we keep accurate records of the foods and commodities left at the door and patiently examine the contents of the dust-bin and endeavour to deduce from such data the events occurring within the closed doors.

-Marjory Stephenson (*Bacterial Metabolism*, 1930)

DECLARATION

I, Abish Samuel Stephen, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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SUMMARY

Oral malodorous gases such as hydrogen sulfide and methanethiol are positively associated with plaque-induced periodontal diseases. It is well known that these Volatile Sulfur Compounds (VSCs) are produced by the oral microbiota during proteolysis, and the specific association of methanethiol in the breath with periodontal diseases was investigated in a clinical study described in this thesis. The association between breath methanethiol and clinical parameters of disease, abundance and prevalence of putative peridontopathic bacteria in periodontal niches were confirmed. A gas chromatographic method was developed to measure subgingival VSCs and associations with clinical parameters, microbial abundance in a range of oral niches and inflammatory markers in saliva and gingival crevicular fluid (GCF) were found. Breath methanethiol was also found to be more closely associated with a range of disease associated inflammatory markers in GCF. Tongue biofilm, subgingival and interdental plaque samples were analysed by Human Oral Microbiome Identification by Next Generation Sequencing (HOMINGS) methodology. Microbial diversity in the tongue was positively associated with breath VSCs in health, but subgingival and interdental niche diversity was more strongly associated with breath VSCs in gingivitis and chronic periodontitis. Tongue ecology in the disease associated cohorts was markedly different compared to health, especially with the increase in abundance and prevalence of putative VSC producing species. The ecology of VSC producing bacteria is described for the niches studied, finding that the methanethiol producing species are almost exclusively periodontopathic, with more dynamic population differences in these group of organisms observed from health to disease. The VSC producing potential in the periodontal niches was also found to be more dynamic compared to the tongue. The role of methanethiol production by the keystone species, *P. gingivalis* was investigated using a 10-species biofilm co-culture model by substituting a methionine gamma lyase (*mgl*) deficient strain for the wild type and marked changes in the overall biofilm composition was observed in terms of community evenness and biomass. The wild type biofilm displayed a more insidious phenotype whereas the higher biomass mutant biofilm exhibited an overtly pathogenic phenotype. *P. gingivalis mgl* is proposed as a potential keystone virulence factor.

ACKNOWLEDGEMENTS

I'm so glad to have seen the other end of this project and I count myself as lucky to have been part of it. Firstly, I would like to thank my academic supervisor Prof. Robert Allaker. Thanks for the freedom, encouragement and thanks for looking out for me. Without your support and assurance it would have been an impossibly uphill task given that I came fresh to this subject and didn't know a whole lot about a lot of the things I got involved with, in this project. I would say your professional and personal guidance throughout the duration of the project has made me all round, a better person and scientist. I would also like to thank two great people who have been a source of wisdom and inspiration throughout this project. Dr. David Bradshaw and Dr. Gary Burnett: you guys have been great sport during the project meetings, and I learned a lot from you two, especially the confidence to do things I could only dream of attempting. Thanks for that impetus.

I would like to thank my colleagues at QMUL: Cecilia for helping me get up to speed on a lot of things at the start of the project, Joe for being such a star in every way, Kaveh for being a good friend and looking out for me, and of course Dr. David Gillam for being an absolute rock from the clinical side. I would like to thank the very special dentists I worked with who helped collect the clinical samples, Narinder, Vam and Bryar, your help is much appreciated. I would also like to thank all the folk at CDOS who I don't have the space to name individually for making the whole experience of this project memorable.

I had a lot of great experiences during this project, and one was driving from Glasgow to Inverness in my 944 via the stunning A82 and I would like to thank Prof. Gordon Ramage for helping to make that happen. I will never forget that journey. Thanks also to the crew at Glasgow Dental School for being so warm and accommodating—Leighann, Emma, Chris, David and *namma ooru* Ranjith.

A special mention to GSK who have made a positive impact in the project development by helping establish the collaboration with Glasgow and also in doing some extra sample analyses that have inspired me and helped me to gain a real appreciation of my subject.

I would like to thank my parents, sister, uncle and aunt for accommodating me during my studies and for being sources of great strength during tough times. Finally, thanks to my loving wife Chrisma, for putting up with me constantly writing and completely ignoring everything else and for looking out for my general wellbeing.

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1 INTRODUCTION

The mouth is one of the most important parts of the human body that serves as an interface to the external environment, with unique hard and soft tissue surfaces that form numerous ecological niches that support dynamic micro successions of commensal microbial communities that exist in a homeostatic relationship with the immune functions of the body (Zijnge et al. 2010).

The mouth is used for mastication and consumption of food, which makes for a relatively unstable environment for microbial colonisation in terms of nutrients. In addition, there is a tendency for introduction of different and 'unfamiliar' microbes into the oral environment. This state of flux in the oral environment and the microbiome leads to the possibility of a diverse array of microbe-associated oral pathologies that can have a lasting adverse effect on the quality of life of individuals (Sbordone & Bortolaia 2003).

1.1 Etiopathogenesis of Periodontal diseases

Periodontal diseases form a group of pathologies of the periodontium with a host of different etiologies (Highfield 2009). The most prevalent of periodontal diseases are gingivitis and chronic periodontitis caused by the host response to bacterial plaque accumulation in the gingiva, resulting in terminal attachment loss of the periodontal ligaments and alveolar bone loss, leading to loss of teeth in chronic periodontitis (Table 1-1). Gingivitis is a milder, reversible form of periodontitis exhibited in the early stages of the disease. The reported prevalence of chronic periodontitis in the developed countries such as UK and USA ranges from 5% of the total population to 35%. Existing

heterogeneity in the diagnostic criteria used by epidemiological studies in the literature prevent an accurate estimate of this disease, however socio-economic factors appear to be a major influence in the prevalence of chronic periodontitis, clearly shown by the WHO Disability Adjusted Life Years statistic for this disease worldwide (Marcenes et al. 2013).

The pathogenetic changes in the periodontium leading up to chronic periodontitis can be classified into four main histological phases (Ohlrich et al. 2009):

- Initial lesion
- Early lesion
- Established lesion
- Advanced lesion

1.1.1 Initial lesion

It is generally agreed that the initial lesion corresponds to clinically healthy gingiva, as the presence of bacterial plaque biofilm subgingivally necessitates the normal host response mechanisms to be activated. This low-level of chronic inflammation in the initial lesion is characterised by increased vasodilation and permeability, enabling leukocytes such as neutrophils and monocytes to migrate into the gingival sulcus via the gingival connective tissue and the junctional epithelium. As a result of increased leakage in the gingival microvasculature, a fluid with characteristics similar to that of serum is observed to flow into the gingival sulci. This fluid, called the Gingival Crevicular Fluid (GCF), has the effect of diluting bacterial products and having a flushing effect on the subgingival biofilm. The initial lesion is said to develop within 2-4 days of plaque accumulation.

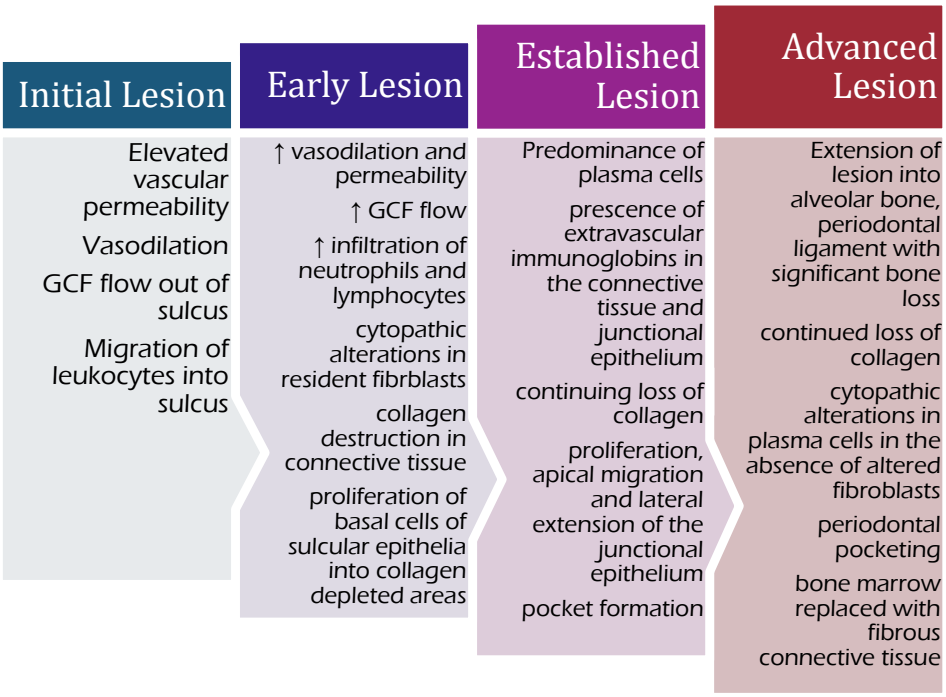


Table 1-1 Histopathological stages of periodontal disease progression

1.1.2 Early Lesion

The early lesion describes the changes in the gingiva that occur after about a week of plaque accumulation and is primarily characterised by erythematous and oedematous gingiva due to increased vascular permeability and the accompanied GCF flow. An enhanced activity of the immune cells including the presence of a large number of lymphocytes and neutrophil infiltration into the gingival sulci is also observed. This coincides with a considerable depletion of collagen in the connective tissue, due to its breakdown by the inflammatory activity of the host immune cells and the products of the proliferating bacterial biofilm.

Fibroblasts appear altered, exhibiting electron-lucent nuclei, swollen mitochondria, vacuolization of the rough endoplasmic reticulum and rupture of their cell membranes, appearing up to three times the size of normal fibroblasts and found in association with moderately-sized lymphocytes.

1.1.3 Established Lesion

Termed ‘clinical gingivitis’, the established lesion displays an increase in the intensity of histopathological symptoms associated with the early lesion with an increased involvement of the humoral and cell-mediated immune response. Progression to the

established lesion is dependent on plaque build up and systemic or local host susceptibility factors.

Similar to the initial and early lesions, the established lesion features an inflammatory reaction confined to the area near the base of the gingival sulcus, but unlike prior stages, displays plasma cells clustered around blood vessels and between collagen fibres outside the immediate area of the reaction site. While most of the plasma cells produce IgG, a significant number do produce IgA (and rarely, some produce IgM). The presence of complement and antigen-antibody complexes is evident throughout the connective and epithelial tissue.

In health, the junctional epithelium creates the most coronal attachment of the gum tissue to the tooth at or near the cementoenamel junction. In the established lesion of periodontal disease, the connective tissue lying subjacent to the junctional epithelium is disintegrated, failing to properly support the epithelium against the tooth surface. In response to this, the junctional epithelium proliferates and grows into the vacant underlying spaces, effectively causing the level of its attachment to migrate towards apically, revealing more tooth structure than is normally evident supragingivally (above the level of the gumline) in health.

While many established lesions continue to the advanced lesion (below), most either remain as established lesions for decades or indefinitely; the mechanisms behind this phenomenon are not well understood.

1.1.4 Advanced Lesion

Many of the features of the advanced lesion are described clinically rather than histologically:

- Periodontal pocket formation
- Gingival ulceration and suppuration
- Destruction of the alveolar bone and periodontal ligament
- Tooth mobility, drifting and eventual loss

Because bone loss makes its first appearance in the advanced lesion, it is equated with periodontitis, while the preceding two lesions are classified as gingivitis in levels of increasing severity.

The advanced lesion is no longer localized to the area around the gingival sulcus but spreads apically as well as laterally around a tooth and perhaps even deep into the gum tissue papilla. There is a dense infiltrate of plasma cells, other lymphocytes and macrophages. The cluster of perivascular plasma cells still appears from the established lesion. Bone is resorbed, producing scarring and fibrous change.

1.2 Microbial role in Periodontitis

Historically, periodontitis has been thought of as an invasive disease, with the bacterial biofilm playing a major role in the pathogenesis (Schneider 1965). This has been due to the observation that effective plaque control appeared to reverse clinical gingivitis and that professional cleaning of the tooth slowed (and in some cases) halted the progression of destructive periodontal disease (Socransky 1977; Ramfjord et al. 1968). It was generally accepted that in health, Gram positive bacteria predominate in the subgingival plaque, whereas, Gram-negative bacteria begin to predominate in diseased states as the lesion progresses through gingivitis to periodontitis (Baker et al. 1976; Löe 1981). Efforts to find specific bacterial species responsible for causing periodontitis according to Koch's postulates led researchers to propose the concept of 'bacterial complexes' of two or more interacting species of bacteria that occur in disease states and indeed are responsible for the pathogenetic changes observed in periodontitis and the preceding lesions (Socransky et al. 1998; Socransky et al. 1988). Among the bacteria involved reported to be associated with periodontal disease, the species grouped together as the 'red complex', namely *Porphyromonas gingivalis, Tanerella forsythia* and *Treponema denticola* were reported to be strongly associated with increasing pocket depth and bleeding on probing (Figure 1-1). The bacterial species grouped under 'orange complex' namely *Fusobacterium nucleatum* and *Prevotella intermedia* also show a strong association with clinical measurement indices of periodontal disease such as pocket depth and bleeding on probing.

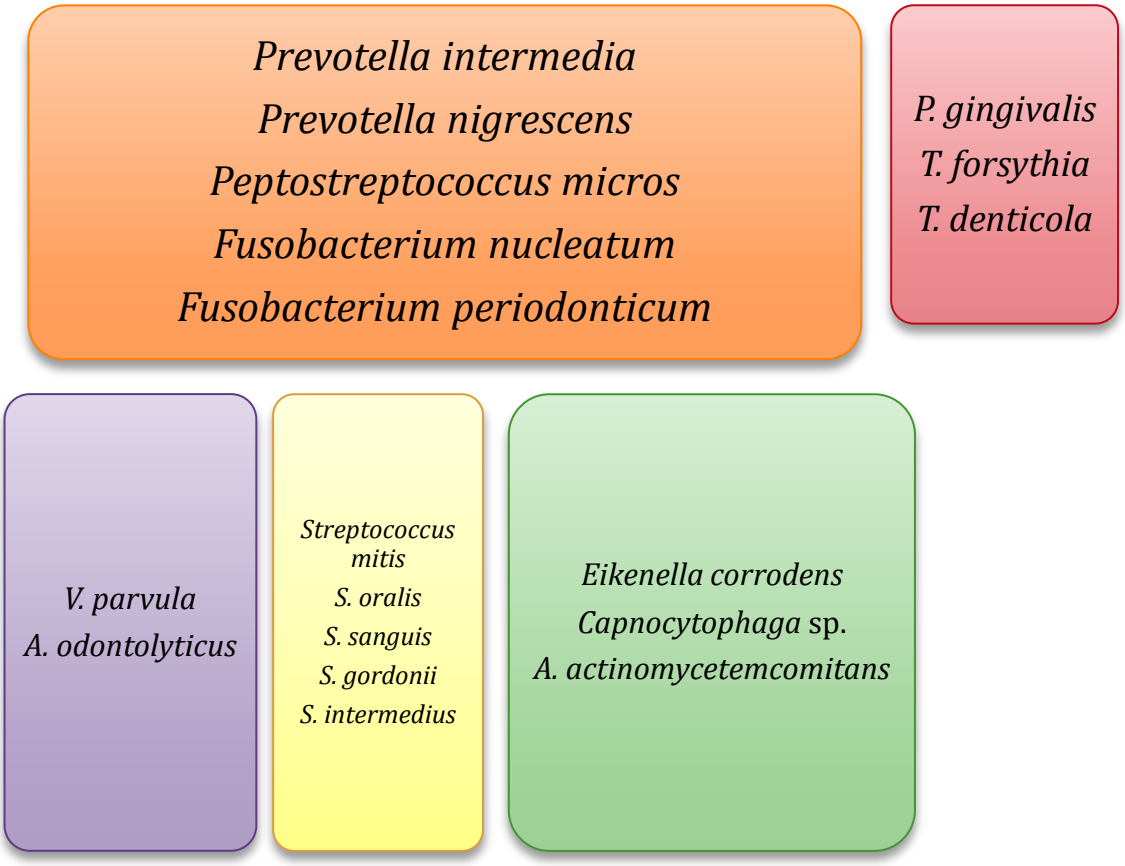


Figure 1-1 Socransky's Bacterial Complexes adapted from (Socransky et al. 1998)

1.2.1 Microbial models of plaque-induced chronic periodontitis

Recent technological advances bringing molecular biological techniques to study the bacterial microbiota in health and periodontal disease has made it increasingly clear that there is no uniformity in the subgingival bacterial species profile of patients with periodontitis (Paster et al. 2001). While presence of the classic 'periodontopathogens' in the subgingival plaque, do not necessarily entail a predictable pattern of breakdown of the periodontal tissues due to the episodic nature of the disease, microbial involvement in chronic periodontitis is essential but not sufficient for pathogenesis (Colombo et al. 2009).

1.2.1.1 Ecological plaque hypothesis

The Ecological Plaque Hypothesis was proposed previously, taking into account emerging evidence from classical microbiology and molecular biological approaches used to study the microbiota in periodontal health and disease (Marsh 1994). This hypothesis unified elements from previous schools of thought that held either that specific bacterial species or the bacterial plaque in general to be responsible for causing

periodontitis, in addition to bringing the concepts of ecological succession and Natural Selection to explain the pathogenetic phenomena associated with periodontal disease from a microbiological perspective. This hypothesis recognises the environmental changes that favour 'pathogenic' bacteria to proliferate and this in turn changing the environment to suit bacteria with 'pathogenic' traits to proliferate in ecological competition, and therefore moving away from a specific bacterial hypothesis, into genetic traits inherent in the normal microbiota that enable survival and in turn act as virulence factors in the advanced stages of disease, fuelling chronic inflammation (Figure 1-2)

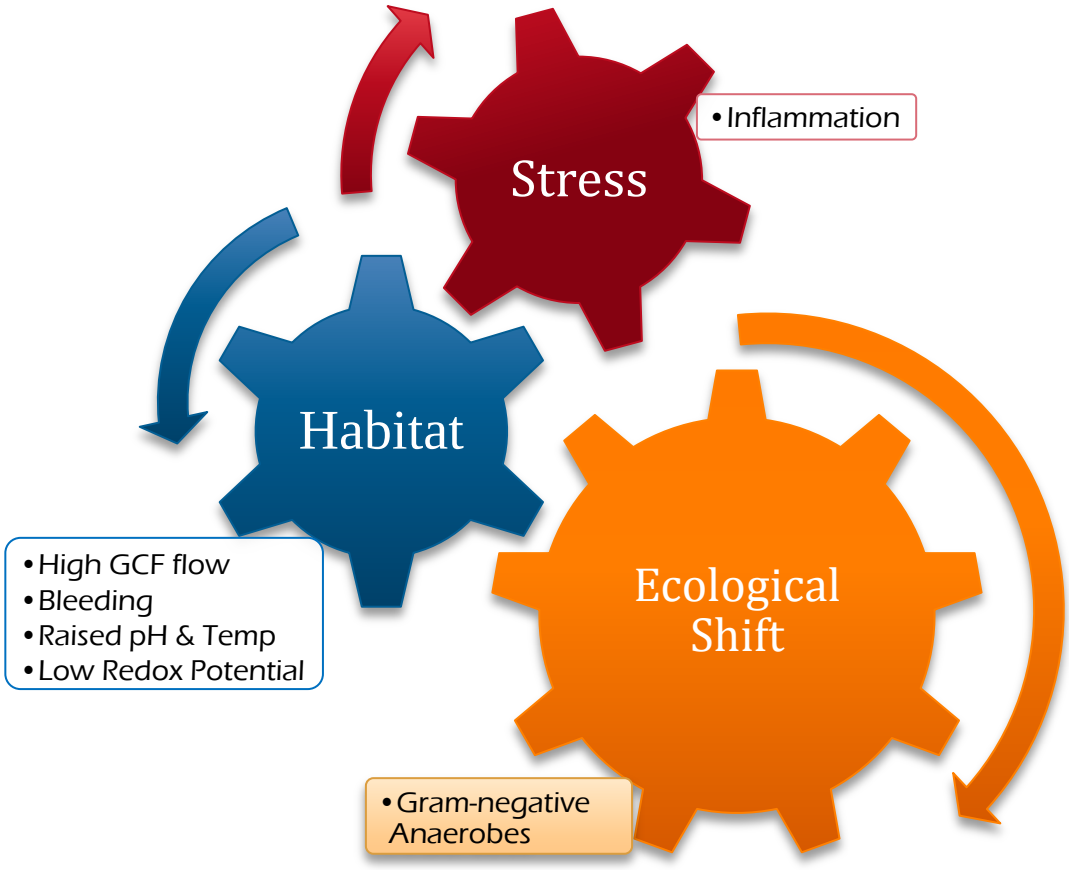


Figure 1-2 The Ecological Plaque Hypothesis adapted from (Marsh 1994)

1.2.1.2 The Polymicrobial Synergy and Dysbiosis model

Although previous hypotheses endeavoured to develop a framework to understand the role of the microbiome in periodontal disease, a new hypothesis has been proposed recently that takes into account a key aspect of periodontal disease progression namely the host-microbiome interaction. This hypothesis named *The Polymicrobial Synergy and Dysbiosis* model was proposed to take into account the effects observed of specific bacterial species though in low abundance appearing to exert control over the overall biofilm composition by manipulating the host response to the developing biofilm and thereby creating a more favourable environment for the survival of the microbial species (Hajishengallis & Lamont 2012). These specific low abundance microbes are termed *keystone species*, as unlike other species that may begin to exert a change in the biofilm community structure simply due to dominance in occupying a large percentage of the total plaque biomass, the keystone species tend to exhibit a similar or greater level of control whilst occupying a small fraction of the total biomass. Another aspect of this hypothesis is that, these 'keystone' species can exhibit overtly pathogenic phenotypes that ensure continual supply of nutrients from the host, whilst not proliferating enough to exceed a particular biomass threshold that may lead to wholesale activation of the host immune system resulting in clearance of the keystone species—this is often termed a subversive phenotype in this model (Hajishengallis 2015).

The advent of Next Generation Sequencing, while increasing the understanding of the known microbial diversity in the oral cavity, has also largely confirmed the more historical associations with the putative periodontopathogens, in addition to finding a number of novel disease associated phylotypes and species (Perez-Chaparro et al. 2014; Wade 2013). To date, only *P. gingivalis* has been proposed as a keystone species with the most extensive evidence available (Table 1-2), while other species such as *A. actinomycetemcomitans* and the novel periodontopathogen *Filifactor alocis* are potential keystone species candidates (Aruni et al. 2014; Fine et al. 2013). Other species such as *T. forsythia, T. denticola* and *F. nucleatum* are also thought to be involved in the ecological changes associated with periodontal disease, with some of the known mechanisms of virulence listed in Table 1-2.

Virulence mechanisms	Review/References
P. gingivalis Suppression of CXCL8 mediated innate immune reponses by dephosphorylation of NFκB via serine phosphatase (SerB) secretion; activation of anti-apoptotic pathways after cell invasion; inhibition of E-selectin secretion; stimulate inflammatory response by C5aR/TLR2 crosstalk; arrest complement cascade by gingipain mediated proteolysis of C3; multimodal signalling by LPS to disable TLR4 mediated responses.	(Hajishengallis & Lamont 2012)
T. forsythia Stimulation of CXCL8 expression by a cysteine protease (PrtH); stimulation of TNF-α secretion by macrophages and inhibition of complement cascade by a matrix metalloprotease like enzyme, karilysin; stimulation of fibrinolysis via a plasminogen activator enolase.	(Sharma 2010; Lee et al. 2015; Ksiazek et al. 2015)
T. denticola Formation of sulfhaemoglobin and hemolysis by the H_2S producing cystalsin; degradation of IL6, TNF-α, IL1β, CCL2 and CXCL8 by cell surface bound protease, dentilisin.	(Dashper et al. 2011)
F. nucleatum Neutrophil and T-cell apoptosis via adhesin mediated aggregation; interferon induction via activation of retinoic acid inducible gene I by NFκB.	(Han 2015; Huynh et al. 2011; Lee & Tan 2014)
A. actinomycetemcomitans Macrophage apoptosis mediated by the leukotoxin (LtxA) resulting in release of considerable IL1β stimulating osteoclastogenesis.	(Åberg et al. 2014)

Table 1-2 listing a few of the known virulence mechanisms of bacteria involved in periodontal disease progression.

1.3 Role of the host immune response in periodontitis

From the findings of numerous studies on the interaction between the oral microbiota and the host immune system in the gingiva, it is clear that the host immune system plays a critical role in maintaining homeostasis and in periodontal disease progression (Garlet 2010). At a basic level, the imbalanced alveolar bone resorption that signifies the advanced stage of disease is mostly host mediated and the microbial role is realised in encouraging the dysregulated immune processes that lead to this stage (Table 1-2; Figure 1-3).

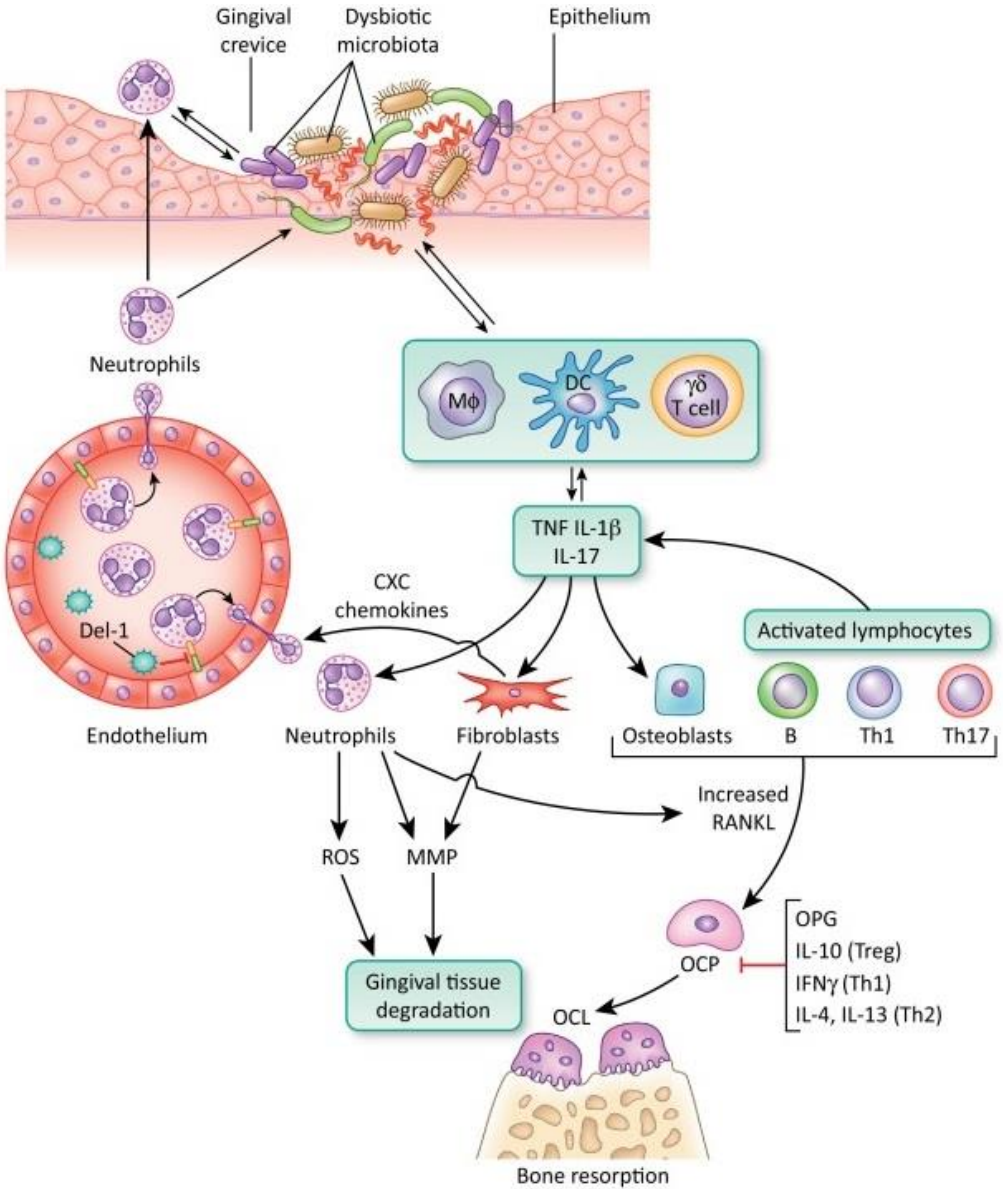


Figure 1-3 a diagram of the mechanisms involved in plaque induced periodontal disease that lead to alveolar bone resorption. Reproduced from (Hajishengallis 2014)

It is generally accepted that the most acute or the most specific response to microbial plaque accumulation is not necessarily better suited for maintenance of gingival health than a well regulated and appropriate immune response that minimises self-damaging processes and effectively addresses the microbial challenge. The human oral epithelial cells constitutively express a number of cytokines and chemokines, with a local gradient of the neutrophil chemokine CXCL8 (aka IL-8) existing in the normal gingiva helping to recruit neutrophils in response to microbial challenge (Darveau 2010). In addition, low numbers of monocytes transmigrate into tissues mediated by intercellular adhesion molecule-1 (ICAM1) expressed by endothelial cells and are resident normally in the gingiva, with constant replenishment of their numbers (Lawson & Wolf 2009).

Microbial biofilm formation in the gingival crevice is recognised by the receptors of the innate immune system such as mannose-binding lectins (MBL), Complement and toll-like receptors (TLR) that recruit cell mediated immune processes. While Complement and MBLs are soluble proteins present in tissue fluids, TLRs are cell surface proteins that are expressed on neutrophils, macrophages, monocytes and B-cells, and these receptors recognise specific ligands that are common to all bacteria and viruses such as gram negative bacterial cell walls containing lipopolysaccharides, peptidoglycans and nucleic acids (Gaffen & Hajishengallis 2008; O'Mahony et al. 2008). Activation of the complement cascade can lead to opsonisation of the bacteria, in addition to some of the cleaved complement proteins acting as chemotactic signals in recruiting monocytes and neutrophils. These cells then recognise gram-positive and gram-negative bacterial cell wall LPS via two important cell surface receptors namely, TLR2 and TLR4 and binding leads to a number of effector functions in these cells, which include secretion of cytokines and chemokines that recruit more numbers of these cells and others such as T-cells and B-cells (Takeuchi et al. 1999). Cytokines and chemokines secreted by activated neutrophils and macrophages include IL1, IL6, TGF-b, TNFa, CXCL8, CXCL10, CCL2, CCL3 and CCL4 which serve as chemotactic signals and result in activation of different types of immune cells and their effector functions (Tecchio et al. 2014; Arango Duque & Descoteaux 2014). Natural killer T-cells are important cells in the innate immune response in detecting and eliminating host cells harbouring invasive organisms, in addition to secretion of cytokines such as interferon-ɣ and TNFα that can influence development of other T-cells toward distinct lineages that are crucial in the type of adaptive response elicited (Vivier et al. 2011). In addition to activated macrophages, dendritic cells are another innate immune cell type that can interact with a

wide array of T-cell subsets by way of antigen presentation and influencing the type and magnitude of the adaptive immune response (Merad et al. 2013). It is notable that many of the bacterial strains termed ‘early colonizers’ and ‘bridging colonizers’ such as *Streptococcus sanguinis, Streptococcus gordonii, Veillonella atypica, Actinomyces naeslundii, Fusobacterium nucleatum, Prevotella nigrescens, Prevotella intermedia* and *Parvimonas micra* that form part of the oral biofilm that adhere to and form primary ecological successions in oral gingival niches do not elicit a strong CXCL8 response from human gingival epithelial cells and display an enhanced susceptibility to neutrophil phagocytosis compared to some of the late colonisers which include the ‘red complex’ bacterial species (Ji et al. 2014). The observed invasive ability of these strains also follows a similar trend, with the early colonizers displaying poor invasiveness compared to bridging and late colonizers (Ji et al. 2007; Dickinson et al. 2011).

1.3.1 The T_H1-T_H2-T_H17 Paradigm

Naïve T-cells homing toward the periodontium are activated by antigen presenting cells (APCs) such as dendritic cells and macrophages, which imparts a number of functional changes in the T-cell, dependent on the co-stimulatory cytokine environment on activation. These activated cells are known as T-helper cells (T_H-cells), and these cells are thought to secrete a number of different cytokine signals that control subsequent immune processes. A number of different T-helper cell phenotypes are currently known such as Th1, Th2, Th9, Th17, Th22 and Treg, with each phenotype secreting specific cytokine markers and displaying a degree of plasticity (Figure 1-4; Geginat et al. 2014). It should be noted that T-cells are observed to be present in histological studies of the healthy gingiva, and it is thought that the regulatory T-cell (T_{reg}) subset likely dominates in health and periodontitis, as this subset is thought to have regulatory control over the developing inflammatory response (Kinane & Lappin 2002; Nakajima et al. 2005; Belkaid 2007).

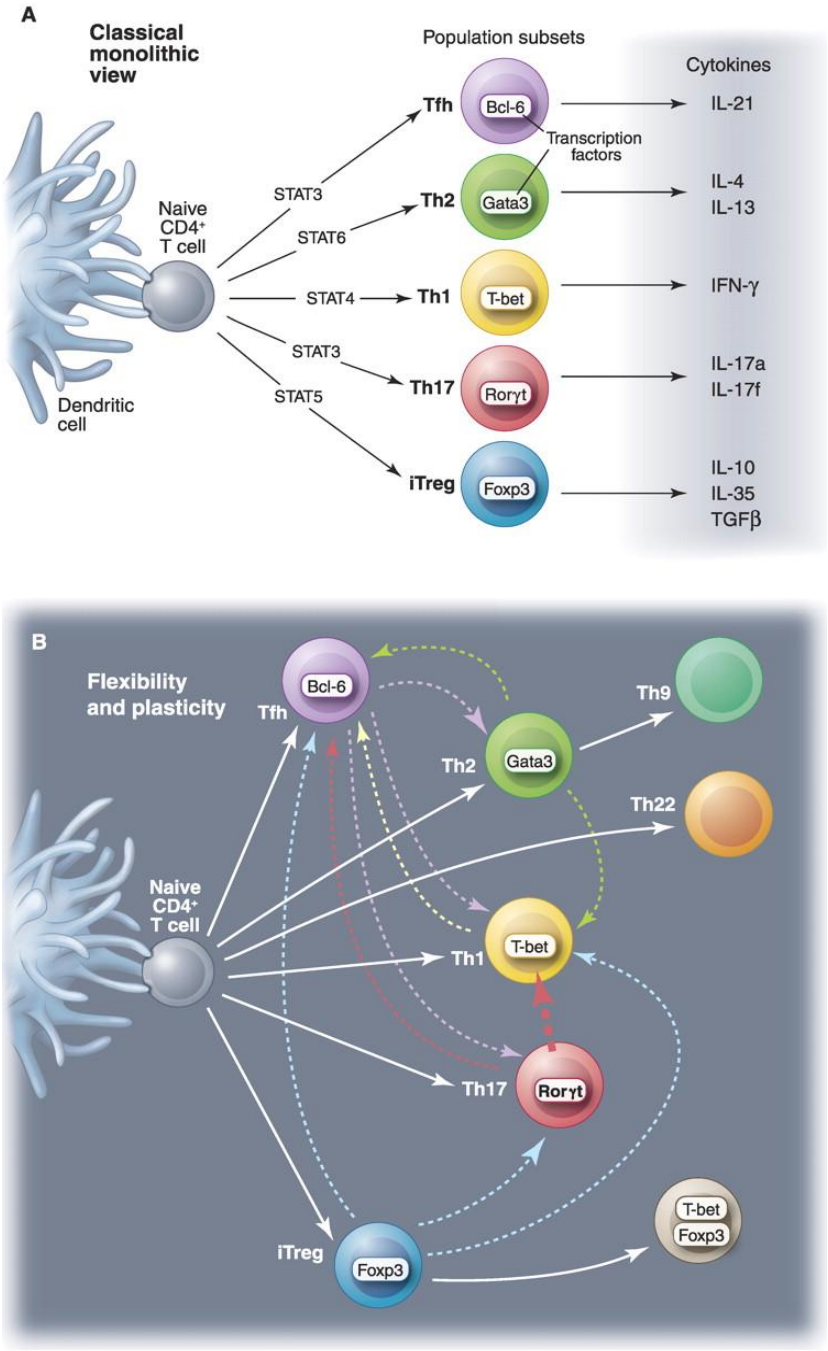


Figure 1-4 showing a schematic of the classical (A) and novel views (B) of the T-helper cell differentiation and plasticity. Reproduced from (O'Shea & Paul 2010)

In classical immunology, periodontitis has been thought of as primarily a 'B-cell lesion' owing to the dense plasma cell infiltrate observed in the periodontium of patients and with a more basic model that only included Th1 or Th2 immune response, which did not explain all available data, especially pertaining to secretion of the Interleukin-17 and the associated T-cell subset, Th17 (Steinman 2007). The previous Th1/Th2 model held that a skewing of T_H-cell subset towards a Th2 response characterised by cytokines such as IL4 and IL6 is responsible for disease progression and enhanced B-cell activation whereas a Th1 response characterised by increased interferon-ɣ production is indicative of a stable lesion (Gemmell & Seymour 1994). However, a rigid interpretation of this supposed 'Th1-protective/Th2-destructive' dichotomy was not consistent with the heterogeneity observed in studies reporting the presence of cytokines that characterise both Th1 and Th2 responses in equal abundance or Th1 response alone as determined in periodontal lesions (Gaffen & Hajishengallis 2008). A further layer of control and complexity is added with the introduction of the separate Th17 T-cell subtype that are thought to have specific differentiating signals including IL12 during APC mediated priming and unique cytokine secretion signatures after activation (Steinman 2007). Current understanding of the Th17 subset suggests that development of the Th17 lineage is inhibited by cytokine signalling characteristic of Th1 and Th2 cells, however in the periodontal inflammatory response, Th17 development is thought to be stimulated by Tranforming Growth Factor-β, IL6 and IL21 co-stimulation suggesting that while Th17 could further promote Th2 type response owing to its development in a Th2-like cytokine environment, it could also have regulatory roles owing to its association with TGF-β signalling and promotion of IL10 production (Bettelli et al. 2006; Raphael et al. 2015).

1.3.1.1 Polarisation of the M1/M2 macrophage phenotypes

Analogous to the development of Th1/Th2 subsets, it is thought that different macrophage subsets also differentiate based on their cytokine environment during activation. The M1 macrophage phenotype is reported to develop under stimulation from interferon-ɣ, bacterial LPS and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), and secreting considerable amounts of TNF-α and IL6 on activation in addition to induction of reactive oxygen and nitrogen intermediates (ROI/RNI) in response intracellular pathogens, and further promoting a Th1 response by production of IL12 (Martinez & Gordon 2014). Three subsets of the M2 phenotype are currently characterised namely, M2a, M2b and M2c: M2a and M2b are thought to drive Th2

immune processes as they are stimulated by IL4, IL13 and TLR signalling, whereas M2c is thought to be a regulatory phenotype stimulated by IL10. The shift in phenotype between M1 and M2 is reported to have important regulatory roles primarily due to a shift in metabolic activity of the macrophages with the M1 phenotype exhibiting microbicidal effects, whereas the M2a phenotype displays increased catabolism of arginine and collagen synthesis. Macrophage invasion by pathogenic bacteria are thought to subvert the M1 phenotype towards the less microbicidal M2a phenotype and hence promote intracellular survival and persistence (Muraille et al. 2014). The M2b and M2c macrophage phenotypes are believed to play important roles in producing fibrosis, extracellular matrix formation and tissue remodelling (Sindrilaru & Scharffetter-Kochanek 2013).

1.3.1.2 Dysregulated Osteoclastogenesis

Whilst the immune mechanisms that drive towards the pathological *terminus* as regards the unregulated alveolar bone loss observed in chronic periodontitis are yet to be fully understood, it is known that inflammatory mediators secreted by the activated macrophages and the resident gingival fibroblasts play an important role in activating osteoclast development which ultimately causes alveolar bone resorption (Graves 2008). The microbicidal activity of neutrophils and secretion of matrix metalloproteinases by activated macrophages in earlier stages of inflammation can lead to extensive collagen and extracellular matrix degradation, in addition to fibroblast apoptosis and this would depend on the intensity of the inflammatory response stimulated by the dental plaque microbiota. Further, inability to control invasive bacteria (Th1) and/or prevent extracellular plaque accumulation (Th2) could lead to a response that may have phases of activation and suppression of pro-inflammatory processes that cannot be resolved completely leading to dysregulated osteoclastogenesis characterised by high Receptor Activator of Nuclear Factor κB Ligand to Osteoprotegrin ratio (RANKL:OPG) resulting in net bone loss (Darveau 2010).

Cytokine	Source	Activity
Interleukin-1α	macrophages, neutrophils epithelial & endothelial cells	fibroblast proliferation, acute phase response
Interleukin-1β	epithelial, dendritic cells, macrophages	osteoclastogenesis, acute phase response
Interleukin-4	γδ T cells, eosinophils, basophils, NKT cells, mast cells and naive CD4 T cells	↑ B-cell and T-cell development (Th2)
Interleukin-6	T cells, B cells, macrophages, fibroblasts, endothelial cells	↑ Th17 ↓Treg
Interleukin-8 (CXCL8)	Monocytes, lymphocytes, granulocytes, fibroblasts, endothelial cells	neutrophil and basophil chemoattractant
Interleukin-10	Activated CD4 & CD8 T cells	↑ B cells, mast cells ↓Th1 T cells
Interleukin-12p70	Macrophages & Dendritic cells	↑Th1 differentiation ↑Interferon-ɣ by T cells
Interleukin-13	Activated T cells, Mast cells and Natural killer cells	↑Th2
Interleukin-17A	CD4 T cells	↑Th17
Granulocyte-Colony Stimulating Factor	macrophages	neutrophil development
Interferon-α	lymphocytes, dendritic cells & macrophages	resistance to viral infection, inhibits cell proliferation
Interferon-ɣ	CD4 & CD8 T cells, NK cells	↑development of T cells, B cells, macrophages and NK cells. ↑Th1
Tumour Necrosis Factor-α	monocytes, macrophages, T cells, neutrophils and fibroblasts	potent mediator of inflammation and bone resorption
CXCL10	monocytes, fibroblasts, endothelial cells	macrophage/T cell/NK cells and dendritic cell chemoattractant
CCL2	macrophages, dendritic cells	macrophage and basophil chemoattractant
CCL3 & CCL4	monocytes, macrophages, neutrophils, T cells & B cells	T cells, B cells and eosinophils
Transforming Growth Factor-β	macrophages	osteoclast induction
E-selectin	endothelial cells	leukocyte adhesion and extravasation
Intracellular Adhesion Molecule-1	transmembrane protein expressed in leukocytes and endothelial cells	leukocyte transmigration

Table 1-3 listing some important molecules involved in periodontal inflammation, their source and activity.

1.4 Volatile sulfur compounds in periodontal disease

Volatile Sulfur Compounds (VSCs) are thought to be the main contributors to malodour originating from the mouth and a variety of anti-malodour treatments and products target VSCs and the bacteria responsible for their production in the mouth (Peruzzo et al. 2008; Greenman & Saad 2009). The correlation between oral malodour and periodontal disease is well documented, with numerous studies reporting an increase in the concentration of VSCs or malodour as detected organoleptically in the breath alongside the presence of periodontal disease in different regional populations (Table 1-4).

Regional Population	Reference
South Africa	(Ayo-Yusuf et al. 2011)
Switzerland	(Bornstein et al. 2009)
Turkey	(Kara et al. 2006*; Nalçaci & Sönmez 2008*)
China	(Liu et al. 2006)
Japan	(Miyazaki et al. 1995; Takeshita et al. 2010; Takeuchi et al. 2010; Amou et al. 2014)
Belgium	(Quirynen et al. 2009)
Taiwan	(Tsai et al. 2008)
Brazil	(Figueiredo et al. 2002)
Canada	(Bosy et al. 1994)
Sweden	(Johansson 2005)
India	(Matthew & Vandana 2006)
Israel	(Amir et al. 1999)*

Table 1-4 listing clinical studies in the literature that showed an association between VSCs and/or oral malodour and clinical periodontitis parameters in different regional populations. Asterisk indicates studies in children/adolescents.

Studies have also reported that the severity of periodontitis as measured by standard clinical parameters is higher in individuals with malodour and periodontitis than individuals with periodontitis but no malodour (Kurata et al. 2008). Oral malodour and VSC concentration is also reported to decrease significantly in periodontitis patients who receive periodontal treatment, and interestingly tongue hygiene alone is reported to have less effect on oral malodour in patients than periodontal treatment, suggesting a

role for periodontal microbiota in oral malodour associated with disease (Pham et al. 2012; Takeuchi et al. 2010; Quirynen et al. 2005). However, other studies also report that oral malodour in periodontal disease is more directly related to the level of tongue biofilm present in individuals, but these studies are not interventional, unlike the former studies that report a greater effect of periodontal treatment (Stamou et al. 2005; Calil et al. 2009). It is likely that both tongue biofilm and periodontal microbiota have a role to play in malodour, in disease and health (Bordas et al. 2008; Yasukawa et al. 2010; Danser 2003).

The chief source of VSCs in the mouth is thought to be protein metabolism of the oral microbiota, with the Gram-negative bacteria thought to be the most significant contributors to VSC production (Nakano et al. 2002). Sulfur-containing amino acids such as cysteine and methionine are the primary substrates in the VSC production by the oral microbiota with complex substrates such as the saliva, sloughed oral epithelia & cells, trapped food particles and dead bacteria providing the majority of the proteinaceous raw material to the oral biofilm (Krespi et al. 2006).

The most important VSCs involved in oral malodour include hydrogen sulfide (H_2S), methanethiol (CH_3SH) and dimethyl disulfide, with H_2S and CH_3SH being the predominant VSCs detected in the breath of individuals with oral malodour in the presence of periodontal disease (Van den Velde et al. 2009). Other VSCs such as dimethyl sulfide and dimethyltrisulfide are reported to be detected in the breath air of patients with advanced stages of periodontitis. Also, the breath of individuals suffering from oral malodour with no obvious symptoms of periodontal disease can present with an appreciable amount of VSCs such as H_2S and CH_3SH, with the concentrations of the detected VSCs increasing with an increase in the organoleptic score (van den Velde et al. 2007).

1.4.1 VSCs in the periodontal pocket

Studies have also reported on the VSCs produced in the periodontal pockets and their association with clinical and microbial aspects of periodontitis. A gas chromatographic method was used to detect H_2S and CH_3SH in periodontal pockets and the concentration of these VSCs are reported to correlate positively with periodontal pocket depths, with a stronger association with methanethiol (Persson 1992). A few studies have used a periodontal probe with an attached ‘sulfide’ sensor to measured sulfides in the periodontal pocket, and one such study reported the concentrations measured to be

significantly associated with clinical disease parameters such as plaque index, bleeding on probing and gingival index in an experimentally induced gingivitis model in a split-mouth study (Pavolotskaya et al. 2006). The concentrations of sulfides measured in periodontal pockets of smokers with periodontitis were reported to be significantly higher than non-smokers with periodontitis (Khaira et al. 2000). Torresyap et al. (2003) studied the microbiota in oral sites in association with sulfide concentrations in periodontal pockets using the probe based sulfide sensor, and found higher counts of the anaerobic red-complex and orange-complex bacteria in sulfide-positive sites compared to sulfide-negative sites in the same individual, independent of probing depths. Another study that measured salivary thiols found that the sulfur containing amino acids and peptides such as cysteine, cysteinylglycine and glutathioine increased significantly in saliva of periodontitis patients compared to health, suggesting more available substrate for VSC production in the oral cavity in relation to disease (Zappacosta et al. 2007). This is particularly relevant, since free cysteine or methionine is not normally detected in the saliva of a healthy individual (Nakamura et al. 2010).

1.4.2 VSCs and microbial ecology

The presence of VSCs in the breath is more closely related to the microbial ecology in the different oral sites such as the tongue and periodontal niches. It has been demonstrated that the rate of VSC production by the putative periodontopathic bacterial species is much higher than the more health-associated species (Persson et al. 1990). In particular, the bacteria associated with a dysbiotic microbiota are more efficient in producing copious amounts of VSCs from more complex proteinaceous substrate such as the serum than free amino acids, suggesting that VSC production could be an important mechanism involved in the proteolytic activity of the periodontopathic bacteria in association with disease activity in the periodontal niches (Persson et al. 1989). However, a molecular study of the tongue microbiota suggested that the H_2S producing species detected were not necessarily associated with gingival inflammation (Washio et al. 2005). This study detected the most abundant species in the tongue of healthy individuals to be from the genera *Veillonella, Prevotella* and *Actinomyces,* although these findings were from a culture dependent method, so may not represent the full diversity of the tongue microbiota. At present only one study in the available literature has investigated the tongue microbiota by using a culture independent, metagenomic methodology in association with oral malodour as defined by the VSC concentration in the breath (Yang et al. 2013). The findings reported in this study is

largely consistent with Washio et al. (2005), whereby the predominant genera and species that showed an association with H_2S concentrations in the breath were different to those that are normally prevalent in the periodontal niches. However, this study only reported differences observed among species or genera that were of high prevalence with no information on rare taxa which more often involves periodontopathic bacteria such as *P. gingivalis, T. forsythia* and *T. denticola.* This study also did not find any relationships with other important VSC producing genus namely, *Fusobacterium* spp. Another study that utilised a next generation sequencing approach to characterise the salivary microbiota found more peridontopathic bacterial species predominating in individuals with severe malodour with a high H_2S or CH_3SH concentration (Takeshita et al. 2012). However, while this study did not report the full periodontal assessment such as the plaque coverage of the enrolled participants, the individuals with higher organoleptic scores were reported to have higher mean periodontal probing depths, suggesting possible shedding of periodontal microbiota in saliva that could explain these results. There are no metagenomic ecological surveys of the tongue in relation to health and disease available in the literature to assess if the tongue ecology changes significantly in association with disease.

1.4.3 Effect of VSCs on host response

Due to the association of VSC production with oral microbiota, a number of studies have investigated the potential adaptive and functional role of VSC production in the oral cavity for the oral microbiota—a number of destructive roles are shown for both H_2S and CH_3SH, with both VSCs equally potent in *in vitro* experiments (Yaegaki 2008). Some of the direct effects reported include apoptosis, genomic DNA damage, collagen degradation, increased mucosal permeability, irreversibly binding to key respiratory enzyme cytochrome c oxidase and forming sulfhaemoglobin that can cause erythrocyte membrane instability (Yaegaki 2008). The *in vivo* evidence for the effects of VSCs is provided by toxicology studies that document occupational or community exposure to VSCs and their systemic effects (Lambert et al. 2006; Inserra et al. 2004). It could be argued that given some of these systemic effects are observed at concentrations not too dissimilar to that found in the breath of individuals, VSCs produced by the oral microbiota likely has local effects that may be involved in periodontal disease progression.

1.4.3.1 Signalling effects of hydrogen sulfide on host tissue

The emergence of literature on H_2S as a signalling molecule with anti-inflammatory and other beneficial effects casts some doubt on the role of VSCs produced by the oral microbiota in general as a toxic agent in the oral cavity (Kimura 2011). Hydrogen sulfide is thought to be produced in the neural tissue where it is thought to suppress synaptic potentials and help relax vascular smooth muscle not unlike the effects of nitric oxide and these effects are thought to be cytoprotective in lowering oxidative stress (Kimura 2002; Lo Faro et al. 2014). Interestingly, anti-inflammatory effects have been observed in models of gastric inflammation where hydrogen sulfide was observed to reduce TNFα and ICAM1 expression, thereby preventing leukocyte extravasation and reducing inflammation (Fiorucci et al. 2005). There is also evidence for NO-H_2S interactions in inflammation. For example, H_2S delivered in gaseous form to LPS activated macrophages was able to inhibit NO production and iNOS expression, and this was attributed to NFκB attenuation (Oh et al. 2006). Indeed, Sen et al. (2012) demonstrate that endogenous H_2S can bind to a cysteine residue in NFκB via sulfhydration and this stimulates transcription of antiapoptotic genes in TNFα stimulated macrophages. Given the ubiquity of NFκB mediated signalling in inflammation, these studies have potentially identified a major mechanism of host-microbiome interaction mediated by H_2S in inflammation (Lawrence 2009). Further, murine macrophages cultured without RANKL, when exposed to gaseous H_2S at concentrations well below that was observed in GCF in prior studies were observed to express osteoclastogenic activity (Ii et al. 2010). These observations suggest that hydrogen sulfide production by anaerobic microbial proteolysis could dampen the immune response, while simultaneously facilitating periodontal tissue destruction. In addition, a number of studies report of the importance of cysteine moieties in proteins and inflammatory cytokines, with redox labile disulfide bonds in particular that could be reduced by VSCs produced by the oral microbiota, inducing functional changes that are not necessarily destructive (Metcalfe et al. 2011; Butera et al. 2014; Kellett-Clarke et al. 2015). However, the increased severity of periodontal disease observed in individuals with malodour compared to without malodour (or low VSC concentration) suggests that this host-microbial interaction could ultimately encourage disease progression (Li et al. 2011; Zanardo 2006)

1.4.3.2 Effects of methanethiol on host tissue

In light of the positive association between breath concentrations of methanethiol and periodontal disease, there are a limited number of studies on the effects of this VSC on host tissue. Production of the Th2 cytokine IL6 by tooth pulp fibroblasts were observed to be significantly upregulated in the presence of methanethiol when stimulated with LPS (Coil et al. 2004). Interestingly, the cytokines IL1 and TNFα were not affected, suggesting specific activation. This specificity in stimulating cytokine secretion was also observed in another study with gingival fibroblasts and monocytes (Ratkay et al. 1995). In the latter study, it was reported that methanethiol was able to induce production of procollagenase via a prostaglandin-cAMP mediated pathway in human gingival fibroblasts, and while it stimulated increased activity of CathepsinB in macrophages, it did not affect elastase. Methanethiol also displays a more potent activity in increasing oral mucosal permeability compared to hydrogen sulfide (Ng & Tonzetich 1984).

1.5 Project aims

The overall aims of this project were:

- To study the microbial, inflammatory and clinical aspects of periodontal disease in relation to Volatile Sulfur Compounds (VSCs) present subgingivally and in the breath.
- Conduct an ecological survey of the tongue and periodontal niches in relation to oral malodour (as defined by VSC concentrations in the breath) and periodontal disease to describe the ecological shifts associated with disease in putative VSC producing species.
- To investigate the role of VSC production in host-microbial interactions *in vitro*

2 MATERIALS AND METHODS

The majority of this thesis involves an exploratory clinical study that included collection of human biological samples and laboratory analyses of these samples. Ethical approval for the study protocol was obtained from the London Central Research Ethics Committee (Ref: 12/LO/1611) in December 2012 and the clinical part of the study commenced in February 2013.

2.1 Study design and clinical protocol

This is a case control study with three groups of participants: (i) Health (ii) Gingivitis (iii) Chronic periodontitis. Suitable participants for the chronic periodontitis cohort were recruited from patients identified from various Consultant, Treatment and Maintenance clinics at the Dental Hospital, Barts Health NHS Trust. Students and staff from Queen Mary University of London were screened and recruited for the health and gingivitis cohorts. All participants were informed of the study procedures with a 1 week interval before the clinic visit when written consent was obtained and clinical assessments were carried out.

2.1.1 Inclusion and exclusion criteria.

Only individuals aged 18 years or above and able to give informed consent were screened and recruited. Specific health related exclusion criteria for participation in this study were as follows:

1) Individuals with general / systemic diseases such as
    a) Diabetes mellitus type 1 and 2

b) Presence or recent history of infectious diseases such as AIDS, Hepatitis and TB or other respiratory infection including those that can be transmitted in saliva

2) Women intending to be pregnant or are pregnant or breast feeding
3) Cigarette smoking or chewing of tobacco or paan, drugs and alcoholism
4) Recent use of antibiotics and/or anti- inflammatory medication (within 4 weeks of visit)
5) Periodontal treatment received within the last 3 months.
6) Medications resulting in xerostomia
7) Denture wearers
8) Other exclusion criteria were: patients who were deemed suitable for the study but withheld consent or were unable to participate (time, availability), and also individuals who were screened for the healthy or gingivitis cohort but were diagnosed with periodontitis on oral examination.

Inclusion criteria pertaining to the oral health of individuals for the different cohorts are listed below:

- Health
    - Good oral health
    - Absence of carious lesions and periodontal disease
    - Presence of at least 20 teeth
    - No more than four periodontal pockets with a maximum of 4 mm of depth excluding 3rd molars.
    - ≤20% sites bleeding on probing (BOP)
    - ≤20% gingival bleeding index (GBI)
- Gingivitis
    - Presence of at least 20 teeth
    - Generalised gingival inflammation with ≥20% of sites bleeding on probing
    - Presence of more than four periodontal pockets with probing depths ≥4mm excluding 3rd molars.

- Absence of any obvious signs of periodontitis such as generalised periodontal pocketing >5mm excluding 3rd molars.

- Chronic periodontitis
  - Presence of at least 10 teeth
  - >2mm Clinical Attachment Loss
  - Presence of at least two periodontal pockets ≥6mm excluding 3rd molars
  - Radiographic evidence of bone loss

The main criteria used for inclusion into the health and gingivitis cohorts were the clinical parameters such as Bleeding on Probing (BOP), Gingival Bleeding Index (GBI) and number of probing sites deeper than 4mm. In case of conflicting scores between GBI and BOP, the latter was given precedence in determining diagnosis due to it preceding GBI in the clinical protocol sequence and as such would be a more accurate measure of periodontal health. However, presence of more than four periodontal pockets ≥4mm excluding 3rd molars determined a diagnosis of gingivitis regardless if BOP and/or GBI scores were ≤20%.

2.1.2 Clinical protocol

The clinical protocol sequence is shown below for collection of samples and oral examination (Figure 2-1). At the arrival of the participants for their dental appointment, the clinical procedures were explained to them again, informing that they can withdraw any time without explanation. Participants were given a copy of the information sheet and consent forms, to enable them to contact the investigator in the future for any feedback. It was also explained that the information sheet given to the participants also contained the unique number assigned to them, which can be used to identify themselves to the investigator at a later date, if they would like their samples to be withdrawn from the study. After consent, a general screen was conducted by way of a medical history questionnaire and the timing of their last oral activity such as eating or oral hygiene. Only participants who had no oral activity in the last two hours were considered for further sample collection. In general, participants were informed not to eat or perform any oral hygiene measures for at least 2 hours prior to the appointment. Samples that are not dependent on diagnosis, such as saliva, tongue scrape and breath samples were collected immediately after enrolment (see section 2.1.3 for more detail in the sample collection procedures).

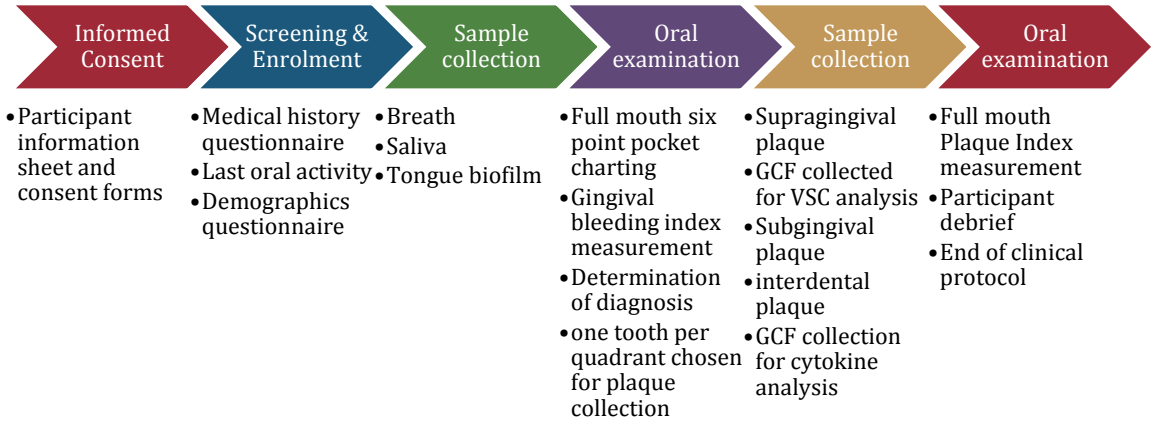


Figure 2-1 showing a flow chart of the clinical protocol sequence with the oral examination and the different sampling aspects.

The six point pocket charting was carried out using a manual Williams 14W periodontal probe (Henry Schein Rexodent, Dorset, UK), determining probing depths (PD) at six sites around each tooth in all teeth present. Any bleeding observed on sites after probing were recorded for calculating the percentage of sites bleeding after probing and reported as BOP scores. After a rinse, the periodontal probe was inserted 1mm beneath the gingival margin and run along the gingival perimeter, with the presence of bleeding at four sites per tooth (labial, mesial, distal and lingual) within 20 seconds of this procedure were recorded. The percentage of sites that bled was calculated as the full mouth Gingival Bleeding Index or Marginal Gingival Bleeding score (GBI/MGB) (Ainamo & Bay 1975). The diagnosis of the participant was determined at this point in the sequence, based on the probing depths and gingival bleeding observed using the BOP and GBI scores, with the BOP and PD scores overruling GBI scores in case of conflict in determining health or gingivitis. For further sample collection procedures, one tooth per quadrant was chosen based on the deepest probing depths recorded. In case of quadrants with probing depths ≤3mm, a tooth was picked at random. The third molars were not included in determining diagnosis and in sampling plaque as false pockets can occur more frequently around this tooth and more plaque accumulation due to restricted access for toothbrushes (Smart 2005). However, bleeding, probing depths and plaque coverage in this tooth was recorded in all cases. Plaque and GCF samples were collected on and around the selected tooth per quadrant, and finally the amount of plaque present in the oral cavity was assessed by disclosing the teeth for the presence or absence of visible plaque on the labial, mesial, distal and lingual surfaces. The Plaque

Index scores were calculated as the percentage of the total surfaces that showed visible plaque (O' Leary et al. 1972).

2.1.3 Clinical sample collection methods

The following descriptions of the sample collection methods occur in the order of the clinical protocol sequence:

2.1.3.1 Breath Sample

Before the collection of the breath sample, the participant was seated upright and asked to breathe through their nose with their mouth closed for approximately one minute. A sterile FEP mouthpiece (Cole-Parmer, London, UK) with a 20ml PTFE syringe (NORM-JECT®, Henke-Sass Wolf, Tuttlingen, Germany) attached was then inserted into the mouth till the mouthpiece reached the back half of the tongue (Figure 2-2). It was ensured that the participants held the mouthpiece with the lips closed (but not sealed) around the mouthpiece to prevent creating a vacuum in the oral cavity during breath sampling. The participants were then instructed to take a deep inhalation through their nose when ready and hold for 5 seconds, during which approximately 15ml of mouth air sample was withdrawn. A 1ml aliquot of the collected breath sample was injected into a portable gas chromatograph (Oral Chroma™; Abimedical Corporation, Japan) for measuring the presence of hydrogen sulfide, methanethiol and dimethyl sulfide. The data output of the Oral Chroma™ was recorded in the included data manager software (Abimedical Corporation).

An organoleptic assessment was approximated by calculating a Malodour Score based on the following formula:

$$Malodour\ Score = \sum \frac{[Odorant_a]}{10 \times Rt_a} + \frac{[Odorant_b]}{10 \times Rt_b} + \cdots + \frac{[Odorant_n]}{10 \times Rt_n}$$

The Malodour Score is equal to the sum of the concentration of each odorant (in this case, a VSC) divided by 10 times its recognition threshold, *Rt*. This formula is proposed based on the logarithmic nature of the relationship between the organoleptic scale and the concentration of odorants and the need for a clear threshold whereby an untrained individual would recognise the odour as malodorous (Greenman et al. 2004). While this formula takes into account the differential stimulation of the olfactory senses by different odorants, it is assumed that as the concentration of each odorant increases, the stimulation of the olfactory sense is additive. However, this may not be valid for

odorant concentrations approaching saturation. The recognition threshold used in the present study for calculating the Malodour Score was 4.7ppb for H_2S and 2.1ppb for CH_3SH (Leonardos et al. 1969). Whilst this value was determined using a trained judge, in the current study, a Malodour Score of 1 is defined as the malodour threshold and this score indicates the presence of one or both VSCs at concentrations that cumulatively exceeded the quoted recognition threshold by ten-fold which should compensate for the potential lack of olfactory acuity in untrained individuals. A breath sample with calculated Malodour Score >1 would be considered to be malodorous, while a score of >5 is defined as moderate to severe malodour.

2.1.3.2 Saliva

The saliva sample was obtained with the participant instructed to tilt their head slightly forward and asked to rest for five minutes and minimise orofacial movements. Unstimulated saliva was allowed to accumulate in the floor of the mouth and the patient was then asked to expectorate about 3ml of saliva into a 20ml sterile universal tube.

2.1.3.3 Tongue biofilm

Tongue biofilm was sampled by scraping a Whatman Omniswab (General Electric Healthcare, Little Chalfont, UK) three times along the tongue dorsum, from as close to the circumvallate papillae as possible towards the tip, with the swab held perpendicular to the lingual plane. The Omniswab pad was then ejected into 500µl of Tris-EDTA (TE) buffer (pH 7.2; Sigma-Aldrich, Poole, UK) in a 5ml sterile universal tube.

2.1.3.4 Supragingival plaque

Supragingival plaque samples were obtained by swabbing the buccal and lingual facial surfaces along the gingival margins using one regular sized Microbrush® per tooth (Patterson Dental Supply Inc, Minnesota, USA) and all four samples pooled together in 500µl TE buffer (Sigma-Aldrich) in a sterile microcentrifuge tube (Eppendorf, Hamburg, Germany).

2.1.3.5 Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) samples for gas chromatographic headspace analysis were collected by inserting three sterile absorbent paper points (size #40; Sybron Dental Specialities, Charlotte, USA) into the site with the deepest periodontal pocket among the four teeth chosen for sampling. The paper points were removed after 10 seconds to minimize serum and salivary contamination and placed in glass crimp top vials (with

300µl inserts; Supelco, Sigma) and sealed with crimp caps (PTFE/silicone septa; Sigma). Sterile Periocol filter paper strips (Oralflow, New York, USA) and sterile Whatman 3MM filter paper (General Electric Healthcare) cut to dimensions (2mm x 25mm) were also used in sampling GCF for VSC analysis (Figure 2-3).

2.1.3.6 Subgingival plaque

Subgingival plaque samples were collected by inserting two size #40 sterile absorbent paper points (Sybron Dental Specialities) in the deepest pockets in the buccal and lingual sites in each tooth. Samples from all four chosen teeth were pooled into 500µl of TE buffer (Sigma) in a microcentrifuge tube (Eppendorf).

2.1.3.7 Interdental plaque

Interdental plaque was collected from the mesial and distal aspects of all four teeth supragingivally, using an interdental brush (Pink Tepe® brush, size=0, wire size=0.4mm; Tepe Oral Hygiene Products, Malmö, Sweden) and the brush stored immediately in 500µl of TE buffer (Sigma) in a microcentrifuge tube (Eppendorf).

2.1.3.8 Gingival Crevicular Fluid

GCF samples for cytokine analysis were collected using sterile Whatman 3MM or Periocol filter paper strips as described in 2.1.3.5, but using two strips per tooth (buccal and lingual) and pooling the strips together into a swab storage tube (Salimetrics, Pennsylvania, USA).

All samples were placed in ice after collection before transportation to the laboratory.

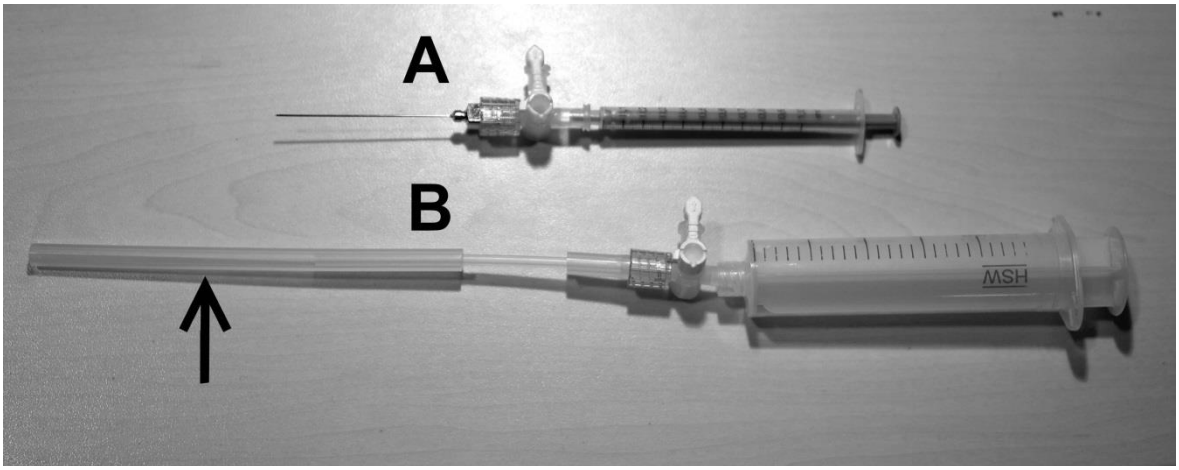


Figure 2-2 photo showing breath sampling syringe (B) and gas sample injection syringe (A) used in this study. Participant mouthpiece indicated with an arrow.

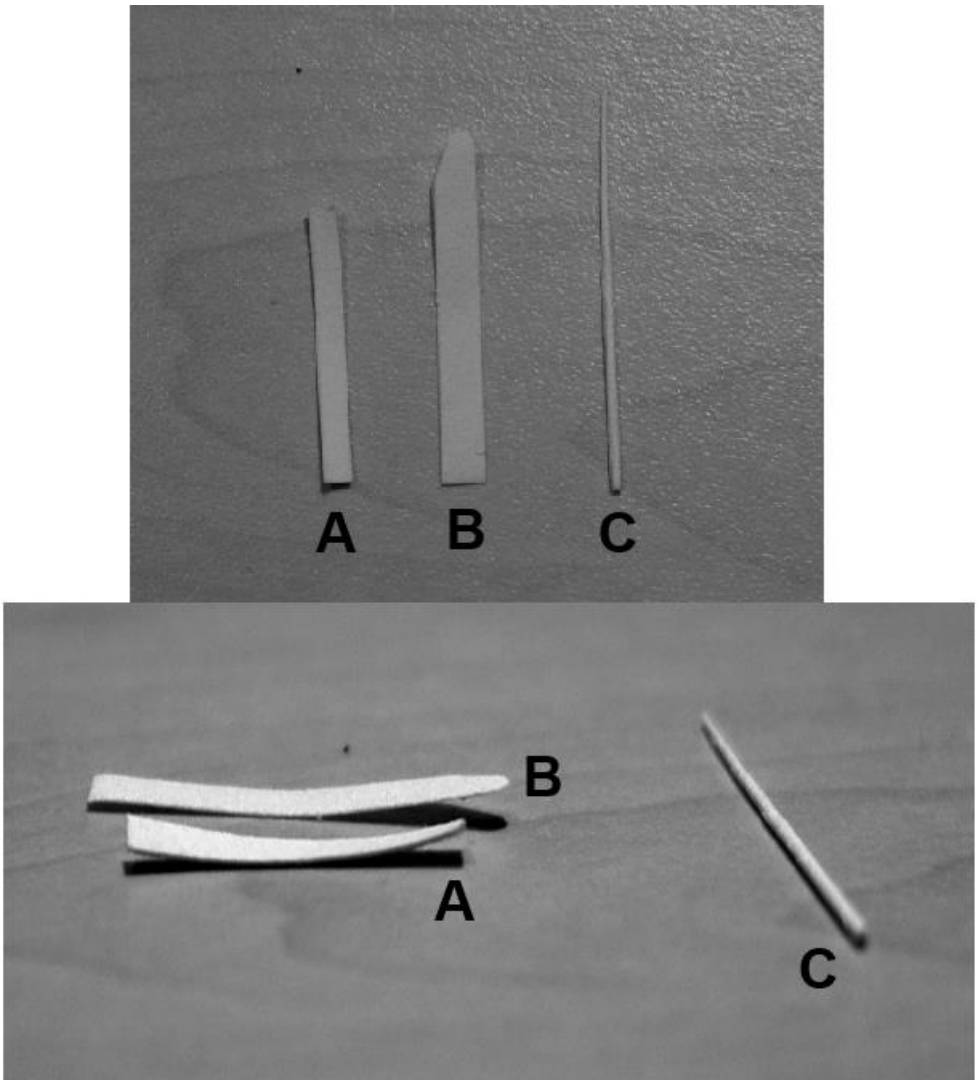


Figure 2-3 photos showing shape and sizes of the devices used to sample GCF in this study. A=Whatman 3MM; B=Periocol strip; C=SybronEndo paper points.

2.2 Laboratory protocols for clinical samples

The plaque and tongue biofilm samples were disrupted from their respective sampling devices by transferring a few sterile glass beads into the sample containing tubes and vortexing vigorously for 20s; the plaque samples were then transferred to separate microcentrifuge tubes. A 1ml aliquot of the saliva sample was microcentrifuged (>10,000 *g*) for 10 mins at 4°C, and the supernatant was separated from the pellet and 500µL of TE buffer added to the pellet only. The salivary cells/debris and plaque samples were stored at -20°C until DNA extraction or incubation for headspace analysis. The salivary supernatant was stored immediately at -80°C until cytokine analysis.

The GCF sample was eluted from the filter strips by adding 150µl of TE to the swab storage tube, ensuring to fully wet all the filter strips and leaving for 1 min on ice, then centrifuging at 3500 *g* and 4°C for 5 mins. The GCF eluted into the collection tube was then pipetted into a sterile microcentrifuge tube and stored at -80°C until use for cytokine analysis. The GCF sample collected for VSC analysis was also stored at -80°C until use.

2.2.1 Quantitative PCR analysis

The genomic DNA extracted from the salivary pellet, tongue biofilm and plaque samples was analysed using quantitative Polymerase Chain Reaction (qPCR), to detect specific oral bacterial species. The protocol for genomic DNA extraction in both clinical samples and bacterial cultures used for construction of standard curves for qPCR were as follows.

2.2.1.1 Genomic DNA extraction

The HOMIM Protocol (2008) was followed for DNA extraction from the clinical samples with the majority of the solutions used from the MasterPure™ Gram-positive DNA purification kit (Epicentre, Madison, USA). Briefly, cells were lysed by adding 1µl of Ready-Lyse™ lysozyme (Epicentre) and incubating at 37°C overnight, adding 250µl of the supplied 2 x T&C lysis solution (Epicentre) to each sample after incubation and mixing well by pipetting up and down to complete the cell lysis process. Protein digestion was carried out by adding 1.5µl of Proteinase K solution (Epicentre) to each sample and incubating at 65°C for 30 mins, vortexing briefly every 5 minutes during

incubation. The samples were then cooled to 37°C and placed on ice for 3-5 mins ready for DNA clean up.

The protein in the lysed sample was then precipitated by adding 250µl of the supplied MPC protein precipitation solution and mixed by vortexing vigorously for 10 seconds. The samples were then pelleted by centrifugation at >10,000 *g* for 10 mins at 4°C and placed immediately on ice before discarding the pellet. DNA was then precipitated by adding 750µl of 2-propanol (Sigma) to the recovered supernatant and mixed by inverting the tube 30 – 40 times. The samples were then placed on ice for approximately 10 minutes followed by pelleting the DNA at >10,000 *g* for 10 mins at 4°C. The DNA pellet was then rinsed with 500µl of 75% v/v ethanol and the residual ethanol removed before the DNA pellet was resuspended in 25µl of TE buffer. Extracted DNA was stored at -20°C till use.

2.2.1.2 Bacterial cultures for standard curves and primer validation

Six bacterial species namely, Fusobacterium nucleatum, Tannerella forsythia, Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Veillonella parvula and Solobacterium moorei were detected in the clinical samples employing qPCR with primers that detected specific sequences in the 16S or RNA polymeraseB genes. Apart from new primers developed for V. parvula and S. moorei, primers used for detecting other species were previously validated (Decat et al. 2012). The amounts of DNA detected by qPCR were related to the number of Colony Forming Unit equivalents, by using standards of DNA extracted from pure cultures of the bacteria detected. All bacterial strains were grown in an anaerobic chamber (MAC MG500; Don Whitely Scientific Ltd, Shipley, UK) with an atmosphere containing 80% N_2, 10% H_2 and 10% CO_2 maintained at 37°C. Specific growth media and the primer sequences used are listed in Table 2-1 and Table 2-2, respectively.

2.2.1.3 Primer design for *S. moorei* and *V. parvula*

qPCR primers were designed for both *V. parvula* and *S. moorei* based on DNA sequences for the RNA PolymeraseB and 16S genes, respectively using online tools such as geneFisher2 and Primer-BLAST (Giegerich et al. 1996; Ye et al. 2012). Primers were also validated using an online tool probeCheck and their specificity checked by conducting qPCR and conventional PCR (with the same annealing temperatures and times to the qPCR method) on genomic DNA templates extracted from closely related bacterial strains (Loy et al. 2008). The amplicons were visualized on an agarose gel for

conventional PCR and melting curves determined for qPCR to ensure specificity. Both primers showed good specificity, sensitivity and no cross-reactivity for human DNA.

Species	**Strain**	**Broth**	**Agar**
Aggregatibacter actinomycetemcomitans	ATCC 43718	-	Blood Agar (5% v/v defibrinated horse blood) – BA
Fusobacterium nucleatum ssp *polymorphum*	NCTC 10562	3.7% w/v Brain Heart Infusion (BHI) + 0.0005% w/v hemin	BA
Tannerella forsythia	ATCC 43037	4% v/v Tryptone Soya Broth + 0.0005% w/v hemin + 0.001% w/v N-acetylmuramic acid	BA
Porphyromonas gingivalis	W50, W83	BHI + 0.5% v/v hemin	BA
Veillonella parvula	NCTC 11809	BHI	BA
Veillonella atypica	NCTC 11830	BHI	BA
Veillonella dispar	NCTC 11831	BHI	BA
Solobacterium moorei	DSM 22971	-	BA
Bulleidia extructa	DSM 13220	-	BA
Erysipelothrix rhusiopathiae	-	-	BA

Table 2-1 listing the bacterial species, strains and culture media used to grow the respective strains for qPCR analysis in this study.

Species	Primer Sequences	T_a	Reference
Aggregatibacter actinomycetemcomitans	F: CTTACCTACTCTTGACATCCGAA R: ATGCAGCACCTGTCTCAAAGC		(Maeda et al. 2003)
Fusobacterium spp.	F: AAGCGCGTCTAGGTGGTTATGT R: TGTAGTTCCGCTTACCTCTCCAG		(Martin et al. 2002)
Tannerella forsythia	F: GGGTGAGTAACGCGTATGTAACCT R: ACCCATCCGCAACCAATAAA		(Shelburne et al. 2000)
Porphyromonas gingivalis	F: GGAAGAGAAGACCGTAGCACAAGGA R: GAGTAGGCGAAACGTCCATCAGGTC		(Park et al. 2011)
Veillonella parvula	F: TTTCATCGAAGCACCATACC R: GTTCAGTTGTGAAGTACCCC		This study
Solobacterium moorei	F: GGCCTGTTAAGTAAGTGGT R: CGTCAGTCTTTGGCTAGC		This study
Universal	F: GTGSTGCAYGGYTGTCGTCA R: ACGTCRTCCMCACCTTCCTC		(Maeda et al. 2003)

Table 2-2 listing primer sequences and their respective annealing temperatures used in the qPCR assays of clinical samples in the present study.

2.2.1.4 SYBR Green qPCR Assay

96-well and 384-well plates (Roche Diagnostics, Mannheim, Germany) were used with final reaction volumes of 20µl and 10µl respectively, with each well containing 2 x LightCycler® FastStart DNA Mastermix (Roche), 1 µM of each primer, 2 µl of the template and de-ionised water to a final reaction volume. DNA extracted from the clinical samples was diluted 10-fold before using as the template in the qPCR assay. All multiwell plates were sealed, centrifuged at 1500 g for 10 seconds, and then amplified in the LightCycler LC480 Real-Time PCR system (Roche), with activation of polymerase (95°C for 5 min), followed by 40 cycles of 10s at 95°C, annealing for 5s at T_a (determined for each set of primers), and an extension time at 72°C. In general, the T_a was determined as 3 to 5°C below the T_m of the primer with the lowest T_m. Extension time was based upon the expected fragment length and was calculated as 1 second for every 25bp. Double-stranded PCR product was measured at 76°C for 1 second by detection of fluorescence associated with the binding of SYBR Green I to the product. Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 30 seconds at 95°C, 30 seconds at 65°C and acquisition at 99°C. Temperature change rates were 4.4°C⁄ s, except in the final step, which was 0.1°C⁄ seconds. Fluorescence curves and melting curves were analysed and the absolute amount of DNA determined by the LightCycler® software v. 1.5 (Roche).

Standard curves were generated separately, and a few template dilutions that were used to generate the standard curves were included as internal controls in each run, used for melt curve analysis and relative quantification. No-template and negative sample

controls were used in every run with all reactions set up in duplicate. The Absolute Fit-Points method was used in the LightCycler® software for relative quantification, with 35 cycles set as the last cycle cut off in all analyses. Melt curve analysis was used to determine positive samples, and the fluorescence baseline adjusted manually to maximize the sensitivity of C_t value determination in the positive samples.

Genomic DNA extracted from *P. gingivalis* cultures were used to generate standard curves for the universal primer. The data calculated as CFU from the qPCR analysis for general bacterial load and the specific bacteria were then converted into proportions for statistical analysis. However, the relative proportions determined by this method will vary dependent on how closely the 16S copy number of *P. gingivalis* relates to the mean (±SD) of the total 16S copies present in each sample as determined using the universal primers, in addition to the universal primer's breadth of sensitivity to all organisms in all oral phyla found in each sample (Horz et al. 2005). Therefore, the proportions were determined only as a way of normalising to the total 16S copies present in each sample in relation to the bacterial species measured as CFU, and not as an absolute value.

2.2.2 Human Oral Microbe Identification using Next Generation Sequencing

DNA extracted from a subset of clinical samples (tongue biofilm, subgingival and interdental plaque) collected from all participants were screened for DNA quality (A280/A260>1.8) and sent to the Forsyth Institute, USA for 16S rDNA sequencing and determination of the diversity of oral microbiota in these samples using HOMINGS, which employs a high throughput barcoded Next Generation Sequencing approach to characterise the oral microbiome. The sequencing method involved amplifying the V3-V4 region of 16S rDNA in each sample, using custom 341F-806R primers, with each sample library tagged with a unique barcode. The samples were then multiplexed and sequenced using a 2x250bp paired-end read methodology applied by the Illumina Miseq sequencer (Illumina, San Diego, USA).

2.2.2.1 Bioinformatic methodology

The output sequence data was demultiplexed and a high pass filter applied to the sequences (quality>20). The resulting paired-end sequences were then merged using the online tool *fastq-join* in the web based platform *Galaxy*, with a minimum overlap of 70bp between the forward and reverse reads specified (Goecks et al. 2010). The merged sequences were then binned to Human Oral Taxa (HOT) based upon 638 species-level

and 129 genus-level unique target sequences based on the 16S reference library found in the online Human Oral Microbiome Database (HOMD). Assigning sequences to HOT was carried out by a custom R-script (ProbeSeq) programmed to analyse the sequences as follows:

1) Each sequence was searched for exact matches of the species-level probe sequences; the sequence was counted if only one probe sequence was found, discarded if multiple were found, or passed to Step 2 if none was found
2) The read was searched for genus-level probe sequences and counted if only one probe sequence was matched.

The relative abundances of unique sequences whether assigned to species level or genus level taxa were then calculated.

The merged sequences supplied by Forsyth Institute were also independently analysed using a separate web platform named Visualisation and Analysis of Microbial Population Structures (VAMPS) that presents a web based operation of bioinformatic sequence processing tools on phylotype or operational taxonomic unit approaches (Huse et al. 2014). Using VAMPS, the Ribosomal Database Project classifier was employed to bin sequences to genus-level phylotypes and the resulting data used to calculate diversity metrics such as Shannon diversity, Simpson diversity, Inverse Simpson diversity, Observed species richness, Abundance Coverage Estimator and Chao I.

The Quantitative Insights In to Microbial Ecology (QIIME) pipeline available in VAMPS was also utilized to perform analyses based on OTUs, mainly to generate rarefaction curves to assess the depth of sequencing required to describe the observed diversity in each niche. OTUs were picked using the UClust tool with a 97% sequence similarity cut off for sequence clustering. The OTUs were aligned using the PyNAST tool, with a minimum sequence length set as 60% of median input sequence length and minimum percent sequence identity as 75%. Taxonomy was assigned to OTUs using RDP (Confidence=0.8), and a phylogenetic tree built using the *fasttree* script. Based on OTUs, beta diversity analyses were conducted, using Bray-Curtis, Canberra, Kulczynski, Weighted UniFrac and Jaccard distance matrices and Principal Coordinate plots generated from these matrices to visualize differences between cohorts and niches.

Putative VSC producing species were identified by searching annotated genomes available on HOMD and NCBI databases, using key words ‘methionine gamma lyase’, ‘cystathionine gamma synthase’, ‘cystathionine beta lyase’ and ‘cystathionine’. All

protein hits displayed were then screened for the conserved amino acid sequence that determines affinity for cysteine or methionine namely, YGG or YGC or LYGCT or YGGS, with the cysteine residue indicating methionine gamma lyase activity (Sato & Nozaki 2009; Ferla & Patrick 2014).

2.2.2.2 Multivariate analytic methodology

Multivariate analyses of the data output of HOMINGS in the form of relative abundances of the species detected were carried out using a Microsoft Excel add-in, Multibase package (Numerical Dynamics, Japan). Differences between niches within each cohort were visualised using Principal Component Analysis. The raw data were not scaled but the average of each variable from all samples centered to the origin. The null hypothesis being, there is no difference in the community composition of the different niches within a cohort that share similar clinically measured periodontal parameters. Owing to limited variation observed between cohorts in the same niche, a Multibase implementation of Orthogonal Projections to Latent Structures (OPLS) namely Partial Least Squares Regression-Discriminant Analysis was used to analyse for differences in the microbial ecology (Sæbø et al. 2008). In this instance, the raw data were not scaled but to help visualise shifts in the microbial population structure of the gingivitis and chronic periodontitis cohorts from health, the average of the variables in the healthy cohort were centred to the origin.

2.2.3 Multiplexed Fluorimetric Immunoassay

Twenty analytes including cytokines, chemokines and adhesion molecules involved in human inflammatory response were detected in gingival crevicular fluid and salivary supernatant samples employing a multiplexed fluorimetric bead immunoassay (FlowCytomix Human Inflammation 20plex, eBioscience, San Diego, USA) as analysed using a flow cytometer (BD FACS Canto II, Becton Dickinson Biosciences, San Jose, USA).

Samples were prepared according to the kit manufacturer's protocol using the reagents provided. Briefly, a standard solution series was prepared using the lyophilized standard provided, while thawing the clinical samples. A homogenized stock solution of the provided bead mixture was prepared utilizing buffer solutions provided as diluents, and 25µl of this bead mixture was added to sample, standard or blank solutions in round bottomed flow cytometry test tubes (BD Falcon), adding 50µl of the provided Biotin-Conjugate solution mixture, and incubating at room temperature for 2 hours, protecting

from light. The tubes were washed by adding 1ml of the provided assay buffer and centrifuging at 200 *g* for 5 mins. The supernatant was carefully discarded, leaving 100µl of liquid in each tube, and repeating this again after which 50µl of the prepared diluted Streptavidin-PE solution was added to all tubes, mixing the contents well and incubating for 1 hour. After this incubation, the tubes were washed twice as described above and finally 500µl of the assay buffer was added to each tube, and the tubes then analysed using the flow cytometer set up according to the manufacturer's instructions. Standard curves were constructed utilizing a five-parameter logistic model and the unknown analyte concentration in the samples calculated using the software provided by the manufacturer (FlowCytomix Pro 3.0; eBioscience, San Diego, USA).

2.2.3.1 Multivariate analysis

Multivariate OPLS analysis was carried out as described in section 2.2.2.2, except the data were scaled according to observed values and not centered, to visualize the changes observed in the different cohorts within the cytokine concentration ranges observed. Variables associated with particular cohorts were separately analysed via non-parametric Kruskal-Wallis tests with Dunn's multiple comparisons to explore significant differences.

2.2.4 Gas chromatographic headspace analysis

The Gas Chromatograph (Agilent G6890N, Agilent Technologies, Edinburgh, UK) was calibrated by gas standards of H_2S and CH_3SH generated from permeation tubes by a gas standards generator (Kin-tek 491M).

The headspace of each vial was sampled manually using a gas tight syringe (NORM-JECT®, Henke-Sass Wolf) after incubating at 80°C for 2 minutes. 2 ml of the headspace samples were introduced on to a Chromosil 330 packed column (8' x 1/8" OD Teflon® (FEP) tubing with central 6' packed) through a sulfinert-treated sample loop connected to a sampling valve with helium carrier gas flowing through the column at a constant flow rate of 45ml/min, via an inlet at 120°C. The packed column was maintained throughout the runs isothermally at 60°C. The Flame Photometric Detector was maintained at 175°C with H_2 and air flow at 50 ml/min and 75 ml/min respectively, with N_2 makeup gas at flow rate of 15ml/min.

2.2.4.1 Method development for analysis of gingival crevicular fluid

In order to construct a standard curve that simulated GCF sampling and the manual headspace sampling method, a dilution range was made up from stock solutions of sodium sulfide nonahydrate (Sigma; 290mM) and sodium thiomethoxide (Sigma; 230mM) and either the absorbent paper points or filter paper strips were dipped 5mm below the meniscus of a 2ml aliquot of each standard dilutions for 10s and then sealed within the gas chromatographic headspace vials (glass crimp top vials with 300µl inserts; Supelco, Sigma). It was determined that each absorbent paper point took up approximately 1.3µg (n=20) of fluid and therefore three paper points were deemed necessary to bring headspace levels of VSCs above detection limits at the lower end of the dilution range (5-10µM). However, only one filter paper strip (Whatman 3MM or Pericol) was necessary to absorb a similar amount of fluid in the same time. This determined the number of each of the devices used in collection of clinical samples. All samples were acidified by injecting 40µl of 1M Phosphoric acid (Sigma) on top of the paper points or strips in the sealed vials, before sampling headspace.

In order to determine the varying absorbing capacity of the filter strips or paper points, 5µl of the standard dilutions were pipetted into headspace vials, and either three paper points or one filter strip added to triplicate vials before sealing with crimp caps and acidified before headspace analysis.

2.2.4.2 Analysis of samples incubated with cysteine & methionine

20µl aliquots of the clinical samples namely saliva, tongue biofilm, subgingival plaque, supragingival plaque and interdental plaque were incubated with amino acid substrate solutions separately to determine the amount of H_2S or CH_3SH produced in the headspace from each sample. Substrates used were L-cysteine (Sigma) or L-methionine (Sigma) at 0.5% w/v in TE buffer (pH 7.4), and incubation times were 1 hour and 24 hours, respectively at 37°C. Each 10ml GC headspace vial contained 20µl of the clinical sample in 0.5ml of TE buffer solution, added to 0.5ml of substrate solution along with 0.5ml of TE. The vials were sealed immediately with an aerobic headspace and incubated with gentle shaking (~80 rpm), before adding 1ml of absolute ethanol (Fluka) to each vial using a syringe to halt bacterial metabolism of the substrate. The headspace of the vials were sampled and analysed for VSCs by GC-FPD as described above in 2.2.4, except a 250µl sample loop was used for H_2S detection and a 1ml sample loop for CH_3SH detection in this experiment.

Protein concentrations of the clinical samples used for this analysis were determined using the colorimetric bicinchoninic acid assay according to the manufacturer's protocol in a microplate format using the supplied albumin protein standards (Pierce BCA Assay, ThermoFisher Scientific, Waltham, USA).

2.3 Biofilm Coculture Model

The Biofilm Co-culture Model is an *in vitro* model enabling study of host pathogen interactions, and the model used in this thesis has been developed in the Glasgow Dental School (Millhouse 2015). This model involves growing a consortium of ten oral bacterial species as biofilms on cover slips, and using this biofilm to stimulate a monolayer of oral cells in multiwell plates (Figure 2-4). This enables study of mRNA and cytokine expression of the cells in response to biofilm challenge. Additionally, this model was applied in this thesis to also study the changes in biofilm composition, when a methanethiol negative mutant *P. gingivalis* strain was substituted for the wild type strain.

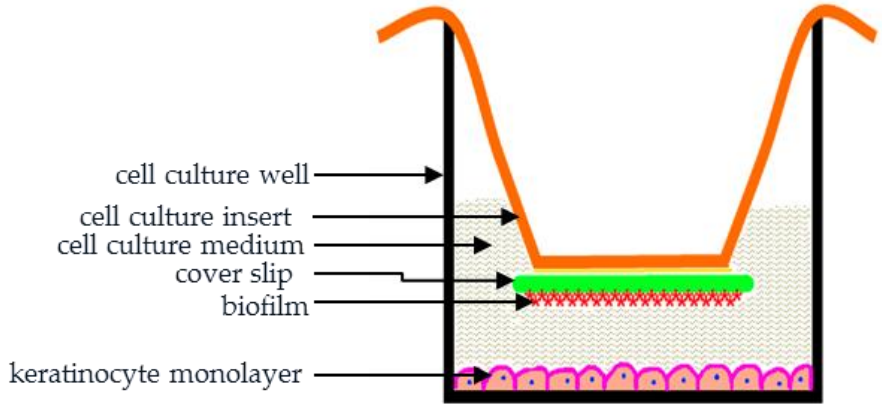


Figure 2-2 showing a schematic of the biofilm co-culture model employed in the study of the role of methanethiol production in host-biofilm interactions.

2.3.1 Mutagenetic manipulation of *P. gingivalis*

Porphyromonas gingivalis W50 was maintained on Fastidious Anaerobe agar (+5% v/v defibrinated horse blood; FAA) in an anaerobic atmosphere (80% N2, 10% H2 and 10% CO2) at 37℃. Liquid culture of *P. gingivalis* was carried out in Brain Heart Infusion (3.7% w/v) broth supplemented with 0.0005% w/v Haemin.

2.3.1.1 ORF identification, validation and DNA manipulation

The open reading frame coding for methionine gamma lyase (*mgl*) in *P. gingivalis* W50 was identified as per (Yoshimura et al. 2000). The ORF was identified and amplified by

using the following oligonucleotide primers (irrelevant sequence in italics and restriction enzyme recognition sequence in bold):

PG0343F1 -- 5'-CATAGACGATCCTCGGTCG-3'

PG0343R1 -- 5'-*atatat***gagctc**ATATTGGGGTTGGCCGGAG-3' (SstI)

PG0343F2 -- 5'-*atatat***tctaga**TCACGGGGGCCAATATGAG-3' (XbaI)

PG0343R2 -- 5'-TGTCCTCCACGTTCTCCAG -3'

The amplicons were purified (Qiagen) and their sizes were confirmed by agarose gel electrophoresis. The purified amplicons were digested with their respective Sstl and XbaI enzymes (New England Biolabs, Ipswich, USA). The digested amplicons were then purified and ligated to a pre-digested *ermF-ermAM* cassette from the plasmid pVA2198. The ligation mixture was then purified and the product reamplified (ReddyMix Extensor PCR MasterMix 1, ThermoFisher Scientific), with the resulting ~3kb fragment used to electroporate exponential *P. gingivalis* W50 cells (Bio-Rad Gene Pulser, Bio-Rad Laboratories, Hercules, USA) to promote mutagenesis by allele exchange. Following overnight recovery under anaerobic conditions, the cell suspension aliquots were plated on to Blood Agar (containing 5µg/ml clindamycin-HCl) and incubation was allowed to continue, anaerobically. The resultant colonies were screened by PCR (ReddyMix Extensor PCR MasterMix 1) to confirm incorporation of the *ermF-ermAM* cassette. Mutagenesis was further confirmed by incubating suspensions of *P. gingivalis* W50 or mutant strain in headspace vials with L-methionine for 1 hour and analysing the headspace for the presence of methanethiol according to the method discussed in 2.2.4.2. The mutant strain will henceforth be referred to as PG343.

2.3.2 Growth of the ten species biofilms

Agar used in culture of organisms were Fastidious Anaerobe agar (+5% v/v defibrinated horse blood; FAA) and Columbia Blood agar (+5% v/v defibrinated horse blood; CBA). Broths used were Schaedler's Anaerobic broth (SCH), Brain Heart Infusion broth (BHI) and Tryptic Soy broth (+0.8% w/v glucose and 0.6% w/v yeast extract; TSB). In maintaining and building up the 10-species biofilms, the medium Artificial Saliva (AS; (Millhouse et al. 2014) was exclusively used.

Bacterial strains were: *Porphyromonas gingivalis* W50 (FAA; SCH), *Streptococcus mitis* NCTC 12261 (CBA; TSB), *Streptococcus intermedius* DSM 20573 (CBA; TSB), *Streptococcus oralis* NCTC 11427 (CBA; TSB), *Aggregatibacter*

actinomycetemcomitans ATCC 43718 (CBA; TSB), *Veillonella dispar* NCTC 11831 (FAA; BHI), *Actinomyces naeslundii* DSM 17233 (FAA; BHI), *Fusobacterium nucleatum* ssp *polymorphum* ATCC 10953 (FAA; SCH), *Fusobacterium nucleatum* ssp *vincentii* DSM 19507 (FAA; SCH), *Prevotella intermedia* DSM 20706 (FAA; BHI).

Streptococcus spp. and *A. actinomycetemcomitans* were maintained and cultured in an aerobic atmosphere containing 5% CO_2, whilst all other strains were maintained in an anaerobic atmosphere (80% N_2, 10% H_2 and 10% CO_2) at 37℃.

Ten-species biofilms were built up on Thermanox™ (Nalge Nunc International, Rochester, USA) coverslips by the following method: 24 hr agar cultures of *S. mitis, S. intermedius* and *S. oralis* were inoculated into 10ml TSB and grown for 1 day, when the cells were harvested by centrifugation (10 min at 3000 rpm) and washed in sterile PBS (pH 7.2±0.2), twice, making suspensions at OD550 equal to 0.5. These suspensions were diluted in AS to the optical density equivalent of 10^7 CFU/ml each into a total volume of 500µL AS, which is added on the coverslip resting in a 24 well plate and cultured in a 5% CO_2 atmosphere at 37°C, overnight.

On the second day, 48 hr broth cultures of *V. dispar* (BHI), *A. naeslundii* (BHI), *F. nucleatum* ssp *polymorphum* (SCH) and *F. nucleatum* ssp *vincentii* (SCH) were harvested and standardised as described above in AS. The spent AS supernatant from the previous day was removed and 500 µL AS suspension containing the newly standardised bacteria was added to the well plate and incubated anaerobically overnight at 37°C.

Finally, 48 hr broth cultures of *P. gingivalis* (SCH), *P. intermedia* (BHI) and *A. actinomycetemcomitans* (TSB) were harvested and standardized as above to make a mixed bacterial suspension in AS, 500µL of which was added to the overnight biofilm culture after removing the spent AS supernatant. This culture was allowed to grow for 4 days with renewal of the AS medium every 24 hrs. After this maturation phase, the AS supernatant was removed and the biofilms stored at -80°C till use.

2.3.3 Keratinocyte growth and co-stimulation

The immortalized oral keratinocyte cell line OKF6-TERT2 (Rheinwald Laboratory, Brigham and Woman's Hospital, Boston, USA) was grown as a monolayer in 24-well cell culture plates and challenged with the 10-species biofilms grown with the wild type or mutant *P. gingivalis* for 4h and 24h as described previously (Millhouse et al. 2014).

Six stimulations were performed on three occasions using independent batches of biofilms, with every two supernatant samples pooled to obtain triplicate samples per batch.

2.3.4 Sample analyses

Three techniques were employed to analyse samples collected from the model:

- qPCR assays of DNA extracted from bacterial biofilms
- Fluorimetric multiplex cytokine assay on the cell culture supernatants
- mRNA expression of cells by employing a qPCR array

2.3.4.1 Bacterial biofilm composition

Triplicate biofilms grown thrice independently, were used for the biofilm composition study. Biofilms on the Thermanox coverslips were revived in AS overnight and washed with PBS (pH 7.2±0.2) thrice before biofilm disruption by sonication for 10 minutes in PBS. DNA extraction from the recovered sonicate was performed as per instructions from the MasterPure GramPositive DNA purification kit (Epicentre Biotechnologies, Madison, USA; see also section 2.2.1.1)

qPCR was performed by using a SYBR Green I fluorophore (Roche) in the LightCycler 480 (Roche). Assays were performed in 20µL total volume per well in 96 well plates. Recommended thermal cycling conditions were used: initial denaturation at 95℃ for 5 mins followed by 40 cycles of 3s at 95℃, 30s at 55℃, 2s at 72℃ and 10s at 76℃. Standard curves were obtained by running decimal dilutions of bacterial DNA extracted from pure cultures with known CFU/ml. Primer pairs used in detection of the species used in the biofilm model are given in table below.

Species	Primer Sequences	Reference
Aggregatibacter actinomycetemcomitans	F: GAACCTTACCTACTCTTGACATCCGAA R: TGCAGCACCTGTCTCAAAGC	(van der Reijden et al. 2010)
Fusobacterium spp.	F: AAGCGCGTCTAGGTGGTTATGT R: TGTAGTTCCGCTTACCTCTCCAG	(Sánchez et al. 2014)
Actinomyces naeslundii	F: GGCTGCGATACCGTGAGG R: TCTGCGATTACTAGCGACTCC	(Periasamy et al. 2009)
Porphyromonas gingivalis	F: GGAAGAGAAGACCGTAGCACAAGGA R: GAGTAGGCGAAACGTCCATCAGGTC	(Park et al. 2011)
Veillonella dispar	F: CCGTGATGGGATGGAAACTGC R: CCTTCGCCACTGGTGTTCTTC	(Periasamy & Kolenbrander 2009)
Prevotella intermedia	F: CGGTCTGTTAAGCGTGTTGTG R: CACCATGAATTCCGCATACG	(Loozen et al. 2011)
Streptococcus spp.	F: GATACATAGCCGACCTGAG R: CCATTGCCGAAGATTCC	(Periasamy et al. 2009)

Table 2-3 listing qPCR primers used to detect the species present in the 10-species biofilm co-culture model.

2.3.4.2 Cell supernatant cytokine analysis

Cell culture supernatants were collected at the end of biofilm stimulation and cytokines measured in 100µL of each sample by a multiplexed bead immunoassay (FlowCytomix, eBioscience) whereby the samples were prepared according to the kit manufacturer's instructions (see section 2.2.3). Standard curves were setup with the reagents supplied by the manufacturer, with the samples and standards analysed with a flow cytometer (BD FACSCanto II). The raw data was then processed using the kit manufacturer's software (FlowCytomix Pro v3.0) to determine the concentration of twenty cytokines: E-selectin, G-CSF, ICAM-1, TGF-β, IFN-α, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17a, CXCL10, MCP-1, MIP-1α, MIP-1β and TNF-α.

2.3.4.3 Determination of mRNA expression by a custom qPCR array

After collection of the cell culture supernatant, the monolayer of cells were lysed and RNA extracted using the RNeasy kit (Qiagen), using the provided spin columns, and performing on-column DNase digestion (Qiagen). Equimolar quantities of RNA (280 ng) from all samples were converted to cDNA with the RT2 First-Strand kit (Qiagen) and then rt-PCR was conducted in a custom 384-well array format in the LightCycler 480 (Roche) with RT2 SYBR Green qPCR MasterMix (Qiagen). Cytokines measured were TNF-alpha, IL-1alpha, IL-6, IL-8, IL-18, IL-13, MIF, CCL20 and CXCL10. Reference genes used to normalize measured Cp values were Actin-β, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase 1 (HPRT1). Relative gene expression was calculated and expressed as ΔCt relative to the

most stable reference gene, while genomic DNA contamination, RT-PCR efficiency and positive PCR controls run for each sample in the array.

2.4 Statistical methods

All statistical analyses were performed using the software GraphPad Prism 6.0 (GraphPad Software Inc, La Jolle, USA) for Microsoft® Windows. Comparisons between cohorts or niches given more than two groups of data (health/gingivitis/periodontitis or saliva/tongue/subgingival/supragingival/interdental), unless specified were always performed via the non-parametric Kruskal-Wallis test, with the posthoc Dunn's multiple comparison with multiplicity adjusted P-values to test for differences in multiple comparisons between any two group combinations. Given two groups of data, the Mann-Whitney U test was used to test differences in medians and the Kolmogorov-Smirnov test used to analyse for differences in the cumulative distributions of the data sets. All correlations were explored using the non-parametric Spearman's correlation.

3 CLINICAL INVESTIGATIONS INTO THE RELATIONSHIP BETWEEN VSCS AND PERIODONTITIS

The association between volatile sulfur compounds in the breath and periodontitis has been extensively reported in different regional populations of the world, in both adults and children (Stamou et al. 2005; Nadanovsky et al. 2007; Eldarrat et al. 2008; Takeuchi et al. 2010; Bornstein et al. 2009; Nalçaci & Sönmez 2008; Kara et al. 2006; Liu et al. 2006; Quirynen et al. 2009). The principal deductions from these studies has been that the prevalence and intensity of oral malodour in individuals with clinically diagnosed periodontitis or gingivits is higher than that of healthy individuals, and this observation holds despite a range of methodological differences in assessing malodour, whether hedonic methods or more objective sulfide monitors were used. Studies have also explored the microbial link between malodour and periodontitis, by measuring putative periodontopathogens in tongue and periodontal niches with qPCR methods and found an association between periodontopathic bacteria in healthy individuals and chronic periodontitis patients with malodour and VSC concentrations in the breath (Amou et al. 2014; Figueiredo et al. 2002; Kurata et al. 2008; Yasukawa et al. 2010). Whilst studies that focussed on oral malodour predominantly only included the tongue as a niche of choice, and studies focussing on periodontal disease were more concerned with the periodontal niches such as subgingival and supragingival plaque, studies that combined these two approaches did not have an assessment of inflammation present in the oral cavity through the use of cytokine analysis. Fewer still are studies measuring VSCs in the periodontal niches (Torresyap et al. 2003; Persson 1992). Therefore, the

present study aimed to combine these different approaches including microbial, breath and inflammatory assessments of the oral cavity to explore potential relationships between VSCs present in the breath and periodontal pockets in health and disease and their link with putative periodontopathic bacteria in a range of oral niches and the inflammatory status in saliva and the periodontal pocket.

3.1 Participant and Cohort Demographics

Participants were recruited to three different cohorts based on the clinical assessment of their oral health: health, gingivitis and chronic periodontitis. The following sections describe the clinical characteristics of each cohort and further describe the demographics with regard to age, sex, ethnicity and parameters relevant to the study such as the oral hygiene habits, oral malodour and the self-perception of oral malodour.

3.1.1 Clinical Demographics

Of the 41 participants recruited to the healthy cohort, 21 were identified as having good periodontal health with ≤30% plaque coverage, ≤20% gingival bleeding index (GBI), ≤20% sites bleeding on probing (BOP) and no more than four periodontal pockets deeper than 4mm excluding 3rd molars. Plaque Index (PI) exceeded 30% for 19 individuals (Mean±SD=46.1%±13.8), 17 of whom had BOP and GBI below 20%, and only two had GBI exceeding 20% (Figure 3-2). As discussed in the methodology, in case of conflicting scores between BOP and GBI, the former was given precedence in determining diagnosis. The clinically healthy individuals with high plaque were included in some analyses where necessary such as assessment of inflammation by cytokine analysis and for sequencing the bacterial 16S rDNA in the microbiome studies.

A total of 26 individuals recruited to the study were considered to have gingivitis. The primary clinical criteria used to assign individuals to this cohort were >20% BOP and/or >4 pockets of ≥4mm depth. Of these, 15 participants had >20% BOP whilst 11 participants had more than four periodontal pockets ≥4mm deep but >20% BOP sites. Additionally, 20 of the 26 individuals had plaque coverage >30% (Figure 3-2). The chronic periodontitis cohort had more heterogeneity in terms of the clinical symptoms as measured by BOP, GBI and PI, although all individuals had radiographic evidence of bone loss and had at least 2 periodontal pockets in excess of 5mm (Figure 3-1). All participants recruited to this study had >20 teeth present with means (±SD) of 29±2 in the healthy, 29±2 in gingivitis and 28±2 in patients with chronic periodontitis.

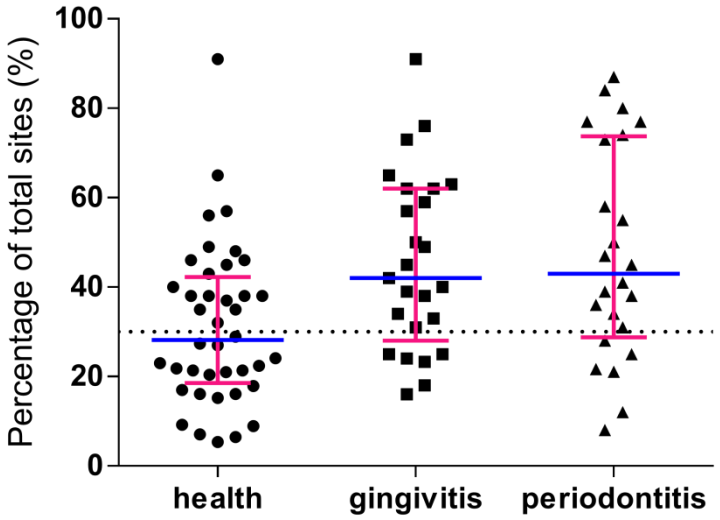


Figure 3-2 showing the distribution of plaque coverage in the healthy, gingivitis and chronic periodontitis cohorts. Median, interquartile range and a line indicating the 30% cut off for classification are indicated.

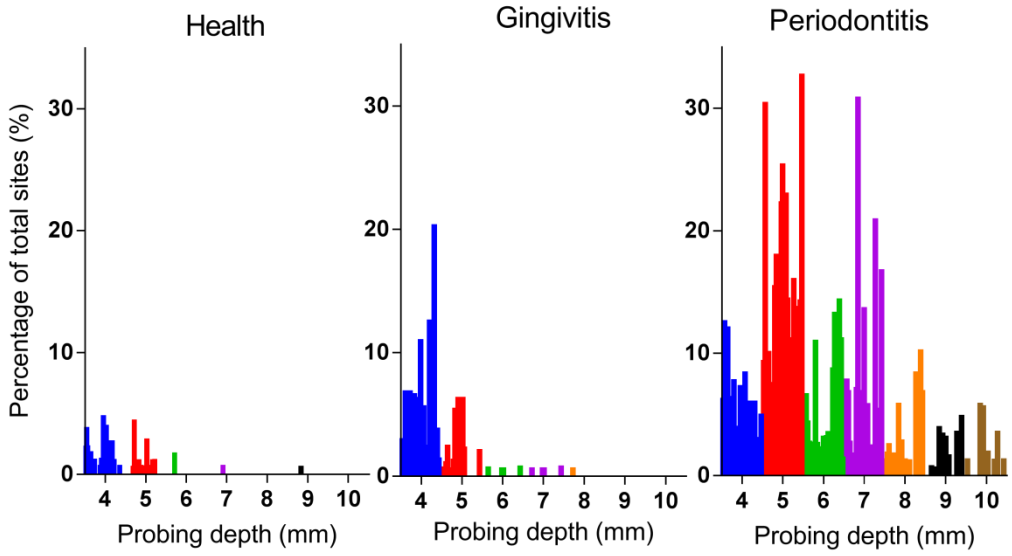


Figure 3-1 showing the distribution of periodontal probing depths ranging 4mm-10mm in the healthy, gingivitis and chronic periodontitis cohorts. Data shown includes 3rd molar probing depths.

3.1.1.1 Age, Gender and Ethnicity

The age ranges in the chronic periodontitis cohort were significantly higher than the healthy or the gingivitis cohort, while the male to female ratio was the highest in the healthy cohort, and the gingivitis cohort having the lowest number of males to females recruited (Table 3-1). In terms of the ethnic diversity of the participants recruited to the cohorts, the most diverse were the healthy and gingivitis cohorts, with individuals of Asian, African and Caucasian ethnicities equally represented in the chronic periodontitis cohort compared to the other cohorts (Figure 3-3).

Cohort	Mean age ±SD (yrs)	Male: Female Ratio
Health	33.17±8.56	1.05
Gingivitis	34.36±9.16	0.60
Chronic Periodontitis	43±7.4	0.85

Table 3-1 listing mean age of the participants in the different cohorts and the ratio of males to females in each cohort.

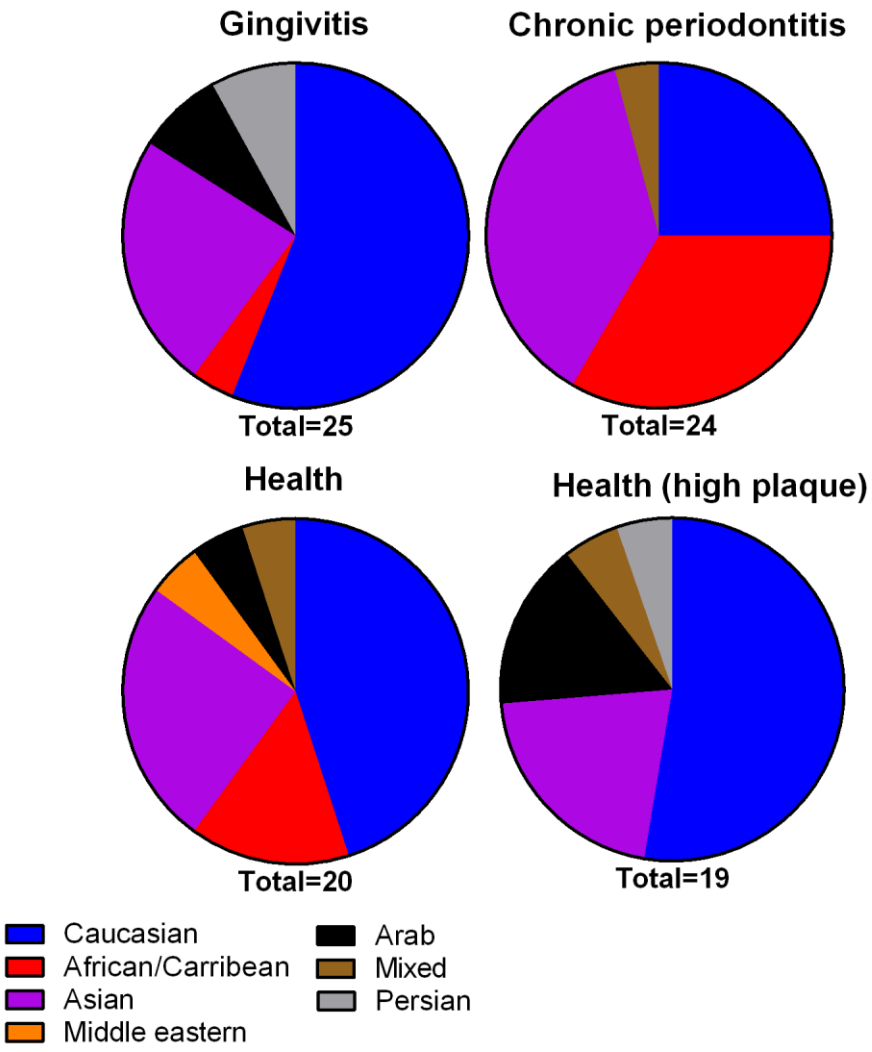


Figure 3-3 showing the composition of the different cohorts of this clinical study in terms of ethnicity.

3.1.1.2 Dental visits and oral hygiene habits

All participants were questioned about their frequency of dental visits with the six-monthly and yearly options popular amongst the healthy and gingivitis cohorts (Figure 3-4). More patients in the chronic periodontitis cohort reported visiting a dental surgery every three months, and this could be because they were from the maintenance clinics. No differences in the dental surgery visiting frequencies were observed between healthy individuals with plaque coverage <30% vs >30%.

There was no difference in the self-reported brushing frequency of individuals in the healthy and gingivitis cohort. As some patients in the chronic periodontitis cohort had

already received some oral hygiene instructions, the self-reported brushing frequency of three or four times a day was higher in this cohort compared to the healthy or gingivitis cohorts (Figure 3-5). The self-reported use of interdental brushes and/or flossing was also higher in the chronic periodontitis cohort compared to health or gingivitis, and it is suspected that these individuals, likely received oral hygiene instructions regarding their periodontal condition in a previous visit (Figure 3-6). Interestingly, a higher proportion of individuals in the healthy cohort reported flossing and/or interdental brushing than the gingivitis cohort. A recent systematic review concluded that there is low quality evidence that interdental brushing and not flossing, in addition to tooth brushing can reduce gingivitis (Poklepovic et al. 2013). However, general awareness of interdental brushing or indeed tongue brushing was low in both the healthy and gingivitis cohorts, and very few reported using both flosses and interdental brushes.

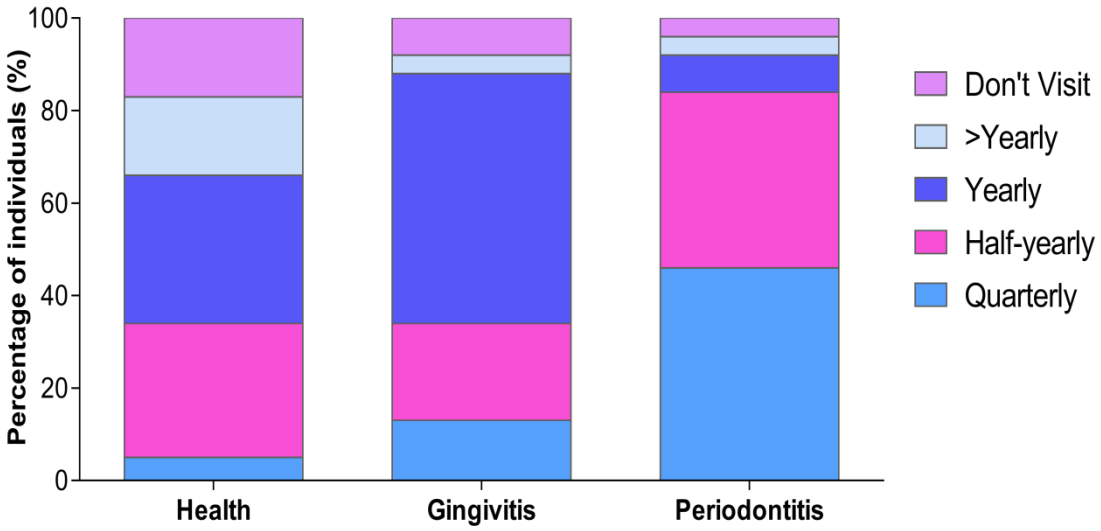


Figure 3-4 showing the dental surgery visiting frequency of individuals recruited to the different cohorts.

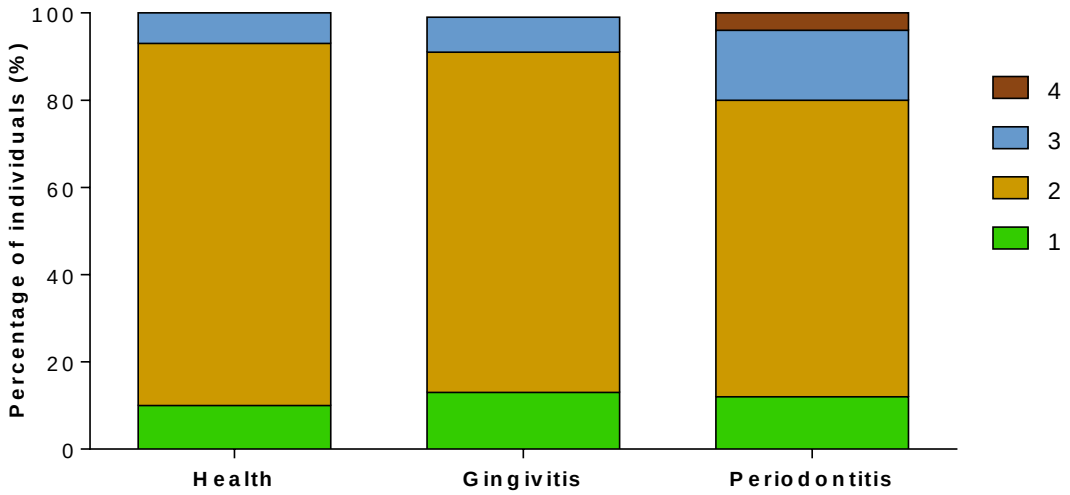


Figure 3-5 showing the brushing frequency per day of the individuals recruited to the different cohorts.

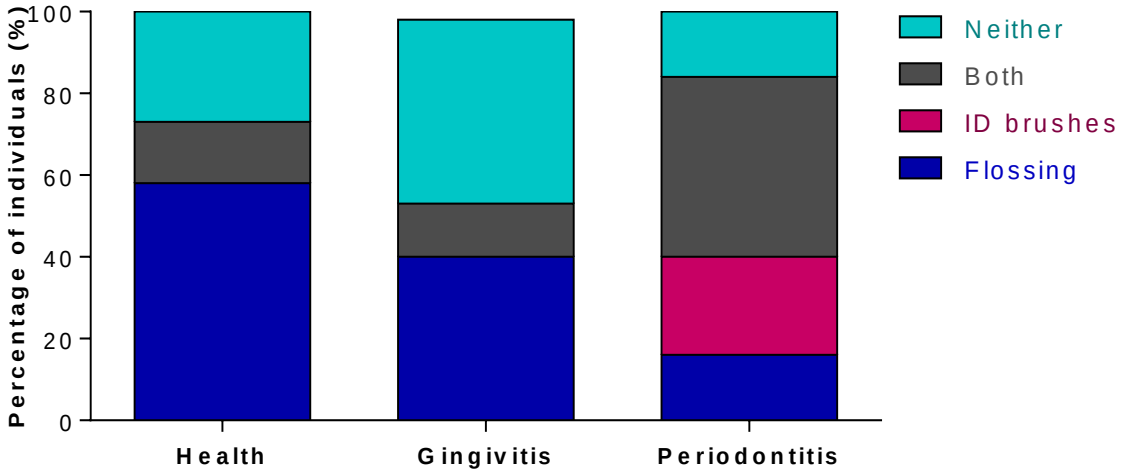


Figure 3-6 showing the reported use of flossing and interdental brushing among the individuals recruited to the healthy, gingivitis and chronic periodontitis cohorts.

Although participants were asked to not perform any kind of oral hygiene before attending the clinic for sample donation, it was endeavoured to ascertain if the participants are using potentially anti-VSC mouthwashes or toothpastes in their day-to-day oral hygiene routines. Participants who used or did not use mouthwashes were equally represented in the health and periodontitis cohorts, with the gingivitis cohort showing a greater proportion of individuals who do not use mouthwashes (Figure 3-7). A small minority in all the cohorts reported using mouthwashes but were unable to name the brand—the lack of awareness may be due to infrequent use of the mouthwash.

A particular observation was the general inability of the individuals across cohorts to name the full product name of the mouthwash. Whilst most of the participants were able to remember the major brand names of products they use such as Listerine®, Sensodyne® or Corsodyl®, a number of products exist within some of these brands that are marketed towards different activities such as anti-malodour, anti-plaque and anti-caries and very few individuals were able to remember the actual product names (Figure 3-8). This was an important consideration to determine if the product used by the individual has zinc containing compounds which display potent anti-VSC activity particularly within the first few hours of product use (Young et al. 2001; Newby et al. 2008; Burnett et al. 2011). More individuals were able to name specific product names for toothpastes, and this perhaps reflects the more common use of toothpastes as an oral hygiene product compared to mouthwashes (Figure 3-9). The responses to these questions were not complete enough to determine if more individuals in the healthy cohort used zinc containing anti-VSC toothpastes and mouthwashes compared to the gingivitis or chronic periodontitis cohorts.

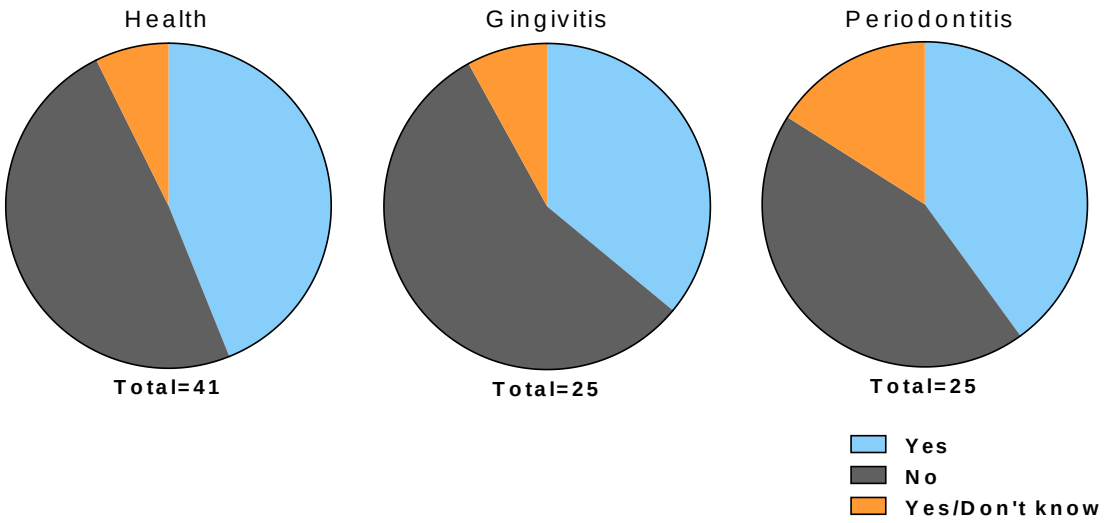


Figure 3-7 pie charts showing the reported use of mouthwashes in the different cohorts.

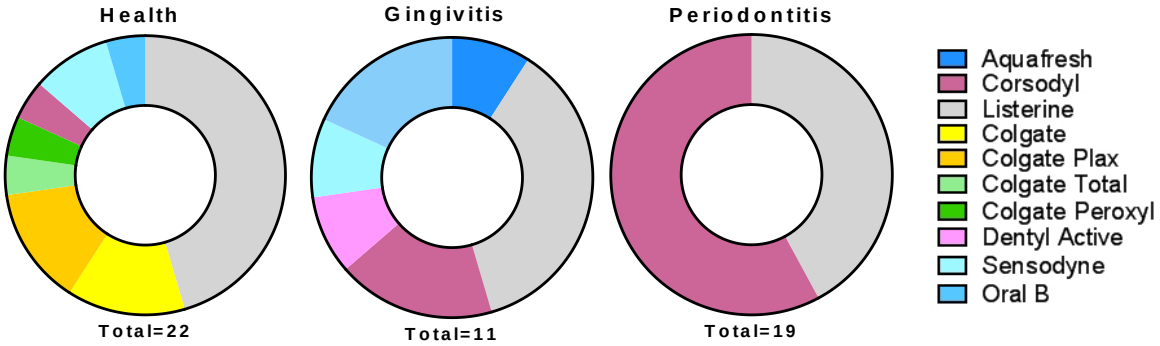


Figure 3-8 showing charts of the use of different mouthwash brands by the individuals recruited to the different cohorts. Legend colours cycle clockwise.

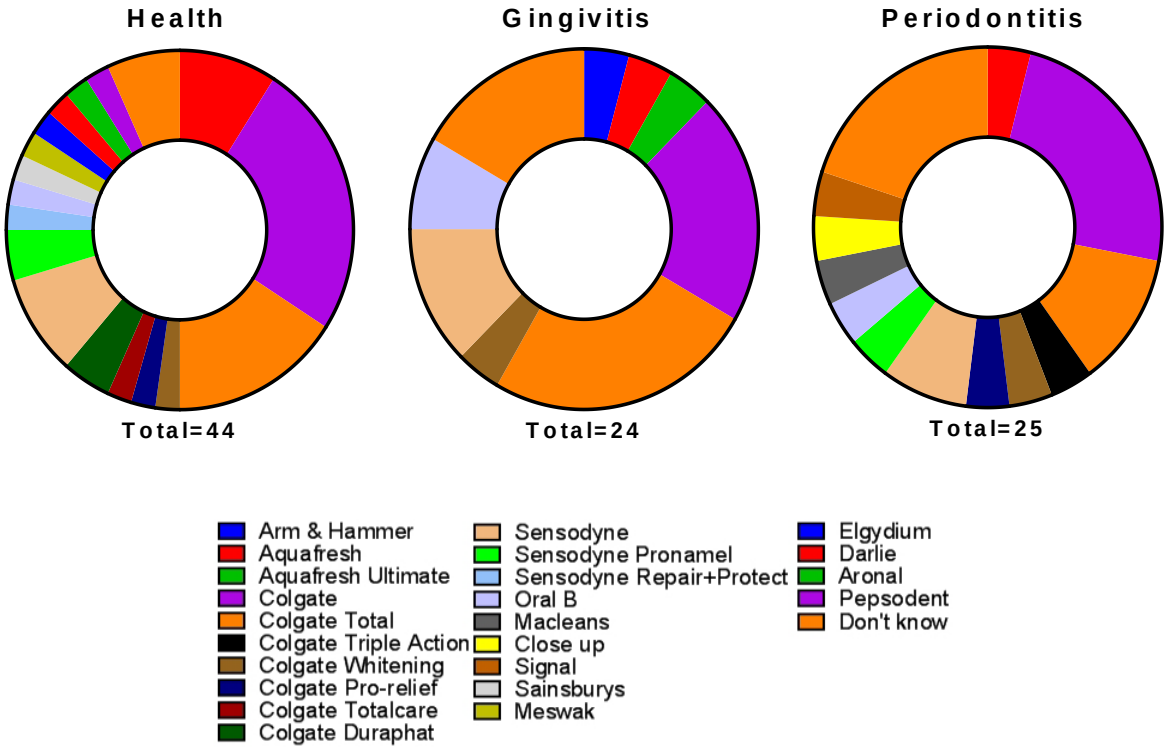


Figure 3-9 showing charts depicting the use of different toothpaste brands in the different cohorts. Legend colours cycle clockwise.

3.2 Breath VSCs and Chronic Periodontitis

VSCs present in the breath of individuals were measured using a portable gas chromatograph, Oral Chroma, which is able to distinguish between three different VSCs namely hydrogen sulfide, methanethiol and dimethyl sulfide. All three gases were generally prevalent (i.e. above detection limits) in the breath samples of individuals classified into the different cohorts, with the prevalence of methanethiol being higher in the gingivitis and chronic periodontitis cohorts compared to health (54% and 71% vs 39%). Elevated concentrations of hydrogen sulfide and methanethiol were observed in the breath of individuals in the chronic periodontitis cohort compared to health, but not between health and gingivitis or gingivitis and chronic periodontitis (Figure 3-10; Figure 3-12). The ratio of CH_3SH: H_2S was also higher in the chronic periodontitis and gingivitis cohorts compared to health, but not between periodontitis and gingivitis (Figure 3-11). Although elevated concentrations of hydrogen sulfide and methanethiol were observed in healthy individuals with >30% plaque coverage compared to <30%, in addition to an increase in the CH_3SH: H_2S ratio, the difference was not significant at $p<0.05$ (Figure 3-13). The Malodour Scores calculated based on the concentrations of hydrogen sulfide and methanethiol measured using Oral Chroma, were significantly elevated in the chronic periodontitis cohort compared to health, but comparisons between health and gingivitis or gingivitis and chronic periodontitis cohorts showed that the differences were not statistically significant (Figure 3-14).

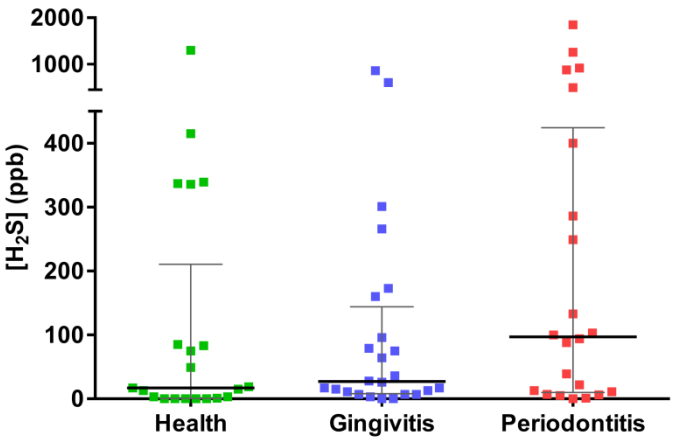


Figure 3-10 showing scatter dot plots of H_2S detected in the breath of individuals in the different cohorts (only individuals with <30% plaque coverage in the healthy cohort are shown). Median and interquartile ranges are indicated.

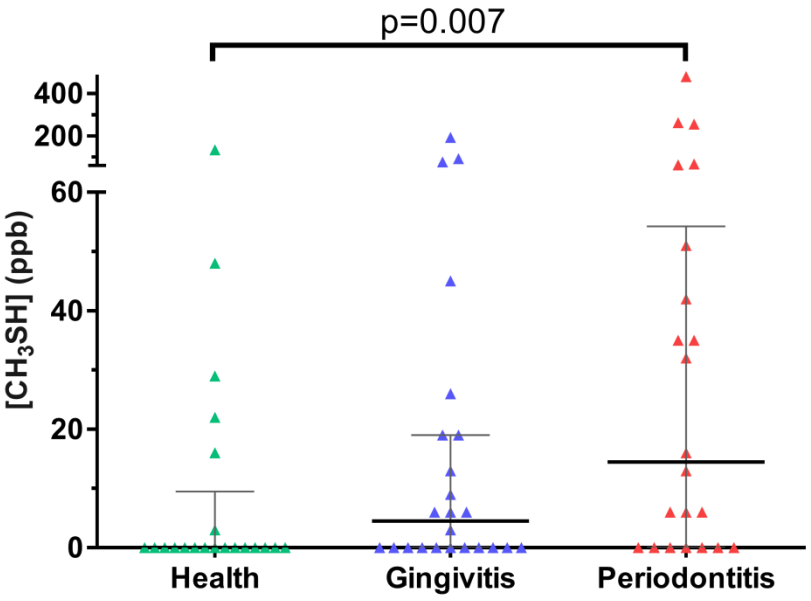


Figure 3-12 showing scatter dot plots of CH_3SH detected in the breath of individuals in the different cohorts (only individuals with <30% plaque coverage in the healthy cohort are shown). Median, interquartile ranges and comparisons that yielded statistical significance are indicated.

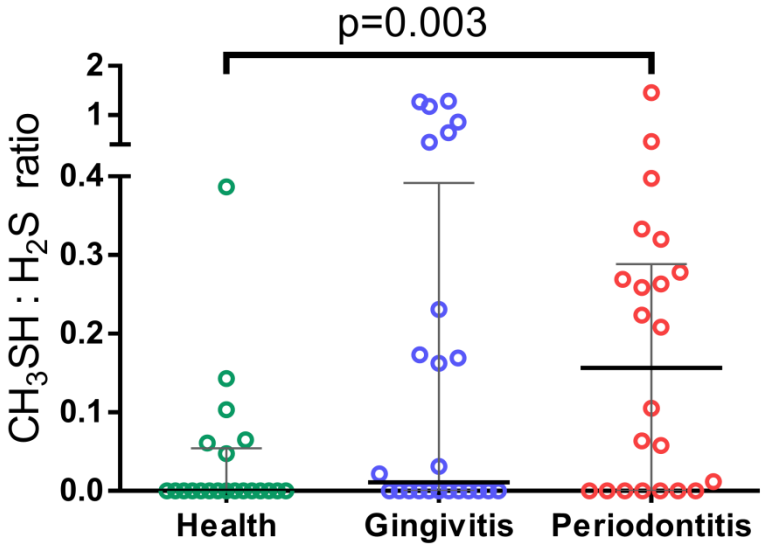


Figure 3-11 showing scatter dot plots of the ratio of CH_3SH: H_2S in the breath of individuals in the different cohorts (only individuals with <30% plaque coverage in the healthy cohort are shown). Median, interquartile ranges and comparisons that yielded statistical significance are indicated.

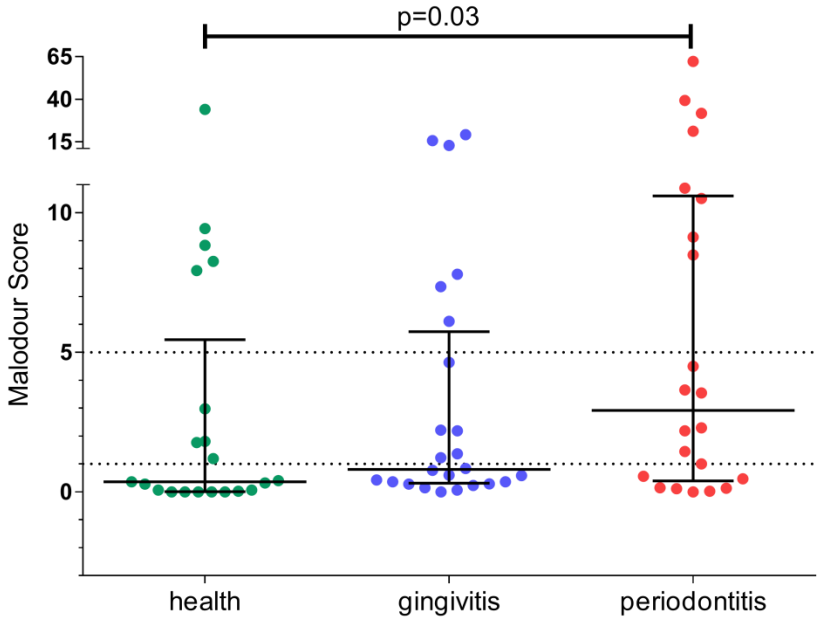


Figure 3-14 showing scatter dot plots of the malodour scores calculated from the breath analysis of individuals in the different cohorts (only individuals with <30% plaque coverage in the healthy cohort are shown). Median, interquartile ranges and comparisons that yielded statistical significance are indicated.

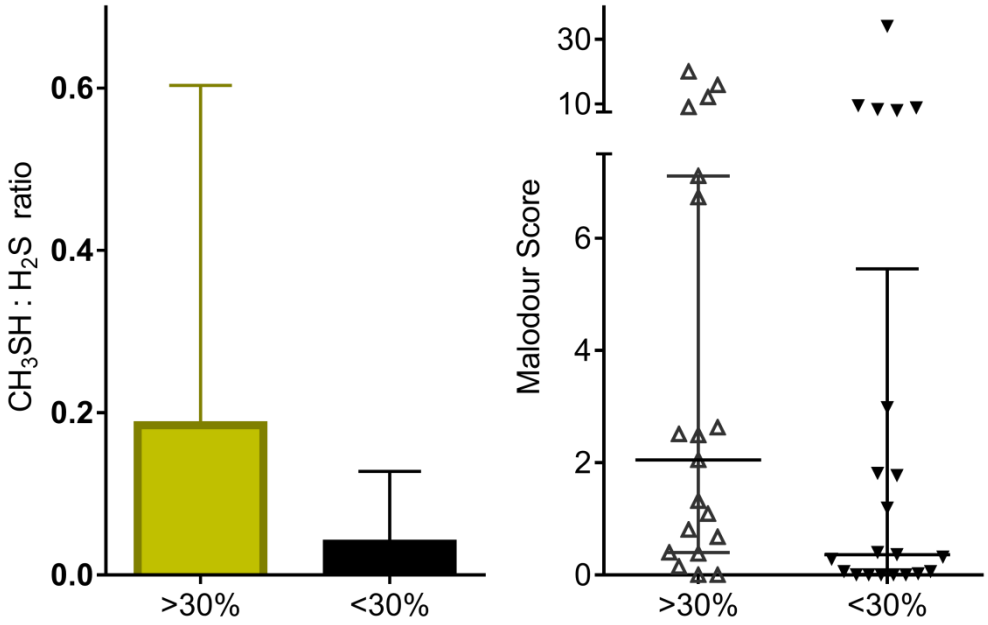


Figure 3-13 Left: Average (±SD) ratio observed in healthy individuals with >30% versus <30% plaque coverage. Right: Malodour scores from the low (<30%) and high (>30%) plaque coverage groups in the healthy cohort. Median and interquartile range indicated.

The CH_3SH: H_2S ratio, malodour score and the concentration of methanethiol are consistent with the findings reported of the association of VSCs with periodontal disease, despite a number of methodological differences (Takeuchi et al. 2010; Tsai et al. 2008). While other studies have reported significantly elevated concentrations of hydrogen sulfide in chronic periodontitis patients compared to health, the current study only found a non-significant increase in the H_2S concentrations (Tsai et al. 2008; Pham et al. 2011). It is possible that this is partly due to the less stringent criteria in terms of plaque coverage (30% vs 20%) or not taking probing depths of the 3rd molar into account when determining diagnosis in the healthy cohort. More importantly, tongue coating was not assessed in this study and the variable tongue coating likely to be present in the healthy cohort could impact on the VSC concentration observed. However, while most other studies aimed to recruit individuals complaining of oral malodour and found an association with periodontal disease in a subset of individuals with malodour, the present study aimed to recruit individuals with disease/health and attempted to ascertain if a greater proportion of individuals with disease had intra-oral malodour associated with increased presence of VSCs in the breath. The findings of the present analyses also confirm that a greater proportion of individuals with disease have increased VSCs in the breath.

An organoleptic assessment of oral malodour was not made in this study, however, the malodour scores calculated from the VSC concentrations serves as a surrogate measurement of malodour as experienced by a human owing to its incorporation of the human recognition threshold in the method. In terms of the relationship between the malodour scores and the organoleptic scale, malodour scores of 1-5 are equivalent to 1-2 in a scale of 0-5, and malodour scores above 5 are equivalent to 3 or higher on the organoleptic scale. The degree of malodour as measured by the malodour scores also increased in the disease groups compared to health. For example, the proportion of individuals with malodour scores higher than 1, which represents the cumulative presence of hydrogen sulfide and/or methanethiol at concentrations 10-fold higher than the recognition threshold for each molecule, were similar in health (43%) and gingivitis (46%) but were higher in chronic periodontitis (58%; Figure 3-14). The proportion of individuals with malodour scores higher than 5 were also higher in the chronic periodontitis cohort (33%) compared to health (24%) or gingivitis (23%).

A few studies in the literature report methanethiol being the VSC more associated with periodontal disease, and the elevated concentrations of this VSC and the CH_3SH: H_2S

ratio observed in the chronic periodontitis and gingivitis cohorts compared to health are consistent with these observations (Figure 3-12; Figure 3-11; Yaegaki & Sanada 1992; Sopapornamorn et al. 2007). Interestingly, clinically healthy individuals with high plaque coverage also had elevated CH_3SH: H_2S ratio, in addition to median malodour score >2 suggesting possible increased disease activity in these individuals (Figure 3-13).

Comparison	Rho	95% CI	P (two-tailed)
% sites ≥6mm vs Ratio	0.211	-0.00211 to 0.4058	0.0459
GBI vs CH_3SH	0.257	0.04523 to 0.4466	0.0151
GBI vs Ratio	0.273	0.06266 to 0.4605	0.0096
PI vs CH_3SH	0.229	0.01599 to 0.4229	0.0306

Table 3-2 listing Spearman's rho values for the most significant comparisons between breath VSC measurements and clinical indices (n=90 with all comparisons).

To ascertain if the CH_3SH: H_2S ratio or the concentration of methanethiol in the breath of individuals could identify periodontal disease activity, correlational analyses were conducted with these breath measures and the conventional clinical measures of disease such as plaque index, gingival bleeding index , bleeding on probing and periodontal pocket depth. Confirming this hypothesis, positive correlations were observed between methanethiol concentrations in the breath and the clinical measures namely gingival bleeding index and plaque index when individuals recruited to the different cohorts were pooled together (Table 3-2). Increased CH_3SH: H_2S ratios were also positively associated with higher percentage of periodontal pockets ≥6mm and GBI. No correlations were observed with H_2S concentrations in the breath of individuals. The weak positive correlations observed between these measurements possibly reflects the impact of periodontal conditions on physiological processes involved in oral malodour that are predominantly linked to the tongue, which plays a major role in oral malodour. This would entail that this correlation is likely to indicate the strength of microbial association between the periodontal and tongue niches as it relates to the VSC concentration. A prediction would be that the abundance of methanethiol producing microbial species in the tongue and the periodontal niches would increase in association with periodontal disease as measured by the clinical parameters such as percentage of periodontal pockets ≥6mm, gingival bleeding index and plaque index.

3.2.1 Demographics, oral hygiene habits and malodour

Studies conducted in some regional populations report a positive association between oral malodour and age, while also associating higher prevalence of malodour in males compared to females (Nadanovsky et al. 2007; Quirynen et al. 2009). However, studies in other regional populations do not support these observations (Miyazaki et al. 1995; Liu et al. 2006). In the present study, a simple Spearman correlation analysis of the VSC measurements with age in the different cohorts did not yield statistically significant correlations, but H_2S concentrations in the healthy cohort that included individuals with high plaque coverage approached significance (r=0.256; p two-tailed=0.107; n=41). No significant differences in the VSC measures could be observed in the different cohorts between male and female sexes. However, an elevated CH_3SH: H_2S ratio could be observed in males (mean=0.33±0.4) compared to females (mean=0.098±0.13) in the chronic periodontitis cohort. The possible effect of different ethnicities on the prevalence of VSCs could not be determined owing to low numbers of the different ethnic minorities in each cohort. However, periodontal diseases are often associated with socioeconomic status, more often than not is associated with ethnic minorities and therefore could have a role to play in oral malodour in the ethnic minorities (Rayman & Almas 2008; Mason et al. 2013). While, socioeconomic factors were not ascertained in this study, these are likely to be more normalized for the healthy and gingivitis cohorts as these individuals were recruited from the staff and students of Queen Mary, University of London. No difference was observed in the prevalence of moderate or severe malodour as determined by the VSC measurements in the gingivitis and high plaque healthy cohorts between Caucasian and non-Caucasian ethnicities. However, in healthy individuals with low plaque (<30%), malodour (>1 malodour score) was more prevalent in individuals belonging to Caucasian ethnicities (67% vs 33%), however prevalence of severe malodour (malodour score >5) was higher in ethnic minorities compared to Caucasians (33% vs 17%).

Association of VSCs with brushing frequency could not be determined due to low numbers of participants in the healthy cohort reporting brushing once or thrice a day. In the healthy and gingivitis cohorts, slight to moderate malodour (malodour score >1<5) and severe malodour (malodour score >5) were equally prevalent in individuals who reported using mouthwashes and those who did not. With regards to flossing or interdental brushing, the prevalence of VSCs above the malodour threshold (malodour score >1) in the breath was higher in individuals in the healthy cohort (including high

plaque individuals) who reported flossing alone or in combination with interdental brushing (56%) compared to individuals who used neither (36%). The prevalence of moderate to severe malodour (malodour score >5) was similar in the floss and/or interdental brush using individuals (10 of 30) compared to individuals who used neither (3 of 11). It is possible that flossing or interdental brushing disturbs mature plaque accumulating interdentally, and aids in transmission of microbiota from the interdental space to the tongue and this could impact on malodour. However, this hypothesis could not be tested based on the available data in the present study as the frequency of flossing or interdental brushing was not recorded.

To assess if the frequency of dental visits have an effect on the prevalence of oral malodour, individuals in the healthy and gingivitis cohorts were analysed. Only those individuals who reported visiting their dentist quarterly, half-yearly or yearly were included due to low number of respondents in the other groups. More numbers of individuals who reported visiting their dentist once a year were classified in the gingivitis group (50%) than half-yearly and quarterly visitors (36%). However, while the prevalence of moderate to severe malodour (malodour score >5) was similar in both the groups (23% vs 27%), the prevalence of slight to moderate malodour (malodour score >1<5) was higher in the individuals who report more frequent visits to their dentist (32% vs 8%). It is notable that more clinically healthy individuals with high plaque coverage reported visiting their dentist half-yearly or quarterly (36% vs 23%) and this could partly explain the observed higher prevalence of slight to moderate malodour in this group (Figure 3-13).

3.2.2 Self-perception of oral malodour

Participants of this study were asked if they thought that they had bad breath in the questionnaire, to assess the level of awareness in the different groups, as oral malodour is a condition that affects social interaction (Mckeown 2003). It has been reported that individuals who perceive a worsening of ‘gum health’ are more likely to believe they have oral malodour (Azodo & Ao 2013). Due to the association of periodontal disease with malodour and by extension the concentration of VSCs in the breath, this perception is more likely to be accurate in correlating with VSCs in the breath of individuals. Data from the current study appears to confirm this notion, with almost 75% of chronic periodontitis patients with breath VSCs exceeding the malodour threshold accurately determining that they had oral malodour (Figure 3-15). In contrast, the majority of

individuals in the gingivitis and healthy cohorts whose breath VSC concentrations exceeded the malodour threshold (malodour score >1), did not believe they have oral malodour. This observed perception among the healthy and gingivitis cohort is not likely to be related to the severity of oral malodour, as 28% of individuals who did not believe they had malodour in the gingivitis cohort, would be classified as having moderate to severe malodour (malodour score >5). In addition, the same is true for 43% of the negative responses among healthy individuals with high plaque coverage and 28% of negative responses for healthy individuals with low plaque coverage. A small subset of individuals in all the cohorts, believed they had oral malodour when their breath VSC concentrations were below the malodour threshold (malodour score <1), suggesting a diagnosis of pseudo-halitosis (Seemann et al. 2006). It is thought that if the individual continues to believe that they have oral malodour when objective measurements such as VSC concentrations are shown, it could lead to a more serious psychosomatic disorder namely, halitophobia (Zürcher et al. 2014). However, all individuals in this study who displayed this inaccurate self-perception of pseudo-halitosis were more relieved when shown results from the Oral Chroma analysis that they did not have malodorous breath.

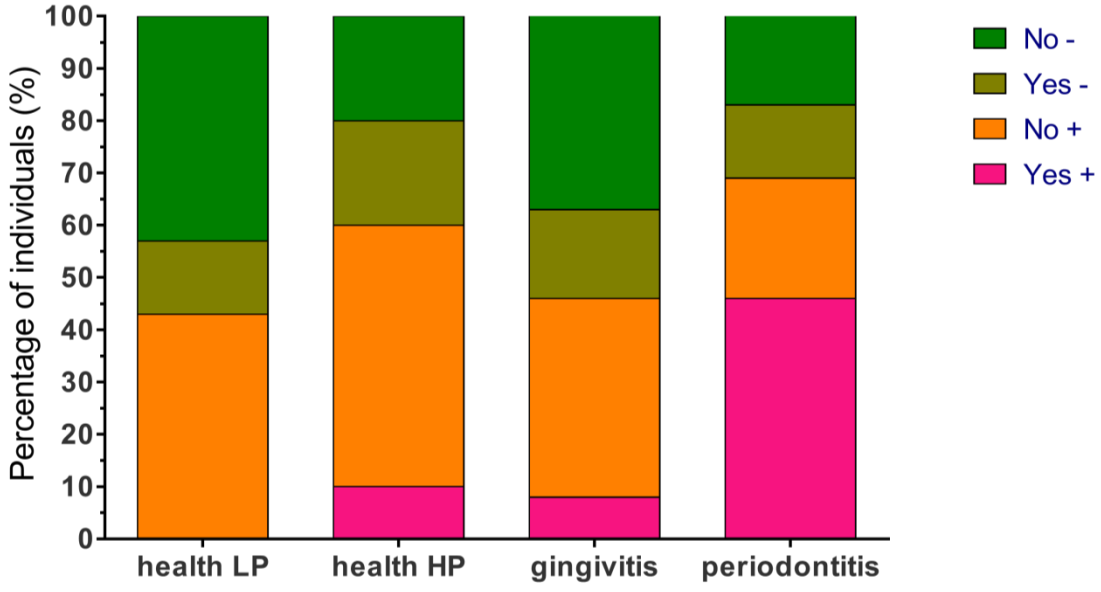


Figure 3-15 showing self-perception of oral malodour among individuals in the different cohorts. LP=<30% PI; HP=>30% PI; Yes/No indicates participants belief of whether they have oral malodour, +/- signs indicate if the VSC concentrations in the breath sample exceeded the malodour threshold.

3.3 Sulcular VSCs and periodontal disease

The association between breath VSCs and periodontal disease is well established, leading investigators to measure VSCs in the periodontal environment to determine if VSCs in these niches correlate with greater disease activity (Torresyap et al. 2003; Persson 1992). These studies took different methodological approaches to measuring VSCs. Torresyap et al (2003) utilized a non-specific 'sulfide' sensor incorporated into a periodontal probe to measure amount of sulfide and hydrosulfide ions in the periodontal pocket whereas Persson (1992) collected GCF via filter paper strips placed in sealed gas chromatographic vials, and detected VSCs in the headspace after acidification of the strips using sulfur specific gas chromatography. Whilst the former methodology is advantageous in measuring sulfide ions *in situ*, its lack of specificity is a limitation, especially given that the VSC methanethiol in the breath was more associated with periodontal disease than hydrogen sulfide. The latter method was chosen in the current study to detect VSCs in the gingival sulci, owing to its greater specificity.

3.3.1 Notes from method development

Initially, a few changes to the published methodology were evaluated, including GCF sampling paper and the gas chromatographic method employed. Due to the anticipated very low amounts of VSCs recovered from GCF, a capillary column method with sulfur specific photometric detection after headspace sampling using a thermal desorber was evaluated. Gas standards generated from permeation tubes were used in evaluation of the thermal desorption compared to direct injection, and reduced recovery of H_2S and CH_3SH was observed in terms of their expected peak heights relative to dimethyl sulfide standards with the thermal desorber compared to direct injection (Figure 3-16). Due to this lowered assay sensitivity for the two main VSCs previously detected by Persson et al (1992) in GCF, a manual injection method was followed for subsequent GC analyses, with high sample volume (2ml) injections via a sulfinert sample loop on to a packed column to enable maximum available sensitivity in this method.

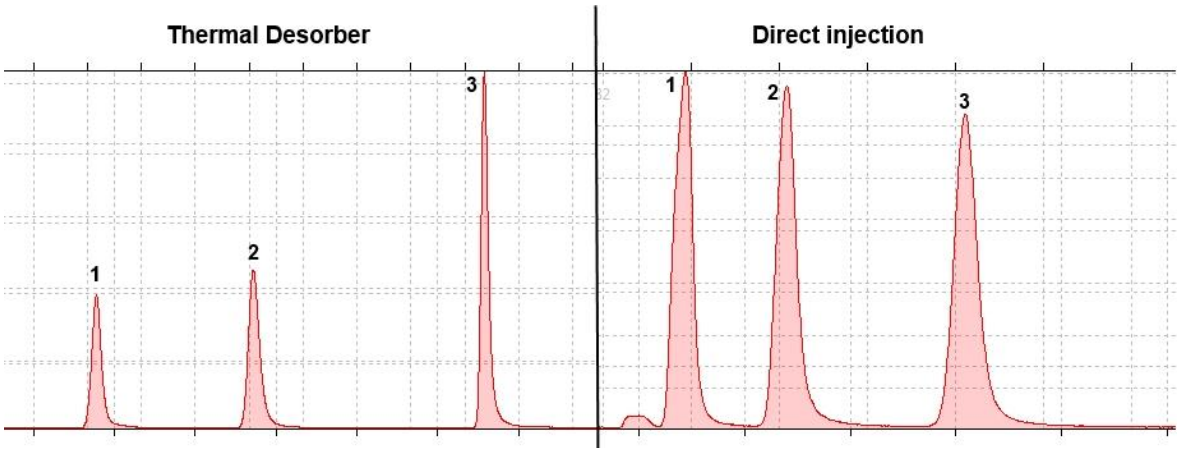


Figure 3-16 showing chromatograms of gas standards obtained using an injection from the thermal desorber (left) and directly via a sample loop (right). Labelled peaks: 1=H_2S; 2=CH_3SH; 3=$(CH_3)_2S$.

Filter paper sampling is the preferred method for collecting GCF to carry out protein or cytokine analyses and Persson et al (1992) used the Whatman No. 3 chromatography paper to sample GCF for headspace analysis, in contrast to another paper namely Whatman 3MM that was previously shown to yield good recovery of protein (Griffiths et al. 1988). Whilst the Whatman 3MM filter papers have to be cut manually to dimensions leading to some variability in size, they were also found to be less amenable in practice for collecting GCF from the more healthy participants owing to the thickness of the paper and the smaller gap or periodontal probing depths in these individuals. Absorbent paper points were more convenient in this regard, as they are precut, rolled and shaped like a point, as were Periocol strips which are thinner filter paper strips than Whatman 3MM, with a curved end enabling easier insertion in to gingival sulci of probing depths 1-4mm. Whilst recognizing that Persson et al. (1992) used gas standards generated from permeation tubes to measure VSCs in headspace of GCF samples, attempting a simulation of the GCF sampling method for calibration purposes using standard solutions of sodium sulfide (Na_2S) and sodium thiomethoxide (CH_3SNa) to generate H_2S and CH_3SH in the headspace led to finding higher than acceptable variability in the detected concentration of the VSCs in the headspace using the absorbent paper points or filter strips. Further investigations by adding paper points or strips to vials preloaded with standard solutions revealed that a portion of sodium sulfide and sodium thiomethoxide absorbed by the paper points and the filter paper strips is not acid-labile and the variance observed due to this was determined (Figure 3-17). These data suggested that the absorbing capacity of the Whatman and Periocol

paper strips were comparable and the absorbent paper points showed greater variance at higher concentrations (Figure 3-19). The Whatman and Periocol strips however exhibited this absorbance only at higher concentrations, with their Lower Limit of Detection (LLOD) not affected as much as the absorbent paper points—approximately 7µM of H_2S in the standard solution could be reliably detected with the filter paper strips compared to about 20µM for the absorbent paper points. The majority of GCF samples were obtained using endodontic paper points, with some using Periocol and Whatman strips.

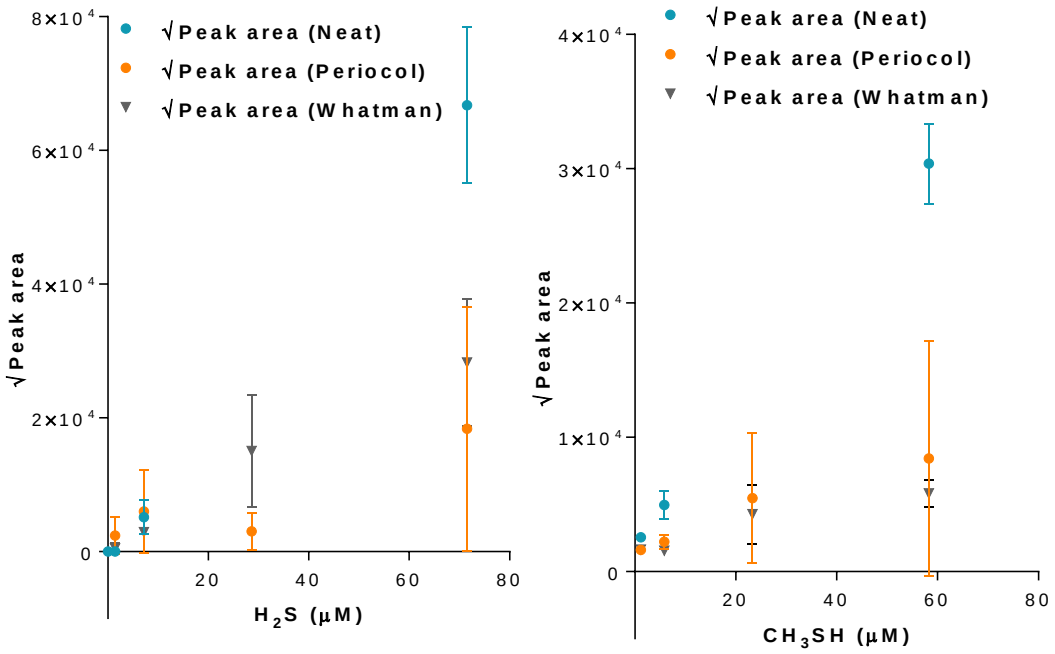


Figure 3-17 showing comparison between the Whatman 3MM and Periocol papers in absorbing sodium sulfide and sodium thiomethoxide relative to standard solutions without the presence of the paper. Data points shown are mean values from triplicate measurements; Standard deviation shown as error bars.

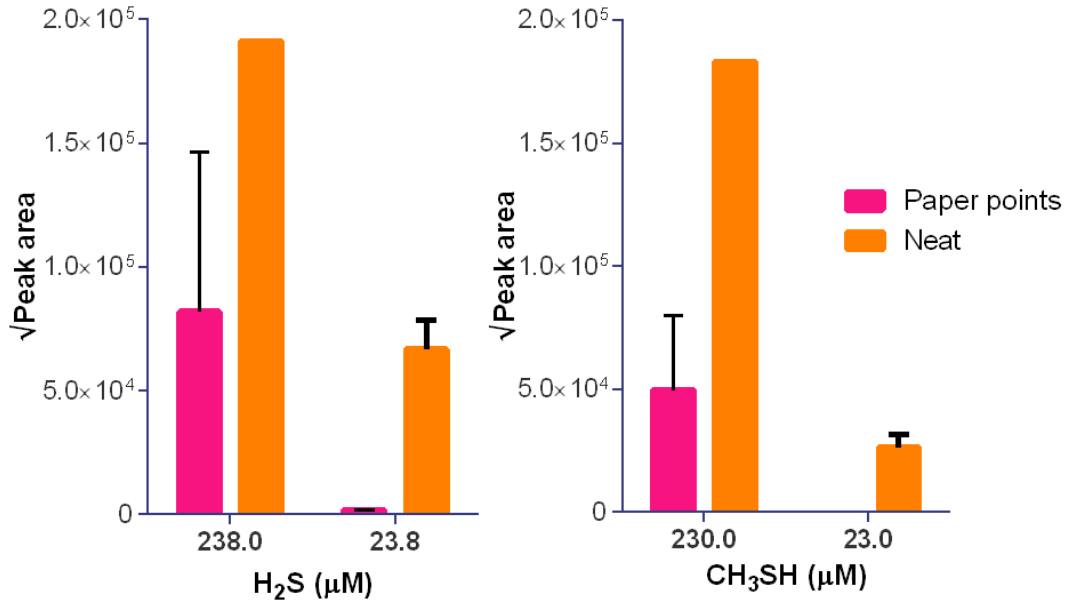


Figure 3-19 showing differences in peak areas (Mean±SD) detected corresponding to H_2S and CH_3SH, with solutions added neat or with paper points. Left: 238μM neat solution saturated the detector, 23.8μM solution with added paper points showed peak areas close to LLOD. Right: No peaks were observed for the 23μM CH_3SNa solution with paper points.

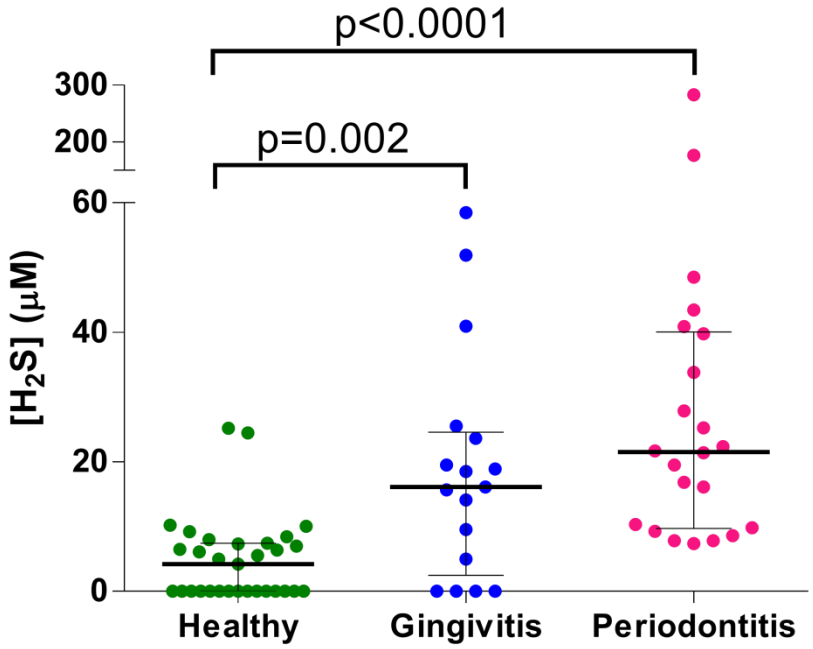


Figure 3-18 showing scatter dot plots of hydrogen sulfide concentrations detected in the headspace of GCF samples collected using absorbent paper points. Comparisons that showed statistical significance, median and interquartile ranges are indicated.

3.3.2 Volatile sulfur compounds in gingival crevicular fluid

Hydrogen sulfide was the most prevalent VSC detected in the GCF across the cohorts, with a significant increase detected in the gingivitis and chronic periodontitis cohorts compared to healthy individuals (including individuals with high plaque; Figure 3-18). Prevalence of methanethiol was low in all samples (7% of the total samples) with only trace amounts detected in the samples that showed the presence of methanethiol. However methanethiol was only detected in the chronic periodontitis and gingivitis patients. A few samples from the different cohorts were analysed with long assay run times, but no other higher molecular weight VSCs such as dimethyl disulfide or dimethyl trisulfide were detected in the samples. Another VSC was found to be more prevalent than methanethiol (22% of all samples), at trace amounts compared to H_2S, and this was tentatively identified to be carbon disulfide (Figure 3-20). This VSC was more frequently detected among the periodontitis cohort (61% prevalence among periodontitis patients) although one sample from the healthy cohort (2.5% prevalence) and three samples from the gingivitis cohort (14% prevalence) also contained this VSC at trace amounts.

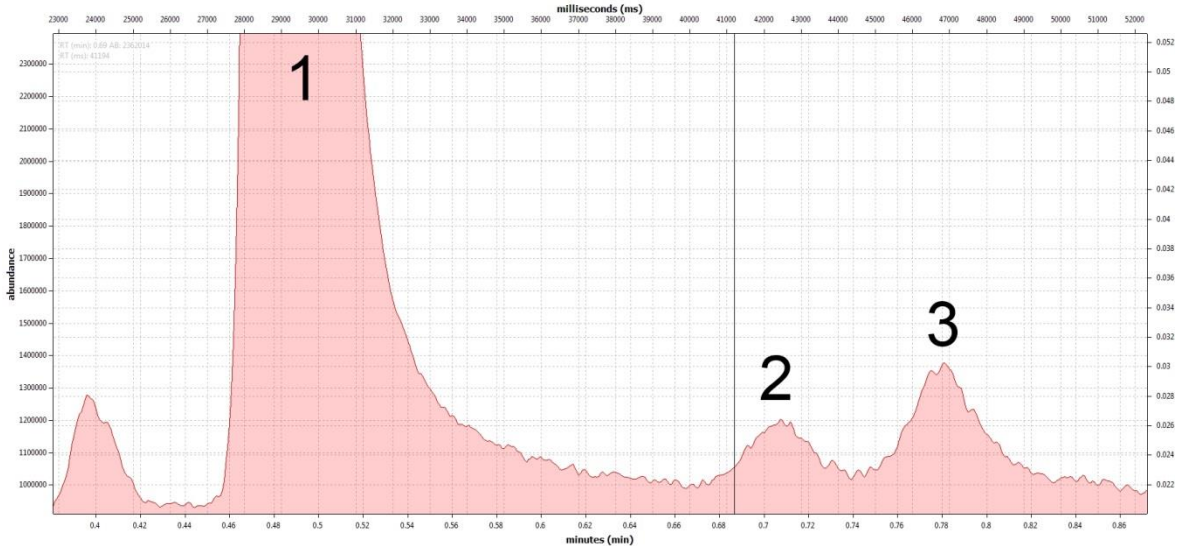


Figure 3-20 showing a scaled chromatogram of the VSCs detected in the headspace of the GCF sample collected from a 7mm periodontal pocket in a patient with severe chronic generalized periodontitis. Peak labelled 1=H_2S, 2=CH_3SH and 3=CS_2.

A few differences could be observed between the results of these analyses compared to Persson et al. (1992), who observed that the amounts of H_2S detected in the samples ranging 10µM to 1mM, is considerably higher than expected, particularly with the

potency of H_2S in disabling key enzymes such as cytochrome oxidase. In view of the capacity of the paper points used in this study to irreversibly bind VSCs, in particular H_2S, the amounts detected in the present study were comparable to Persson et al. (1992). The authors report the prevalence of methanethiol to be much higher than what was found in the present study, detecting this VSC in the range of >10μM to 160μM in periodontal pockets ranging 4mm to 10mm. The LLOD for methanethiol was higher in the present study for the paper points (~35μM), and this could partly explain these results as the majority of the values reported by Persson et al (1992) fall below this threshold. The finding of carbon disulfide in the GCF samples was not reported by Persson et al. (1992) and another study had detected this VSC in the breath of individuals but the authors suggested environmental contamination (van den Velde et al. 2007). However, the detection of this VSC in GCF suggests that endogenous sources are also relevant, since a number of studies also report associations of this VSC in the breath with pathologies including cystic fibrosis, liver disease and even psychological maladies such as dissociative identity disorder (Miekisch et al. 2004; Kamboures et al. 2005; Phillips et al. 1993). Possible endogenous sources of this VSC could be sulphate reducing bacteria which may be present in the oral cavity, and incomplete methionine transamination by hepatocytes (Vianna et al. 2008; Scislowski & Pickard 1994). Carbon disulfide is also a component of cigarette smoke and alcoholic beverages, and the participants recruited to this study have never smoked previously (Gerbersmann et al. 1995; Dong & DeBusk 2009). These exogenous factors can be ruled out due to the prevalence of this VSC being strongly biased towards the chronic periodontitis cohort. CS_2 concentration is also thought to be highly elevated in the breath of individuals taking the anti-alcoholism drug disulfiram, and this too could be ruled out in the chronic periodontitis cohort due to access to a more complete medical history before recruitment. In addition, only trace amounts were observed which would be more in line with the disulfiram negative hypothesis.

Clinical parameter	Rho	95% CI	P (two-tailed)
% ≥4mm PD	0.6384	0.4819 to 0.7554	< 0.0001
% ≥5mm PD	0.6451	0.4906 to 0.7602	< 0.0001
% ≥6mm PD	0.5948	0.4261 to 0.7236	< 0.0001
Gingival Bleeding Index	0.2883	0.0666 to 0.4828	0.0095
Bleeding on Probing	0.3432	0.1271 to 0.5283	0.0018
Plaque Index	0.2452	0.0203 to 0.4464	0.0284

Table 3-3 listing Spearman's Rho values for the measured clinical parameters and H_2S detected from the GCF samples of all participants recruited to this study (n=80 in all these comparisons).

All clinical parameters used to measure disease were found to correlate positively with the concentration of H_2S detected in the GCF of individuals; with the strongest being the percentage of periodontal pockets ≥5mm (Table 3-3). The GCF samples were collected from one tooth with the deepest periodontal pocket in each individual, unlike the more generalized sampling by Persson et al (1992), and these results suggest that the amount of H_2S found in the individual site is correlated with periodontal disease prevalence in the oral cavity. With the use of more sensitive GCF sampling devices, this could be a useful indicator for monitoring active periodontal disease in multiple sites within the oral cavity.

3.4 Periodontopathogens and Oral malodour

A small number of studies investigating the relationship between specific bacterial species and malodour have found those bacterial species associated with periodontal disease to be more abundant in the oral cavity of individuals with malodour compared to those without malodour (Kurata et al. 2008; Tanaka et al. 2004; Kuroshita et al. 2010b). While the classic red complex species namely *P. gingivalis, T. forsythia* and *T. denticola* have all been demonstrated to degrade free cysteine, methionine and serum peptides to VSCs (Persson et al. 1990; Persson et al. 1989), fewer bacterial species are known to degrade methionine, and most of these species currently known to degrade methionine to methanethiol are suggested to be periodontopathic (see section 4.5). Therefore in this study, qPCR was employed to detect six species namely *P. gingivalis, T. forsythia, A. actinomycetemcomitans*, *F. nucleatum*, *V. parvula* and *S. moorei* in five different oral niches namely, saliva, tongue biofilm, subgingival plaque, supragingival plaque (facial surfaces) and interdental plaque of individuals to study

their association with VSCs. All six species are capable of producing H_2S in serum and also from free cysteine substrate, whilst only *P. gingivalis* and *F. nucleatum* are known to produce CH_3SH from methionine, with *P. gingivalis* producing methanethiol at a higher rate compared to *F. nucleatum* from a serum substrate as opposed to free methionine and vice versa (Persson et al. 1990). Some strains of *A. actinomycetemcomitans* have also been reported to produce CH_3SH in broth cultures (Kuroshita et al. 2010a).

3.4.1 Periodontopathogens in the different niches in health and disease

Among the species detected, *P. gingivalis*, *T. forsythia* and *A. actinomycetemcomitans* displayed the most dynamic changes in the different niches between the cohorts. These are low prevalence, low abundance species, and were found to be significantly more abundant in the periodontitis cohort in the different niches studied. *P. gingivalis* was found to be significantly elevated in saliva, subgingival, supragingival and interdental plaque of periodontitis patients compared to health (Figure 3-21). Interestingly, healthy individuals with higher plaque coverage showed lower prevalence of *P. gingivalis* in all the niches compared to healthy individuals with low plaque coverage. This could be related to the 'evenness' of the microbial community where numbers of *P. gingivalis* remain similar in healthy individuals with high plaque score vs lower scores, but individuals with high plaque have greater abundance of other species involved in secondary colonization leading to lower proportions of *P. gingivalis* in individuals with higher plaque coverage, which also implies increase in biofilm thickness. However, normalizing these data using total bacterial load as determined using universal primers revealed a similar distribution of data with similar results, except for a significant increase in the proportions of *P. gingivalis* being observed with the supragingival plaque of the periodontitis cohort compared to healthy individuals with low plaque coverage. While *P. gingivalis* is thought to prefer the subgingival environment, these data indicate that this species is equally prevalent in the supragingival (buccal and lingual/palatal facial surfaces), and interdental plaque.

	Niches				
	Saliva	**Tongue**	**Subgingival**	**Supragingival**	**Interdental**
BOP	0.276	0.321	0.347	0.304	-
GBI	-	0.253	0.354	0.322	-
% ≥4mm PD	0.462	0.270	0.359	0.396	0.322
% ≥5mm PD	0.392	-	0.263	0.266	0.223
% ≥6mm PD	0.334	-	0.344	0.294	0.216

Table 3-4 listing significant (p<0.05) Spearman's Rho values computed for the number of *P. gingivalis* cells detected in the different niches and the clinical parameters.

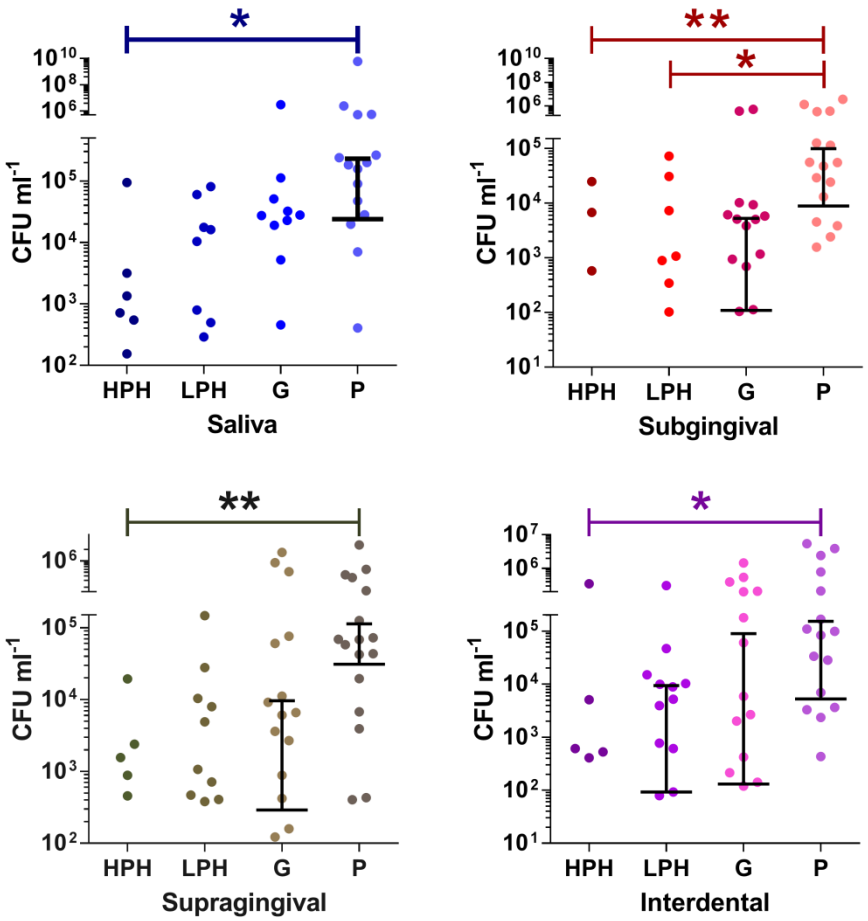


Figure 3-21 showing scatter dot plots of numbers of *P. gingivalis* CFU ml^{-1} detected in the niches that showed significant differences. Comparisons that showed statistical difference, median and interquartile ranges are indicated. HPH=High plaque health; LPH=Low plaque health; G=Gingivitis; P=Chronic periodontitis.

Positive correlations were also observed with the abundance of *P. gingivalis* in at least one niche and clinical parameters of disease such as BOP, GBI and percentage of probing depths greater than or equal to 4mm (Table 3-4). No correlations were observed with increasing plaque index scores, and these relationships hold true for the proportions of *P. gingivalis* present in these niches. Despite no significant differences being observed with the different cohorts of *P. gingivalis* abundance on the tongue, the positive association observed with increasing BOP, GBI and percentage of periodontal pockets ≥4mm suggests that although the tongue is the least dynamic niche studied, periodontal disease has an effect on the tongue ecology and this could partly explain the elevated methanethiol concentrations observed in the breath of the periodontitis cohort, and the increase in CH_3SH: H_2S ratio in association with the clinical parameters such as GBI and PD (Table 3-2). Similarly, only a non-significant increase of *T. forsythia* was observed on the tongue of the chronic periodontitis cohort, compared to health in the subgingival and supragingival niches (Figure 3-22). However, *T. forsythia* on the tongue was positively correlated with an increase in percentage of probing depths ≥4mm and ≥5mm (Table 3-5). *A. actinomycetemcomitans* in different niches was found to be more closely associated with an increase in plaque scores and % of probing depths ≥6mm (Table 3-5). Additionally, due to its association with plaque coverage, *A. actinomycetemcomitans* prevalence was also elevated in the healthy individuals with plaque coverage ≥30%, and this was reflected in the comparisons between healthy individuals with low plaque coverage and the gingivitis, chronic periodontitis cohorts in all the niches (Figure 3-23). These results further support the hypothesis that the tongue ecology changes in association with disease and this could result in a change in VSC profile observed in the breath of individuals.

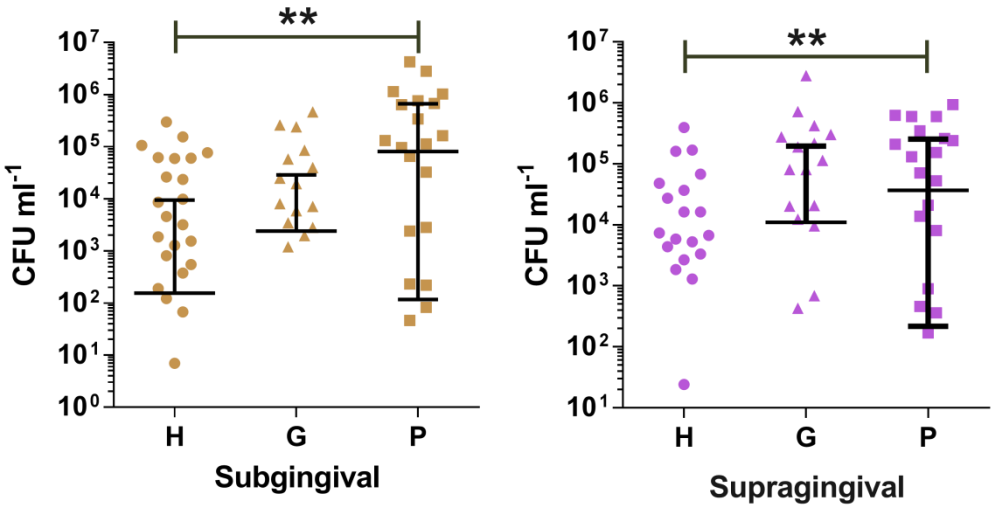


Figure 3-22 showing scatter dot plots of *T. forsythia* CFU ml^{-1} detected in niches that showed statistically significant differences between cohorts (indicated). No distinctions were observed between healthy individuals with low or high plaque coverage, H=Healthy including high plaque individuals; G=Gingivitis, P=Chronic periodontitis. Median and interquartile ranges are indicated.

	Tannerella forsythia				
	Saliva	**Tongue**	**Subgingival**	**Supragingival**	**Interdental**
BOP	-	-	0.279	0.393	0.262
GBI	-	-	-	0.256	-
% ≥4mm	0.299	0.298	0.315	0.407	-
% ≥5mm	0.402	0.335	0.345	0.387	-
% ≥6mm	-	-	0.315	0.348	-
	Aggregatibacter actinomycetemcomitans				
PI	0.256	-	0.244	0.264	0.272
% ≥4mm	0.239	-	-	-	-
% ≥6mm	0.234	-	0.251	0.227	-

Table 3-5 listing significant (p<0.05) Spearman's rho values computed for the relationship between *T. forsythia* and *A. actinomycetemcomitans* CFUs detected in the different niches and the clinical parameters in all individuals.

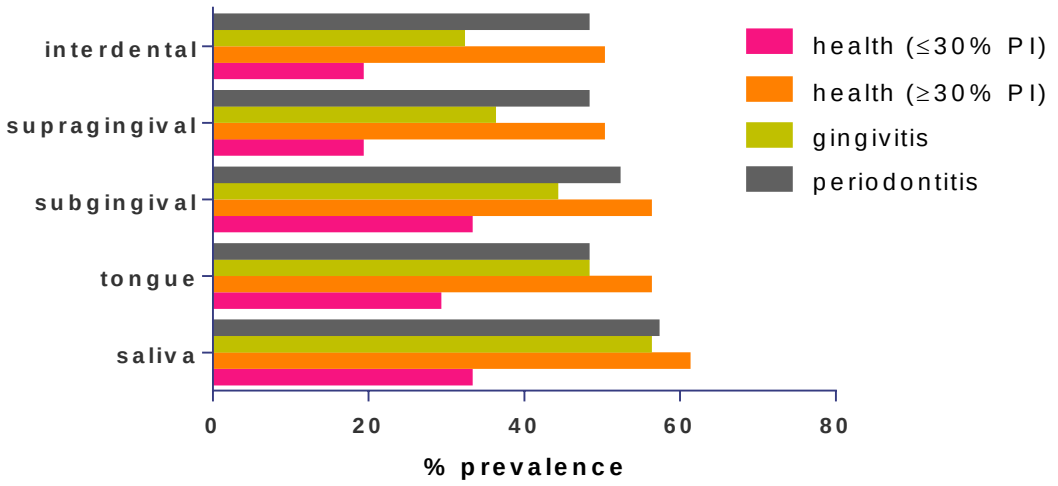


Figure 3-23 showing the prevalence of *A. actinomycetemcomitans* in the different niches among the cohorts. Data includes individuals in the healthy cohort with ≤30% PI (n=21) and ≥30% PI (n=18), gingivitis (n=25) and periodontitis (n=23).

F. nucleatum is one of the most prevalent species in the oral cavity and is an important VSC producer which is also associated with periodontal disease. Sub species exist within this microbial species that may be health or disease associated, but to date no qPCR method exists to determine the subspecies composition based on 16S primers and the data pertaining to this organism in the current study includes all subspecies (Kistler et al. 2013). More importantly, all subspecies of *F. nucleatum* have the homologs necessary to produce both H_2S and CH_3SH. However, while prevalence was high (>98%) in all niches, and differences could be observed between cohorts, these were not statistically significant (Figure 3-24). Increasing amounts of *F. nucleatum* could be observed in the saliva of individuals from health to periodontitis, and the abundance of *F. nucleatum* in terms of number of CFUs detected and in proportions, was higher in the interdental plaque of healthy individuals with low plaque coverage compared to all other cohorts. No significant correlations were observed with proportions of *F. nucleatum* in the oral niches and clinical parameters. Higher resolution in determining the different subspecies and indeed, other species such as *F. periodonticum* should yield a greater level of insight in to the ecological dynamics of this group of species in the different niches in health and disease (see section 4.5).

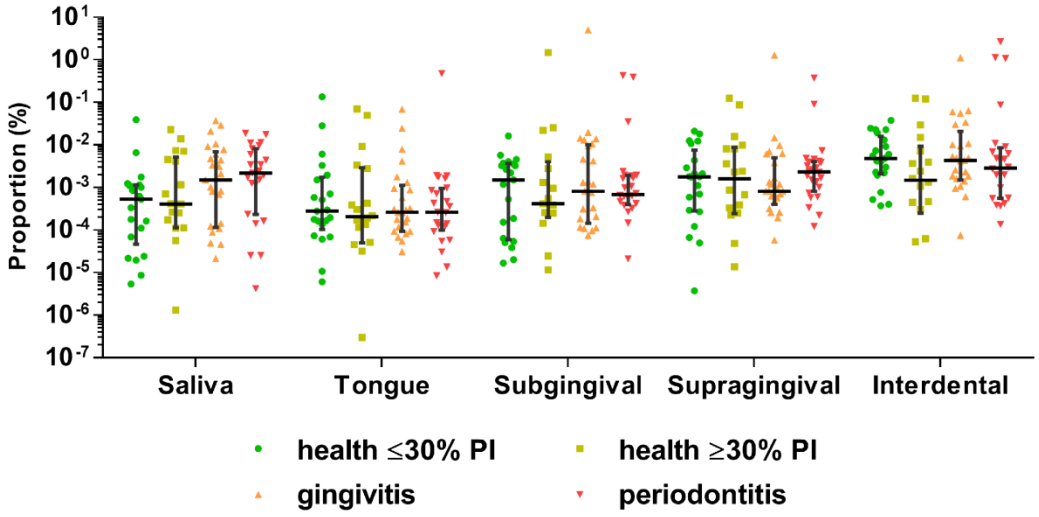


Figure 3-24 showing scatter dot plots of *F. nucleatum* proportions in the different niches among the cohorts. Median and interquartile ranges indicated.

V. parvula and *S. moorei* are important species in relation to oral malodour and while *Veillonella* spp are some of the most prevalent species in the oral cavity, and have been associated with malodour and periodontal disease, *S. moorei* was reported to be selectively prevalent on the tongue of individuals with halitosis (Belda-Ferre et al. 2012; Haraszthy et al. 2008; Washio et al. 2005). QPCR assays were developed to detect both *V. parvula* and *S. moorei* in clinical samples in the present study, based on the RNA Polymerase B and 16S genes respectively. Since the start of this project, another study has reported qPCR detection of *S. moorei* in clinical samples, but the *V. parvula* assay remains the only optimised qPCR assay detecting this species in clinical samples (Vancauwenberghe et al. 2013). Among the veillonellae, the species *V. parvula* was chosen due to its membership in the classical 'purple' complex, its high abundance on the tongue in particular and also its reported high rate of H_2S production in serum and free cysteine substrate (Persson et al. 1990; Socransky et al. 1998; Socransky & Haffajee 2005). Although no statistically significant changes in absolute numbers or proportions of *V. parvula* were detected among the niches, the most marked shifts among the different cohorts occurred on the tongue, supragingival plaque and saliva in terms of proportions (Figure 3-25). Negative relationships were observed with the proportions of this species in the subgingival plaque with the percentage of probing depths ≥4mm, ≥5mm and ≥6mm, while a positive correlation was observed with plaque score and proportions of *V. parvula* on the tongue (Table 3-6). These data suggest that

V. parvula could be an important source of hydrogen sulfide in individuals with high plaque coverage owing to the association of this clinical parameter to the proportions of *V. parvula* in the tongue. This observation is particularly significant in light of the ecological stability of the tongue as observed in metagenomic studies, which implies that smaller changes in the ecology of the tongue would likely have a greater effect owing to the high surface area of the tongue compared to the other niches studied (Caporaso et al. 2011; Yang et al. 2013).

S. moorei prevalence in all the individuals was similar in the tongue and interdental niches (92%), whereas its prevalence in the subgingival and supragingival niches were lower (78% and 85%), and a 100% prevalence was observed in saliva. Increase in the proportions of *S. moorei* were observed with the saliva, tongue, subgingival and supragingival niches from health to disease, with a statistically significant shift observed in the supragingival plaque (Figure 3-26). Significant positive correlations were also observed with the increase in proportions of *S. moorei* in the subgingival, supragingival and salivary niches in association with the clinical parameters (Table 3-6). It is notable that this species appears to increase in niches where it is normally less prevalent, in association with disease. This is possibly a hallmark of an opportunist and the available literature on *S. moorei* supports this view (Detry et al. 2006; Zheng et al. 2010; Schirrmeister et al. 2009).

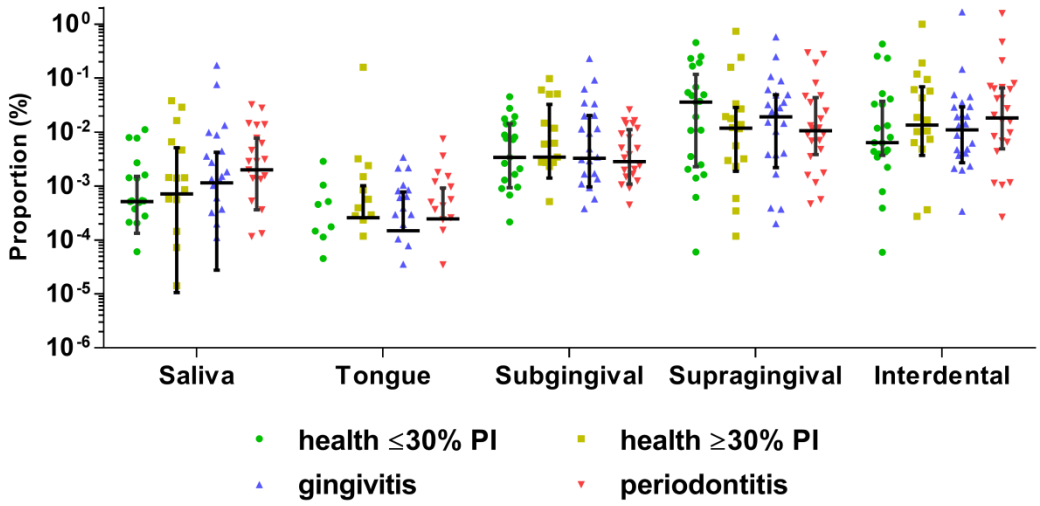


Figure 3-25 showing scatter dot plots of *V. parvula* proportions in the different niches among the cohorts. Median and interquartile ranges indicated.

	Saliva	Tongue	Subgingival	Supragingival
Veillonella parvula				
PI	-	0.256	-	-
≥4mm PD	-	-	-0.245	-
≥5mm PD	0.239	-	-0.332	-
≥6mm PD	-	-	-0.243	-
Solobacterium moorei				
BOP	-	-	0.258	-
GBI	0.340	-	0.289	0.292
PI	0.341	-	-	0.303
≥4mm PD	-	-	-	0.257
≥6mm PD	-	-	0.314	0.261

Table 3-6 listing Spearman's Rho values for comparisons that showed significant relationships (two tailed p<0.05) between proportions of *V. parvula* and *S. moorei* detected in the different niches and the clinical parameters.

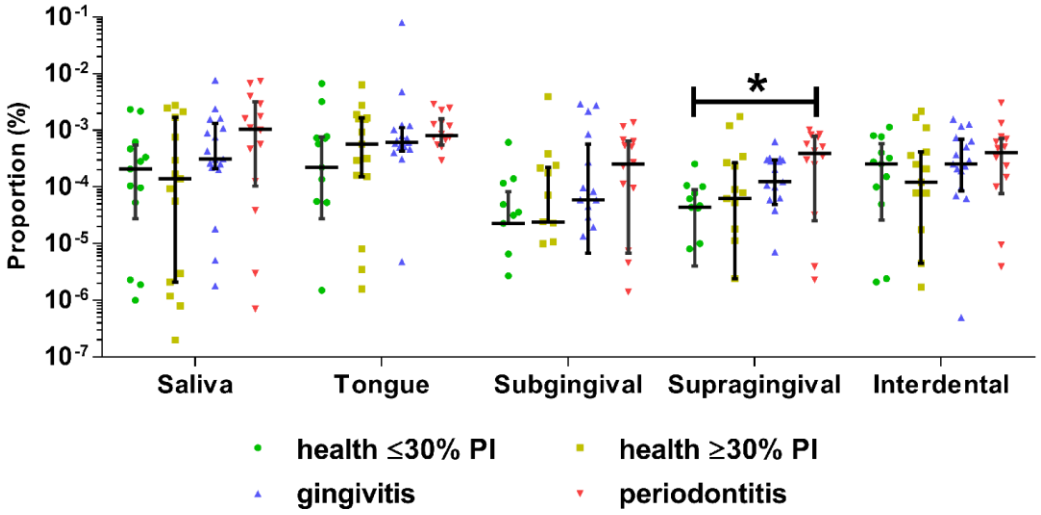


Figure 3-26 showing scatter dot plots of *S. moorei* proportions in the different niches among the cohorts. Comparison that showed statistical significance, median and interquartile ranges are indicated.

3.4.2 VSCs and Periodontopathogens

The bacterial species detected in the different oral niches in individuals are all known VSC producers and given their associations with specific niches in disease, correlational analyses were conducted on the breath VSCs, sulcular H_2S and associated parameters. Greater numbers of correlations were observed between the bacterial species detected in the different niches and the concentration of hydrogen sulfide measured subgingivally than any other VSC parameter (Table 3-7; Table 3-8). In terms of breath VSCs, significant correlations were observed between breath methanethiol concentrations, CH_3SH: H_2S ratio and the periodontal niches. These observations support the hypothesis that subgingival VSCs and indeed, breath methanethiol concentration and the associated parameter namely, CH_3SH: H_2S ratio are related to disease activity.

While it is believed that tongue biofilm is responsible for the presence of breath VSCs, if tongue biofilm alone is responsible for the change in VSC profile, one would expect these correlational analyses to identify prevalence and/or abundance of bacterial species in tongue rather than the periodontal niches as those that are associated breath VSCs (Amou et al. 2014; Calil et al. 2009). Instead, due to breath methanethiol and CH_3SH: H_2S ratio being more associated with VSC producers and periodontopathogens increasing in numbers and proportions in periodontal niches such as interdental, subgingival and supragingival plaque, it is likely that these breath parameters identify a general periodontal inflammation or disease activity as in the case of H_2S in the sulci. However in the present study, sampling of the tongue biofilm was not carried out on the dorsal area posterior to circumvallate papillae of the tongue which is thought to harbour a more diverse microbiota and it is possible that periodontal ecological changes affect the composition of this aspect of the tongue and thus bring about changes in the VSC concentration in the breath (Allaker et al. 2008). However, there is evidence that periodontal treatment is more effective at reducing malodour and indeed breath VSC concentration in individuals with gingivitis or chronic periodontitis than tongue cleaning combined with periodontal treatment, and this is consistent with the findings of this study (Pham et al. 2012).

	Tannerella forsythia				
	Saliva	**Tongue**	**Subgingival**	**Supragingival**	**Interdental**
GCF H_2S	0.377	0.288	0.430	0.309	-
	Porphyromonas gingivalis				
CH_3SH (ppb)	0.294	-	0.224	0.277	0.223
CH_3SH: H_2S	0.285	-	-	0.286	0.254
GCF H_2S	0.277	-	0.236	0.311	0.269
	Aggregatibacter actinomycetemcomitans				
CH_3SH: H_2S	-	-	0.204	-	0.206
GCF H_2S	0.291	0.251	0.213	0.321	0.287
	Veillonella parvula				
GCF H_2S	-	-	-0.271	-	-
	Fusobacterium nucleatum				
GCF H_2S	0.206	-	-	-	-
	Solobacterium moorei				
CH_3SH (ppb)	-	-	-	-	-0.241
CH_3SH: H_2S	-	-	-		-0.248

Table 3-7 listing Spearman's Rho values that showed significant (two tailed p<0.05) relationships between the proportions of bacterial species detected in the different niches and the VSCs measured in the breath and periodontal pocket.

	Porphyromonas gingivalis				
	Saliva	**Tongue**	**Subgingival**	**Supragingival**	**Interdental**
CH_3SH	0.289	-	0.289	0.304	0.237
CH_3SH: H_2S	0.275	-	0.259	0.305	0.261
GCF H_2S	-	-	0.229	0.279	-
	Aggregatibacter actinomycetemcomitans				
Malodour Score	0.216	-	0.213	-	-
CH_3SH: H_2S	-	-	0.237	-	0.248
GCF H_2S	0.293	0.255	0.251	0.330	0.299

Table 3-8 listing Spearman's Rho values for significant comparisons (two tailed p<0.05) between CFUs of *P. gingivalis* or *A. actinomycetemcomitans* detected in the different niches and VSCs measured in the breath and periodontal pocket.

3.4.3 Latent VSC producing capability in oral sites

To investigate if the different plaque samples from the individuals with gingivitis or chronic periodontitis are inherently more capable of producing VSCs than health, a small subset of samples collected from this study were incubated aerobically with cysteine or methionine substrate and the headspace analysed for VSCs. Only about 6% of the total samples analysed did not have any detectable H_2S in the headspace and these were mostly samples from healthy individuals. In contrast, methanethiol was observed in a very limited number of samples across the different cohorts, with the frequency of methanethiol detection being higher in the samples from the gingivitis and chronic periodontitis cohorts than health (Figure 3-27). Interestingly, methanethiol was not detected in the headspace of any tongue biofilm samples when incubated with methionine. While it is possible that an anaerobic environment is favourable for methionine degradation in the tongue, methanethiol detected in other samples suggest that it is not a necessary condition for all niches. However, a higher percentage of samples might have displayed methanethiol production under anaerobic conditions than observed in this experiment.

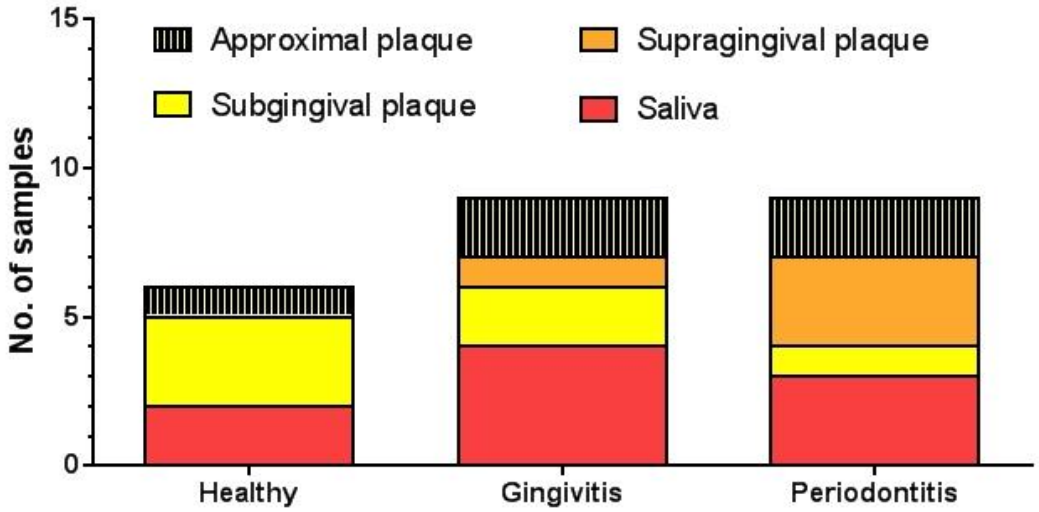


Figure 3-27 showing stacked bars of the number of samples from the different niches in health, gingivitis and chronic periodontitis cohorts that showed the presence of methanethiol in the headspace.

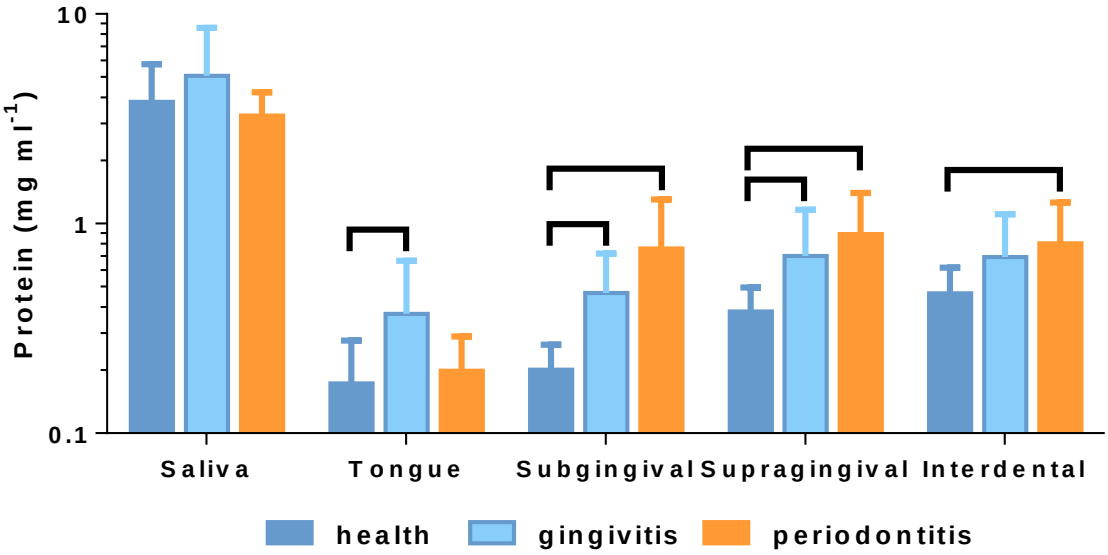


Figure 3-28 plotting mean (±SD) protein concentrations determined in the different oral samples in individuals with gingivitis, chronic periodontitis and oral health. Statistically significant comparisons indicated.

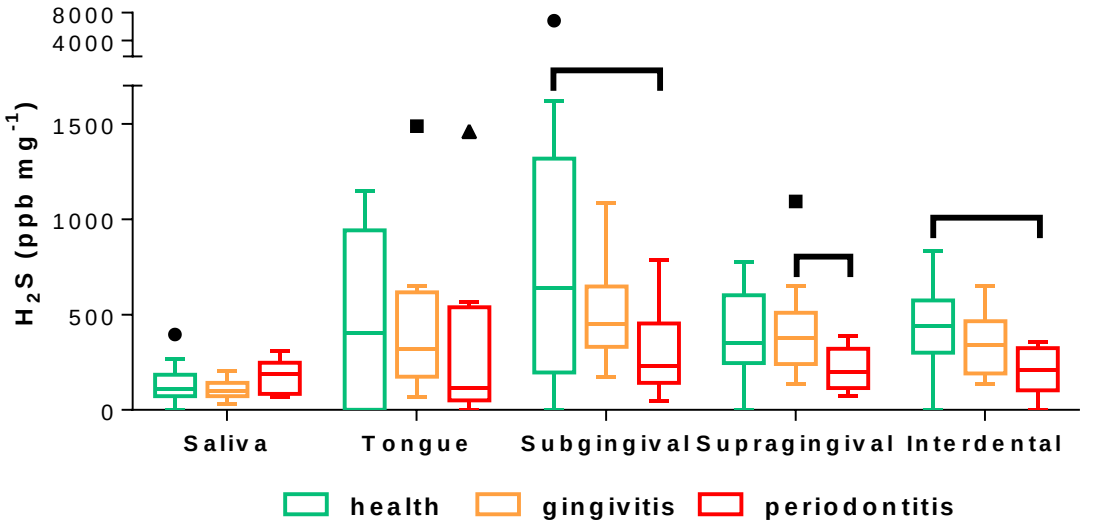


Figure 3-29 showing box plots of the H_2S generated in the headspace of clinical samples incubated with cysteine as a function of the protein concentration of the samples. Midline is median, boxes extend from 25th to 75th percentile and the whiskers and outliers plotted by the Tukey method. Comparisons that showed statistical significance are indicated.

Determining the protein concentrations of the different samples revealed that except for saliva, the tongue biofilm and plaque samples from the gingivitis and chronic periodontitis cohorts had significantly higher concentration of protein than those from healthy individuals (Figure 3-28). Normalizing the observed H_2S concentrations using the protein measured in each sample revealed significant differences between the health and disease cohorts in the periodontal niches which reflected the observed protein concentration in the samples (Figure 3-29). These data suggested that despite the higher protein concentration observed in the periodontal niches, the healthy samples from the same niches produced similar or higher amounts of H_2S in the headspace, with the subgingival plaque and tongue biofilm showing the highest amounts of H_2S produced. However, taking in to account the amount of protein measured in saliva of the different cohorts being similar, higher amounts of VSC production from saliva of the periodontitis patients was observed compared to health or gingivitis (Figure 3-28; Figure 3-29). The total bacterial load determined by the universal primers was also used to normalize these data, revealing further relationships (Figure 3-30). These data indicated that the interdental plaque displayed a significantly elevated rate of H_2S production in individuals with chronic periodontitis compared to health or gingivitis, with the supragingival plaque also showing a similar non-significant increase. These results further support the hypothesis that the periodontal niches display the most dynamic shifts in VSC production in association with disease.

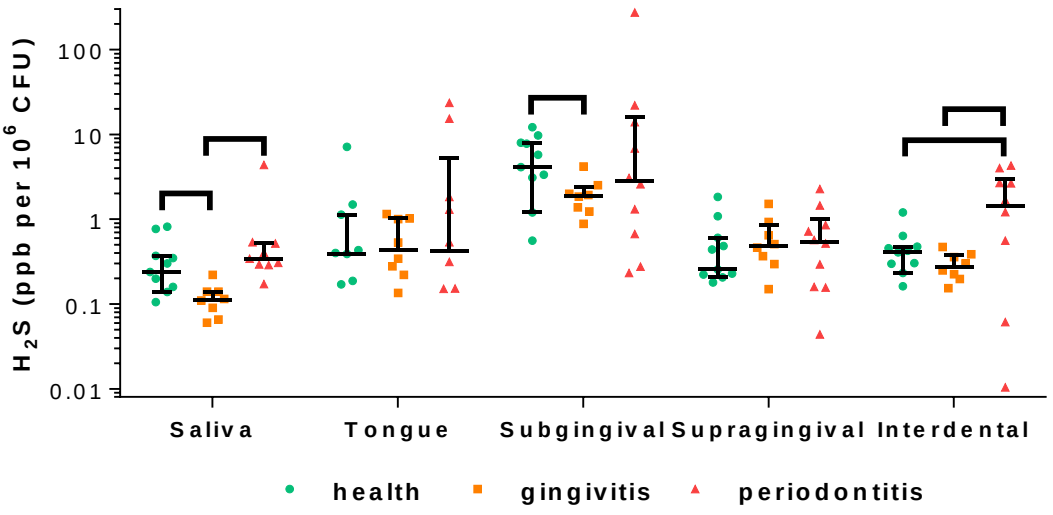


Figure 3-30 scatter dot plots showing H_2S generated in the headspace of clinical samples incubated with cysteine as a function of the total bacterial load in samples. Median, interquartile range and comparisons that showed statistical significance are indicated.

3.5 Inflammation in the oral cavity and oral malodour

The destructive sequelae arising from periodontal disease are primarily mediated by the host's immune system and as such, the cytokine profile present in the healthy gingiva and the changes associated with gingivitis and chronic periodontitis are of particular interest. As found in the present study, the concentration of VSCs present in the breath was associated with periodontal disease, and it is possible that malodour as it relates to disease might identify particular cytokine markers of periodontal disease to which it might be closely related to in terms of disease progression. No previous investigations have attempted to relate malodour with inflammation as defined by the concentration of cytokines in the fluids of the oral cavity, and available evidence for the possible role of VSCs in the pathogenesis of periodontitis as it relates to inflammation and inflammatory markers secreted by the gingival tissue has been entirely *in vitro* to date. Therefore, in the present study, the relationship between malodour as defined by the VSC concentration in the breath and the cytokine profile present in the gingival crevicular fluid and saliva in the oral cavity of individuals with good oral health, gingivitis and chronic periodontitis was investigated.

3.5.1 Crevicular cytokine profile in health and disease

Analysis of the measured concentrations of the different cytokines with a Partial Least Squares regression based model yielded distinct cytokine profiles for the healthy individuals and chronic periodontitis patients, with an overlap of the gingivitis patients between these two groups (Figure 3-31). Cytokines that showed increased concentrations in the GCF of healthy individuals were IL1 alpha, TNF alpha, IL12p70, IL4, IL10, the chemokines CXCL10 and CCL2; and the adhesion molecules E-selectin and Intercellular Adhesion Molecule-1 (ICAM-1). Cytokines that showed more of an association with the periodontitis cohort were Granulocyte-Colony Stimulating Factor (G-CSF), IL6, IL13, IL1 beta, IL-17a, Interferon alpha (IFNa), Latency Associated Peptide (LAP), and the chemokines CXCL8 (IL8), CCL3 and CCL4 (Figure 3-32). Samples from individuals with gingivitis showed profiles that were either health or disease associated, clustering closely with either of the aforementioned cytokine profiles—no trends based on clinically measured plaque score, bleeding on probing or gingival bleeding index could be observed with regards to the more health associated or disease associated samples from this cohort.

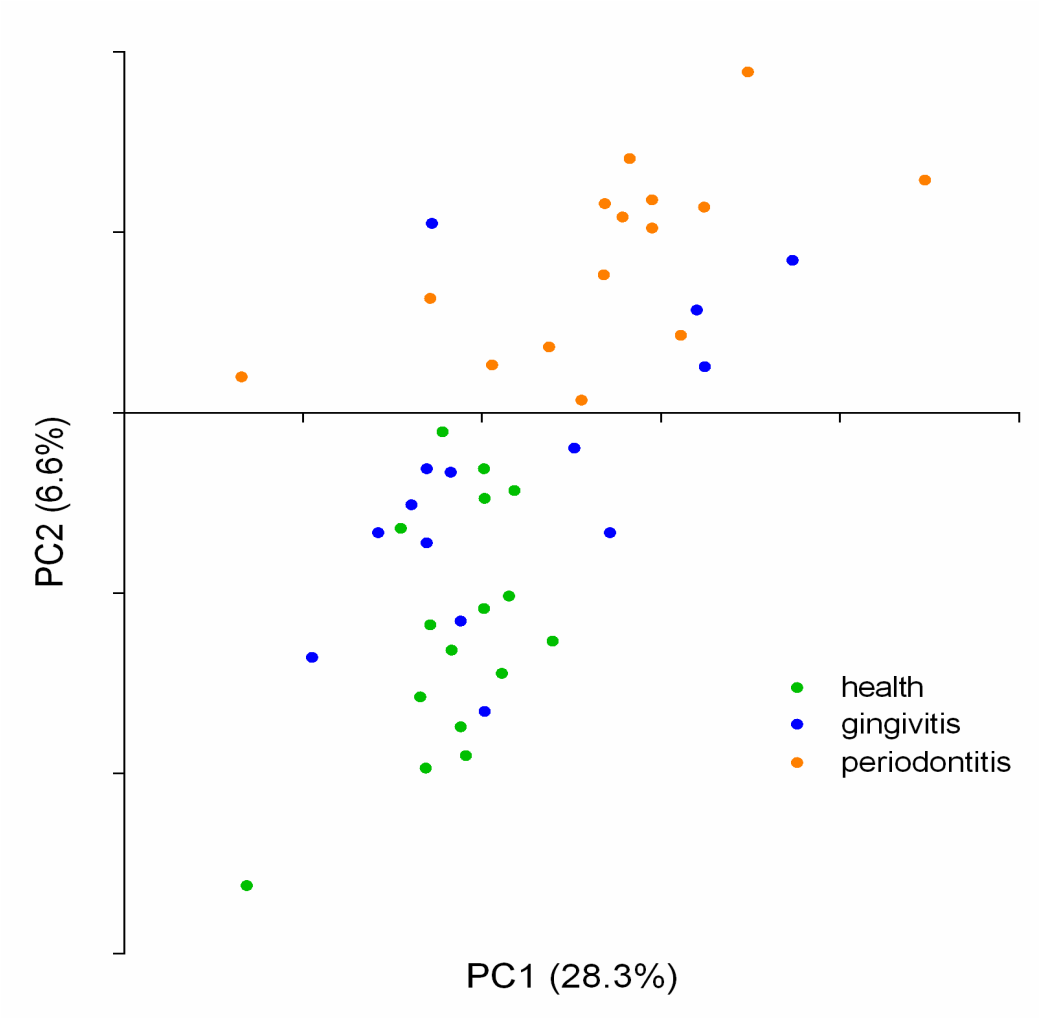


Figure 3-31 showing a score plot of the cytokine profiles in the GCF samples from healthy, gingivitis and chronic periodontitis cohorts. The associated loading plot is shown in Figure 3-32.

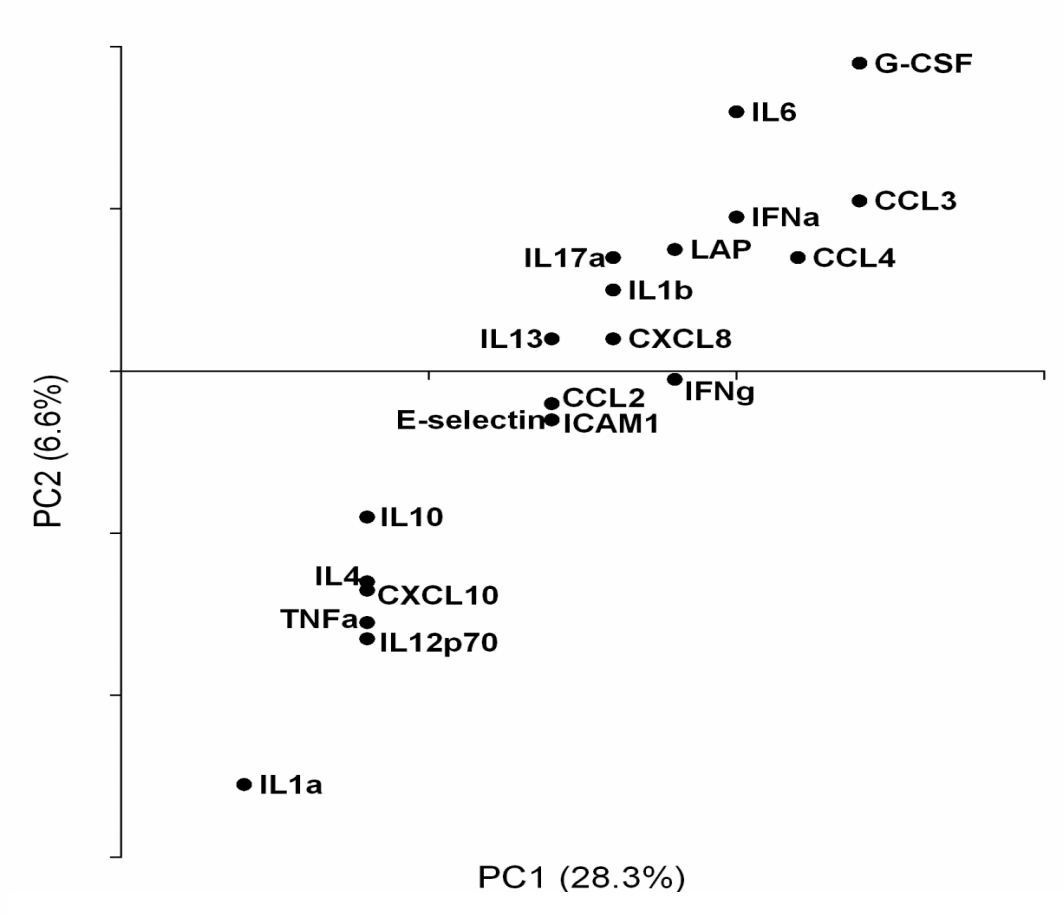


Figure 3-32 showing the loading plot for the measured cytokines in the GCF, associated with the score plot shown in Figure 3-31.

Data for the healthy and gingivitis cohort in the present study appears to suggest that the traditionally identified osteoclastogenic cytokines such as IL1 alpha and TNF alpha are more abundant in the GCF of individuals with clinically healthy gums or gingivitis than periodontitis (Figure 3-31; Figure 3-32). IL1 alpha is thought to be constitutively secreted by normal oral keratinocytes and upregulated in response to TNF-alpha stimulation and while it is possible that these cytokines drive pro-inflammatory processes evident in healthy gingiva or gingivitis, the association observed with the anti-inflammatory T-reg cytokine, IL10 points to a homeostasis that exists in the gingival crevice of these individuals (Ng et al. 2013; Perrier et al. 2002; Lappin et al. 2001). A key cytokine associated with the healthy and gingivitis cohort is IL4 which is thought to help differentiate naïve T-cells into a Th2 lineage and inhibit development of a Th1 response, as the latter is thought to play a role in the development of persistent inflammation whereas Th2 cell differentiation and the associated immune cell recruitment is thought to help in microbial clearance and maintaining homeostasis. Indeed, IL4 is thought to selectively increase expression of IL1 alpha in oral

keratinocytes and reported data in the literature from GCF measurements suggest a role for IL4 in gingival health and in remission of periodontal disease (Pradeep et al. 2008). The association of IL4 with the healthy and gingivitis cohorts also supports the role for IL1 alpha and TNF alpha in the healthy gingiva (Perrier et al. 2002). However, IL12p70, the active heterodimeric form of IL12p40 and IL12p35 is thought to encourage T-cell differentiation towards a Th1 lineage and it may well be that health is characterized by the dynamic equilibrium between IL12 and IL4 mediated responses as suggested by these data (Hölscher 2004).

The association of IL1 beta, IL6, IL17a, G-CSF and IFN alpha and the chemokines CXCL8, CCL3 and CCL4 with the chronic periodontitis cohort in the present study is in broad agreement with another study that used multiplexed bead-based immunoassay to detect cytokines and chemokines in the GCF collected from periodontitis patients before and after therapy (Thunell et al. 2010). The authors report elevated concentrations of IL1 alpha, IL1 beta, IL6 and the chemokines CCL2, CCL3 and CXCL8 in diseased sites vs healthy sites of generalized severe chronic periodontitis patients. Given that both localized and generalized, and also moderate and severe chronic periodontitis patients were included in the present study, the previously reported data correlate well with what is observed in the present study with regards to the health associated and disease associated cytokine profiles. Thunell et al. (2010) also reported an increase in CXCL10 in the GCF after periodontal therapy in healthy sites and this is consistent with data from the present study finding this chemokine to be more health associated, with a role in wound healing being postulated.

The glycoprotein G-CSF had the largest loading associated with the chronic periodontitis cohort in the present study and this cytokine is known to be involved in stimulating neutrophil proliferation and persistence in the gingiva by inhibiting its apoptotic mechanisms, while also suppressing osteoblast activity (Greenbaum & Link 2011). This highlights the role of neutrophils in chronic inflammation as much as it is thought to be an important component of the initial immune response to microbial antigens. A study that measured CCL3 (MIP-1a) and CCL4 (MIP-1b) in health, gingivitis, chronic and aggressive periodontitis and found similar concentrations among the different groups, and consistent with this study more GCF samples in chronic periodontitis patients had elevated concentrations of these macrophage chemoattractants than health (Emingil et al. 2005). The role of the IL17 family of cytokines in periodontitis is a subject of ongoing investigations, and current knowledge suggests

IL17A to be the most pro-inflammatory of the IL17 family and is thought to be a signature cytokine of the Th17 response, which in itself is thought to have both protective and destructive roles in periodontitis depending on other co-stimulatory cytokine signals and the findings of the present study on the association of IL17A to chronic periodontitis is consistent with these observations (Awang et al. 2014; Gaffen & Hajishengallis 2008).

Comparison between the different cohorts for the individual cytokines revealed that IL6 and LAP were significantly elevated while IL4 was significantly reduced (at $p<0.05$) in the chronic periodontitis cohort when compared to health but not gingivitis (Figure 3-33). Elevated concentrations of IL17A and IL13 were also observed in chronic periodontitis compared to gingivitis but not health (Figure 3-34). These observations are consistent with the PLS analysis and highlight the inflammatory mechanisms active in the different cohorts, in particular in chronic periodontitis. For example, elevated levels of IL6 in the periodontitis cohort suggests that a T-reg inhibitory and Th17 stimulatory environment prevails subgingivally in this cohort and blocking IL6 signaling is reported to lead to positive outcomes in treating various chronic inflammatory diseases in experimental models (Neurath & Finotto 2011). In regards to IL4, two sub groups with similar numbers of individuals were observed in the CP cohort: one with IL4 below detection limits and the other with values similar to the gingivitis cohort (Figure 3-33). Interestingly, individuals with detectable IL4 had higher mean levels of IL13 and IL10 than those with no IL4 in the CP cohort suggesting two separate mechanisms are in play in these individuals. IL4 and IL13 signaling is characteristic of M2 type macrophage activation, and the higher presence of IL10 also in the same individuals is possibly indicative of a better prognostic outcome owing to the M2 macrophage phenotype involved in ligament repair and fibrosis leading to a regenerative response (Martinez & Gordon 2014; Chamberlain et al. 2011). LAP is a surrogate marker for active Transforming Growth Factor-beta, and increased levels of LAP in the CP cohort reinforces the observation that regenerative mechanisms are more active in chronic periodontitis compared to health or gingivitis (Steinsvoll et al. 1999).

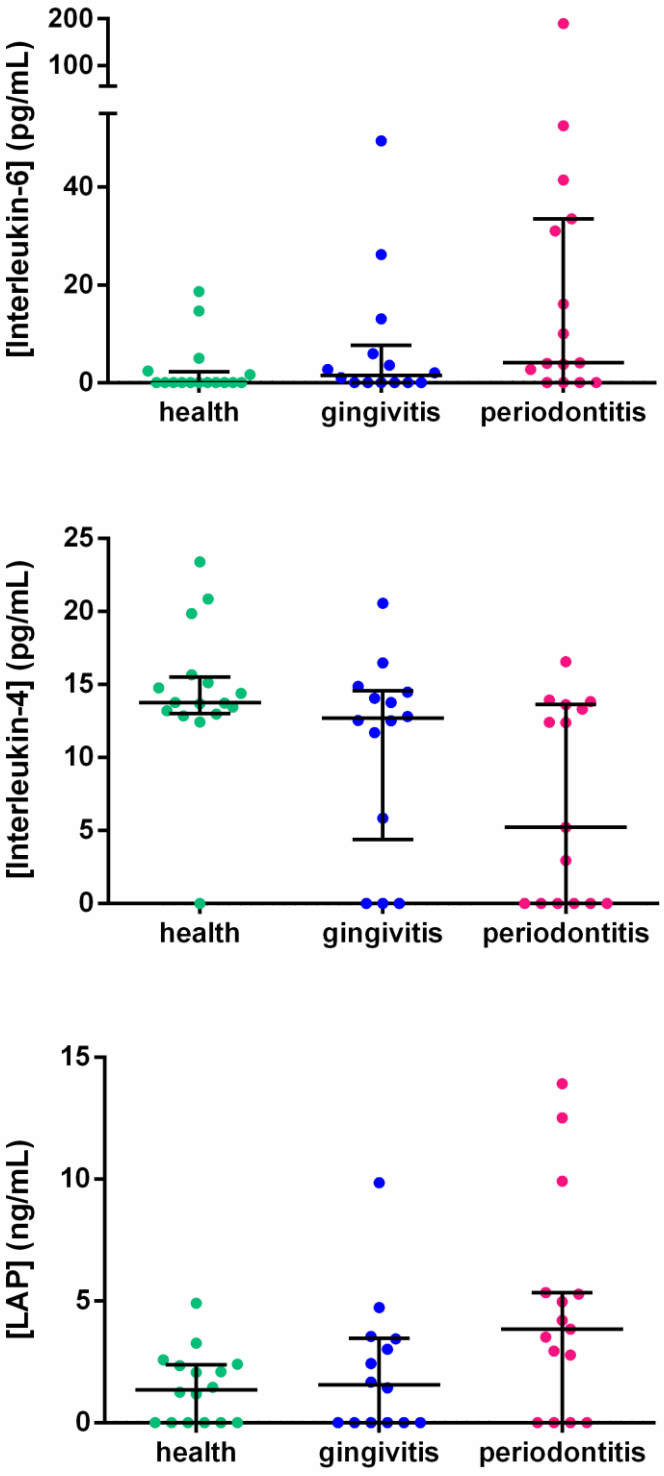


Figure 3-33 showing scatter dot plots of concentrations of cytokines that showed statistically significant differences only between health and chronic periodontitis cohorts ($p<0.05$). Median and interquartile ranges indicated.

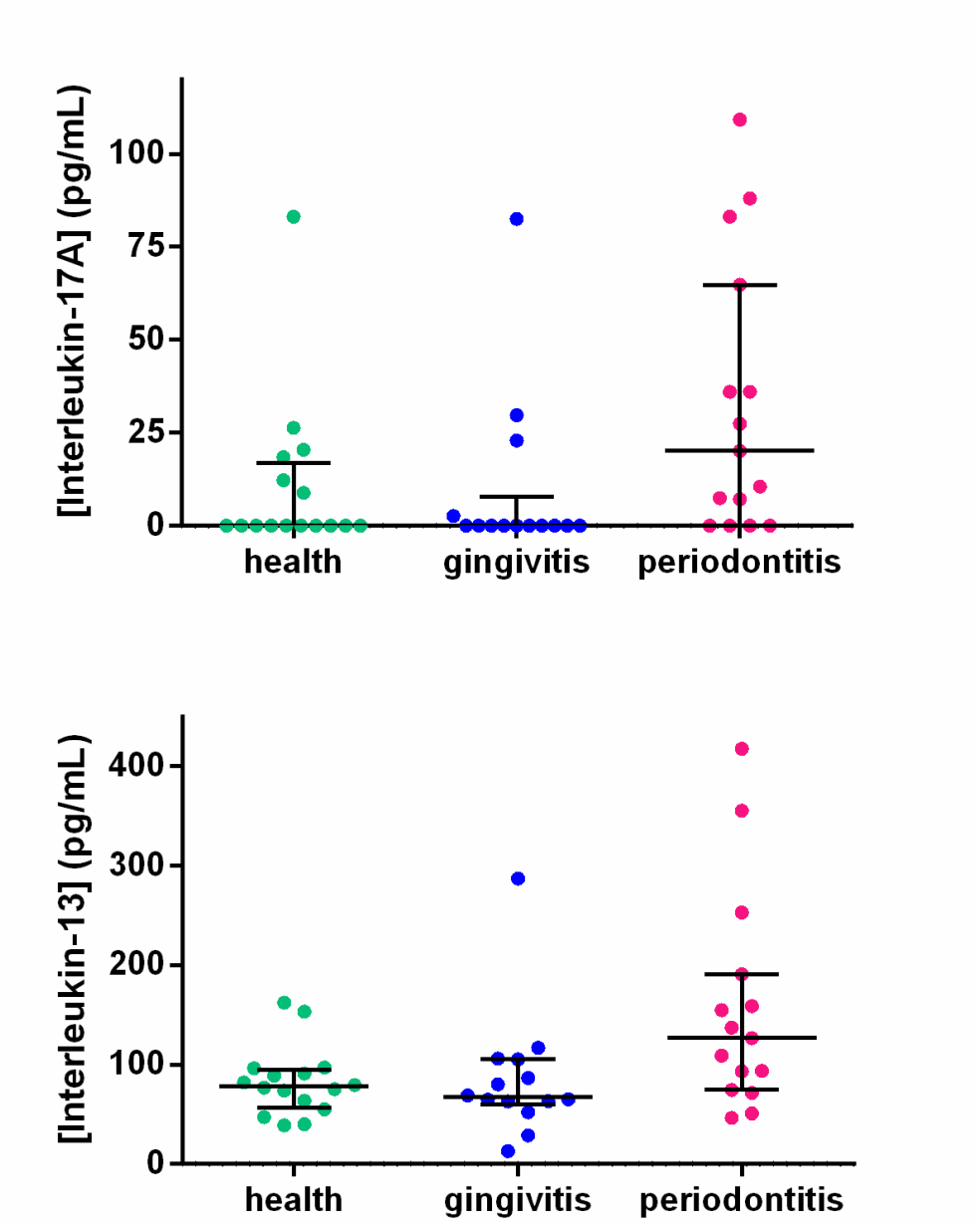


Figure 3-34 showing dot plots of the concentrations of the cytokines IL13 and IL17A that showed statistically significant differences only between the gingivitis and chronic periodontitis cohorts ($p<0.05$). Median and interquartile ranges indicated.

3.5.1.1 Correlations with clinical parameters

Given the different inflammatory cytokine profiles found in the different cohorts, correlations between the measured clinical parameters (Gingival Bleeding Index, Plaque Index and Bleeding on Probing) and the cytokine concentrations within the healthy, gingivitis and chronic periodontitis cohorts were explored and a number of relationships were found (Table 3-9; Table 3-10; Table 3-11). Probing depths were not included in these analyses as GCF samples from multiple sites were pooled in all individuals to get the volume required for the cytokine analysis. More numbers of cytokines were found

to be correlated with the percentage of sites bleeding on probing (BOP) in individuals in health, gingivitis and chronic periodontitis cohorts compared to the other clinical measures (Table 3-9). This is likely due to the fact that this is the first clinical measurement carried out along with probing depths in the oral health assessments. GBI and PDI are carried out later than BOP in the clinical protocol sequence and this may obscure some relationships with regards to the inflammatory status of the gingiva in general.

Cytokine	**Rho**	**95% CI**	**p-value**
E-selectin	0.5588	0.07133 to 0.8308	0.0244
G-CSF	-0.196	-0.6401 to 0.3461	<0.0001
IL17A	0.6112	0.1020 to 0.8666	0.0202
G-CSF	0.6094	0.09929 to 0.8659	0.0207
ICAM1	0.5604	0.02505 to 0.8460	0.0371
IL4	0.6529	0.1952 to 0.8770	0.0103
Interferon-ɣ	-0.4171	-0.7726 to 0.1375	0.0067

Table 3-9 listing cytokines measured in GCF of individuals in health (green), gingivitis (yellow) and chronic periodontitis (red) cohorts that showed significant Spearman's correlations against their BOP values.

Cytokine	Rho	95% CI	p-value
CCL2	0.5214	0.01853 to 0.8137	0.0403
E-selectin	0.5199	0.01651 to 0.8130	0.041
Interferon-ɣ	0.7598	0.3691 to 0.9223	0.0016
Interferon-α	0.7112	0.2739 to 0.9048	0.0043
G-CSF	0.5626	0.02815 to 0.8469	0.0362
IL4	-0.5629	-0.8470 to -0.02867	0.0361
E-selectin	0.5157	-0.01210 to 0.8187	0.0491

Table 3-10 listing cytokines measured in GCF of individuals in the health (green), gingivitis (yellow) and chronic periodontitis (red) cohorts that showed significant Spearman's correlations against their Gingival Bleeding Index values.

Cytokine	Rho	95% CI	p-value
IL17a	0.5775	0.09872 to 0.8392	0.0191
CCL3	0.5319	0.03306 to 0.8185	0.034
E-selectin	0.5129	0.0069 to 0.8098	0.0422
IL1α	-0.641	-0.8783 to -0.1502	0.0135

Table 3-11 listing cytokines measured in GCF of individuals in the health (green) and gingivitis (yellow) cohorts that showed significant Spearman's correlations against their Plaque Index values. No significant correlations were observed in the chronic periodontitis cohort.

BOP scores in the healthy group were positively correlated with E-selectin and IFN-g (p=0.058), and negatively correlated with IL10 (p=0.057) indicating that gingival bleeding is related to lower levels of the anti-inflammatory cytokine IL10 in the GCF even in the healthy cohort where the cut-off is at <20% of the total sites with bleeding upon probing, while increased concentrations of E-selectin and IFN-g are certainly considered to be pro-inflammatory (Table 3-9). Interestingly, increased BOP in individuals who were classified as having gingivitis is more associated with increased concentrations of IL17A, G-CSF and ICAM1 pointing to possibility that the dominant mechanism prevalent in the cohort being the activation of Th17 response and neutrophil proliferation and anti-apoptosis. While a significant reduction in the levels of IL4 was detected in the GCF of the chronic periodontitis patients, bleeding on probing was found to be more positively correlated with IL4 levels in contrast to the healthy cohort. Given the involvement of IL4 and IL10 regenerative mechanisms, this suggests that higher levels of bleeding on probing in chronic periodontitis patients may indicate a more favourable outcome as bleeding in this context relates to the same mechanism as observed in the healthy cohort but in an inverse manner. Also, a decrease in IFN-g in the CP cohort in association with BOP scores suggests that higher bleeding as it relates to increased IL10 is likely to result in inhibition of IFN-g production and the converse of this as observed in the healthy cohort further corroborates the relationship between BOP and GCF cytokines in the healthy and chronic periodontitis cohorts (Cope et al. 2011).

As discussed above, GBI and PI were carried out much later in the clinical protocol sequence, and though the number of cytokines related to GBI is much lower than BOP scores, the findings are consistent with this, and forming a smaller subset of the relationships observed with the cytokines and BOP scores (Table 3-10). With regards to

the plaque scores, when individuals with plaque coverage >30% but with <20% BOP and % sites with <4mm PD were included in the Spearman's correlation analysis, the Th17 cytokine IL17a and macrophage chemokine CCL3 were positively associated with an increase in plaque coverage (Table 3-11).

3.5.2 Salivary cytokine profile in health and disease

The salivary cytokine profile is thought to reflect the inflammatory condition of the oral cavity and it is suggested that GCF contributes substantially toward the cytokine concentrations in the saliva, in addition to a systemic influence (Ruhl et al. 2004; Wozniak et al. 2002). Whilst there are a few studies associating a number of pro-inflammatory cytokines in saliva with periodontitis, a clear consensus with regards to the salivary cytokine profile as observed with GCF in health and periodontitis is not found in the literature (Teles et al. 2010). In the present study, a small subset of the total number of saliva samples collected from healthy, gingivitis and chronic periodontitis patients were analysed to ascertain if differences between health and disease could be observed, in addition to associations with malodour.

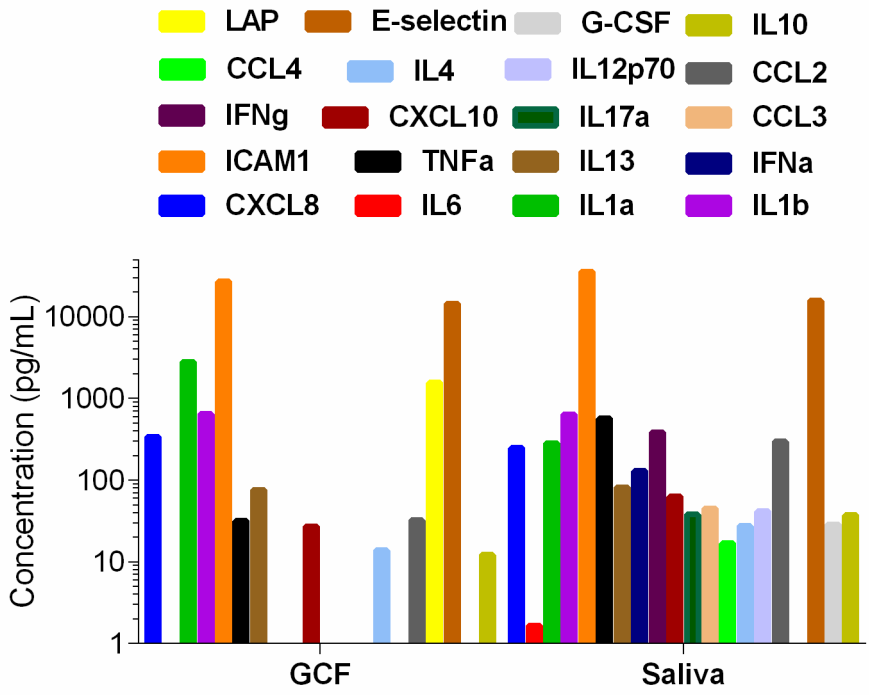


Figure 3-35 showing the different cytokine profile in GCF and Saliva in this study (median value of each cytokine concentration across all cohorts plotted as bars).

Paired samples when analysed by the Wilcoxon's signed rank test for differences in the concentrations of the cytokines between saliva and GCF across all cohorts showed IL1a and LAP to be significantly elevated in GCF, whereas higher concentrations of TNFa, IFNa, IFNg, G-CSF, CCL2, CCL3, CCL4, CXCL10, IL17A, IL10, IL12p70 and IL4 were observed in saliva compared to GCF in the same individuals (Figure 3-35). Teles et al. (2010) investigated salivary cytokines in healthy individuals and chronic periodontitis patients and did not find any differences between the cohorts and attributed the results to dilution of GCF contributing to the overall cytokine profile in saliva. Interestingly, Wozniak et al. (2002) investigating potential biomarkers and their inhibition in saliva reported that concentration of the eight cytokines they measured were much lower in whole saliva than parotid saliva, and attributed these results to the sequestering effect of salivary mucins on the cytokines. Of the cytokines tested, the authors found only IL1a levels not affected when added exogenously to parotid or whole saliva and this was also the only cytokine found to be significantly elevated in saliva of chronic periodontitis patients compared to health ($p<0.05$) in the present study. The observation that this cytokine was also more elevated in GCF than saliva in all individuals in the present study possibly suggests that GCF contributes to salivary IL1a levels. However, while GCF concentrations of IL1a were elevated in the chronic periodontitis patients compared to health, this was not statistically significant. This could be due to only a limited number of sites sampled for GCF in this study (2 per quadrant), and though these generally are the deepest gingival pockets in each quadrant, the more generalized chronic periodontitis patients had more sites with equally deep pockets in each quadrant that were not sampled—and GCF flow from these sites could further enhance salivary IL1a levels.

Unlike GCF, PLS analysis revealed that saliva samples in the gingivitis and chronic periodontitis patients clustered more closely and had greater separation from health, with five variables namely IL1a, IL1b, CXCL8, CXCL10 and CCL2 showing the largest loadings associated with the gingivitis and chronic periodontitis samples (Figure 3-36; Figure 3-37). However, receiver operating characteristic (ROC) analysis of the sum of all cytokines positively associated with the second principal component revealed an Area under Curve (AUC) value of 0.68 ($p=0.08$) for distinguishing health from gingivitis and chronic periodontitis, indicating that these salivary cytokines are only a marginally useful model for diagnostics or monitoring cytokines relating to periodontal disease.

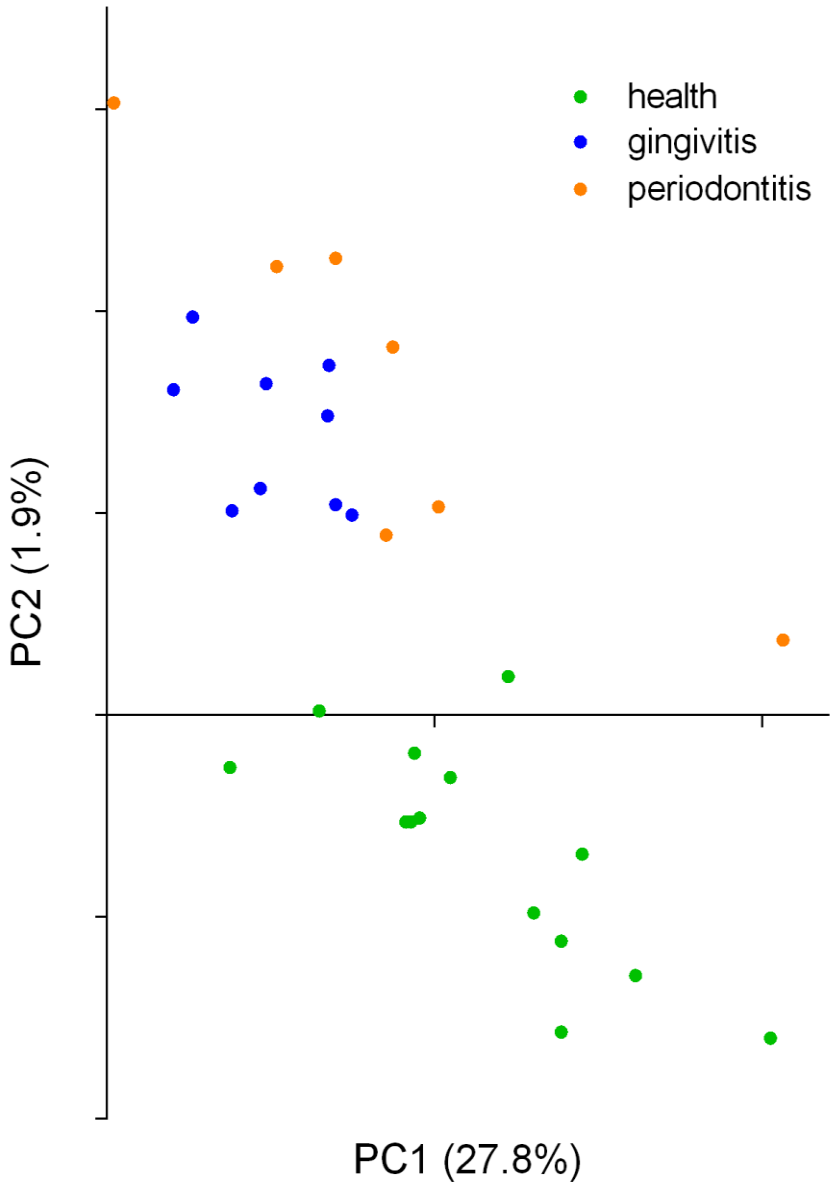


Figure 3-36 score plot from Partial Least Squares analysis of the measured cytokines in saliva samples of individuals showing distinct clustering between health and disease cohorts. Loading plot shown in Figure 3-37.

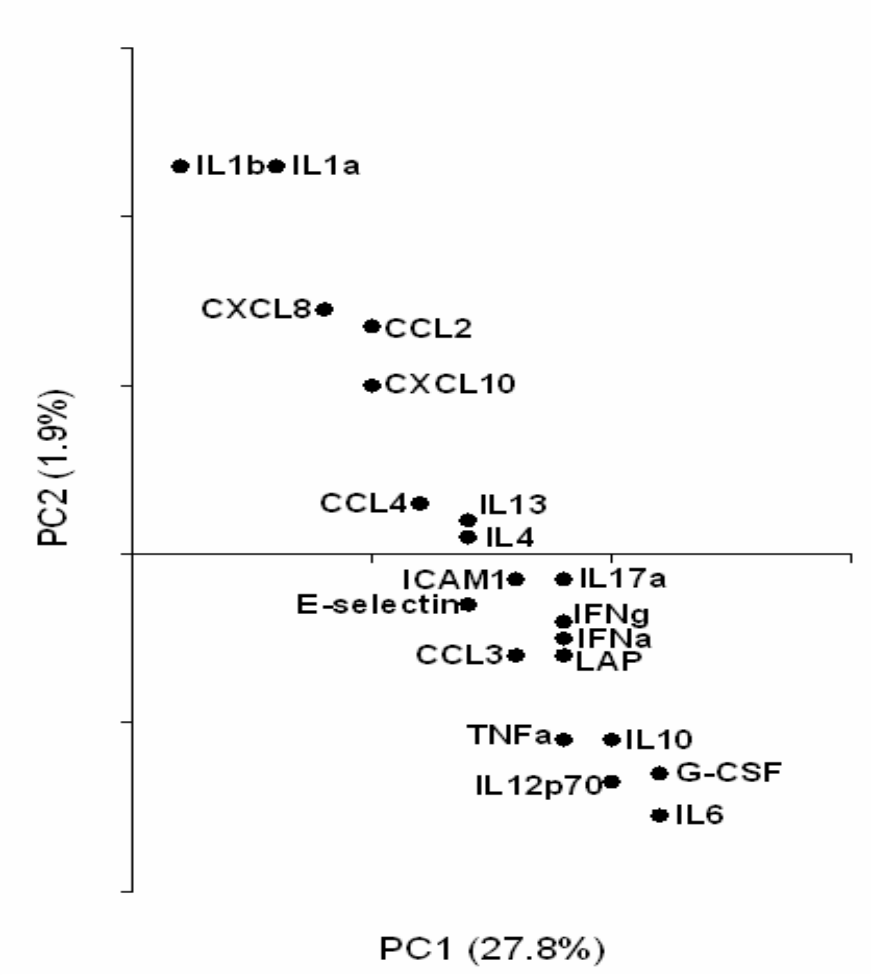


Figure 3-37 loading plot showing cytokines responsible for the clustering pattern shown in Figure 3-36.

3.5.2.1 Correlations with clinical parameters

Given that the clinical parameters such as PI, BOP, GBI and PD were used as diagnostic criteria, associations between salivary cytokines and these parameters in all individuals not split into cohorts were explored. More cytokines correlated significantly with plaque scores than any other clinical parameter, unlike those cytokines from GCF. These included positive associations with E-selectin, Interferon-α, CCL3, IL12p70 and IL17A (Table 3-12). Also, due to saliva being collected before any of the clinical assessments (BOP/GBI/PI) in the clinical protocol sequence, in principle should lead to less ambiguous relationships with these measurements provided that salivary cytokines are sufficiently influenced by periodontal disease to overcome the inhibitory nature of whole saliva to the cytokines measured. Whilst no statistically significant relationships were observed with salivary cytokines and BOP, the closest was the classic pro-inflammatory cytokine IL1b with a fair correlation (r=0.35, p=0.056), whereas GBI was

positively associated with IL6, IL13 and IFN-a (Table 3-12). The strongest correlations observed were IL6 with GBI and E-selectin with PI. As discussed for GCF, the association with GBI and IL6 indicates that GBI as a clinical measurement to grade periodontal disease is valid, and while plaque coverage is thought to be more variable, its correlation with the more periodontitis-associated cytokines as observed with the GCF samples suggests that the overall plaque present in the mouth has an effect on salivary cytokines and a combination of these clinical measurements will allow one to make a good assessment of the periodontal condition.

Gingival Bleeding Index			
Cytokine	**Rho**	**95% CI**	**P (two-tailed)**
IL6	0.4872	0.1431 to 0.7262	0.0063
IL13	0.4627	0.1119 to 0.7110	0.01
Interferon-α	0.4189	0.0579 to 0.6830	0.0212
Interferon-ɣ	0.3598	-0.0116 to 0.644	0.0508
Plaque Index			
E-selectin	0.506	0.1673 to 0.7378	0.0043
Interferon-α	0.4546	0.1018 to 0.7059	0.0116
CCL3	0.4183	0.05723 to 0.6826	0.0214
IL12p70	0.374	0.0048 to 0.6535	0.0417
IL17A	0.3675	-0.0028 to 0.6492	0.0457
Interferon-ɣ	0.3607	-0.0106 to 0.6446	0.0502

Table 3-12 listing Spearman's correlation coefficients for salivary cytokines that showed an association with GBI and PI with the samples not divided into cohorts (n=30; only cytokines yielding relationships with p<0.06 are listed).

3.5.3 Relationship between VSCs and cytokines in saliva and GCF

The association between breath VSCs and cytokines in saliva and GCF were explored, following from the observation that there was an overall increase in the breath methanethiol concentrations, the CH_3SH:H_2S ratio and Malodour Score in the chronic periodontitis cohort compared to health in the present study. Whilst the clinical parameters such as GBI, PI and BOP measure different characteristics of periodontal disease, they were found to be associated with different cytokines in GCF and saliva, perhaps identifying the mechanisms of the disease that they are more closely related with. For example, increase in plaque coverage was positively associated with an increase in the measured E-selectin levels in the saliva of individuals. It is well known that E-selectin plays an important role in neutrophil recruitment and leukocyte extravasation as an adhesion molecule and these data are consistent with the explanation that the greater coverage of plaque around the teeth would lead to higher stimulation of E-selectin production and this would be reflected in salivary levels of E-selectin. Similarly, IL6 is known to be a Th17 differentiating, Treg inhibiting cytokine and the method by which a clinician assesses GBI would more easily associate with underlying inflammatory processes that cause swelling or edema, and IL6 is thought to be a key cytokine that drives the inflammatory processes towards a more chronic inflammatory condition if unregulated. Finally, BOP is more related to depth of the periodontal pocket in addition to inflammation, and the association with the classic osteoclastogenic cytokine IL1b further illustrates this point, as a deeper pocket indicates greater alveolar bone resorption. It was hypothesized that because malodour as defined by the VSC concentrations measured showed a significant difference with health and chronic periodontitis, it might also be positively associated with inflammatory processes that lead to and are active in periodontitis, in a similar manner to GBI, PI and BOP.

3.5.3.1 VSCs and GCF cytokines

The cytokines TNFa and IL12p70 were negatively correlated with Malodour Scores while weak positive associations were observed with IFNa and IL6 in GCF (Table 3-13). Given that different immune mechanisms are more dominant in health, gingivitis and chronic periodontitis, the malodour scores in the different cohorts were analysed separately, yielding only one significant correlation, namely an inverse relationship with IL12p70 in the chronic periodontitis cohort. The association with IL12p70, IFNa and IL6 is consistent with the hypothesis that malodorous VSC concentration in the breath is associated with inflammation in the periodontium. While TNFa negatively correlating

with malodour scores is not compatible with this view, higher TNFa levels was associated with the healthy cohort in the present study and in this sense, these data are just internally-consistent with the findings of the health and disease associated cytokine profiles in GCF (Figure 3-31; Figure 3-32). There is evidence that suggests that a sustained release of H_2S can inhibit formation of TNFa from LPS stimulated macrophages in a concentration dependent manner, and another study reports that H_2S delivered in gaseous form to stimulate macrophages at concentrations much lower than usually found in the mouth air is sufficient to induce osteoclast differentiation and TNFa production (Whiteman et al. 2010; Ii et al. 2010). In reality, endogenous H_2S production could also play a part and it is possible that these opposing mechanisms could exist in a dynamic equilibrium in the oral cavity. The lack of association between sulcular H_2S measured in this study and GCF TNFa points to this possibility, with a weak positive relationship between sulcular H_2S and TNFa in the healthy cohort (r=0.33) and a weak negative relationship observed with chronic periodontitis cohort (r=-0.3) indicating that opposing mechanisms are at play in the different cohorts.

When the individual disease cohorts were not separated, the correlational analysis revealed that methanethiol and the methanethiol: hydrogen sulfide ratio yielded the strongest relationships even though H_2S and malodour scores were also found to be associated with the same cytokines (Table 3-13). Therefore methanethiol concentrations and the VSC ratios were further analysed in the gingivitis and chronic periodontitis groups; the healthy cohort was not analysed in this instance due to low detection frequency of methanethiol in this cohort leading to less meaningful statistical analyses. These analyses yielded negative associations with IL17A in the gingivitis cohort and the cytokines IL12p70, TNFa and IL1b in the chronic periodontitis cohort (Table 3-14). While some of these relationships are consistent with breath VSCs being associated with periodontal inflammation, the negative correlations with cytokines such as IL17A and IL1b were less compatible with this hypothesis. This could be partly due to GCF being sampled from limited sites in the mouth and one would expect breath VSCs to be more consistent with the overall inflammatory status of the oral cavity and by extension, salivary cytokines. Indeed, associations with sulcular VSCs gave a more consistent picture of the positive relationship between VSCs and the localized inflammation detected by the GCF cytokine profile (Table 3-15).

Malodour Score			
Cytokine	**Rho**	**95% CI**	**p-value**
IL12p70	-0.382	-0.6154 to -0.08699	0.0105
TNFa	-0.3874	-0.6193 to -0.09337	0.0094
Hydrogen sulfide (ppb)			
IL12p70	-0.3356	-0.5812 to -0.03392	0.026
TNFa	-0.3698	-0.6065 to -0.07297	0.0135
Methanethiol (ppb)			
IFNa	0.3767	0.08084 to 0.6115	0.0117
IL6	0.3053	0.0001691 to 0.5584	0.0439
IL4	-0.3638	-0.6021 to -0.06607	0.0152
IL12p70	-0.4016	-0.6296 to -0.1099	0.0069
TNFa	-0.4101	-0.6357 to -0.1200	0.0057
Methanethiol: hydrogen sulfide			
IFNa	0.3516	0.05212 to 0.5931	0.0193
IL4	-0.3183	-0.5682 to -0.01461	0.0352
TNFa	-0.4038	-0.6311 to -0.1125	0.0066
IL12p70	-0.4404	-0.6572 to -0.1563	0.0028

Table 3-13 listing Spearman's correlation coefficients for GCF cytokines that showed an association with sulcular H_2S, breath H_2S and CH_3SH, ratio of CH_3SH to H_2S and malodour score with the samples not divided into disease cohorts (n=44).

Methanethiol (ppb)			
Cytokine	**Rho**	**95% CI**	**P (two-tailed)**
IL12p70	-0.418	-0.7832 to 0.1617	0.0131
Methanethiol: hydrogen sulfide ratio			
IL17a	-0.3013	-0.7256 to 0.2890	0.0251
IL12p70	-0.542	-0.8383 to 0.001493	0.0009
TNFa	-0.5654	-0.8481 to -0.03232	0.035
IFNg	-0.318	-0.7342 to 0.2720	0.043

Table 3-14 listing Spearman's rho values for GCF cytokines that showed an association with CH_3SH: H_2S ratio and methanethiol concentrations in the breath of gingivitis (yellow) and chronic periodontitis cohorts (red).

Cytokine	Rho	95% CI	P (two-tailed)
IL17a	-0.3625	-0.7566 to 0.2248	0.005
G-CSF	-0.4204	-0.7844 to 0.1589	< 0.0001
LAP	-0.5048	-0.8224 to 0.05270	0.0315
CCL3	-0.5305	-0.8334 to 0.01754	0.0024
IFNg	-0.6418	-0.8786 to -0.1517	< 0.0001
IFNa	-0.7587	-0.9219 to -0.3668	0.001
G-CSF	0.6044	0.1169 to 0.8572	0.017

Table 3-15 listing Spearman's rho values for GCF cytokines that showed an association with sulcular H_2S concentrations in the healthy (green), gingivitis (yellow) and chronic periodontitis cohorts (red).

Associations that were found with sulcular H_2S and GCF cytokines may not always be due to a simple stimulation of a proinflammatory response due to bacterial VSC production. It is well known that VSCs and in this case hydrogen sulfide can influence cytokine levels by sulfhydrylation of key cysteine residues in the peptide chains of the cytokines, in addition to other mechanisms such as transcriptional modulation and also helping in the invasion of periodontopathic bacteria and proteolytic breakdown of cytokines (Li et al. 2011). All of these mechanisms could influence cytokine levels in GCF and these are not always synergistic, while endogenous hydrogen sulfide production further complicates interpretation, as it is not possible to distinguish the relative contribution towards the sulcular VSCs detected in this study having a bacterial or potentially dysregulated host sulfur metabolism in the gingiva as a source. For example, endogenous H_2S is thought to activate the transcription factor NF-κB by way of sulfhydration leading to an anti-inflammatory state, in addition to encouraging GAPDH mediated apoptotic mechanisms by direct sulfhydration of GAPDH (Mir et al. 2014; Sen et al. 2012). It is quite possible that bacterial H_2S production also contributes to these mechanisms, and it may well be that bacterial VSC production in the gingival milieu could be one of the main mechanisms of the commensal interaction between the oral microbiota and host immune pathways.

A biphasic effect of H_2S signalling is observed in studies that use NaHS as a H_2S donor, where an inhibitory effect is observed at lower concentrations (100-200μM) and a stimulatory effect at higher concentrations (>500μM) is reported. This cannot explain the observed pattern as clearly, the amounts of H_2S detected in the gingivitis cohort is

similar to the chronic periodontitis cohort (Whiteman et al. 2010; Figure 3-18). It could be that other VSCs such as methanethiol and in particular carbon disulfide detected in higher frequency in the chronic periodontitis cohort could have a role to play in these signalling events, resulting in a markedly different relationship. While the VSC profile in the subgingival microenvironment is difficult to interpret in conjunction with its relationship with the cytokine profile, VSCs in the breath have a more direct relationship with the oral microbiota. Therefore, the presence or absence of methanethiol in the breath could reveal important relationships with regards to the immune processes in the subgingival environment. Given the markedly different immune mechanisms prevalent subgingivally in chronic periodontitis, only the healthy and gingivitis cohorts were included in this analysis. This is also a more valid comparison as the VSC concentrations present in the breath of the gingivitis cohort did not differ significantly with health (Section 3.2). It was found that individuals with the presence of methanethiol in their breath had reduced levels of TNFa and IL12p70, and significantly higher levels of interferon-alpha in GCF (Figure 3-38). Furthermore, the CH_3SH: H_2S ratio in this data set yielded fair correlations with IFNa and IL12p70 confirming the previous findings (Table 3-16). However, individuals with clinically classified gingivitis were overrepresented in the methanethiol positive group (64%) compared to the negative group (31%). For the cytokines interferon-alpha and IL12p70 distinct groupings were observed but no clinical difference with regards to individuals belonging to health or gingivitis cohorts could be ascertained (Figure 3-38).

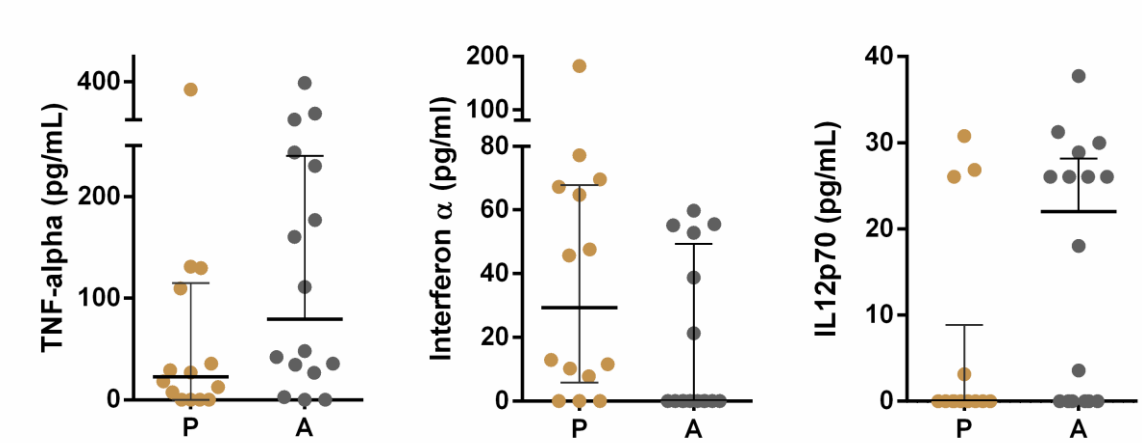


Figure 3-38 showing scatter dot plots of cytokines that showed differences between methanethiol positive (P) or negative (A) individuals. Only IFN-a showed statistical significance at p=0.043.

Methanethiol: hydrogen sulfide ratio			
Cytokine	**rho**	**95% CI**	**P (two-tailed)**
Interferon α	0.3884	0.02160 to 0.6631	0.0339
IL12p70	-0.3595	-0.6438 to 0.01200	0.051

Table 3-16 listing Spearman's rho values for the cytokines that showed the strongest associations with the CH_3SH: H_2S ratio in healthy and gingivitis cohort GCF.

3.5.3.2 VSCs and Salivary cytokines

The cytokines present in saliva were hypothesized to be more closely associated with breath VSCs and potential correlations were explored. As discussed in section 3.5.2, any relationships observed will be despite the inhibitory effect of whole saliva on the detection of these cytokines, although IL1a is one cytokine for which no inhibitory effect is known. Accordingly, moderately positive correlations were observed for the chemokine CCL2 and IL1a in association with malodour score, hydrogen sulfide and methanethiol concentrations in the breath (Table 3-17). A stronger relationship was also observed between these cytokines, in addition to the chemokine IL8 (CXCL8) with increasing CH_3SH: H_2S ratio in the breath of individuals. These cytokines were more associated with gingivitis and periodontitis cohorts as found in the multivariate analysis of these salivary cytokines and these observations support the hypothesis that VSCs in the breath as it relates to health and chronic periodontitis, are associated with a pro-inflammatory state in the oral cavity (Figure 3-36; Figure 3-37). Further, the VSC methanethiol was found to be largely responsible for these observations. For example, when all samples were split into methanethiol positive or negative groups, the methanethiol positive samples showed significantly elevated IL8, IL1a and CCL2 concentrations than the methanethiol negative samples (Figure 3-39). To minimize the contribution of the saliva samples from the chronic periodontitis cohort towards these results, samples from just the healthy and gingivitis cohorts were analysed and saliva from methanethiol positive individuals still showed significantly elevated IL1a, and though IL8 and CCL2 levels were not statistically different at $p<0.05$, elevated concentrations were still observed (Figure 3-39). Although, individuals with clinically classified gingivitis were over represented in the methanethiol positive group (55%) compared to the methanethiol negative group (25%), elevated levels of IL1a, CCL2 and IL17A could still be observed in the saliva samples of individuals in the healthy cohort whose breath samples were positive for methanethiol (Figure 3-39).

The observation that methanethiol concentrations and CH_3SH: H_2S in the breath were largely associated with pro-inflammatory cytokines in saliva and to some extent in GCF

supports the hypothesis that methanethiol is the VSC that identifies increased disease activity. This is because unlike hydrogen sulfide, methanethiol production in the oral cavity is entirely bacterial and increased concentration of methanethiol suggests that bacteria that can produce this VSC are more active in the oral biofilm relative to other species. Indeed, as discussed in the next chapter, bacteria that have the capability to produce methanethiol are almost exclusively putative periodontopathogens and the observed increase of the classic neutrophil chemokine IL8 and macrophage chemo attractant CCL2 (MCP-1) in saliva in the methanethiol positive individuals suggests wholesale activation of immune mechanisms taking place in the oral cavity in response to the activity of the few methanethiol producing bacteria. Furthermore, a striking difference between GCF and salivary cytokine profiles was the low prevalence of the Latency associated Peptide (LAP) in saliva of healthy individuals, and the positive association of LAP in saliva with breath methanethiol and the CH_3SH: H_2S ratio indicates that immune regulatory mechanisms are much more active when higher methanethiol concentrations or CH_3SH: H_2S was observed in the breath of individuals.

Malodour Score			
Cytokine	**rho**	**95% CI**	**P (two-tailed)**
CCL2	0.3971	0.02450 to 0.6729	0.0329
IL1a	0.3657	-0.01226 to 0.6523	0.0511
Methanethiol			
CCL2	0.4062	0.03528 to 0.6787	0.0288
Methanethiol: hydrogen sulfide ratio			
CCL2	0.4903	0.1398 to 0.7316	0.0069
IL1a	0.4046	0.03341 to 0.6777	0.0295
LAP	0.3906	0.01671 to 0.6686	0.0362
IL8	0.3821	0.006828 to 0.6631	0.0408

Table 3-17 listing Spearman's correlation coefficients for salivary cytokines that showed significant associations (p>0.05) with breath H_2S and CH_3SH, ratio of CH_3SH to H_2S and malodour score with the samples not divided into disease cohorts (n=29).

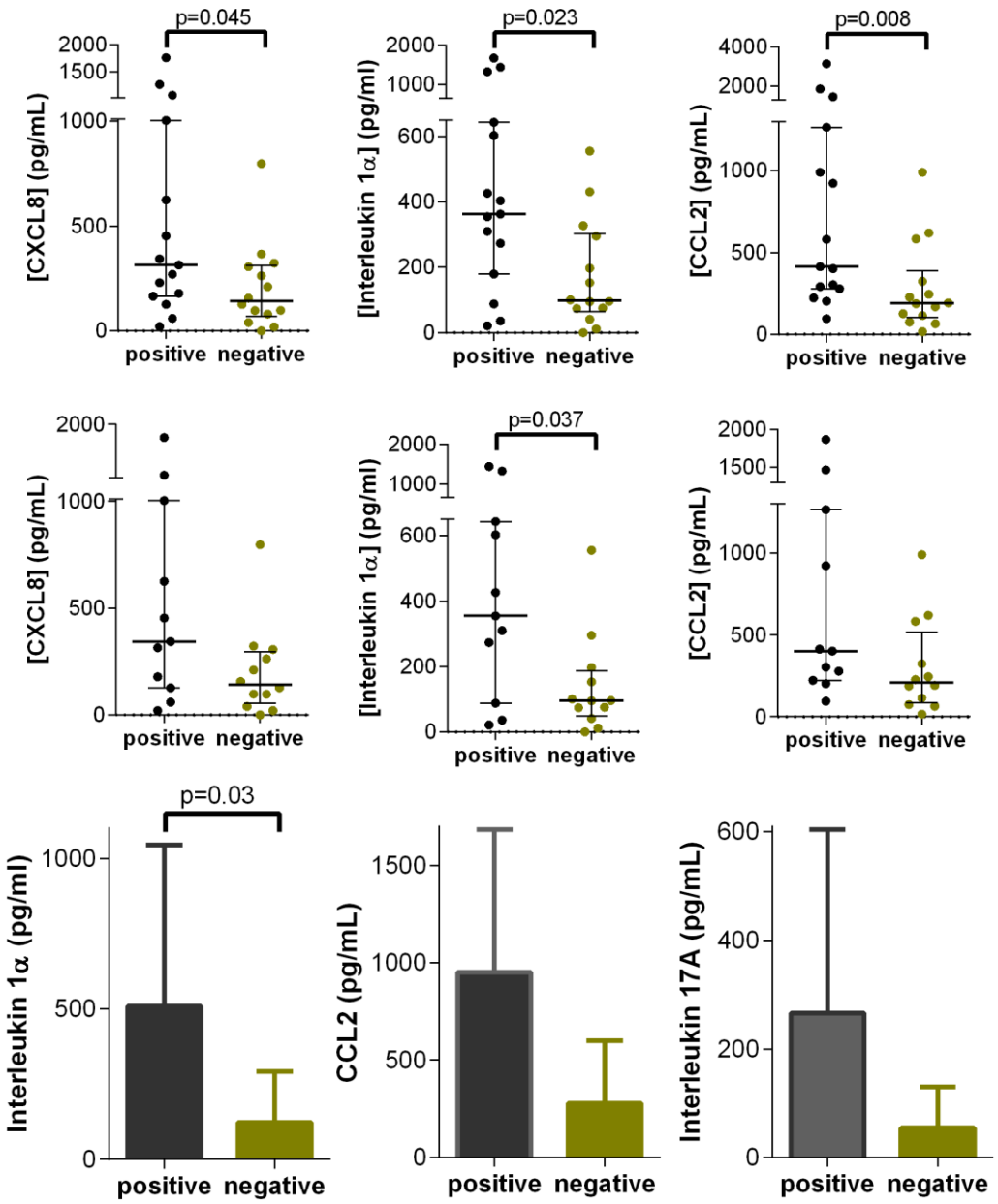


Figure 3-39 showing salivary cytokines that differed between individuals whose breath samples were positive or negative for methanethiol. Cytokines that showed statistically significant differences are indicated. Top row: individuals from all cohorts split into methanethiol positive and negative groups. Median and interquartile ranges indicated. Middle row: individuals from only healthy and gingivitis cohorts were included. Median and interquartile ranges indicated. Bottom row: Mean (±SD) levels of cytokines that showed differences in methanethiol positive (n=5) and negative (n=9) individuals within the healthy cohort.

3.6 Summary

The microbial and inflammatory aspects of the association between VSCs in the breath and periodontal disease were investigated by employing a number of different techniques. Breath analysis confirmed the different VSC profile in the breath of periodontitis patients with an increased presence of methanethiol in the breath. Methanethiol concentration and methanethiol to hydrogen sulfide ratio in the breath were found to correlate positively with clinical parameters of periodontal disease. A gas chromatographic method was developed to detect VSCs in the periodontal pocket, leading to finding significantly elevated concentrations of hydrogen sulfide in the periodontal pockets of individuals with gingivitis and periodontitis. The microbial aspect of periodontitis was studied using a qPCR methodology with specific samples from the periodontal niches, tongue and saliva to find a number of positive associations between the prevalence and abundance of putative periodontopathogens which are known VSC producers in the niches studied. The observed changes of periodontopathogens were also found to correlate positively with breath methanethiol and subgingival hydrogen sulfide concentrations. qPCR assays were developed to detect specific microbial species that were associated with malodour and periodontal disease, helping elucidate the ecological shifts observed with these species in health and disease. The VSC producing capability of the samples from different oral niches were studied to reveal periodontal niches as the most dynamic in VSC production in free cysteine substrate in health and disease, suggesting the importance of VSC production in these niches. The inflammatory cytokine profile in health and disease was determined by analysis of saliva and gingival crevicular fluid samples of individuals, finding that a more disease associated inflammatory profile correlated with breath and subgingival VSCs.

4 THE VOLATILE SULFUR COMPOUND PRODUCING MICROBIOME

Advances in DNA sequencing technologies have accelerated the characterisation of the oral microbiome in health and disease in recent years. Familiar concepts of the microbial role in health and disease have been refined and extended in the search for a unified pathogenetic theory of oral diseases involving microbial plaque (Rosier et al. 2014). Ecological dynamics involving different bacterial taxa have been known to play a part in the aetiopathogenesis of gingivitis and chronic periodontitis and efforts have been focused on identifying the microbial taxa most responsible for altering the overall microbiota of the oral soft and hard tissue niches in the different disease states, in addition to characterising individual differences (Kistler et al. 2013; Lourenço et al. 2014; Abusleme et al. 2013; Liu et al. 2012).

Intra-oral halitosis and its relation to chronic periodontitis, depends on the sulfur metabolism of the oral microbiome, and an accelerated sulfur metabolic cycle in the oral microbiome confers selective advantages in the face of the mounting host inflammatory challenge (Yaegaki 2008). Mechanisms by which this could help create a dynamic shift in the ecology of the oral microbiota are still being investigated with many being proposed. Although, the majority of the gram-positive and gram-negative bacterial species have orthologs of the enzyme family responsible for cysteine and/or methionine degradation, it was found that fewer species are efficient at degrading free cysteine and methionine in the fluids surrounding the oral mucosa such as the saliva and gingival crevicular fluid (Persson et al. 1990; Persson et al. 1989). Fewer still, are able to produce the volatile sulfur compounds from peptides in the niches relevant to

periodontal diseases such as the subgingival and interdental niches. Indeed, if sulfur metabolism of the microbiota is an important contributor to periodontal diseases, one would expect to find bacterial taxa that are known to be particularly efficient VSC producers to be causing the ecological shift in the niches relevant to disease at the various stages of disease progression. The aims of this investigation were therefore to characterise the native bacterial taxa at the niches relevant to periodontitis and oral malodour namely subgingival plaque, interdental plaque and tongue, in healthy individuals. Then, to compare this to individuals with clinically identified gingivitis and chronic periodontitis in terms of the bacterial taxa causing the ecological shift and hence to determine the proportions of VSC producing taxa that are involved in the changes to the microbial community.

4.1 Samples & sequencing summary

A total of 113 samples from 53 participants overall passed DNA quality control and were sequenced, with the number of samples in each niche per cohort listed in Table 4-1. An aggregate of 11,473,531 sequences were assembled after initial quality control, yielding a total of 3,483,725 unique sequences with 407bp mean length. The mean (±SD) number of sequences from the different samples overall were: interdental plaque 82,136(±28,704); subgingival plaque 106,516(±38,363); tongue 117,732(±35,209). HOMINGS analysis resolved on average 87% of the total sequences from each sample to human oral species and genus listed in the curated HOMD (Human Oral Microbiome Database), whilst the VAMPS based RDP-classifier also reached a similar sequencing depth with classification, except resolving only up to genus level (Table 4-2). Rarefaction curves plotted against alpha-diversity measures such as Phylogenetic Diversity, Chao I, Shannon and Simpson indices based on Operational Taxonomic Units (OTU) clustering in QIIME, suggested a sub-sampling depth of 8000 sequences per sample would give sufficient coverage of the observed diversity in all the niches (Figure 4-1).

Cohort	Interdental	Subgingival	Tongue	No. of sample triads
Health	18	12	17	9
Gingivitis	12	8	13	7
Chronic Periodontitis	12	10	8	6

Table 4-1 listing the number of samples analysed from each cohort and the number of individuals who contributed all three niche samples that passed quality control.

Identification level	Percentage of total sequences (Mean±SD %)					
	HOMINGS			VAMPS		
	Interdental	Subgingival	Tongue	Interdental	Subgingival	Tongue
Genus	31.5±9.5	32.9±18.3	29.3±12.2	79.3±10.3	89.1±3.9	87.7±7.1
Species	54.4±11.4	54.8±20.0	57.6±14.7	n/a	n/a	n/a
Unmatched	14.1±7.4	12.4±8.7	13.2±12.7	6.8±2.9	5.1±1.6	4.8±1.0

Table 4-2 comparing the percentage of sequences assigned by HOMINGS and VAMPS to their respective maximum identification level.

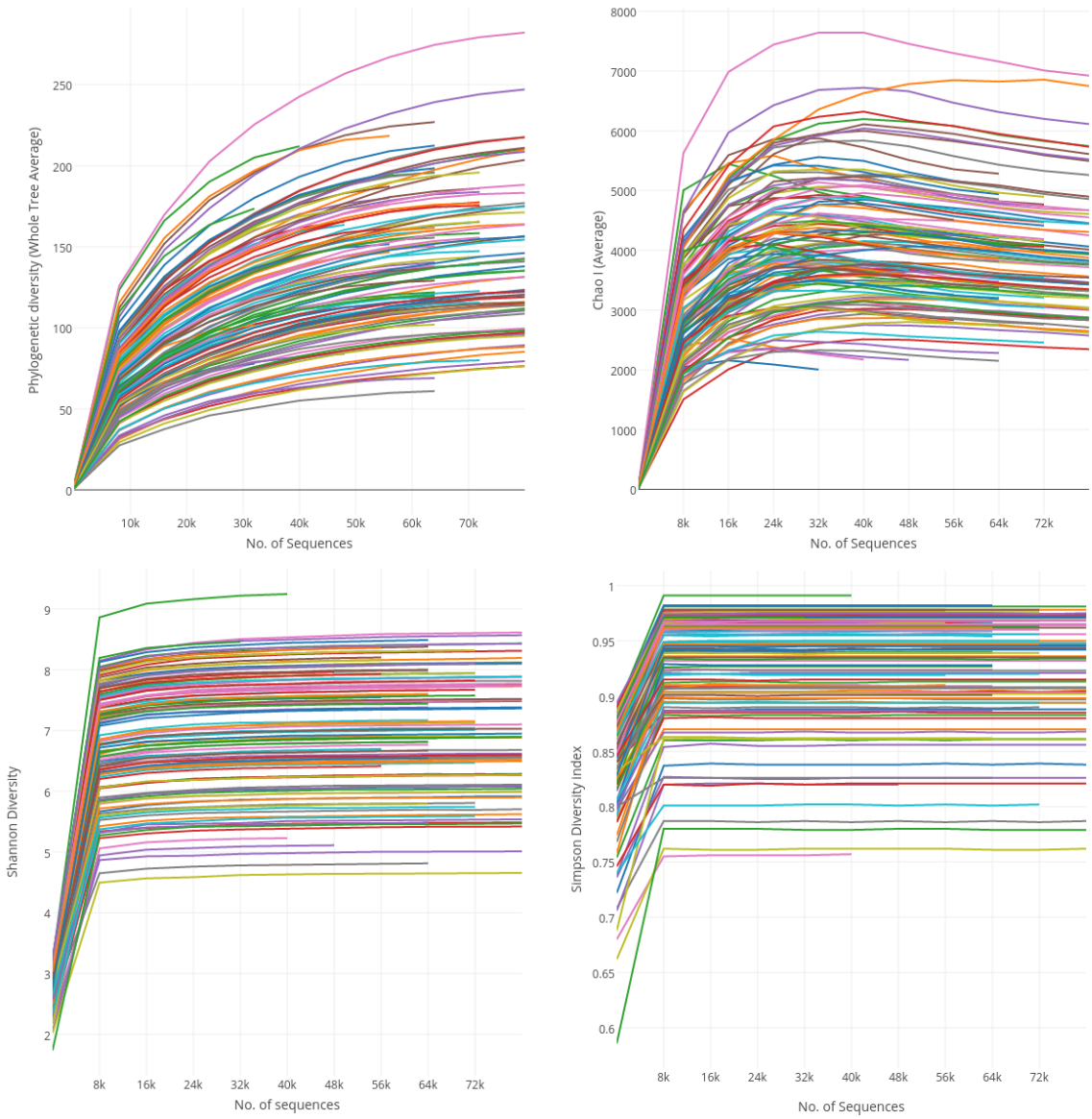


Figure 4-1 showing rarefaction curves for all samples plotted against Phylogenetic Diversity, Chao I, Shannon and Simpson diversity indices at a sub-sampling depth of 8000 sequences.

4.2 Species diversity, chronic periodontitis and oral malodour

An increase in ecological diversity and species richness in the oral cavity is often reported in association with periodontal disease, for example in gingivitis and chronic periodontitis (Kistler et al. 2013; Ge et al. 2013; Griffen et al. 2012; Liu et al. 2012). However, owing to much methodological heterogeneity in sequence processing pipelines and indeed, participant selection criteria and sampling strategies at the more basic level, this observation has not been consistently supported in the literature (Galimanas et al. 2014; Jünemann et al. 2012).

4.2.1 Microbial diversity between niches and cohorts

In the present study, a triad of oral samples collected from the same participants in healthy, gingivitis and chronic periodontitis cohorts were analysed with alpha-diversity measures such as Chao 1, Shannon-Weaver and Simpson Diversity indices with the RDP classified data at the genus-level. Comparing these metrics between the niches and cohorts yielded useful relationships (Table 4-3). For example, the interdental plaque was consistently richer than the subgingival plaque in healthy individuals but not in gingivitis and chronic periodontitis. Whilst, the richness between the niches were not significantly different in gingivitis and chronic periodontitis, the diversity metrics were able to distinguish between the subgingival and interdental niches in both health and gingivitis but not in chronic periodontitis. This indicates that while the richness of the subgingival plaque increases in gingivitis, the overall diversity remains the same in comparison to the interdental plaque, even at the genus-taxonomic level.

Further, comparing these measures in the same niche between the cohorts did not reveal any significant differences, though non-significant differences were found, such as increase in the interdental plaque diversity in gingivitis compared to health and CP, and increase in subgingival diversity in CP compared to health or gingivitis. Determining the richness and diversity of microbial plaque in the interdental and subgingival niches of individuals may be a useful marker to distinguish gingivitis from periodontitis and health as it accounts for individual variation. Additionally, whilst the richness estimators were able to distinguish the interdental niche from the tongue in the healthy and gingivitis cohorts, the diversity as measured by the Shannon, Simpson and Inverse Simpson indices failed to do so. However, in the CP cohort, no differences were observed with any of these measures when the different niches were compared. Together these data suggest that in the gingivitis stage, the subgingival niche experiences an increase in richness, as more genera colonise the niche relative to the interdental plaque, which culminates in the changes observed in the subgingival plaque of chronic periodontitis patients where the diversity matches the interdental plaque indicating a dysbiotic state, given that the interdental niche is the least disturbed in terms of prevalent oral hygiene practices and possibly represents a ‘proto-dysbiotic’ microbial reservoir in the healthy individuals.

Indices	Interdental vs Subgingival			Interdental vs Tongue			Subgingival vs Tongue		
	H	G	CP	H	G	CP	H	G	CP
Observed Richness	+	ns	ns	++	++	ns	ns	ns	ns
Abundance Coverage Estimator	+	ns	ns	++	++	ns	ns	+	ns
Chao I	ns	ns	ns	+	ns	ns	ns	ns	ns
Shannon Diversity	++	++	ns	ns	+	ns	ns	ns	ns
Simpson Diversity	+	++	ns	ns	ns	ns	ns	ns	ns
Inverse Simpson	+	++	ns	ns	ns	ns	ns	ns	ns

Table 4-3 showing differences in the diversity and richness estimates of plaque collected from the different niches within the same individual (Friedman's test with Dunn's multiple comparison; significance indicated as +; H=Health, G=Gingivitis, CP=Chronic periodontitis).

4.2.2 Microbial diversity and oral malodour

A metagenomic study that investigated the tongue microbiota in relation to oral malodour did not find an association with microbial diversity (Yang et al. 2013). However, this study did not use an organoleptic assessment of the breath of subjects to determine malodour, but rather relied on an arbitrary H_2S concentration in 100 ppb steps for absence of malodour and moderate/severe malodour. In the present study, the malodour score used to distinguish individuals with or without malodour employed both VSCs measured in conjunction with the recognition threshold for the VSCs and enabled a more realistic classification criterion. Within the healthy cohort, a non-significant increase in diversity of the tongue samples of individuals with malodour was observed, compared to individuals without malodour at the genus-level (Figure 4-2). In addition, a non-significant decrease in the diversity of the interdental plaque was also observed with regards to individuals who had malodorous breath compared to those that did not have malodour. Although limited in the number of observations, healthy individuals with severe malodour also displayed a clearer difference with regards to diversity and richness in the tongue compared to healthy individuals without malodour.

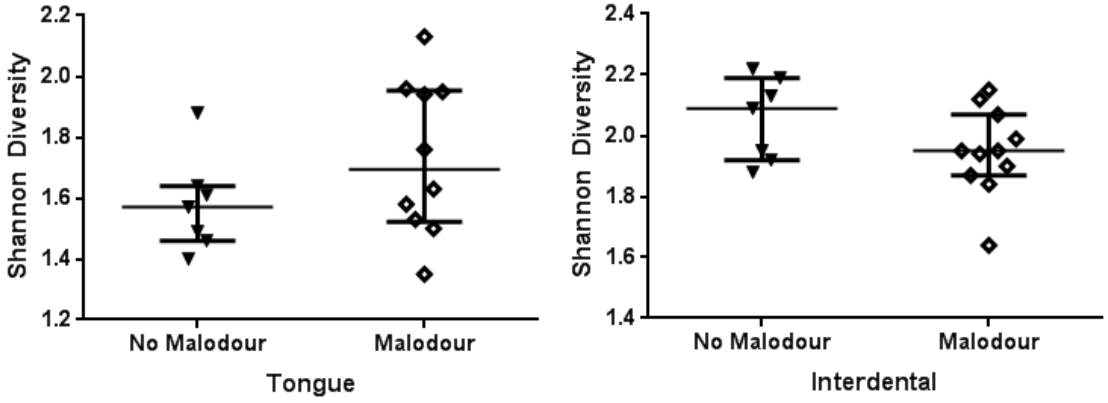


Figure 4-2 plotting Shannon diversity index for healthy individuals with or without malodour in the tongue and interdental plaque (Median and interquartile range is shown).

Exploring possible associations between oral malodour as measured by the malodour score in the participants in the different cohorts revealed that interdental and subgingival niches too have a relationship with malodorous breath (Table 4-4**)**. The strongest associations between malodour and alpha-diversity were observed for the subgingival niche in chronic periodontitis and interdental plaque in the gingivitis cohort. Whilst malodour in the healthy cohort was associated an increase in diversity and richness in the tongue, a negative relationship was observed with the interdental plaque. The latter association was observed to be stronger in the gingivitis cohort in relation to the diversity measures, with the richness and diversity of subgingival plaque showing a stronger positive association with malodour, than in health. As established in the previous section (4.2.1), a general equalisation of the richness and diversity is observed in chronic periodontitis between the different niches. However, unexpectedly, higher malodour scores within this cohort were still associated with an increase in richness and diversity of all three niches—the strongest association being with the subgingival plaque (Table 4-4).

Previous studies have established the association between oral malodour, volatile sulfur compounds in the breath and plaque-induced periodontal diseases (Takeuchi et al. 2010; Quirynen et al. 2009), and available ecological surveys of the human oral cavity have tended to focus on the tongue or saliva in relation to oral malodour, as those two niches are thought to play important roles in halitosis (Yang et al. 2013; Takeshita et al. 2012). The present study is the first to consider the ecology of the periodontal niches in relation to oral malodour and has found important associations between microbial diversity in the periodontal environment and malodour exhibited in

individuals with gingivitis, chronic periodontitis and periodontal health. Whilst changes occurring in the tongue ecology may be important in the malodour present in individuals with good oral health, periodontal habitats such as the subgingival and interdental niches proved to be more associated with malodour found in individuals with gingivitis or chronic periodontitis. Although, the tongue may be the dominant niche responsible for malodour in the gingivitis and chronic periodontitis cohorts due to its surface area, the associations found in this analysis suggests that subgingival microbiota may have a role to play in influencing the composition of the tongue microbiota and hence, oral malodour. These data highlight the interconnected nature of the different oral niches and the importance of niches other than the tongue to inform the relationship of the oral microbiota with malodour in health and disease.

Indices	**Health**			**Gingivitis**			**Chronic Periodontitis**		
	T	S	I	T	S	I	T	S	I
Observed Richness	0.38	0.03	-0.10	0.01	0.24	-0.08	0.40	0.81	0.40
Abundance Coverage Estimator	0.29	0.15	-0.11	0.03	0.17	-0.06	0.40	0.72	0.38
Chao I	-0.06	0.28	0.01	0.05	0.29	0.02	0.62	0.55	0.24
Shannon Diversity	0.39	-0.13	-0.40	0.08	0.33	-0.66	0.31	0.72	0.07
Simpson Diversity	0.22	-0.20	-0.51	0.03	0.30	-0.45	0.22	0.66	0.01

Table 4-4 listing Spearman's rho values computed for the malodour scores linked with the alpha-diversity metrics for the different niches from the RDP-classifier (T=tongue; S=Subgingival; I=Interdental) within each cohort.

4.3 General bacterial ecology in health and disease

The human oral microbiota is one of the most extensively characterised human microbiome in terms of health and disease, and studies have emphasized the unique nature of each niche within the oral cavity (Belda-Ferre et al. 2012; Abusleme et al. 2013; Zaura et al. 2009). Attempts to describe the inter-individual variation present in the microbiota of oral niches have suggested that while a particular strain or species in a niche may be shared by different individuals, and even among different niches in the same individual, variation is observed in the relative abundances of the species or strain, and this is due to the functional adaptations required to colonise a specific niche (Huse et al. 2012; Caporaso et al. 2011; The Human Microbiome Project Consortium 2012). Thus, the higher resolution afforded by the HOMINGS methodology in identifying particular species and strains enables better identification of species within a genus that may be health or disease associated.

4.3.1 Health

Analysis of the tongue, subgingival plaque and interdental plaque in the healthy cohort enabled extraction of three principal components that accounted for 75.9% of the total variation observed (Figure 4-3). The score plots revealed that the three niches in health are distinct in their community structure with the greatest distance between the interdental and tongue microbiota (Figure 4-4). With little or no overlap between the communities in these two niches, 46% variation (PC1) observed between the samples accounted for this difference.

The major difference of interdental plaque from tongue and subgingival plaque can be explained by a significant decrease in the *Rothia mucilaginosa* ($p<0.0001$), and an increase of *Fusobacterium* spp. ($p<0.0001$; Figure 4-5). Further associations that differentiate interdental plaque from the other niches include *Streptococcus intermedius*, TM7 genus and *Parvimonas micra*. The community structure of interdental plaque also exhibited the least variation among samples. These observations are in line with previous studies that have explored the interdental and tongue microbiota with regards to the most abundant taxa identified in the interdental plaque and tongue being *Fusobacterium* sp and the nitrate-reducing *R. mucilaginosa*, respectively; and TM7 genus also being particularly abundant in the interdental plaque (Zaura et al. 2009; Takeshita et al. 2012; Kazor et al. 2003)

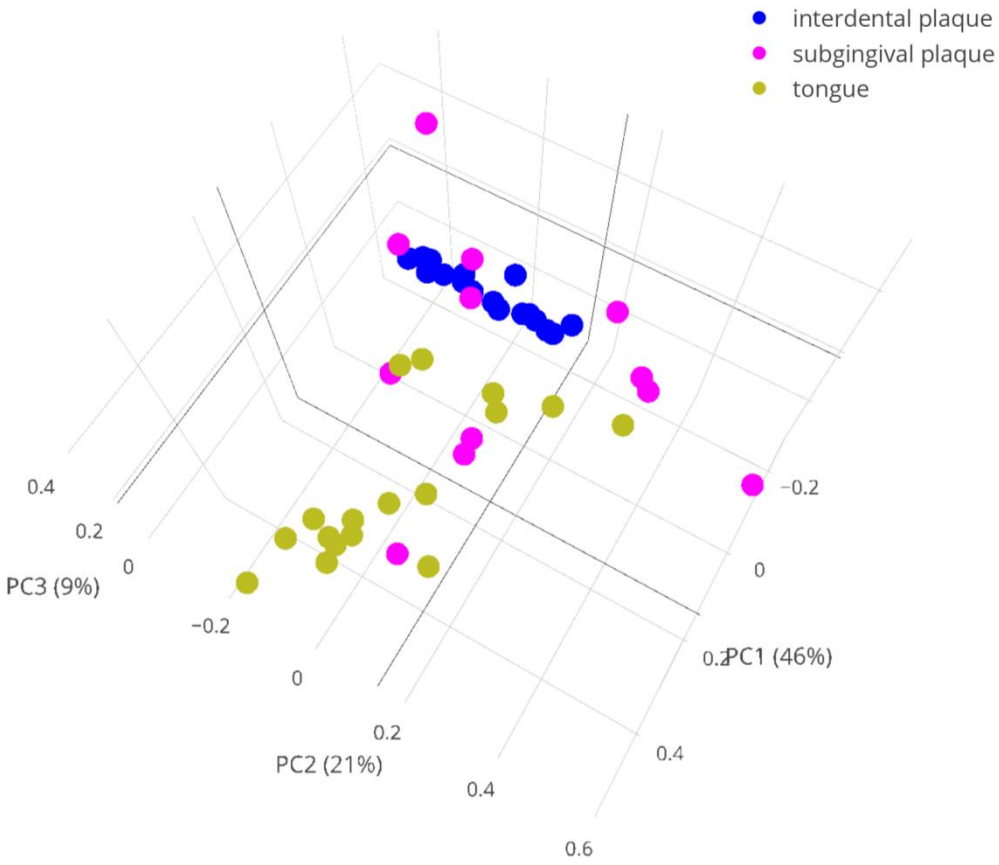


Figure 4-3 PCA score plot showing interdental niche from the healthy cohort lying furthest away from tongue and subgingival plaque, correlating negatively with 1st, 2nd and 3rd principal components with variance explained 46%, 21% and 9% respectively.

Greater heterogeneity of the subgingival microbiota was observed in health—a few samples were more similar to the tongue microbiota (Figure 4-4), whilst a third of the samples were more similar to the interdental microbiota in the taxa that exhibited the largest loadings in that axis, for example *Fusobacterium* sp. A few others varied along the third principal component with an associated increase of *Rothia dentocariosa* (PC3=0.69). This heterogeneity may be partly due to the sampling strategy of buccal and lingual subgingival samples being pooled, and the resultant community structure perhaps reflecting the interface of the tongue and interdental niches in that individual.

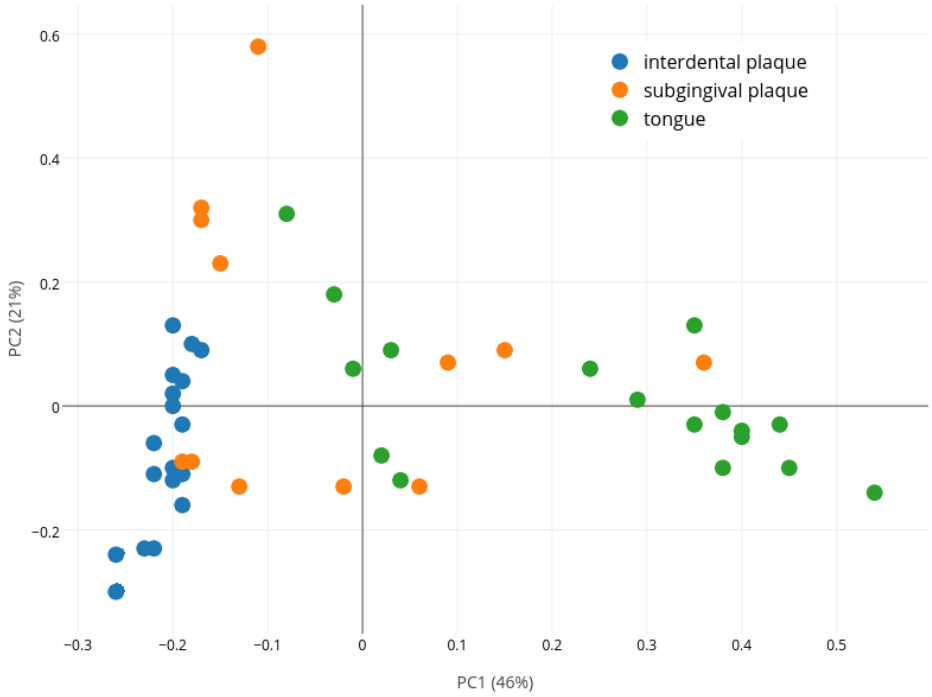


Figure 4-4 showing variance of the subgingival niche from tongue and interdental niches in health, along the PC1 and PC2 axes.

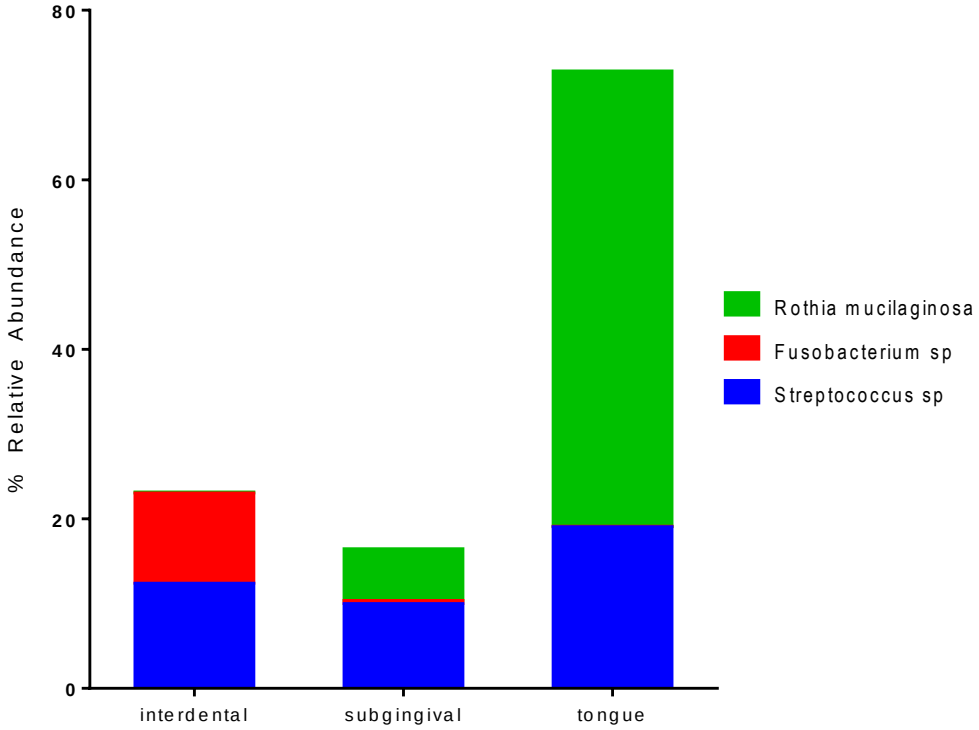


Figure 4-5 showing median values (plotted as stacked bars) of the three taxa that showed the largest loadings with the different niches within the healthy cohort. *R. mucilaginosa* and *Fusobacterium* sp showed statistically significant differences.

Whilst the tongue and interdental plaque have distinctive microbial communities in the healthy cohort, subgingival plaque showed a general conglomeration of species, possibly reflecting the dynamic nature of this niche in health. However, a few species could still be identified as being particularly associated with this niche in comparison to the other niches in this cohort. These relate to a preponderance of streptococci including *S. sanguinis, S. gordonii, Neisseria elongata* and *Lautropia mirabilis.* Species such as *S. sanguinis* and *S. gordonii* have been previously reported to be associated with subgingival plaque accumulating in the sites with shallow probing depth in chronic periodontitis patients (Ge et al. 2013), and the present study indicates that these species may just be associated with the subgingival niche in health regardless of other affected teeth as these bacteria may have adaptations to a shallow subgingival niche. The species *L. mirabilis* has also been previously associated with health in the subgingival niche (Kistler et al. 2013).

4.3.2 Gingivitis

In gingivitis, the interdental plaque community was still as distinct from the other niches as in health (Figure 4-6), with a similar community structure, especially with *Fusobacterium* spp. being the most dominant taxon compared to subgingival and tongue niches which in turn had *Streptococcus* spp. and *R. mucilaginosa* as being their most dominant taxons, respectively. However additional species not associated with the studied niches in health were also found to distinguish the different niches from each other within the gingivitis cohort. For example, *Atopobium rimae* displayed significant shifts with regards to the interdental plaque compared to the subgingival and tongue niches (Figure 4-7). Whilst *A. rimae* has been associated with chronic periodontitis in previous studies in relation to the subgingival microbiota and with periradicular lesions (Paster et al. 2001; Kumar et al. 2003; Schirrmeister et al. 2009), it may well be that this species first proliferates in the interdental plaque at the gingivitis stage before gaining access to the subgingival environment as inflammation persists, particularly as its general prevalence and relative abundance in the tongue is also comparatively higher.

A large overlap of the subgingival and tongue microbiota was observed in gingivitis, in particular, due to the subgingival microbiota exhibiting an increase in abundance of *Granulicatella adiacens* and *Granulicatella paradiacens* compared to the interdental niche (Figure 4-8). As with *A. rimae*, a direct route of transmission from the tongue is indicated, as this genus is highly prevalent and abundant in the tongue and it is possible

that with an increase in inflammatory load, *Granulicatella* spp. find colonisation of the subgingival niche more favourable.

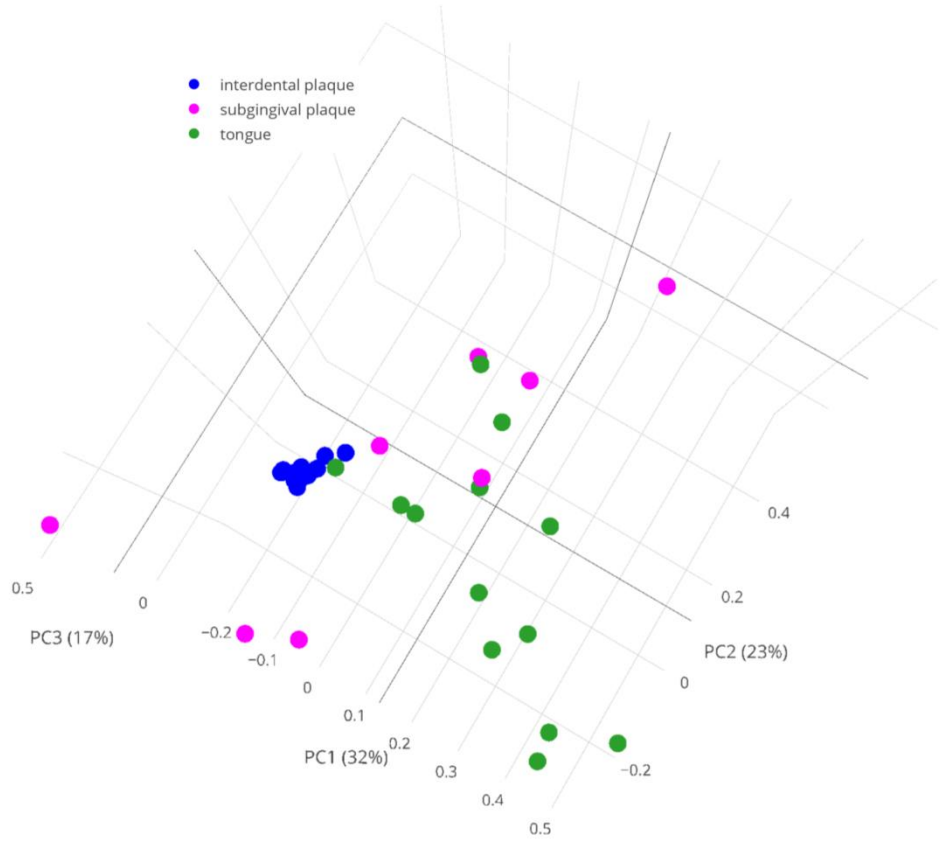


Figure 4-6 showing PCA sample score plots of interdental, subgingival and tongue communities in gingivitis. The variances explained are: PC1=32%, PC2=23%, PC3=17%.

As observed with the subgingival and interdental niches, the inherent variation present in tongue samples in the gingivitis cohort could be explained by shifts in taxa associated with the tongue in health in comparison to the subgingival and interdental niches, in addition to a few taxa not observed in the healthy cohort. These gingivitis-associated taxa include a phylotype identified as *Streptococcus salivarius/vestibularis* ($p<0.0001$), *Gemella sanguinis* ($p<0.0001$) and *Prevotella melaninogenica* ($p=0.0003$; Figure 4-9). *S. salivarius* has been associated with the tongue in studies that have explored the different oral niches, with cloning-sequencing and Next Generation Sequencing approaches (Aas et al. 2005; Eren et al. 2014), both in terms of prevalence and abundance. Whilst this phylotype has generally been associated with a 'healthy' subgingival plaque in comparison to chronic periodontitis (Lourenço et al. 2014), studies have also associated *S. salivarius* with the subgingival niche, particularly in the deep sites of moderate/high carious lesions (Ge et al. 2013). It is possible that the

associations observed in other studies with the subgingival plaque could simply due to an opportunistic response to the inflammatory stress in the subgingival environment leading to 'overspill' of this species from tongue and colonisation of the subgingival plaque.

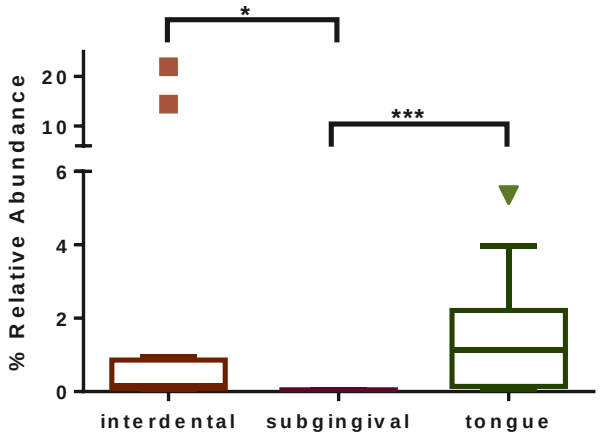
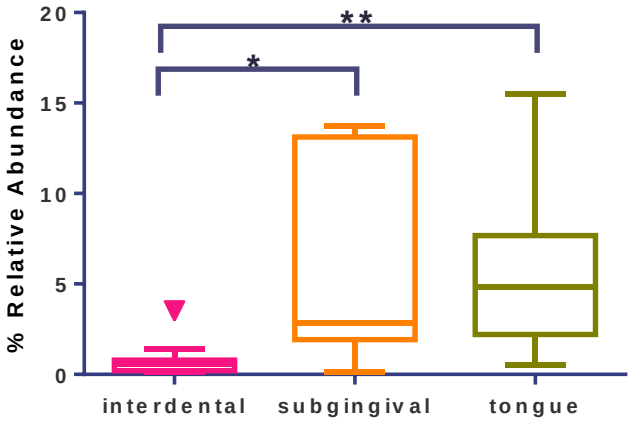


Figure 4-7 showing relative abundance of *A. rimae* in the different niches within the gingivitis cohort. Significant differences between niches are indicated; boxes represent 25th to 75th percentile; whiskers and outliers are plotted per the Tukey



method.

Figure 4-8 showing relative abundance of the phylotype identified as *G. adiacens*/ *paradiacens* in the subgingival, tongue and interdental niches. Significant differences between the niches are indicated; boxes represent 25th to 75th percentile; whiskers and outliers.

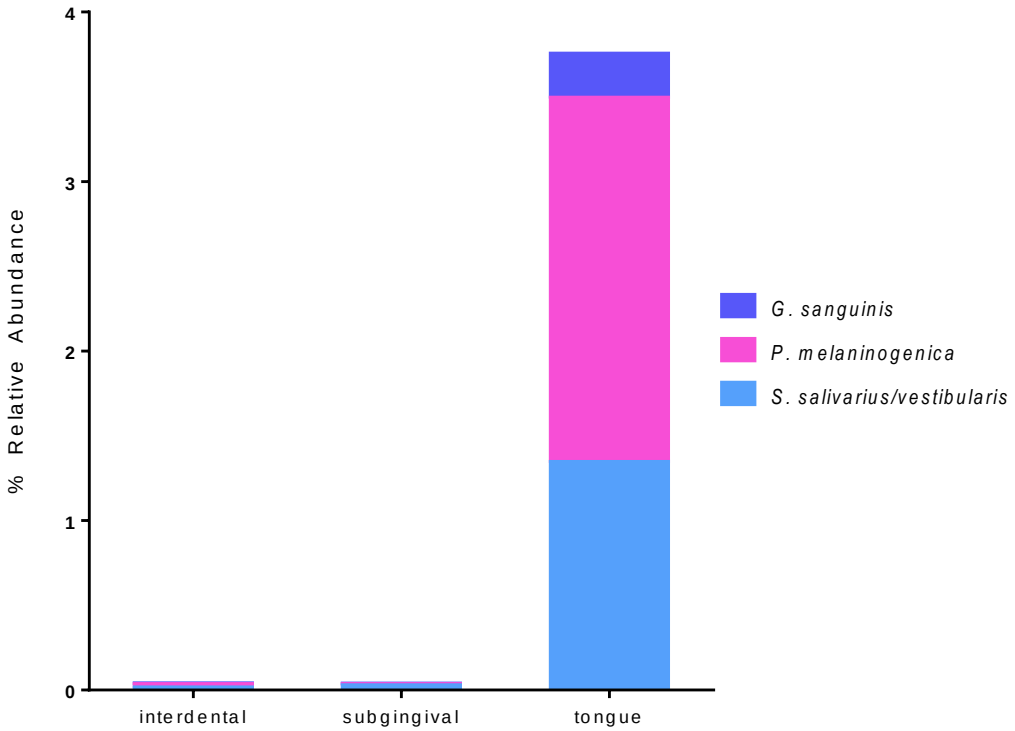


Figure 4-9 showing taxa that differentiate the tongue from the subgingival and interdental plaque in the gingivitis cohort (median values plotted as stacked bars). Statistically significant differences ($p<0.001$) were observed with all three taxa when compared with subgingival or interdental niches

The genera *Gemella* and *Prevotella* are some of the most prevalent taxa in the oral cavity and in particular on the tongue dorsum (Huse et al. 2012), so it is not unexpected that this study found the *G. sanguinis* and *P. melaninogenica* to selectively increase in the tongue compared to the other niches in the gingivitis cohort. However, these species are also known to be opportunistic (Ruoff 2002), and these data suggest that an increase in inflammation observed in the periodontal environment in the gingivitis cohort also perhaps has an effect on the tongue microbiota resulting in proliferation of *G. sanguinis* and *P. melaninogenica*. Interestingly, the abundance of *P. melaninogenica* in subgingival plaque was found to have a fairly negative correlation with the chronic periodontitis patients whose periodontal pocket depths improved in response to treatment (Schwarzberg et al. 2014). This is consistent with the findings of the present study, associating this species as a 'native' of the tongue microbiota and as a possible opportunist in the periodontal environment as inflammation persists subgingivally.

4.3.3 Chronic periodontitis

A much larger overlap and heterogeneity of the microbial communities across the oral niches was observed in chronic periodontitis (CP), with the interdental niche in the different individuals clustering more similarly to the subgingival niches (Figure 4-10). However, the loading plots for the variables accounting for this clustering pattern suggest that the 'core' taxa underpinning the interdental niche such as *Fusobacterium* sp, has not changed significantly from health, through to disease—with additional taxa such as *Porphyromonas endodontalis* and *Tanerella forsythia* exhibiting an association with this niche in the CP cohort (Figure 4-11).

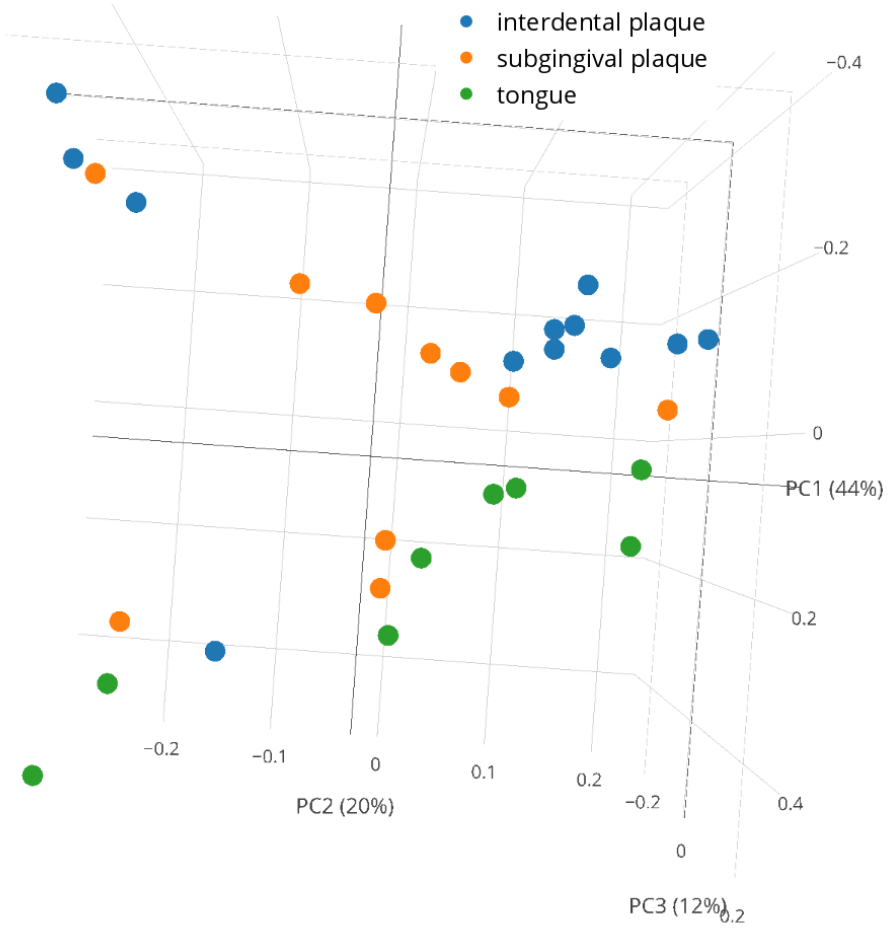


Figure 4-10 showing clustering of microbiota in the interdental, subgingival and tongue niches with respect to the 1st (44%), 2nd (20%) and 3rd principal components in chronic periodontitis patients

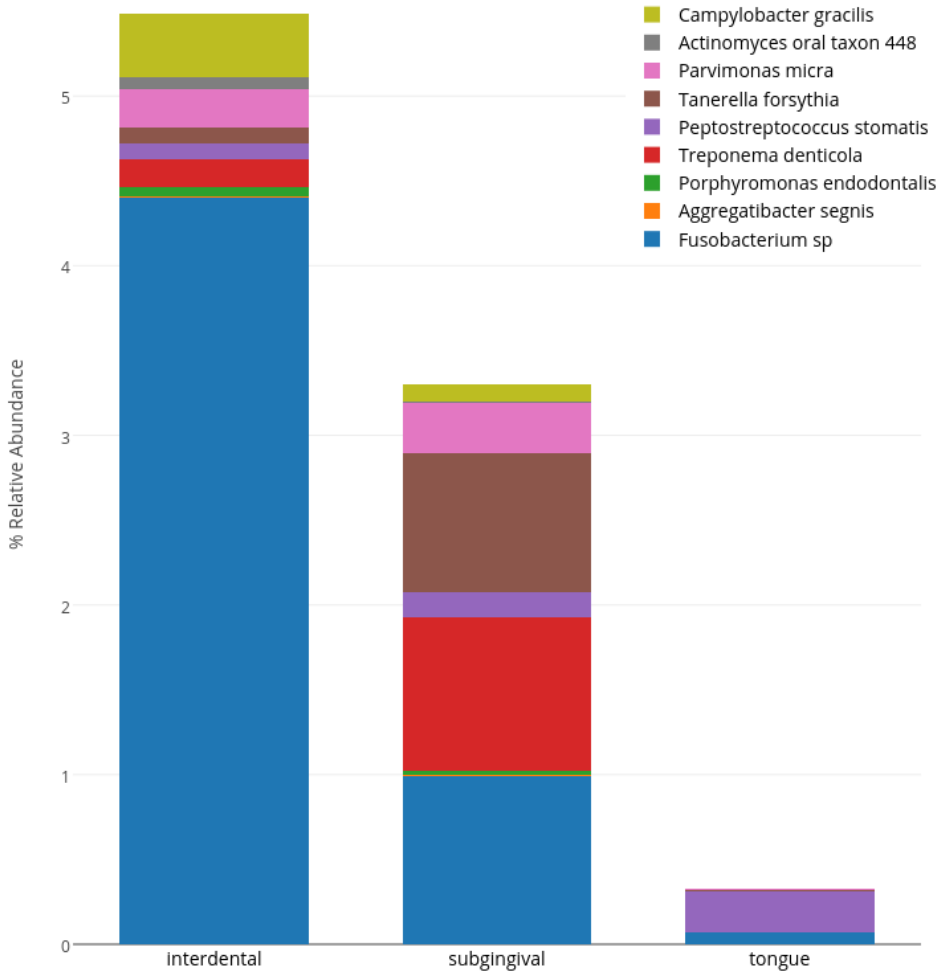


Figure 4-11 showing median values (plotted as stacked bars) of the top taxa that showed the largest loadings with respect to the interdental niche within the chronic periodontitis cohort.

Similarities between subgingival and interdental microbiota were observed in chronic periodontitis, with regards to the taxa that showed largest loadings with the interdental niche, highlighting the overlap between the two niches, in particular with the species *T. denticola* and *T. forsythia* (Figure 4-11). The role of these species in the pathogenesis of chronic periodontitis is well documented and in this case, their presence in the interdental niche could be due in part to transmission from the subgingival niche as clearly, they are more abundant subgingivally in the CP cohort (Sharma 2010; Frederick et al. 2011; Figure 4-11). The genera *Rothia, Granulicatella* and *Streptococcus* showed the largest shifts in relation to the subgingival niche compared to the interdental or the tongue niches in the CP cohort—not unlike the gingivitis cohort (Figure 4-12). Additionally however, species such as *Leptotrichia honkongensis, P. melaninogenica* and *Corynebacterium durum* were found to decrease in the subgingival niche in CP

compared to the other niches, in particular the interdental niche. The species *R. dentocariosa* has been associated with healthy subgingival plaque and the present study suggests that owing to the high relative abundance of this species in the periodontal environment (Figure 4-12), a larger shift in its abundance from health to disease is necessary for it to be considered a health-associated species (Kistler et al. 2013). Indeed, this has been recognised in a study by (Abusleme et al. 2013) who suggest that *R. dentocariosa* be considered part of the 'core' microbiota in the subgingival niche due to its high relative abundance in health and disease.

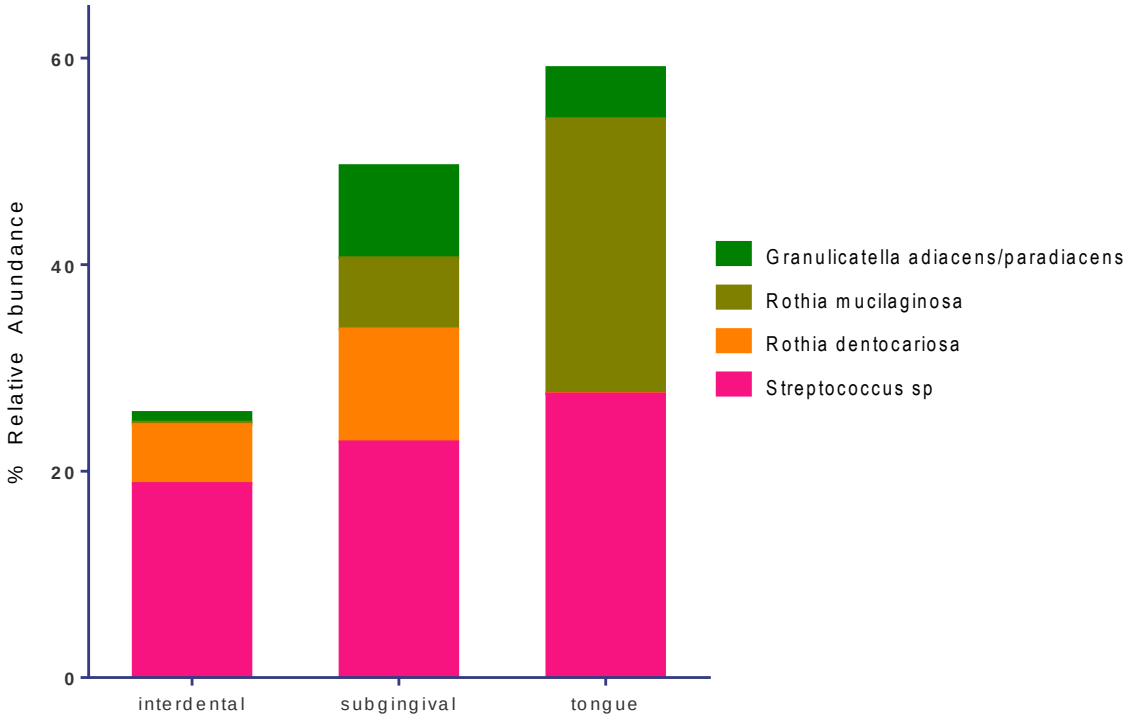


Figure 4-12 showing median values (plotted as stacked bars) of the taxa that showed the largest loadings with respect to the subgingival niche within the CP cohort.

The tongue microbiota in the CP cohort exhibited variation similar to that of the gingivitis cohort with species such as *R. mucilaginosa*, *Streptococcus* spp, *G. sanguinis* and *P. melaninogenica* forming the 'core' taxa whose relative abundances differentiated this niche from the subgingival and interdental niches. In addition, taxa such as *Haemophilus parainfluenzae*, *Leptotrichia* sp HOT417, *Actinomyces odontolyticus*, *Actinomyces* sp HOT172 and HOT449 showed large loadings for individual tongue samples.

4.4 Ecological variation in specific niches

Comparing the different niches within the healthy, gingivitis and chronic periodontitis cohorts in this study has revealed that each niche has a few 'core' species that dominate or account for a large percentage of the bacterial biomass observed, and this largely remained the same from health through to disease. This fundamental stability of the oral niches has been observed in a much larger scale in other investigations that have studied the temporal variation in selected oral niches (Yang et al. 2013; Xu et al. 2015). However, it is also understood that the microbial diversity found within a human niche could account for its temporal stability, such that the more diverse a niche, the more temporal stability was observed in a cross-sectional study (Flores et al. 2014). This feature of the human oral microbiome allows one to compare the same niche among different cohorts separated by the degree of inflammation in the mouth such as gingivitis and chronic periodontitis, to look for patterns of microbial composition that influence or is affected by the inflammation observed.

4.4.1 Interdental plaque

A dimension reduction technique using Partial Least Squares regression enabled identification of phylotypes that displayed differences in relative abundance amongst the three cohorts. This analysis is biased toward the more prevalent and abundant taxa, so larger fluctuations in relative abundance of a particular taxon would result in higher loadings assigned to it. As discussed in the previous section in relation to the chronic periodontitis cohort (Figure 4-12), microaerophiles such as *R. dentocariosa* and *R. mucilaginosa* exhibited large loadings associated with the interdental niche in the CP cohort (Figure 4-13; Figure 4-14). Whilst *R. dentocariosa* is more abundant in the subgingival niche, the accumulation of *R. mucilaginosa* in the interdental plaque of chronic periodontitis patients is unusual. As described in the previous sections, *R. mucilaginosa* is the most dominant phylotype in the tongue and this finding suggests that the tongue microbiota may have a major role to play in chronic periodontitis, by acting as a reservoir for organisms that can colonise the subgingival and interdental niches. Additionally, this finding follows the clinical observation that the interdental spaces in affected teeth of CP patients are typically larger than healthy individuals, leading to the interdental niche in the CP patients being much less anaerobic than the healthy (Imai et al. 2012).

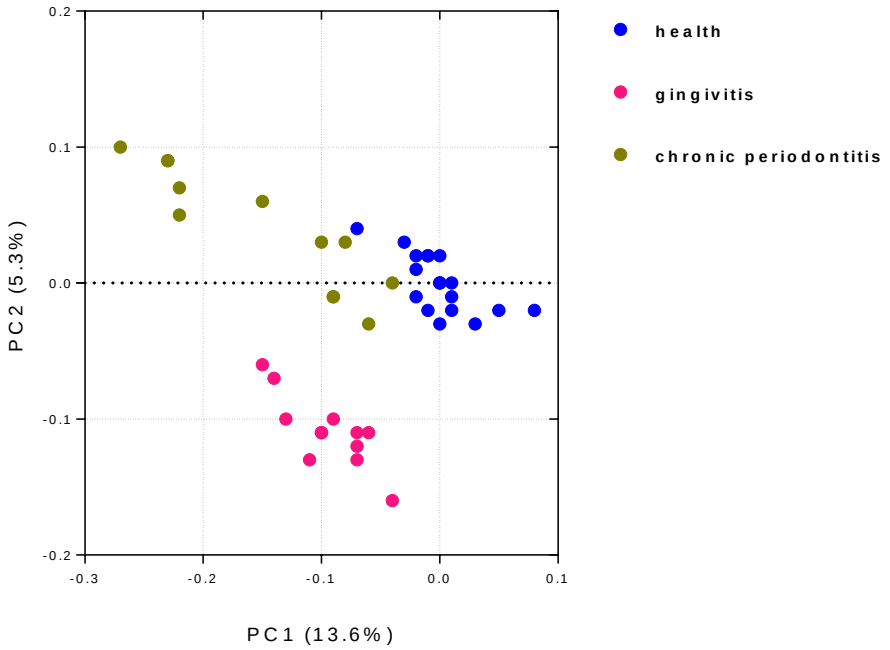


Figure 4-13 showing score plots of the interdental plaque communities in health, gingivitis and chronic periodontitis showing deviation of the different cohorts.

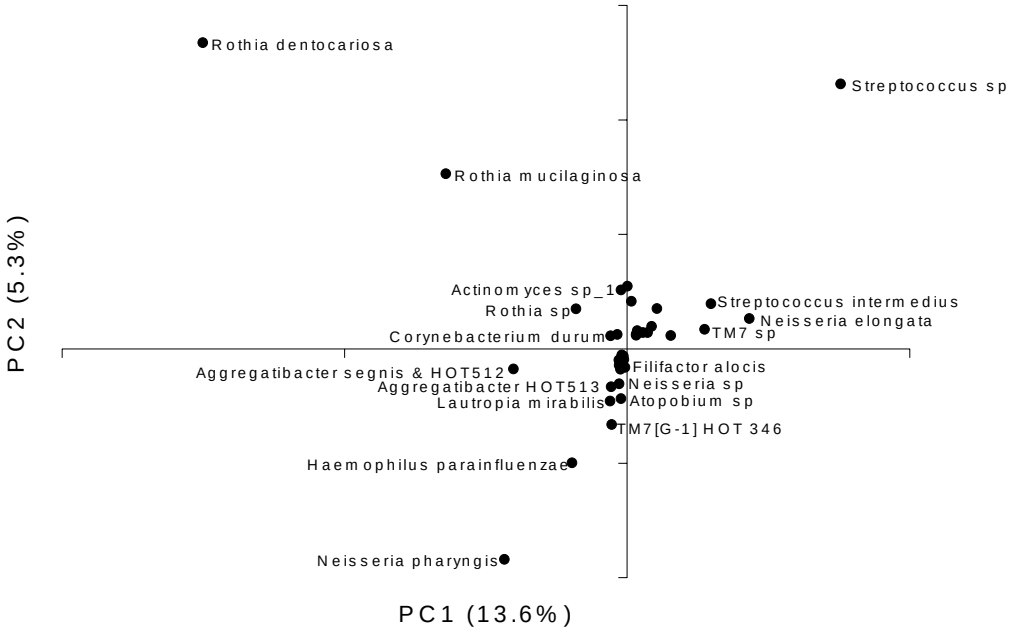


Figure 4-14 showing taxons that had large loadings with respect to the principal components shown in Fig. 4-13.

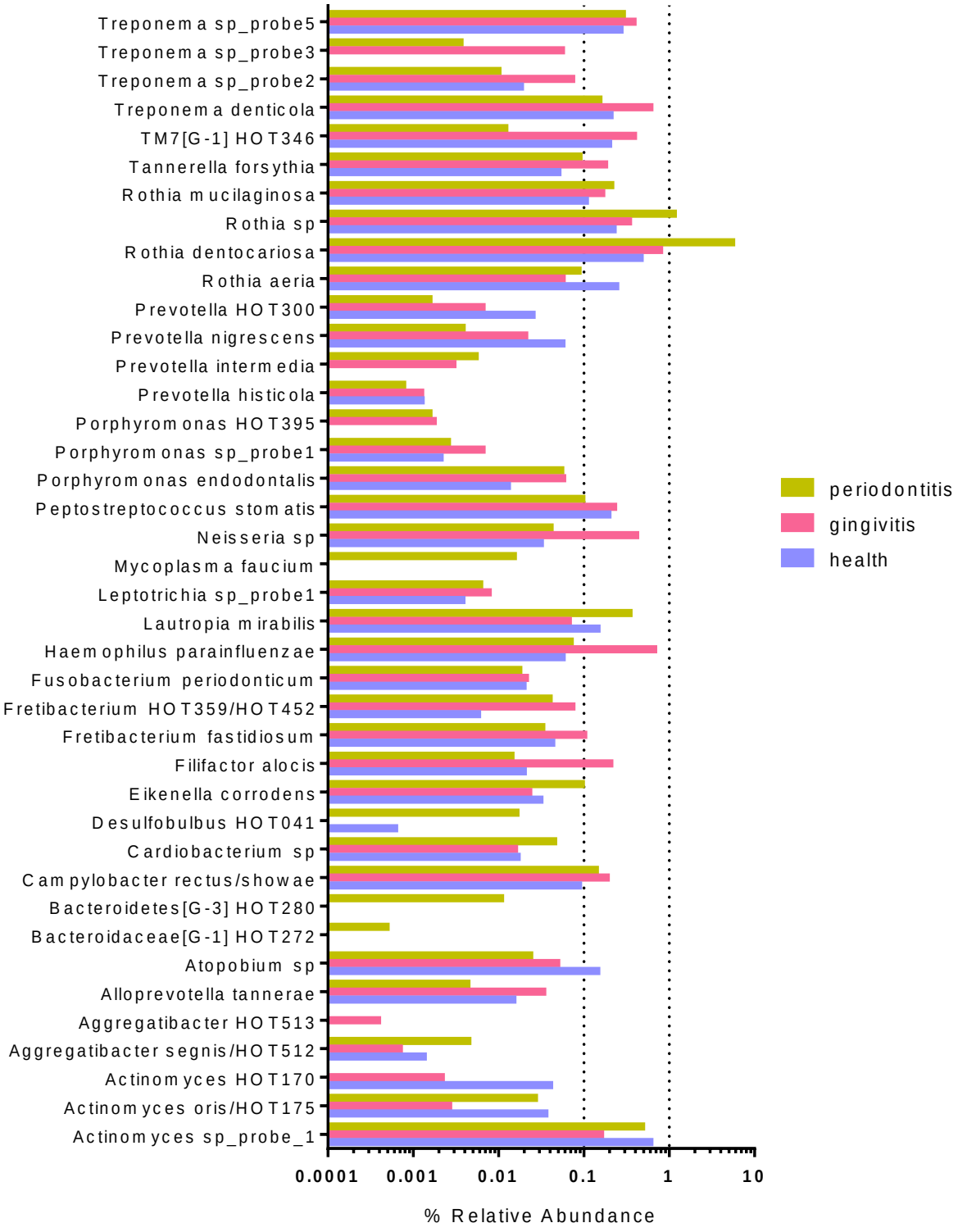


Figure 4-15 bar plot showing median values of taxa that showed an association with the shift of the interdental samples in the gingivitis and chronic periodontitis cohort as shown in Fig. 4-13 and 4-14 (1% cut-off for dominant taxa and 0.1% cut-off for rare taxa are indicated in the plot).

Forty targets in total were identified as influencing the community shift from health to gingivitis and periodontitis in the interdental plaque with the most relevant plotted in Figure 4-15. The majority of these targets are measured as 'rare' taxa (<0.1% relative abundance), and a few species such as *Mycoplasma faucium*, *Bacteroidetes* [G-3] HOT280, *Bacteroidaceae* [G-1] HOT272 were found to be exclusively associated with CP. The classical periodontopathogens such as *P. gingivalis* and *T. forsythia* also most commonly occur as rare phylotypes and all detected targets were searched for a shift in the median values from nil in the healthy and gingivitis cohorts to >nil in the CP cohort revealing statistically significant shifts in abundances of *Aggregatibacter paraphrophilus*, *Eubacterium saphenum*, *Peptostreptococcaceae* HOT383, *M. faucium* and *P. gingivalis* (Figure 4-16).

Receiver operating characteristic (ROC) curves generated for the taxa identified as linked with the shift of the gingivitis cohort from health in Figure 4-13 and Figure 4-14 yielded two targets with Area under Curve (AUC) values of 0.72 at $p<0.05$, namely *Filifactor alocis* and *Fretibacterium* HOT359/HOT452 (Figure 4-17). In addition, the sum of taxa that showed statistical significance as plotted in the heat map in the individual participants (Figure 4-16), gave an AUC value of 0.8 at $p<0.005$ in distinguishing health from chronic periodontitis, but the comparisons between health-gingivitis and gingivitis-chronic periodontitis pairs were not significant (Figure 4-17).

Species such as *F. alocis* and *E. saphenum* are relatively newer associations with periodontitis, but at the subgingival niche (Kumar et al. 2003; Bruce J Paster et al. 2001; Griffen et al. 2012). *M. faucium*, *Fretibacterium* HOT359 and the *Bacteroidetes* phylotypes HOT272 and HOT280 have been associated with periodontitis recently by studies that have used NGS technologies to survey the subgingival ecology in relation to periodontitis (Abusleme et al. 2013; You et al. 2013; Griffen et al. 2012). *A. paraphrophilus* and *Peptostreptococcaceae* HOT383 are novel associations with periodontitis, and most of the discussed taxa are novel associations with gingivitis or chronic periodontitis in the interdental niche. In particular, species such as *F. alocis* and *E. saphenum* are considered to be putative periodontopathogens in the subgingival niche not unlike *P. gingivalis* or *T. forsythia* and the present study has identified a significant increase of *F. alocis* abundance in the interdental niche of the gingivitis cohort compared to health, suggesting that this species is involved at an earlier stage of the etiopathogenesis of periodontitis than previously recognised. Although the pathogenicity of *M. faucium* and *A. paraphrophilus* is largely unexplored in relation to

periodontitis, studies of these organisms on other contexts indicate that these species have latent virulence factors that may enable a pathobiont-like role in the development of chronic periodontitis (Kwon et al. 2004; Sen Yew et al. 2014).

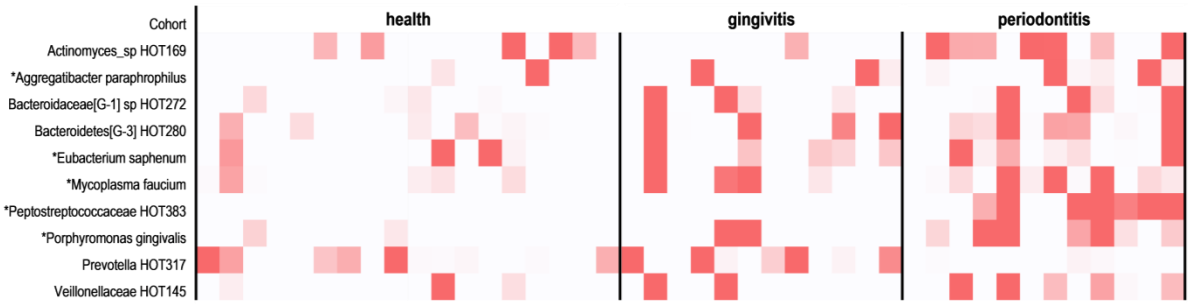


Figure 4-16 showing a heat map of rare taxa that showed strong associations with the CP cohort. Asterisk before taxon name indicates statistical significance between health and CP; darkest red in each row indicates >90th percentile; white denotes absence of detection.

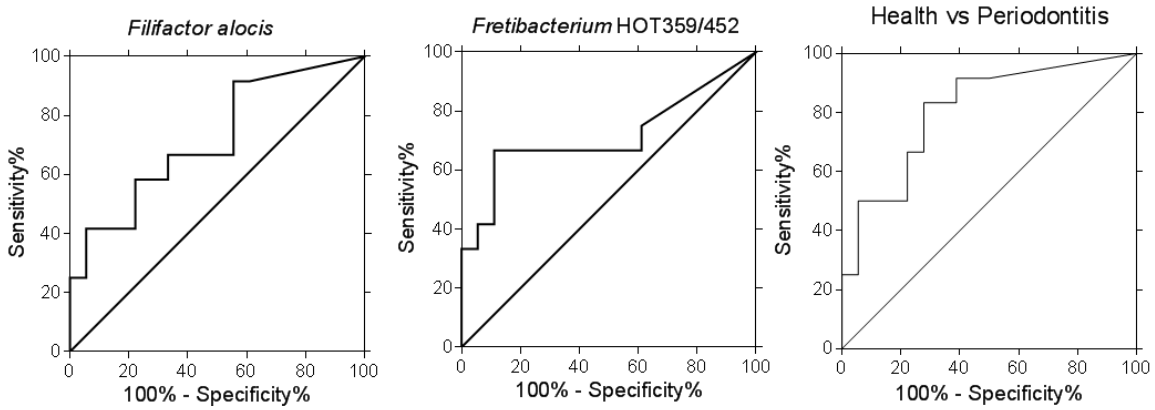


Figure 4-17 showing significant ROC curves in distinguishing gingivitis from health (*F. alocis* and *Fretibacterium* sp), and periodontitis from health (sum of *P. gingivalis, E. saphenum, A. paraphrophilus, M. faucium* and *Peptostreptococcaceae* HOT383) in the interdental plaque.

4.4.2 Subgingival Plaque

The subgingival plaque is the most widely studied niche in relation to gingivitis and chronic periodontitis, including using the next sequencing approaches, and in the present study a variation of less than 5% of the total relative abundances of 52 taxa measured in the subgingival plaque were identified as influencing the shift from health to gingivitis or chronic periodontitis with only about 2.4% variation in 43 taxa between gingivitis and chronic periodontitis (Figure 4-18). The species that were found in higher abundance in the healthy subgingival plaque compared to that of gingivitis or chronic periodontitis were *Rothia mucilaginosa*, *Rothia aeria*, *Streptococcus* sp and

Leptotrichia wadei (Figure 4-19). Interestingly, *R. dentocariosa* was found in higher average abundances in the gingivitis cohort compared to health. This could be due to the dynamic relationship between the most abundant taxa observed in the subgingival plaque of individuals in this study namely *Streptococcus* sp, *R. mucilaginosa*, *R. dentocariosa*, *Neisseria* sp and *H. parainfluenzae*, whereby only one of those species dominate by occupying the largest biomass, especially between *Rothia* sp and *Streptococcus* sp. The taxa *Neisseria* sp and *H. parainfluenzae* were only present in comparable relative abundances in a minority of individuals and so cannot be used to infer relationships with health or disease (Figure 4-20). However, a more even community could be observed in the CP cohort with regards to *Rothia* sp, *Streptoccoccus* sp and *Granulicatella* sp compared to health or gingivitis where a larger variance in the abundance of the different taxa that dominate the ecological landscape is observed (Figure 4-20).

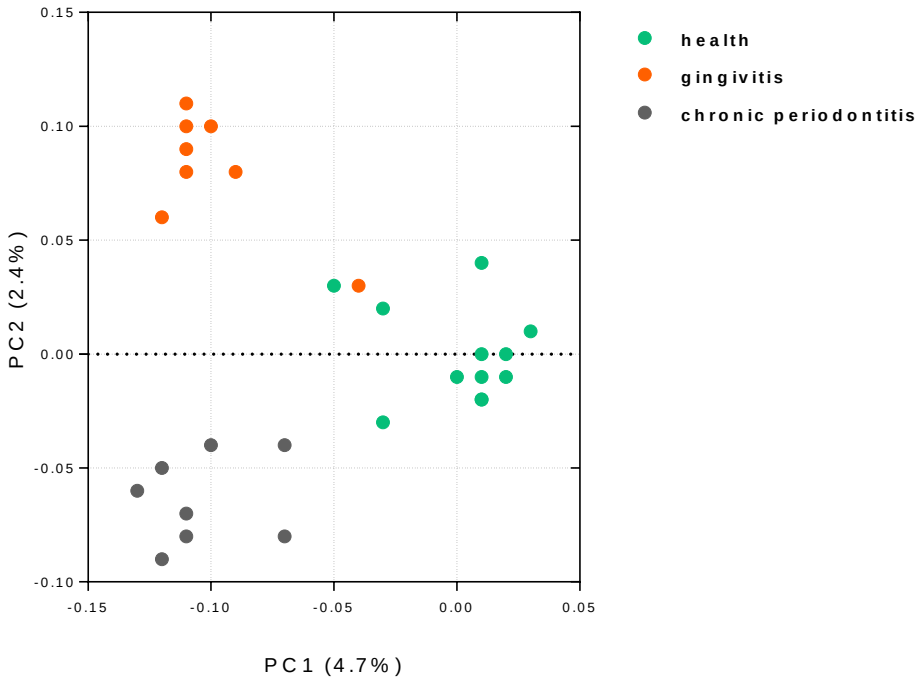


Figure 4-18 showing subgingival communities from health, gingivitis and chronic periodontitis against the extracted principal components.

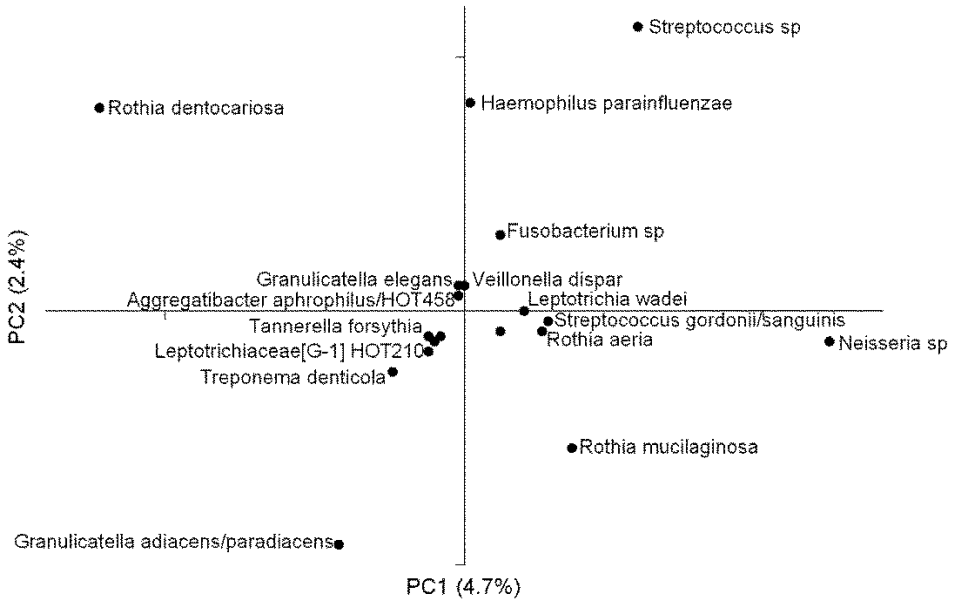


Figure 4-19 showing taxa that had the largest loadings associated with the score plot shown in Fig. 4-18.

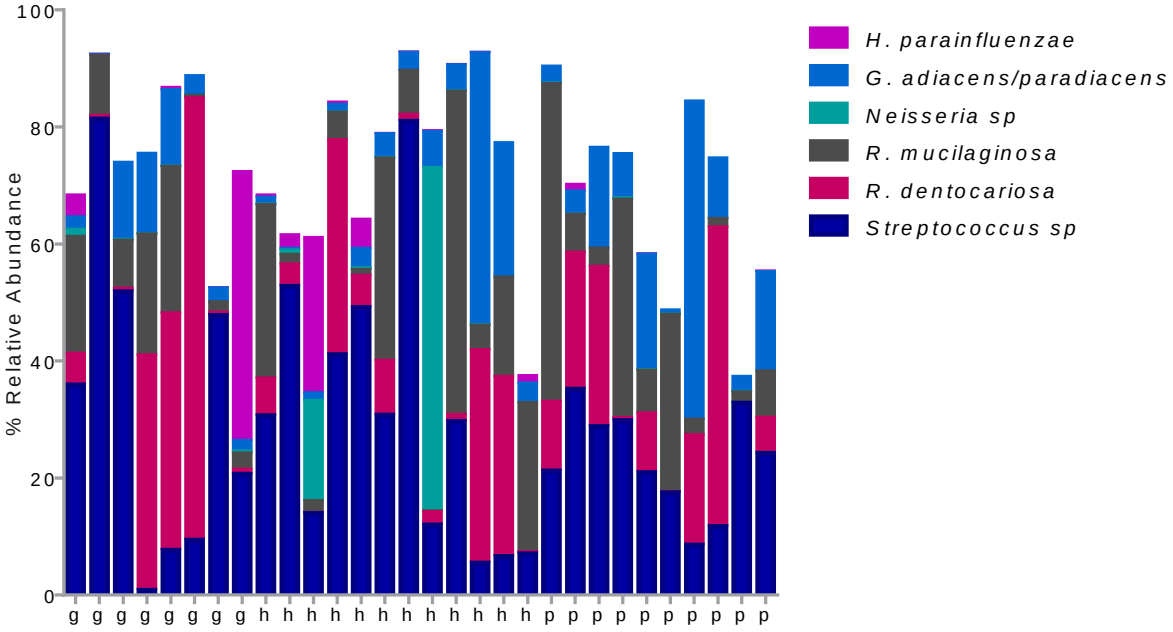


Figure 4-20 showing the taxa that collectively occupied the largest biomass in the individual subgingival niches in health and disease (h=health; g=gingivitis; p=chronic periodontitis).

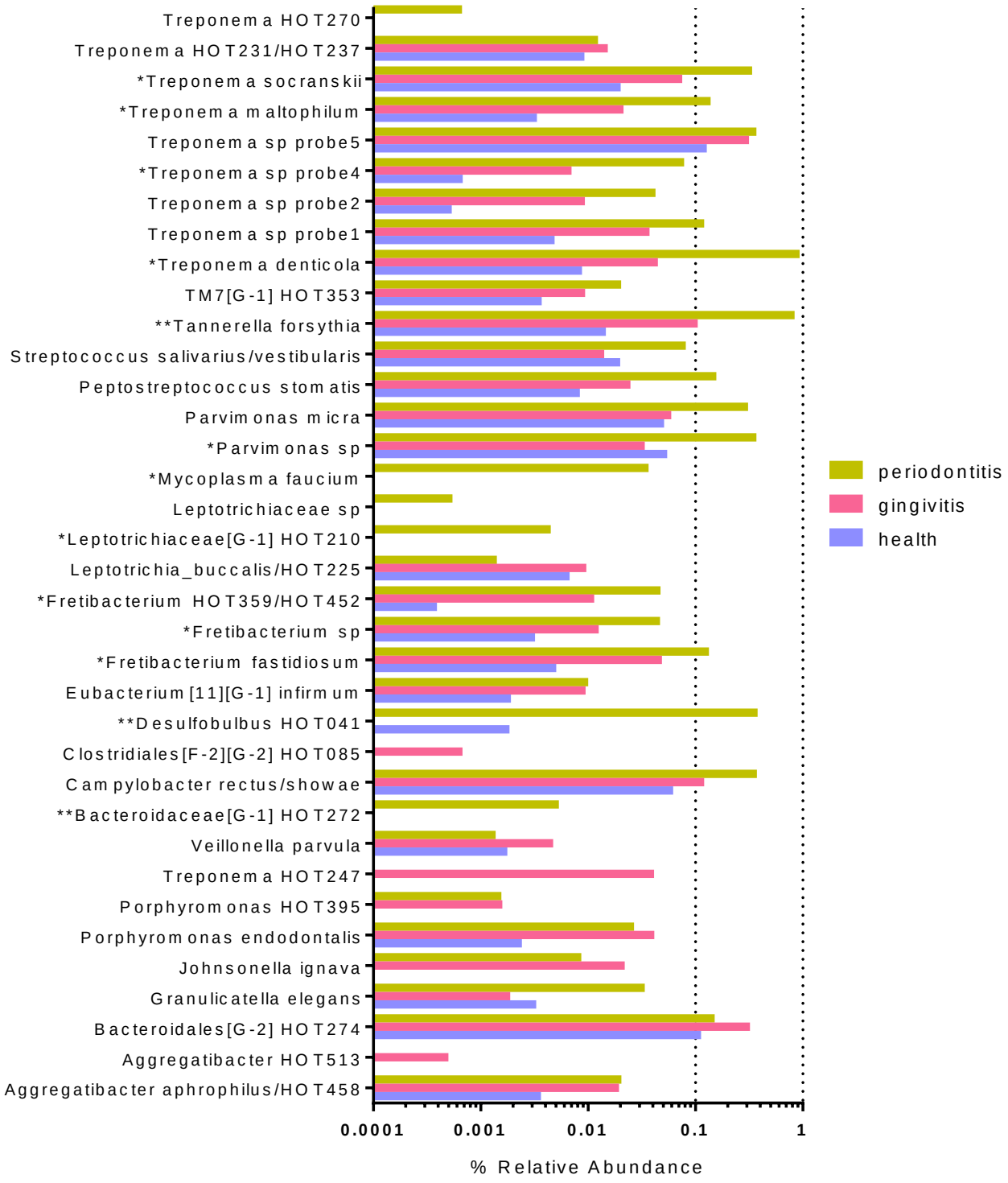


Figure 4-21 bar plot showing median values of rare taxa (<0.1% relative abundance) that showed an association with the shift of the subgingival samples in the gingivitis and chronic periodontitis cohort as shown in Fig. 4-18 and 4-19 (taxa with statistically significant differences are indicated with an asterisk).

In the subgingival plaque, a higher prevalence and abundance of phylotypes belonging to the family *Synergistetes* [F-2] and genus *Treponema* sp were observed in relation to gingivitis and chronic periodontitis and this has also been observed by other investigators (Figure 4-21; You et al. 2013; Abusleme et al. 2013). *Treponema* sp HOT247 and *Aggregatibacter* sp HOT513 were observed to be prevalent in just the gingivitis cohort, while *Treponema* sp HOT270, *M. faucium, Leptotrichia* sp HOT210/HOT220 and *Bacteroidaceae*[G-1] HOT272 were associated with an increased prevalence and abundance in the chronic periodontitis cohort.

When all the measured taxa were screened for a shift in the median values from nil within the healthy or gingivitis cohort, to >0 in the CP cohort, *M. faucium, P. gingivalis* and *Eubacterium minutum* showed the largest changes exclusively associated with the CP cohort (Figure 4-22). A separate cluster of taxa that showed incremental increase in the median values from nil in health through to >0 in gingivitis and CP were *E. saphenum, Chloroflexi* sp HOT439, *Bacteroidetes* sp HOT280 and *Treponema parvum* (Figure 4-22). The sum of all these taxa was able to distinguish between health and periodontitis and gingivitis from health, while the *M. faucium, P. gingivalis* and *E. minutum* cluster only differentiated between health and CP. However, the second cluster was able to distinguish more accurately between health, gingivitis and CP (Figure 4-23).

It is notable that *E. saphenum, P. gingivalis. M. faucium* and *Bacteroidetes* HOT280 exhibited similar shifts in abundance in both the interdental and subgingival niches across the cohorts and these may be due to these taxa playing the role of the tertiary colonisers in both the sites (Figure 4-16 and Figure 4-22). However, the prevalence of these species was higher in the interdental niche of the healthy cohort compared to the subgingival niche, suggesting that perhaps these putative periodontopathic strains are normally present at low levels in the interdental niche of healthy individuals and this follows the clinical observation that this niche is a critical area of the periodontium linked with the origin of carious lesions and periodontal bone loss due to microbial and host induced destruction (Berchier et al. 2008). While these taxa are normally prevalent at very low abundances in the subgingival niche, the classical periodontopathogens such as *T. forsythia* and *T. denticola* were much more abundant and exhibited changes from gingivitis to periodontitis to such an extent that both these taxa would be considered to be dominant in this niche in periodontitis (>1% relative abundance; Figure 4-21). Indeed, the diverse array of species from the genus *Treponema* implicated in the aetiopathogenesis of periodontitis would likely make this

group of species one of the most important in the overall ecology and pathogenicity of the microbiota found in the periodontal environment (Meng You et al. 2013).

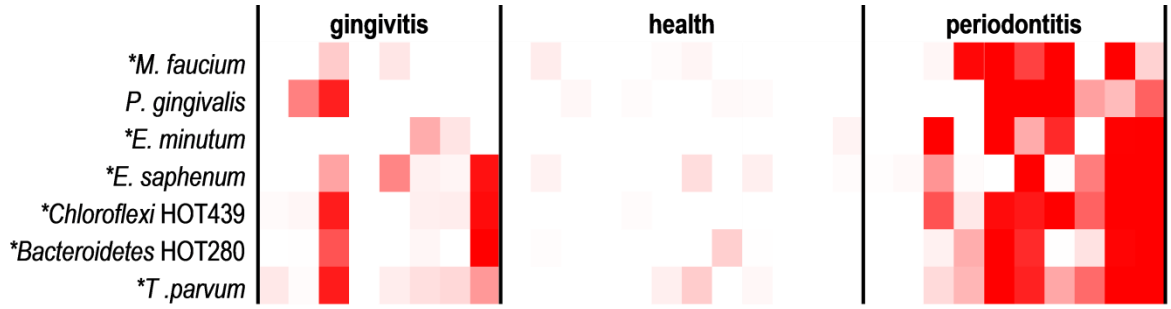


Figure 4-22 showing a heat map of rare taxa that showed strong associations with the CP cohort in the subgingival plaque. Asterisk before taxon name indicates statistical significance between health and CP; darkest red in each row indicates >90th percentile; white denotes absence of detection.

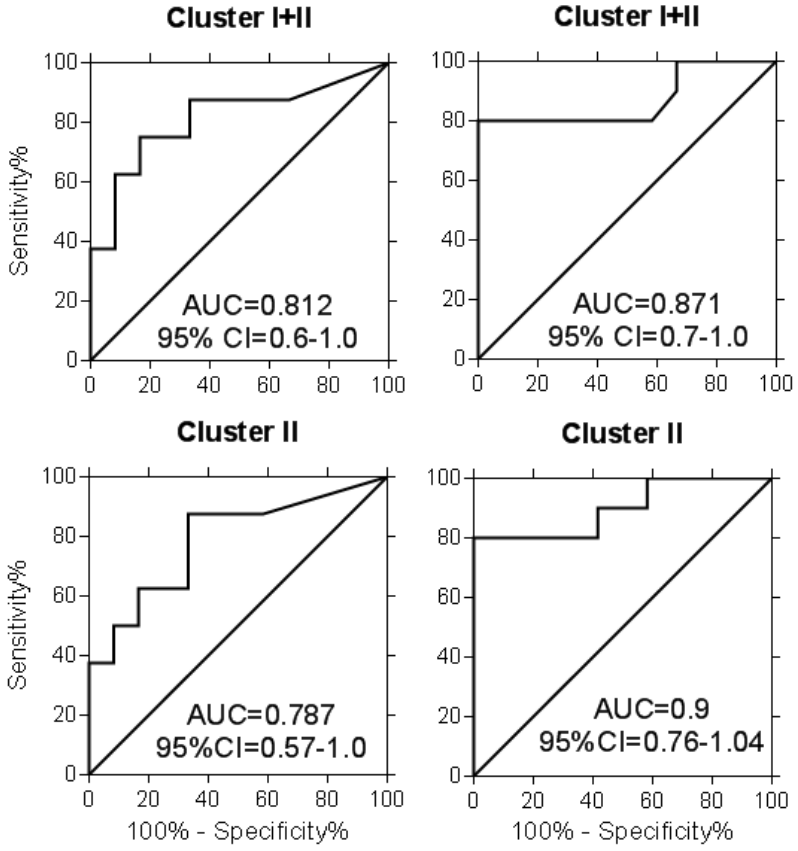


Figure 4-23 showing ROC curves for Cluster II (*E. saphenum*/*Chloroflexi* HOT439/*Bacteroidetes* HOT280/*T. parvum*) and sum of taxa in Cluster II and I (*M. faucium*/*P. gingivalis*/*E. minutum*). Plots on the left are for health vs gingivitis and the right are for health vs chronic periodontitis.

4.4.3 Tongue

Of the niches studied, limited data as regards the tongue ecology exists in association with periodontal disease (Galimanas et al. 2014), while another investigation had also used the NGS approach to study tongue ecology in relation to halitosis (Yang et al. 2013). The present study has indicated that tongue has an important role to play in the ecology of the periodontal niches due to its nature of being a potential reservoir for periodontopathic microbiota and also its close proximity to the interdental and subgingival niches in the lingual aspect of the periodontal environment, leading to free transmission of microbiota that may colonise the subgingival and interdental niches. Conversely, the alpha diversity analyses of the different niches with respect to the breath VSCs in the participants of the present study has indicated that the changes occurring in the periodontal environment in relation to gingivitis and periodontitis influences the tongue microbiota and hence, may explain the changes observed in the concentrations of the different breath volatiles as the tongue is thought to be the largest contributor to the presence of malodorous volatiles in the breath of individuals with halitosis. Unlike the periodontal niches, however, the tongue ecology was less well defined across the different cohorts with the PLS analysis, finding larger variance in the tongue communities from the different cohorts and leading to an appearance of a more stable ecology with respect to the different disease states (Figure 4-24).

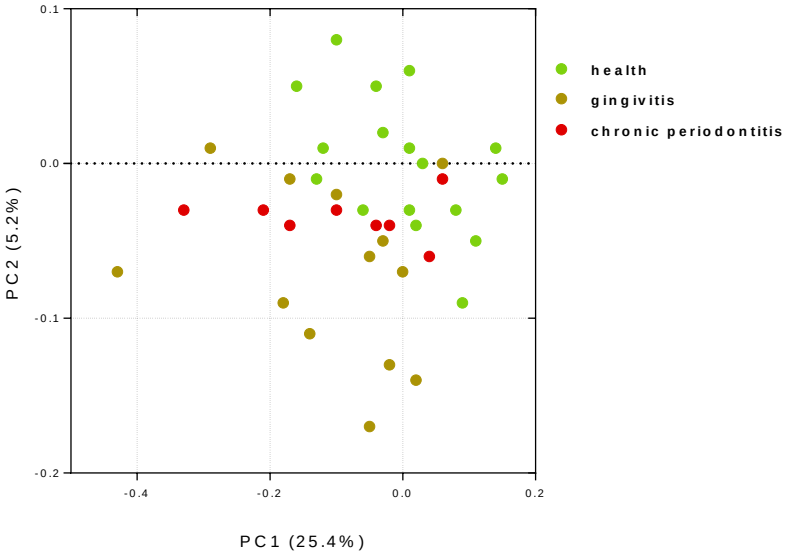


Figure 4-24 showing score plot of tongue samples from the different cohorts against the first two principal components.

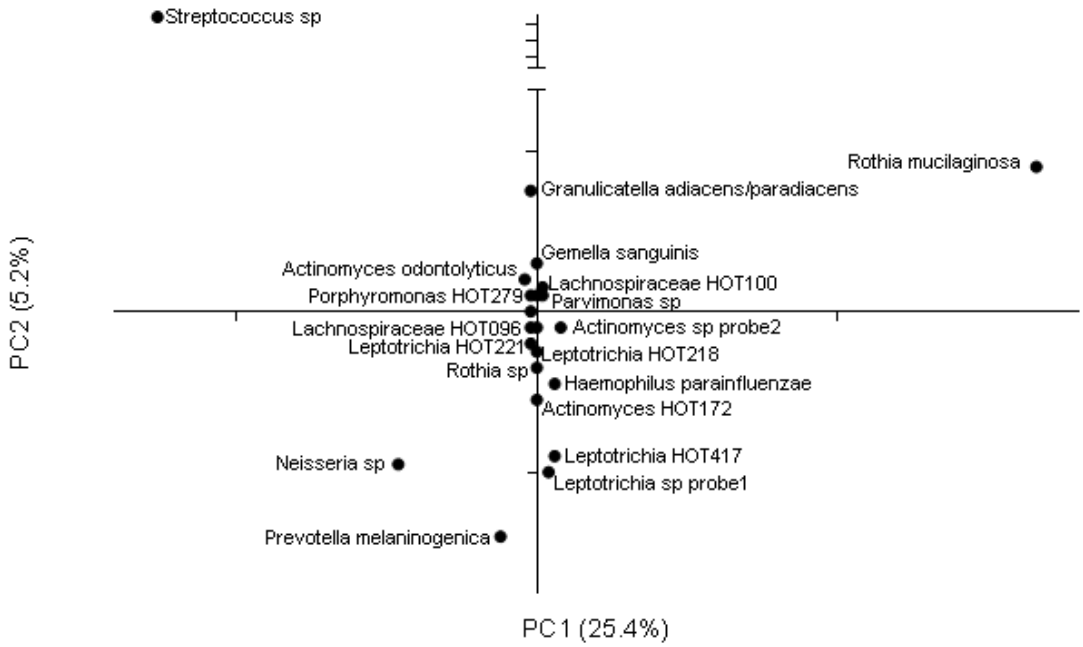


Figure 4-25 showing taxa that had substantial loadings on the tongue communities observed in Fig. 4-24.

The principal components extracted by the analysis showed the tongue ecology of the gingivitis cohort to be more dissimilar to health than chronic periodontitis; however a small shift in the periodontitis samples could still be observed (Figure 4-24). Whilst the tongue was observed to be the least diverse of the niches considered in this study, smaller changes in relative abundances of the observed species present in the tongue will likely result in a much larger impact in overall ecology and indeed the composition and concentration of the breath volatiles, owing to the large surface area of the tongue. A small subset of targets were identified in the tongue samples that increased from health to gingivitis or chronic periodontitis and these include *Prevotella melaninogenica, Alloprevotella tannerae, Atopobium rimae, Streptococcus salivarius/vestibularis, Actinomyces* HOT172, *Prevotella pallens, Prevotella veroralis* and *Fusobacterium* sp from health to gingivitis (Figure 4-25 and Figure 4-26).

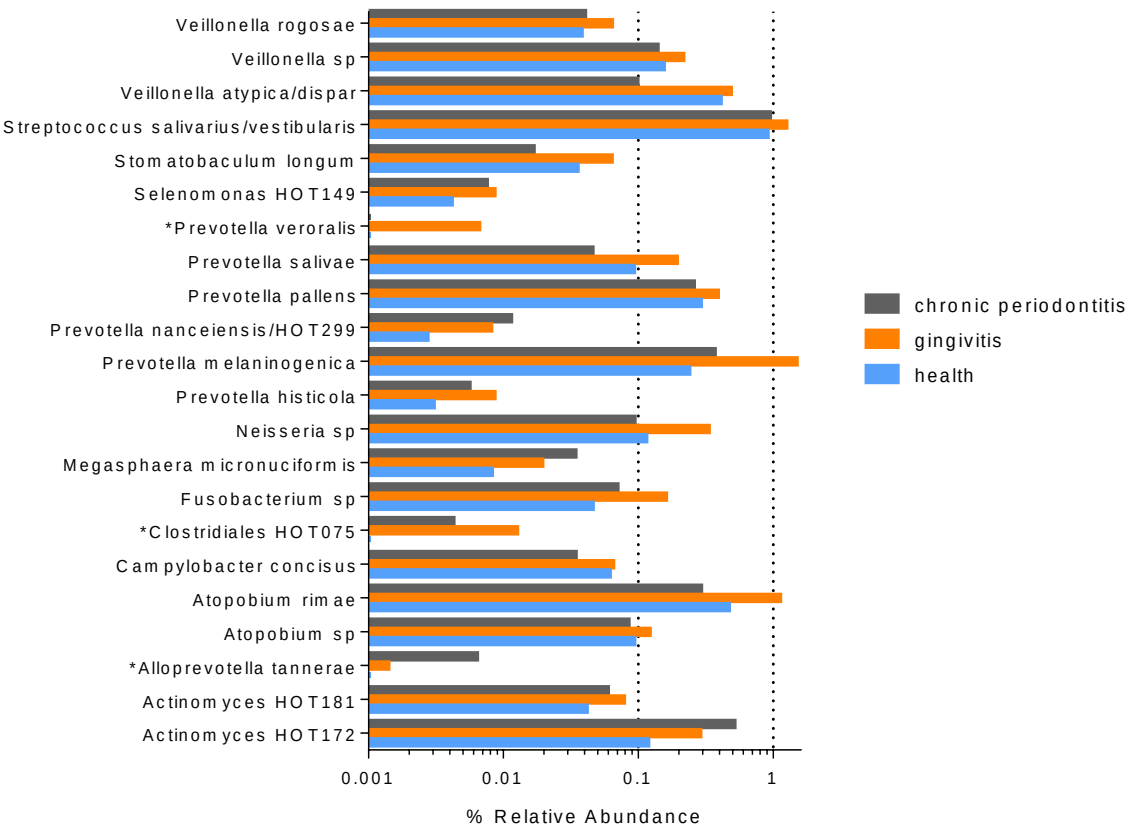


Figure 4-26 bar plot showing median values of relevant taxa that showed an association with the shift of the tongue samples in the gingivitis and chronic periodontitis cohort as shown in Fig. 4-24 and Fig. 4-25 (taxa with statistically significant differences are indicated with an asterisk)

Due to the variation observed in the different taxa among the cohorts, all measured taxa were screened for the largest incremental shifts in the median values from health to gingivitis and chronic periodontitis, and 33 targets were found to increase in gingivitis and CP, while 45 targets were found to increase in gingivitis but decrease in CP. These include *P. melaninogenica*, *Eubacterium* sp, *S. gordonii/sanguinis*, *Fusobacterium* sp and *Parvimonas* sp (Figure 4-27). In addition, searching for taxa that displayed a considerable increase in prevalence in the tongue of gingivitis and chronic periodontitis patients compared to healthy individuals, revealed taxa such as *Eubacterium brachy*, *Streptococcus constellatus*, *Peptostreptococcaceae* HOT113 and *Desulfobulbus* HOT041 involved in the ecological shifts observed (Figure 4-28).

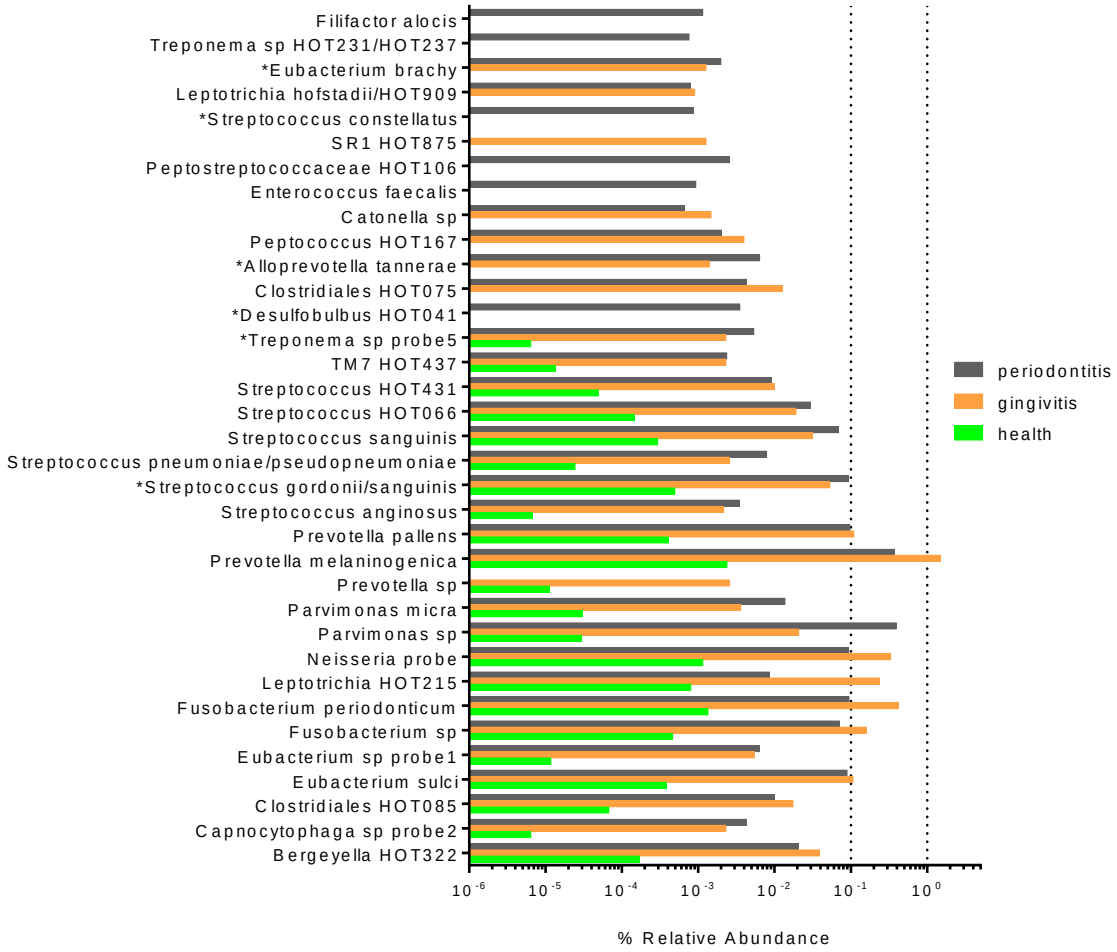


Figure 4-27 plot of the median values of taxa that showed largest increase in prevalence and abundance from health to gingivitis and periodontitis in the tongue samples (taxa with statistically significant differences are indicated with an asterisk).

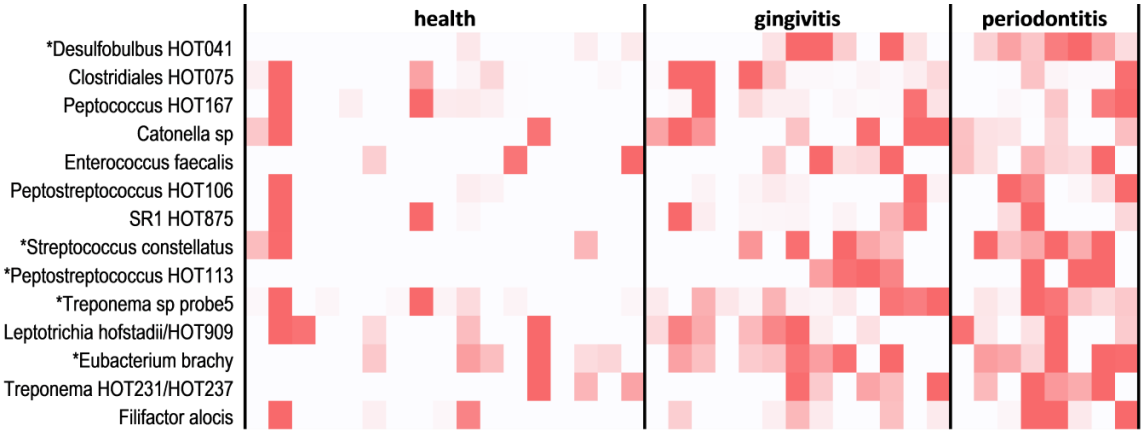


Figure 4-28 showing heat map of rare taxa present in the tongue that showed positive associations with gingivitis and chronic periodontitis cohorts. Asterisk before taxa name indicates statistical significance between cohorts; darkest red in each row indicates >90th percentile; white denotes absence of detection.

The tongue ecology was observed to differ in both the dominant and rare taxa from health to disease. The dominant genera that increased in abundance were *Prevotella* sp, *Fusobacterium* sp and *Streptococcus* sp, and species such as *P. melaninogenica, F. periodonticum* and *S. salivarius/vestibularis* in particular within these genera. Galimanas et al. (2014) studied tongue ecology in relation to periodontitis and reported a shift in composition in association with periodontitis with an increase in abundance of genera such as *Synergistetes*, *Treponema* and *Clostridiales*—largely agreeing with the findings of this study pertaining to the rare taxa. Whilst no data are available in the literature with regards to shift of the dominant taxa in association with disease, existing data confirm *Prevotella* sp, *Fusobacterium* sp and *Streptococcus* sp to be the most prevalent and dominant taxa on the tongue (Paster et al. 2006; Eren et al. 2014). Though an increase in the numbers of dominant and rare taxa detected in the tongue samples was observed from health to gingivitis and chronic periodontitis, the first indication that the tongue ecology changes in association with inflammation in the periodontium is suggested by the rare taxa that increase in abundance and prevalence in the gingivitis and CP cohorts (Figure 4-27 and Figure 4-28). For example, the streptococcal species *S. gordonii, S. sanguinis* and in particular *S. constellatus* are thought to be preferentially abundant and prevalent in the subgingival and supragingival niches (Paster et al. 2006; Eren et al. 2014). This is consistent with that observed in the subgingival and interdental samples of the healthy cohort in this study, and these species exhibited an increased prevalence and abundance in the tongue samples of the gingivitis and CP cohort individuals (Figure 4-27 and Figure 4-28). Further evidence that the tongue ecology changes with periodontal disease or indeed that free transmission occurs between the tongue and periodontal niches is suggested by the increase in the tongue of TM7 sp HOT437 that was particularly abundant and prevalent in the interdental niche in health or disease (Figure 4-27). In addition, periodontopathic species such as *P. gingivalis, T. forsythia* and *Eubacterium* sp also increase in prevalence and abundance on the tongue of individuals with gingivitis or chronic periodontitis. Taken together, these data suggest that while the tongue is a unique niche with specific periodontitis associated taxa different from the subgingival and interdental niches increasing in prevalence and abundance, a change in ecology of the tongue is also observed in relation to rare taxa that are not normally associated with the tongue but have been observed to increase in the subgingival and interdental niches.

4.5 Ecology of the VSC producers

Volatile sulfur compound producing bacteria are some of the most prevalent in the oral cavity and anaerobic degradation of proteinaceous substrate in the oral cavity is thought to be linked with periodontitis and malodour in health and periodontitis (Kurata et al. 2008; Zappacosta et al. 2007). As observed in the present study, an association is also reported with the prevalence and increased concentration of methanethiol in the breath of chronic periodontitis patients (Takeuchi et al. 2010). This is consistent with the observation that gram-negative anaerobic bacteria are generally the more active VSC producers in the oral cavity in disease, and these bacteria also tend to increase in abundance in the periodontal niches in association with oral inflammation (Torresyap et al. 2003). In this study, the association of oral malodour and periodontal disease was investigated in terms of the putative VSC producing species present in the oral cavity. The exponential growth in understanding of the existing ecological diversity in the oral cavity through NGS approaches has limited one's ability to generalise previous surveys of VSC producing bacteria in the oral cavity in elucidating the full diversity of VSC producing species present (Washio et al. 2005; Persson et al. 1990). However, a bioinformatic appraisal of the available genomes of the oral microbiota in the current study helped extend the diversity of the VSC producing potential in the oral cavity. Of the 573 species level targets detected by HOMINGS, 295 species had full genomic sequences available and were searched for the presence of homologs capable of degrading cysteine or methionine to produce VSCs. Only 24 species were determined to have a homolog of methionine gamma lyase capable of degrading methionine to CH_3SH, in contrast to the majority of the available bacterial genomes containing cysteine desulfhydrases capable of producing H_2S from cysteine (218 of 295; See Appendix for full list). The ecological changes observed in these species in association with disease in the different niches are discussed in the following sections.

4.5.1 Health

A consistent observation across the different niches in the PLS analysis was that, the majority of taxa that were observed to increase in the different niches associated with gingivitis or chronic periodontitis were VSC producing organisms. A further observation is that the 24 species having the potential to produce methanethiol from methionine are often disease associated species and these include *Fusobacterium* sp, *Porphyromonas* sp *Treponema* sp and *Megasphaera micronuciformis*. Correlation

analysis of all the targets detected in relation to the Malodour Score as measured from the VSCs present in the breath samples of individuals in the healthy cohort revealed potential associations with malodour in the different niches. More taxonomic groups in the tongue were associated with the malodour scores compared to the interdental or subgingival niches, suggesting that tongue ecology is more relevant to malodour in healthy individuals (Table 4-5). Periodontopathic species such as *A. actinomycetemcomitans* and *T. lecithinolyticum* were observed to show a positive association in the interdental and subgingival niches respectively and the species *Johnsonella ignava* showed a positive association with malodour in both the niches. *J. ignava*, a likely H_2S producer, has been associated with oral squamous cell carcinoma (Pushalkar et al. 2012), however, due to the lower prevalence of this species (<40% of the total samples), its associations with the periodontal niches and malodour is primarily due to an increase in prevalence rather than abundance.

Interdental plaque		**Tongue**	
Taxon	**rho**	**Taxon**	**rho**
Johnsonella ignava	0.567	*Parvimonas sp*	0.710
Streptococcus salivarius/vestibularis	0.557	*Tannerella* HOT808	0.686
Aggregatibacter actinomycetemcomitans	0.521	*Streptococcus sp*	0.624
TM7 HOT352	0.495	*Gemella sp*	0.573
Veillonellaceae HOT155	-0.491	*Bergeyella* HOT322	0.573
Selenomonas sputigena	-0.526	*Stomatobaculum* HOT097	0.559
Prevotella veroralis	-0.566	*Peptococcus* HOT167	0.557
Subgingival plaque		*Gemella haemolysans*	0.545
Taxon	**rho**	*TM7* sp	0.503
Mollicutes HOT504	0.670	*Peptostreptococcus stomatis*	0.493
Treponema lecithinolyticum	0.664	*Neisseria elongata*	-0.525
Johnsonella ignava	0.616	*Aggregatibacter aphrophilus/*HOT458	-0.544
Prevotella pallens	0.606	*Rothia mucilaginosa*	-0.546
Porphyromonas HOT279	-0.624	*Selenomonas noxia*	-0.589
		Actinomyces massiliensis	-0.629
		Rothia sp	-0.645

Table 4-5 listing taxa that showed the strongest correlations (Spearman's rho) with particular niches as malodour scores increased in the healthy cohort.

An increase in *Parvimonas* sp and *Streptococcus* sp, in addition to a decrease in *Rothia* sp abundance on the tongue in association with oral malodour as measured by VSCs in the breath is consistent with the ecological changes observed on the tongue from health to chronic periodontitis in the present study, suggesting that the tongue ecology of healthy individuals with malodour is possibly more biased towards a disease associated ecology. However, no consistent clustering pattern could be established with regards to disease associated ecology in the niches studied—the healthy cohort samples from individuals with malodour clustered both closer to the gingivitis/periodontitis cohort(s) and further away from disease towards a more health associated ecology (Figure 4-29). This finding also held true with non-euclidean clustering of the samples using Bray-Curtis, Jaccard and Kulczynski distances determined by OTU-based analysis in QIIME.

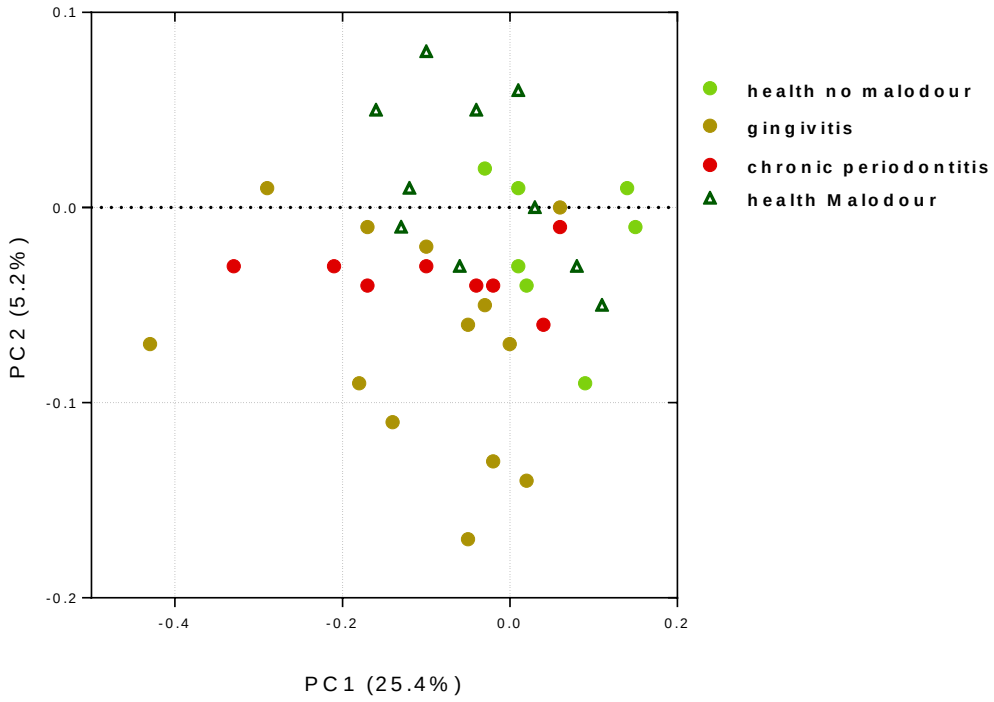


Figure 4-29 PCA score plot of tongue samples from healthy individuals with and without malodour as determined by the Malodour Score along with gingivitis and chronic periodontitis samples.

Different streptococcal species increasing in the tongue, subgingival and interdental niches in the disease cohorts in comparison to health and in association with malodour scores underlines the role of these species in both disease and malodour. Whilst streptococci are not the most prolific producers of VSCs from proteinaceous substrate, it has been demonstrated that *S. salivarius* can encourage degradation of mucin to produce malodorous volatiles including VSCs by other gram-negative bacteria by the activity of β-galactosidases inherent to streptococci and *S. salivarius* is present even in the most anaerobic areas of the tongue such as the dorsal posterior to circumvallate papillae, co-existing with gram-negative anaerobic bacteria (Sterer & Rosenberg 2006; Sterer et al. 2009; Allaker et al. 2008). Further, given the association of VSCs with periodontal disease, increase of streptococci in the tongue and other niches in association with increase of VSCs in the healthy cohort could be taken to mean that increase of these streptococci could facilitate ecological succession for more periodontopathic bacteria, and the increased presence of VSCs in the breath could be a consequence of increase in prevalence and abundance of periodontopathic bacteria in the tongue of individuals with malodour. This hypothesis is supported by data from this study pertaining to the classic red complex bacteria—it was found that these bacteria collectively are more abundant and prevalent in the different niches of healthy individuals with malodour as opposed to no malodour ( ). Whilst no statistical differences could be demonstrated owing to less sample numbers, increase in prevalence and abundance of *T. forsythia* and *A. actinomycetemcomitans* was observed in all three niches in individuals with malodour scores >1 compared to <1. All of these organisms have the enzyme homologs to produce H_2S while only *T. denticola* and *P. gingivalis* can produce CH_3SH.

Of the 24 species identified as methanethiol producers only 14 species were observed in all of the niches in the healthy cohort, with the interdental niche showing the highest prevalence of methanethiol producing species (mean=11±2), compared to the subgingival niche (mean=9±2) or the tongue (mean=6±2). These observations also hold true for the observed richness of the H_2S producing species in the different niches. The species *T. denticola, F. naviforme/nucleatum* ssp *fusiforme* were the most abundant in the interdental niche whereas *F. periodonticum* and *M. micronuciformis* were the most prevalent and abundant in the tongue of the healthy cohort (Figure 4-31). These data suggest that the interdental niche in the healthy individuals is a potential reservoir for VSC producers and *F. periodonticum* and *M. micronuciformis* are likely involved in the production of methanethiol in the breath of the healthy cohort in this study, given

that the tongue ecology is the most important in malodour present in healthy individuals. A weak positive association was found with abundance of *F. periodonticum* in the tongue and methanethiol concentration in the breath (r=0.33). It is notable that species such as *T. denticola, P. endodontalis* and *F. nucleatum* are more abundant and prevalent in the periodontal niches whereas *F. periodonticum* and *M. micronuciformis* are more abundant in the tongue, particularly as the former species have been shown to be more prolific in degrading serum substrate to VSCs, whereas the tongue associated *F. periodonticum* is more efficient at producing methanethiol from free methionine and less so at degrading serum proteins (Persson et al. 1990). This further illustrates the nature of adaptations inherent in these species toward the different habitats they occupy, as the periodontal niches are more likely to provide serum proteins in the form of gingival-crevicular fluid, whereas free methionine or cysteine is more likely to be available in the tongue, by degradation of proteinaceous material from saliva and mucosal debris from tongue biofilm.

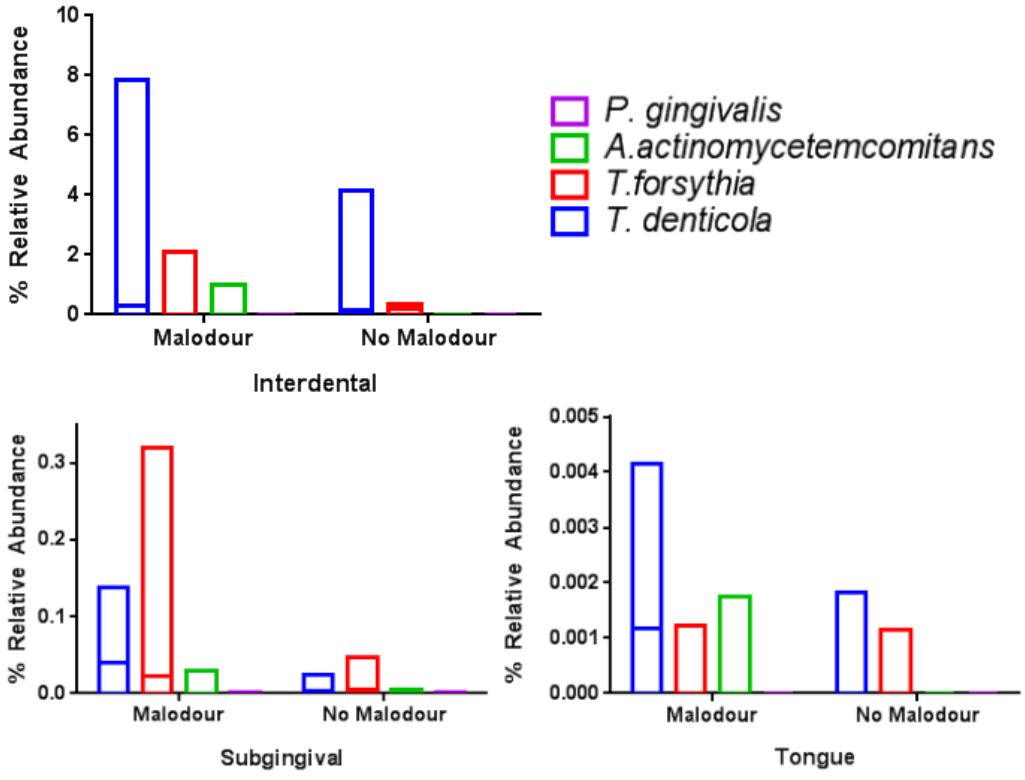


Figure 4-30 showing relative abundances of the red complex bacteria and *A. actinomycetemcomitans* in the different niches between healthy individuals with and without malodour as determined by the malodour score. Boxes represent Min-Max range and the mid line is the median.

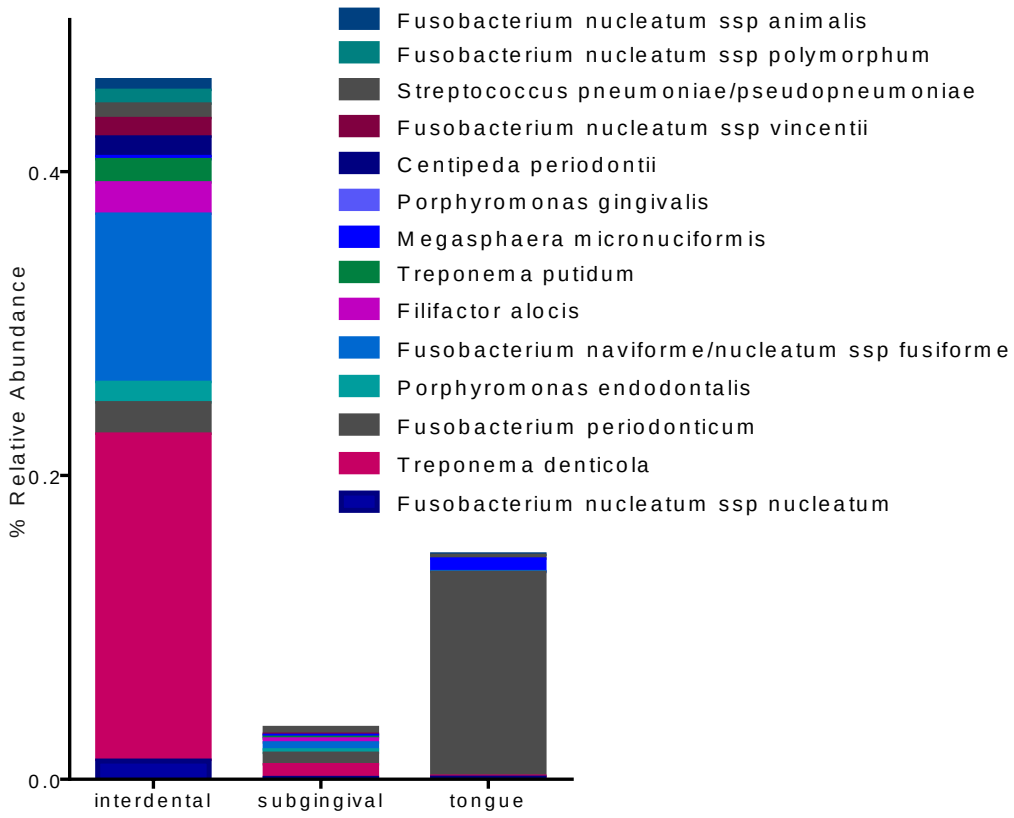


Figure 4-31 showing median values (plotted as stacked bars) of the methanethiol producing taxa in the tongue, interdental and subgingival niches of the healthy cohort

4.5.2 Gingivitis

Metagenomic studies have so far explored the association of oral malodour with the ecology of saliva and tongue in healthy individuals (Yang et al. 2013; Takeshita et al. 2012), and the present study is the first to explore oral malodour with periodontal disease. Findings from this study pertaining to the VSCs present in the breath of the health and disease cohorts are consistent with findings reported in the literature, particularly of the association of malodour as defined by the concentration of the VSCs in the breath increasing in the disease cohort, and the role of methanethiol as the more dominant VSC in the breath of individuals with gingivitis and chronic periodontitis, further supported by the increase of the ratio of CH_3SH to H_2S in these cohorts compared to health.

Interdental plaque		Subgingival plaque	
Taxon	**rho**	**Taxon**	**rho**
Porphyromonas HOT275	0.741	*Atopobium sp*	0.881
Olsenella HOT809	0.631	*TM7[G-1]* HOT347	0.873
Enterococcus faecalis	0.588	*Granulicatella elegans*	0.762
Solobacterium moorei	0.580	*Clostridiales* HOT075	0.762
*Leptotrichia hofstadii/*HOT909	-0.592	*Neisseria pharyngis*	-0.577
Leptotrichia goodfellowii	-0.608		
*Catonella morbi/*HOT164	-0.629	*Eubacterium yurii*	-0.592
Selenomonas noxia	-0.634	*Veillonella atypica/dispar*	-0.655
Oribacterium HOT078/HOT372	-0.650	*Enterococcus faecalis*	-0.655
Porphyromonas endodontalis	-0.676	*Prevotella nanceiensis/*HOT299	-0.764
Leptotrichia spp probe2	-0.748	*Oribacterium* HOT108	-0.764

Tongue

Taxon	rho	Taxon	rho
Leptotrichia HOT215	0.714	*Streptococcus constellatus*	0.617
Stomatobaculum HOT097	0.703	*Corynebacterium* spp	0.604
Neisseria elongata	0.674	*Eubacterium infirmum*	0.587
Actinomyces spp probe1	0.659	*Prevotella micans*	0.577
Rothia aeria	0.654	*Actinomyces odontolyticus*	-0.613

Table 4-6 listing taxons that showed the strongest correlations with the three niches in the gingivitis cohort. Only variables with 95% confidence intervals in line with the spearman's correlation coefficient values are listed.

The relationship between malodour and the periodontal niches were not similar in the gingivitis and chronic periodontitis cohort to that of health as shown in previous section discussing the association between overall richness and diversity and malodour (4.2.2). For example, species richness and diversity in the tongue had the least positive relationship with malodour scores in the gingivitis cohort, compared to the health and chronic periodontitis groups, whilst a more dynamic relationship was observed with regards to the subgingival and interdental niches in the gingivitis and chronic periodontitis cohorts in comparison to health. This supports the contention that malodour as it relates to VSCs in the breath of individuals has a deeper relationship with inflammation in the oral cavity and in particular, the microbial role in inflammation of the periodontium. This suggests that the associations observed with malodour in the gingivitis and chronic periodontitis cohorts include changes in microbial ecology due to the disease and/or a more direct relationship with VSC production.

In the gingivitis cohort, malodour scores were associated with an increase of *Porphyromonas* HOT275, *Olsenella* HOT809 and *E. faecalis* in the interdental plaque; *Atopobium* sp, TM7 HOT347 and *G. elegans* in the subgingival plaque*; Leptotrichia* HOT215, *Stomatobaculum* HOT097 and *N. elongata* in the tongue. All of these species that have full genome sequences available have enzyme homologues that can produce H_2S. It is interesting to note that the strongest correlation observed with malodour and the subgingival plaque in the gingivitis cohort was for the genus probe detecting a collection of species namely *A. fossor, A. minutum, A. parvulum, A. rimae* and *Atopobium* HOT199 (Spearman's rho=0.881; Table 4-6). These species are less abundant in the subgingival plaque than the tongue or the interdental plaque and as observed in the comparison between the niches in this cohort (4.3.2), they increased in the interdental plaque, and it was suggested that this species is likely an opportunist in the subgingival plaque in response to inflammation. Another example of a non-native species that proliferates in a different niche in the gingivitis cohort compared to health and that being associated with malodour is *S. constellatus* in the tongue (Figure 4-28; Table 4-6). These observations indicate the potential relationship between VSCs and inflammation in gingivitis, as clearly if VSC concentration in the breath has a mechanistic link with inflammation, one would expect to find these associations with ecological changes occurring as part of the aetiopathogenesis of the disease and this may not always relate to VSC production. This is further illustrated by the negative correlation observed between abundance of *P. endodontalis*, a prolific H_2S and CH_3SH producer in the interdental plaque of the gingivitis cohort, suggesting that the change of ecology occurring in the interdental niche in relation to gingivitis is related to malodour, but in an inverse manner, given that in the healthy cohort *P. endodontalis* is more abundant in the interdental niche than any other niches studied. However, a negative relationship was still observed with respect to this species and malodour score in the subgingival plaque of this cohort, whilst a non-significant increase in prevalence and abundance was observed in comparison to health.

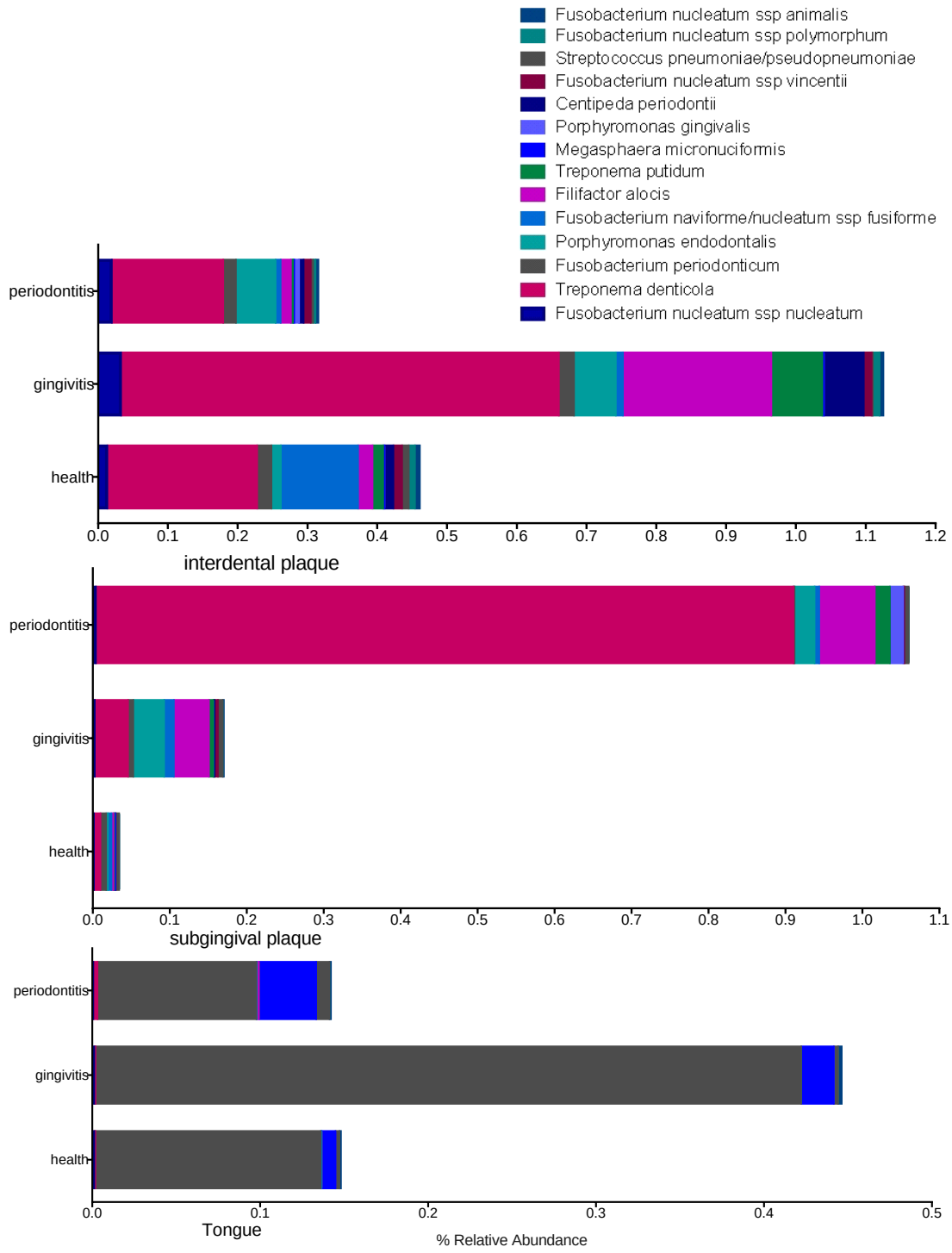


Figure 4-32 showing stacked median values of putative methanethiol producing species in the oral niches in health and disease

The ecology of methanethiol producing bacteria in the periodontal niches could be a microcosm of disease progression from health to chronic periodontitis, and these niches were observed to be the most dynamic, with the interdental niche found to be most active in gingivitis (Figure 4-34). Species such as *T. denticola, F. alocis, T. putidum, P. endodontalis* and *Centipeda periodontii* displayed an increase in abundance in this niche (in that order), with a representative increase of abundance observed in the subgingival plaque in *T. denticola, P. endodontalis* and *F. alocis.* It is notable that *T. denticola* and *F. alocis* appear to play a strong role in the changes observed in this niche in gingivitis, and *T. denticola* is shown to be very active in producing copious amounts of methanethiol and hydrogen sulfide from serum proteins (Persson et al. 1990). In addition, the methionine gamma lyase present in *T. denticola* responsible for producing methanethiol has been shown to be so efficient that its rate of methanethiol production from degrading free methionine is almost 100-fold higher at physiological concentrations than *P. gingivalis* (Fukamachi et al. 2005). *T. denticola* is likely to be more important in methanethiol production in the periodontal niches.

An increase in abundance of *F. periodonticum, M. micronuciformis* and *F. nucleatum* ssp *animalis* was observed on the tongue, however no change in richness was observed. *M. micronuciformis* and *F. periodonticum* levels in saliva were positively associated with high methanethiol and hydrogen sulfide content in the breath of healthy individuals, respectively, whereas *P. endodontalis* only displayed a weak correlation to VSCs in a metagenomic study by Takeshita et al. (2012) with a sample size of 43. Observations in the present study suggest that the associations observed by Takeshita et al (2012) are predominantly due to microbial shedding from the tongue and whilst saliva is not as specialised as the niches considered in the present study, a positive correlation would still be expected with respect to the subgingival or interdental niches and *P. endodontalis.* However, with the overall diversity estimates in connection with malodour scores only displaying a fair positive association with subgingival niche and a moderate *negative* correlation with the interdental niche (Table 4-4), any real relationships will be due to species that display more dramatic shifts in abundances or prevalence in association with VSCs.

4.5.3 Chronic periodontitis

The total number of significant correlations found between malodour scores and the different niches were the highest in the chronic periodontitis cohort compared to health or gingivitis. Stronger relationships were found too with the subgingival and tongue niches showing predominantly positive associations whilst the interdental niche showing more negative associations with microbial species (Table 4-7). The group of species detected by the *Rothia* spp probe and *R. dentocariosa* in the subgingival and interdental niches were found to correlate negatively with malodour scores. Whilst greater abundance of *Rothia* spp in general and *R. dentocariosa* in particular in the periodontal niches is considered to be more health associated, this correlation suggests that the periodontal ecology of periodontitis patients with malodour is likely to be more disease associated than patients without malodour (Kistler et al. 2013; Abusleme et al. 2013). Indeed, the positive associations observed with putative periodontopathic species and phylotypes such as *Fretibacterium* HOT360, *Bacteroidetes* HOT280, *T. denticola, F. alocis* and *E. saphenum* in the subgingival plaque supports this hypothesis.

The strongest correlations with malodour scores in this cohort was observed with TM7 phylotypes and tongue, with a collection of species detected by the TM7 spp probe showing a strong positive correlation with regards to the tongue of the chronic periodontitis patients (rho=0.976). The phylotype most responsible for this is likely to be TM7 HOT352, as that displayed the next strongest relationship with the tongue (rho=0.952). This is unlikely to be a spurious relationship as TM7 phylotypes feature heavily in the periodontal niches as being positively associated with increasing malodour scores, and due to the high prevalence of these phylotypes, this relationship is not due to increase in prevalence but due to abundance in the different niches as part of disease progression (Table 4-7). Positive associations were also found between malodour scores and TM7 prevalence in the tongue, interdental and subgingival niches in the healthy and gingivitis cohorts (Table 4-5; Table 4-6). TM7 are a major phylum of uncultivated microorganisms, until recently when a species named TM7x was cultivated as a strict epibiont of a particular *A. odontolyticus* strain (He et al. 2015). This species was found to have a reduced genome devoid of amino acid synthesising capability and a parasitic symbiosis with the host bacterial strain. Assuming that this finding is an archetype of the phylum TM7, it is possible that the TM7 phylotypes that show correlations with malodour score and consequently with VSC concentration in the breath are linked with microbial strains that are key VSC producers themselves and/or

are disease associated and thus stimulate VSC production by way of their epibiotic associations.

Subgingival plaque		**Tongue**	
Taxon	**rho**	**Taxon**	**rho**
Fretibacterium HOT360	0.833	*TM7* spp	0.976
Bacteroidetes HOT280	0.772	*TM7* HOT352	0.952
Treponema denticola	0.768	*Gemella morbillorum*	0.91
Stomatobaculum HOT097	0.76	*Porphyromonas* HOT279	0.874
Chloroflexi HOT439	0.759	*Gemella* spp	0.857
Filifactor alocis	0.754	*Lachnoanaerobaculum umeaense*	0.833
Treponema sp probe5	0.746	*Oribacterium* HOT108	0.833
Capnocytophaga granulosa	0.746	*Clostridiales* HOT085	0.814
Treponema sp probe4	0.736	*Clostridiales* HOT075	0.802
Eubacterium saphenum	0.733	*Parvimonas* spp	0.786
Prevotella nigrescens	0.717	*Peptococcus* HOT167	0.779
Alloprevotella tannerae	0.717	*Eubacterium sulci*	0.762
TM7 HOT353	0.709	*Solobacterium moorei*	0.762
Peptostreptococcaceae HOT369	0.697	*Campylobacter rectus/showae*	0.738
TM7 HOT437	0.687	*Stomatobaculum* HOT097	0.738
Rothia spp	-0.746	*Actinomyces odontolyticus*	-0.709
Interdental plaque			
Taxon	**rho**	**Taxon**	**rho**
TM7 HOT353	0.832	*Veillonella atypica/dispar*	-0.648
TM7 HOT349	0.743	*Gemella haemolysans*	-0.65
Clostridiales HOT075	0.669	*Rothia* sp	-0.657
TM7 sp	0.587	*Leptotrichia hongkongensis*	-0.69
Veillonella parvula	-0.559	*Actinomyces* sp probe1	-0.741
Actinobaculum HOT183	-0.585	*Rothia dentocariosa*	-0.811
Campylobacter concisus	-0.626		

Table 4-7 listing taxons that showed the strongest correlations with the three niches and malodour scores in the chronic periodontitis cohort. Only the strongest correlations found in species with ≥75% prevalence in this cohort is reported.

Whilst TM7 has been detected in tongue and saliva in previous metagenomic malodour studies that were carried out in healthy individuals, no associations were reported (Yang et al. 2013; Takeshita et al. 2012). Though associations were observed in the present

study with the tongue in the healthy cohort, they were of moderate strength compared to the disease cohorts and the associations were more due to increase in prevalence than abundance. This suggests that the ecological shift observed with regards to TM7 is more disease associated with VSCs being a marker of disease that identifies this relationship, as TM7 was more prevalent in the interdental niche in the healthy cohort and was found to increase in prevalence in the other niches in gingivitis and chronic periodontitis.

Methanethiol producing species were more abundant in the subgingival niche of the chronic periodontitis cohort compared to all the other niches, suggesting a potential pathogenetic role for methanethiol production in active periodontal disease. *T. denticola* was found to be the most abundant of the CH_3SH producing species in this niche with *P. endodontalis* and *P. gingivalis* also showing an increase in abundance (Figure 4-34). The prevalence of *P. gingivalis* was observed to increase in both the interdental and subgingival plaque in this cohort. An increase in prevalence of *T. denticola* and *F. alocis* was observed in the tongue of the chronic periodontitis patients compared to health, and the 'core' species of *F. periodonticum* remained similar in abundance, with an increase in abundance of *M. micronuciformis* also observed. It is notable that with all of the methanethiol producing species diversity is conserved in the interdental plaque of periodontitis patients with the periodontopathic *P. gingivalis* being carried by this niche, suggesting that active disease could be precipitated by transmission of the latent virulence from the interdental to the subgingival niche, possibly explaining the episodic nature of periodontal disease.

4.5.3.1 Ecology of the sulphate reducers

Perhaps the most direct evidence of VSC production in the periodontal niches is provided by a class of bacteria called Sulphate reducing bacteria (SRB) known to use sulphate as an electron acceptor in anaerobic respiration forming a number of malodorous VSCs including hydrogen sulfide, carbon disulfide, and trace amounts of methane-, ethane- and propanethiol (Le et al. 2005). The major genera of oral SRBs belong to class deltaproteobacteria and epsilonproteobacteria and are some of the least studied group of bacteria in relation to oral malodour, but consistently show an association with periodontal disease (Vianna et al. 2008; Figure 4-15; Figure 4-21; Figure 4-27; Figure 4-28). Limited numbers of these species are detected by HOMINGS, namely *Desulfomicrobium orale, Desulfovibrio fairfieldensis, Desulfovibrio* HOT040, *Desulfobulbus* HOT041 and a group of species belonging to the genus *Desulfobulbus* namely *D. elongatus, D. mediterraneus, D. propionicus* and *D.*

rhabdoformis. Prevalence of these species were high in the interdental niche in general, but also showed a significant increase in prevalence and abundance in the subgingival niche of chronic periodontitis patients (Figure 4-35). Only *Desulfobulbus* spp was detected in the tongue, which also showed a significant increase in prevalence in chronic periodontitis patients compared to health but not gingivitis.

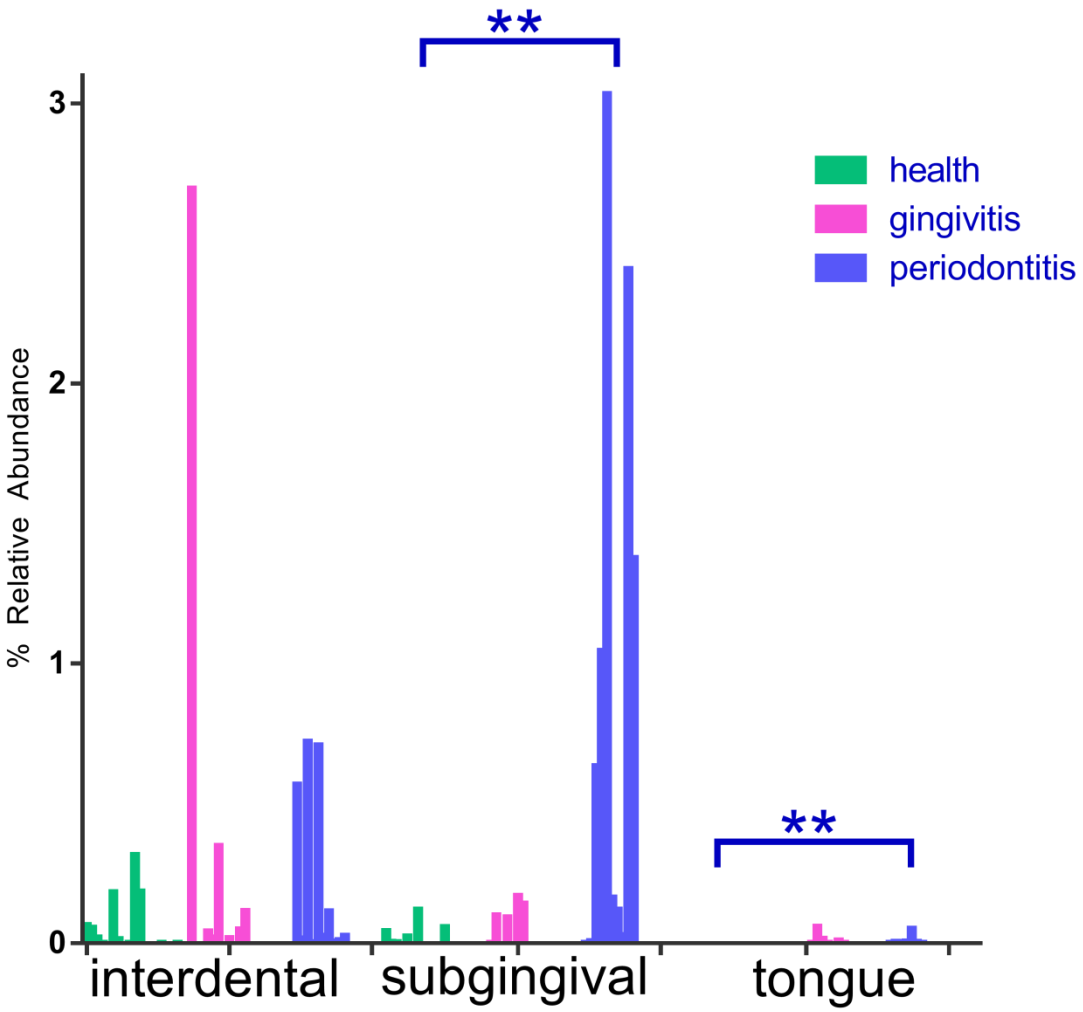


Figure 4-35 showing abundances of the sum of all sulfate-reducing taxa detected in the three niches of the different cohorts as individual bars. Statistical differences found using one-way ANOVA between the cohorts are indicated.

The RDP-classifier displayed a higher resolution than HOMINGS, detecting eight different genera of sulphate reducing bacteria in all the niches, with the tongue showing the most diversity amongst the niches. The genus *Desulfobulbus* spp was found to be the most prevalent in the tongue of periodontitis patients confirming the results of the HOMINGS analysis whilst the genus *Sulfospirillum* spp was more prevalent in the

tongue of healthy individuals (Figure 4-36). Interestingly, though a high prevalence of *Desulfobulbus* spp was observed in the subgingival and interdental samples from the three cohorts, the species richness of the sulphate-reducers increased in both the niches in periodontitis suggesting that the available sulphate levels in the periodontal niches must also increase to sustain this diversity. Indeed, this hypothesis is supported by the gas chromatographic analysis of subgingival paper point samples for the presence of VSCs, showing a significant increase of H_2S from health to gingivitis and periodontitis (Figure 3-19).

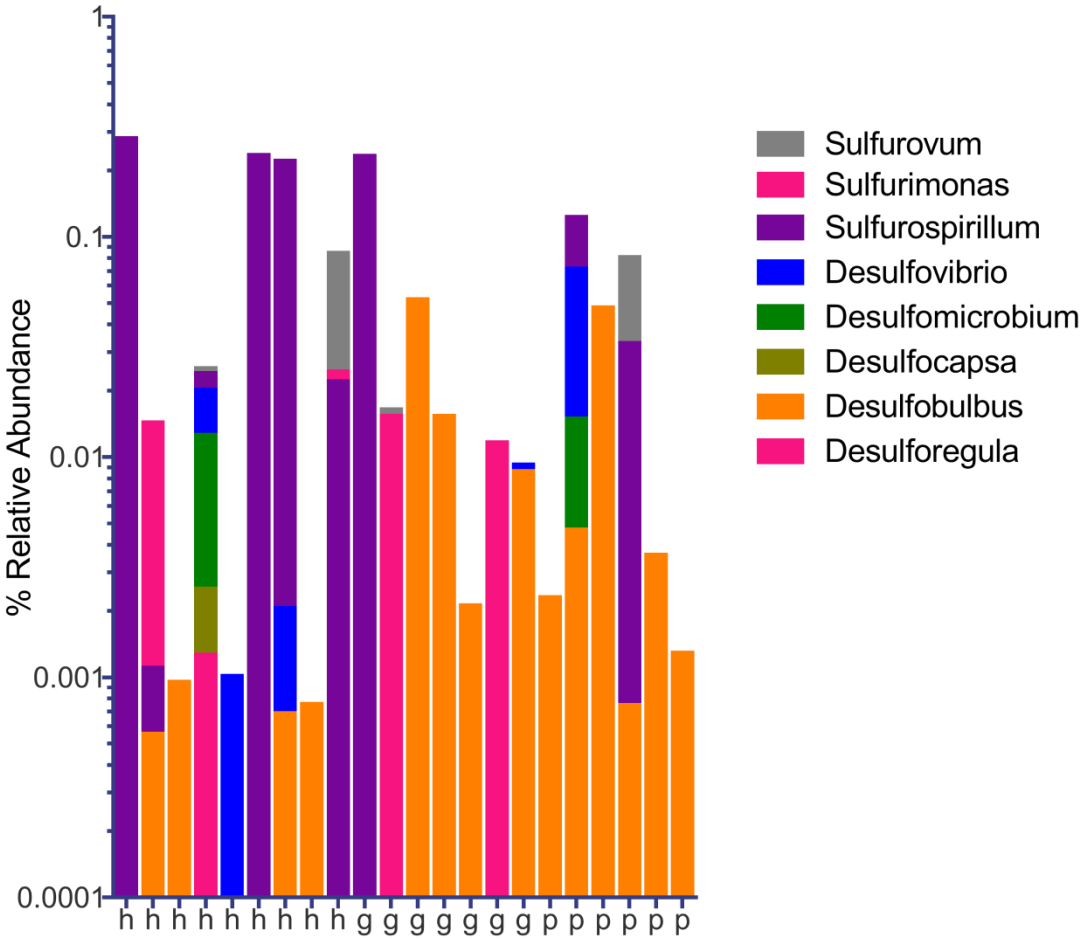


Figure 4-36 showing the diverse genera of SRBs detected in selected tongue samples from the different cohorts. Only samples with detected SRBs are shown (h=health; g=gingivitis; p=periodontitis).

The role of VSC production in chronic periodontitis is well illustrated by the ecological changes observed with the sulphate reducing bacteria present in the oral cavity. Unlike other bacterial species, a direct functional relationship can be inferred with an increase in prevalence and abundance of this group of bacteria in the different niches. Given the association of tongue with malodour and VSC concentration in the breath, the observed diversity of SRBs in the tongue in all the cohorts suggests that available suphate is high in the tongue regardless of disease state. A change in SRB ecology in the interdental and subgingival niches in association with periodontitis indicates that VSC production in these niches is high and that it plays a functional role in the anaerobiosis of the periodontal environment. The observed abundance of SRBs in the subgingival and interdental niches of periodontitis patients suggests that this class of bacteria may even be the most important VSC producer in the subgingival environment in association with inflammation, as sulphate is most likely generated as a by-product of the prevailing oxidative stress in response to microbial challenge and these group of bacteria that can readily metabolise such compounds to lower the redox potential may have functional advantages for the wider bacterial community.

4.6 Summary

To conclude, the study of general ecology in health, gingivitis and chronic periodontitis of three different niches namely tongue, subgingival and interdental plaque using Next Generation Sequencing revealed that the interdental niche was the most diverse in health, with the tongue being the least diverse. Consistent with the available literature, it was found that in chronic periodontitis, there is a general normalisation of diversity and richness among the three niches and this included proliferation of species in niches not normally found in health. The interdental and subgingival niches were found to be the most dynamic in gingivitis and chronic periodontitis in this regard, with changes also observed in the tongue ecology in gingivitis and chronic periodontitis, particularly with the prevalence and abundance of periodontopathic bacterial species. Malodour as defined by the VSC concentration of the breath was found to correlate positively with diversity and richness in the tongue in health, whereas this association was weaker in disease. However, the periodontal niches were found to be more associated with VSC concentration in the breath of the gingivitis and periodontitis cohorts. Observed variation in the microbial ecology of the tongue, subgingival and interdental niches

within and between cohorts were elucidated to find that both classical and novel periodontopathogens involved in changes associated with disease.

Diversity of VSC producing bacteria was described for each niche in the different cohorts, and numerous relationships were found with VSC concentrations in the breath. Periodontopathic species were found to be VSC producers and positive correlations were found with these species in each cohort within the three niches studied, such that increasing VSC concentrations within each cohort was found to correlate more strongly with disease associated ecology. Finally, the ecology of sulphate reducing bacteria in the periodontal and tongue niches is described and important relationships with periodontal disease were elucidated.

5 THE ROLE OF METHIONINE GAMMA LYASE IN INFLAMMATION AND MICROBIAL ECOLOGY

Porphyromonas gingivalis is one of the most widely studied bacterium in relation to plaque-induced inflammatory diseases of the mouth. Classically part of a trio of microbial species (‘red complex’) including *Tanerella forsythia* and *Treponema denticola* that were strongly linked to the etiopathogenesis of periodontitis, *P. gingivalis* has now been proposed as a ‘keystone’ pathogen in the new paradigm of periodontitis (Hajishengallis et al. 2012). This model recognizes that some bacterial species though in low abundance are able to drive the ecology of the biofilm community in response to environmental cues or adaptations by influencing the diversity and evenness of such communities. Studies have elaborated the mechanisms by which *P. gingivalis* could bring about changes in the community structure by manipulating the host response. These include interfering with neutrophil recruitment by deactivating IL-8 production, down regulation of E-selectin production by gingival epithelia, impeding the complement cascade by effecting C5aR/TLR2 crosstalk and gingipain induced degradation of important complement proteins such as C3 and C5 (Bostanci et al. 2007a; Hajishengallis et al. 2011; Takeuchi et al. 2013; Wang et al. 2010). Whilst these mechanisms could elicit a sustained, if ineffective immune response from the host, the adaptation of *P. gingivalis* to the subgingival habitat is thought to be realized in the episodic nature of these mechanisms.

P. gingivalis is known to be a prolific producer of volatile sulfur compounds (VSCs) in serum and also from free sulfur containing amino acids such as cysteine and methionine, however it is reported to be more efficient at producing VSCs from a serum substrate than cysteine and methionine (Persson et al. 1990; Stephen et al. 2014). These VSCs such as hydrogen sulfide, methanethiol and dimethyl disulfide are thought to be useful in lowering the redox potential of the subgingival microenvironment and aid in the invasiveness of the organism and indeed the biofilm, by increasing the permeability of the mucosal cell membrane (Ng & Tonzetich 1984). Given that one of the described mechanisms of IL-8 deactivation by serB protease requires cell invasion for it to occur (Takeuchi et al. 2013), one would expect VSC production to be an important adaptive mechanism for *P. gingivalis* and indeed for other potential keystone pathogens.

Of the genetic complement that can produce VSCs in *P. gingivalis*, a previous study has characterized the methionine γ lyase (mgl) that produces methanethiol by catabolic degradation of methionine, and this enzyme has been reported to confer resistance to an antimicrobial agent 3-chloro-DL-alanine by exhibiting deaminating activity towards it as with methionine, in both *P. gingivalis* and *F. nucleatum* (Yoshimura et al. 2000; Yoshimura et al. 2002a). Virulence studies in a murine abscess model has demonstrated that *P. gingivalis* W83 wild type was markedly more invasive than the mgl-deficient mutant, whilst a drug trifluoromethionine that depends on the activity of *mgl* to release its active form showed considerable increase in the survival of mice when injected along with the wild type *P. gingivalis* (Yoshimura et al. 2000; Yoshimura et al. 2002b). These observations are consistent with the hypothesis that VSC production or indeed mgl-activity could aid invasiveness. However, it is well known that planktonic forms of bacteria often exhibit a different phenotype to biofilms and these studies did not involve biofilms and it is possible biofilm dwelling *P. gingivalis* downregulates VSC producing genes to exhibit a less invasive phenotype. The present study aimed to replicate the knockout of the methionine γ lyase in the strain W50 (as opposed to W83). The mutant and wild type *P. gingivalis* were then grown in a 10-species oral biofilm model and used to stimulate transformed oral keratinocytes in vitro. Biofilm composition was determined to study the effect of the mgl gene knockout in influencing the ecology of the biofilm, and if the possible altered ecology in any way affects the cytokine response of the oral keratinocytes.

5.1 Characteristics of the mutant and biofilms

The methionine gamma lyase in *P. gingivalis* W50 was confirmed to be identical to the strain W83 used by Yoshimura et al. (2000) and the mutant W50 (named PG343 henceforth) produced less than 1% of the total methanethiol in the headspace compared to the wild type W50, when incubated with L-methionine suggesting successful knockout of *mgl* (Figure 5-1). However, it is possible that *P. gingivalis* W50 has other as yet undiscovered gene(s) still capable of producing methanethiol, as the previous study on strain W83 only observed complete absence of methanethiol in the headspace of bacterial cells incubated with L-methionine after mutagenesis but not in the culture supernatants.

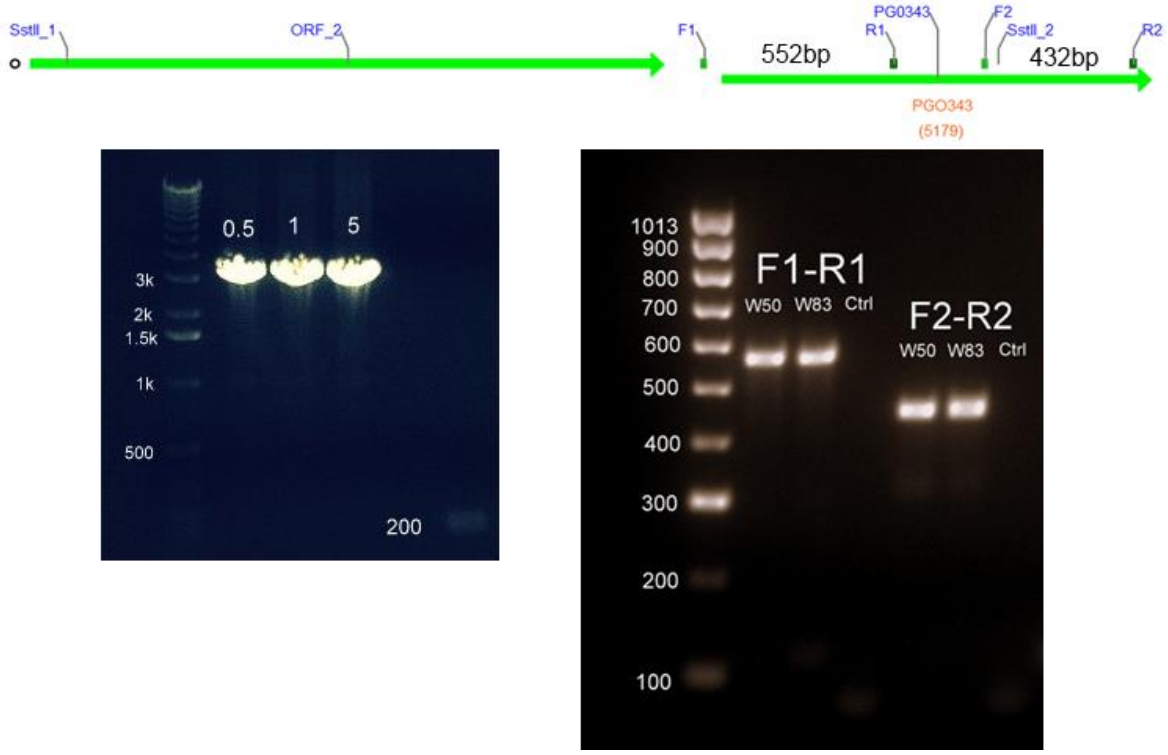


Figure 5-1 top: restriction sites and expected amplicons sizes on the ORF identified to code for *mgl* in *P. gingivalis* W50; bottom right: an agarose gel showing DNA bands for the actual amplicons within the ORF chosen to ligate the *ermF-ermAM* cassette in *P. gingivalis* strains W50 and W83; bottom left: agarose gel showing intense 3kb DNA bands (0.5/1/5µl loads shown) corresponding to the amplified F1-R2 fragment after restriction digest and before electroporation.

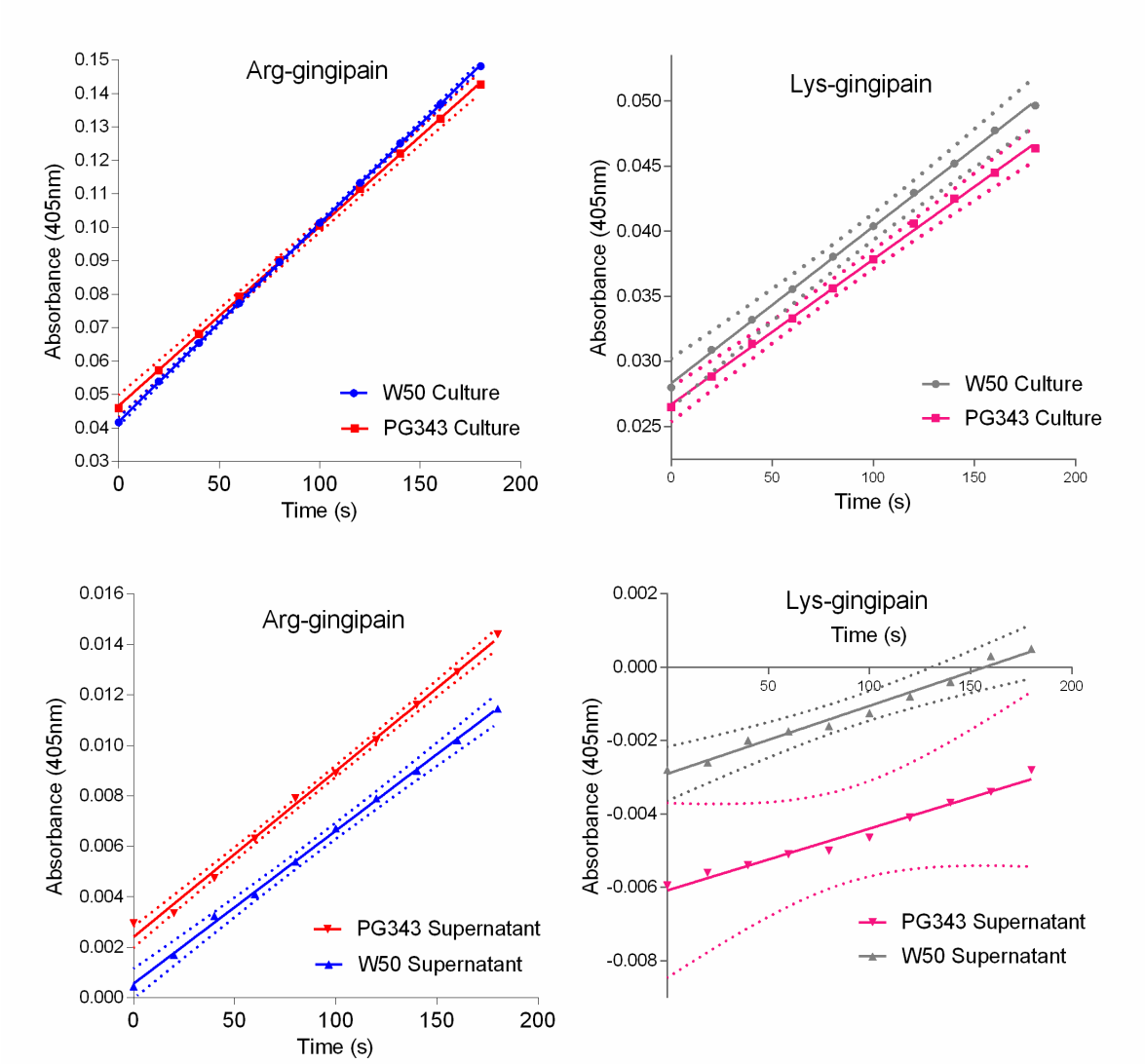


Figure 5-2 showing arg-gingipain and lys-gingipain activities in BHI haemin broth cultures and their respective cell-free culture supernatants of *P. gingivalis* W50 and PG343. Data points are mean values from duplicates with linear regression lines and 95% confidence bands plotted. Only the slopes of PG343 and W50 cultures for the arg-gingipain assay differed significantly (p=0.001).

Morphologically, PG343 colonies resembled W50, with a distinction being, the mutant strain took longer to attain black pigmentation when cells frozen at -80°C were revived on blood agar, but subsequently subcultured generations did not show this difference with the wild type W50. The growth rates of both strains were also similar in BHI broth supplemented with haemin. Colorimetric assays showed similar activities for both arg-gingipain (Rgp) and lys-gingipain (Kgp) in PG343 and W50 broth cultures and supernatants, with only the slopes of Rgp activity measured in the cultures of PG343

differing significantly (Figure 5-2). As reported in the literature, the culture supernatants showed marked reduction in measured enzyme activity for both the gingipains, but this was particularly evident for Kgp (Aduse-Opoku et al. 2000). This is consistent with the observations of the agar colonies, as lys-gingipains are thought to play a key role in the release of heme from haemoglobin and subsequently to black pigmentation. The differences observed of the Rgp activities between PG343 and W50 cultures may only result in a negligible reduction of the Rgp-Kgp complex's function. There is also evidence that methanethiol can compromise the erythrocyte cell membrane, causing it to rupture, in addition to having particular preference for inhibiting erythrocyte associated cytochrome c oxidase, catalases and pyruvate kinases (Ahmed et al. 1984; Valentine et al. 1987; Finkelstein & Benevenga 1986). While lys-gingipains are ultimately responsible for black-pigmentation by providing access to haemoglobin, methanethiol production may accelerate the availability and access to haemoglobin, and therefore to iron uptake and black pigmentation.

5.1.1 Biofilm composition

Growth of biofilms with PG343 and W50 resulted in markedly different biofilm compositions. Firstly, measurement of the total CFU ml^{-1} equivalents in the biofilms by using *P. gingivalis* cultures as standards indicated that the PG343 biofilms had, on average an order of magnitude more cells than the W50 biofilms. Secondly, the proportions of the individual species in the PG343 biofilms were congruent, resulting in a more even community compared to W50 biofilms. Differences were observed in the overall composition of biofilms grown with the W50 or PG343 strains (Figure 5-3). *Streptococcus* spp ($p=0.02$) and *A. naeslundii* ($p=0.004$) proportions decreased significantly in the mutant biofilms compared to wild type, whilst *V. dispar* proportions increased significantly ($p=0.0008$). The cumulative distributions of *A. actinomycetemcomitans, V. dispar, A. naeslundii* and *Streptococcus* spp proportions in the mutant biofilms also differed significantly from the wild type (WT) biofilms. No statistical differences in the proportions of other species were detected. However, when the amounts of DNA extracted from each sample is taken into account (a truer reflection of biomass) without normalizing to the universal primer set, *F. nucleatum, P. intermedia, V. dispar* and *P. gingivalis* increased significantly in the PG343 biofilm compared to W50 both in numbers and cumulative distributions (Figure 5-4). Although not statistically significant, elevated CFU ml^{-1} equivalents of *A. actinomycetemcomitans* were also observed in the PG343 biofilms compared to W50.

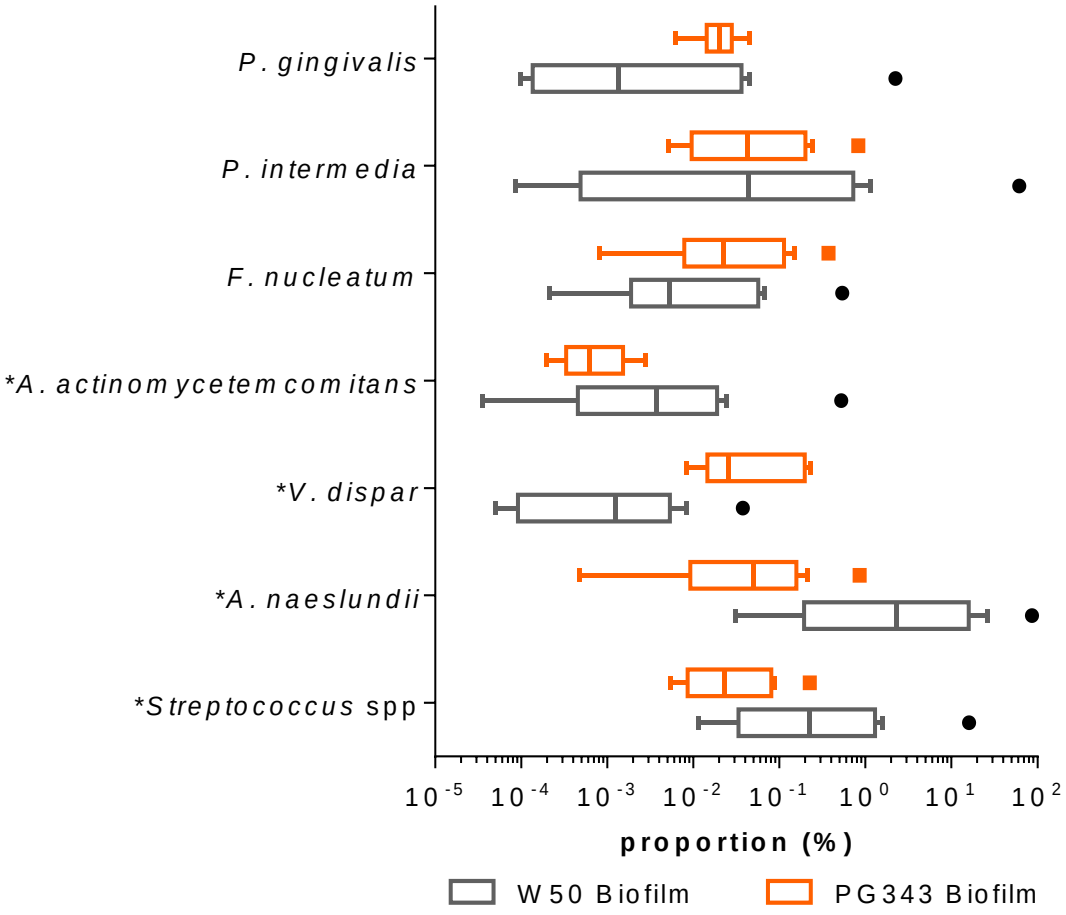


Figure 5-3 showing the proportion of the different species in the PG343 and W50 biofilms (n=9 for both) relative to total CFU ml^{-1} equivalents as measured by the universal primers ineach biofilm. Boxes extend from 25th to 75th percentile, mid line being median; whiskers and outliers plotted by the Tukey method. Asterisk before species indicate statistical significance.

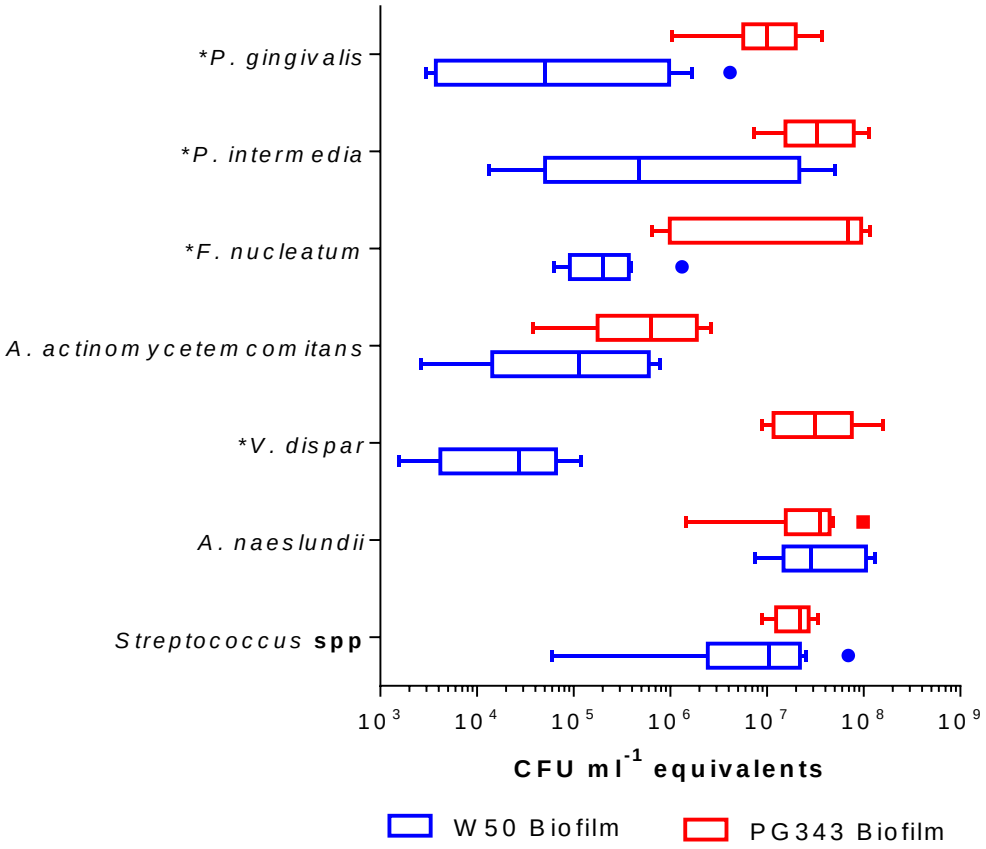


Figure 5-4 showing the composition of the W50 and PG343 biofilms (n=9 for both) as a function of the amounts of DNA extracted from each biofilms. Asterisk before species name indicates statistical significance at p<0.01 for both Mann-Whitney U and Kolmogorov-Smirnov tests. Boxes extend from 25th to 75th percentile, mid line being median. Whiskers and outliers plotted by the Tukey method.

It is well known that *P. gingivalis* is one of the most efficient degrader of free L-methionine and in serum (Persson et al, 1990). It is possible that methionine degradation by *P. gingivalis* prevents other species, in particular, *F. nucleatum* from proliferating by using it as an energy source (Bakken et al. 1989; Gharbia et al. 1989). It may well be that in the PG343 biofilms, more availability of L-methionine encourages growth of *F. nucleatum*, which can further support the proliferation of all the other species as *F. nucleatum* is well known as a bridging-species in the oral biofilm (Bradshaw et al. 1998; Merritt et al. 2009). This could explain the increased overall CFEs as measured in the PG343 biofilms. This hypothesis would suggest that *mgl* of *P. gingivalis* could have a role to play in the overall ecology and community structure of the oral biofilm, as a 'keystone' virulence factor. A possible mechanism by which this could occur is via

control of the activated methionine cycle which in turn regulates *luxS* mediated production of autoinducer-2 like quorum sensing molecules that have been extensively studied in relation to bacterial biofilm formation (Figure 5-5). Given the importance of the methionine cycle in the quorum sensing phenomena, the catabolism of available methionine may play a role in co-aggregation behaviour among bacterial species, and indeed this could be one mechanism by which the periodontopathic bacteria which form a small subset of the oral microbiota that are capable of catabolic degradation of methionine may exert community-wide effects (Geske et al. 2008; Sun et al. 2004). Interestingly, disabling *luxS* in *P. gingivalis* W50 is reported to have a reduction in haemagglutination and cysteine protease (gingipain) activity of the strain but did not affect the overall virulence (Burgess et al. 2002).

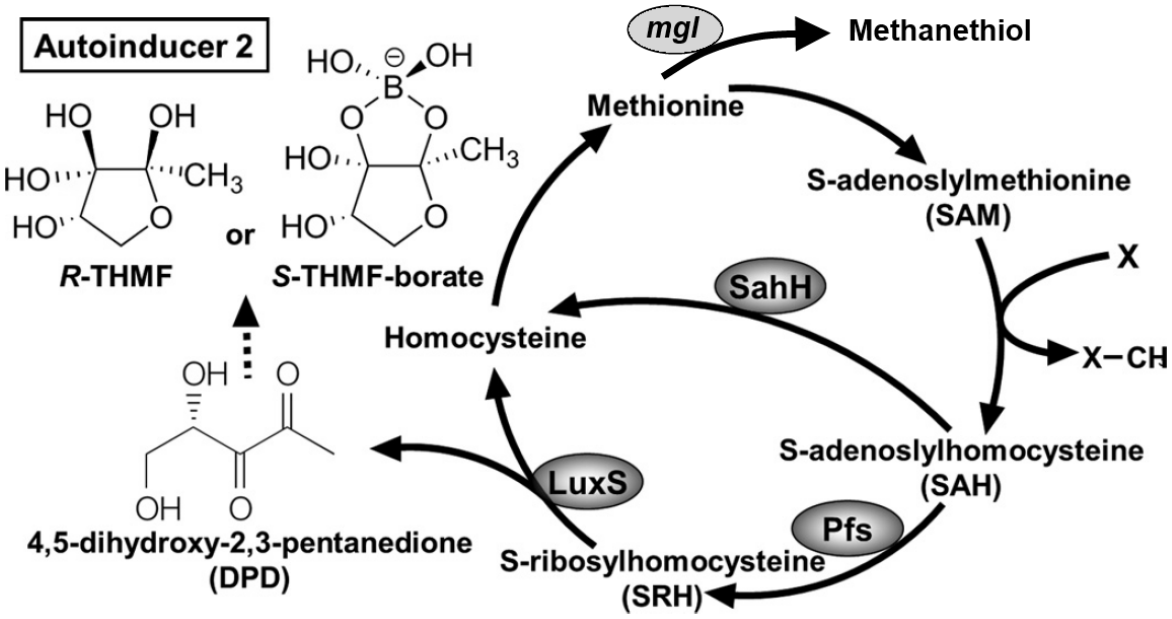


Figure 5-5 showing a schematic of the activated methionine cycle and its role in producing autoinducer-2, with the potential role for methionine gamma lyase in controlling this cycle. Adapted from (Redanz et al. 2012).

If the increased proportions of *Streptococcus* spp and particularly *A. naeslundii* in the W50 biofilms may be thought of as beneficial, owing to their prior associations to healthy plaque, the presence of *P. gingivalis* in low levels in the oral biofilm may help regulate the community structure towards a more health associated ecology (Abusleme et al. 2013; Ge et al. 2013). However, the metagenomic study in this thesis and other such studies have detected disease associated streptococci and one particular strain used in growing the biofilms namely *S. intermedius* is thought to be a more opportunistic pathogen in diseases that involve oral transmission and also periodontitis (Socransky et

al. 2013; Whiley et al. 1992). This species is also shown to co-aggregate synergistically with disease associated species and is also a more efficient H_2S producer than the other streptococcal strains used in this model (Young et al. 1996; Takahashi et al. 2011). The inability to delineate the relative proportions of these species among the streptococci by quantitative PCR in this biofilm model is a limitation, as while this study has found a reduction in the overall proportion of the streptococci in the PG343 biofilms compared to W50 (Figure 5-3), the total number of cells (measured as CFU ml^{-1} equivalents) were higher in the PG343 biofilms (Figure 5-4).

The species that displayed the most substantial change in proportions and CFU equivalents (increase of ~10^3 CFUe) in the PG343 biofilms was *V. dispar*. This species lacks methionine gamma lyase and it is possible that *V. dispar* benefits the most from the *F. nucleatum* vs *P. gingivalis* dichotomy in the ability to degrade methionine within the present *invitro* model. The proportions of *P. gingivalis* measured in both the PG343 and W50 biofilms did not show a difference, suggesting that methionine degradation is not critical to the general metabolism of *P. gingivalis*, however judging by the PG343 biofilm composition in terms of CFU equivalents, a considerable increase was observed (a difference of 2 orders of magnitude). This may also be due to the fact that the CFU equivalents measured of the overall biofilm community was also increased in the PG343 biofilms, possibly an effect of the increased presence of *F. nucleatum* being able to support more biomass (Hendrickson et al. 2014). In another *invitro* multi-species biofilm model, *V. dispar, P. intermedia* and *F. nucleatum* were seen in close proximity to each other and the effect observed of general proliferation of cells in the PG343 biofilm supported by an increase in *Fusobacterium* spp suggests a possible explanation of these data (Guggenheim et al. 2009). Whilst three different *F. nucleatum* subspecies were used in constructing the present 10-species model the available qPCR primers based on the 16S rRNA gene are unable to distinguish between these species, so the dynamics between these species in the PG343 and W50 biofilms were not determined.

A proteomic study on the effect of *A. actinomycetemcomitans* in an *invitro* 11-species biofilm containing *P. gingivalis*, described the antagonistic nature of interactions between these two species (Bao et al. 2015). The authors observed that whilst no change in the overall species proportions were observed in terms of numbers when *A. actinomycetemcomitans* was incorporated into an existing biofilm, a wide array of protein expression profiles among the different species were found with most of the virulence factors in *P. gingivalis* being down regulated. However, it has also been

reported that while *P. gingivalis* can aggregate and develop mutualistic biofilms with *A. actinomycetemcomitans,* in simple *in vitro* models *P. gingivalis* tended to dominate and overwhelm *A. actinomycetemcomitans* and this was a function of the proteolytic ability of the *P. gingivalis* strain used (Takasaki et al. 2013; Periasamy & P. E. Kolenbrander 2009). In the present study *A. actinomycetemcomitans* is the only species observed to be less in CFU equivalents and relative proportions in the PG343 biofilms compared to all the other species in the W50 biofilms (Figure 5-3; Figure 5-4). This suggests that the mutant PG343 is more dominant than W50, possibly due to more available methionine for autoinducer-2 mediated signalling and interactions with other species in the biofilm.

5.2 Cytokine response of the cells to biofilms

Analysis of the cell culture supernatants revealed that compared to the W50 biofilm stimulations, the supernatants from the PG343 biofilm stimulations contained significantly lower IL-8 in both the 4h ($p=0.03$) and 24h ($p=0.005$) conditions, whilst the same was observed for IL-6 in just the 4h stimulations ($p=0.0002$). Statistical differences with a similar pattern were found between the PG343 and W50 biofilm stimulations in the following cytokines (Figure 5-6): IL-1α at 4h ($p=0.048$); ICAM-1 at 4h ($p=0.02$) and 24h ($p=0.004$); TGF-β at 24h ($p=0.03$). Although no statistically significant differences were observed with the concentrations of interferon-gamma, IL-13 or E-selectin between the PG343 and W50 biofilm stimulations, different patterns of stimulation were observed at 4h and 24h (Figure 5-7). For example, the W50 biofilms appear to stimulate more IL-13 production at 4h than the PG343 biofilms, but this pattern was not observed at 24h where all conditions including controls produced a similar concentration of IL-13. A similar pattern at 24h was observed for E-selectin, but in this instance, the PG343 stimulated more E-selectin production at 4h. Except for IL8 and IL6, amounts of other cytokines tended to increase at 24h vs 4h. Differences were also observed within this, for instance IL1 alpha was found consistently across all samples whereas IL1 beta and IL6 were found to be present only at the 24h and 4h stimulations respectively. As regards interferon-gamma and interferon-alpha, more PG343 biofilms stimulated production of the former than the latter compared to the W50 biofilms at 4h. At 24h however, more W50 biofilms stimulated production of interferon-gamma, whereas the interferon-alpha levels were similar for both the biofilm types, but more reduced than controls.

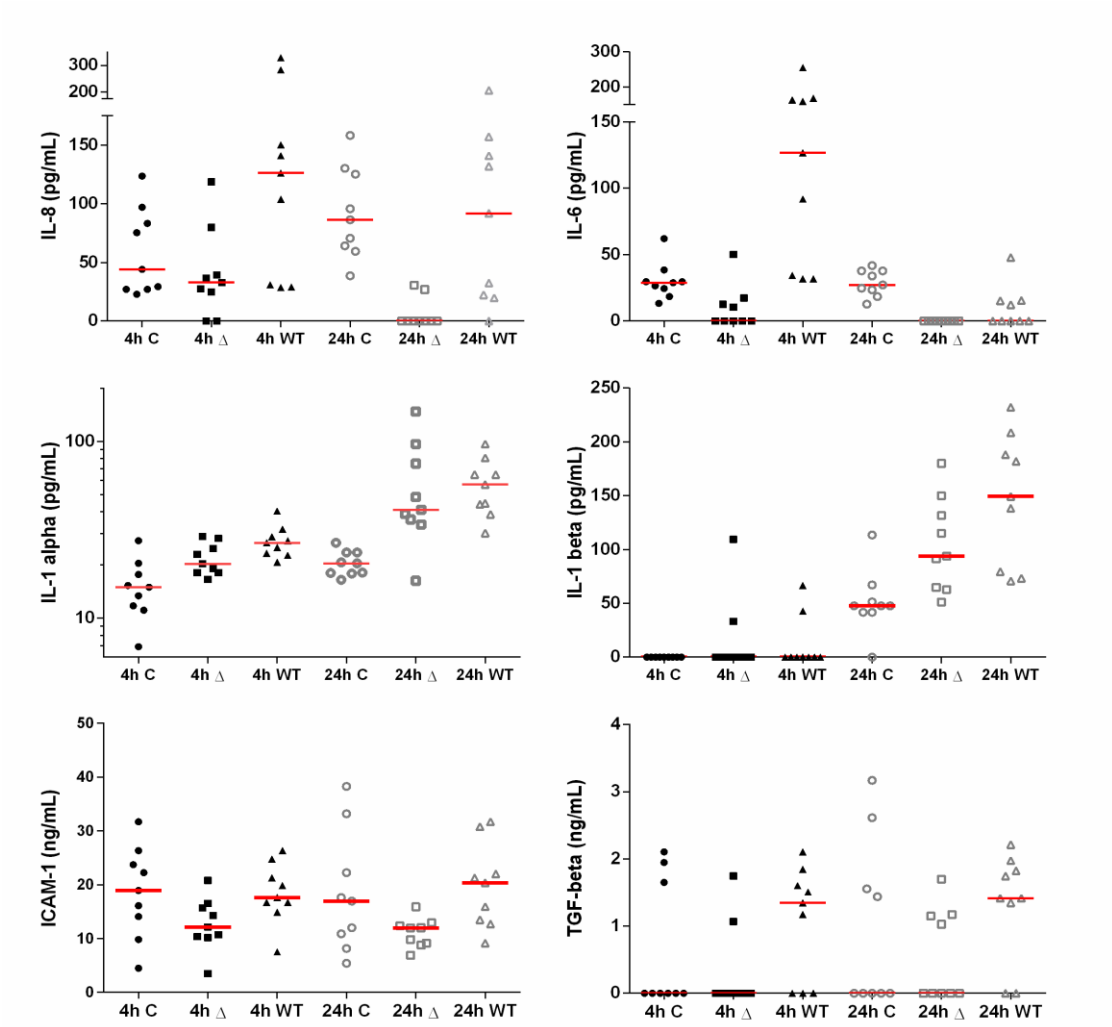


Figure 5-6 showing scatter dot plots of the concentrations of IL8, IL6, IL1 alpha, IL1 beta, ICAM1 and TGF beta measured in the cell culture supernatants after a 4h or 24h stimulation with the PG343 (Δ) or W50 (WT) biofilms (C= Controls). Only statistical significance between the PG343 and W50 biofilms are indicated. Red lines indicate median values.

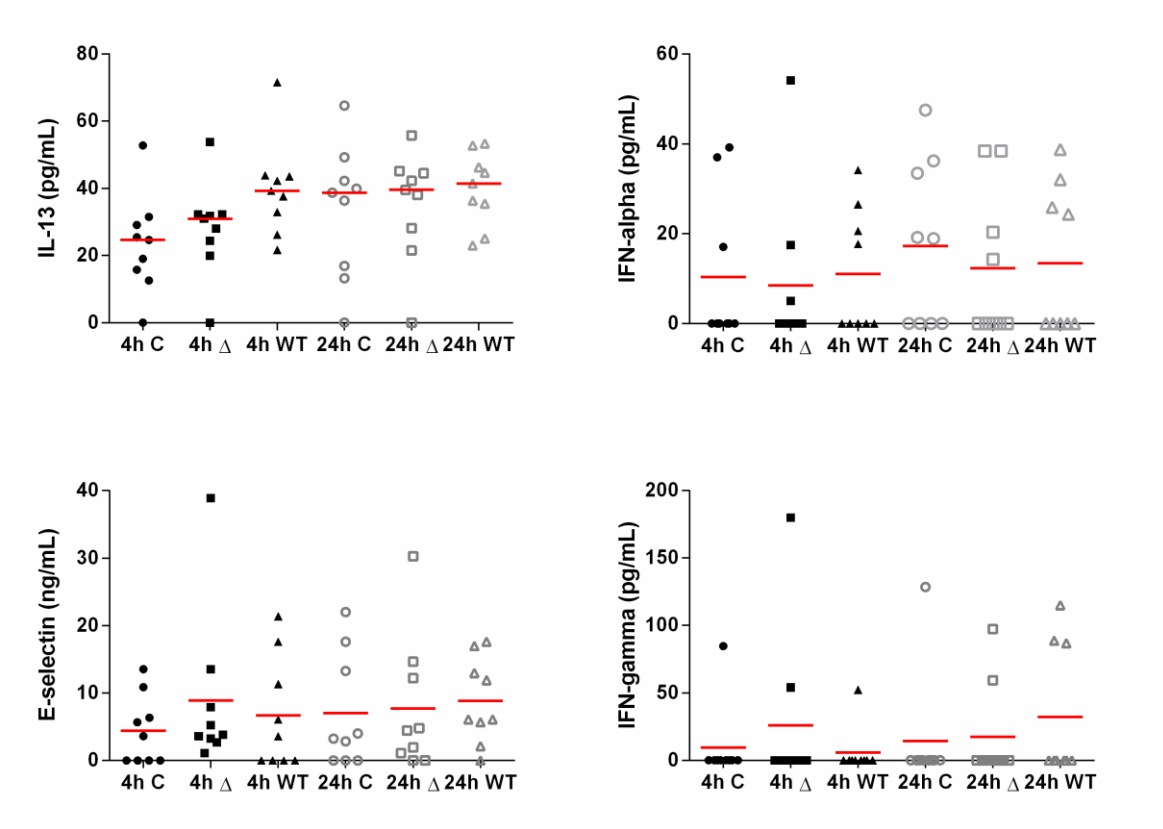


Figure 5-7 showing scatter dot plots of the concentrations of IL13, interferon-alpha, E-selectin and interferon-gamma detected in the cell culture supernatants after 4h and 24h stimulations with the PG343 (Δ) or W50 (WT) biofilms (C= Controls).

A more comprehensive evaluation of the cytokines secreted by the immortalised OKF6 human oral mucosal keratinocyte monolayer was carried out in this study, finding the presence of more than ten signalling molecules changing in response to stimulation with the bacterial biofilms. The cells used in this model are transformed and not from primary cultures, and there is likely to be differences to an *in vivo* response of the oral keratinocytes (Dickson et al. 2000). Indeed, the mRNA expression of the cell line used in this study is reported to resemble primary dysplasic cell lines (Dickman et al. 2014). However, without the methodological constraints of maintaining a primary cell line, the monolayer comprised of transformed oral keratinocytes is a valid model to explore epithelial cytokine response to microbial challenge given appropriate experimental controls.

5.2.1 Interleukin-8 and Interleukin-6

Detectable IL8 and IL6 levels were present at the 24h time point showing differences between the PG343 and W50 biofilms. Detection of IL6 in the control conditions at 4h and 24h were similar for IL6 but the control cells were observed to produce more IL8 at 24h compared to 4h (Figure 5-6). These data suggest a possible dampening of immune

response by the PG343 biofilm compared to the W50 owing to the presence of lower amounts of the potent chemokine IL8 at 24h compared to 4h. However, the ability of *P. gingivalis* to degrade both IL8 and IL6 is extensively reported in the literature and these data are also consistent with *P. gingivalis* or indeed other organisms degrading IL8 and IL6 as the levels of these cytokines in the PG343 biofilm stimulations were lesser than the controls. While IL8 is a chemokine that is primarily concerned with attracting neutrophils to an insulted site, IL6 is thought to be a major regulator of the pro-inflammatory response towards an infectious agent, particularly in linking the innate and adaptive immune responses (Neurath & Finotto 2011; Tanaka et al. 2014). So, by degrading IL6, the oral biofilm may prevent IL6 mediated signalling to recruit the Th2 and Th17 responses to more effectively address the microbial challenge. The mRNA expression data as discussed in the next section sheds some light on the patterns observed of concentrations of cytokines such as IL8 and IL6 which showed inconsistency of mRNA expression in relation to actual concentration of cytokines detected, and the mRNA data indicates that a more consistent interpretation is provided by the cytokine degradation hypothesis and therefore the supposed greater virulence of the PG343 biofilm (see section 5.3).

5.2.2 Interleukin-1α and Interleukin-1β

The levels of IL1 alpha and IL1 beta were higher in the supernatants of W50 biofilm stimulations compared to the controls and PG343 stimulations at both 4h and 24h (Figure 5-6). Unlike IL8 and IL6, the amounts of IL1 alpha and IL1 beta detected in the PG343 biofilm stimulations were higher than controls suggesting a pro-inflammatory effect in both types of biofilms. Studies have reported that *P. gingivalis* can antagonise production of IL1 alpha and IL1 beta when used to co-stimulate with other species in planktonic phase, while a non-degradative hypothesis is advanced with stimulation of *P. gingivalis* on its own in particular its LPS being identified as the main component of IL1 stimulation (Bostanci et al. 2007a; Bostanci et al. 2007b; Hamedi et al. 2009). Peyyala et al. (2013) report that stimulation of oral keratinocytes with 3-species (*S. gordonii/P. gingivalis/F. nucleatum*) biofilms resulted in greater secreted IL1 alpha compared to biofilms that did not contain all three species at once, suggesting synergistic co-stimulation. In another study, secreted IL1 alpha levels are reported to be directly related to the amount of *F. nucleatum* cells present (Peyyala et al. 2012). The observed levels of IL1 alpha and IL1 beta may be a reflection of stimulation by *F.*

nucleatum and *P. gingivalis* and the mRNA data sheds further light on whether the PG343 or W50 biofilms exert a greater pro-inflammatory effect (See section 5.3).

5.2.3 E-selectin

Expression of E-selectin—an important leukocyte adhesion molecule was found to be elevated in the supernatants of cells stimulated with the PG343 biofilms compared to W50 and controls at 4h but not at 24h (Figure 5-7). E-selectin is primarily an extracellular molecule due to its function and is secreted in response to TNF-alpha, IL1 and *P. gingivalis* lipopolysaccharide (LPS), and it is thought that it takes at least 2 hours after cytokine recognition for its expression on the cell surface with the levels returning to baseline by 24h after cessation of stimuli (Leeuwenberg et al. 1992). Furthermore, *P. gingivalis* LPS is thought to display heterogeneous structures that differentially modulate E-selectin expression and it is possible that the *mgl*$^{-}$ *P. gingivalis* in the PG343 biofilms display more of the penta-acylated LPS structures that are thought to be more virulent and/or LPS from other species such as *F. nucleatum* also playing a role (Reife et al. 2006). The more acute response shown by the cells in expressing E-selectin to the PG343 biofilms at 4h suggests this biofilm to be more virulent compared to the W50 biofilms, and this is consistent with the increase in total number of bacterial cells and in turn the numbers of individual species in the PG343 biofilms as revealed by the qPCR data.

5.2.4 Intercellular adhesion molecule-1

In contrast to E-selectin expression, a reduction was observed in the amount of the intercellular adhesion molecule-1 (ICAM-1) detected in the supernatants of cells stimulated with the PG343 biofilms and it is reported that increasing ICAM-1 expression facilitates *P. gingivalis* invasion in gingival epithelial cells in a single species model (Kato et al. 2014). Conflicting with this report is another study that found that the cysteine proteases of *P. gingivalis* (i.e. gingipains) degrade ICAM-1 secreted by human oral epithelia in a dose dependent manner and thereby prevent polymorphonuclear leukocyte attachment, so helping *P. gingivalis* in immune evasion (Tada et al. 2003). The data from the present study is more consistent with the findings of the latter study, however the former study was concerned with *P. gingivalis* invasion of epithelial cells while the latter with immune subversion, and even low levels of ICAM1 did not abolish invasion and it is not clear how these findings may relate to a

biofilm context where other species may influence the ability of the biofilm to stimulate and/or degrade ICAM1.

5.2.5 Transforming growth factor-β, interferons and interleukin-13

A greater prevalence of TGF-beta was observed in cells stimulated by the W50 biofilms compared to the PG343 and controls at both 4h and 24h, with the PG343 biofilm stimulations having similar levels as in controls (Figure 5-6). TGF-beta and IL6 are believed to act in concert to differentiate naïve T-cells into an IL17 secreting Th17 lineage, and this data is consistent with the amount of IL6 detected in PG343 vs W50 biofilm stimulations, as increased presence of IL6 is thought to also induce TGF-beta production (Bettelli et al. 2006; Mangan et al. 2006).

Interferon-alpha (IFN-alpha) was detected more frequently across all samples than interferon-gamma (IFN-gamma) and no significant differences were found with regards to both the cytokines, while a difference in levels of IL13 between the experimental conditions was only observed at 4h (Figure 5-7). Elevated levels of IL13 were observed in stimulations with W50 biofilms compared to PG343 biofilms at 4h and this cytokine is thought to suppress inflammatory responses and has an important role in Th2 response (Bao & Reinhardt 2015).

The keystone pathogen hypothesis perhaps best explains the observed cytokine patterns, whereby biofilm dwelling *P. gingivalis* acts as a keystone species and is able to bring about community-wide effects via its immune subversive mechanisms. The cytokine expression patterns found in this study are consistent with *P. gingivalis* dominating the biofilm community in concert with other species in the biofilm, and the modulation of virulence from being covert to overt in the W50 and PG343 biofilms, respectively precipitated by deletion of methionine gamma lyase suggests that this enzyme has an important role to play in *P. gingivalis* being a keystone species, particularly in its interaction with other species of the biofilm.

5.3 mRNA expression profile of the stimulated cells

Array analysis of the RNA extracts from cells after stimulation showed that at 4h, relative to the unstimulated controls, the cells stimulated with the PG343 biofilms expressed a higher fold change of mRNA corresponding to the pro-inflammatory cytokines such as TNF alpha, IL8, IL6, CCL20 and CXCL10 compared to the W50 biofilms, whereas macrophage inhibitory factor (MIF), IL-18 and IL-13 mRNA showed

greater spread of expression compared to controls in both the W50 and PG343 biofilm stimulations (Figure 5-8). Whilst the 24h stimulations yielded the highest relative fold changes in mRNA expression versus the 4h stimulations, there were differences in the cytokine expression profiles between the mutant and wild type stimulations at 24h (Figure 5-9). Compared to the wild type biofilm, the cells stimulated by the mutant biofilm expressed a greater fold change of mRNAs for IL18 and IL13 whereas IL1 alpha, TNF alpha, IL8, CXCL10 and MIF were expressed to a greater level in the former.

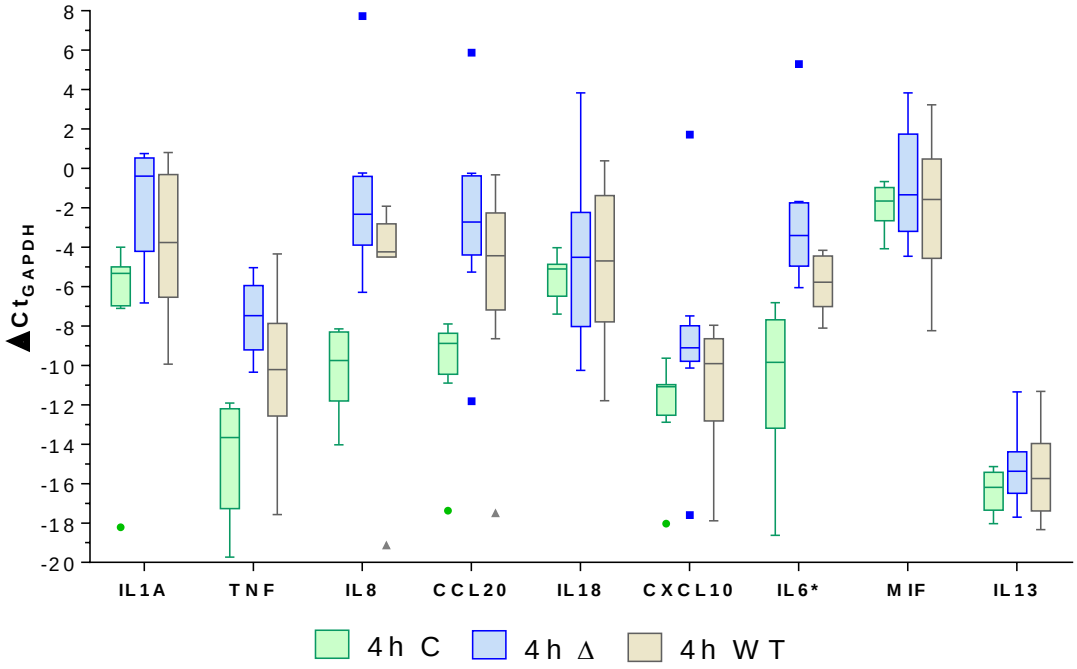


Figure 5-8 mRNA expression at 4h of the different cytokines shown as box plots of ΔCt values against the housekeeping gene GAPDH. Whiskers and outliers determined by the Tukey method; Midline is median. C=Control; Δ=PG343 biofilms; WT=W50 biofilms. Asterisk before cytokine name in the x-axis denotes statistical significance at p<0.05 between Δ and WT.

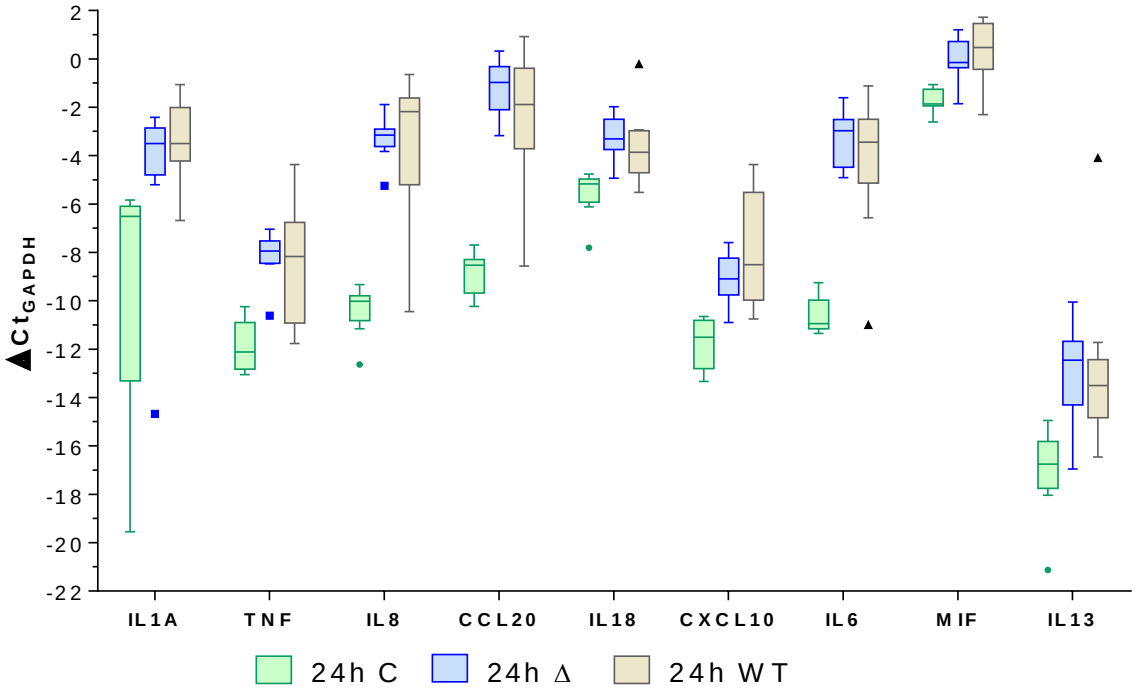


Figure 5-9 mRNA expression at 24h of the different cytokines shown as box plots of ΔCt values against the housekeeping gene GAPDH. Whiskers and outliers determined by the Tukey method; Midline is median. C=Control; Δ=PG343 biofilms; WT=W50 biofilms.

The mRNA expression data indicated a higher-fold upregulation of IL-8, IL-6 and IL1 alpha transcripts in the cells stimulated with PG343 biofilms both at 4h and 24h. The ability of *P. gingivalis* to degrade IL-8, IL-6 and IL-1β has been documented in the literature and this may explain the decreased levels of these cytokines in the cell supernatants but higher mRNA expression in the cells stimulated with the PG343 biofilm compared to W50 biofilms (Fletcher et al. 1998). This suggests that the PG343 biofilm encourages greater mRNA transcription owing to degradation of these cytokines by *P. gingivalis* and possibly other species in the biofilm disturbing the dynamic equilibrium that is more evident with the W50 biofilm, where a negative feedback loop likely prevents a 'runaway' effect of continuous transcription, translation and degradation as is observed with the PG343 biofilm stimulations.

Although little or no TNF-alpha was detected in the cell culture supernatants of both PG343 and W50 biofilms, mRNA expression for this potent cytokine suggested a more acute response by the cells toward the PG343 biofilms at 4h with both biofilm types showing equal stimulation of this cytokine transcription at 24h. *P. gingivalis* cysteine proteases are reported to degrade TNF-alpha rapidly (<10 mins), and the chosen time

points for supernatant harvest of 4h and 24h is likely to be too late to observe the effect of the mRNA transcription as intact TNF alpha in the cell culture supernatants (Calkins et al. 1998).

Transcription of IL18 and IL13 mRNA were upregulated by the PG343 biofilms at 24h compared to non-stimulated controls. IL18 is thought to be a regulatory protein that promotes IFN-gamma production while IL13 is thought to have anti-inflammatory properties. It is notable that IFN-gamma levels in most of the samples measured were below detection limits at both 4h and 24h, but the regulatory IL18 transcription suggests that more of the IFN-gamma production is stimulated by the cells in response to the PG343 biofilms than the W50 biofilms. Indeed, IFN-gamma is known to promote production of ICAM1 and the concentration of ICAM1 detected in both the biofilm stimulation and controls were comparable, suggesting a possible block in IFN-gamma production by the biofilms, possibly by post-translational modulation as IFN-gamma suppression is reported in *T. denticola* and *P. gingivalis* (Lee et al. 2009; Pulendran et al. 2001).

P. gingivalis LPS is reported to stimulate a strong IL13 response in a mouse model and though the cells used in the present study are not immune cells *per se*, stimulation of IL13 mRNA transcription is still observed at both 4h and 24h with a greater effect shown by the PG343 biofilms (Pulendran et al. 2001). However, in terms of the actual cytokines detected, an opposite effect was observed between the PG343 and W50 biofilms at 4h. It is possible that the biofilm degrades IL13, although no studies of this exist. *P. gingivalis* is also thought to inhibit production of the chemokine IP-10 (CXCL10) which is believed to be important in eliciting a Th1 response promoting clearance of bacteria (Hajishengallis & Lamont 2014). Certainly, at 24h the mRNA expression of CXCL10 by cells in response to PG343 would be considered to be down regulated compared to the W50 biofilms, reinforcing the pathogenicity of the PG343 biofilms. Cytokine data also indicated a reduction in the concentration of intact CXCL10 in both the stimulations compared to the unstimulated controls consistent with the literature.

The expression of macrophage migration inhibitory factor (MIF) and CCL20 in the biofilm stimulations were elevated compared to controls but similar to each other. MIF was not measured by the immunoassay and it is also reported to be cleaved by the lys-gingipains; the mRNA expression in this study reveals a marginally acute stimulation at 4h by the PG343 biofilms compared to the W50 biofilms (Klarström Engström et al.

2015). A similar pattern for CCL20 was also observed except the transcription of this chemokine was considerably elevated in relation to the unstimulated controls at both 4h and 24h. The CCL20 observations are consistent with data reported in the literature with regards to human primary gingival fibroblasts and *P. gingivalis* in planktonic phase (Dommisch et al. 2012).

5.4 Summary

The finding that methanethiol producing species are also periodontopathic and the association of methanethiol in the breath with periodontitis suggests that methanethiol or the enzyme responsible for production of methanethiol may have a role to play in inflammation and ecology. As such, an *in vitro* 10-species biofilm coculture model was employed to test this hypothesis. Methionine gamma lyase deficient *P. gingivalis* was constructed in the strain W50 and incorporated into the biofilm co-culture model. Analysis of the biofilms grown with the wild type or mutant *P. gingivalis* (introduced as the last in the sequence during construction of the biofilms) revealed considerable changes in the composition and overall biomass of the biofilms, suggesting a more virulent phenotype in the mutant biofilm. Cytokines measured in the cell culture supernatants after stimulation with both the biofilm types suggested that the mutant biofilms may have a more immunogenic phenotype as the cytokine levels and mRNA expression revealed a more acute pro-inflammatory effect compared to the wild type biofilms. However, the response elicited by the W50 biofilms could in fact be more insidious, by way of the slower accumulation of the cytokines and a much more controlled inflammatory response at the 4h time point. The presence of elevated levels of intact cytokines such as IL-8, IL-6 and IL-1β may help activate inflammatory pathways that may be more desirable for the microbial fitness in the oral cavity, possibly modelling the low grade inflammation that exists in the normal healthy gingiva. Possible mechanisms by which methionine gamma lyase in *P. gingivalis* and indeed other methanethiol producers might help bring about the changes observed in the biofilm composition and hence, the cytokine response are proposed.

6 CONCLUSIONS

The increased incidence of oral malodour in periodontitis patients is an oft reported observation in the literature, with the suggestion that a component of oral malodour that involves volatile sulfur compounds namely hydrogen sulfide and methanethiol being particularly associated with clinical parameters of disease. Investigations have also further reported that methanethiol concentrations in the breath of individuals with periodontitis is markedly elevated compared to health, with the associated parameter namely the ratio of methanethiol to hydrogen sulfide also correlating with clinical indices of disease such as periodontal pocket depth, bleeding on probing and plaque coverage. It has also been established that when there are no systemic confounders, the oral microbiota is largely responsible for producing these breath odours, with the sulfur metabolism of the oral microbiome implicated in VSC production. Whilst the tongue biofilm is thought to be mainly responsible for malodorous VSC concentrations present in the mouth in healthy individuals, the oral niches around the gingiva were thought to be linked to malodour and the observed VSC profile in individuals with chronic plaque-induced periodontitis. It is also well known that the changes occurring in the periodontal niches in terms of the disease process, is more proteolytic and therefore more favourable for VSC production. However, gaps exist in the current understanding of the relationship between VSCs in the breath and plaque-associated periodontal disease. For example, while VSCs in the breath are associated with clinical measurements of periodontal disease, no studies exist of the association between inflammatory mechanisms observed in periodontal disease progression and VSCs, with regards inflammatory markers in the fluids of the oral cavity. Given the closer association of breath VSCs with oral microbiota, and the fact that periodontal tissue destruction is

largely caused by dysregulated host immune mechanisms, inflammation may not necessarily correlate with VSCs in the breath. In addition, the understanding of the oral microbial diversity in health and disease has been accelerated since the advent of high throughput DNA/RNA sequencing technologies, and currently there are no investigations that have studied the microbial ecology of the tongue in context of the periodontal niches in health and disease in association with VSCs in the breath. Therefore, in the investigations described in this thesis, it was endeavoured to study the microbial and inflammatory aspects of periodontal disease in view of the possible role of VSCs as a marker of disease and as a marker of the activity of dysbiotic microbiota.

Consistent with the literature, it was confirmed that individuals with periodontal disease have significantly higher methanethiol concentration in the breath compared to healthy individuals. The methanethiol to hydrogen sulfide ratio in the breath of individuals was also found to correlate with clinical measures of periodontal disease such as periodontal pocket depth, bleeding on probing and plaque coverage. Breath samples collected from individuals was not assessed organoleptically, however a formula based on the human recognition threshold for VSCs was constructed and the resulting scale was used to grade breath samples in terms of malodour intensity, finding that the intensity of malodour was significantly higher in the breath of periodontitis patients compared to health based on the measured VSC concentrations. Malodour self-perception was also assessed, confirming the belief that individuals with poor gum health are more likely to have a true perception that they have malodour. Attempts to measure VSCs in the gingival crevicular fluid using *ex vivo* gas chromatographic methodology highlighted the variation inherent in the different filter paper types used to sample GCF, leading to a reduced sensitivity in detecting VSCs. However, significantly higher concentrations of H_2S were observed in the samples obtained from individuals classified into the gingivitis and chronic periodontitis compared to health. A novel VSC namely carbon disulfide was also observed to be highly prevalent in the GCF of chronic periodontitis patients compared to health. These data suggested that presence of VSCs in the gingival sulcus indicated a more proteolytic and active inflammatory state than absence. Indeed, inflammatory markers determined in GCF samples of the same individuals suggested that H_2S in the GCF was more closely associated with a pro-inflammatory state subgingivally in chronic periodontitis patients.

A qPCR methodology was used to detect key bacterial species in saliva, tongue and three periodontal niches namely subgingival plaque, supragingival plaque and

interdental plaque. These bacteria were associated with periodontitis but also reported to be efficient VSC producers. The role of the putative periodontopathogens namely *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* in periodontal disease and association with malodour were confirmed as these organisms increased in the periodontal niches in association with disease and breath methanethiol concentrations. A stronger association was observed between the abundance of these periodontopathic species and the H_2S concentrations measured in the GCF of individuals. When the different plaque samples were incubated with cysteine or methionine substrate, the periodontal niches proved to be more dynamic in VSC production from health to disease, suggesting a greater latent potential in the periodontal microbiota to produce VSCs than the tongue. However, it has to be recognised that due to the surface area of the tongue, the larger bacterial biomass in the tongue would eclipse the contribution of the periodontal microbiota toward breath VSCs, and these data suggested that VSC production in the periodontal niches could be an important virulence mechanism, either as a direct cause of disease or as a consequence of proteolytic activity due to disease.

An ecological survey of the tongue, subgingival and interdental niches was conducted using a Next Generation Sequencing approach to further characterise putative VSC producing species present in the different niches in different disease states. While malodour intensity was positively correlated with an increase in microbial diversity on the tongue in health, the subgingival niches showed stronger associations with overall microbial diversity and richness at the genus level, in the gingivitis and chronic periodontitis cohorts. These observations suggested that while the tongue may be an important niche for oral malodour, the periodontal niches are more closely associated with malodour or breath VSCs in disease. These data are consistent with interventional studies that showed periodontal treatment to be more effective at reducing malodour than tongue cleaning in chronic periodontitis than health. The tongue ecology was also observed to change in association with disease, with the putative VSC producing periodontopathogens increasing in prevalence and abundance in the tongue in association with disease. A survey of the available bacterial genomes revealed that very few species are capable of producing methanethiol from methionine, and the majority of species identified to produce this VSC are infact, known periodontopathogens. These observations have possibly discovered the cause of the increasing methanethiol to hydrogen sulfide ratio in association with clinical parameters of periodontal disease. It is suggested that an increase in methanethiol concentration in the breath in association

with disease is observed, directly due to the activity of these putative periodontopathogens as this enzyme is exclusive to prokaryotes and not found in humans.

An *in vitro* 10-species biofilm co-culture model was used study the potential role for the methanethiol producing enzyme methionine gamma lyase of *P. gingivalis*, in eliciting an inflammatory response and influencing biofilm composition, especially in light of *P. gingivalis* being a keystone species. Comparison of the composition of the biofilms grown with the wild type *P. gingivalis* or methionine gamma lyase deficient mutant revealed marked differences, with a larger biomass and a more even community observed with the mutant biofilms compared to wild type. The inflammatory response elicited by the biofilms from oral keratinocytes suggested that the wild type biofilms were more insidious compared to the mutant biofilm which displayed a more overtly pathogenic phenotype, the latter possibly due to an increase in biomass. These data illustrated the possible keystone phenotype of *P. gingivalis* with the VSC producing gene, methionine gamma lyase likely to be an important virulence factor, in helping *P. gingivalis* exert community wide effects.

A recent metatranscriptomic study appears to confirm that sulfur metabolism of the oral microbiota, and methionine gamma lyase of *P. gingivalis* in particular are important phenotypes that are upregulated in periodontal disease compared to health (Yost et al. 2015). Further work could focus on uncovering some of the mechanisms of host-pathogen interactions of other methanethiol producing species, and indeed hydrogen sulfide production, in biofilm formation and inflammation.

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8 APPENDICES

8.1 List of species with H_2S and CH_3SH producing homologs

HOT	Organism Name	H_2S	CH_3SH
389	Abiotrophia_defectiva	y	none
	Achromobacter_Genus_probe	y	none
554	Acinetobacter_baumannii	y	none
	Acinetobacter_Genus_probe	y	none
408	Acinetobacter_sp_oral_taxon_408		na
183	Actinobaculum_sp_oral_taxon_183		na
850	Actinomyces_cardiffensis		na
888_449	Actinomyces_dentalis_&_sp_oral_taxon_449	y	n
	Actinomyces_Genus_probe_1		none
	Actinomyces_Genus_probe_2	y	none
617	Actinomyces_georgiae	y	n
618	Actinomyces_gerencseriae	y	n
866	Actinomyces_graevenitzii	y	n
866	Actinomyces_graevenitzii	y	n
645	Actinomyces_israelii	y	n
849	Actinomyces_johnsonii	y	n
852	Actinomyces_massiliensis	y	n
671	Actinomyces_meyeri	n	n
176	Actinomyces_naeslundii	y	n
176	Actinomyces_naeslundii	y	n
701	Actinomyces_odontolyticus	y	n
701	Actinomyces_odontolyticus	y	n
701	Actinomyces_odontolyticus	y	n
708	Actinomyces_oricola	na	na
893_175	Actinomyces_oris_&_sp_oral_taxon_175	y	n
746	Actinomyces_radicidentis	na	na
746	Actinomyces_radicidentis	na	na
169	Actinomyces_sp_oral_taxon_169	na	na

170	Actinomyces_sp_oral_taxon_170	y	n
171	Actinomyces_sp_oral_taxon_171	y	n
172	Actinomyces_sp_oral_taxon_172	y	n
175	Actinomyces_sp_oral_taxon_175	y	n
178	Actinomyces_sp_oral_taxon_178	y	n
180	Actinomyces_sp_oral_taxon_180	y	n
181	Actinomyces_sp_oral_taxon_181	nm	n
414	Actinomyces_sp_oral_taxon_414		na
446	Actinomyces_sp_oral_taxon_446	na	na
448	Actinomyces_sp_oral_taxon_448	y	n
525	Actinomyces_sp_oral_taxon_525	na	na
525	Actinomyces_sp_oral_taxon_525	na	na
848	Actinomyces_sp_oral_taxon_848	y	n
877	Actinomyces_sp_oral_taxon_877	y	n
896	Actinomyces_sp_oral_taxon_896	na	na
897	Actinomyces_sp_oral_taxon_897	na	na
179	Actinomyces_timonensis	y	n
179	Actinomyces_timonensis	y	n
688	Actinomyces_viscosus	y	n
531	Aggregatibacter_actinomycetemcomitans	y	n
545_458	Aggregatibacter_aphrophilus_&_sp_oral_taxon_458	y	n
720	Aggregatibacter_paraphrophilus	na	na
720	Aggregatibacter_paraphrophilus	na	na
762_512	Aggregatibacter_segnis_&_sp_oral_taxon_512	y	n
458	Aggregatibacter_sp_oral_taxon_458	y	n
512	Aggregatibacter_sp_oral_taxon_512	na	na
513	Aggregatibacter_sp_oral_taxon_513	na	na
485	Agrobacterium_tumefaciens	n	n
831	Alloiococcus_otitis	nm	n
	Alloprevotella_Genus_probe	a	none
302	Alloprevotella_rava	y	n
308	Alloprevotella_sp_oral_taxon_308	na	na
473	Alloprevotella_sp_oral_taxon_473	y	n
474	Alloprevotella_sp_oral_taxon_474	na	na
912	Alloprevotella_sp_oral_taxon_912	na	na
913	Alloprevotella_sp_oral_taxon_913	na	na
914	Alloprevotella_sp_oral_taxon_914	na	na
914	Alloprevotella_sp_oral_taxon_914	na	na
466	Alloprevotella_tannerae	n	n
198	Alloscardovia_omnicolens	y	n
	Anaerococcus_Genus_probe	y	y; A. hydrogenalis; A. prevotii; A. vaginalis
859	Anaerococcus_lactolyticus	n	n
738_788	Anaerococcus_prevotii_&_tetradius	n	n
121	Anaeroglobus_geminatus	n	n
	Aquamicrobium_Genus_probe	y	none

660	Aquamicrobium_lusatiense	na	na
	Arcanobacterium_Genus_probe	a	none
811	Arcanobacterium_haemolyticum	nm	n
190	Arsenicicoccus_sp_oral_taxon_190	y	n
	Atopobium_Genus_probe	y	none
674	Atopobium_minutum	nm	n
723	Atopobium_parvulum	nm	n
750	Atopobium_rimae	nm	n
750	Atopobium_rimae	nm	n
199	Atopobium_sp_oral_taxon_199	nm	n
416	Atopobium_sp_oral_taxon_416	na	na
810	Atopobium_sp_oral_taxon_810	nm	n
814	Atopobium_vaginae	nm	n
45	Bacillus_clausii	y	y
272	Bacteroidaceae[G-1]_sp_oral_taxon_272	nm	n
274	Bacteroidales[G-2]_sp_oral_taxon_274	nm	n
911	Bacteroidales[G-3]_sp_oral_taxon_911	na	na
	Bacteroides_Genus_probe	y	none
630	Bacteroides_heparinolyticus	na	na
787	Bacteroides_tectus	na	na
465	Bacteroides_zoogleoformans	na	na
	Bacteroidetes[G-3]_Genus_probe	y	none
280	Bacteroidetes[G-3]_sp_oral_taxon_280		na
281	Bacteroidetes[G-3]_sp_oral_taxon_281		na
365	Bacteroidetes[G-3]_sp_oral_taxon_365		na
436	Bacteroidetes[G-3]_sp_oral_taxon_436		na
503	Bacteroidetes[G-3]_sp_oral_taxon_503		na
899	Bacteroidetes[G-3]_sp_oral_taxon_899		na
509	Bacteroidetes[G-4]_sp_oral_taxon_509		na
	Bacteroidetes[G-5]_Genus_probe	y	none
505	Bacteroidetes[G-5]_sp_oral_taxon_505		na
507	Bacteroidetes[G-5]_sp_oral_taxon_507		na
511	Bacteroidetes[G-5]_sp_oral_taxon_511		na
511	Bacteroidetes[G-5]_sp_oral_taxon_511		na
516	Bacteroidetes[G-6]_sp_oral_taxon_516		na
	Bartonella_Genus_probe	y	none
39	Bdellovibrio_sp_oral_taxon_039		Closest 16S B. bacteriovorus does not have mgl; B. exovorus has MGL
319	Bergeyella_sp_oral_taxon_319		na
322	Bergeyella_sp_oral_taxon_322		na
900	Bergeyella_sp_oral_taxon_900		na
907	Bergeyella_sp_oral_taxon_907		na
407	Bifidobacteriaceae[G-2]_sp_oral_taxon_407		na
895	Bifidobacterium_animalis_subsp_animalis	y	none

895	Bifidobacterium_animalis_subsp_lactis	y	n
889	Bifidobacterium_breve	y	n
588	Bifidobacterium_dentium	y	n
	Bifidobacterium_Genus_probe_1	y	none
	Bifidobacterium_Genus_probe_2	y	none
862	Bifidobacterium_longum	y	n
891	Bifidobacterium_scardovii	na	n
	Bordetella_Genus_probe	y	none
590	Brevundimonas_diminuta	y	n
	Brevundimonas_Genus_probe	y	none
603	Bulleidia_extructa	nm	n
	Burkholderia_Genus_probe	y	none; present in undetected strains
94	Butyrivibrio_sp_oral_taxon_094		na
575	Campylobacter_concisus	y	y?
575	Campylobacter_concisus	y	y?
580	Campylobacter_curvus	y	n
	Campylobacter_Genus_probe	y	none
623	Campylobacter_gracilis	y	n
748_763	Campylobacter_rectus_&_showae	n	n
44	Campylobacter_sp_oral_taxon_044		na
776	Campylobacter_sputorum	y	none
842	Campylobacter_ureolyticus	nm	n
	Capnocytophaga_Genus_probe_1	a	none
	Capnocytophaga_Genus_probe_2	a	none
337	Capnocytophaga_gingivalis	y	n
325	Capnocytophaga_granulosa	y	n
627	Capnocytophaga_haemolytica		na
*_326	Capnocytophaga_infantium_&_sp_oral_taxon_326	n	none
329	Capnocytophaga_leadbetteri		na
700	Capnocytophaga_ochracea	y	none
323	Capnocytophaga_sp_oral_taxon_323		na
324	Capnocytophaga_sp_oral_taxon_324	y	n
332	Capnocytophaga_sp_oral_taxon_332	y	n
334	Capnocytophaga_sp_oral_taxon_334		na
335	Capnocytophaga_sp_oral_taxon_335	y	n
336	Capnocytophaga_sp_oral_taxon_336	y	n
338	Capnocytophaga_sp_oral_taxon_338	y	n
380	Capnocytophaga_sp_oral_taxon_380	n	n
412	Capnocytophaga_sp_oral_taxon_412	y	n
863	Capnocytophaga_sp_oral_taxon_863	y	n
864	Capnocytophaga_sp_oral_taxon_864		na
878	Capnocytophaga_sp_oral_taxon_878		na
901	Capnocytophaga_sp_oral_taxon_901		na
902	Capnocytophaga_sp_oral_taxon_902		na
903	Capnocytophaga_sp_oral_taxon_903		na

775	Capnocytophaga_sputigena	y	n
	Cardiobacterium_Genus_probe	a	none
633	Cardiobacterium_hominis	nm	n
540	Cardiobacterium_valvarum	nm	n
	Catonella_Genus_probe	a	none
165_164	Catonella_morbi_&_sp_oral_taxon_164	y	none
451	Catonella_sp_oral_taxon_451		na
2	Caulobacter_sp_oral_taxon_002		na; *C. crescentus; C. vibrioides; C. segnis*
726	Centipeda_periodontii	y	y
	Chlamydophila_Genus_probe	a	none
733	Chlamydophila_pneumoniae	na	na
439	Chloroflexi[G-1]_sp_oral_taxon_439	nm	n
439	Chloroflexi[G-1]_sp_oral_taxon_439	nm	n
93	Clostridiales[F-1][G-1]_sp_oral_taxon_093		na
402	Clostridiales[F-1][G-2]_sp_oral_taxon_402		na
75	Clostridiales[F-2][G-1]_sp_oral_taxon_075		na
75	Clostridiales[F-2][G-1]_sp_oral_taxon_075		na
85	Clostridiales[F-2][G-2]_sp_oral_taxon_085		na
366	Clostridiales[F-2][G-3]_sp_oral_taxon_366		na
381	Clostridiales[F-2][G-3]_sp_oral_taxon_381		na
876	Clostridiales[F-3][G-1]_sp_oral_taxon_876	y	n
591	Corynebacterium_diphtheriae	y	n
591	Corynebacterium_diphtheriae	y	n
591	Corynebacterium_diphtheriae	y	n
595	Corynebacterium_durum	y	n
	Corynebacterium_Genus_probe	a	none
666	Corynebacterium_matruchotii	y	n
835	Corynebacterium_mucifaciens		na
184	Corynebacterium_sp_oral_taxon_184		na
853	Corynebacterium_urealyticum	n	n
579	Cryptobacterium_curtum	nm	na
23	Delftia_acidovorans	n	n
	Desulfobulbus_Genus_probe	a	none
41	Desulfobulbus_sp_oral_taxon_041	nm	nm
703	Desulfomicrobium_orale		na
605	Desulfovibrio_fairfieldensis		na
40	Desulfovibrio_sp_oral_taxon_040		na
	Dialister_Genus_probe_1	a	none
	Dialister_Genus_probe_2	a	none
118	Dialister_invisus	nm	n
843	Dialister_micraerophilus	nm	n
736	Dialister_pneumosintes		na
119	Dialister_sp_oral_taxon_119		na
502	Dialister_sp_oral_taxon_502		na
502	Dialister_sp_oral_taxon_502		na

502	Dialister_sp_oral_taxon_502		na
502	Dialister_sp_oral_taxon_502		na
	Dietzia_Genus_probe	a	none
368	Dietzia_sp_oral_taxon_368		na
813	Dolosigranulum_pigrum	y	n
	Eggerthella_Genus_probe	a	none
654	Eggerthella_lenta	y	n
569	Eggerthia_catenaformis	y	none
577	Eikenella_corrodens	y	n
11	Eikenella_sp_oral_taxon_011		na
880_*	Enterococcus_durans_&_faecium	y	n
604	Enterococcus_faecalis	y	n
	Enterococcus_Genus_probe	a	y; E. malodoratus
803_802	Enterococcus_italicus_&_saccharolyticus	y	n
904	Erysipelothrichaceae[G-1]_sp_oral_taxon_904		na
905	Erysipelothrichaceae[G-1]_sp_oral_taxon_905		na
	Erysipelothrichaceae_Genus_probe	a	none
	Erysipelothrix_Genus_probe	aa	none
484	Erysipelothrix_tonsillarum	y	n
747	Erythromicrobium_ramosum		na
	Escherichia_Genus_probe	a	y; *E. coli; E. fergusonii*
105	Eubacterium[11][G-1]_infirmum	y	n
467	Eubacterium[11][G-1]_sulci	y	none; *E. acidaminophilum*
557	Eubacterium[11][G-3]_brachy	y	none
759	Eubacterium[11][G-5]_saphenum	y	n
673	Eubacterium[11][G-6]_minutum	y	none
694	Eubacterium[11][G-6]_nodatum	y	none
694	Eubacterium[11][G-6]_nodatum	y	none
377	Eubacterium[11][G-7]_yurii	y	n
377	Eubacterium[11][G-7]_yurii	y	n
	Eubacterium_Genus_probe_1	a	none; present in E. acidaminophilum
	Eubacterium_Genus_probe_2	a	none
655	Eubacterium_limosum	y	n
655	Eubacterium_limosum	y	n
539	Filifactor_alocis	nm	none; Putative MGL with YGSC code?
	Filifactor_Genus_probe	a	y; F. alocis
662	Finegoldia_magna	nm	n
318	Flavobacteriales[G-1]_sp_oral_taxon_318		na
321	Flavobacteriales[G-1]_sp_oral_taxon_321		na
320	Flavobacteriales[G-2]_sp_oral_taxon_320		na
363	Fretibacterium_fastidiosum		na
	Fretibacterium_Genus_probe		none
358_453	Fretibacterium_sp_oral_taxon_358_&_sp_oral_taxon_453		na
359_452	Fretibacterium_sp_oral_taxon_359_&_sp_oral_taxon_452		na

360	Fretibacterium_sp_oral_taxon_360		na
361	Fretibacterium_sp_oral_taxon_361		na
362	Fretibacterium_sp_oral_taxon_362		na
	Fusobacterium_Genus_probe	a	y; various
860	Fusobacterium_gonidiaformans	n	y
860	Fusobacterium_gonidiaformans	n	y
689_*	Fusobacterium_naviforme_&_nucleatum_subsp_fusiforme		y; fusiforme
690	Fusobacterium_necrophorum	y	y
420	Fusobacterium_nucleatum_subsp_animalis	y	y
420	Fusobacterium_nucleatum_subsp_animalis	y	y
698	Fusobacterium_nucleatum_subsp_nucleatum	y	y
202	Fusobacterium_nucleatum_subsp_polymorphum	y	y
200	Fusobacterium_nucleatum_subsp_vincentii	n	y
201	Fusobacterium_periodonticum	y	y
205	Fusobacterium_sp_oral_taxon_205		na
829	Gardnerella_vaginalis	y	n
555	Gemella_bergeri	y	n
	Gemella_Genus_probe	a	none
626	Gemella_haemolysans	y	n
46	Gemella_morbillorum	y	n
46	Gemella_morbillorum	y	n
757	Gemella_sanguinis	y	n
871	GN02[G-1]_sp_oral_taxon_871		na
872	GN02[G-1]_sp_oral_taxon_872		na
873	GN02[G-2]_sp_oral_taxon_873		na
534	Granulicatella_adiacens_&_paradiacens	y	n
596	Granulicatella_elegans	y	n
535_641	Haemophilus_aegyptius_&_influenzae	y	n
821	Haemophilus_ducreyi	n	n
	Haemophilus_Genus_probe	a	none
851_036	Haemophilus_haemolyticus_&_sp_oral_taxon_036	y	n
*	Haemophilus_parahaemolyticus		na
718	Haemophilus_parainfluenzae	y	n
35	Haemophilus_sp_oral_taxon_035		na
	Helicobacter_Genus_probe	a	none
812	Helicobacter_pylori	y	n
	Johnsonella_Genus_probe	a	none
635	Johnsonella_ignava	n	n
166	Johnsonella_sp_oral_taxon_166		na
777	Jonquetella_anthropi	nm	y
582_012	Kingella_denitrificans_&_sp_oral_taxon_012	y	n
582_012	Kingella_denitrificans_&_sp_oral_taxon_012	y	n
646	Kingella_kingae	y	n
706	Kingella_oralis	y	n
459	Kingella_sp_oral_taxon_459		na
	Kytococcus_Genus_probe	a	none

	Lachnoanaerobaculum_Genus_probe	a	none
82	Lachnoanaerobaculum_orale		na
494	Lachnoanaerobaculum_saburreum	y	none
83	Lachnoanaerobaculum_sp_oral_taxon_083		na
89	Lachnoanaerobaculum_sp_oral_taxon_089		na
496	Lachnoanaerobaculum_sp_oral_taxon_496		na
107	Lachnoanaerobaculum_umeaense		na
88	Lachnospiraceae[G-2]_sp_oral_taxon_088		na
88	Lachnospiraceae[G-2]_sp_oral_taxon_088		na
96	Lachnospiraceae[G-2]_sp_oral_taxon_096		na
100	Lachnospiraceae[G-3]_sp_oral_taxon_100		na
100	Lachnospiraceae[G-3]_sp_oral_taxon_100		na
100	Lachnospiraceae[G-3]_sp_oral_taxon_100		na
80	Lachnospiraceae[G-5]_sp_oral_taxon_080		na
455	Lachnospiraceae[G-5]_sp_oral_taxon_455		na
90	Lachnospiraceae[G-6]_sp_oral_taxon_090		na
86	Lachnospiraceae[G-7]_sp_oral_taxon_086		na
163	Lachnospiraceae[G-7]_sp_oral_taxon_163		na
500	Lachnospiraceae[G-8]_sp_oral_taxon_500		na
558	Lactobacillus_brevis	y	n
816	Lactobacillus_coleohominis	n	n
608	Lactobacillus_fermentum	y	n
615_819	Lactobacillus_gasseri_&_johnsonii	n	n
	Lactobacillus_Genus_probe_1	a	none
	Lactobacillus_Genus_probe_2	a	none
	Lactobacillus_Genus_probe_3	a	none
	Lactobacillus_Genus_probe_4	a	none
838	Lactobacillus_iners	n	n
839	Lactobacillus_jensenii	y	n
424	Lactobacillus_kisonensis	y	n
418	Lactobacillus_parafarraginis	y	n
818	Lactobacillus_reuteri	y	n
756	Lactobacillus_salivarius	y	n
52	Lactobacillus_sp_oral_taxon_052		na; L. composti has mgl
51	Lactobacillus_vaginalis	y	n
804	Lactococcus_lactis	y	n
22	Lautropia_mirabilis	n	n
24	Leptothrix_sp_oral_taxon_024		na
25	Leptothrix_sp_oral_taxon_025		na
563_225	Leptotrichia_buccalis_&_sp_oral_taxon_225	y	n
	Leptotrichia_Genus_probe_1	a	none
	Leptotrichia_Genus_probe_2	a	none
845	Leptotrichia_goodfellowii	y	n
224_909	Leptotrichia_hofstadii_&_sp_oral_taxon_909	y	n
213	Leptotrichia_hongkongensis		na

214	Leptotrichia_shahii	y	n
212	Leptotrichia_sp_oral_taxon_212		na
215	Leptotrichia_sp_oral_taxon_215	y	n
215	Leptotrichia_sp_oral_taxon_215	y	n
217	Leptotrichia_sp_oral_taxon_217		na
218	Leptotrichia_sp_oral_taxon_218		na
219	Leptotrichia_sp_oral_taxon_219		na
221	Leptotrichia_sp_oral_taxon_221		na
223	Leptotrichia_sp_oral_taxon_223		na
223	Leptotrichia_sp_oral_taxon_223		na
392	Leptotrichia_sp_oral_taxon_392		na
417	Leptotrichia_sp_oral_taxon_417		na
462	Leptotrichia_sp_oral_taxon_462		na
463	Leptotrichia_sp_oral_taxon_463		na
498	Leptotrichia_sp_oral_taxon_498		na
847	Leptotrichia_sp_oral_taxon_847		na
879	Leptotrichia_sp_oral_taxon_879	y	none
222	Leptotrichia_wadei	y	n
210	Leptotrichiaceae[G-1]_sp_oral_taxon_210		na
220	Leptotrichiaceae[G-1]_sp_oral_taxon_220		na
	Leptotrichiaceae_Genus_probe	a	none
	Listeria_Genus_probe	a	none
614	Lysinibacillus_fusiformis	y	n
	Lysinibacillus_Genus_probe	a	none
122	Megasphaera_micronuciformis	nm	y
123	Megasphaera_sp_oral_taxon_123		na; M. micronuciformis and M. massiliensis have mgl
123	Megasphaera_sp_oral_taxon_123		na
841	Megasphaera_sp_oral_taxon_841		na
185	Microbacterium_sp_oral_taxon_185		na
	Mitsuokella_Genus_probe	a	none
684	Mitsuokella_multacida	y	n
131	Mitsuokella_sp_oral_taxon_131	y	n
521	Mitsuokella_sp_oral_taxon_521		na
521	Mitsuokella_sp_oral_taxon_521		na
521	Mitsuokella_sp_oral_taxon_521		na
	Mobiluncus_Genus_probe	a	none
830	Mobiluncus_mulieris	n	n
593	Mogibacterium_diversum		na
	Mogibacterium_Genus_probe	a	none
742	Mogibacterium_pumilum		na
42	Mogibacterium_timidum	nm	n
504	Mollicutes[G-1]_sp_oral_taxon_504		na
906	Mollicutes[G-2]_sp_oral_taxon_906		na

833_*	Moraxella_catarrhalis_&_nonliquefaciens	n	n
	Moraxella_Genus_probe	a	none
	Mycobacterium_Genus_probe	a	none
823	Mycobacterium_leprae	y	n
561	Mycoplasma_buccale		na; M. putrefaciens has MGL
606	Mycoplasma_faucium		na
607	Mycoplasma_fermentans	nm	n
616	Mycoplasma_genitalium	nm	n
	Mycoplasma_Genus_probe	a	none; M. putrefaciens has MGL
632	Mycoplasma_hominis	nm	n
656	Mycoplasma_lipophilum		na
704	Mycoplasma_orale	nm	n
732	Mycoplasma_pneumoniae	nm	n
754	Mycoplasma_salivarium	nm	n
13	Neisseria_bacilliformis	y	n
598	Neisseria_elongata	y	n
610	Neisseria_flavescens	y	n
610	Neisseria_flavescens	y	n
	Neisseria_Genus_probe	a	none
621	Neisseria_gonorrhoeae	y	n
649	Neisseria_lactamica	y	n
669	Neisseria_meningitidis	y	n
014_016	Neisseria_oralis_&_sp_oral_taxon_016		na
729	Neisseria_pharyngis		na
729	Neisseria_pharyngis		na
764	Neisseria_sicca	y	n
18	Neisseria_sp_oral_taxon_018		na
20	Neisseria_sp_oral_taxon_020	y	n
499	Neisseria_sp_oral_taxon_499		na
523	Neisseria_sp_oral_taxon_523		na
476	Neisseria_subflava	y	n
92	Neisseria_weaveri	y	n
	Olsenella_Genus_probe	a	none
806	Olsenella_profusa	y	n
807	Olsenella_sp_oral_taxon_807		na
809	Olsenella_sp_oral_taxon_809	y	n
38	Olsenella_uli	y	n
457	Oribacterium_sinus	y	n
078_372	Oribacterium_sp_oral_taxon_078_&_sp_oral_taxon_372	y	none
102	Oribacterium_sp_oral_taxon_102		na
108	Oribacterium_sp_oral_taxon_108	y	n
108	Oribacterium_sp_oral_taxon_108	y	n
108	Oribacterium_sp_oral_taxon_108	y	n
894	Ottowia_sp_oral_taxon_894		na

894	Ottowia_sp_oral_taxon_894		na
	Paenibacillus_Genus_probe	a	none
786	Paenibacillus_sp_oral_taxon_786	y	n
586	Parascardovia_denticolens	n	n
	Parvimonas_Genus_probe	a	none
111	Parvimonas_micra	n	n
110	Parvimonas_sp_oral_taxon_110	n	n
167	Peptococcus_sp_oral_taxon_167		na
167	Peptococcus_sp_oral_taxon_167		na
168	Peptococcus_sp_oral_taxon_168		na
548	Peptoniphilus_asaccharolyticus		na; Other Peptoniphilus species have mgl
	Peptoniphilus_Genus_probe	a	y
840	Peptoniphilus_indolicus	nm	y
648	Peptoniphilus_lacrimalis	y	n
375	Peptoniphilus_sp_oral_taxon_375	y	na
386	Peptoniphilus_sp_oral_taxon_386	nm	y
836	Peptoniphilus_sp_oral_taxon_836	y	n
383	Peptostreptococcaceae[11][G-1]_sp_oral_taxon_383		na
91	Peptostreptococcaceae[11][G-2]_sp_oral_taxon_091		na; miscellaneous have mgl
91	Peptostreptococcaceae[11][G-2]_sp_oral_taxon_091		na
91	Peptostreptococcaceae[11][G-2]_sp_oral_taxon_091		na
382	Peptostreptococcaceae[11][G-3]_sp_oral_taxon_382		na
495	Peptostreptococcaceae[11][G-3]_sp_oral_taxon_495		na
103	Peptostreptococcaceae[11][G-4]_sp_oral_taxon_103		na
369	Peptostreptococcaceae[11][G-4]_sp_oral_taxon_369		na
493	Peptostreptococcaceae[11][G-5]_sp_oral_taxon_493		na
81	Peptostreptococcaceae[11][G-7]_sp_oral_taxon_081		na
106	Peptostreptococcaceae[11][G-7]_sp_oral_taxon_106		na
113	Peptostreptococcaceae[13][G-1]_sp_oral_taxon_113	n	n
790	Peptostreptococcaceae[13][G-2]_sp_oral_taxon_790		na
542	Peptostreptococcus_anaerobius	n	n
	Peptostreptococcus_Genus_probe	a	none
112	Peptostreptococcus_stomatis	y	n
547	Porphyromonas_asaccharolytica	n	y
283	Porphyromonas_catoniae	n	n
283	Porphyromonas_catoniae	n	n
283	Porphyromonas_catoniae	n	n
283	Porphyromonas_catoniae	n	n
283	Porphyromonas_catoniae	n	n
283	Porphyromonas_catoniae	n	n
273	Porphyromonas_endodontalis	n	y
	Porphyromonas_Genus_probe_1	a	y
	Porphyromonas_Genus_probe_2	a	y
	Porphyromonas_Genus_probe_3	a	y

619	Porphyromonas_gingivalis	n	y
275	Porphyromonas_sp_oral_taxon_275		na; absent in 278/279
275	Porphyromonas_sp_oral_taxon_275		na
275	Porphyromonas_sp_oral_taxon_275		na
277	Porphyromonas_sp_oral_taxon_277		na
277	Porphyromonas_sp_oral_taxon_277		na
278	Porphyromonas_sp_oral_taxon_278	y	n
279	Porphyromonas_sp_oral_taxon_279	y	n
279	Porphyromonas_sp_oral_taxon_279	y	n
279	Porphyromonas_sp_oral_taxon_279	y	n
284	Porphyromonas_sp_oral_taxon_284		na
285	Porphyromonas_sp_oral_taxon_285		na
395	Porphyromonas_sp_oral_taxon_395		na
785	Porphyromonas_uenonis	nm	y
553	Prevotella_baroniae	n	n
556	Prevotella_bivia	y	n
560	Prevotella_buccae	y	n
562	Prevotella_buccalis	y	n
583	Prevotella_dentalis	y	none
291	Prevotella_denticola	y	n
600	Prevotella_enoeca	y	none
600	Prevotella_enoeca	y	none
782	Prevotella_fusca	y	none
782	Prevotella_fusca	y	none
782	Prevotella_fusca	y	none
	Prevotella_Genus_probe	a	none
298	Prevotella_histicola	y	none
643	Prevotella_intermedia	y	none
643	Prevotella_intermedia	y	none
643	Prevotella_intermedia	y	none
658	Prevotella_loescheii	y	none
658	Prevotella_loescheii	y	none
658	Prevotella_loescheii	y	none
289	Prevotella_maculosa	y	none
665	Prevotella_marshii		na
665	Prevotella_marshii		na
469	Prevotella_melaninogenica	y	n
378	Prevotella_micans		na
378	Prevotella_micans		na
685	Prevotella_multiformis	y	none
794	Prevotella_multisaccharivorax	y	none
*_299	Prevotella_nanceiensis_&_sp_oral_taxon_299		na
693	Prevotella_nigrescens	y	n
705	Prevotella_oralis	y	n
311	Prevotella_oris	y	none

288	Prevotella_oulorum	y	none
714	Prevotella_pallens	y	none
714	Prevotella_pallens	y	none
303	Prevotella_pleuritidis	y	none
781	Prevotella_saccharolytica	y	none
781	Prevotella_saccharolytica	y	none
781	Prevotella_saccharolytica	y	none
307	Prevotella_salivae	y	n
885	Prevotella_scopos	y	none
795	Prevotella_shahii	y	none
292	Prevotella_sp_oral_taxon_292		na
292	Prevotella_sp_oral_taxon_292		na
293	Prevotella_sp_oral_taxon_293		na
296	Prevotella_sp_oral_taxon_296		na
300	Prevotella_sp_oral_taxon_300		na
300	Prevotella_sp_oral_taxon_300		na
301	Prevotella_sp_oral_taxon_301		na
304	Prevotella_sp_oral_taxon_304		na
305	Prevotella_sp_oral_taxon_305		na
306	Prevotella_sp_oral_taxon_306		na
309	Prevotella_sp_oral_taxon_309		na
310	Prevotella_sp_oral_taxon_310		na
315	Prevotella_sp_oral_taxon_315		na
317	Prevotella_sp_oral_taxon_317	y	none
317	Prevotella_sp_oral_taxon_317	y	none
317	Prevotella_sp_oral_taxon_317	y	none
376	Prevotella_sp_oral_taxon_376		na
396	Prevotella_sp_oral_taxon_396		na
443	Prevotella_sp_oral_taxon_443		na
472	Prevotella_sp_oral_taxon_472	y	none
475	Prevotella_sp_oral_taxon_475		na
515	Prevotella_sp_oral_taxon_515		na
526	Prevotella_sp_oral_taxon_526		na
820	Prevotella_sp_oral_taxon_820		na
572	Prevotella_veroralis	y	none
191	Propionibacterium_acidifaciens	y	n
530	Propionibacterium_acnes	nm	n
552	Propionibacterium_avidum	y	none
552	Propionibacterium_avidum	y	none
	Propionibacterium_Genus_probe	a	y
739	Propionibacterium_propionicum	y	none
192	Propionibacterium_sp_oral_taxon_192	y	none
193	Propionibacterium_sp_oral_taxon_193		na
194	Propionibacterium_sp_oral_taxon_194		na
915	Propionibacterium_sp_oral_taxon_915		na
	Proteus_Genus_probe	a	y

676	Proteus_mirabilis	y	y
536	Pseudomonas_aeruginosa	y	n
612	Pseudomonas_fluorescens	y	y
	Pseudomonas_Genus_probe	a	y
834	Pseudomonas_otitidis	y	n
32	Pseudomonas_sp_oral_taxon_032		na
538	Pseudoramibacter_alactolyticus	y	n
357	Pyramidobacter_piscolens	y	none
28	Rhodocyclus_sp_oral_taxon_028		na
188	Rothia_aeria	nm	n
587	Rothia_dentocariosa	n	n
	Rothia_Genus_probe	a	none
681	Rothia_mucilaginosa	y	n
	Sanguibacter_Genus_probe	a	none
	Scardovia_Genus_probe	a	none
642	Scardovia_inopinata	n	n
195	Scardovia_wiggsiae	n	n
	Selenomonas_&_Centipeda_Genus_probe	a	none
124	Selenomonas_artemidis	y	n
124	Selenomonas_artemidis	y	n
139	Selenomonas_dianae	na	na
139	Selenomonas_dianae	na	na
125	Selenomonas_flueggei	y	n
130	Selenomonas_noxia	y	n
130	Selenomonas_noxia	y	n
130_140	Selenomonas_noxia_&_sp_oral_taxon_140		na
133	Selenomonas_sp_oral_taxon_133	y	n
133	Selenomonas_sp_oral_taxon_133	y	n
134	Selenomonas_sp_oral_taxon_134		na
134	Selenomonas_sp_oral_taxon_134		na
134	Selenomonas_sp_oral_taxon_134		na
136	Selenomonas_sp_oral_taxon_136		na
136	Selenomonas_sp_oral_taxon_136		na
137	Selenomonas_sp_oral_taxon_137	y	n
137	Selenomonas_sp_oral_taxon_137	y	n
137	Selenomonas_sp_oral_taxon_137	y	n
138	Selenomonas_sp_oral_taxon_138	y	n
143	Selenomonas_sp_oral_taxon_143		na
146	Selenomonas_sp_oral_taxon_146		na
149	Selenomonas_sp_oral_taxon_149	y	n
388	Selenomonas_sp_oral_taxon_388		na
442	Selenomonas_sp_oral_taxon_442		na
478	Selenomonas_sp_oral_taxon_478	y	none
478	Selenomonas_sp_oral_taxon_478	y	none
501	Selenomonas_sp_oral_taxon_501		na
151	Selenomonas_sputigena	y	n

151	Selenomonas_sputigena	y	n
151	Selenomonas_sputigena	y	n
151	Selenomonas_sputigena	y	n
151	Selenomonas_sputigena	y	n
151_143	Selenomonas_sputigena_&_sp_oral_taxon_143		na
95	Shuttleworthia_satelles	y	n
683	Simonsiella_muelleri	y	n
602	Slackia_exigua	y	n
	Slackia_Genus_probe	a	none; S. heliotrinireducens has MGL
844	Sneathia_amnionii		na
837	Sneathia_sanguinegens		na
678	Solobacterium_moorei	y	n
	Sphingomonas_Genus_probe	a	none; S. parapaucimobilis has MGL
345	SR1[G-1]_sp_oral_taxon_345		na
874	SR1[G-1]_sp_oral_taxon_874		na
875	SR1[G-1]_sp_oral_taxon_875		na
	SR1_Genus_probe	a	none
550_*	Staphylococcus_aureus_&_gallinarum	y	n
	Staphylococcus_Genus_probe	a	none
*_076	Staphylococcus_pasteuri_&_warneri	y	pasteuri not found
663	Stenotrophomonas_maltophilia	y	n
663	Stenotrophomonas_maltophilia	y	n
419	Stomatobaculum_longum	y	none
97	Stomatobaculum_sp_oral_taxon_097		na
373	Stomatobaculum_sp_oral_taxon_373		na
910	Stomatobaculum_sp_oral_taxon_910		na
537	Streptococcus_agalactiae	y	n
543	Streptococcus_anginosus	y	n
576	Streptococcus_constellatus	y	n
578	Streptococcus_cristatus	y	n
594	Streptococcus_downei	y	n
	Streptococcus_Genus_probe	a	y; S. penumoniae
622_758	Streptococcus_gordonii_&_sanguinis	y	n
644	Streptococcus_intermedius	y	n
686	Streptococcus_mutans	y	n
411	Streptococcus_parasanguinis_II	y	n
734_*	Streptococcus_pneumoniae_&_pseudopneumoniae	y	y
745	Streptococcus_pyogenes	y	n
755_021	Streptococcus_salivarius_&_vestibularis	y	n
758	Streptococcus_sanguinis	y	n
768	Streptococcus_sobrinus	y	n
64	Streptococcus_sp_oral_taxon_064		na; S. pneumoniae has mgl

66	Streptococcus_sp_oral_taxon_066	y	n
68	Streptococcus_sp_oral_taxon_068		na
69	Streptococcus_sp_oral_taxon_069		na
431	Streptococcus_sp_oral_taxon_431		na
486	Streptococcus_sp_oral_taxon_486		na
487	Streptococcus_sp_oral_taxon_487		na
435	Syntrophomonadaceae[8][G-1]_sp_oral_taxon_435		na
613	Tannerella_forsythia	n	n
	Tannerella_Genus_probe	a	none
286	Tannerella_sp_oral_taxon_286	y	none
808	Tannerella_sp_oral_taxon_808		na
916	Tannerella_sp_oral_taxon_916		na
916	Tannerella_sp_oral_taxon_916		na
916	Tannerella_sp_oral_taxon_916		na
916	Tannerella_sp_oral_taxon_916		na
346	TM7[G-1]_sp_oral_taxon_346		na
347	TM7[G-1]_sp_oral_taxon_347		na
348	TM7[G-1]_sp_oral_taxon_348		na
348	TM7[G-1]_sp_oral_taxon_348		na
349	TM7[G-1]_sp_oral_taxon_349		na
352	TM7[G-1]_sp_oral_taxon_352		na
353	TM7[G-1]_sp_oral_taxon_353		na
488	TM7[G-1]_sp_oral_taxon_488		na
350	TM7[G-2]_sp_oral_taxon_350		na
351	TM7[G-3]_sp_oral_taxon_351		na
355	TM7[G-4]_sp_oral_taxon_355		na
356	TM7[G-5]_sp_oral_taxon_356		na
437	TM7[G-5]_sp_oral_taxon_437		na
	TM7_Genus_probe		none
541	Treponema_amylovorum		na
584	Treponema_denticola	n	y
584	Treponema_denticola	n	y
	Treponema_Genus_probe_1	a	none
	Treponema_Genus_probe_2	a	yes
	Treponema_Genus_probe_3	a	yes
	Treponema_Genus_probe_4	a	yes
	Treponema_Genus_probe_5	a	yes
653	Treponema_lecithinolyticum	y	n
664	Treponema_maltophilum	y	n
805	Treponema_pallidum	n	n
724	Treponema_parvum		na
725	Treponema_pectinovorum		na; T. phagedenis; T. pedis; T. putidum
743	Treponema_putidum		y
769	Treponema_socranskii	y	none
769	Treponema_socranskii	y	none

769	Treponema_socranskii	y	none
769	Treponema_socranskii	y	none
226	Treponema_sp_oral_taxon_226		na
227	Treponema_sp_oral_taxon_227		na
228	Treponema_sp_oral_taxon_228		na
228	Treponema_sp_oral_taxon_228		na
230	Treponema_sp_oral_taxon_230		na
231_237	Treponema_sp_oral_taxon_231_&_sp_oral_taxon_237		na
232	Treponema_sp_oral_taxon_232		na
234	Treponema_sp_oral_taxon_234		na
235	Treponema_sp_oral_taxon_235		na
236	Treponema_sp_oral_taxon_236		na
238	Treponema_sp_oral_taxon_238		na
239	Treponema_sp_oral_taxon_239		na
242	Treponema_sp_oral_taxon_242		na
246	Treponema_sp_oral_taxon_246		na
247	Treponema_sp_oral_taxon_247		na
249	Treponema_sp_oral_taxon_249		na
250	Treponema_sp_oral_taxon_250		na
252	Treponema_sp_oral_taxon_252		na
253	Treponema_sp_oral_taxon_253		na
254	Treponema_sp_oral_taxon_254		na
255	Treponema_sp_oral_taxon_255		na
256	Treponema_sp_oral_taxon_256		na
257	Treponema_sp_oral_taxon_257		na
258	Treponema_sp_oral_taxon_258		na
260	Treponema_sp_oral_taxon_260		na
262	Treponema_sp_oral_taxon_262		na
262	Treponema_sp_oral_taxon_262		na
263	Treponema_sp_oral_taxon_263		na
264	Treponema_sp_oral_taxon_264		na
265	Treponema_sp_oral_taxon_265		na
268	Treponema_sp_oral_taxon_268		na
268	Treponema_sp_oral_taxon_268		na
269	Treponema_sp_oral_taxon_269		na
270	Treponema_sp_oral_taxon_270		na
271	Treponema_sp_oral_taxon_271		na
490	Treponema_sp_oral_taxon_490		na
508	Treponema_sp_oral_taxon_508		na
508	Treponema_sp_oral_taxon_508		na
517	Treponema_sp_oral_taxon_517		na
518	Treponema_sp_oral_taxon_518		na
29	Treponema_vincentii	n	n
832	Turicella_otitidis	y	n
717	Variovorax_paradoxus	n	n
524	Veillonella_atypica	y	n

524_160	Veillonella_atypica_&_dispar	y	n
887	Veillonella_denticariosi		na
887	Veillonella_denticariosi		na
160	Veillonella_dispar	y	n
	Veillonella_Genus_probe	y	y; V. ratti
161	Veillonella_parvula	y	n
161	Veillonella_parvula	y	n
158	Veillonella_rogosae		na
780	Veillonella_sp_oral_taxon_780	y	n
917	Veillonella_sp_oral_taxon_917		na
129	Veillonellaceae[G-1]_sp_oral_taxon_129		na
132_150	Veillonellaceae[G-1]_sp_oral_taxon_132_&_sp_oral_taxon_150		na
135	Veillonellaceae[G-1]_sp_oral_taxon_135		na
145	Veillonellaceae[G-1]_sp_oral_taxon_145		na
148	Veillonellaceae[G-1]_sp_oral_taxon_148		na
155	Veillonellaceae[G-1]_sp_oral_taxon_155		na
155	Veillonellaceae[G-1]_sp_oral_taxon_155		na
483	Veillonellaceae[G-1]_sp_oral_taxon_483		na
918	Veillonellaceae[G-1]_sp_oral_taxon_918		na
	Veillonellaceae_probe_1	y	none
	Veillonellaceae_probe_2	y	none
	Yersinia_Genus_probe	y	y; Y. pestis